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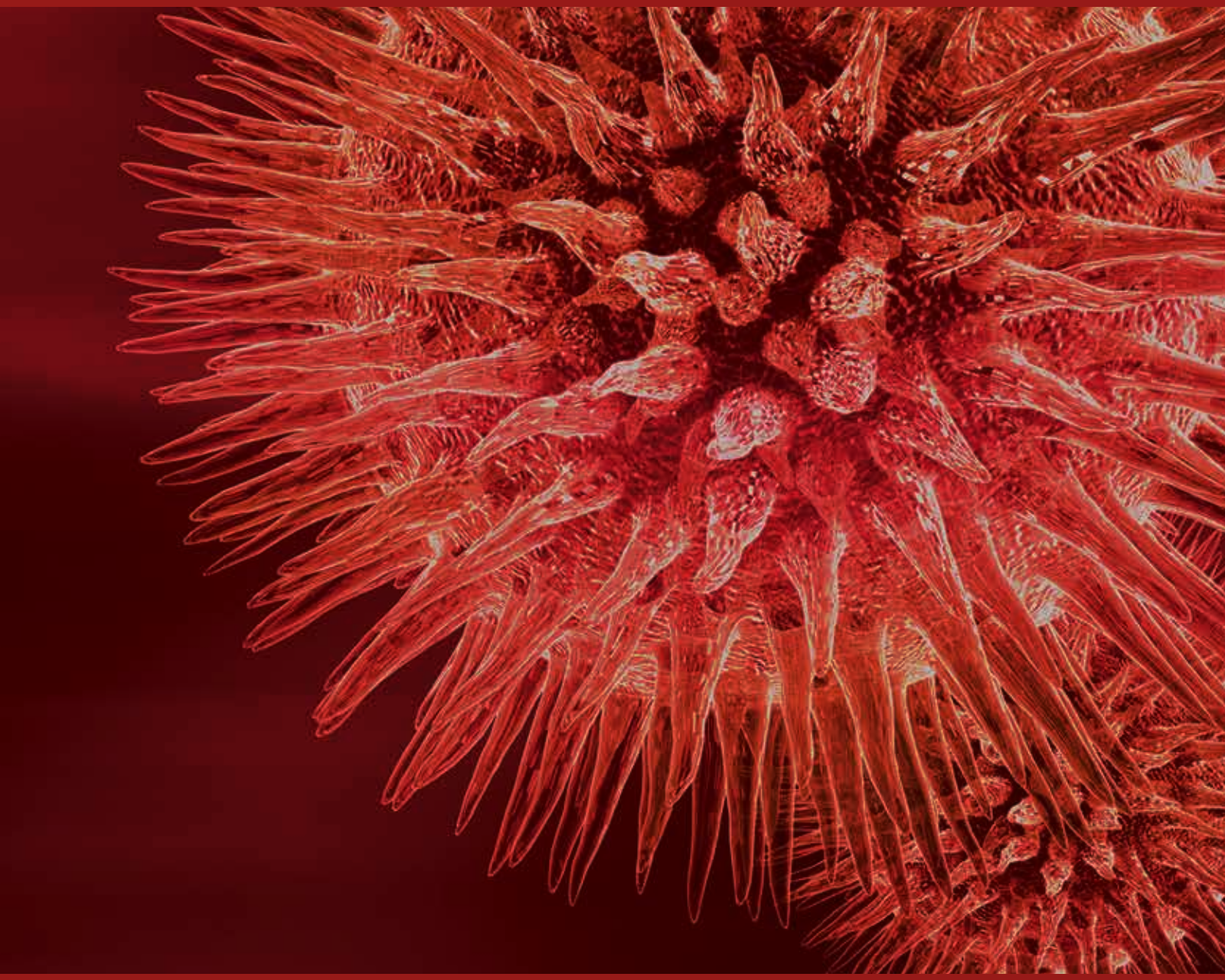
Guest Editors: Clara G. de los Reyes-Gavilán, María Fernández, John Andrew Hudson, and Riitta Korpela



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Editorial

Role of Microorganisms Present in Dairy Fermented Products in Health and Disease

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Thousands of years ago humanity started agricultural practice and the domestication of cattle. Milk from farmed animals represented a good source of nutrients and liquid for hydration. The fermentation of milk provided a simple way to increase its shelf-life while improving its safety. From the initial accidental phenomenon of fermentation, humans learned to control these processes. Incorporating the controlled fermentation of milk in domestic practices of these primitive societies gave rise to a progressive diversification of dairy products, as influenced by habits of different ethnicities, geographical environments, and type of dairy farming. European-derived populations show lactase activity into adulthood, exhibiting selection for a lactase persistence haplotype [1]. The strong positive selective pressure exerted by animal husbandry practices resulted in the best studied phenomenon of gene-culture coevolution in the mutual human and animal symbiosis promoted by the advent of agriculture [2].

Therefore, fermented dairy products have been linked to the human nutrition and progress from ancient times. Nowadays they continue to be fundamental components of a balanced western diet. A huge variety of fermented dairy products are now available for consumers. Although a small proportion of these products are homemade, most of them are produced industrially; indeed, the dairy industry, particularly of fermented products, is economically important in many countries. Fermented products are generally populated by a diverse microbiota that impacts human health. Knowledge of microorganisms inhabiting underexplored natural fermented dairy products and their potential effects in human health, mechanisms underlying beneficial or detrimental effects of such microorganisms, and research in new safe alternative technologies to thermal processes constitute matters of current interest in food and health research. This special issue aimed to shed light on the role that microorganisms present in dairy fermented products play in human health and disease.

This special issue comprises reviews and experimental articles. Editors present first a general overview of the current state of the art. Although the special issue was opened to both beneficial and harmful microorganisms, contributions received focused on "good bugs." Articles cover the following items: mechanisms of beneficial action of probiotics, food safety, and technological aspects of lactic acid bacteria and probiotics.

Relating mechanisms of probiotic action, the contributions address different aspects of microorganisms from the genus *Bifidobacterium*, a subdominant intestinal microbial group, some of whose strains have recognized probiotic effects. The beneficial action of probiotics is related, among others, to their capacity to colonize the host. V. Grimm and coworkers reviewed the mechanisms responsible for host colonization by bifidobacteria and factors involved. Two experimental articles dealing with the study of the mechanisms of

probiotic-host interaction were presented. In one of them, N. Salazar et al. analyzed the capacity to modulate immune response and insulin-dependent glucose homeostasis by two exopolysaccharide-producing *Bifidobacterium* strains in a Wistar rat model. In the other, B. Sánchez et al. studied in an *in vitro* model the modification of the profile of immune mediators and proteins synthesized by the intestinal cell line HT29 in the presence of a strain of *Bifidobacterium breve,* concluding that the presence of bifidobacteria could favor innate immune response and reinforcement of the intestinal physical barrier.

M. J. Saez-Lara et al. reviewed the degree of scientific evidence in randomized human clinical trials for benefits associated with the use of lactic acid bacteria and bifidobacteria in the prevention and treatment of inflammatory bowel disease (Crohn's disease and ulcerative colitis) and other related diseases such as pouchitis and cholangitis.

Polyphenols are characterized by the presence of large multiples of phenol structural units that are synthesized by many vegetables as defense compounds. Many of them have antioxidant and other beneficial effects in human health. Finally the beneficial action of polyphenols greatly depends on the generation of bioactive compounds through their biotransformation by the intestinal microbiota. The addition of polyphenols to fermented dairy products deserves further research and development of technological applications. Two manuscripts reviewed the interaction of dietary polyphenols and the intestinal microbiota. M. Dueñas et al. analyzed the current knowledge on the modulation of the intestinal microbiota by these compounds from the point of view of the experimental approaches used. In contrast, L. Marín et al. explored the potentially beneficial action of dietary polyphenols as antiviral, antibacterial, and antiparasitic agents.

Five articles addressed technological aspects of beneficial microorganisms included in fermented dairy products, either by considering the behavior of probiotics during the manufacture process or by focusing towards the production of specific beneficial compounds by microorganisms during the elaboration of such products. J. M. Castro et al. reviewed aspects related to the potential of cheeses as probiotic carriers and some technological aspects related to the maintenance of the viability of probiotics in cheeses. An experimental contribution presented the development of a potential probiotic fresh cheese using two *Lactobacillus salivarius* strains isolated from human milk (N. Cárdenas et al.), a novel and interesting source for probiotics that is receiving considerable recent attention. The remaining three articles deal with the release of beneficial compounds by lactic acid bacteria and bifidobacteria in fermented dairy products. Thus, one article shows the capacity of lactic acid bacteria isolated from alpine traditional raw cow's milk cheese to produce γ-aminobutyric acid (E. Franciosi et al.) whereas another article (M. Gagnon et al.) analyzed the bioaccessibility of antioxidants during simulated digestion of milk that has been fermented by several strains of *Bifidobacterium longum* subsp. *longum*. In turn, M. A. Villar-Tajadura et al. demonstrated the ability to produce conjugated linoleic and α-linolenic acids by bifidobacteria from human milk in a milk-based medium. These articles open the possibility to use such strains for the development of fermented products with different functional properties.

Finally, three papers deal with the role of lactic acid bacteria in the safety of fermented dairy products. J. L. Arqués and colleagues review the antimicrobial activity against pathogens of lactic acid bacteria in dairy fermented products as well as in the gut after ingestion. In addition, two experimental articles address different aspects of safety in dairy products. Metagenomic analysis was used to characterize the presence of antibiotic resistance genes in the microbiota of a specific dairy fermented product, *Mozzarella di Bufala Campana* manufactured in Italy (C. Devirgiliis et al.). In turn, P. Carasi et al. determined safety aspects and antimicrobial properties of several strains of the species *Lactobacillus kefiri,* one of the most predominant microorganisms present in kefir-fermented milk.

This editorial presents a brief summary of the topics discussed in the articles published in this special issue. We hope readers will find useful information on the topics discussed here.

Acknowledgments

We express our deep gratitude to the reviewers of articles submitted to this special issue. We thank the authors for providing such highly valuable contributions to this special issue.

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Review Article

Antimicrobial Activity of Lactic Acid Bacteria in Dairy Products and Gut: Effect on Pathogens

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The food industry seeks alternatives to satisfy consumer demands of safe foods with a long shelf-life able to maintain the nutritional and organoleptic quality. The application of antimicrobial compounds-producing protective cultures may provide an additional parameter of processing in order to improve the safety and ensure food quality, keeping or enhancing its sensorial characteristics. In addition, strong evidences suggest that certain probiotic strains can confer resistance against infection with enteric pathogens. Several mechanisms have been proposed to support this phenomenon, including antimicrobial compounds secreted by the probiotics, competitive exclusion, or stimulation of the immune system. Recent research has increasingly demonstrated the role of antimicrobial compounds as protective mechanism against intestinal pathogens and therefore certain strains could have an effect on both the food and the gut. In this aspect, the effects of the combination of different strains keep unknown. The development of multistrain probiotic dairy products with good technological properties and with improved characteristics to those shown by the individual strains, able to act not only as protective cultures in foods, but also as probiotics able to exert a protective action against infections, has gained increased interest.

1. Bacteriocins to Improve Dairy Products Safety

1.1. Bacterial Contamination in Dairy Products. Farmed animals represent a major reservoir of pathogens that can be transferred to milk. The predominant human bacterial pathogens that can potentially be transferred to milk include mainly *Listeria monocytogenes*, *Salmonella* spp., *Staphylococcus aureus*, and pathogenic *Escherichia coli*. Raw milk provides a potential growth medium for the development of these bacteria [1]. Although pasteurization destroys potential pathogenic microorganisms, postpasteurization processing can lead to the recontamination of dairy products.

L. monocytogenes can cause illnesses extending from those with mild flu-like symptoms or gastroenteritis to more serious, potentially fatal conditions such as bacteraemia and meningitis and in pregnancy can cause preterm delivery, foetal loss, neonatal infection, or infant death. Between 1998 and 2008 in USA, at least 25% of reported outbreaks of listeriosis were of dairy origin [2]. *Listeria* can contaminate the dairy environment from manure or improperly fermented silage and can be introduced in the human food supply chain. The control of this pathogen in the food industry remains a challenge because of its ubiquitous character and its ability to grow at low temperatures and to survive and persist even in hostile environments. Soft cheeses can support the growth of *Listeria* introduced after processing independently of the use of raw or pasteurized milk. Recalls of dairy products, mainly soft cheeses, contaminated with the pathogen are relatively frequent. Because of its high case-fatality rate, listeriosis is, after salmonellosis, the second most frequent cause of food-borne infection-related deaths in Europe [3].

S. aureus is a causative agent of bovine mastitis capable of producing thermostable enterotoxins. Food-borne illness due to *S. aureus* can cause abdominal cramps, nausea, vomiting, and diarrhoea [4]. *S. aureus* is a common environmental microorganism which is found in raw milk [5]. Dairy products contain low levels of enterotoxigenic staphylococci.

However, temperature abuse above 10°C and poor starter culture activity during fermentation are factors involved in dairy related outbreaks of staphylococcal intoxication [6].

E. coli O157:H7 is a Shiga toxin-producing *Escherichia coli* (STEC) serotype of high virulence (it can cause disease at a dose of 5–50 cells). The number of cases of severe disease caused by STEC in dairy products has remained quite low, probably thanks to the compliance with good hygienic practices at the farm level [1]. The main reservoirs of STEC are ruminants, contaminating milk through subclinical mastitis or faecal routes, and the bacteria can persist in milking equipment. While severe cases of bloody diarrhoea or haemolytic uremic syndrome caused by STEC are uncommon, they do affect mostly vulnerable groups such as young children and elderly people.

Salmonella has long been recognized as an important human health problem of economic significance in animal and humans. *Salmonella* is found in the environment and in the gastrointestinal tract of farmed and wild animals. A total of 108,614 confirmed cases of salmonellosis were reported in the European Union in 2009, although cases attributed to *S.* enteritidis have decreased during the last years [7]. However, *Salmonella* infections have not declined over the past 15 years in USA [8]. Dairy products along with meat and eggs are the most common causes of food-borne infection by *Salmonella*. Salmonellosis from contaminated milk and dairy products has been associated with inadequate pasteurization and postprocess contamination. Most cheeses, including raw or pasteurized milk cheeses, properly manufactured and aged, appear to pose no significant health risk of *Salmonella* infection.

Several factors can increase the risk of food-borne infections and the severity of the diseases, resulting in a reduction in the number of pathogens needed to cause the disease and increased severity. The occurrence of infection in groups of people with a high risk was reviewed by Lund and O'Brien [9]. Susceptible population accounts for 15–20% of the general population in developed countries and includes mainly people with immunodeficiency, pregnant women, children, and the elderly. The risk of food-borne disease should be minimised for these vulnerable groups.

Many control measures in the food industry are provided to prevent or minimise bacterial contamination, including the appearance or growth of food-borne pathogens. Good manufacturing practices, sanitation, and hygiene measurements for raw material, the food industry environment, and so forth do not avoid the occurrence of food-borne outbreaks.

1.2. Applications of Bacteriocins and Bacteriocinogenic Strains in Dairy Products. The application of antimicrobial-producing lactic acid bacteria (LAB) or food-grade ferments in the manufacture of dairy products, which can be incorporated into fermented or nonfermented dairy products, implies a processing additional advantage to improve the safety and increase the quality of dairy products, providing an additional hurdle to reduce the likelihood of food-borne diseases (Table 1).

Bacteriocins are ribosomally synthesized bioactive peptides produced by bacteria displaying antimicrobial activity against related (narrow spectrum) or nonrelated (broad spectrum) bacteria. These peptides are considered natural biopreservatives and their potential application in the food industry has received great interest. On the basis of modifications of their precursor peptides, bacteriocins are classified into class I and class II [21]. Class I bacteriocins or lantibiotics undergo posttranslational modifications which introduce the thioether amino acids: lanthionine and methyllanthionine. Novel bacteriocins with translational modifications atypical of lantibiotics have been recently identified [22]. Class II contain unmodified peptides and are subdivided into four groups [23]: IIa (one-peptide pediocin-like bacteriocin), IIb (two-peptide bacteriocins), IIc (cyclic bacteriocins), and IId (linear non-pediocin-like one-peptide bacteriocins).

Bacteriocins are active against Gram-positive pathogens such as *L. monocytogenes* and *S. aureus* and may be effective against Gram-negatives if the outer membrane is destabilized [24]. Bacteriocins produced *in situ* through the incorporation of producing strains as starters or adjunct cultures in fermented dairy products can be applied to improve the safety of the product. The generally recognized as safe (GRAS) bacteriocin nisin produced by *Lactococcus lactis* was the first antibacterial peptide described in LAB. Nisin and pediocin PA1 are used in biopreservation, and preparations of these bacteriocins are applied commercially. The use of ferments or bacteriocin-producing starter cultures does not require regulatory approval or label declarations and is frequently considered a more attractive strategy to incorporate bacteriocins in foods [25, 26]. The efficacy of bacteriocins used in combination with other antimicrobial treatments or hurdles increasing the opportunity to target Gram-negative pathogens has been summarized [27], where synergistic antimicrobial effects have been demonstrated.

The earliest application of nisin in dairy products was the prevention of spoilage by clostridial species responsible for the late-blowing defect in cheese [28]. Nisin was bactericidal against different strains of *L. monocytogenes*, and its effect was enhanced by addition of NaCl or reduction of pH [29]. Other lantibiotics have been applied in the elimination of *L. monocytogenes* in dairy products. The broad spectrum lacticin 3147 powder produced by *Lc. lactis* DPC 3147 inactivated this pathogen in yogurt and in cottage cheese [10].

Due to its strong antilisterial activity and its stability and activity in a wide range of pH values, pediocin has been applied in dairy products. A dried preparation of pediocin decreased *L. monocytogenes* counts in cottage cheese, cream, and cheese sauce systems [11], although the pathogen restarted growth in the mildly acidic and neutral food systems. Regrowth of *L. monocytogenes* also occurred with piscicolin 126 a class IIa produced by *Carnobacterium piscicola* JG126 in Camembert cheese [12]. Cell-free preparations of enterocins as enterocin CRL 35 reduced *Listeria* up to 9 log units in goat cheese at the end of the ripening period [13].

As direct addition of bacteriocins to food systems could result in some loss of the antimicrobial activity due to the diffusion into the food matrix or the interaction with food components, different strategies of incorporation have been considered. Microencapsulation of bacteriocins in liposomes has been proposed as an alternative to the direct addition of

Table 1: Applications of bacteriocins and bacteriocinogenic strains in dairy products.

Bacteriocin	Bacteriocin-producing culture	Application	Pathogen	Product	Reference
Lacticin 3147	*Lc. lactis* DPC 3147	Spray-dried powder	*L. monocytogenes*	Cottage cheese	[10]
Pediocin	*P. acidilactici* PAC1.0	Dry powder	*L. monocytogenes*	Cottage cheese and yogurt	[11]
Piscicolin 126	*C. piscicola* JG 126	Concentrated supernatant	*L. monocytogenes*	Camembert cheese	[12]
Enterocin CRL35	*E. faecium* CRL 35	Concentrated supernatant	*L. monocytogenes*	Goat milk cheese	[13]
Nisin	*Lc. lactis* CNRZ 150	Starter culture	*L. monocytogenes*	Camembert cheese	[14]
Nisin	*Lc. lactis* TAB 50	Starter culture	*L. monocytogenes*	Semihard cheese	[15]
Lacticin 481	*Lc. lactis* TAB 24	Starter culture	*L. monocytogenes*	Semihard cheese	[15]
Lacticin 3147	*Lc. lactis* DPC 4275	Starter culture	*L. monocytogenes*	Cottage cheese	[16]
Enterocin AS-48	*E. faecalis* TAB 28	Starter culture	*L. monocytogenes*	Semihard cheese	[15]
Enterocin AS-48	*E. faecalis* INIA 4	Starter or adjunct culture	*L. monocytogenes*	Manchego cheese	[17]
Pediocin	*Lc. lactis* MM 217	Starter culture	*L. monocytogenes*	Cheddar cheese	[18]
Pediocin	*Lb. plantarum* WHE 92	Surface sprayed cell suspension	*L. monocytogenes*	Munster cheese	[19]
Pediocin	*Lc. lactis* CL1	Adjunct culture	*L. monocytogenes*	Semihard cheese	[20]
Pediocin	*Lc. lactis* CL1	Adjunct culture	*S. aureus*	Semihard cheese	[20]
Nisin	*Lc. lactis* ESI 515	Adjunct culture	*S. aureus*	Semihard cheese	[20]

free bacteriocin to milk to improve stability and distribution in cheese, while preventing the antimicrobial action on the cheese starter during manufacture [30]. Nisin was encapsulated in nanovesicles from soy lecithin and inactivated *L. monocytogenes* growth in milk at low temperatures over 14 d, being as effective as free nisin [31]. Bioactive packaging with bacteriocins incorporated in different films was applied in sliced Cheddar cheese. Nisin in cellulose-based bioactive inserts reduced levels of *Listeria innocua* and *S. aureus* by approximately 2 logs during storage in modified atmosphere packaging (MAP) at refrigeration temperatures [32]. Sorbitol-plasticized sodium caseinate films containing nisin also reduced *L. innocua* counts on surface inoculated cheese by approximately 1 log unit. Although nisin did not migrate much inside the cheese matrix, films were effective against surface contaminated cheese [33].

Bacteriocinogenic cultures as starter or adjunct cultures in cheese manufacture permit the bacteriocin production *in situ*, reduce the cost of the biopreservation, and do not require regulatory approval. Nisin-producing strains in combination with other nisin resistant or tolerant cultures with desirable properties have been proposed as an alternative to the addition of nisin in commercial form. Selected mixed starter cultures with a nisin Z-producing *Lc. lactis* subsp. *lactis* biovar diacetylactis strain and a commercial starter were successfully developed by Bouksaim et al. [34]. Nisin-producing suitable strains for cheese making have been isolated from natural environments as raw milk and raw milk cheese [35, 36]. Nisin-producing starter cultures inactivated *L. monocytogenes* in Camembert cheese, although regrowth of the pathogen occurred when pH increased in this cheese variety [14]. A decrease in *L. monocytogenes* counts was registered by Rodríguez et al. [15] when nisin-producing *Lc. lactis* subsp. *lactis* ESI 515 and TAB 50 were used as single-starter cultures in the manufacture of raw milk cheese.

Other bacteriocinogenic cultures have been assayed in cheese manufacture. In Cheddar cheese manufactured with lacticin 3147-producing cultures, the bacteriocin was stable over 6-month ripening [37]. Lacticin 3147-producing transconjugant *Lc. lactis* DPC 4275 strain used as starter culture in the manufacture of cottage cheese reduced numbers of *L. monocytogenes* to <10 cells/g within 5 d at 4°C [16]. Lacticin-481 producing *Lc. lactis* subsp. *cremoris* TAB 24 used as single-starter in cheese lead to counts of the pathogen 2.5 units lower than in cheese made with a commercial starter [15]. Nisin A, nisin Z, and lacticin-481 producing lactococci selected by their technological potential as starter cultures [38] were useful to control *L. monocytogenes* in cottage cheese, with a higher antilisterial activity with the nisin A producing strains.

Cell suspensions of pediocin-producing *Lactobacillus plantarum* WHE 92 sprayed on the surface of Munster cheese inhibited *L. monocytogenes* growth [19]. The production of pediocin in heterologous hosts is considered an alternative to extend the application of this bacteriocin in milk and dairy products. Pediocin-producing *Lc. lactis* MM 217 starter culture containing a plasmid coding the pediocin PA1 operon reduced *L. monocytogenes* levels in Cheddar cheese by 3 log units after 92 d of ripening [18]. Food-grade pediocin-producing lactococcal strains developed by Reviriego et al. [39, 40] and used as adjuncts to the starter culture reduced *L. innocua* counts in a cheese model system and *L. monocytogenes*, *S. aureus,* and *E. coli* O157:H7 in cheese [20]. Plantaricin 423-producing *Lb. plantarum* LMG P-26358 isolated from artisanal cheese and used as adjunct to a nisin-producing starter [41] was highly effective against *L. innocua* and compatible with nisin producers, showing interest in cheese technology.

Many enterococcal bacteriocins are class II pediocin-like bacteriocins with strong antilisterial activity. Their utilization in foods would require a case-by-case evaluation of safety of each potential strain [42]. Enterocin AS-48 has an important potential as biopreservative [43]. Enterocin AS-48-producing *Enterococcus faecalis* used as starter or coculture with a

commercial lactic starter in the manufacture of raw milk Manchego cheese decreased *L. monocytogenes* counts by 6 log units after 7 d [17] and completely inactivated the pathogen during the manufacture and ripening of raw milk cheese manufactured without starter culture [15].

Combinations of different preservation methods may act synergistically or provide higher protection than a single method alone. Bacteriocins have been combined with physical or biological treatments to allow the use of lower concentrations or reduce the severity of physical treatment, while achieving a higher lethality. Lacticin 3147 activity increased considerably after pressurization in skim milk or whey at 400–800 MPa [44], and the combination of this bacteriocin with 250 MPa acted synergistically lowering *S. aureus* counts in milk by more than 6 log units. Reductions of *S. aureus* in cheese by high pressure treatments combined with different bacteriocin-producing strains were synergistic [45]. This effect was also observed for *L. monocytogenes* [46] and *E. coli* O157:H7 [47]. Sublethal damage of the outer membrane of Gram-negatives or changes in membrane fluidity by pressurization could facilitate the access of bacteriocins to the cytoplasmic membrane.

Combinations of bacteriocins and reuterin, an antimicrobial compound produced by some strains of *Lb. reuteri*, exhibited a clear synergistic effect on the inhibition of *L. monocytogenes* and *S. aureus* in milk [48, 49]. Nisin did not inactivate five selected Gram-negative pathogens in milk [50], whereas reuterin reduced *E. coli* O157:H7, *S.* enteritidis, *Campylobacter jejuni*, *Aeromonas hydrophila*, and *Yersinia enterocolitica* counts. The combination of nisin and reuterin achieved reductions close to those obtained with only reuterin, without enhancing the antimicrobial effect of reuterin.

2. Bacteriocins in the Prevention and Reduction of Intestinal Pathogens

Gut microbiota play an essential role in digestion, metabolism, and immune function. Changes in the diversity and function of this ecosystem have been associated with a range of diseases including functional bowel disorders, inflammatory immune diseases, insulin resistance, and obesity and infectious diseases as the caused by *Clostridium difficile*. Dysbiosis as a result of antibiotics usage or the presence of different pathogenic organisms can be prevented or reduced by probiotics consumption.

Probiotics, or live microorganisms which when administered in adequate amounts, confer a health benefit on the host, can exert protective effect in the control of intestinal pathogens. Antimicrobial activity is considered a probiotic trait. Several proposals to explain this activity are the production of bacteriocins, competitive exclusion of the pathogen binding, competition for nutrients, or modulation of the immune system [51]. However, the mechanisms of action in the prevention of different gastrointestinal disorders are still poorly understood. Most probiotics applied in food products are lactic acid bacteria, mainly *Lactobacillus* and *Bifidobacterium*.

The role of bacteriocins within the gastrointestinal tract (GIT) on the prevalence of the producing strain and the microbial diversity and the survival of pathogens was reviewed by Dobson et al. [52]. Bacteriocins could contribute to probiotic functionality acting as colonizing peptides that facilitate the introduction or dominance of the bacteriocin-producing strain into the GIT niche. They may act as antimicrobial peptides directly killing other bacteria, as signalling peptides through quorum sensing and cross talk with bacterial communities or as signalling cells of the host immune system [52]. Bacteriocins can inhibit the invasion of competing or pathogen strains in the community or modulate the composition of the microbiota and the host immune system [53]. A review of recent *in vivo* studies on bacteriocin-based treatments of human and animal infections and the potential of bacteriocins in health was published by Hammami et al. [54].

2.1. Purified Bacteriocins in the GIT. Purified bacteriocins can be used in the treatment of pathogenic bacteria and may be employed as alternative to existing antibiotics, limited by the emergence of resistant pathogens and the damage of the human commensal microbiota. The spread of antibiotic resistance particularly in the hospital environments is a significant problem of healthcare and resistant pathogens to multiple antibiotics are a major challenge as antibiotics used to treat some pathogens are no longer effective. This consideration was reviewed by Cotter et al. [55].

Antimicrobial activity of nisin and lacticin 3147 *in vivo* has been recently demonstrated in a murine infection model. Lacticin 3147 was subcutaneously administered to mice infected intraperitoneally (IP) with a luminescent *S. aureus* to analyze *in vivo* imaging. After 6 h of infection, photoluminescence and microbial analyses of liver, kidneys, and spleen revealed that the bacteriocin controlled the systemic spread of *S. aureus* in mice by preventing the dissemination of the pathogen [56]. Similar experiments were carried out by Campion et al. [57] with nisin A and its bioengineered variant with increased bioactivity nisin V [58] against bioluminescent *L. monocytogenes* EGDe in mice infected IP. Antimicrobial effect of nisin V was higher than the one observed with nisin A to control the infection with *L. monocytogenes* in mice, pointing to the interest in this peptide for therapeutic applications.

C. difficile can take profit from the antibiotic broad spectrum associated disruption of the microbiota and grow and produce toxins in the gut. Lacticin 3147 has the potential to be employed in the treatment of *C. difficile* diarrhoea and to eliminate the pathogen when added to an anaerobic fecal fermentation, although levels of the bacteriocin required were much higher than the antibiotic needed [59]. In the same way, other members of the GIT microbiota were affected by this application [59, 60].

The presence of nisin in duodenum, ileum, and faeces of rats treated with pure nisin was reported by Bernbom et al. [61], although nisin inactivation was registered when the concentrations estimated by ELISA were compared with a biological assay. These authors investigated the ability of pure nisin, a nisin-producing *Lc. lactis* CHCC 5826 and the isogenic non-nisin-producing *Lc. lactis* CHCC 2862 to modify

the composition of the intestinal microbiota of human microbiota-associated rats. Both microbial cultures affected the composition of the intestinal microbiota increasing bifidobacteria levels and decreasing *Enterococcus/Streptococcus* populations in faeces, but the effect was not observed when purified nisin was administered.

Pediocin PA-1 producing strain *P. acidilactici* UL5 [62], able to inhibit *L. monocytogenes in vitro,* did not reduce the pathogen in the intestine of mice when administered intragastrically at high levels and was not detected in faeces. However, repeated doses of the purified pediocin PA-1 provided up to 2-log reductions in fecal listerial counts compared to the infected control group and slowed pathogen translocation into the liver and spleen, leading to the disappearance of *L. monocytogenes* infection in these two organs within six days. Pediocin PA-1 did not affect the composition of the mouse intestinal flora [62].

Bacteriocin-producing *Lb. salivarius* NRRL B-30514 or *Paenibacillus polymyxa* NRRL B-30509 inhibited *Campylobacter jejuni in vitro*, but the strains did not affect the pathogen in chickens. When the purified bacteriocin was encapsulated and administered to chickens colonized with the pathogen [63], *C. jejuni* was reduced by at least 6 log units. According to these authors, the bacteriocin was produced *in situ* in limiting quantities to kill *C. jejuni* when the strains were administered.

2.2. Bacteriocin-Producing Probiotics. The production of bacteriocins *in situ* by probiotics selected by their ability to survive in the GIT may be advantageous as proteolysis during gastric transit would be avoided. Although the protective effect of probiotics through bacteriocin production *in situ* has been studied, the determination of the fate of these peptides *in vivo* and the bacteriocin detection in complex environments present important limitations. Whereas studies detected the lack of efficacy *in vivo* of some bacteriocins, others provide evidence that bacteriocins can be produced and retain bioactivity in the GIT.

Although the lantibiotic lacticin 3147 was highly effective to inhibit pathogens, the producing lactococci were not able to confer protection against *L. monocytogenes* in a mouse model [64]. The bacteriocin-producing *Lc. lactis* DPC 6520 was able to survive the GIT passage in simulated conditions and *in vivo* survived the intestinal transit in mice and pigs, although the excretion rate was low (10^2–10^5 cfu/g) and the bacteriocin was not detected in faeces. When this strain was investigated against *C. difficile* in a simulated human distal colon using a bacteriocin negative variant as control, no reduction in the pathogen counts was registered. Previous data showed that lacticin 3147 delivered orally was rapidly degraded in the GIT [65].

Administration of human intestinal isolates pediocin PA1-producing *P. acidilactici* MM33 and nisin Z-producing *Lc. lactis* MM 19 increased total LAB and anaerobes in mice, and *P. acidilactici* also decreased Enterobacteriaceae levels. Both strains were resistant to acid and bile and reduced vancomycin resistant *Enterococcus* (VRE) intestinal colonization when administered orally with the two bacteriocin-producing cultures or the *P. acidilactici* M33A, a mutant without the capacity to produce bacteriocin. The eradication of VRE was attributed to pediocin activity as the pediocin negative derivative did not exhibit this antimicrobial effect against VRE [66].

Protective activity *in vivo* was not detected when pediocin AcH-producing *Lb. plantarum* DDEN 11007 or its non-producing isogenic variant was studied [67] in gnotobiotic rats colonized with *L. monocytogenes*. Higher levels of the pathogen were detected in liver and spleen of animals colonized with the bacteriocin or the non-bacteriocin-producing strains. According to these authors, inoculating germ-free rats with the probiotic will induce immune responses facilitating *L. monocytogenes* to cross the epithelial barrier.

The antimicrobial activity of bacteriocin-producing probiotics in the GIT was observed with class II bacteriocin abp-118-producing *Lb. salivarius* UCC118 [68]. The administration of 10^9 cfu/d during 3 days before infection reduced *L. monocytogenes* levels in mice compared with a variant bacteriocin-negative. The impact of this strain on the intestinal microbiota of mice and pigs was investigated by Riboulet-Bisson et al. [69]. *Lb. salivarius* UCC118 or a mutant lacking bacteriocin production survived throughout the pig GIT and colonized the ileum. The bacteriocin-producing strain led to a significant decrease in Spirochaetes levels and affected Firmicutes genus members. This last effect was not observed when the mutant strain was administered and was thus associated with bacteriocin production. *Lb. salivarius* UCC118 administration has a significant but subtle impact on mouse and pig microbiota by a mechanism that seems, at least partially, bacteriocin-dependent.

At the GIT level, a probiotic mixture of *Lactobacillus* and *Pediococcus* of porcine intestinal origin alleviates *Salmonella* infection in a porcine model [70]. Salivaricin P-producing *Lb. salivarius* DPC6005, the only bacteriocin-producing strain in the mixture of probiotics administered to pigs, dominated over the rest of strains in the ileum digesta and mucosa. It was suggested that the predominance of this strain could be related to a competitive advantage attributed to bacteriocin production [71]. The increased efficacy of multistrain probiotics against pathogens may be caused by the greater variety of antimicrobial capacities associated with mixed preparations, such as production of weak organic acids, bacteriocins, hydrogen peroxide, coaggregation molecules and/or biosurfactants, and the stimulation of sIgA production and mucus secretion by the host [72]. According to Chapman et al. [73], multistrain probiotics show higher efficacy than single strains, although the studies published do not demonstrate whether synergistic interactions or higher probiotic doses are responsible for this effect.

Although production of bacteriocins by intestinal bacteria has been recognized, its prominent role within gut ecology has not been elucidated. In part, this could be due to the high metabolic costs expended by bacteria to elaborate and secrete these nonstructural polypeptides. It is likely that bacteriocins play additional roles in regulating the intestinal flora, such as signaling within and among microbial species.

Bacteriocins might act as quorum-sensing molecules or autoinducing peptides in the intestinal environment. Nisin acts as a secreted signal molecule that induces the

transcription of the genes involved in its biosynthesis [74]. Cocultivation of *Lb. plantarum* DC400 with *Lb. sanfranciscensis* DPPMA174 leads to the induction of the synthesis of plantaricin A. As a response, *Lb. sanfranciscensis* increased the expression of proteins involved in stress response, amino acid metabolism, energy metabolism, membrane transport, nucleotide metabolism, and regulation of transcription [75]. Cultivation of *Lb. plantarum* DC400 with plantaricin A or with other lactobacilli increased the capacity to adhere to Caco-2 cells and to prevent the adhesion of potential intestinal pathogens. The adhesion or competition of *Lb. plantarum* DC400 was also mediated by the peptide plantaricin A and by cocultivation with other species in the ecosystem [76].

The specific probiotic cell products involved in immunomodulation are not well known. van Hemert et al. [77] studied a number of genes of *Lb. plantarum* that might influence the immune response of peripheral blood mononuclear cells, detecting specific genes encoding components of the plantaricin biosynthesis and transport pathway that might be responsible for the stimulation of anti- or proinflammatory immune responses in the gut. In fact, deletion of these genes from *Lb. plantarum* WCFS1 resulted in changes in IL-10 and IL-12 cytokine profiles compared with the wild type.

The identification of bacteriocin-producing potentially probiotic bacteria from the intestinal microbiota has been summarized by O'Shea et al. [22]. Considering the high proportion of intestinal bacteria that are nonculturable and the biased results of cultured-based screening procedures, emerging high throughput sequencing technologies and functional metagenomics-based approaches will be crucial to the identification of genes potentially encoding novel bacteriocins [22].

The effects of multistrain probiotics keep unclear. Although the number of studies is limited, multiple-strain cultures appear to exhibit greater efficacy than single strains, even when the strains are integrating the mixture. The development of multistrain probiotic dairy products with good technological properties, able to act as protective cultures in foods and as probiotics exerting a protective action against infections, has gained increased interest.

3. Future Trends

Bacteriocin effectiveness as biopreservatives in food may be hindered by the proteolytic activity of food or microbial enzymes, their adsorption to fat, and the appearance of resistant variants in sensitive strains. Food legislation for their approval and acceptance as food preservatives has also restricted their use, as only nisin and pediocin PA-1 are commercially available. In cheese manufacture, the activity of combined starters including both technological strains and bacteriocin-producing cultures is rather difficult to control for correct acidification, bacteriocin production, and quality of cheese. Compatible combinations of lactic starters and bacteriocin-producing strains may help to solve the problem. More research is needed for the optimization of bacteriocin production and activity in dairy products.

The simultaneous application of more than one bacteriocin or multiple bacteriocin producers may reduce the emergence of resistances in target strains. Bacterial cultures exhibiting overexpression of bacteriocins or multiple heterologous bacteriocin producers have received particular interest by researchers, although their industrial use would be limited by the restrictive legal regulations and the lack of acceptance by consumers. Combined treatments of bacteriocins with physical processes or other biopreservatives offer a wide scenario of practical future applications.

In vitro and animal studies have confirmed that the production of bacteriocins contributes to probiotic functionality in the GIT. The ability of a bacteriocin to function *in vivo* is influenced by the strain survival, the specific activity of the bacteriocin, the dosing regimen, the animal model, and the target organism. The factors controlling bacteriocin production in the GIT are not well understood and bacteriocin production in the GIT is difficult to assess. For that reason, standardized methods of assessing bacteriocin activity would be useful since variations in animal models, dosage, and quantification have made the comparison of data between laboratories difficult. This information will lead to human trials in which health properties will be accurately assessed.

The emergence of resistant pathogens is another area that deserves investigation. The application of bacteriocins in human health will depend on the knowledge of the mechanisms of action. The development of strategies for bacteriocin production at sufficient quantity and the performance of clinical trials to determine the efficacy of bacteriocins *in vivo* are areas that also would need to be addressed.

Multistrain probiotics appear to show higher efficacy than the single strains. Dairy products would be an effective vehicle for multistrain probiotic cultures, with good technological properties and improved characteristics to those shown by the individual strains, able to act not only as protective cultures in foods, but also as probiotic.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Review Article

Impact on Human Health of Microorganisms Present in Fermented Dairy Products: An Overview

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Fermented dairy products provide nutrients in our diet, some of which are produced by the action of microorganisms during fermentation. These products can be populated by a diverse microbiota that impacts the organoleptic and physicochemical characteristics foods as well as human health. Acidification is carried out by starter lactic acid bacteria (LAB) whereas other LAB, moulds, and yeasts become dominant during ripening and contribute to the development of aroma and texture in dairy products. Probiotics are generally part of the nonstarter microbiota, and their use has been extended in recent years. Fermented dairy products can contain beneficial compounds, which are produced by the metabolic activity of their microbiota (vitamins, conjugated linoleic acid, bioactive peptides, and gamma-aminobutyric acid, among others). Some microorganisms can also release toxic compounds, the most notorious being biogenic amines and aflatoxins. Though generally considered safe, fermented dairy products can be contaminated by pathogens. If proliferation occurs during manufacture or storage, they can cause sporadic cases or outbreaks of disease. This paper provides an overview on the current state of different aspects of the research on microorganisms present in dairy products in the light of their positive or negative impact on human health.

1. The Microbial World Diversity in Fermented Dairy Products

Fermented dairy products are an important part of our diet and can contain a diverse microbiota. Lactic acid bacteria (LAB) are the main players during milk fermentation, converting lactose to lactic acid, which results in an increased acidity that makes growth conditions of microorganisms other than LAB increasingly unfavourable. The LAB involved in fermented dairy processing belong to diverse microbial groups that are characterized by different nutritional, metabolic, and culture requirements as well as different technological properties. The most common LAB present in milk includes species belonging to the genera *Lactobacillus*, *Streptococcus*, *Leuconostoc*, *Enterococcus,* and *Lactococcus* [1].

Lactococcus lactis ssp. *lactis* and *Lactococcus lactis* ssp. *cremoris*, in particular, are primarily known because of their role as starter cultures for the cheese industry. The genus *Lactobacillus* currently consists of 174 different species. Lactobacilli play two main roles in fermented dairy products, as starters or as secondary microbiota. *Lactobacillus delbrueckii* ssp. *bulgaricus* and *Lactobacillus delbrueckii* ssp. *lactis* are used worldwide as starters in yoghurt production. In contrast, other lactobacilli initially present in raw milk increase in number during the manufacture of dairy products and can become particularly dominant during cheese ripening [2].

These populations, which are often referred to as nonstarter LAB (or NSLAB), are able to carry out proteolysis and lipolysis, subsequently producing many end products that contribute to the development of flavour and texture of cheeses [3]. The species more frequently involved include *Lactobacillus helveticus*, *Lactobacillus casei*, *Lactobacillus paracasei, Lactobacillus plantarum/paraplantarum, Lactobacillus rhamnosus, Lactobacillus curvatus, Lactobacillus brevis, Lactobacillus sake, Lactobacillus pentosus, Lactobacillus acidophilus, Lactobacillus reuteri, Lactobacillus johnsonii, Lactobacillus crispatus, Lactobacillus fermentum, Lactobacillus buchneri,* and *Lactobacillus gasseri*. While the analysis of the presence and levels of these species in food products can be underestimated with the use of culture-dependent methods, the development of culture-independent techniques for the study of microbial communities, such as PCR-DGGE, PCR-TTGE, qPCR, 16S rRNA gene sequencing, and metagenomic approaches, is contributing to a deeper knowledge of the fermented dairy products microbiota. Although many streptococcal genera are pathogenic, *Streptococcus thermophilus* carries a "GRAS" status [4]. *S. thermophilus* is a thermophilic LAB widely used as starter culture in the manufacture of dairy products, notably in the yoghurt production, and is considered as the second most important industrial dairy starter after *La. lactis*. Enterococci are the most controversial group of food-associated LAB and they could act either as starter cultures, probiotics, spoilage, or pathogenic organisms depending on the strain considered [5]. *Leuconostoc*, in particular the species *Leuconostoc mesenteroides* and *Leuconostoc pseudomesenteroides*, have the ability to produce CO_2 which is responsible for the eye formation in some types of cheeses [6]. Other microbial groups comprising Gram-positive and Gram-negative bacteria, as well as yeasts and moulds, also contribute to the organoleptic and physicochemical properties of dairy products. In this regard, Gram-positive bacteria like *Corynebacterium* spp., *Arthrobacter* spp., and *Brevibacterium* are essential in smear-ripened cheeses. *Propionibacterium freudenreichii* subsp. *shermanii* carries out the propionic fermentation through the conversion of lactic acid formed by acidifying bacteria to acetate, propionate, and CO_2, the latter being responsible of the eye formation in Swiss-type and other cheeses.

Bifidobacteria represent an important group of nonstarter microorganisms that are included in some dairy products, mainly fermented milks, because of the health-promoting properties attributed to some of them. Although they usually have a considerably slower growth-rate than starter cultures, their proliferation will contribute to increase levels of lactate and acetate in final products.

Regarding undesirable microorganisms in dairy products, special attention should be focused on the spore-former bacteria which are important contaminants in the dairy industry. Thus, microorganisms belonging to the genus *Clostridium*, such as *Clostridium tyrobutyricum* or *Clostridium butyricum*, are considered the main organisms responsible for the late-blowing of cheese [7]. Pathogenic clostridia will be commented on below. The presence of contaminating Gram-negative bacteria, mainly enterobacteria, is rather common in dairy foods, sometimes reaching levels up to 10^6-10^7 CFU g^{-1} in cheeses and they can contribute to a worsening of sensory quality of dairy products [8].

Yeast and moulds are important microbial populations in dairy products, especially in some types of cheeses. As with bacteria, the development of culture independent DNA-based analytical methods has allowed detection of genera and species not previously found in dairy environments, such as *Torrubiella* and *Malassezia* [9]. In cheese, yeasts and moulds play a key role in the development and enhancement of texture and flavour through the activity of some microbial extracellular enzymes in the food matrix. The yeast species most frequently found in dairy products include *Kluyveromyces lactis*, *Debaryomyces hansenii, Candida* spp., *Geotrichum candidum,* and *Yarrowia lipolytica*. Among moulds *Penicillium*, *Geotrichum*, *Aspergillus*, *Mucor,* and *Fusarium* are the most common genera [10].

2. Beneficial and Toxic Compounds Released by LAB, Yeasts, and Moulds during Fermentation

Some health-promoting properties of fermented dairy products are due to the synthesis or to the release from the food matrix of bioactive compounds as a result of the metabolic activity of LAB, propionibacteria, yeast, and moulds. Worth mentioning are among others, conjugated linoleic acid (CLA), exopolysaccharides (EPS), bioactive peptides, vitamins, gamma-aminobutyric acid (GABA), and oligosaccharides [11].

Although milk contains vitamins, fermentation by LAB often leads to the enrichment of some of them, as it is the case for vitamin B_{12}, folic acid, and biotin produced by propionibacteria [12] or the higher synthesis of folate in milk fermented with some LAB with respect to nonmilk complex culture media [13]. CLA is a native component of milk fat. Its content can be increased in fermented milk through bioconversion of unsaturated fatty acids such as linoleic and linolenic acids by different LAB [14, 15]. The functionality of CLA has been well documented with respect to its anti-inflammatory [16], antiatherogenic, and antioxidant properties [17].

Bioactive peptides are specific fragments of milk proteins that are released by proteolytic activity from caseins predominantly and also from whey proteins. Antihypertensive, antimicrobial, antioxidative, and immune-modulatory activities have been described for peptides released as a result of the activity of LAB in fermented milk products [18]. In general, their bioactive characteristics are based on the specific amino acid sequence and chain length (generally from two to twenty residues) as well as on their resistance to hydrolysis. The most studied mechanism of bioactive peptides is the antihypertensive action displayed by the inhibition of the angiotensin-I-converting enzyme (ACE; peptidyldipeptide hydrolase, EC 3.4.15.1) which regulates blood pressure [19]. Some *Lactobacillus*-fermented milks and cheeses with added probiotic lactobacilli revealed ACE-inhibitory activity [20, 21]. GABA is another compound with blood pressure repressing properties; it has been demonstrated to be produced in

Table 1: Some examples of outbreaks caused by fermented dairy products and the pathogen involved.

Pathogen	Fermented dairy products	Outbreak details	Reference
Brucella	Pecorino cheese	7 cases. Made from raw milk and insufficiently aged.	[105]
Clostridium botulinum	Yoghurt	27 cases, 1 death. Insufficient processing of hazelnut conserve used as a flavour.	[106]
Listeria monocytogenes	Hard cheese	12 cases, 4 deaths. Postmanufacture contamination.	[107]
Salmonella	Hard cheese	Estimated 3000 cases. Cheese made from raw milk.	[108]
Staphylococcus aureus	Sheep milk cheese	25–27 cases. Raw milk used in production.	[109]
STECO157:H7	Gouda cheese	41 cases. Raw milk used to make cheese and numerous production/handling problems including insufficient ageing.	[110]
	Yoghurt	16 cases, 13 hospital admissions, 5 haemolytic uraemic syndrome. Possible improperly cleaned pump.	[111]

There are numerous other reports in the literature but many of them do not provide details on the dairy product involved. The table above includes data only for dairy products made with a starter culture. A more comprehensive list of outbreaks involving any kind of cheese is given elsewhere [112].

fermented milk by *Lb. casei* Shirota and *La. lactis* through transformation of glutamic acid derived from milk proteins [22]. Bacteriocins are also among the beneficial peptides intrinsically synthesized by some LAB during milk fermentation and their usefulness in preventing growth of undesirable and pathogenic microorganisms during milk fermentation has been commented on above.

Galactooligosaccharide (GOS) synthesis by LAB is due to a transgalactosylation side-line activity by β-galactosidase on lactose, the main sugar of milk. GOS have recognized prebiotic effect on intestinal microbiota, promoting selective growth of bifidobacteria [23, 24]. EPS are complex extracellular carbohydrate polymers produced by some microorganisms. They can protect the producer strains against environmental adverse factors and some of them positively interact with the colonic microbiota and with the immune system of the host [25, 26].

Special mention is deserved of bioactive peptide components of proteins secreted by LAB and probiotic bacteria. This is the case of the enriched serine/threonine peptide derived from one of the main extracellular proteins produced by *Lb. plantarum*, which displayed immunomodulatory properties after being released during digestion [27].

Although the metabolic activity of microorganisms during dairy fermentation yields mostly beneficial compounds, in some cases metabolic activities result in the release of toxic substances for the consumer. Two types of toxic compounds have been identified in dairy products, mycotoxins produced by some fungi, and biogenic amines (BA) mainly due to the metabolic activity of some LAB.

Mycotoxins are chemical hazards synthesized primarily by three genera of filamentous fungi: *Aspergillus*, *Fusarium,* and *Penicillium* [28]. They are termed secondary metabolites, because they are not essential for normal growth and development. Although fungi can collectively produce hundreds of mycotoxins, only trichothecenes, fumonisins, and zearalenone (produced by *Fusarium* species) and aflatoxins, ochratoxins, and patulin (produced by *Aspergillus* and *Penicillium* species) are of note from a health point of view [28]. These secondary metabolites are products of multistep biochemical pathways. The genes encoding the synthase, the modifying enzymes, the transporters, and the transcriptional regulators are typically located next to one another in a gene cluster [29]. In milk and dairy products mycotoxins mainly come from feed contaminated either in the field or during drying and storage. One of the most economically important mycotoxins worldwide is aflatoxin. This polyketide produced mainly by *Aspergillus flavus* and *Aspergillus parasiticus* is a potent carcinogen [28]. Aflatoxin M1, that results from the metabolic conversion of aflatoxin B1, can occur in milk and milk products from animals consuming feedstuffs contaminated with B1 aflatoxins. Aflatoxin-producing *Aspergillus* can contaminate grain before harvest or during storage; favourable conditions of temperature, humidity, and mechanical kernel damage during harvesting, among other factors, may favour the active production of aflatoxin B1 in contaminated grains. Aflatoxin B1 is transformed to aflatoxin M1 in the liver of lactating animals and is excreted by the mammary gland. The potential occurrence of mycotoxins in dairy products and mainly in milk makes them a particular risk for humans because of their negative effects for adults and, especially, children [30].

The other toxic compounds mainly associated to the metabolism of some bacteria are BA. These are low-molecular weight nitrogenous organic bases with biological activity, mainly synthesized by decarboxylation of the corresponding amino acids. The most important and frequent BA found in dairy products are histamine, tyramine, and putrescine, which are produced by decarboxylation of histidine, tyrosine, and ornithine, respectively. Putrescine can also be synthesized by deimination of agmatine. Cadaverine (originating from lysine decarboxylation) is found less frequently in dairy products [31]. BA are naturally present in vegetables, animals, and humans, being involved in important biological processes. Many bacteria of different genera and species have the capacity to produce BA. Gram-negative bacteria (mainly Enterobacteriaceae) that can be present in milk are able to produce histamine, putrescine, and cadaverine [32–34]. However, the main BA producers in dairy products are mostly LAB of the genera *Enterococcus*, *Lactobacillus*, *Leuconostoc*, *Lactococcus,* and *Streptococcus* [35–39]. These bacteria can be (i) present in milk, (ii) introduced by contamination

Table 2: Beneficial and detrimental microbial compounds that can be released in fermented dairy products during fermentation and the main producer microorganisms.

Compounds	Main producer microorganisms in dairy products	Reference
	Beneficial	[11, 12, 15, 77]
Conjugated linoleic acid (CLA)	BAL (*Lactobacillus, Lactococcus,* and *Bifidobacterium*)	
Microbial exopolysaccharides (EPS)	BAL (*Lactobacillus, Lactococcus, Pediococcus*, *Streptococcus thermophilus,* and *Bifidobacterium*)	
Oligosaccharides	BAL (*Bifidobacterium and Lactobacillus*) and *Kluyveromyces lactis*	
Vitamins (B_{12}, biotin, and folic acid)	BAL (*Lb. plantarum, Bifidobacterium, S. thermophilus, Lb. delbrueckii, and Propionibacterium*)	
Gamma-aminobutyric acid (GABA)	BAL (*Lactococcus, Enterococcus, Lactobacillus, Pediococcus, Streptococcus*, and *Leuconostoc*)	
Bioactive peptides:		
Immune modulatory	*Lactobacillus* GG	
Antihypertensive	*Lactobacillus* GG, *Lb. helveticus, and S. thermophilus,*	
Antimicrobial	*Lb. helveticus and Lb. acidophilus*	
Antioxidative	*Bifidobacterium longum and Lb. delbrueckii*	
Bacteriocins	BAL (*Lactococcus, Enterococcus, Lactobacillus, Pediococcus, Streptococcus, Bifidobacterium*, and *Leuconostoc*)	[101]
	Detrimental	
Mycotoxins:		[28]
Aflatoxins, ochratoxin, and patulin	*Aspergillus* and *Penicillium*	
Trichothecenes, fumonisins, and zearalenone	*Fusarium*	
Biogenic Amines:		[31, 38]
Tyramine	BAL (*Enterococcus, Lb. curvatus, and Lb. brevis)*	
Putrescine	BAL (*Enterococcus, Lb. curvatus, Lb. brevis*, and *La. lactis*) and Enterobacteriaceae	
Cadaverine	Enterobacteriaceae	
Histamine	BAL (*Lb. buchner and, S. thermophilus*)	

throughout the entire process of cheese production, (iii) and even part of starter or adjunct cultures [40]. Among the fermented dairy products, cheeses can have the highest BA concentrations, because of their complex microbiota and the availability of precursor amino acids from casein proteolysis. In fact, BA concentrations up to 2,000 mg per kg of cheese have been reported [41, 42]. The intake of such contaminated foods could cause serious health problems. For this reason, during the recent past the metabolic pathways involved in the synthesis of these compounds and the environmental conditions favouring their accumulation in foods have been studied in depth [40], in parallel with the development of reliable detection methods either for BA or for the microbial BA producers [43, 44].

A general picture of beneficial and detrimental compounds produced by microorganisms present in dairy products is indicated in Table 2.

3. Probiotics and Mechanisms of Beneficial Action

Probiotics are live microorganisms which confer a health benefit on the host when administered in adequate amounts [45]. The most commonly investigated and commercially available probiotics are mainly microorganisms from species of the genera *Lactobacillus* and *Bifidobacterium*. In addition, several others such as *Propionibacterium*, *Streptococcus*, *Bacillus*, *Enterococcus*, *Escherichia coli*, and yeasts are also used [46, 47]. Probiotics must be able to survive in the gastrointestinal tract and be resistant to gastric juices and bile. They should exert benefits to the host through their activity in the human body. In order to confer health benefits, they should be nonpathogenic and nontoxic and provide protection against pathogenic microorganisms by means of multiple mechanisms [45]. In addition, probiotics should be lacking transferable antibiotic resistance genes. Different bacterial strains of the same genus and species may exert different effects on the host. The most promising health effects of probiotics in human intervention studies include amelioration of acute diarrhoea in children, reduction of the risk of respiratory tract infections, relief of children's milk allergy/atopic dermatitis, and alleviation of irritable bowel syndrome. Probiotics may exert their beneficial health effects by normalization of the host's microbiota, by inhibition of pathogens, by interaction with the immune system of the host, and through their own metabolic activity. Probiotics

may also enhance the resilience of microbiota against detrimental outside factors. However, the molecular mechanisms behind the effects are largely unknown.

3.1. Inhibition of Pathogens. Clinical and animal studies have demonstrated that specific probiotics are effective in alleviating infections, but the mechanisms of action are not completely understood. Additionally, beneficial properties and efficacy can vary considerably among different strains from the same species. Possible mechanisms of probiotic action include (1) hindering the adsorption, (2) cell internalization of the pathogen, (3) production of metabolites and substances with a direct effect on the pathogen, and (4) crosstalk (immunomodulation) with the cells in establishing the protection [47, 48]. The possible mechanisms by which probiotics may act against infections are presented in Table 3.

The gastrointestinal and respiratory tracts are covered by mucosal epithelial surfaces which are constantly exposed to numerous microorganisms and serve as primary ports of entry for most infectious viruses. Pathogen attachment to a host cell is the first step in the disease process, and, therefore, interruption of this attachment could be beneficial to the host. Probiotic bacteria may bind directly to the pathogen and inhibit pathogen attachment to the host cell receptor. For instance, there is evidence that, *in vitro*, specific strains of lactobacilli and bifidobacteria are able to bind and inactivate rotavirus [49] and vesicular stomatitis virus [50]. In addition, adhesion of probiotics on the epithelial surface [51–53] may block pathogen attachment by steric hindrance, cover receptor sites in a nonspecific manner, or inhibit binding of pathogens to specific carbohydrate receptors. Luminal secretions (mucus, glycolipids, and protective peptides) and antimicrobial peptides (defensins) may also protect epithelial cells from infections. Intestinal mucins may bind to pathogens through specific mucin-bacterial/viral interaction and inhibit their adherence to the epithelial cells [54]. Probiotics may induce mucosal regeneration by increasing mitosis rate in the small intestine and increasing the numbers of cells in the villi [55, 56]. They can also promote intestinal epithelial homeostasis via soluble proteins [57]. Probiotics also show direct activity against pathogens by producing antimicrobial substances such as organic acids, hydrogen peroxide, diacetyl, short chain fatty acids, biosurfactants, and bacteriocins. It is widely known that intestinal permeability increases in gastrointestinal infections, as pathogens attach to cell receptors below the tight junctions on the basolateral membrane, thus modifying tight junctions and disturbing the barrier. A possible mechanism of probiotics beneficial action is the reinforcement of gut defence barrier by normalizing permeability and disturbed gut microbial ecology [47, 58, 59].

3.2. Interaction with the Immune System. An optimally functioning immune system is important for the maintenance of physiological integrity and health. The immune system provides defence against infections caused by pathogenic microorganisms. It also modulates our health and well-being in many ways sometimes by up- or downregulating the defence system. An effectively functioning immune system is fundamental for protection against infectious diseases. One possible probiotic mechanism against infections could be the stimulation of the gut immune system. In the gut epithelial cells, probiotics can be recognized by toll-like receptors [60–63]. Probiotics may, therefore, modulate cytokine expression patterns through epithelial cells [64] and/or through macrophages and dendritic cells [65–70]. Many experimental studies *in vitro* show that certain strains of probiotics are capable of providing protection against infections by stimulating antiviral, cytokine, and chemokine responses in gastrointestinal and respiratory epithelial cells or immune cells. Administration of lactobacilli to mice may affect respiratory infections by reducing virus titre in the lungs and increasing survival rate of the animals via stimulating innate immune responses [47, 71].

4. Strategies to Improve Viability and Functionality of Probiotics in Fermented Dairy Products and the Gastrointestinal Tract

Probiotics are generally added as adjunct cultures in fermented dairy products. Their viability in foods should ensure the minimum daily dose able to provide the health benefits attributed to the specific functional food product in which they are included. However, probiotics often show poor survival in the food matrix, due to factors such as low pH, oxygen content, temperature, and the presence of other microorganisms. In addition, probiotics should remain viable at sufficient levels through the gastrointestinal transit in order to arrive alive to the site of action, the intestine. During digestion, they have to face different harsh physiological barriers, including digestive enzymes, the acidic pH of the stomach, and bile salts in the intestine and then compete with members of the resident intestinal microbiota for scarce fermentable substrates. In addition, not only the viability but also the maintenance of the metabolic activity and the beneficial properties of strains are important [46].

Some strategies targeting the food product and/or the composition of starter cultures have been used to improve viability of microorganisms in fermented dairy products. The selection and combination of appropriate LAB strains [72, 73], the control of the final pH and postacidification phenomenon by different approaches [74, 75], or the addition of protectors and oxygen scavengers [6, 76] are some examples.

Other strategies affecting the microorganism itself are useful to increase survival in the food matrix and during the gastrointestinal transit. For example, the selection of EPS-producing probiotics could be an appropriate way to obtain strains with adequate viability, since these polymers can act as protectors of the producing bacteria, contributing to their viability [77, 78]. Resistant derivatives to technological or physiological conditions are easy to obtain by exposing the probiotic to sublethal stressing factors (freezing, heat, drying, oxygen, acid, bile, NaCl, etc.). Usually, the resistant microorganisms present a stable phenotype with higher viability, but they often develop cross-resistances to other stresses [79]. Adaptation to stress may also influence physiological

Table 3: Summary of the possible mechanisms by which probiotics exert healthy effects.

Mechanisms
(1) By inhibiting the adhesion of pathogens to the epithelium in a nonspecific manner or by competing for specific receptors and nutrients
(2) By producing antimicrobial agents against pathogens
(3) By inducing mucin production in the epithelial cells
(4) By strengthening the mucosal barrier through the regeneration of epithelial cells and reduction of permeability
(5) By modulating the immune system through the antigen-presenting cells
(6) By inducing cytokine production from the epithelial and immune cells, resulting in enhanced cell-mediated immune responses and the activation of cytotoxic T cells, phagocytic cells, and NK cells
(7) By increasing the proliferation of B cells through the induction of cytokines, which travel to secondary lymphatic organs in mucosa-associated lymphoid tissue and differentiate into immunoglobulin-producing plasma cells that may return to gut-associated lymphoid tissue by inducing the production of specific antibodies such as secretory IgA

characteristics of microorganisms that could hence impact technological and sensory aspects as well as probiotic-related properties [80–82]. Gene modification is another way to increase stress tolerance. However, the use of such genetically modified microorganisms is limited by current regulation in several countries [83, 84].

Addition of some food ingredients to food could enhance survival of probiotics, as is the case of prebiotics. These can be defined as "a selectively fermented ingredient that results in specific changes in the composition and/or activity of the gastrointestinal microbiota, thus conferring benefits upon host health" [85]. Most prebiotics are complex carbohydrates from plant origin. Probiotics have been employed in combination with prebiotics (synbiotics) to improve their viability; prebiotics often act as entrapping matrices during the gastrointestinal transit, further releasing the microorganism in the intestine and then serving as fermentable substrates [86]. Microencapsulation of probiotics on different materials has been also used to enhance the viability [87].

5. Opportunistic and Pathogenic Microorganisms and Mechanisms of Detrimental Action in the Host

Gram-positive bacteria associated with food poisoning comprise mainly nonsporulating microorganisms from the genera *Staphylococcus* and *Listeria,* as well as sporulating *Clostridium tertium*, *Clostridium perfringens, Clostridium botulinum,* and members of the *Bacillus cereus* group [88]. Some Gram-negative bacteria contaminating dairy foods are considered as indicators of poor hygiene and may constitute a health risk if pathogenic species are present. These include the species *E. coli*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, and *Citrobacter freundii* and the genera *Enterobacter*, *Proteus*, *Psychrobacter*, *Halomonas,* and *Serratia* [89]. Specific pathogens are mainly particular enterotoxigenic *E. coli* pathotypes and *Salmonella*.

The proportion of foodborne disease outbreaks and sporadic cases that can be attributed to the consumption of dairy products was approximately 4–7% of outbreaks in the USA from 1998 to 2008 [90] and in 2009-2010 this figure was 13% [91]. Only a proportion of these would be attributable to fermented dairy products. In the EU, cheese was identified as the vehicle of transmission in 41 of 763 (5.4%) outbreaks and other dairy products (excluding milk) and in only 4 (0.5%) during 2012 [92]. Inspection of the data shows that many of these outbreaks are, in fact, associated with coagulated dairy products that have not been fermented but produced by direct acidification. The behaviour of pathogens is different in cheeses produced with or without a starter culture [93]. Of six dairy-associated outbreaks of listeriosis recorded in the USA from 1998 to 2008, four were caused by Mexican-style queso fresco/queso blanco which are soft cheeses produced without starter cultures [94].

Raw (unpasteurized) milk can contain a variety of bacterial pathogens which may cause disease if not eliminated during production [95]. Disease can be caused by two major mechanisms: infection by the organism or ingestion of preformed toxin. *Listeria monocytogenes* [96], *E. coli* O157:H7, and other shiga toxigenic *E. coli* (STEC) [97] can cause significant clinical outcomes. Around 20–30% of listeriosis cases are fatal while STEC infections can cause kidney failure and, more rarely, death, especially in young people. Salmonellosis is usually a diarrhoeal disease, while brucellosis is a systemic infection causing symptoms such as fever, fatigue, and myalgia. In contrast, both *Staphylococcus aureus* and *C. botulinum* can grow in foods to form toxins [98, 99]. Staphylococcal enterotoxin can result in emesis when ingested, while botulinum toxin can result in paralysis and death in an estimated 17.3% of domestically acquired foodborne cases of botulism in United States [100].

In cheesemaking using raw milk, initial production steps can involve periods where the milk is held at >30°C, temperatures which may allow contaminating bacteria to proliferate. However, in general, subsequent steps result in inactivation of bacterial pathogens. The use of a starter culture is critical because of the resulting low pH concomitant with the production of lactic acid [101]. During fermentation, milk and curd may rapidly reach a pH at which pathogens will not grow and subsequently their levels will decline as long as the pH remains low. The potential for pathogens to survive manufacture and ripening to contaminate the retail product made from raw milk depends mainly on (1) the initial levels of the pathogen, (2) growth and entrapment in the curd during manufacture, (3) the rate of microbial population decrease during ripening, (4) antagonistic activity of LAB present in

milk or added as starters, (5) physicochemical parameters, such as pH, salt content, and water activity, and (6) the length of ripening.

In cheeses which are mould-ripened or bacteria smear-ripened (e.g., smear cheeses), the fungi or bacteria used to achieve the particular characteristics of the product cause a rise in the pH during ripening and so potentially allowing surviving pathogens to grow. The fate of various pathogens in cheese production has been reviewed [102].

Pasteurisation is the common method to eliminate pathogens from milk prior to the manufacture of dairy products, and so when contamination occurs it is a result of poor hygiene practices postpasteurisation or pasteurisation failure. While there has been much public debate about the relative merits of consuming dairy products made with raw milk versus pasteurised milk, when consumption volumes are considered, raw milk products cause a disproportionately large proportion of cases of foodborne disease compared to those made with pasteurised milk [91, 103, 104].

As a whole, despite the overall excellent safety record of fermented dairy products, outbreaks and incidents of disease still can result from their consumption [105–112]. Table 1 gives some examples of outbreaks, the pathogens that caused them, and the reasons why they occurred.

6. Strategies for Counteracting Pathogens and Harmful Microorganisms in Fermented Dairy Products

The most common approach to guarantee the safety of fermented dairy products is to ensure that the milk used in their manufacture is pathogen-free (or contains an acceptably low level of some pathogens like *S. aureus*) followed by the prevention of recontamination during production, distribution, and retail sale. With current technology, pathogen-free raw milk is difficult to produce, but using food quality milk from a source that submits animals to a strict pathogen testing regime and has good hygiene practices in place may help to meet this goal.

Pasteurisation (usually, the exposure to 72°C for 15 seconds, or 63°C for 30 min) is considered to be sufficient to remove bacterial pathogens from milk intended for the use in fermented dairy products. An alternative to pasteurisation of milk, which is implemented in several countries, is to age cheeses made from raw milk for 60 days as a minimum. However, this has been shown to be ineffective under some circumstances such as when pathogenic strains are resistant to low pH or in postprocessing contamination of surface-mold-ripened cheeses in which a rise of pH occurs during maturation [113, 114].

Alternatives to pasteurisation have been sought in order to produce safe milk for processing yet not producing the perceived organoleptic changes resulting from pasteurisation. Some examples of this are ultrahigh pressure treatment [115], pulsed electric field (PEF) technology, and ultrasonication [116]. High hydrostatic pressure has been applied to both milk used to make cheese [117] and cheese itself [118] where significant reductions in *S. aureus* were recorded. PEF is not particularly effective with bacterial spores but kills vegetative cells, typically by 4-5 $\log_{10}$ CFU/mL, through the production of pores in bacterial membranes. There may also be an improved curd quality in cheese made using PEF milk. Ultrasonication works primarily by cavitation which causes shear stress and physical damage to cells, but the effects are only significant at temperatures above 50°C. It can be used in combination with other physicochemical treatments [115].

There are also a number of nonphysicochemical measures which could broadly be termed biocontrol, including the use of bacteriophages, bacteriocins/protective cultures, and naturally-occurring chemicals, such as essential oils. Bacteriophages (phages) are bacterial viruses. They have been shown to control *Salmonella* in cheddar cheese production [119], *S. aureus* in fresh and hard cheese production [120], and *E. coli* O157 in fermented milk production [121]. After 90 days of storage levels of *Salmonella* were consistently 2-3 $\log_{10}$ CFU g^{-1} higher in untreated cheeses compared to those in phage-treated cheeses. Control of *L. monocytogenes* by phages has been similarly reported for smear-ripened soft cheeses [122]. The cheese was ripened at 14°C for 16 days, packaged, and then stored for five more days at 6°C. The levels of *L. monocytogenes* reached 10^5 CFU cm^{-2} in untreated cheeses at 16 days and $>10^7$ CFU cm^{-2} by day 21. Application of the phage preparation eliminated *L. monocytogenes* and no further growth occurred during storage. Similar results have been reported elsewhere [123]. Starter and nonstarter LAB can act as a protective culture [124], inhibiting the growth of pathogens through competition (pH reduction, production of hydrogen peroxide, etc.) and/or by the production of bacteriocins [101]. Bacteriocins are a heterogeneous group of antimicrobial peptides that inhibit the growth of other bacteria. These compounds generally display action on a narrow range of organisms. Whereas some of them only act against other LAB, others are also able to inhibit the growth of some foodborne pathogenic bacteria [125], serving as natural biopreserving agents in fermented dairy products. Nisin, a commercially available bacteriocin, has found use in the prevention of the outgrowth of spores, particularly those of *Clostridium* species [101, 125], allowing flexibility in the formulation of dairy products such as processed cheese. NSLAB producing bacteriocins can be used singly and in combination with high pressure to kill pathogens in cheese [126].

A novel idea is to use plant-derived essential oils to control pathogens. For example, oregano and thyme essential oils have been shown to increase the rate of inactivation of *L. monocytogenes* and *E. coli* O157:H7 in Feta cheese [127], the cheeses being accepted by taste panellists.

7. Concluding Remarks and Future Trends

Although the manufacture of fermented dairy products by humanity began in prehistory, we continue innovating production even today. Figure 1 presents a schematic overview of the main areas of scientific and technological interest in relation with microorganisms present in fermented dairy products and human health.

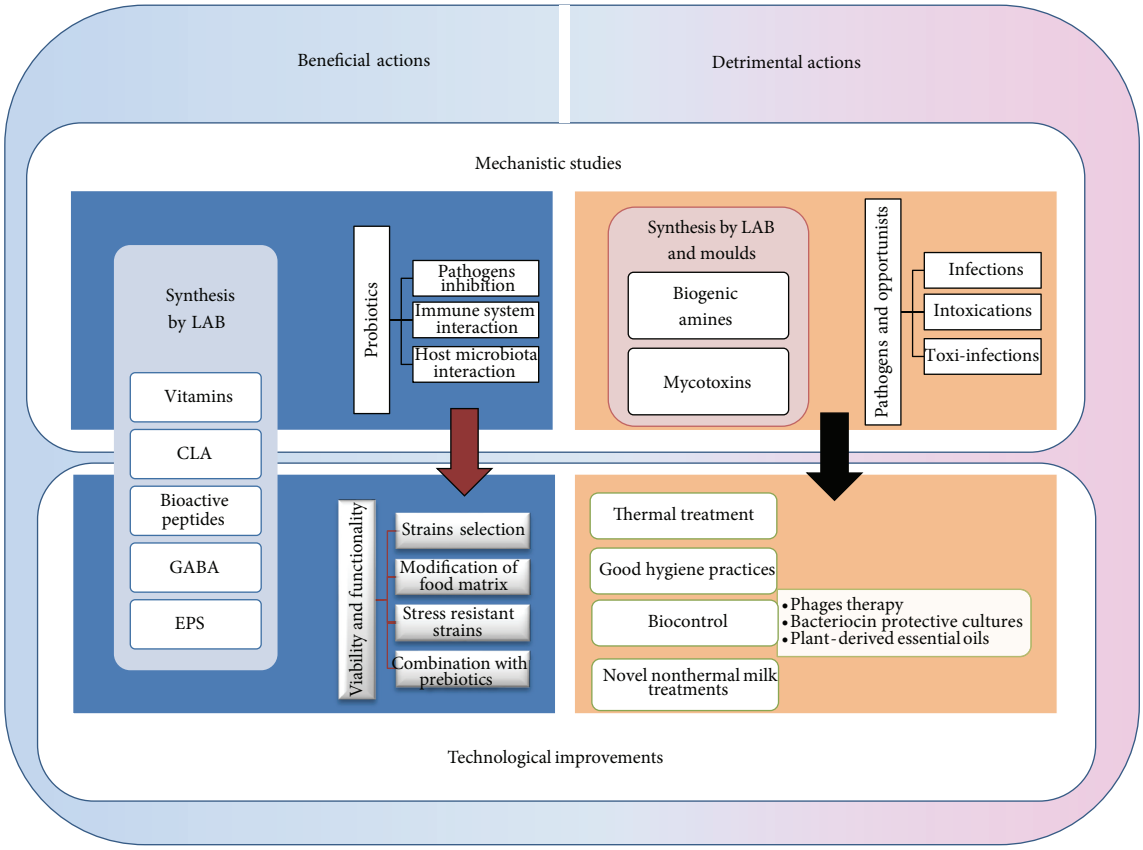

Figure 1: Overview of the main scientific and technological areas of interest relating microorganisms present in fermented dairy products and human health. LAB: lactic acid bacteria; CLA: conjugated linoleic acid; GABA: gamma-aminobutyric acid; EPS: exopolysaccharides.

The extraordinary recent development of next generation sequencing (NGS), functional genomics (with their related dynamic techniques such as metabolomics, proteomics, and transcriptomics), and systems biology will facilitate in the coming years a better understanding of microbial population dynamics occurring in fermented dairy products, as well as a more accurate prediction of the biochemical processes occurring in fermented milk products as depending on the microbiota which is present. Cell biology techniques are necessary tools for deciphering the interaction mechanisms between pathogens and probiotics with the host, with respect to their detrimental or beneficial action. In the case of probiotics, this knowledge will help in the selection of the best strains targeting specific human populations with defined needs. While mechanistic research advances, it is necessary to continue and improve surveillance programs of diseases caused by fermented dairy products; vigilance must remain in maintaining the hygienic conditions of dairy processing. Finally, research in new technologies providing safe alternatives to milk thermal processes, such as pasteurisation, may allow the development of safer products with organoleptic properties more to the liking of some consumers. In spite of the scientific advances, our knowledge on the effects of fermented dairy products and the accompanying microorganisms on human health remains incompletely understood.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Research Article

Bioaccessible Antioxidants in Milk Fermented by *Bifidobacterium longum* subsp. *longum* Strains

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Bifidobacterium longum subsp. *longum* is among the dominant species of the human gastrointestinal microbiota and could thus have potential as probiotics. New targets such as antioxidant properties have interest for beneficial effects on health. The objective of this study was to evaluate the bioaccessibility of antioxidants in milk fermented by selected *B. longum* subsp. *longum* strains during *in vitro* dynamic digestion. The antioxidant capacity of cell extracts from 38 strains, of which 32 belong to *B. longum* subsp. *longum*, was evaluated with the ORAC (oxygen radical absorbance capacity) method. On the basis of screening and gene sequence typing by multilocus locus sequence analysis (MLSA), five strains were chosen for fermenting reconstituted skim milk. Antioxidant capacity varied among the strains tested ($P = 0.0009$). Two strains of *B. longum* subsp. *longum* (CUETM 172 and 171) showed significantly higher ORAC values than the other bifidobacteria strains. However, there does not appear to be a relationship between gene sequence types and antioxidant capacity. The milk fermented by each of the five strains selected (CUETM 268, 172, 245, 247, or PRO 16-10) did not have higher initial ORAC values compared to the nonfermented milk samples. However, higher bioaccessibility of antioxidants in fermented milk (175–358%) was observed during digestion.

1. Introduction

Probiotic microorganisms, by definition, have proven their beneficial functionality for human health [1–3]. Within the large collection of microorganisms used in probiotic dairy products, bifidobacteria are interesting members, as they are natural inhabitants of the human gastrointestinal tract (GIT) and their presence has been associated with healthy colon microbiota [4, 5]. Although the diversity of colon microbiota changes dramatically throughout life [6], *Bifidobacterium longum* is an important inhabitant of both the infant and adult colon [7, 8], with *B. longum* subsp. *longum* representing the most common subspecies [7, 9].

Dairy products are widely used as a delivery mode for probiotics into the colon. However, to provide health benefits, the probiotics present in dairy products need to survive the harsh conditions of the GIT and arrive in the colon in sufficient quantities [10]. Bacteria passing the GIT are subjected to several stress conditions, such as stomach acidity and high concentrations of bile salts in the duodenum [11, 12]. As for most colon bacteria, *B. longum* is a strict anaerobe [13], so the presence of oxygen in the GIT (highest concentration at the beginning of the GIT) is an important additional stress factor with which this species has to cope. Oxygen, due to incomplete reduction, produces reactive oxygen species (ROS) that damage cellular macromolecules, for example, by breaking peptide bonds and inducing oxidation of membrane lipids [14]. Bacteria are known to have distinct mechanisms to protect themselves against oxygen. For instance, as for lactic acid bacteria [15–17], *B. longum* produces antioxidant molecules in order to scavenge free oxygen radicals [18]. However, not much information is available in the literature about this antioxidant capacity and its relation with the oxidative stress response in *B. longum*.

Several genes present in bifidobacteria encode proteins related to the oxidative stress response. Alkyl hydroperoxide reductase C (AhpC) is a NADH-oxidase homolog that reduces oxygen to hydrogen peroxide [13, 19, 20]. Complete

genome sequencing of *B. longum* NCC2705 has revealed the presence of a gene (*trx*) encoding a thioredoxin reductase-like protein that is believed to cooperate with AhpC to eliminate hydrogen peroxide [4]. Other enzymes include ribonucleotide reductase alpha subunit (NrdA) and NTP pyrophosphohydrolase (MutT1) that are involved in DNA damage protection and repair after oxidative stress [19]. Moreover, polyphosphate granules (poly P) are formed in response to oxidative stress. The putative polyphosphate kinase gene (*ppk*) present in bifidobacteria is thought to be responsible for this poly P synthesis [21].

Oxidative stress also affects human health. Several diseases and disorders, such as inflammatory bowel disease [22, 23] and cardiovascular diseases [24], have been related to the presence of ROS. Improving the blood antioxidant status has been proposed as a way to reduce the occurrence of these diseases. Studies have demonstrated that a change in diet increases the antioxidant capacity of blood [25, 26]. For this, antioxidants present in the food matrix first need to be absorbed in the GIT and then utilized by human metabolism, which represents antioxidant bioavailability. Bioavailability is related to bioaccessibility which represents the ingested antioxidants that are available for absorption in the gut after digestion [27]. Several models have been used to study the bioaccessibility of antioxidants. One of these is the TNO *in vitro* model for digestion (TIM-1), which is a dynamic model for the upper GIT (stomach to ileum) [28–30]. Furthermore, this model can be used to evaluate survival of probiotics in the GIT [11, 31–33].

Within the *B. longum* species, several metabolic characteristics (such as the ability to degrade prebiotics [34]) display strain-dependent differences [35, 36], so antioxidant capacity should also be expected to differ among strains. The goals of this study were first to evaluate the antioxidant capacity of 32 *B. longum* subsp. *longum* strains in order to link this capacity with the diversity of genes related to oxidative stress responses. Secondly, the bioaccessibility of antioxidants in milk fermented with five selected strains of *B. longum* subsp. *longum* showing a range of antioxidant capacities of milk was assessed using the TIM-1 model.

2. Material and Methods

2.1. Screening of *B. longum* subsp. *longum* Strains

2.1.1. Bacterial Strains, Growth Conditions, and Viable Counts. The 32 strains of *B. longum* subsp. *longum* are listed in Table 1. For the ORAC assay, other bacterial strains than *B. longum* subsp. *longum* were used for comparison purposes, namely, *B. adolescentis* ATCC 15703, *B. breve* ATCC 15698, *B. catenulatum* CUETM 174, *B. longum* subsp. *suis* ATCC 27533, *B. longum* subsp. *infantis* ATCC 15702, and *B. animalis* subsp. *lactis* BB-12. The stock cultures were kept at −80°C in MRS broth supplemented with 20% (v/v) glycerol (EMD Chemicals, Fisher Scientific, Ottawa, ON, Canada). For each experiment, the strains were subcultured in MRS broth (Sigma-Aldrich, Oakville, ON, Canada) supplemented with 0.05% cysteine (Sigma-Aldrich) and 0.1% Tween 80 (Sigma-Aldrich) by adding 2% of the frozen stock. After 24 h of incubation at 37°C in a glove box anaerobic chamber (Plas-Labs Inc., Lansing, MI, USA), 1% of the first subculture was added to fresh medium and incubated for another 24 h at 37°C.

Table 1: Origin of *Bifidobacterium longum* subsp. *longum* strains.

Strain	Origin	Reference or source
ATCC 15707	Adult intestine	American Type Culture Collection, Manassas, VA
ATCC 15708	Child feces	American Type Culture Collection, Manassas, VA
ATCC 51870	Child feces	American Type Culture Collection, Manassas, VA
DSM 20097	Calf feces	Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany)
NCC 2705	Infant feces	Nestlé, Lausanne, Switzerland
CUETM 171	Child feces	Bahaka et al. [63]
CUETM 172	NA[1]	Bahaka et al. [63]
CUETM 177	Child feces	Bahaka et al. [63]
CUETM 186	Child feces	Bahaka et al. [63]
CUETM 193	Child feces	Bahaka et al. [63]
CUETM 239	Child feces	Bahaka et al. [63]
CUETM 245	Child feces	Bahaka et al. [63]
CUETM 247	Child feces	Bahaka et al. [63]
CUETM 259	Child feces	Bahaka et al. [63]
CUETM 260	Child feces	Bahaka et al. [63]
CUETM 263	Child feces	Bahaka et al. [63]
CUETM 268	Child feces	Bahaka et al. [63]
CUETM 281	Child feces	Bahaka et al. [63]
CUETM 287	Child feces	Bahaka et al. [63]
CUETM 290	Child feces	Bahaka et al. [63]
PRO 16-10	Adult feces	Savard et al. [64]
PRO 42-1	Adult feces	Savard et al. [64]
PRO 42-10	Adult feces	Savard et al. [64]
PRO 42-2	Adult feces	Savard et al. [64]
PRO 42-8	Adult feces	Savard et al. [64]
RW 001	Commercial preparation	Roy et al. [65]
RW 008	Commercial preparation	Roy et al. [65]
RW 009	Commercial preparation	Roy et al. [65]
RW 019	Commercial preparation	Roy et al. [65]
RW 020	Commercial preparation	Roy et al. [65]
RW 023	Commercial preparation	Roy et al. [65]
RW 024	Commercial preparation	Roy et al. [65]

[1]Not available.

After two subcultures, 1 mL of culture was centrifuged at 12,000 ×g for 10 min at 4°C. The pellet for DNA extraction was kept at −80°C. Also with the second subculture, 1% was added to 20 mL of MRS broth and incubated for 24 h. To determine viable counts, expressed as colony forming units (CFU), 0.1 mL of the appropriate dilution was added to molten MRS agar (MRS-based broth supplemented with 0.05% cysteine, 0.1% Tween 80, and 2% dextrose) by pour plating and incubated for 48 h at 37°C in a glove box anaerobic chamber containing an atmosphere of 80% N_2, 10% H_2, and 10% CO_2 (Praxair, Quebec, QC, Canada). Dilutions for viable counts were performed with peptone water (1% of Bacto Peptone (BD Biosciences, Mississauga, ON, Canada) and 0.05% cysteine) with pH adjusted to 6.8.

2.1.2. Oxygen Radical Absorbance Capacity Assay. The ORAC assay was performed on cell-free extracts in triplicate for each strain. Optical density at 600 nm of each culture was measured against MRS broth as blank with a VIS spectrophotometer Genesys 20 (Thermo Scientific, Waltham, MA, USA). Viable counts were carried out as described above. First, the 20 mL 24 h culture was centrifuged at 12,000 ×g for 10 min at 4°C. Then, the pellet was washed three times with 20 mL phosphate buffer (75 mM) and finally suspended in 20 mL of the same buffer. After incubating for 30 min at 37°C, cells were mechanically lysed with a XL-2020 sonicator (Misonix Inc. Farmingdale, NY, USA) at 50 watts, five times for 1 min with a cooling step on ice for 5 min between each sonication step. Next, to obtain the cell-free extract, lysed cells were centrifuged at 12,000 ×g for 10 min at 4°C. The supernatant (cell-free extract) was finally diluted in a 1 : 1 ratio with phosphate buffer. The ORAC assay was performed based on the technique described by Dávalos et al. [37], Saide and Gilliland [15], and Bazinet et al. [38]. The diluted cell-free extracts were analyzed in triplicate in a 96-well plate in the Fluostar Galaxy (BMG Labtechnologies, Durham, NC, USA). To each well, 200 μL of fluorescein (Sigma-Aldrich) solution (0.036 mg/L), 20 μL of diluted sample, and 75 μL of 2,2′-azobis-2-aminopropane dihydrochloride (AAPH) (Sigma-Aldrich) solution (8.6 mg/L) were added. The ORAC assay quantifies the inhibition (expressed in percentage and time) of fluorescence produced by peroxyl radicals generated at a constant rate by thermal decomposition of AAPH. The antioxidant capacity is expressed in μM Trolox Equivalent (TE) calculated from the Trolox (Sigma-Aldrich) standard curve.

2.1.3. Multilocus Sequence Analysis. DNA extraction was performed with the DNeasy Blood & Tissue Kit: gram positive bacteria DNA extraction protocol (Qiagen, Mississauga, ON, Canada) with some modifications. To the lysis buffer 10 μL/mL of 5 U/mL mutanolysin (Sigma-Aldrich) was added. Primers (see Supplementary Table S1 in the Supplementary Material available online at http://dx.doi.org/10.1155/2014/169381) were designed using Geneious Pro R6 software (Biomatters, San Francisco, CA, USA) based on the *B. longum* sequences available for each gene locus obtained from GenBank through the Geneious Pro R6 software. The PCR amplification volume of 50 μL contained 1 μL of DNA, 1 μL of dNTP mix (10 mM), 2 μL of each primer (10 mM), 5 μL of 10X Taq buffer, 0.25 μL of Taq DNA polymerase (Feldan, Quebec, QC, Canada), and 38.75 μL of nuclease-free water. PCR amplification of the five genes for each strain was performed with a Tgradient (Biometra, Montreal Biotech, Montreal, QC, Canada) using the following program: one cycle at 94°C for 5 min, 30 cycles with denaturation at 94°C for 30 s, primer annealing at 58°C for 30 s, and DNA extension at 72°C for 30 s, and a final extension step at 72°C for 5 min. Next, DNA sequence analysis was carried out on both strands of the purified PCR products with the BigDye Terminator v3.1 cycle sequencing kit and 3100 Genetic Analyzer (Life Technologies, Burlington, ON, Canada).

The sequences of the forward and reverse strands were aligned using Geneious R6 software. The allele number for each distinct sequence variant was determined with nonredundant databases (NRDB) program (http://pubmlst.org/analysis/). Then a sequence type (ST) number was given to each distinct combination of alleles for the five genes with START2 software [39]. Finally, for each strain, individual gene sequences were concatenated and phylogenetic trees were built using Jukes-Cantor neighbor-joining with bootstrapping as statistical method.

2.2. Dynamic In Vitro Gastrointestinal Digestion of Fermented Milk

2.2.1. Bacterial Strains and Growth Conditions. Five strains of *B. longum* subsp. *longum* (CUETM 172, CUETM 245, CUETM 247, CUETM 268, and PRO 16-10) were tested for their capacity to ferment reconstituted skim milk. The strains were subcultured in MRS-based broth (MRS without glucose; Rosell Institute, Montreal, QC, Canada) supplemented with 0.05% cysteine, 0.1% Tween 80, and 0.5% dextrose (EMD Chemicals) by adding 2% of the frozen stock culture. After 24 h of incubation at 37°C in a glove box anaerobic chamber, 1% of the first subculture was added to the MRS supplemented with 0.5% lactose (EMD Chemicals) instead of dextrose and incubated for 24 h at 37°C. After two subcultures as for the growth curves, 1% was added to 350 mL of reconstituted milk and incubated for 18 h at 37°C in a glove box anaerobic chamber. The milk was composed of 12% low heat skim milk powder (Agropur, Granby, QC, Canada), 0.6% yeast extract (BD Biosciences), and 2% dextrose. Yeast extract and dextrose were added to ensure optimal growth of the strains in milk.

2.2.2. Dynamic In Vitro Digestion. The intake (300 g of fermented milk) was added to the TIM-1 (TNO Nutrition and Food Research Institute, Zeist, The Netherlands) and digested for 5 h at 37°C. TIM-1 run was performed as described by Fernandez et al., [31] which was based on Minekus et al. [40]. The fermented milk passed through four compartments connected in series to simulate the stomach, duodenum, jejunum, and ileum, separated by valve segments that were computer controlled. Description of gastric and ileal deliveries, initial contents, secretions, and dialysis fluid are provided in the Supplementary Material (Table S2). Before adding the fermented milk, initial contents and secretions were

deaerated by bubbling nitrogen gas for 90 s. Throughout the digestion experiment, jejunal and ileal compartments and effluent were maintained under anaerobic conditions with nitrogen gas flow (Praxair). The container for ileal effluent was maintained on ice to prevent the multiplication of cells. Dialysis of the contents of jejunal and ileal compartments was performed with Purema polyethersulfone membrane (hollow fibres) Xenium 110 Dialyzer (Baxter, Deerfield, IL, USA).

2.2.3. Survival Evaluation and ORAC Analysis. Bacterial growth was measured by viable counts as described above and by propidium monoazide treatment in combination with quantitative PCR with (PMA-qPCR). Samples were taken from fermented milk at the start and from the TIM-1 at the following points: 30 and 60 min from the gastric compartment, at 60, 120, 180, and 240 min from the duodenal compartment, at 300 min from the combined jejunal and ileal compartments, and at 60, 120, 180, 240, and 300 min from the ileal effluent. PMA treatment was carried out as follows. One mL of sample was mixed with 42.4 μL of 50% (w/v) sterile trisodium citrate solution (BDH Chemicals, Toronto, ON, Canada) and centrifuged 12,000 ×g for 10 min at 4°C. Cell pellets were suspended in 500 μL of 2X TE (20 mM Tris HCL pH 8.0, and 2 mM EDTA). PMA (Biotium, Hayward, CA, USA) was added to the samples at a final concentration of 50 μM and the samples shaken in the dark for 5 min were placed in the PMA lamp apparatus (LED-Active Blue, Ingenia Biosystems, Barcelona, Spain) for 15 min. Finally, the PMA-treated cell suspensions were centrifuged 12,000 ×g for 10 min at 4°C and the cell pellets were stored at −80°C until DNA extraction.

DNA extraction was performed based on the protocol of Licitra et al. [41]. Briefly, the DNeasy Blood & Tissue Kit: gram positive bacteria DNA extraction protocol was used with some modifications. The cell pellets were suspended in 400 μL (for milk and stomach samples) or 180 μL (for other samples) of enzymatic lysis buffer (20 mM Tris HCl pH 8.0, 2 mM EDTA, 1.2% Triton X-100, 20 mg/mL lysozyme (Sigma-Aldrich), and 10 μL/mL of 5 U/mL mutanolysin (Sigma-Aldrich)) and incubated at 37°C for 1 h. Next, 25 μL of proteinase K and 200 μL of AL buffer were added and incubated at 70°C for 30 min. The suspensions were transferred to 2 mL microtubes containing 0.3 g of 1 mm diameter zirconium beads (Biospec Products, Bartlesville, OK, USA) and shaken twice for 90 s in a Mini-BeadBeater-16 (Biospec Products). Then, samples were centrifuged at 10,000 ×g for 10 min. Finally, 200 μL of ice-cold absolute ethanol was added and DNA purification was performed according to the Qiagen protocol. The samples were stored at −20°C until qPCR amplification.

DNA quantification was performed with Applied Biosystems 7500 Fast Real-Time PCR System with software version 2.0.1 (Life Technologies). Primers tuf_F (5′-ACCTGGCCACGCTCGACATC-3′) and tuf_R (5′-AGACCATGGACGCCTGCGAG-3′) were used for the amplification of a 85-bp region of the *B. longum* elongation factor Tu gene (*tuf*). The PCR amplification volume of 25 μL contained 10 μL of Fast SYBR Green Master Mix (Life Technologies), 5 μL of DNA, 1 μL of each 2.5 μM primer, and 8 μL of nuclease-free water. Duplicate qPCR amplifications were carried out consisting of a 20 s denaturation step at 95°C, followed by 40 cycles of 3 s at 95°C and 30 s at 60°C. Finally, viable cells/mL were obtained from the C_t values using the corresponding standard curve. The standard curve and detection limit were determined using a pure culture of *B. longum* CUETM 172. One mL of culture was serially diluted eight times in sterile reconstituted milk. Next, 1 mL of each dilution was treated with PMA as described before. DNA extraction and quantification were performed as for the TIM-1 samples. After qPCR amplification, C_t results were plotted against the corresponding viable count (CFU/mL).

The ORAC analysis was also performed as described before on fermented milk samples after dilution in a 1 : 500 ratio with phosphate buffer and on dialysate samples of the *in vitro* digestion experiments after dilution in a 1 : 50 ratio with phosphate buffer.

2.3. Statistical Analysis. All statistical analyses were performed using JMP version 9 Software (SAS Institute, Cary, NC, USA). ORAC values of the different bifidobacteria strains were compared with analysis of covariance (ANCOVA) with optical density at 600 nm as covariate. The means separation was done using the pairwise comparisons of least squares means using Student's *t*-tests (LSMeans Student's *t*). ORAC values of the nonfermented milk and the milk fermented by the five *B. longum* subsp. *longum* were compared with analysis of variance (ANOVA).

3. Results

3.1. Antioxidant Capacity of Cell-Free Extracts. ORAC results were weighted with the optical density at 600 nm as covariate, as there was a linear relationship between the ORAC values and this parameter ($F = 38.2226$; $P < 0.0001$) (Table 2). The ORAC values ranged between 76.5 ± 38.2 and 274.3 ± 38.4 μmol TE/L and differed among species and strains ($F = 2.2141$; $P = 0.0009$). The pairwise comparisons divided the 38 strains into three groups. Three strains exhibited ORAC values higher than 250 μmol TE/L, of which two strains CUETM 172 and CUETM 171 belong to *B. longum* subsp. *longum*. The last strain, CUETM 174, belongs to *B. catenulatum*. *B. longum* subsp. *infantis* ATCC 15702, *B. animalis* subsp. *lactis* BB-12, and *B. adolescentis* ATCC 15703 possessed the lowest antioxidant capacity (lower than 100 μmol TE/L).

3.2. Genetic Analysis of Oxidative Stress Response Genes. MLSA based on five genes (*mutT1, ahpC, trx, nrdA,* and *ppk*), which are predicted to be involved in the oxidative stress response of bifidobacteria, was performed to evaluate the genetic diversity of the 32 tested *B. longum* subsp. *longum* strains. The allele numbers and ST numbers were determined for all strains (see Supplementary Material, Table S3). For the 32 strains, there are 22 different STs based on the concatenated sequences of the five sequenced loci, a total of 2,079 bp. Despite the high percent of identity (96.2%) of the concatenated sequences of the 32 strains, polymorphic nucleotides

Table 2: Antioxidant capacity of cell-free extracts evaluated by the oxygen radical absorbance capacity (ORAC) assay.

Genus and species	Strain	ORAC (μM TE[1]) ± SE[2]
B[3]. *adolescentis*	ATCC 15703	76.5 ± 38.2^{M}
B[3]. *animalis* subsp. *lactis*	BB-12	79.8 ± 38.4^{LM}
B. longum subsp. *infantis*	ATCC 15702	85.9 ± 38.9^{KLM}
B. longum subsp. *longum*	RW 024	101.0 ± 39.7^{JKLM}
B. longum subsp. *longum*	PRO 42-2	109.5 ± 39.1^{IJKLM}
B. longum subsp. *longum*	CUETM 290	115.0 ± 38.2^{HIIJKLM}
B. longum subsp. *longum*	RW 008	117.0 ± 33.1^{IIJKLM}
B. longum subsp. *longum*	RW 009	118.7 ± 39.2^{HIIJKLM}
B. longum subsp. *longum*	CUETM 239	122.7 ± 33.6^{HIIJKLM}
B. longum subsp. *longum*	PRO 42-8	125.0 ± 38.2^{GHIIJKLM}
B. longum subsp. *longum*	RW 020	127.5 ± 39.1^{FGHIIJKLM}
B. longum subsp. *longum*	RW 023	129.2 ± 40.1^{EFGHIIJKLM}
B. longum subsp. *longum*	CUETM 186	132.4 ± 38.6^{DEFGHIIJKLM}
B. longum subsp. *longum*	PRO 42-1	135.6 ± 38.1^{DEFGHIIJKLM}
B. longum subsp. *longum*	CUETM 247	137.5 ± 38.0^{DEFGHIIJKLM}
B. longum subsp. *longum*	ATCC 15708	140.5 ± 12.5^{IJKLM}
B. longum subsp. *longum*	CUETM 193	144.4 ± 39.1^{CDEFGHIJKLM}
B. longum subsp. *longum*	CUETM 177	149.2 ± 38.5^{CDEFGHIJKLM}
B. longum subsp. *longum*	CUETM 260	154.1 ± 33.1^{CDEFGHIJKLM}
B. longum subsp. *longum*	RW 019	157.2 ± 39.0^{BCDEFGHIJKLM}
B. longum subsp. *longum*	ATCC 15707	158.2 ± 38.5^{BDEFGHIJKLM}
B. longum subsp. *longum*	ATCC 51870	158.5 ± 39.5^{BCDEFGHIJKLM}
B. longum subsp. *longum*	DSM 20097	162.0 ± 38.6^{BCDEFGHIJKLM}
B. longum subsp. *suis*	ATCC 27533	175.7 ± 38.2^{ABCDEFGHIJKLM}
B. longum subsp. *longum*	NCC 2705	187.3 ± 38.3^{ABCDEFGHIJKL}
B. longum subsp. *longum*	CUETM 245	187.4 ± 33.1^{ABCDEFGHIJ}
B. longum subsp. *longum*	CUETM 263	191.0 ± 38.5^{ABCDEFGHIJK}
B. longum subsp. *longum*	CUETM 268	195.3 ± 38.1^{ABCDEFGHIJ}
B. longum subsp. *longum*	CUETM 281	218.2 ± 39.5^{ABCDEFGHI}
B. longum subsp. *longum*	PRO 16-10	223.5 ± 39.1^{ABCDEFGH}
B. longum subsp. *longum*	CUETM 287	227.1 ± 38.0^{ABCDEFG}
B. longum subsp. *longum*	PRO 42-10	232.6 ± 38.1^{ABCDEF}
B[3]. *breve*	ATCC 15698	237.0 ± 38.1^{ABCDE}
B. longum subsp. *longum*	RW 001	241.5 ± 39.0^{ABCD}
B. longum subsp. *longum*	CUETM 259	245.7 ± 38.1^{ABC}
B. longum subsp. *longum*	CUETM 171	265.1 ± 39.1^{AB}
B[3]. *catenulatum*	CUETM 174	266.4 ± 33.5^{A}
B. longum subsp. *longum*	CUETM 172	274.3 ± 38.4^{A}

Means with different capital letter superscripts were significantly different ($P < 0.05$).
[1]Trolox equivalent.
[2]Results were expressed as means ± standard error ($n = 3$).
[3]*Bifidobacterium*.

were found in all five genes (see Supplementary Material, Table S4). A phylogenetic tree of the concatenated sequences of the five loci for the 32 *B. longum* subsp. *longum* strains was constructed and compared to the antioxidant capacities of these strains (Figure 1). *B. longum* subsp. *longum* CUETM 171 and CUETM 172, both having high ORAC values, did not belong to the same cluster in the phylogenetic tree. The allele for *ahpC* was the only allele the two strains had in common. The four *B. longum* subsp. *longum* PRO 42 strains, isolated from the same human donor, had the same ST number (Table S3), but three had low antioxidant capacity, while the value observed for PRO 42-10 was higher. Five strains spanning the varying antioxidant capacities and different genetic groups were selected to perform experiments with fermented milk (Figure 1). More details about strain selection are available in Supplementary Material, Table S5.

3.3. Dynamic In Vitro Gastrointestinal Digestion (TIM-1) of Fermented Milk

3.3.1. Fermentation of Milk. All five strains (CUETM 172, CUETM 268, CUETM 245, CUETM 247, and PRO 16-10) acidified the milk until a mean pH of 4.5 and reached cell counts of 10^9 CFU per mL.

3.3.2. Bacterial Survival. During the first 30 min of digestion, viability of the five *B. longum* subsp. *longum* strains remained high (Figure 2). After 60 min, the viable cell counts decreased for CUETM 245 and PRO 16-10. However, the cell

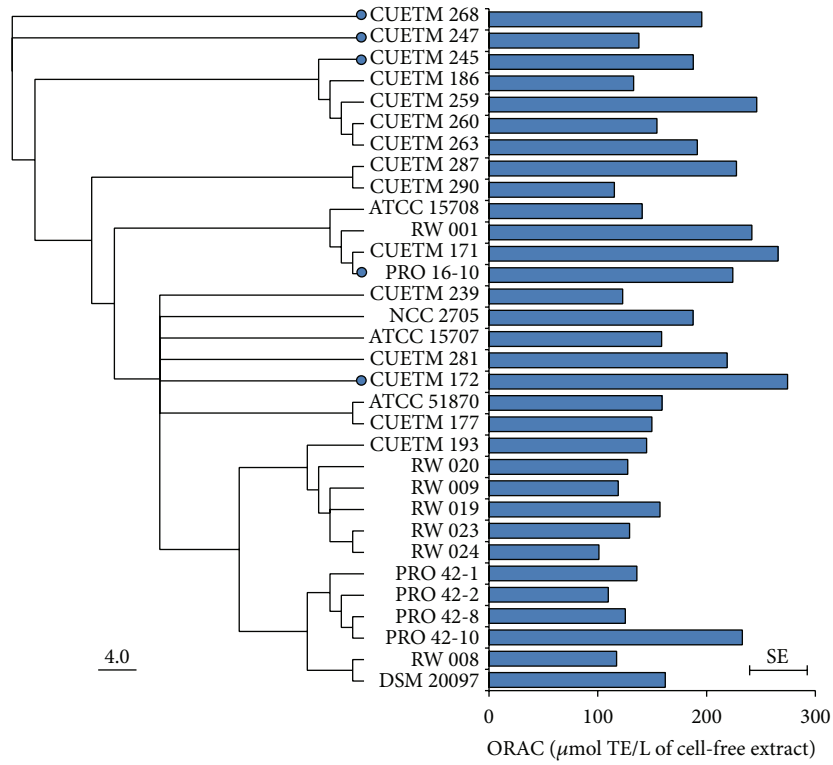

Figure 1: Antioxidant capacity of *B. longum* subsp. *longum* strains paired with the MLSA dendrogram. On the left, Jukes-Cantor neighbor-joining dendrogram constructed using the concatenated sequences of five loci (*mutT1, ahpC, trx, nrdA*, and *ppk*). Strains marked with a blue dot are the strains selected for milk fermentation. The length of the branches expressed in units of substitutions per site of the sequence alignment is indicated by the scale bar. On the right, oxygen radical absorbance capacity (ORAC) values correspond to the weighted means determined by ANCOVA. The error bar represents the standard error (SE).

concentrations evaluated with PMA-qPCR remained stable over this period for all strains. After 120 min, the PMA-qPCR counts were higher than the viable counts (CFU/mL) in the duodenal compartment. *B. longum* subsp. *longum* CUETM 172, CUETM 247, and CUETM 245 showed the smallest decline in viability with a loss of about 1 log cells/mL between 60 and 240 min of digestion in the duodenal compartment. *B. longum* subsp. *longum* CUETM 268 and PRO 16-10 were more affected by the conditions of the duodenal compartment, as cell concentrations decreased from 8 log to 6.5 log of viable cells/mL.

In the effluent, total number of cells evaluated with PMA-qPCR was at least 10^9 viable cells for all strains (CUETM 172: 2.64×10^{10} cells, CUETM 268: 4.09×10^{10} cells, CUETM 245: 4.25×10^9 cells, CUETM 247: 1.26×10^{10} cells, and PRO 16-10: 4.99×10^9 cells). Survival rates of cells in the TIM-1 effluent estimated by PMA-qPCR were higher than those determined with viable counts (Figure 3). *B. longum* subsp. *longum* CUETM 172, 268, and 247 exhibited survival rates higher than 3% according to the PMA-qPCR results. In contrast, the survival rate of *B. longum* subsp. *longum* PRO 16-10 was lower than 1%.

3.3.3. Bioaccessibility of Antioxidants in Fermented Milk. Before digestion (Table 3), there was no significant difference between the antioxidant capacity of nonfermented milk and milk fermented by each of the five bifidobacteria strains ($F = 0.9870$; $P = 0.4649$).

Table 3: Comparison of antioxidant activity (ORAC) for a portion of 100 g of different food types.

Food description	ORAC value (μmol TE[1]/100 g)
Blueberries, wild, raw[2]	9621
Wine, table, red, Cabernet Sauvignon[2]	4523
Cranberry juice, unsweetened[2]	1452
Fermented milk (CUETM 245)	1318
Fermented milk (PRO 16-10)	1312
Fermented milk (CUETM 247)	1255
Fermented milk (CUETM 268)	1175
Fermented milk (CUETM 172)	1076
Nonfermented milk	1174
Commercial UHT skimmed cow milk[3]	1270
Apple juice, canned or bottled, unsweetened, without added ascorbic acid[3]	414

[1]Trolox equivalent.
[2]Haytowitz and Bhagwat [66].
[3]Zulueta et al. [43].

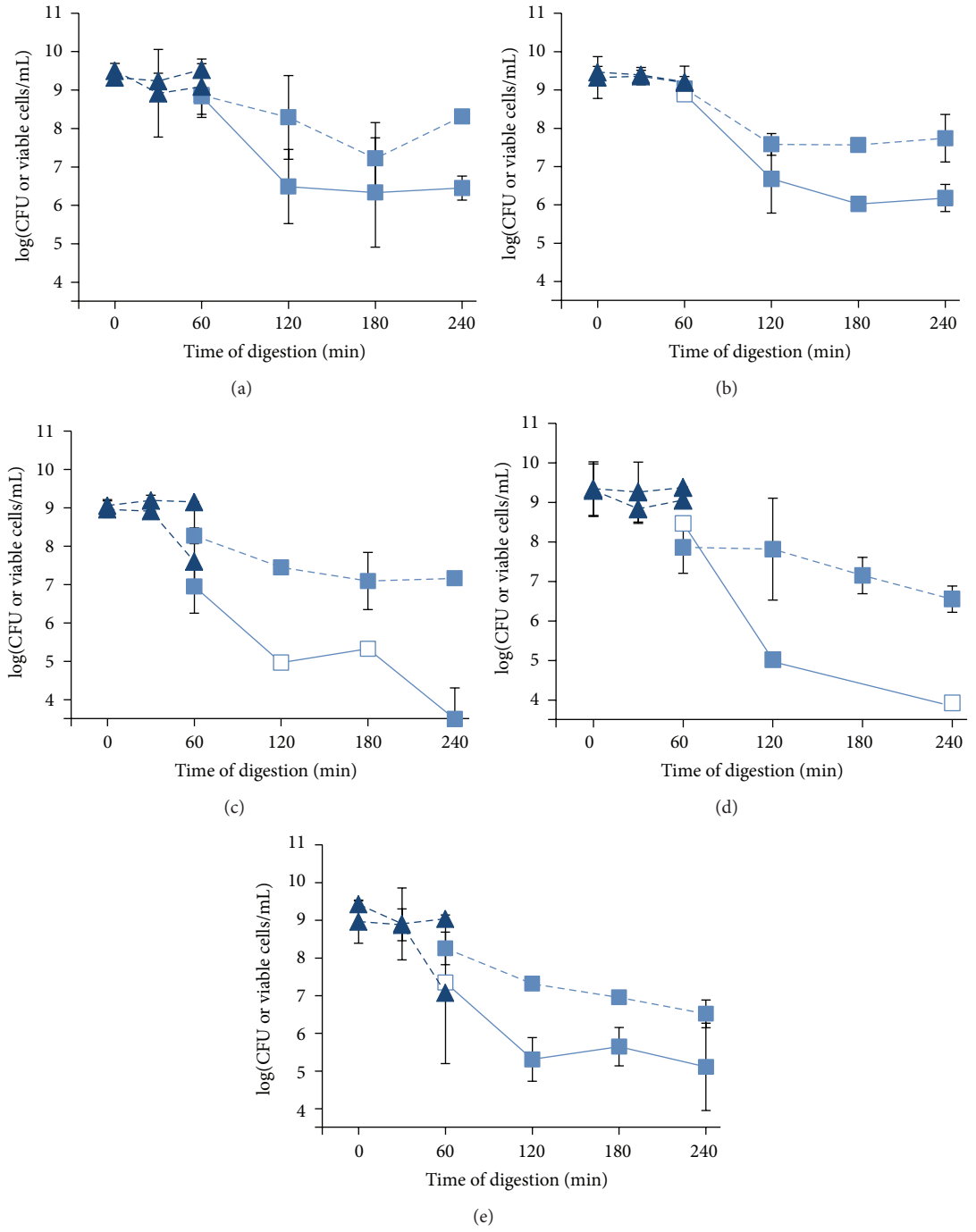

Figure 2: Survival curve during *in vitro* digestion (TIM-1) of fermented milk with *B. longum* subsp. *longum* CUETM 172 (a), CUETM 247 (b), CUETM 245 (c), CUETM 268 (d), and PRO 16-10 (e). The cell concentrations were determined by viable counts in CFU/mL (solid line) and by PMA-qPCR in viable cells/mL (dashed line). Samples were taken in gastric (▲) and duodenal (■) compartments. Empty symbols indicate that only one value was obtained. The limit of detection of PMA-qPCR was 3.51 log of viable cells/mL. The error bars represent the standard deviation.

During digestion, the antioxidant capacity remained higher in the jejunal compartment than the ileal compartment at each sampling point (data not shown). The quantity of bioaccessible antioxidants delivered was determined by multiplying the antioxidant capacity from the jejunal and ileal compartments at each hour of digestion by the volume of dialysate (Figure 4(a)). The largest delivery of antioxidants was obtained between 60 and 120 min of digestion in both jejunal and ileal compartments. After five hours of digestion, the milk fermented with *B. longum* subsp. *longum* PRO 16-10 showed the highest quantity of antioxidants at 16,383 μmol TE. The lowest quantity of antioxidants (8,080 μmol TE) was

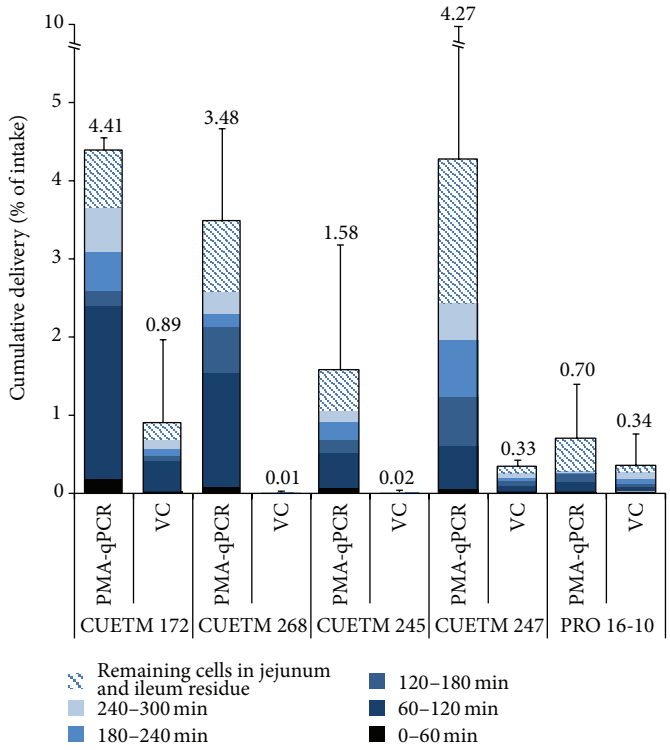

Figure 3: Survival rate of the five strains used for milk fermentation during *in vitro* digestion (TIM-1). The viable cells were determined by PMA-qPCR and by viable counts (VC) in the effluent after each hour of digestion. The remaining cells in the jejunum and ileum residue after 300 min of digestion were included in the survival rate. The results are presented relative to the total cells in the fermented milk at the start (% of intake). The error bars represent the standard deviation.

obtained by milk fermented with *B. longum* subsp. *longum* CUETM 172. Antioxidant bioaccessibility was expressed as a percentage of the intake of antioxidant in the meal (300 g of fermented milk) before digestion (Figure 4(b)). By the end of digestion, the antioxidants in fermented milk possessed a bioaccessibility ranging from 175% for *B. longum* subsp. *longum* CUETM 172 to 358% for *B. longum* subsp. *longum* PRO 16-10.

4. Discussion

As the antioxidant capacity of cell-free extracts of 32 *B. longum* subsp. *longum* strains is highly strain specific, it is thus possible to classify bifidobacteria strains according to this characteristic. However, in the present study, the sequence types of five genes coding for responses to oxidative stress were not correlated with antioxidant capacity among these 32 strains. Although *B. longum* subsp. *longum* CUETM 172 showed the highest antioxidant capacity during the screening of 32 *B. longum* subsp. *longum* strains, this was not reflected in the antioxidant capacity of the fermented milk. The antioxidant capacity of nonfermented milk and fermented milk in this study is similar to reconstituted milk (15% skim milk powder) [42] and a commercial UHT skimmed cow milk [43]. The development of radical scavengers during fermentation of milk can be explained in part by proteolysis [17], but bifidobacteria have low proteolytic activities [44, 45]. Indeed, antioxidant molecules can be located in the cytoplasm of bacteria [46]. If the cell membrane is intact, the antioxidant capacity of these molecules will not be detected with the ORAC assay. Even though the antioxidant capacity of the fermented milk before digestion is lower than blueberries and red wine (Table 3), this does not mean that they are less suitable sources of antioxidants. The quantity of bioaccessible antioxidant compounds is variable in foods such as fruit and vegetables [27]. For instance, the total bioaccessibility of anthocyanins in wild blueberries during TIM-1 digestion was less than 10% of the intake [28]. Furthermore, Lila et al. [28] have shown that bioaccessibility data overestimate *in vivo* (rodent) bioavailability, since TIM-1 hollow fibres for dialysis do not perfectly simulate the endothelial cells of the GIT. Moreover, the bioavailability of antioxidants is affected by many factors, such as food microstructure and chemical interactions with other phytochemicals and biomolecules [27]. In the future, antioxidants produced by bacteria such as *B. longum* subsp. *longum* strains will need to be tested *in vivo* in order to evaluate whether the antioxidants are absorbed in the same way as in the TIM-1 model and whether they are metabolized or not.

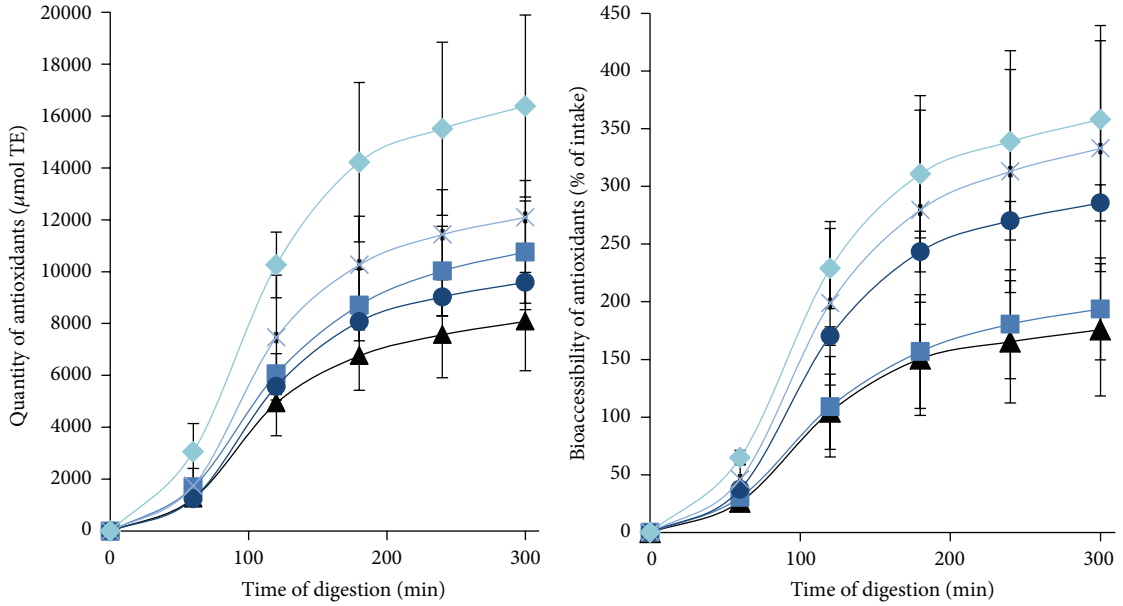

FIGURE 4: Bioaccessibility of antioxidants (in dialysates) evaluated with oxygen radical absorbance capacity (ORAC) during *in vitro* digestion (TIM-1) of fermented milk (300 g) by *B. longum* subsp. *longum* CUEMT 172 (▲), CUETM 268 (■), CUETM 245 (●), CUETM 247 (×), and PRO 16-10 (✦). (a) The cumulative quantity of bioaccessible antioxidants is expressed in μmol Trolox equivalent (TE). (b) The bioaccessibility of antioxidants expressed as a percentage of intake (antioxidants in 300 g of fermented milk before digestion). The error bars represent the standard deviation.

We hypothesize that the bioaccessibility of antioxidants produced by *B. longum* subsp. *longum* could be improved by the harsh conditions of the GIT. These conditions can stress or kill bifidobacteria present in the fermented milk, even though *B. longum* strains are well adapted to the colon ecosystem [13]. However, it is difficult to evaluate the difference of these two states with viable counts because stress can lead to viable but noncultivable cells (VBNC state) [47]. The PMA-qPCR method can enumerate both viable and VBNC cells [48]. The five strains were not affected by the high acidity of the stomach compartment in the TIM-1 for 60 min, according to viable counts and PMA-qPCR results. All five *B. longum* subsp. *longum* strains were affected to varying degrees by the bile salts in the duodenum compartment, despite the presence in the genome of *B. longum* of the *bsh* gene encoding a bile salt hydrolase [49]. As for acid tolerance, resistance to bile salts seems to be a strain-specific characteristic and together they have a major influence on the final survival rate through the GIT [12]. Saide and Gilliland [15] have in fact suggested that the encounter with bile could improve the delivery of antioxidants to the intestine.

Data on pharmacokinetics of bifidobacteria in different parts of the intestinal tract and in colon simulation models are mainly based on comparison of bacterial strains before and after ingestion rather than on precise data on bacterial survival rates [50]. *Bifidobacterium* sp. can survive transit through the intestinal tract with recovery rates in faeces ranging from 20 to 22% for the fermented milk and lyophilized form, respectively [51, 52]. Among bifidobacteria, *B. animalis* subsp. *lactis* strains displayed the highest survival rates during *in vivo* ileal perfusion and simulated gastric transit with an estimated survival rate ranging from 23.5% to 37.5% [53, 54] with a faecal recuperation of 30% [55]. Only single strains of *B. longum* subsp. *longum* (LMG 13196) exhibited survival rates comparable with those observed for the *B. animalis* subsp. *lactis* strains during *in vitro* assessment of the transit tolerance [56]. Fujiwara et al. [57] noted that *B. longum* subsp. *longum* SBT2928 was found in good proportions in the faeces.

The survival rate obtained in this study can best be compared to other studies using dairy products as a delivery mode for probiotics in TIM-1 as milk is known to provide protection to probiotic bacteria [58]. The survival of the five *B. longum* subsp. *longum* strains determined by viable counts is very low (0.8–0.01%) compared to *Lactobacillus amylovorus* DSM 16698 (survival rate up to 100%) [32]. *Bifidobacterium bifidum*, *L. acidophilus*, and *Pediococcus acidilactici* UL5 have also demonstrated better survival rates (10–20%) [11, 31]. The survival rates of the five *B. longum* subsp. *longum* strains seem to be more comparable to those of *Lactococcus lactis* ATCC 11454 (0.00073%) [31], *Streptococcus thermophilus* ST20, and *Lactobacillus delbrueckii* subsp. *bulgaricus* LB9 (close to the detection limit) [11]. However, the results presented here show that viable counts underestimate cell survival and *in vivo* the presence of other food components could enhance protection of the bacteria.

Without the use of PMA-qPCR, we would assume that all five strains in this study had a low survival rate. However, the VBNC state is revealed by the difference between PMA-qPCR

estimates and viable counts. For *B. longum* subsp. *longum* PRO 16-10, the absence of difference between PMA-qPCR and viable cell counts indicates that cells did not reach the VBNC state and only a small portion survived after digestion in the TIM-1. Adams [59] suggested that variable amounts of dead cells might contribute to the differences in effects observed when administering live probiotics. Even though some probiotics have low survival rates, the number of cells that would reach the colon alive may be sufficient. For milk fermented by all five strains in this study, there was a greater amount of antioxidants present in the dialysate than in the milk before digestion (1.5–3.5-fold higher). For *B. longum* subsp. *longum* PRO 16-10, the quantity of bioaccessible antioxidants delivered by the fermented milk was higher at the end of digestion, which was accompanied by a low survival rate (0.70%).

The evaluation of antioxidant capacity in cell-free extracts must be complemented by cell survival assays in order to properly select strains for fermentation of milk with the best bioaccessibility of antioxidants. This is the first time that strains with low survival rate in fermented milk are shown to deliver more bioaccessible antioxidants during *in vitro* dynamic digestion. In addition to the liberation of antioxidants, dead bacteria provide other health benefits such as immunomodulation and anti-inflammatory effects [60–62]. In order to provide other kinds of benefit to the host, it is still important to ensure that a portion of the intake of probiotics survive the GIT passage. It has been suggested that the antioxidant effect from probiotics reaching the colon can be explained by the scavenging of oxidant compounds or the prevention of their generation in the colon [62]. However, the presence of antioxidants in the dialysate suggests that a major portion of antioxidants produced by *B. longum* strains may be absorbed in the small intestine and could thus be transported in the blood.

5. Conclusion

Milk fermented by different strains of *B. longum* subsp. *longum* provided bioaccessible antioxidants during digestion. However, the characterization of antioxidant capacity of cell-free extracts cannot be used as a selection criterion for antioxidant probiotic strains because survival rate in the GIT had more influence on the bioaccessibility of antioxidants. The improved bioaccessibility probably comes from the death of a portion of *B. longum* subsp. *longum* cells. The milk fermented with the strain with the lowest survival rate in the upper GIT (*B. longum* subsp. *longum* PRO 16-10) had the highest bioaccessibility of antioxidants. On the contrary, the milk fermented with the strain with the best survival rate (*B. longum* subsp. *longum* CUETM 172) had the lowest bioaccessibility of antioxidants. Probiotics are usually defined as "live microorganisms, which, when administered in adequate amounts, confer a health benefit on the host" (FAO/WHO) but variable amounts of dead cells during digestion of fermented milk may contribute to health benefits by providing bioaccessible antioxidants. These antioxidants could lead to the improving antioxidant capacity of human blood.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Research Article

Biodiversity and γ-Aminobutyric Acid Production by Lactic Acid Bacteria Isolated from Traditional Alpine Raw Cow's Milk Cheeses

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"*Nostrano*-cheeses" are traditional alpine cheeses made from raw cow's milk in Trentino-Alto Adige, Italy. This study identified lactic acid bacteria (LAB) developing during maturation of "*Nostrano*-cheeses" and evaluated their potential to produce γ-aminobutyric acid (GABA), an immunologically active compound and neurotransmitter. Cheese samples were collected on six cheese-making days, in three dairy factories located in different areas of Trentino and at different stages of cheese ripening (24 h, 15 days, and 1, 2, 3, 6, and 8 months). A total of 1,059 LAB isolates were screened using Random Amplified Polymorphic DNA-PCR (RAPD-PCR) and differentiated into 583 clusters. LAB strains from dominant clusters ($n = 97$) were genetically identified to species level by partial 16S rRNA gene sequencing. LAB species most frequently isolated were *Lactobacillus paracasei*, *Streptococcus thermophilus*, and *Leuconostoc mesenteroides*. The 97 dominant clusters were also characterized for their ability in producing GABA by high-performance liquid chromatography (HPLC). About 71% of the dominant bacteria clusters evolving during cheeses ripening were able to produce GABA. Most GABA producers were *Lactobacillus paracasei* but other GABA producing species included *Lactococcus lactis*, *Lactobacillus plantarum*, *Lactobacillus rhamnosus*, *Pediococcus pentosaceus*, and *Streptococcus thermophilus*. No *Enterococcus faecalis* or *Sc. macedonicus* isolates produced GABA. The isolate producing the highest amount of GABA (80.0 ± 2.7 mg/kg) was a *Sc. thermophilus*.

1. Introduction

Traditional alpine raw milk cheeses are commonly produced in alpine regions including the province of Trentino in North-Eastern Italy. Here they are called "*Nostrano*-cheeses" and are semicooked cheese made by mixing approximately in 1 : 1 ratio the raw cow's milk from two different milking. The first milking is carried to dairy factory the evening before the cheese-making and is stored in large shallow tank for 9–11 hours where a spontaneous creaming occurs. After this overnight stage, the partially skimmed milk under the cream in the tank is manually drained from the cream fat and placed in the cheese-making vat. The whole milk from the morning milking, the second milking, is then added to the skimmed milk. No commercial lactic starters are added and the natural milk microbiota obtained from the overnight skimmed milk initiates the acidification process. The vat milk is coagulated by commercial rennet and, after the manual cutting, the curd is cooked at about 48°C. After moulding and salting, the ripening is held at about 18°C for 3 to 8 months.

The milk for "*Nostrano*-cheeses" typically comes from Holstein Friesian and/or Brown Swiss cattle breeds, which are fed differently during the year. The cows are typically fed on hay during the cold season in the valleys and from late June to middle September (summer season) are grazed on high mountain alpine pasture.

It has been reported that the use of commercial starters in raw milk cheeses may modify the characteristics of the cheese microbiota, in particular lowering the microbial biodiversity [1] and it is also well known that mainly LAB microbiota developing during ripening influences the typical organoleptic characteristics of the cheese [2]. Thus, LAB represent a fundamental process factor for the final attributes and quality of artisan dairy products such as alpine cheeses. Several studies have focused on the genotypic and technological characterization of LAB isolated from different traditionally fermented cheeses [3–6], but little work has so far been done on "*Nostrano*-cheeses."

In addition to the technological relevance of LAB in cheese, there is currently much research and industry interest in the potential biological activity of dairy LAB, either for use as probiotics in their own wright or as bioactive agents capable of modulating the health functionality of cheese and other dairy products [7]. Raw milk cheeses have already been identified as a useful source of microbial biodiversity and new LAB strains with health promoting properties [8].

Since caseins are rich in glutamate which is released by proteolytic action, the decarboxylation of this amino acid into γ-aminobutyric acid (GABA) can have an important effect on the formation of eyes in cheese [9]. Besides its technological effect in cheese, GABA has several well-characterized physiological functions in mammals including neurotransmission, induction of hypotension, diuretic and tranquilizer effects, and stimulation of immune cells [10–12]. Some studies have reported also that GABA derived from the gut may be a neuroactive molecule within the gut-brain axis [13], which is a complex communication highway linking the gut environment with both the central and peripheral nervous systems. Strains of *Lb. buchneri* [14], *Lb. brevis*, *Lb. paracasei,* and *Lb. plantarum* [15] isolated from traditional cheeses have been shown to produce GABA. GABA-producing LAB have not been isolated and extensively characterised from traditional alpine cheeses produced in Trento, though the presence of GABA in these cheeses has been confirmed and its concentration at the end of ripening reported at between 120 and 1,739 mg/kg [16], which is high compared to other Italian cheese varieties (typically 0.260 to 391 mg/kg) [15]. Therefore, the objective of this study was to analyze the diversity and the successional development of LAB in traditional "*Nostrano*-cheeses" from the Trento alps during cold and summer seasons and to extensively screen and identify GABA-producing LAB isolates.

2. Materials and Methods

2.1. Cheese Factories and Milk Sampling. Cheeses were sampled in three dairy factories (called B, C, and D according to a previous paper [17]) located throughout the Trentino region and producing traditional alpine cheeses called "*Nostrano*-cheeses." Each factory collected milk from farms within a 15 km radius.

Two cheese batches from each dairy factory, one in February and the other in July, were sampled, making a total of six batches subjected to microbiological analyses. All factories processed milk obtained from stabled cows fed with hay during the "cold season" from October to May and high mountain pasture fed cattle in the summer season from June to September. For each of the six batches, at least five cheese samples at different stages of ripening were collected (24 hours, 15 days, 1 month, 2 months, and 3 months and for five batches also 6 and 8 months) making a total of 40 cheese samples per factory.

2.2. Enumeration and Isolation of Microorganisms. Cheese samples (25 g) were homogenized (2 min at 260 rpm) using a stomacher (laboratory blender stomacher 400, Seward, London, UK) in 225 g peptone water (0.1% mycological peptone (Oxoid, Basingstoke, UK)) and serially diluted. Dilutions were plated and incubated as follows: onto MRS agar acidified to pH 5.5 with 5 mol/L lactic acid, anaerobically, for 2 days, at 30°C and 45°C for mesophilic and thermophilic rod-shaped LAB, respectively; onto MRS agar added with vancomycin (8 μg/mL; Sigma-Aldrich, Saint Louis, MI,US) [18] and acidified to pH 5.5 with 5 mol/L lactic acid, anaerobically, for 72 h at 30°C for mesophilic heterofermentative rod-shaped LAB; onto M17 agar for 2 days, aerobically, at 30°C and anaerobically at 45°C for mesophilic and thermophilic coccoid LAB, respectively; onto KAA aerobically, for two days, at 37°C for enterococci; onto PCA added with 10 g/L skimmed milk aerobically, for 24 h, incubated at 30°C for total bacterial count (TBC). All culture media were purchased from Oxoid.

At least three colonies were picked from each countable plate; Gram-positive colonies (as determined by KOH method; [19]) and negative to the catalase test (as determined by transferring fresh colonies from agar medium to a glass slide and adding 5% H_2O_2) were isolated. Cell morphology was determined by microscopic observation. Each isolate was purified by subsequent culturing onto M17 or MRS and pure cultures were stored at −80°C in glycerol (20% v/v) stocks.

2.3. DNA Extraction and RAPD-PCR. DNA was extracted from overnight broth cultures of isolated strains. Cells were centrifuged at 10,000 ×g for 5 min and the pellets were washed twice in sterile distilled water and suspended in 1 mL of distilled water. Cell lysis was achieved using the *Instagene* Matrix (Bio-Rad, Hercules, CA, USA) following the manufacturer's instruction.

RAPD-PCR was carried out in a total volume of 25 μL using primer PC1 [20]. Cluster analysis of DNA patterns was carried out using GelCompar II-BioNumerics software (package version 6.0; Applied Maths, Belgium), exploiting the unweighted pair group method arithmetic averages (UPGMA). Similarity of PCR fingerprinting profiles was calculated based on Pearson product-moment correlation coefficient. The threshold breakpoint value was fixed to 80%; isolates with similarity coefficient higher than 80% were classified into the same cluster, according to Gatti et al. [21].

2.4. Genotypic Identification of LAB. One isolate representative of each LAB cluster was genotypically identified by 16S rRNA gene analysis. All isolates from M17 45°C were tested by

Table 1: Bacterial counts from cheese ($n = 6$ for each time ripening) sampled at 24 h, 15 days, 1 month, 2 months, 3 months, 6 months, and 8 months.

Ripening time	Agar media (log cfu/g)						
	PCA	MRS 45	MRS 30	MRS VAN	M17 45	M17 30	KAA
24 h	8.5 ± 0.8	5.2 ± 0.6	5.3 ± 0.6	4.6 ± 1.4	8.5 ± 0.6	6.6 ± 0.6	5.6 ± 1.4
15 d	9.0 ± 0.5	5.1 ± 0.7	8.1 ± 0.8	7.7 ± 0.6	7.9 ± 1.2	7.8 ± 0.6	6.2 ± 1.0
1 month	8.8 ± 0.4	6.2 ± 1.0	8.3 ± 0.5	8.1 ± 0.5	7.8 ± 0.9	7.8 ± 0.8	5.7 ± 1.2
2 months	8.7 ± 0.3	6.3 ± 0.8	8.0 ± 0.4	8.0 ± 0.4	7.2 ± 0.7	8.1 ± 0.3	5.7 ± 1.4
3 months	8.4 ± 0.6	5.7 ± 0.7	7.8 ± 0.7	7.5 ± 0.7	7.1 ± 0.9	6.7 ± 1.6	5.7 ± 0.6
6 months[a]	7.7 ± 0.6	5.6 ± 1.5	7.0 ± 0.4	7.5 ± 0.9	6.9 ± 1.0	5.9 ± 1.4	5.7 ± 0.6
8 months[a]	6.9 ± 0.7	5.2 ± 1.0	6.5 ± 0.9	6.5 ± 0.6	6.0 ± 1.1	4.6 ± 1.5	4.6 ± 1.0

[a]$n = 5$.

Sc. thermophilus species specific PCR according to Lick et al. [22]; isolates from M17 at 30°C by *Lc. lactis lactis/cremoris* species specific PCR according to Delorme et al. [23]; and all the other isolates from MRS at 30°C by *Lb. casei*, *Lb. paracasei,* and *Lb. rhamnosus* species specific PCR with the primers Y2, Casei, Para, and Rham described by Ward and Timmins [24]. If the species specific PCR gave a negative result, identification was carried out by using 16S rRNA gene sequencing. The sequence analysis of a 16S rRNA fragment gene was performed using 27f (5′-GAGAGTTTGATCCTGGCTCAG-3′) and 1495r (5′-CTACGGCTACCTTGTTACGA-3′) primers, designed by Grifoni et al. [25].

The obtained PCR products (ca. 30 ng) were purified with Exo-SAP-IT kit (USB Co., Cleveland, OH) and sequenced through the BigDye Terminator v1.1 cycle sequencing kit (Applied Biosystems, Foster City, CA) as reported by the manufacturer in an ABI PRISM 3100 sequencer (Applied Biosystems). Species were assigned after comparison of the obtained sequences by BLAST alignment (http://www.ncbi.nlm.nih.gov/BLAST).

All the amplicons were analyzed by electrophoresis on 2.5% (w/v) agarose gel (Gibco BRL, Cergy Pontoise, France) at 100 V for 90 minutes in 1X TAE buffer and were revealed by staining with ethidium bromide (0.5 μg/L). All amplifications were performed with a T100 Thermal Cycler (Bio-Rad Laboratories).

2.5. γ-Aminobutyric Acid (GABA) Production and Quantification. Glutamate decarboxylase (GAD) activity of LAB isolates and the production of GABA were checked using the method of Nomura et al. [26], with some modifications; cultures were centrifuged (9,000 rpm for 15 min at 4°C), washed twice with sterile PBS, and suspended in sterile 0.85% NaCl solution in order to achieve the $A_{620\,nm}$ value of 2.5. Afterward; 100 μL of cell suspension was mixed with 900 μL of 50 mM sodium acetate buffer (pH 4.7) containing 7.0 mM L-glutamate and 0.1 mM pyridoxal phosphate. The reaction mixture was incubated for 24 h at the same temperature of isolation (30°C for mesophilic and 45°C for thermophilic isolates) and filtered through a 0.22 μm pore size filter (Minisart, Sartorius Stedim Biotech, Goettingen, Germany). The sample, diluted 10 times with sodium tetraborate 0.1 M (pH adjusted to 10.5) and added to glycine, as internal standard to a final concentration of 10 mg/L, was stored at −20°C before the analysis. L-Glutamic acid, glycine, and GABA were quantified as o-phthalaldehyde (OPA) adducts modifying the method proposed by Lehtonen [27] in order to notably reduce the time of separation to only 2.7 minutes but without worsening selectivity and accuracy. This was possible in the light of the specifically designed and perfectly known matrix.

The measures were performed using an UHPLC Ultimate 3000 (Thermo Fisher Scientific, Waltham, MA, USA) equipped with a fluorescence detector (Ex = 336 nm, Em = 445 nm). Separation was carried out with sodium acetate 0.05 M (pH adjusted to 7.5; eluent A) and methanol (eluent B) using a column Chromolith Performance RP-18e (100 × 4.6 mm; Merck, Darmstadt, Germany) with Guard Cartridge Chromolith RP-18e (10 × 4.6 mm; Merck) at 40°C. The flow rate was set at 2 mL/min. The analytical gradient for eluent B was as follows: 40% for 30 sec, 25% for 90 sec, 100% for 30 sec and 60% for 15 sec. The sample (10 μL), kept at 10°C by the autosampler, was automatically introduced into the loop, added with 10 μL derivatising solution, mixed for 1 min, and injected. The derivatising mix was 4.5 g/L of OPA (Sigma-Aldrich) in sodium tetraborate 0.1 M, corrected to pH 10.5, 10% methanol, and 2% 2-mercaptoethanol (Sigma-Aldrich). The detection limit for GABA was estimated at 0.025 mg/L (3 times the standard deviation of the GABA contents measured repeating 10 times the analysis of a sample at unquantifiable content).

3. Results

3.1. Microbial Cell Counts. The microbial populations of *Nostrano*-cheese samples were estimated on different selective media (Table 1). The total bacterial counts were in the range of 8-9 log cfu/g from 24 h to 3 months of ripening and decreased after 6 and 8 months by 1 order of magnitude. The thermophilic cocci reached the highest counts after 24 h of ripening (mean values of 8.5 log cfu/g); mesophilic cocci reached the highest counts after 2 mo of ripening (mean values of 8.1 log cfu/g). Enterococci were never dominant and reached their highest count after 15 days of ripening (mean values of 6.2 log cfu/g). The lactobacilli group (counts onto MRS at 45 and 30°C) was higher after 1 mo of ripening. The growth dynamic of the different microbial

groups was different (Table 1); thermophilic cocci counts (onto M17 45°C) were dominant in the first 24 hours; after 15 days to 3 months of ripening, mesophilic cocci (onto M17 30°C) and lactobacilli counts (onto MRS and MRS VAN 30°C) started to increase and were dominant together with thermophilic cocci; finally, at the end of ripening (6 and 8 months) mesophilic lactobacilli and thermophilic cocci maintained the dominance within the alpine cheeses microbiota.

On average, three colonies, for each colony morphology, were isolated in pure culture from each medium. For summer season at 6 and 8 months, only two types of cheese were available and sampled in dairy factories C and D. A total of 1,105 isolates were collected. From the total number of isolates, 46 were discarded from further analysis as nonlactic acid bacteria (they were found positive to catalase and negative to KOH tests). The remaining 1,059 strains were characterised for cell morphology; 677 were cocci and 382 were rods (Table 2).

3.2. Molecular Clustering of LAB Isolates and Species Identification. All putative LAB isolates were analyzed by RAPD-PCR as a first grouping into clusters. The isolates from the same kind of cheese showing a RAPD similarity coefficient of at least 80% were considered as belonging to a single cluster. The RAPD-PCR analysis grouped 1,059 LAB into 583 clusters with 80% similarity index (results not shown). From these clusters, 276 isolates were selected for further analysis because they belonged to the dominant microbial populations as enumerated by plate counts on MRS, MRS VAN at 30°C, M17 at 30, and 45°C. The RAPD-PCR analysis of these 276 dominant isolates discriminated 97 different clusters defined at a minimum similarity level of 80% (Figure 1). The 97 clusters were designated using a progressive number followed by the letters B, C, or D to indicate the dairy of origin of the clustered isolates (Figure 1; clusters 1D to 97C).

Species identification was performed by species specific PCRs or partial 16S rRNA gene sequencing. Table 3 shows the results of bacteria identification for each ripening time. The highest diversity within a single species was found for *Lb. paracasei* and *Sc. thermophilus* with 35 and 23 different genotypes, respectively.

Lb. paracasei was the dominant species (72 isolated on MRS and 16 on M17 agar plates), followed by *Sc. thermophilus* (50 isolated on M17 agar) and *Ln. mesenteroides* (27 isolated on MRS and 18 on M17 agar). A different dominant species successional development was observed in cheeses at different ripening time: *Sc. thermophilus* was always dominant in the first 24 hours and one of the codominant species for up to 2 months of ripening; *Lc. lactis* species was also found codominant in cheese at 24 h ripening with *Sc. thermophilus*. Streptococci and enterococci species were recorded in abundance in the first three months but largely disappeared after six months and at the end of ripening (Table 3). We did not find difference in species distribution between cheeses sampled in February and July. The same species were recorded both in cold and in summer season (data not shown).

After the genotypic characterization, 97 strains, one representative of each dominant cluster, were processed for the detection of GABA production.

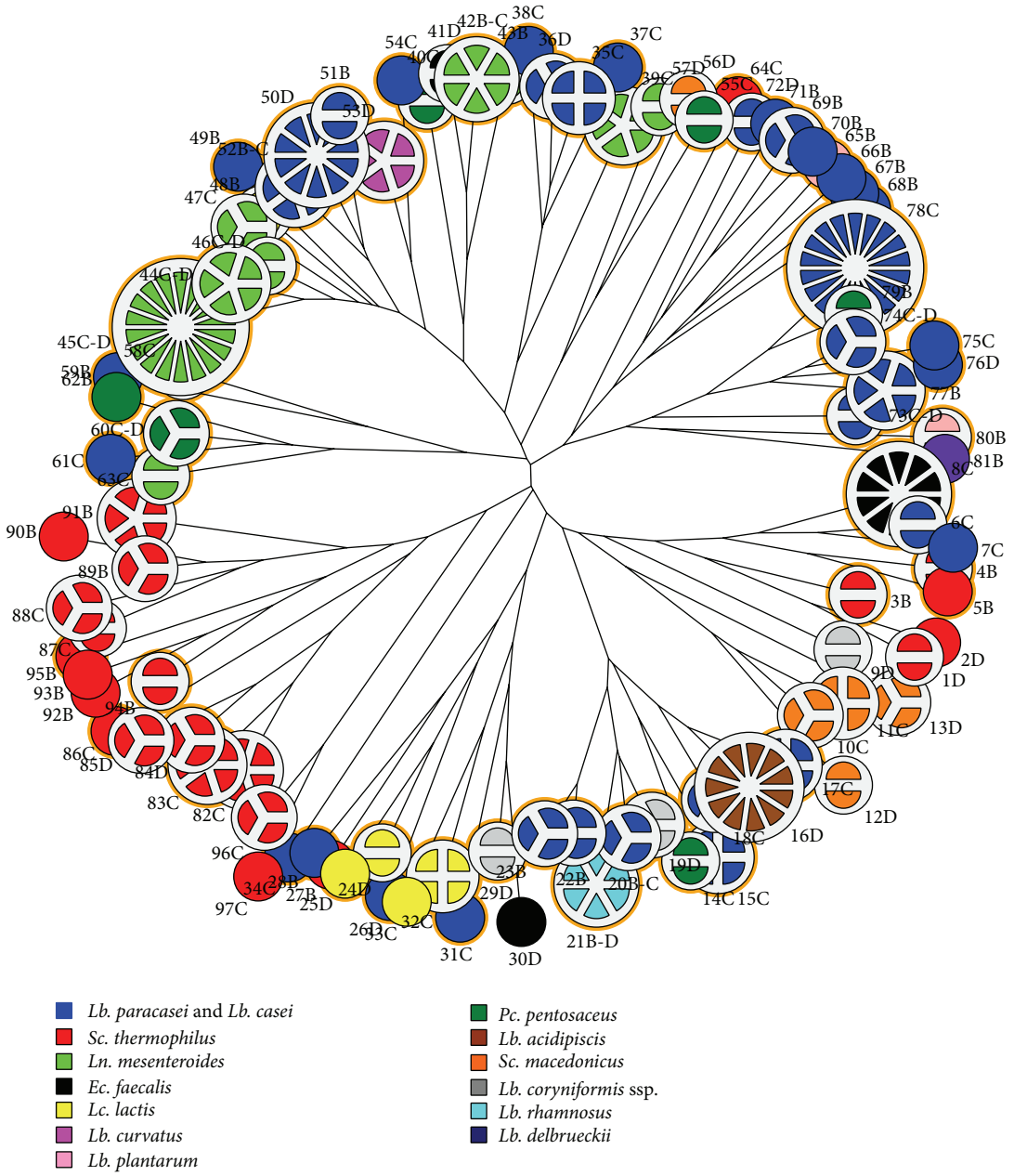
3.3. GABA Production. Sixty-eight isolates out of the 97 different clusters synthesized GABA (GABA amount > 0.25 mg/kg) after 24 h of incubation at 30 or 45°C in presence of glutamic acid (Table 4). They grouped 195 of the dominant isolates (71% of the tot) and in particular three (1 *Lb. paracasei*, 1 *Lb. rhamnosus,* and 1 *Sc. thermophilus*) were able to produce GABA concentrations higher than 10 mg/L (Table 4, lines in bold).The *Sc. thermophilus*, cluster 84C showed the highest glutamate decarboxylase activity generating a mean value of 80 mg/L of GABA (Table 4, first line). No *Ec. Faecalis* or *Sc. macedonicus* isolate was able to produce amount of GABA higher than 0.25 mg/kg.

4. Discussion

In Italy the province of Trento has a long dairy history with various dairy biotechnological traditions arising from the geographical challenges of transport and communication between different alpine valleys and a diverse cultural heritage. A wide range of cheeses coexist, each with their own specific biotechnological processes, organoleptic characteristics, and history. A previous review has discussed the importance of preserving this type of traditional artisan cheese, usually made from raw cow's milk, because of their high microbial biodiversity and in particular high species richness of "wild" LAB with diverse metabolic activities and of great potential as dairy starters or even probiotic agents [8]. Previous work has shown that the "*Nostrano*-cheeses" contain high concentrations of GABA compared to other Italian cheeses [16] and it is known that LAB is responsible for producing GABA in cheese [15]. We, therefore, selected Trento "*Nostrano*-cheeses" for the screening and isolation of GABA-producing LAB.

The successional development of the lactic microbiota of six"*Nostrano*-cheeses" from 24 hours to 8 months of ripening was characterised. 276 isolates belonging to dominant lactic microbiota were grouped into 97 clusters, identified to the species level, and screened for their GABA production. The milk used to produce these cheeses was the subject of a previous report [17] but in summary, the microbiological characterization was in agreement with microbial counts reported for other traditional Italian cow raw milk cheeses [6, 28, 29]. M17 and MRS were not perfectly selective, in agree with previous works [6, 30] in fact some nontarget isolations were recorded; for example, 2 *Lc. lactis* isolates amongst 13 were found on MRS agar plates and about 14% of all rod-shaped isolates were isolated on M17 agar plates.

As commonly found in many raw milk cheeses [28–30], the microbial composition of the "*Nostrano*-cheeses" was dominated by LAB. *Lb. paracasei* was the most abundant species (31.9% of the isolates), followed by *Sc. thermophilus* and *Ln. mesenteroides* (18.1% and 16.3%, resp.). These species were amongst the dominant microbiota at all production stages; in particular, *Sc. thermophilus* dominated after 24 h



Lb. paracasei and *Lb. casei*
Sc. thermophilus
Ln. mesenteroides
Ec. faecalis
Lc. lactis
Lb. curvatus
Lb. plantarum
Pc. pentosaceus
Lb. acidipiscis
Sc. macedonicus
Lb. coryniformis ssp.
Lb. rhamnosus
Lb. delbrueckii

Figure 1: Unrooted dendrogram of the 276 dominant isolates obtained from RAPD-PCR patterns using the Pearson product moment correlation coefficient (r) and the unweighted pair group algorithm with arithmetic averages (UPGMA). Each circle-pie is a cluster and the number of slices represents the number of isolates for each cluster. GABA producer clusters are indicated by a yellow circle. Each color is a different species.

until 2 months of ripening, while *Lb. paracasei* and *Ln. mesenteroides* reached their highest levels in the cheese after 15 days and remained at high levels until 3 months of ripening with a similar trend observed in microbial counts on MRS which started to decrease after 6 months of ripening, probably the result of microbial autolysis [31, 32]. Another 11 different LAB species were found in the cheese samples but none at a relative abundance higher than 5%. All species identified were previously recorded and very common in the dairy environment [4, 6, 28–30], with the exception of *Lb. acidipiscis*, which is a species described by Tanasupawat et al. [33] and isolated from fermented fish and has also been isolated more recently from traditional Greek cheeses [34].

RAPD analysis displayed a great genetic diversity amongst the isolates. In fact about 33% of the RADP clusters were singletons (one cluster for one isolate). This genetic biodiversity may reflect a real picture of the high species richness amongst the isolates collected from the cheeses but could also be consequence of the large number of strains analysed in this study. A similar result was found

Table 2: Number of putative LAB strains (cocci/rods) isolated from each plate at different sampling times in traditional alpine cheeses ($n = 6$).

Ripening time	Agar media (cocci/rods)						TOT number of LAB isolates (cocci/rods)
	MRS 45	MRS 30	MRS VAN	M17 45	M17 30	KAA	
24 h	12 (7/5)	19 (15/4)	8 (8/0)	18 (18/0)	20 (18/2)	22 (21/1)	99 (88/11)
15 d	8 (8/0)	38 (10/28)	15 (1/14)	18 (17/1)	31 (24/7)	27 (22/5)	137 (82/55)
1 month	19 (10/9)	43 (15/28)	42 (19/23)	31 (28/3)	34 (28/6)	21 (20/1)	190 (120/70)
2 months	28 (16/12)	54 (17/19)	49 (20/39)	36 (35/1)	46 (33/13)	22 (19/3)	235 (140/95)
3 months	18 (13/5)	48 (21/27)	46 (24/22)	25 (24/1)	23 (22/1)	24 (17/7)	184 (121/63)
6 months[a]	8 (4/4)	30 (18/12)	25 (8/17)	16 (13/3)	22 (22/0)	12 (9/3)	113 (74/39)
8 months[a]	15 (5/10)	20 (5/15)	16 (4/12)	25 (19/6)	16 (15/1)	9 (5/4)	101 (48/48)
TOT	108 (63/45)	252 (101/151)	201 (84/117)	169 (154/15)	192 (162/30)	137 (113/24)	1059 (677/382)

[a]$n = 5$.

in a previous work, where 206 isolates from spontaneously fermented cheeses were analysed by RAPD PCR [30].

We compared all the clusters recovered from "*Nostrano*-cheeses" with those found in the corresponding milk samples and reported in a previous report [17] and no milk RAPD pattern was found amongst the 586 cheese clusters. This may be because the fermentation is not spontaneous but driven by a starter culture from the overnight skimmed milk that, even if natural and not commercial, may inhibit milk microbiota growth and development. It is worth highlighting that some isolates from different dairies grouped within the same cluster. The 9 species occurring in different dairies were *Lb. paracasei*, *Lb. rhamnosus,* and *Ln. mesenteroides*. These few coincident clusters occurred often in different dairy environments and might represent part of an endemic geocentric cheese microbiota, not necessarily coming from milk, but adapted to the cheese-making practice, ripening, and local microclimate and environmental conditions specific to the Trento alps. On the other hand, 34 of the 97 clusters were RAPD-PCR singletons and some species like *Lb. coryniformis* ssp. *torquens, Lb. acidipiscis, Lb. curvatus,* and *Lb. delbrueckii* were peculiar only for one of the three dairy factories. These aspects suggest that each manufacturing facility may also be characterized by a unique microbial population.

Considering the recent interest in the gut-brain axis, the potential role of neurotransmitters like GABA in the periphery, and the immunological potential of systemic GABA, we screened the 97 dominant clusters for GABA producing strains [35]. A total of 68 GABA producing strains were identified. Previous studies by Siragusa et al. [15] and more recently by Diana et al. [36] found that sheep milk cheeses contained higher levels of GABA than cow's milk cheeses and consequently had higher numbers of GABA-producing LAB. The raw cow's milk cheeses subject of this current study showed higher amounts of GABA at the end of ripening [16] and a higher percent of GABA producer strains (71%) than these two previous studies where GABA producing strains were less than 14%. This difference may be due to the peculiar traditional environment of production of these Trento cheeses. However, it may also be the result of the cheese production times sampled. We screened the isolates starting at 24 h and followed the cheese LAB microbiota until the end of ripening. It is probable that microbial GABA production follows the same trend as the bacterial growth with higher number of GABA producing strains in the first 3 months followed by a rapid decrease in LAB numbers.

Amongst the 68 positive strains, 13 GABA producing strains gave more than 4 mg/kg and belonged mainly to *Lb. paracasei* species but also to *Lc. lactis*, *Lb. plantarum*, *Pc. pentosaceus, Lb. rhamnosus,* and *Sc. thermophilus*. The ability to produce GABA has been reported in various LAB, in particular *Lactobacillus* sp. isolated from fermented food [37]. *Lc. lactis* and *Sc. thermophilus* were also found to produce GABA in different Italian cheeses screened by Siragusa et al. [15] and *Pc. pentosaceus* was isolated as high GABA producing strain from a Thai fermented meat [38]. From our screening, no *Ec. faecalis* or *Sc. macedonicus* strain was able to produce GABA (less than 0.25 mg/kg) and, to our knowledge, these species have never previously been identified as GABA producers.

GABA is a desired bioactive compound because of its physiological functions such as neurotransmission, induction of hypotension, diuretic and tranquilizer effects, and stimulation of immune cells [10–12]. For these beneficial effects, GABA has been introduced in the diet as an oral supplement; the Japanese government defines the foods enriched with GABA as "foods for specified health use" [3]. Fermented milk enriched in GABA produced by lactobacilli may have commercial potential as a health-oriented dairy product.

Siragusa et al. [15] observed that *Lb. paracasei*, *Lb. delbrueckii* subsp. *bulgaricus*, *Lc. lactis*, *Lb. plantarum*, and *Lb. brevis* strains isolated from different Italian cheese varieties

Table 3: Number of isolates and species found at each of the sampling time in traditional alpine cheeses ($n = 6$).

Ripening time	*Lb. paracasei*	*Sc. thermophilus*	*Ln. mesenteroides*	*Ec. faecalis*	*Lc. lactis*	*Pc. pentosaceus*	*Lb. acidipiscis*	*Sc. macedonicus*	*Lb. casei*	*Lb. coryniformis*	*Lb. rhamnosus*	*Lb. curvatus*	*Lb. plantarum*	*Lb. delbrueckii*	Total dominant isolates
24 h	—	19	—	—	6	—	—	2	1		—	—	—	—	28
15 d	20	8	3	1	1	1	—	—	4	—	2	—	3	—	43
1 month	20	10	12	3	2	2	—	—	—	—	2	5	—	—	56
2 months	14	10	13	8	—	2	—	4	3	2	2	1	—	1	60
3 months	15	3	13	1	—	5	—	5	2	3	—	—	—	—	47
6 months[a]	12	—	1	—	1	2	6	—	—	—	—	—	—	—	22
8 months[a]	7	—	3	—	3	—	5	—	—	2	—	—	—	—	20
TOT	88	50	45	13	13	12	11	11	10	7	6	6	3	1	276

[a]$n = 5$.

Table 4: GABA production (Mean ± sd) of the dominant clusters in sodium acetate buffer containing 7.0 mM L-glutamate after incubation for 24 h at the same temperature of the isolation medium. Clusters are ordered from the highest to lowest GABA producer.

Cluster	Species	Isolate number	Ripening time	Isolation medium and temperature	GABA production (mg/kg)
84C	***Sc. thermophilus***	**3**	**24 h, 1-2 months**	**M17 45**	**80.0 ± 2.7**
15C	***Lb. paracasei***	**4**	**3–8 months**	**MRS 30**	**14.8 ± 5.3**
21D-B	***Lb. rhamnosus***	**6**	**24 h, 1-2 months**	**MRS (30, VAN)**	**11.3 ± 0.72**
24D	*Lc. lactis cremoris*	2	24 h, 6 months	M17 30	9.0 ± 0.21
62B	*Pc. pentosaceus*	1	1 month	MRS 30	6.7 ± 0.61
80B	*Lb. plantarum*	2	15 d	MRS 30	5.2 ± 0.56
59B	*Lb. paracasei*	1	15 d	MRS VAN	4.9 ± 0.20
70B	*Lb. paracasei*	1	15 d	MRS VAN	4.6 ± 0.21
22B	*Lb. paracasei*	3	15 d	MRS (30, VAN)	4.2 ± 0.41
49B	*Lb. paracasei*	1	1 month	MRS 30	4.0 ± 0.8
54C	*Lb. paracasei*	1	3 months	MRS 30	4.0 ± 0.69
73D-C	*Lb. paracasei*	3	1–6 months	M17 30, MRS 30	4.0 ± 0.29
20B-C	*Lb. paracasei*	3	15 d, 3 months	MRS 30	4.0 ± 0.18
66B	*Lb. paracasei*	1	15 d	MRS 30	4.0 ± 0.18
77D	*Lb. paracasei*	18	1-2–6 months	M17 30, MRS (30–45, VAN)	3.7 ± 0.71
68B	*Lb. casei*	3	2 months	MRS 30	3.6 ± 0.32
25D	*Lc. lactis lactis*	1	15 d	M17 30	2.8 ± 0.10
71B	*Lb. paracasei*	1	15 d	MRS VAN	2.5 ± 0.52
28B	*Lb. paracasei*	1	15 d	MRS VAN	2.5 ± 0.44
69B	*Lb. paracasei*	1	15 d	MRS VAN	2.4 ± 0.31
34C	*Lb. paracasei*	1	15 d	MRS VAN	2.3 ± 0.37
50D	*Lb. paracasei*	10	15 d, 2-3–6–8 months	M17 30, MRS (30, VAN)	2.3 ± 0.25
76D	*Lb. casei*	5	24 h, 15 d	MRS 30	2.2 ± 0.71
36C	*Lb. paracasei*	3	1-2 months	M17 30, MRS 30	2.2 ± 0.41
58C	*Lb. casei*	2	3 months	MRS VAN	2.2 ± 0.14
75C	*Lb. paracasei*	1	1 month	M17 30	2.2 ± 0.14
51B	*Lb. paracasei*	2	15 d	MRS 30	2.1 ± 0.96
72D	*Sc. thermophilus*	2	2 months	MRS 30	2.1 ± 0.11
65B	*Lb. paracasei*	1	15 d	MRS 30	1.9 ± 0.69
38C	*Lb. paracasei*	1	2 months	MRS 30	1.8 ± 0.40
79B	*Lb. paracasei*	2	1–3 months	MRS 30	1.6 ± 0.39
52B-C	*Lb. paracasei*	5	15 d, 2–6 months	MRS (30, VAN)	1.6 ± 0.32
37D	*Lb. paracasei*	1	1 month	MRS VAN	1.6 ± 0.15
4B	*Lb. paracasei*	2	2–6 months	M17 30, MRS 30	1.5 ± 0.13
78B	*Pc. pentosaceus*	2	1-2 months	MRS 30	1.46 ± 0.08
53D	*Lb. curvatus*	5	1 month	MRS (30, VAN)	1.4 ± 0.98
14C	*Pc. pentosaceus*	2	3 months	MRS VAN	1.4 ± 0.89
29D	*Lb. coryniformis ssp. torquens*	2	8 months	MRS 30	1.4 ± 0.11
35C	*Lb. paracasei*	4	1 month	MRS (30, VAN)	1.33 ± 0.066
32C	*Lc. lactis lactis*	4	24 h	MRS 30, M17 45	1.3 ± 0.37
31C	*Lb. paracasei*	1	2 months	MRS VAN	1.3 ± 0.13
17C	*Lb. paracasei*	4	2–8 months	MRS (30, VAN)	1.2 ± 0.94
74D-C	*Lb. paracasei*	1	2 months	MRS 30	1.1 ± 0.22
55C	*Pc. pentosaceus*	2	3 months	MRS 30	1.06 ± 0.044
43B	*Pc. pentosaceus*	2	6 months	MRS 30	1.00 ± 0.099
23B	*Lb. paracasei*	3	15 d, 3 months	MRS VAN	1.0 ± 0.54
67B	*Lb. paracasei*	1	1 month	MRS 30	1.0 ± 0.15
5B	*Lb. paracasei*	1	3 months	MRS 30	1.0 ± 0.12
61C	*Lb. paracasei*	2	8 months	MRS 30	0.88 ± 0.081

Table 4: Continued.

Cluster	Species	Isolate number	Ripening time	Isolation medium and temperature	GABA production (mg/kg)
39C	*Ln. mesenteroides*	5	3 months	MRS (30, VAN)	0.8 ± 0.64
18C	*Lb. paracasei*	3	3 months	MRS (30, VAN)	0.8 ± 0.20
60C	*Pc. pentosaceus*	3	15 d, 2-3 months	MRS (30, VAN)	0.72 ± 0.058
19D	*Lb. coryniformis ssp. torquens*	3	2-3 months	MRS (30, VAN)	0.6 ± 0.12
95B	*Sc. thermophilus*	1	15 d	M17 45	0.6 ± 0.10
94B	*Sc. thermophilus*	2	15 d, 2 months	M17 45	0.57 ± 0.071
40C	*Ln. mesenteroides*	6	1-2 months	MRS (30, VAN)	0.57 ± 0.013
8C	*Sc. thermophilus*	1	15 d	M17 45	0.56 ± 0.045
45D-C	*Ln. mesenteroides*	18	15 d, 1-2-3–8 months	M17 30, MRS (30, VAN)	0.54 ± 0.060
3B	*Sc. thermophilus*	2	24 h	M17 45	0.52 ± 0.041
26D	*Lb. paracasei*	1	8 months	M17 45	0.50 ± 0.42
83C	*Sc. thermophilus*	5	15 d, 3 months	M17 45	0.50 ± 0.046
87C	*Sc. thermophilus*	2	24 h	M17 45	0.5 ± 0.24
44D-C	*Ln. mesenteroides*	5	2–6 months	M17 30, MRS (30, VAN)	0.48 ± 0.017
42B-C	*Ln. mesenteroides*	2	1 month	MRS 30	0.4 ± 0.13
64C	*Lb. plantarum*	1	15 d	MRS 30	0.39 ± 0.027
63C	*Ln. mesenteroides*	2	1 month	M17 30	0.37 ± 0.033
86D	*Sc. thermophilus*	1	3 months	M17 45	0.35 ± 0.055
46D-C	*Ln. mesenteroides*	2	3 months	M17 30. MRS 30	0.34 ± 0.072
1D	*Sc. thermophilus*	2	15 d	M17 45	<0.25
2D	*Sc. thermophilus*	1	15 d	M17 45	<0.25
6C	*Ec. faecalis*	10	15 d, 1-2-3 months	M17 30-45	<0.25
7C	*Sc. thermophilus*	2	24 h	M17 45	<0.25
9D	*Lb. coryniformis ssp. torquens*	2	2 months	M17 30	<0.25
10C	*Sc. macedonicus*	3	3 months	M17 45	<0.25
11C	*Sc. macedonicus*	4	2 months	M17 45	<0.25
12D	*Sc. macedonicus*	2	24 h	M17 30-45	<0.25
13D	*Sc. macedonicus*	3	8 months	M17 45	<0.25
16D	*Lb. acidipiscis*	11	6–8 months	MRS (30, VAN)	<0.25
27B	*Sc. thermophilus*	1	2 months	M17 45	<0.25
30D	*Ec. faecalis*	1	2 months	M17 30	<0.25
33C	*Lc. lactis cremoris*	1	24 h	M17 30	<0.25
41D	*Ec. faecalis*	2	2 months	M17 30	<0.25
47C	*Ln. mesenteroides*	3	15 d, 2 months	M17 30	<0.25
48B	*Lc. lactis cremoris*	2	1 month	M17 30	<0.25
56D	*Sc. macedonicus*	2	3 months	M17 45	<0.25
57D	*Ln. mesenteroides*	2	1 month	M17 30	<0.25
81B	*Lb. delbrueckii*	1	2 months	MRS 30	<0.25
82B	*Sc. thermophilus*	5	24 h, 1 month	M17 45	<0.25
85D	*Sc. thermophilus*	3	1 month	M17 45	<0.25
88C	*Sc. thermophilus*	3	2 months	M17 45	<0.25
89C	*Sc. thermophilus*	3	24 h	M17 30	<0.25
90B	*Sc. thermophilus*	1	24 h	M17 45	<0.25
91B	*Sc. thermophilus*	5	1-2 months	M17 45	<0.25
92B	*Sc. thermophilus*	1	1 month	M17 45	<0.25
93B	*Sc. thermophilus*	1	1 month	M17 45	<0.25
96B	*Sc. thermophilus*	3	24 h	M17 45	<0.25
97C	*Sc. thermophilus*	1	24 h	M17 45	<0.25

were the best GABA-producers during the fermentation of reconstituted skimmed milk. A *Lb. casei* and *a Lc. lactis* subsp. *lactis* were used for the manufacture of a GABA-enriched fermented milk: the first strain hydrolyzed milk protein into glutamic acid and the second converted glutamic acid into GABA, respectively [39]. This current study suggests a real potential of the *Sc. thermophilus* isolate from cluster 84C to produce GABA in fermented dairy products. A daily intake of fermented milk with an amount of 10 mg of GABA for 12 weeks has been shown to decrease blood pressure by 17.4 Hg in hypertensive patients [39]. *Sc. thermophilus* belonging to the cluster 84C in this current study produces 80 mg/kg of GABA. 125 mg of milk fermented with this strain could, therefore, be enough to obtain the daily intake necessary for a potential antihypertensive effect observed by Inoue et al. [39].

We are now examining the ability of this *Sc. thermophilus* strain to produce GABA in fermented milk, either alone or in association with other milk protein hydrolyzing LAB and under simulated gastrointestinal conditions.

5. Conclusions

This study describes the diverse lactic microbiota of traditional semihard "*Nostrano*-cheeses" from the Trento alps in Italy and how this microbiota changes during ripening. We have also characterised the potential of selected LAB isolates to produce GABA under controlled conditions, a molecule newly recognised as a putative food bioactive. We identified one *Sc. thermophilus* strain as a "high GABA producer" with considerable biotechnological potential for the development of new and attractive dairy products, an important commercial objective for increasing the potential of cheese as *multifunctional* dairy product.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Review Article

Biocheese: A Food Probiotic Carrier

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This review describes some aspects related to the technological barriers encountered in the development and stability of probiotic cheeses. Aspects concerning the viability of probiotic cultures in this matrix are discussed and the potential of cheese as a biofunctional food carrier is analyzed, outlying some points related to health and safety. In general, the manufacture of probiotic cheese should have little change when compared with the elaboration of cheese in the traditional way. The physicochemical and technological parameters influencing the quality of these products have also to be measured so as to obtain a process optimization.

1. Introduction

Functional foods are those which contain some health-promoting components which go beyond the traditional nutrients [1]. One way in which foods can be modified to become functional is by adding probiotics. A probiotic food is a processed product which contains viable probiotic microorganisms in a suitable matrix and in sufficient concentration [2].

Traditionally, the most popular food delivery systems for probiotic cultures have been freshly fermented dairy foods such as yoghurts and fermented milks. However, their survival and viability may be adversely affected by processing conditions as well as by the product environment and storage conditions. From a regulatory point of view, probiotic population must be stated on the product label [1]. Most current national legislations establish minimum viable quantities of 10^6-10^7 CFU/g or CFU/mL of probiotic cultures present in the food taking into account a daily consumption of 100 g or 100 mL. In order for a cheese to be recognized as probiotic, appropriate probiotic added microorganisms have to retain quantity and quality throughout the process steps involved in the manufacture, which is not all that easy, beginning with the fact that competition will more than likely be exerted by starter cultures and ending with the challenge to obtain a proper delivery in the gastrointestinal tract (GIT).

For products such a cheese where the probiotic displays an active metabolism the stability depends on the inherent aptitudes of the strain involved and on the physical properties of the matrix. The matrix has a large impact on the probiotic viability and shelf-life.

Many compositional and process factors significantly affect the viability of probiotics in cheese including the kind and the amount of probiotic inoculation, flavouring supplementation, microbiota competition, possible presence of bacteriocines or other antimicrobials, pH, redox potential, incubation and storage temperature, salt and water activity and packaging materials, and other factors.

It is apparently clear from the data collected that survival fitness is linked to a particular strain and not to a particular species or genus. Therefore, it is crucial to study case by case to see whether the characteristics are properly maintained in the cheese matrix or not. An illustrative generic example could be *Lactococcus lactis* ssp. *lactis*, which is a candidate probiotic for use in aquaculture [3]. The significative higher halotolerance in strains from marine fish compared to those used as cheese starter indicates that each strain has adapted to its particular environment confirming the need to carry out a careful strain selection depending on the purpose. It is hard to visualize how the behaviour of a culture will turn out. Therefore, in-depth studies have to be carried out to ensure proper characteristics. Hence, it is mandatory to confirm the stability in order to ensure that the characteristics are retained.

The incorporation of probiotics into a wide range of food products is conditioned by the food matrix. Probiotic bacteria

have been incorporated into a wide range of foods, including dairy products (such as yoghurt, cheese, ice cream, desserts, cultured milks, or pasteurized unfermented milk). Although dairy products are currently the most common platform for delivery of probiotics we should remember that probiotics are also sold in nondairy products (such as chocolate, cereals, and juices) and other different non-food-related formulations. Either way the viability of cells is of crucial importance because they have to stay alive until reaching their action site. Many reports indicated that there is poor survival of probiotic bacteria in products containing free probiotic cells [5].

There are still people who debate whether the beneficial probiotic cultures actually survive in cheese. Many cheeses are presently being developed as probiotic and scientific data show that the probiotic culture is still present in numbers high enough to be marketed as such. The microencapsulation would seem to offer a good technological alternative for use in the cheese industry [6] receiving considerable interest. However, this increases the cost [7]. Based on the level of probiotic bacteria needed to provide a health benefit it should be possible to lower the amount that needs to be ingested in cheese by up to a factor of 10^3 compared with other fermented dairy foods or when consumed as supplements [8]. The panoply of probiotic cheese available will increase significantly in the near future, although many of these will not be sold on the market even if they do have additional advantages such as lower fat or cholesterol content because they will not have a great flavour nor will they show desirable characteristics, for example, in rheology.

Most studies of strains being used as probiotics are based on functional properties and less knowledge is available concerning their capacity to withstand stress related to food manufacturing and storage. Cheese as a probiotic food carrier represents a good choice for the dairy industry showing potential advantages over other dairy fermented products but it is also a technological challenge at the same time. Strain viability and maintenance of desirable characteristics during processing steps and storage are a must to assure a beneficial effect [9]. In this review some of the aspects related with the probiotic strains and food technology parameters involved in the elaboration of probiotic cheese are discussed. Some trends and perspectives for the near future are also discussed.

2. Probiotic Strains: Culture Production and Viability in Cheese

In practice, probiotic *Lactobacillus* spp. and *Bifidobacterium* spp. are the most common microorganisms included in cheese. Because of their physiology, they are very well suited to this matrix. Of course bacteria other than these may play essential roles in probiotic cheeses [10]; therefore other microbial candidates are expected to and will surely increase the number in the future (see Section 4). Table 1 shows a noninclusive list of the most relevant species/subspecies used or to be used as probiotics in cheese.

It is important to emphasize that an initial and frequently serious problem for any probiotic strain to be included in a dairy food elaboration is the large-scale biomass production [11]. Although it is not an aim of this review to focus on the probiotic production, it is noteworthy to mention that scale-up has to solve appropriate fermentation processes, adequate inexpensive ingredients, and growth conditions as well as tests for the presence of possible allergens. The biomass is then concentrated by centrifugation or ultrafiltration and preserved using freeze-drying steps and cryoconservants to maximize later cell recovery. Final steps include proper milling and packaging.

Table 1: Most relevant species/subspecies of probiotic bacteria successfully added to cheese. *Species including candidate strains or of possible potential use as probiotics. P = *Propionibacterium*. Data collected and adapted from Karimi et al. [4].

Lactobacillus	*Bifidobacterium*	Others (∗)
L. acidophilus	*B. animalis*	*Enterococcus faecalis*
L. casei	*B. animalis* ssp. *lactis*	*E. faecium*
L. casei ssp. *pseudoplantarum*	*B. breve*	*Lactococcus lactis*
L. casei ssp. *rhamnosus*	*B. infantis*	*Leuconostoc paramesenteroides*
L. delbrueckii ssp. *bulgaricus*	*B. lactis*	*P. freudenreichii* ssp. *shermanii*
L. delbrueckii ssp. *lactis*	*B. longum*	*Streptococcus thermophilus*
L. gasseri		
L. paracasei		
L. plantarum		
L. rhamnosus		
L. salivarius		

Despite the fact that a particular strain of interest for cheese making can be available in sufficient amounts to cover industrial requirements, it has to be stable irrespective of whether it is designed as a starter culture or a probiotic supplement or an adjunct culture. In fact, they all have many properties in common since they obviously can play different roles. Starter cultures are used at one or more stages in the cheese manufacturing process, which develop the desired metabolic activity during the fermentation or ripening which confer unique characteristics mainly taste, aroma, colour, texture, safety, preservation, nutritional value, and, perhaps, possible health benefits according to the definition of microbial food culture by the European Food and Feed Cultures Association (http://www.effca.org/).

One of the traditional trends in cheese research has been to screen among the existing starter or nonstarter strains and determine whether they could have potential health benefits. The survival of the autochthonous microbiota of samples collected during Pecorino di Carmasciano cheese manufacturing was evaluated when a model mimicking the GIT was used. One of the conclusions was very suggestive; the bacterial survival appeared to be more affected by experimental conditions than strain inherent; thus while some strains showed an acceptable survival when resuspended in skim milk, but not in ewe's milk, the opposite was seen for others [12]. The results support the idea that a screening among

autochthonous bacteria with this aim is feasible and possibly useful. However, probiotic strains are most frequently of intestinal origin where they are expected to exert health benefits.

At first glance, the use of GIT strains might be considered a handicap for cheese making since it is hard to manufacture specific cheeses with peculiar and genuine properties when large amounts of adjunct cultures are added. GIT strains are commonly oxygen sensitive and complex nutrients demanding. This represents one of the important reasons why for a new strain it is essential to show reasonable survival behaviour when added during the cheese manufacturing process and also to check effects on carbohydrate, protein, and fat usage as well. A good survival of probiotic microorganisms in simulated gastrointestinal conditions of probiotic strains added to cottage cheese was found as well as a good metabolic behaviour and the generation of potentially antioxidant peptides and antilisterial activity [13].

There are techniques adapted to enhance the viability of probiotic bacteria in cheese including the selection of oxygen-tolerant, acid-tolerant, and bile-resistant strains, but it must always be kept in mind that one of the most important aspects, from the food technology point of view, is the need to develop good sensory properties without changing textures or flavours. It is clear that many of the properties are inherent to the particular strain, but long-term industrial processing and storage conditions may influence them. Thus both technologically relevant and functional properties should be taken into consideration in quality-control measures [14].

Cheese compared to other fermented products shows a better buffering capacity for probiotics, less water activity (usually >0.90) depending of the ripening time, and a low storage temperature (4–8°C) with a storage time of weeks or even years. These values are very variable according to the type of cheese considered; that is, water activity (a_w) during first stages of cheese manufacture is >0.99, which it is suitable for the growth and activity of the starter culture. After whey drainage, salting and during ripening the prevailing a_w levels are increasingly lower and below the optimal requirements for most starter bacteria. Therefore, more than likely a_w levels contribute to the control of their metabolic activity and multiplication. Some lactic acid bacteria (LAB) generally display higher a_w than other cheese bacteria such as *L. lactis*, *S. thermophilus*, *L. helveticus,* and *P. freudenreichii* ssp. *shermanii* (displaying optimal a_w values of between 0.93 and 0.98) [15]. High values should promote cell counts probiotic maintenance at least for fresh or short ripened cheeses. The water loss through evaporation, the salting stage, and the hydrolysis of proteins and triglycerides cause a fall in a_w throughout ripening. The solution to control moisture loss is usually the increase of the relative humidity in the ripening chambers or the packaging in wax or plastic. Moreover, the lack of homogeneity could be a problem as different in-depth areas of the cheese show different a_w values. It is well known for many cheeses such as Camembert that the pH increases continuously during ripening from acidic to slightly alkaline, whereas a_w decreases displaying a wide range of values depending on the particular nonstandardized conditions used. In general, brine-salted hard and semihard cheeses show higher values towards the centre, whereas in cheddar cheese no loss of moisture and no change in a_w values occur since the salt is uniformly distributed in the cheese and it is vacuum packed. Most probiotic cheeses are protected by a proper wrapping to ensure moisture content; this circumstance does not in any way exclude the requirement to systematically analyse these parameters for the many different kinds of the proclaimed probiotic cheeses present worldwide.

3. Cheese Making and Probiotics

Cheese manufacture is essentially a dehydration of milk combined with other preservative effects, such as culturing, salting, packaging, ripening, and/or storage. Some major hurdles associated with the addition and viability maintenance of probiotic bacteria in the development and processing of functional cheese are discussed here.

3.1. Milk Culturing, Probiotic Inoculation, and Cheese Processing. Milk is where the history of cheese begins. In large-scale cheese manufacture, the milk is generally pasteurized, for example, 73°C for 15 seconds. It has been described that non-starter lactic acid bacteria (NSLAB) can survive pasteurization at low numbers and slowly grow during cheese ripening up to 10^6-10^7 CFU/g, depending on the ripening period and temperature [16]. Most NSLAB species are lactobacilli, pediococci, and micrococci. This is interesting because at least in the first two cases diverse species involved include NSLAB as well as probiotic strains, that is, *L. casei*, *L. paracasei*, *L. plantarum*, and so forth. The use of both kinds of strains could improve flavour intensity of the cheese and provide suitable technological properties such as longer storage periods. The addition of *L. plantarum* I91 and *L. paracasei* I90 as selected strains of NSLAB exerted a technological and probiotic role in the elaboration of cheese showing satisfactory properties for their use as adjunct cultures, achieving the dual role of being secondary starters and probiotic cultures [16].

An alternative to the traditional thermal milk treatment is a hyperbaric treatment through high-pressure homogenization (HPH). This approach was used in the elaboration of Crescenza cheese using *S. thermophiles* as a starter and commercial probiotic lactobacilli [17]. The authors carried out compositional, microbiological, physicochemical, and organoleptic analysis from 1 to 12 days of refrigerated storage (4°C). No significant differences were found in comparative analysis with adequate cheese controls for gross composition and pH. On one hand, there was a good technological behaviour since HPH-milk increased the cheese yield to about 1% and positively affected the viability during the refrigerated storage of the probiotic bacteria. On the other hand a significant positive effect on free fatty acids release and cheese proteolysis was observed. No significant differences were found for diverse sensory descriptors.

The use of ultrafiltered milk (UF) in cheese making is reasonably well established and attracting considerable worldwide attention. Probiotic Iranian ultrafiltered feta cheese was produced by inoculating the heat treated retentate with a probiotic *L. casei* strain [18]. UF cheese has been traditionally

produced as full-fat cheese, but lately UF cheeses are low-fat dietetic products. A reduced fat UF cheese was manufactured following the established production procedure by mixing milk protein powder, skim milk, and cream with adjunct probiotics [19]. The authors found enhanced secondary proteolysis, maintenance of adjunct culture population over a two-month ripening period, a remarkably improved aroma compared with the control, and an overall high count of probiotic *L. acidophilus* throughout the ripening period.

A combined addition of probiotic bacteria and starter culture requires testing appropriate proportions to solve viable probiotic loss during draining. Probiotics can be added as a primary starter or adjunct culture. In the first case, the low capability of probiotics to generate lactic acid during fermentation could be considered a handicap while a joint addition would be a more suitable solution.

Two-step fermentation for cultured dairy products has been shown to be effective in increasing the viability of probiotic bacteria by allowing probiotics to become dominant prior to the addition of the starter cultures. Since starter bacteria could produce inhibitory substances against probiotic bacteria and grow quicker during fermentation, the viability of probiotics could be reduced. Fermentation with probiotic bacteria initially for 2 h followed by fermentation with starter cultures may be helpful in improving the viability of the former and result in higher counts. This has allowed the probiotic bacteria to be in their final stage of lag growth phase or early stage of log phase and thus could dominate the microbiota, resulting in higher counts. The initial counts of probiotic bacteria have been found to increase by four to five times in the product elaborated by the two-step fermentation process. The probiotic bacteria could also be totally added at the end of fermentation [14].

Two types of inoculation methods were compared [20]; in one type of experimental cheese, probiotic bacteria were directly added to the milk as a lyophilized culture, while in the other they were preincubated in a substrate composed of milk and milk fat and then added to the milk. As a result, the direct addition as a lyophilized culture was considered more efficient as direct addition was easier, quicker, and less vulnerable to contamination. Although preincubation in the substrate increased the probiotic population in the inoculum by almost one log cycle, which can be considered more cost-effective for industry, the addition of probiotics after preincubation in the substrate did not improve their survival during cheese ripening. The substrate did not only enhance the protection of probiotic bacteria; but it was also a more complex methodology than direct addition of lyophilized culture. Firstly, it was more time consuming and secondly preincubation could be a sensitive step when considering issues related to contamination and phage attack.

In the case where the probiotic is added later than the starter, a cooling step is normally included to reduce both metabolic activities; later coagulant agents such as lemon juice, plant rennet, or proteolytic enzymes such as chymosin (rennin) or even mold derived are added. The coagulation then occurs under controlled temperature conditions when the previously mentioned enzymes display optimum activity. The slightly acidic environment under which LAB releases enough lactic pH is reduced creating an appropriate environment for optimum activity of rennin. As the processing continues, lower values create a nonsuitable atmosphere for unwanted microorganisms.

There are some serious problems to solve in order to obtain survival improvement of probiotics and procedures to help probiotics overcome the above-mentioned hurdles. Probiotics are also very often placed into cheese in slightly different ways from those present in industrial protocols. One clear trend is the microencapsulation (ME) of probiotic bacteria. Alginate-based or other types of coatings are valid carriers of probiotics and prebiotics because of their nontoxicity, biocompatibility, and low cost [21]. For example, cells immobilized in calcium alginate gels have been added to Crescenza cheese in an effort to improve the survival of bifidobacteria in the final product [22].

Lamb rennet pastes containing encapsulated *L. acidophilus* and a mixture of *B. longum* and *B. lactis* were designed for the manufacture of Pecorino cheese from Gentile di Puglia ewe's milk [23]. On one hand, *L. acidophilus* retained its viability for a few days and then showed a quick reduction. On the other hand, *B. longum* and *B. lactis* showed an initial death slope, followed by a tail effect owing to acquired resistance. After an initial period in which the lowest levels were observed, the highest levels were reached after one month of ripening and then remained so until the end for *L. acidophilus*, whereas bifidobacteria underwent a decrease of about 1 log CFU/g. Greater enzymatic activities and positive correlation were found between enzymatic activities and water-soluble nitrogen and proteose-peptone in probiotic cheeses because of its release from alginate beads.

In another interesting study a fairly good survival rate was obtained using the alginate-microencapsulation of a probiotic *L. paracasei* ssp. *paracasei* strain during the manufacture of Mozzarella cheese, a *pasta filata* cheese in which the curd was heated to 55°C and stretched in 70°C-hot brine followed by a 6-week storage at 4°C [8].

The probiotic cheddar cheese deserves a special mention as it is currently the most widely produced and consumed hard cheese in the world. Different reports indicate, yet again, that different species/strains of *Lactobacillus* and *Bifidobacterium* display different survival ability, although some reports clearly describe that factors such as salt, oxygen, and temperature negatively affect the viability [24].

An improvement of survival from freezing and simulated gastrointestinal conditions of *B. longum* 15708 was confirmed by ME in alginate beads although salting of the curd had a negative effect [25]. The authors observed a 100 times lower viability loss with ME during the technological steps. However, there was a 2 log CFU/mL reduction after 21 days of storage, still unsuitable levels for commercial leading purposes. A clear conclusion is that some probiotics could be highly sensitive cultures. Results were promising since polymers produced showed a relatively good survival as compared to *B. longum* free cells with 3-4 log CFU/mL reductions in addition to an increased resistance to simulated gastric and intestinal environments by a factor of 30.

A sensory acceptance for any food must be ensured after an initial interest for health claims by the consumer

[26]. Thus, it is important to use probiotic bacteria with mild acidifying ability to prevent an excessive formation of organic acid. An excessive proteolysis is also linked to inadequate storage and ripening temperature, which in turn could change the organoleptic properties of the final product [4].

Probiotic cultures may change the flavour or texture sometimes in a positive way, as has been reported in petit-Suisse cheese [27]. It has been published elsewhere [28] that probiotic bacteria should remain viable but not metabolically active as reported with *B. longum* in cheddar cheese without affecting their sensory properties. It is of course possible to develop probiotic dairy foods with similar acceptance as conventional products. The addition of increasing amounts of probiotics is apparently a simple solution to ensure a proper microbial viability. Either way sensorial analysis has to be made along with other analyses as well. No negative effect on gastrointestinal welfare was observed in an animal model after an intake of probiotic semihard Edam-type cheese containing *L. plantarum* at a daily dose of 10 log CFU for 3 weeks but the consumption of 100 g/d caused hard stools from the second week of the assay [29].

The processing of cheese can also be affected when a high level of supplementation is used. Some reports have shown a few negative sensory effects with a probiotic *L. acidophilus* strain during the processing of Minas fresh probiotic cheese when high counts (>9 log CFU/g) were present throughout shelf-life [30]. The probiotic cheese presented lower pH values and a greater production of organic acids but lower scores for appearance, aroma, and texture. The same authors have reported that the development of a probiotic cheese requires the handling of different technological options to guarantee a proper functionality throughout during shelf-life.

Some manufacturing procedures include a heating or a cooking stage of the curd. A heating generally between 37 and 45°C affects the rate at which whey is expelled as well as the growth of the starter culture. Curds and whey are often stirred to separate particles. Once curd particles have become firm and a correct acid development has taken place, the whey is removed allowing the curd particles to join together. Once the curd has reached the desired texture it is broken up into small pieces to enable it to be salted in cheeses such as cheddar. Milling the curd can be carried out either by hand or mechanically. In Fior di Latte cheese manufacturing after a proper curd-ripening phase, the drained curd is stretched in hot water. A previous selection of heat-resistant probiotic lactobacilli resulted in a good choice to obtain adequate survival rates under heat conditions, which mimicked the stretching of the curd. After a screening to heating resistance (65 or 55°C for 10 min) in 18 probiotic strains the addition of specific probiotic heat-adapted *L. delbrueckii* ssp. *bulgaricus* and *L. paracasei* strains enhanced shelf-life and cheese flavour formation [31].

Cottage cheese is an unripened, particulate, and acidic cheese made from skim milk. The curd is cut and heated to 55°C; then a cream and salt dressing is added as well as the probiotic. This procedure appeared to be desirable once the adverse effects of the high scalding step were avoided. It is also important to consider their physiological state in order to form an idea of their survival throughout ripening and/or storage. In terms of the growth curve, microbial cells between the late exponential and the stationary phase are the favourite option and the preparation of a previous substrate to inoculate the strain may be sometimes beneficial.

The form of the probiotic inoculants and their viability and maintenance represents an important technological challenge. Milk powder containing a probiotic *L. paracasei* strain as adjunct starter was spray-dried during cheddar cheese manufacturing with a low loss of viability and no adverse effects after three months of ripening [32]. In a semihard cheese the use of either a freeze-dried powder dispersed in milk or a substrate containing milk and milk fat has been proposed for improving survival of probiotic bacteria [33].

3.2. Salting and Packaging. It is well known that probiotic bacteria are sensitive to high salt concentrations. The viability of probiotic bacteria decreased drastically in cheeses with salt concentration of over 4% [18]. This implies the need to optimize production conditions in order to incorporate functional characteristics. Almost without exception a dry salting of the milled curd, a surface dry salting after moulding or a brine immersion [34] are used in cheese elaboration after rennet coagulation and curd formation to enhance taste of the curd, safety, and shelf-life. Possible solutions include microencapsulation, cell incubation under sublethal conditions, and careful strain selection. This has to be carried out without negative effects on texture, aroma, and/or acceptance by the consumers. An excellent review on the encapsulation applications in probiotic dairy products and cheese technology is now available [35].

The viability of encapsulated probiotic *B. bifidum* BB-12 and *L. acidophilus* LA-5 was studied in white brined cheese and using Na-alginate by either an extrusion or an emulsion technique. The authors found effective both techniques being the probiotic population higher than the therapeutic limit [36]. The counts for nonencapsulated and microencapsulated probiotic bacteria decreased approximately by 3 and 1 log, respectively. In other cases, results were not so promising. The microencapsulation of probiotic *L. acidophilus* DD910 and *B. lactis* DD920 in calcium-induced alginate-starch capsules did not improve their viability in a Feta cheese matrix during storage in brine solution, possibly because of the high salt concentrations [37].

While the viability of probiotics in dry salted cheese varieties has been well documented [38], limited data are available on the probiotic viability in cheeses salted with NaCl and KCl mixtures. New salting procedures include the possibility to substitute at least partially NaCl with KCl. This was shown in Akawi cheese with probiotic bacteria for 30 days of storage at 4°C without apparent significant differences in sensory attributes among experimental Akawi cheeses at the same storage period [39]. The addition of KCl enhanced syneresis in probiotic Iranian Feta cheeses (3% salt and 3-month ripening period) and only those with a 25% replacement by KCl had similar sensory acceptability to those containing NaCl alone [3]. Very similar results were previously reported in Minas fresh cheese [38].

Most probiotic strains are microaerophilic and anaerobic. For this reason permeability to oxygen exposure during manufacturing and storage is an important issue and to choose a suitable packing and vacuum system is relevant. Probiotic cheese is usually packaged in plastic films with low permeability to oxygen or by using vacuum based procedures. An interesting review on packaging systems has already been published [40]. For the elaboration of Turkish white cheese best scores in flavour and texture were detected when probiotic cheese (including *L. acidophilus*) was vacuum packed following salting compared to the control stored in brine following salting [41].

3.3. Ripening and Storage. The ripening process is example of a complex biofilm development in which microbiological and biochemical changes occur in the curd mainly related to the metabolism of residual lactose, lactate, and citrate besides lipolysis and proteolysis [42]. Again a major concern is the probiotic survival over a long essential ripening period devoted to the development of aroma and flavour by the activity of many enzymes. The presence of ripening periods during cheese processing is an additional problem for the stability of a probiotic culture as it is not easily predicted due to the biochemical changes which occur as water activity decreases, often together with further decreases in pH, creating an unsuitable environment for the adjunct cultures. Again a possible solution is the ME and the careful optimization of the ripening conditions.

An additional problem is the proliferation of other nonpathogenic adventitious populations which often become the dominant microbiota in cheese; thus NSLAB establishes a competition for nutrients which makes the quantitative determination of probiotic viability more difficult. Lactobacilli and pediococci represent some of the few contaminant bacteria capable of growing in cheese after manufacture as NSLAB. Both as starter or as NSLAB, these may play different roles in the primary metabolic events during cheese ripening with the proteolysis being the major and most complex biochemical event taking place in most cheese varieties. The casein is broken down into low molecular weight peptides and amino acids. This happens while the cheeses are stored under controlled temperature and humidity conditions.

Generally speaking, probiotic bacteria enzymes act mainly in the secondary proteolysis increasing the aminoacid pool, which contributes decisively to cheese flavour and could be precursors for the synthesis of other flavours or volatile aroma, resulting in off-flavours [43]. On the contrary, lipolytic enzymes have a lower activity when compared to starters and NSLAB. Nevertheless, there are already many examples of probiotic cheeses developed with minimum, even undetectable changes in proteolytic and lipolytic profiles, exerting a positive effect on the overall quality of the cheese as well as the production of bioactive peptides [44].

Cheese companies should provide the required viable probiotic population when the product is sold but also guarantee that this situation continues throughout a time-labelled storage. Probiotic cultures could produce antimicrobial substances thus contributing to inhibit development of pernicious microbiota and subsequently acquire a prolonged shelf-life. In other cases preservative agents such as NaCl are added. Nonadequate temperature storage, for example, 12°C, often present in many retailed food markets could reduce this population and increase consumer rejection due to changes in sensory qualities. Some publications, for example, report survival of probiotics through the relatively hard technological phases of pasta filata cheese production in the elaboration and ripening of Scamorza ewe milk cheese. However, texture and appearance attributes differentiated probiotic from control cheeses [45, 46]. Interestingly enough, the authors described specific criteria that should be implemented in order to monitor the quality of probiotic cheeses.

Studies of cheese as a source of new interesting isolates are quickly accumulating. For example, technologically relevant properties of candidate probiotic *L. plantarum* strains make them especially suitable for dairy products such as the long term survival at refrigerated temperatures, the growth viability in the presence of widely used preservatives, and the acidifying, coagulating, and enzymatic activities [47]. In another study a culture containing probiotic *L. fermentum* strains derived from human faeces was suitable and did not adversely affect Turkish Beyaz cheese quality in the four months of ripening [48].

Suitable probiotic properties can be screened *in vitro* before application; that is, potential probiotic strains from Feta, Kasseri, and Graviera cheeses were tested searching for those showing good levels of β-galactosidase, low proteolytic and coagulation activities, and antibacterial activities which could be properly exported to to get improvement during longer storage periods [49].

Other activities present in potential probiotics can be used to obtain prolonged ripening and improved storage such as antifungal and anti-Listeria activities as potential preservatives. This approach could provide useful elements for the development of probiotic adjunct cultures producing natural biopreservatives during food fermentations. For example authors detected antimicrobial and antifungal activities of *L. curvatus* strain isolated from homemade Azerbaijani cheese. These authors evaluated probiotic properties of this strain, as well as its safety regarding antibiotics resistance [50].

4. Trends and Perspectives

It is clear that the development of new probiotic cheese varieties and derived products will be the leading force in the near future. Some specific aspects and concerns are as follows.

4.1. Nutritional Facts. A leading focus in probiotic cheese development is based on the nutritional facts. There is an increasing demand for *diet* or *light* foods. A good example of this is the study of the influence of sweeteners in probiotic petit-Suisse cheese in concentrations equivalent to that of sucrose. Of great interest is the conclusion that none of the assayed sweeteners exerted negative consequences on the viability either on the starter or on the probiotics [51].

Manufacturing companies are providing consumers with cheese derived products containing reasonable levels of sodium, including natural cheeses, processed cheeses, dips, dressings, and spreads. A leading technology is to replace

sodium by potassium chloride, both reduce free water and microbial growth and the onset of pathogens. Traditional potassium chloride contributes to undesirable bitterness, but some formulations present in the market seem to overcome this drawback.

Only a few studies have considered the effect of probiotic adjuncts on fatty acids and conjugated linoleic acid (CLA) composition. It has been reported that LAB can produce CLA from linoleic acid, which is a biofunctional lipid [52].

A positive correlation between the CLA and linoleic acid contents of *L. paracasei* and *L. acidophilus* cheeses was observed when Pickle white cheeses with five different probiotic cultures have been studied [33]. The CLA content during the storage period increased because the lipolysis of free linoleic acid by LAB. Another interesting contribution was the elaboration of a probiotic caprine coalho cheese naturally enriched in CLA as a vehicle for *L. acidophilus* and beneficial fatty acids [52].

4.2. Safety Aspects. Strain safety will continue to be a concern especially in relation to young and elderly people. A premarket safety assessment of food microbes is based on four mainstays (establishing identity, body of knowledge, possible pathogenicity, and final use) according to the European Food Safety Authority (EFSA) [53]. A representative example is *Enterococcus faecalis* which includes commensal, starter culture, and probiotic strains [54]. However, *E. faecalis* is a highly diverse species that also includes pathogenic strains. A great emphasis on the importance and challenge of precisely characterizing strains from various sources has been made [55]. In fact, these authors have developed a typing scheme and found that a specific genetic cluster included most probiotic and cheese-derived strains. Therefore, strains clustered to this genetic group are more likely to have potential for safe usage as cheese starters and/or probiotics. Available data does not support inclusion of the genus *Enterococcus* within the Qualified Presumption of Safety (QPS) concept of the European Food Safety Authority (EFSA), as safety aspects cannot be determined at the genus or species level but have to be evaluated specifically for each strain [55, 56]. In these cases it seems to be clear that a case-by-case approach should be adopted.

The finding that enterococci are present as a normal microbiota in the human mammary gland during breast breeding [57] opens new interesting perspectives and the possibility to modify QPS status provided genetic fingerprinting techniques unambiguously guarantee the strain identification.

Pediococci and propionibacteria are also frequently used as cheese starters (mainly Swiss cheese or Emmental) and hence consumed in large quantities with apparently no side-effects. Some species/strains have a long history of apparent safe usage in the food chain and consequently some species/strains will be used for cheese manufacture.

4.3. Strain Screening. An area of active research is to search for strains showing desirable characteristics such as the ability to produce antimicrobial compounds, the absence of antibiotic resistances, and the capacity to survive the imposed technological hurdles during cheese manufacture. Almost all studies have been focused on *Lactobacillus* sp. and *Bifidobacterium* sp. but of course other bacteria may play essential roles in probiotic cheeses.

There is a recent and growing interest in the probiotic potential propionibacteria (PAB); they are known not only for their ability to produce propionic acid with antimicrobial properties particularly against fungi but also to produce a variety of bacteriocins as well, with a wider antimicrobial spectrum covering other Gram-positive bacteria including LAB, Gram-negative bacteria, and yeasts and filamentous fungi, in some cases [58]. The dairy species are involved in the ripening of the widely consumed Swiss-type cheeses such as Emmental where their concentration reaches 10^9 bacteria per gram. The dairy propionibacteria show technological properties very suitable for their uses as probiotics in cheese; that is, they display tolerance to technological stresses such as reconstitution in milk, fermentation of a wide range of carbohydrate substrates, microencapsulation, spray-drying, freeze-drying, and storage at low temperatures. Of particular interest is that the β-galactosidase activity remained after withstanding the cooking temperature of Swiss-type cheeses and remained stable during storage at low temperatures.

Propionibacteria in cheese have better tolerance to acid challenge than free cultures and they produce propionic acid, a natural biological acid which benefits the bifidus microbiota and displays a good constitutive survival under digestive stress [59]. However, supplementation with dairy propionibacteria has mainly involved mixtures with probiotic bacteria from other genera. There are available dietary supplement capsules designed to maintain the intestinal ecosystem balance which includes two *P. freudenreichii* strains (Sécuril, http://www.swansonvitamins.com) on the market. The use of propionibacteria as adjunct probiotic or in combination with LAB and/or bifidobacteria is a matter of time. It has been reported that a cheese containing a mixture of probiotics (lactobacilli and *P. freudenreichii* ssp. *shermanii* JS) reduces the risk of high yeast counts, especially *Candida* sp. in the elderly [60]. An excellent recent review on probiotic propionibacteria is available [61].

With regard to *Pediococcus*, only *P. acidilactici* and *P. pentosaceus* are relevant dairy strains found in cheeses as adventitious cultures occasionally used during cheese manufacturing. However, there is a potential interest in some of their properties such as the ability to produce antimicrobial compounds (pediocins) and the modification of the texture due to their capability of producing exopolysaccharides. Some probiotic candidates produce lactate crystal formation through the formation of a mixture of L- and D-lactate isomers which is normally considered as a cheese defect. Furthermore, the addition of pure bacteriocins has so far only a few and limited authorized uses in foods. In 1988, nisin produced by *Lactococcus lactis* received the US-FDA approval as food additive for the first time and is being used in the European Union in some cheeses.

Pediocin PA-1 from *Pediococcus* is now on the market. The use of pediococci producing pediocin PA-1 has the potential to be used to improve sensorial properties and to

avoid development of undesirable microbiota. For example, Danisco commercializes in lyophilised *P. acidilactici* (Choozit Lyo. Flav 43) to be included as adjunct starter in the elaboration of cheddar cheese and other semihard cheeses to potentiate aroma and flavour [62]. Other strains will be added in the near future in a similar way.

4.4. Daily Intake. A relevant problem for cheese acting as carrier of probiotics results from the high fat and salt content and the relatively low recommended daily intake. The concentration of probiotics in cheese should be about four to five times higher than in other dairy fermented products such as yoghurt. However, this does not apply to fresh cheese, such as Cottage cheese, which can easily be adjusted to low fat and salt contents and for which recommended daily intake is higher. Low-fat fresh cheese may thus serve as a food with a high potential to be applied as a carrier of probiotics. In a recent report flavour profiles of reduced-fat and semihard cheeses manufactured with *L. paracasei* ssp. *paracasei* (strains CHCC 2115, 4256, and 5583) were analyzed [63]. The authors observed that reduction in fat content did not affect the population of *Lactobacillus* strains reaching 10^8 CFU/g throughout the storage period. Because reduced and low-fat cheeses contain more moisture and are generally produced using lower cooking temperatures, lactic acid bacteria are capable of growing high populations.

4.5. Synbiotic Cheese. One of the latest trends is to add simultaneously prebiotic and probiotic to fresh cheese (the so-called synbiotic cheese). Knowledge on synbiotics is fairly limited despite the fact that they will probably be one of the next most featured subjects in probiotics research.

In the Fior di late cheese the elaboration was carried out after a proper curd-ripening phase using an edible coating as carrier of probiotic (*L. rhamnosus*) and fructooligosaccharide (FOS). The combination improved the final taste of the product extending its shelf-life [64]. The addition of FOS and inulin did not affect probiotic viability growth and viability of *L. casei* 01 and *B. lactis* B94 during manufacture or a two-month ripening period. However, they generated an improved free fatty acid profile. Another example is the recent work on tagatose which is an epimer of fructose naturally present in small amounts in dairy products [65]. This low reduced-calorie monosaccharide enhanced the growth and probiotic functions of *L. casei* 01 and *L. rhamnosus* strain GG.

Transcriptomic studies and quantitative real-time polymerase chain reaction tests showed induction of a large number of genes associated with carbohydrate metabolism including the phosphotransferase system (PTS) in *L. rhamnosus* strain GG. This is the first confirmation of the catabolism of tagatose by a lactobacilli strain as a prebiotic substrate via tagatose-specific PTS. This study reflects the kind of molecular studies to be possibly demanded in the near future to highlight the potential application of a synbiotic partner in functional dairy foods such as yoghurt and cheese.

4.6. Cheese and Microbiota Behaviour. The study of the survival of autochthonous microbiota in cheese to select those with potential ability to arrive metabolically active to the colon will continue being another active area of research. An example of this has been described earlier [12]. The isolation and screening of microorganisms from cheese have been the most powerful means to obtain useful and genetically stable strains and will be so in years to come. Thus, many efforts are being made to screen NSLAB from cheese elaborated with raw milk searching for high tolerance to the different hostile technological processes. This occurs in the elaboration of Parmigiano Reggiano cheese [66] in which NSLAB fraction comes from untreated milk and it is not a part of the normal whey starter. This fraction is particularly attractive as a bioreservoir for potential probiotic strains suitable to survive to GIT condition.

There are many probiotic cheese varieties available on the market and it is essential to verify the behaviour and the performance of the microbial cultures in this environment. In fact, technological control processes and adaptations of the existing manufacturing ones are usually necessary. Testing parameters are decisive for the marketing of the product, such as organic acid profile, typical aroma compounds, or other sensorial attributes.

4.7. Health Benefits. The development after in-depth studies of products designed to improve wellness will be strongly supported. For example, some publications indicate the potential function as a dietary item of the probiotic cheese with specific *L. plantarum* [67] or *L. acidophilus* and *Bifidobacterium longum* [68] strains to reduce the risk of cardiovascular diseases. In the first case, the inclusion of *L. plantarum* K15 in cheddar cheese lowered cholesterol effects in a mouse model. In the second case, a popular Brazilian probiotic fresh cheese (Minas Frescal) attenuated exercise-induced immune suppression in Wistar rats thus opening an alternative to enhance the immune system and to prevent infections.

Recent studies on probiotics are leading to their administration combined with diets focused on the control of the metabolism of carbohydrates and lipids and the amino acid turnover. Of particular interest are the results found by Sharafedtinov et al. [69]. They found that the consumption of a hypocaloric diet supplemented with protein-rich full-fat cheese resulted in the lowering of blood glucose levels by 18% without increasing levels of total cholesterol, low density lipoprotein, or triglycerides. These authors concluded that the combination of a hypocaloric diet supplemented and probiotic cheese could help to reduce body mass index, arterial blood pressure, and the risk of metabolic syndrome in obese patients with hypertension.

5. Concluding Remarks

Although a number of ready-to-use probiotic strains are now commercially available worldwide as probiotics in cheese, new strains claimed as probiotic or beneficial adjunct cultures will surely increase the present-day list. Main considerations have to include the ability to grow in different economical media such as milk or cheese whey to be available in large quantities and to be adapted to the technological challenges (mainly high temperature and salt content) involved in

manufacturing. Many sensorial and nonsensorial aspects are involved in consumer acceptance and have to be considered: flavour texture and of course, last but not least, the price.

The dairy sector has a major advantage in the probiotic foods sector and cheese offers initial advantages as a probiotic carrier. Thus some steps forward seem to be the development of new varieties, the incorporation of new probiotic and well characterized strains, or the manufacture of synbiotic cheeses.

Although it seems apparently obvious, it is noteworthy to mention that the effects on health improvement are strain dependent. No probiotic strain is available and capable of providing all the benefits previously reported [2]. A probiotic cheese is a food not a medicine, which means it is not an alternative treatment for any health condition. Consult your doctor.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Review Article

Bioavailability of Dietary Polyphenols and Gut Microbiota Metabolism: Antimicrobial Properties

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Polyphenolic compounds are plant nutraceuticals showing a huge structural diversity, including chlorogenic acids, hydrolyzable tannins, and flavonoids (flavonols, flavanones, flavan-3-ols, anthocyanidins, isoflavones, and flavones). Most of them occur as glycosylated derivatives in plants and foods. In order to become bioactive at human body, these polyphenols must undergo diverse intestinal transformations, due to the action of digestive enzymes, but also by the action of microbiota metabolism. After elimination of sugar tailoring (generating the corresponding aglycons) and diverse hydroxyl moieties, as well as further backbone reorganizations, the final absorbed compounds enter the portal vein circulation towards liver (where other enzymatic transformations take place) and from there to other organs, including behind the digestive tract or via blood towards urine excretion. During this transit along diverse tissues and organs, they are able to carry out strong antiviral, antibacterial, and antiparasitic activities. This paper revises and discusses these antimicrobial activities of dietary polyphenols and their relevance for human health, shedding light on the importance of polyphenols structure recognition by specific enzymes produced by intestinal microbial taxa.

1. Bioavailability of Dietary Polyphenols

1.1. Structural Diversity. Flavonoids are very abundant 15C secondary metabolites in plants, containing two aromatic rings (connected by a heterocycle pyrone ring), which are tailored with diverse hydroxyl moieties. Some are produced at chloroplasts as defense against oxidative damage generated during photosynthesis [1]; others are produced at the sexual organs as defense against solar UV [2], at the root area as attractants for bacterial and fungal symbionts [3], or as defense against virus, bacteria, fungi, and herbivores [4].

All flavonoids derive from L-phenylalanine, due to diverse transformations taking place at the phenylpropanoid pathway. Initial common steps are conversion of L-Phe in cinnamic acid (by phenyl ammonia lyase (PAL)), its conversion in *p*-coumaric acid (by cinnamate-4-hydroxylase (C4H)), and its transformation in *p*-coumaroyl-CoA (by 4-coumaroyl-CoA ligase (4CL)) [5]. Both *p*-coumaric acid and *p*-coumaroyl-CoA are building blocks for hydroxycinnamic acids and flavonoids, respectively (Figure 1) [5].

In flavonoid biosynthesis, one molecule of *p*-coumaroyl-CoA and three molecules of malonyl-CoA are used by the chalcone synthase (CHS) in order to generate a bicyclic chalcone as naringenin chalcone (Figure 1) [4]. Chalcones are substrates for chalcone isomerase (CHI), which carries out the B-ring closure at these compounds, rendering flavanones (as naringenin from citrus fruits) (Figure 2). All flavonoid subfamilies derive from these 15C flavanones (Figure 2 shows the atom numbering and ring denomination). Other phenylpropanoid enzymes will generate diverse final products as shown in Figure 2 [4]. Flavone synthase (FNS) will generate flavones (as apigenin from celery). Isoflavone synthase (IFS) will generate isoflavones (as genistein from soy). Flavanone-3-hydroxylase (F3H) will generate dihydroflavonols (as aromadendrin from pine trees). Flavonol synthase (FLS) will generate flavonols (as quercetin from

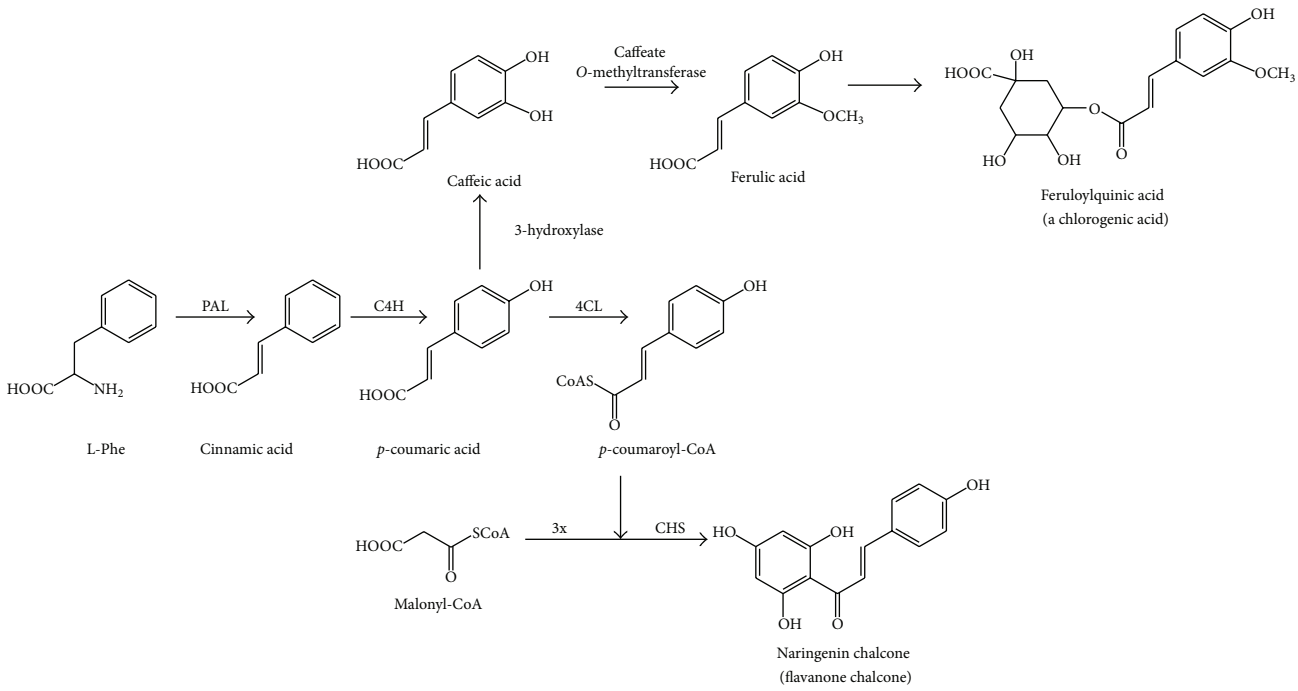

Figure 1: Initial common steps during hydroxycinnamic acids and flavonoids biosynthesis in plants.

onion or kaempferol from capers). Dihydroflavonol reductase (DFR) and anthocyanin synthase (ANS) will generate anthocyanidins (as pelargonidin from diverse red flowers). Anthocyanidin reductase (ANR) will generate flavan-3-ols (as epicatechin from cocoa).

Flavonoids are usually present and stored in plant tissues in the form of diverse derivatives, mostly as sugar O-conjugates at C2 (chalcones), at C3 (flavonols, anthocyanidins, and flavan-3-ols), or at C7 (flavanones, flavones, and isoflavones) positions. Most common bound sugars are glucose, galactose, rhamnose, xylose, rutinose, arabinopyranose, and arabinofuranose [6]. These modifications (and others as methylations and gallate tailoring) add extra structural stability to flavonoids during storage in vacuoles and chloroplasts [7–9]. Once the plant, fruit, or seed is recollected, flavonoids usually have good stability in this conjugated state and keep high concentrations in food and beverages. All these modifications in chemical structure and sugar binding will determine their absorption and bioavailability [10–13].

1.2. Intestinal Absorption. The study of flavonoids metabolism in human body is crucial to determine which ones are better absorbed and which ones lead to formation of bioactive metabolites. Following the ingestion of flavonoids, sugar moieties (as in quercetin-3-glucoside) are cleaved from the phenolic backbone in the small intestine and absorbed here. Enzymes as lactase phlorizin hydrolase (LPH) (at enterocyte membrane) or β-glucosidase (CBG) (cytosolic, for polar glycosides) hydrolyze glycosylated flavonoids and then aglycones enter epithelial cells by passive diffusion [14–16] (Figure 3). However, flavonoids linked to a rhamnose moiety must reach the colon and be hydrolyzed by the α-rhamnosidases secreted by the colon microbiota (as *Bifidobacterium dentium*), in order to proceed to its absorption [17] (Figure 3). Flavan-3-ols, such as (−)-epicatechin, are never glycosylated but often acylated by gallic acid. These compounds are absorbed at enterocyte level without any deconjugation or hydrolysis [18]. Proanthocyanidins are polymers of high molecular weight, and therefore oligomers larger than trimers are unlikely to be absorbed in the small intestine in their native form [19].

The other main family of polyphenols, hydroxycinnamic acids, are commonly esterified to sugars, organic acids, and lipids. There are no esterases in human tissues able to break these ester links, so the main site for its metabolism is colonic microbiota, although up to one third of their absorption can also take place in the small intestine [46–49]. Some hydroxycinnamic acids, as ellagitannins, are polymers (Figure 4). These are resistant to the action of LPH or CBG and consequently cannot be absorbed in the small intestine, reaching the colon, where its microbiota cleaves the conjugating moieties. The resultant aglycones are extensively metabolized by this microbiota, leading to the production of various hydroxyphenylacetic acids [50, 51].

Once a final derivative or aglycon has been absorbed (at small intestine or colon), it undergoes some degree of phase II metabolism at enterocyte level, as methylation (at C3′ or C4′ by catechol-O-methyltransferase (COMT)), sulfation (at C3′, C4′, C5, or C7 by sulfotransferases (SULT)), and glucuronidation (by UDP-glucuronosyltransferases) (Figure 3). Then these products enter the blood stream by the portal vein, reaching the liver, where they may be subjected to more phase II metabolism, hence becoming conjugated and

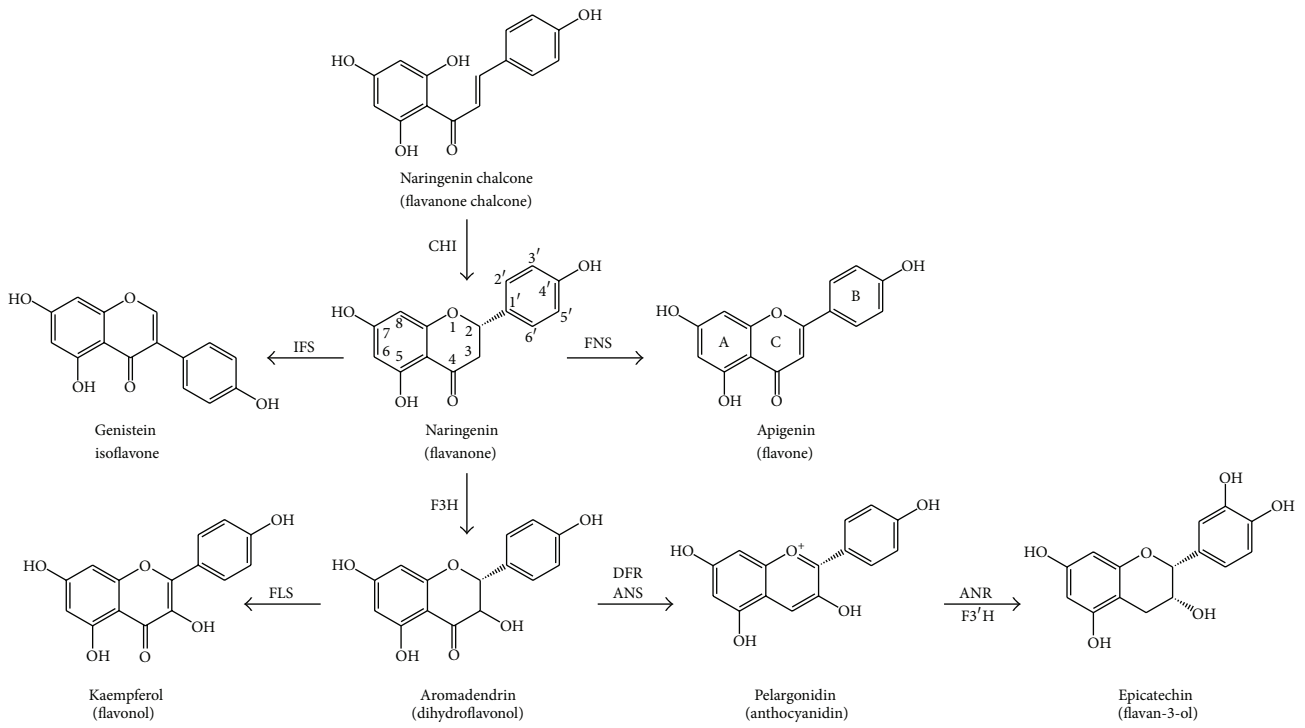

Figure 2: Biosynthetic steps for generation of flavonoid subfamilies. Naringenin structure shows atom numbering and apigenin structure shows rings denomination.

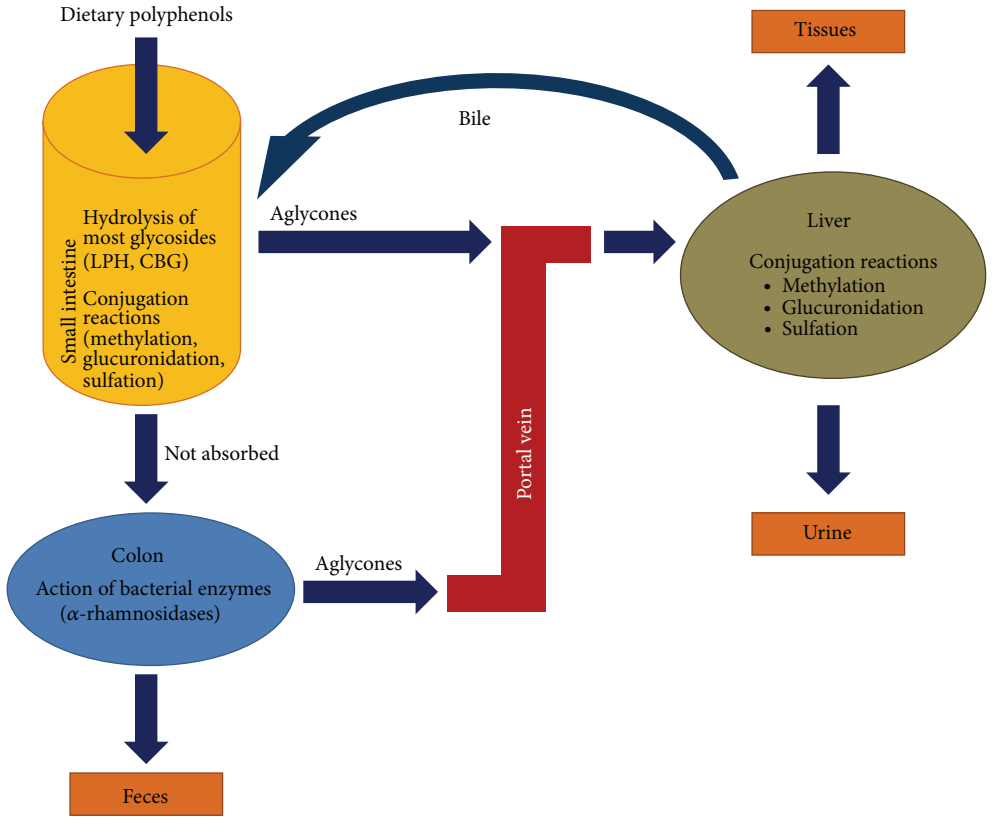

Figure 3: Absorption and metabolism routes for dietary polyphenols and their derivatives in humans.

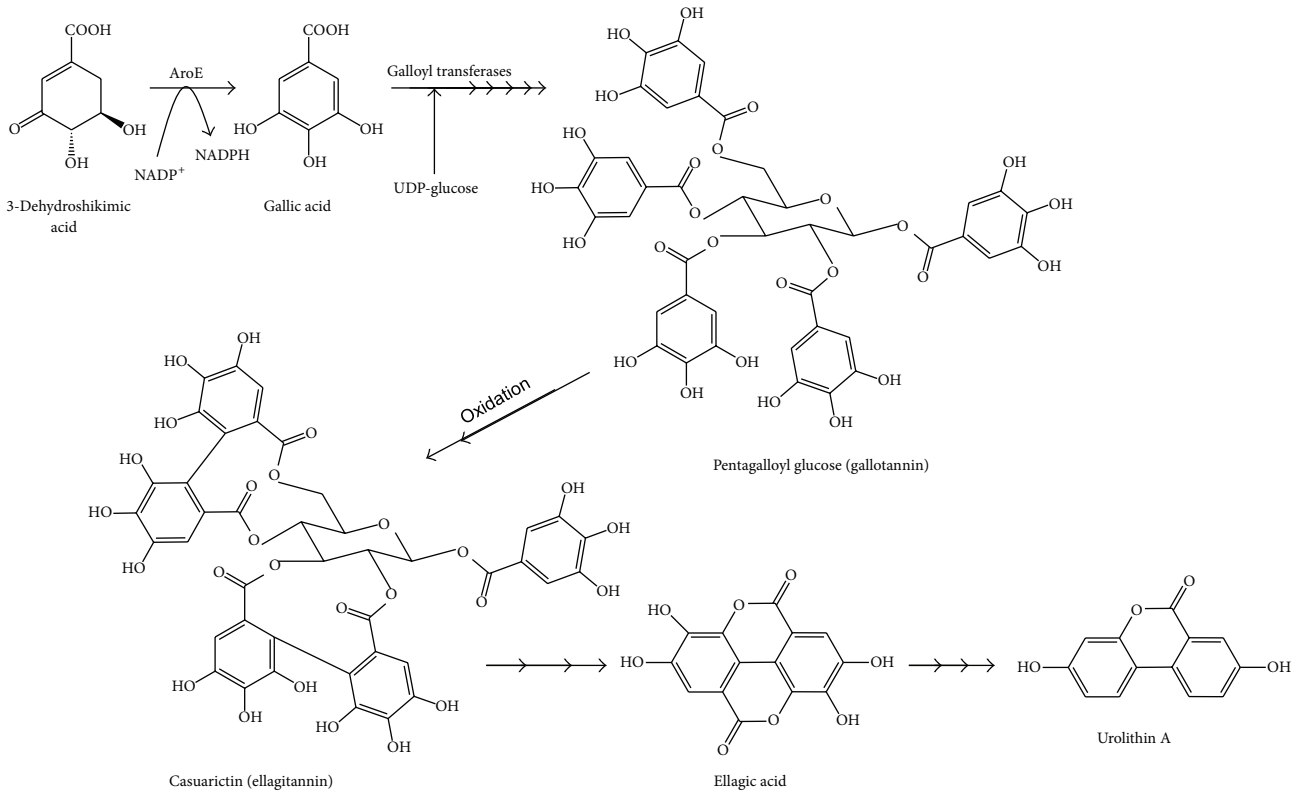

Figure 4: Biosynthetic steps for generation of two hydroxycinnamic acid polymers: ellagitannins and gallotannins.

transported to the bloodstream again until they are secreted in urine (Figure 3) [25, 52–61]. Some of the liver conjugates are then excreted as bile components back into the intestine (enterohepatic recirculation) and deconjugated compounds are regenerated by gut microbial enzymes before being reabsorbed again [21, 62, 63]. The unabsorbed metabolites are eliminated via faeces (Figure 3). All these conjugation mechanisms are highly efficient, and free aglycones are generally absent or present in low concentrations in plasma after nutritional doses. An exception is green tea catechins, whose aglycones constitute a significant proportion of the total amount in plasma, as they are nonglycosylated flavonoids in food and are readily absorbed at the small intestine without extra modifications [12].

2. Antimicrobial Effects of Dietary Polyphenols and Their Gut Microbiota Metabolites

The level of biotransformations suffered by a specific dietary polyphenol along the gastrointestinal tract is determined by two main factors. One is the specific structural subfamily of the polyphenol, as its scaffold will allow only some transformations, to be carried out by intestinal enzymes and gut microbiota species. This chemical structure will therefore, at this initial level, restrict the range of possible final bioactive products to be absorbed and consequently the scale of possible antimicrobial properties generated as a result of these biotransformations on dietary polyphenols. The second factor is the individual richness at the level of intestinal microbiota, as some specific biotransformations on dietary polyphenols can be carried out by a vast array of gut microbial species and genera (as deglycosylations), but other more specific chemical reactions on polyphenols require the presence of particular species or strains gifted with special genes coding for precise enzymes (as those responsible for intestinal generation of urolithins or (*S*)-equol).

Along the next sections, intestinal transformations of dietary polyphenols by diverse microbiota species (and the antimicrobial bioactivities of those derivatives) are organized according to their different structural subfamilies.

2.1. Flavonols. Flavonols (kaempferol, quercetin, and myricetin) (Figure 2) share the 3-hydroxyflavone backbone. Different positions for the phenolic OH moieties give diversity to this subgroup. They are found as glycosylates in many common foods as onion, capers, apples, broccoli, grapefruit, and plums. One of the most important diet flavonols is quercetin, whose 4′-O-glucoside and 3,4′-O-diglucoside, among others, are abundant in onion and propolis, for example [64, 65].

The type of initial glycosylation pattern affects flavonols degradation rates in the gut. Metabolism of di- and trisaccharides is much slower compared to that of flavonol monosaccharides. Position of the hydroxyl groups may also influence their degradation, as recent studies indicate that flavonoids without hydroxyl groups at the C5, C7, and C4′ positions

are degraded slower. Some gut microbiota species that have been involved in this hydrolysis are *Bacteroides distasonis*, *Bacteroides uniformis*, *Bacteroides ovatus*, *Enterococcus casseliflavus*, and *Eubacterium ramulus* [20, 21, 66, 67]. Also, the type of glycosidic bond (C- or O-glycosides) has influence on their degradation rates. Metabolism of a *C*-glycosidic bond seems to be much slower than the hydrolysis of an O-glycosidic bond. This is of interest from a nutraceutical point of view, as the slow degrading compounds may be more bioavailable, because they have greater opportunity to be absorbed than the ones that are degraded at a quicker rate at colon level [66].

Once flavonols have been metabolized in their aglycones, they are extensively degraded by other colonic microbiota, generating simpler phenolic compounds derived from A- and B-ring metabolism, after the flavonoid C-ring has been broken down [64] (Table 1, Figure 5). C-ring breakdown takes place at different positions (breaking the bond between C1 and C2 positions, between C3 and C4, or between C4 and C10) giving rise to a high number of simple phenolics (Table 1, Figure 5). Some gut microbiota involved in this C-ring breakdown is *Eubacterium oxidoreducens*, *E. ramulus*, *E. casseliflavus*, *Clostridium orbiscidens*, and others belonging to *Butyrivibrio* genus [20–23].

Following the C-ring fission, dehydroxylation occurs at the two remaining free phenolic rings (Figure 5). The hydroxylation pattern of A- and B-ring affects therefore the type of phenolic compounds produced, which will be finally absorbed at colon level. For example, the primary gut microbiota metabolites of quercetin are 2-(3,4-dihydroxyphenyl)-acetic acid (from A-ring) and protocatechuic acid (from B-ring), and those ones of myricetin are 2-(3,5-dihydroxyphenyl)-acetic acid (from A-ring) and gallic acid (from B-ring) (Figure 5). Further dehydroxylation results in the formation of 2-(3-hydroxyphenyl)-acetic acid from both metabolites [22, 23, 68].

With respect to bioactivity, flavonols have been described as antiviral, inhibiting HIV-1 integrase, although in a non-specific way, but also against HSV, respiratory syncytial virus, and poliovirus [69, 70]. Quercetin has been shown to potentiate the action of acyclovir against HSV infection [71]. With respect to its antibacterial activity, oral administration of quercetin protected against *Shigella* infection in an animal model using a 140 mg/kg doses [72]. *Escherichia coli* gyrase is inhibited by quercetin and other flavonols, by inhibiting the ATPase GyrB subunit [73]. In *in vitro* assays, quercetin increases the bacterial cell membrane, giving rise to dissipation in membrane potential, and diminished cell motility, which is an important factor in bacterial virulence [74].

2.2. Flavanones. This class of flavonoids (hesperetin, naringenin) (Figure 2) has a 2,3-dihydro-2-phenylchromen-4-one structure. They are very abundant in citrus fruits and tomatoes. They seem to be more bioavailable than other close flavonoids such as flavonols or flavan-3-ols. This can be due to the fact that these compounds are less degraded by colonic microbiota and therefore they are more available for absorption [24]. The reason for this can be their common presence in food as rutinosides (bound to the disaccharide rutinose: 6-O-α-L-rhamnosyl-D-glucose) and neohesperidosides (bound to the disaccharide neohesperidose: 2-O-α-L-rhamnosyl-D-glucose), a tailoring that seems to be resistant to some colon microbiota species. In both cases, these disaccharides are attached at position C7. By contrast, flavanone glucosides are rare.

Flavanones deglycosylation and further degradation by colonic microbiota pathway is similar to that observed in flavonols (Figure 5), with the main difference being C-ring cleavage between C1 and C2 positions or between C4 and C10 ones. *Clostridium* species and *E. ramulus* are able to carry out these transformations in the colon [21, 24].

The flavanone hesperetin aglycon (e.g., from citrus fruits) shows a notable inhibitory activity against vancomycin-intermediate *Staphylococcus aureus* (VISA) and against *Helicobacter pylori* [75, 76]. It possesses also a synergistic effect on VISA when combined with antibiotics like vancomycin and oxacillin [75]. It inhibits also intracellular replication of diverse virus (herpes simplex virus type-1, poliovirus type-1, parainfluenza virus type-3, influenza A virus, and respiratory syncytial virus) [77, 78].

Its glycosylated flavanone, hesperidin, shows antibacterial activity against *Aeromonas hydrophila*, an emerging human pathogen that causes both intestinal and extraintestinal infections. In a murine model, hesperidin showed inhibition of bacterial colonization and a significant increase in anti-LPS IgM levels and reduction of anti-LPS and anti-ECP IgA levels to their normal values [79]. Hesperidin also shows activity against infection with human rotavirus [80] and against influenza virus replication *in vitro* by inhibition of the viral sialidase activity [81]. Growth of fungus *Phytophthora citrophthora* has been inhibited *in vitro* with this glycosylated flavanone, suggesting its role as antifungal toxin in the fruits of *Citrus sinensis,* a big source of this flavanone [82]. Also antiparasitic activity of hesperidin *in vitro* and *in vivo* has been shown against adult worms of *Schistosoma mansoni*, the causative agent of schistosomiasis [83].

Sulphonated hesperidin, one of its plasma metabolites, inhibits pathogens like *Chlamydia trachomatis* and *Neisseria gonorrhoeae in vitro* [84]. This conjugate also inhibits the enveloped viruses herpes simplex virus type-2 and human immunodeficiency virus (HIV) to the point that it has been suggested as a contraceptive antimicrobial agent against HIV transmission [84].

2.3. Flavan-3-Ols. Flavan-3-ols (Figure 2) form a very complex group of flavonoids consisting of simple flavan-3-ols (catechin and epicatechin; gallocatechin, epigallocatechin, and the corresponding gallate esters) and their polymeric forms. They are abundant in green tea, cocoa, kola, banana, and pomegranate.

Such broad polymerization degree and galloylation determine their bioavailability, as oligomers with a degree of polymerization >3 are not absorbed in the small intestine, and therefore they are metabolized in the colon [19, 25]. Their gallate esters are catabolised by colon microbiota, as, for example, epicatechin gallate and epigallocatechin gallate,

Table 1: Main metabolites derived from flavonoids and identified bacteria involved in their transformation.

Precursors		Main metabolites identified	Bacteria	References
Flavonols	Kaempferol	2-(4-Hydroxyphenyl)propionic acid	*Clostridium orbiscidens*	[20]
	Quercetin	2-(3,4-Dihydroxyphenyl)acetic acid 2-(3-Hydroxyphenyl)acetic acid 3-(3,4-Dihydroxyphenyl)propionic acid 3-(3-Hydroxyphenyl)propionic acid	*C. orbiscidens, Eubacterium oxidoreducens* *Eubacterium ramulus* *Enterococcus casseliflavus*	[21–23]
	Myricetin	2-(3,5-Dihydroxyphenyl)acetic acid 2-(3-Hydroxyphenyl)acetic acid	*C. orbiscidens, E. oxidoreducens*	[20, 22, 23]
Flavanones	Naringenin	3-(4-Hydroxyphenyl)propionic acid	*Clostridium* strains *E. ramulus*	[21, 24]
Flavan-3-ols	Catechin Epicatechin	3-(3-Hydroxyphenyl)propionic acid 5-(3′,4′-Dihydroxyphenyl)-γ-valerolactone 5-(3,4-Dihydroxyphenyl)valeric acid 3-(3,4-Dihydroxyphenyl)propionic acid	*Clostridium coccoides, Bifidobacterium* spp.	[25–28]
	Epigallocatechin	5-(3′,4′-Dihydroxyphenyl)-γ-valerolactone 5-(3′,5′-Dihydroxyphenyl)-γ-valerolactone		
Anthocyanins	Cyanidin Peonidin Pelargonidin Malvidin	3,4-Dihydroxybenzoic acid 3-Methoxy4-hydroxybenzoic acid 4-Hydroxybenzoic acid 3,4-Dimethoxybenzoic acid	*Lactobacillus plantarum, Lactobacillus casei, Lactobacillus acidophilus* LA-5, *Bifidobacterium lactis* BB-12	[29, 30]
Isoflavones	Daidzein	(*S*)-Equol	*Bacteroides ovatus*, *Streptococcus intermedius*, *Ruminococcus productus*, *Eggerthella* sp.Julong 732, *Enterococcus faecium* EPI1, *Lactobacillus mucosae* EPI2, *Finegoldia magna* EPI3	[31–33]
		O-Demethylangolensin	*Clostridium* spp. HGHA136	[34]
	Genistein	6′-Hydroxy-*O*-desmethylangolensin		[35]
	Formononetin	Daidzein		[36]
	Biochanin A	Genistein		[36]
Flavones	Luteolin, apigenin	3-(3,4-Dihydroxyphenyl)-propionic acid, 3-(4-hydroxyphenyl)-propionic acid, 3-(3-hydroxyphenyl)-propionic acid, and 4-hydroxycinnamic acid, phloretin	*C. orbiscindens, Enterococcus avium*	[37]

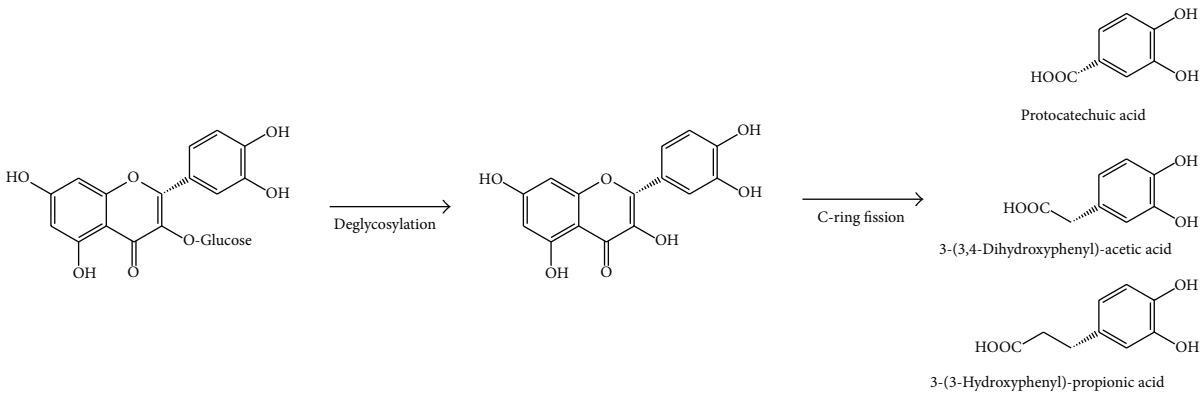

Figure 5: Colonic degradation of quercetin glycosides, as an example of flavonol glycosides.

generating aglycones and gallate, which is further decarboxylated into pyrogallol [25, 26, 85].

Flavan-3-ols aglycones lack a carbonyl group at C4 (as present in flavonols and flavanones). This may be the reason to avoid its transformation by colonic microbiota which modifies other types of flavonoids, as *E. ramulus* [27].

Once the initial gallate esters have been metabolized, the aglycones suffer C-ring opening, giving rise to

diphenylpropan-2-diol, which is further converted into 5-(3′,4′-dihydroxyphenyl)-γ-valerolactone. This lactone ring opens and gives rise to 5-(3,4-dihydroxyphenyl)valeric acid. Further transformations generate OH-phenylpropionic and hydroxy-benzoic acids [25, 27] (Figure 6) (Table 1). Bacteria responsible for these metabolic reactions belong to the genera *Bifidobacterium* (as *Bifidobacterium infantis*) and *Clostridium* (as *Clostridium coccoides*). Actually, colonic populations of *Bifidobacterium* are increased in subjects consuming high doses of flavan-3-ols [28], which further enhance the benefits of flavan-3-ols consumption. These bacteria are resistant to these compounds because they do not use heme-containing enzymes, and these flavan-3-ols are important iron-chelating compounds [86].

In recent years, several studies have reported that the main catechin of green tea leaves, epigallocatechin-3-gallate (EGCG), has anti-infective properties [87]. Inhibition effect of EGCG on the capacity to infect cells by several viruses has been reported by different authors, who found that EGCG inhibits entry of hepatitis C virus by impairing virus binding to the cell surface [88–90]. EGCG also shows antiviral effects against HIV-1, interfering with several aspects of its life cycle. It interacts with the viral envelope destroying viral particles [91], prevents attachment of virions to cells downregulating CD4 cell surface receptor expression [92, 93], affects viral replication via inhibition of reverse transcription [94], and inhibits proviral genome integration by binding between the integrase and the viral DNA [95]. The antiviral activity of EGCG against influenza virus infection in cell culture was attributed to agglutination of virus particles thus preventing virus from adsorbing to cells [96]. EGCG also inhibits the acidification of endosomes and lysosomes required for the fusion of viral and cellular membranes [97] as well as of neuraminidase activity responsible for preventing self-aggregation of virus particles [98]. Clinical studies performed to investigate the preventive effect of catechins consumption on influenza infection in humans found this statistically significant [99, 100]. Enterovirus 71 [101], human hepatitis B virus [102], adenovirus [103], Epstein-Barr virus [104], and herpes simplex virus [105] are also clearly affected by EGCG.

With regard to antibacterial activity, there are multiple mechanisms by which EGCG exerts this activity against *Staphylococcus*, including damage to the lipid bilayer of the cell membrane [106], decrease slime production and inhibition of biofilm formation [107], binding and neutralization of enterotoxin B [108], and working with a synergistic effect in combination with β-lactams [109] or carbapenems [110]. Other bacteria killed by the action of EGCG are *Streptococcus pyogenes* [111], *Bacillus* spp. and *Clostridium* spp. [112], *Salmonella typhi* [113], and enterohemorrhagic *E. coli* [114]. The general antibacterial property of flavan-3-ols explaining these effects can be their chelating properties on iron, an important oligoelement for heme-utilizing bacteria [25]. EGCG inhibits growth of *Legionella pneumophila* inside macrophages not by any direct antibacterial effect on the pathogen, but due to selective changes in the immune response of macrophages and enhanced production of cytokines [115].

The antimicrobial effect of EGCG is also extended to eukaryote microorganisms, as against *Candida* spp. and the dermatophytes *Cryptococcus neoformans* and *Trichophyton mentagrophytes* [116]. EGCG specifically inhibits the germination of *T. mentagrophytes* conidia and subsequent hyphal growth [117]. These positive led to establishing *in vivo* research with EGCG in a murine model of disseminated candidiasis, showing its antifungal activity *in vivo* and its synergistic effect when combined with amphotericin B [118].

EGCG inhibits epimastigotes growth of *Trypanosoma cruzi* and increases mice survival rates in EGCG-treated animals that point out to a potential new compound for chemotherapy of Chagas disease [119]. EGCG also inhibits 37%–80% of binding of various isolates of *Plasmodium falciparum* to the ICAM-1 cellular receptor related to cerebral malaria [120]. The lethal mitochondrial damage that EGCG causes to *Leishmania donovani* [121] and *Leishmania amazonensis* [122] has been explained by its inhibition in the enzymatic activity of the parasite arginases [123].

2.4. Anthocyanidins. Unlike other flavonoids that are absorbed and secreted, anthocyanins, the glycosylated versions of anthocyanidin aglycons (as cyanidin, pelargonidin, and malvidin), do not appear to undergo extensive metabolism of the parent glycosides to glucuronic, sulfo or methyl derivatives, and therefore their bioavailability is very low [24]. Procyanidins occur in monomeric as well as in oligomeric and polymeric forms and are the most abundant and bioactive dietary polyphenols, as they are responsible for most red, blue, and purple color in fruits (specially berries), flowers, and leaves, besides having an important antioxidant activity [29, 30].

Since only a small part of ingested anthocyanins is absorbed at small intestine, large amounts of these compounds are likely to enter the colon, where they are deglycosylated by gut microbiota [124]. The gut microbiota has a high hydrolytic potential and ring scission properties so several anthocyanins degradation products have been identified. Some of them include vanillic, phloroglucinol, and protocatechuic acid [124, 125]. For example, incubation of malvidin-3-glucoside (from grape extracts) with fecal bacteria results in formation of gallic, syringic, and *p*-coumaric acids (Figure 7) (Table 1). Some species responsible for this degradation are *Lactobacillus plantarum*, *Lactobacillus casei*, *Lactobacillus acidophilus*, and *Bifidobacterium lactis* [29, 30]. All the anthocyanins and their metabolites tested significantly enhance growth of *Bifidobacterium* spp., *Lactobacillus* spp., and *Enterococcus* spp. Therefore anthocyanins and their metabolites could perform a positive modulation of intestinal bacterial populations [126].

There are different mechanisms that can explain the antimicrobial activity of anthocyanins, as they can cause localized disintegration of bacterial outer membrane, leaking of cytoplasm (with the presence of significant amounts of cytoplasmic material and membrane debris outside the cells), and irregular shape [127]. The mechanisms thought to be responsible for the toxicity of pure anthocyanidin compounds to microorganisms include enzyme inhibition

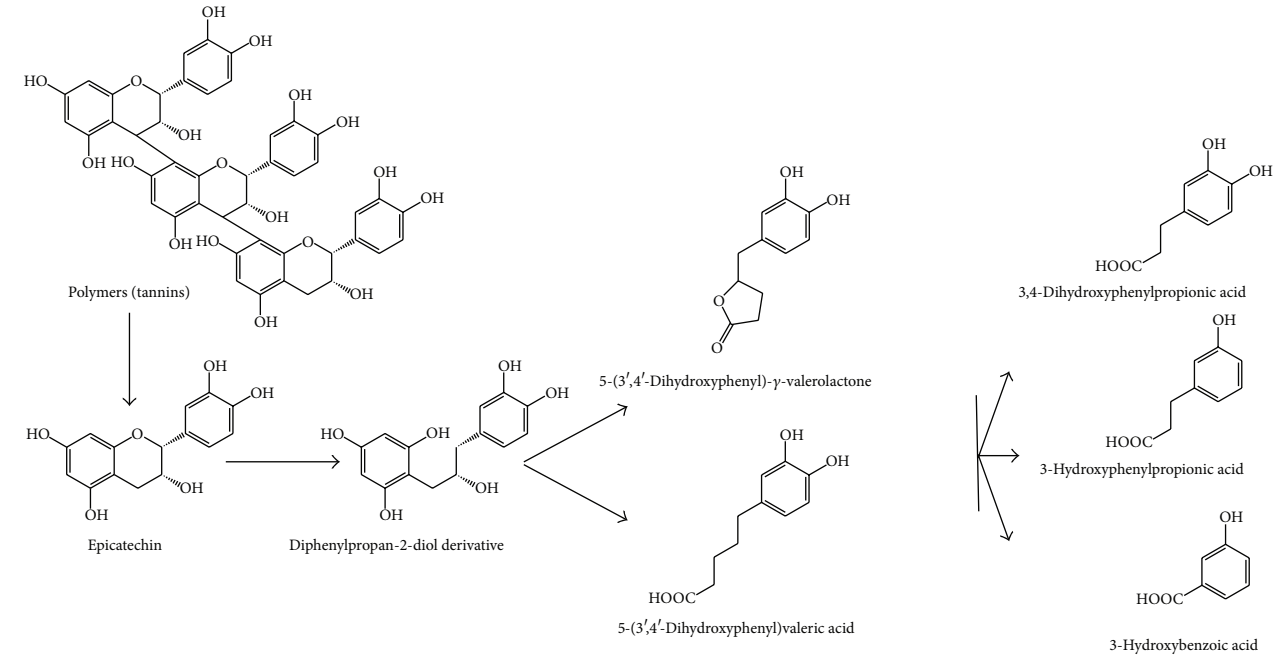

Figure 6: Colonic degradation of epicatechin tannins, as an example of flavan-3-ol polymers.

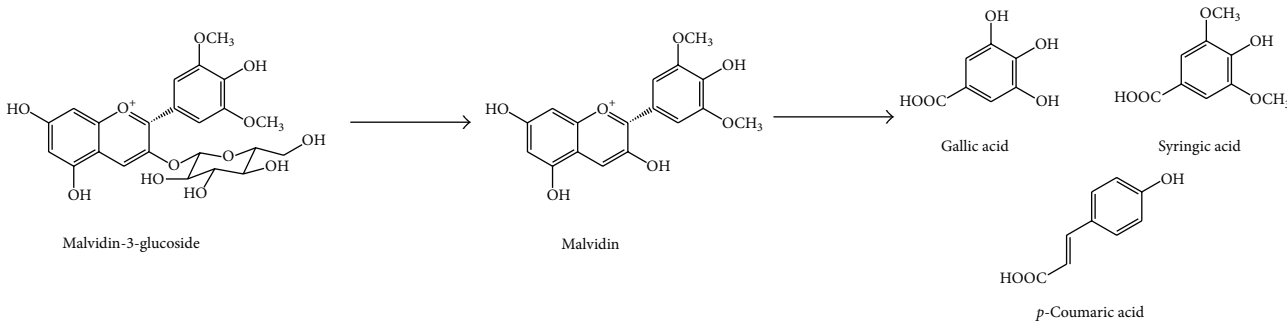

Figure 7: Colonic degradation of malvidin-3-glucoside, as an example of anthocyanin.

by the oxidized compounds, possibly through reaction with sulfhydryl groups or through more nonspecific interactions with proteins often leading to inactivation of the membrane protein and loss of function. Probable targets in the microbial cell are surface-exposed adhesions, cell wall polypeptides, and membrane-bound enzymes. Anthocyanidins may also render substrates unavailable to microorganisms, as some oligoelements [128, 129].

Many studies have shown the antimicrobial activities of the crude extract, fractions, and pure anthocyanidins from different berries. In bilberries (*Vaccinium myrtillus*), anthocyanins comprise 90% of the phenolic compounds. Extracts from bilberry and blueberry (*Vaccinium corymbosum*) showed inhibitory effects on the growth of Gram-positive bacteria (*Listeria monocytogenes*, *S. aureus*, *Bacillus subtilis*, and *Enterococcus faecalis*) and Gram-negative ones (*Citrobacter freundii*, *E. coli*, *Pseudomonas aeruginosa*, and *Salmonella enterica* ser. *Typhimurium*). However yeasts are resistant to these berry extracts [130].

Cyanidin-3-O-glucoside (C3G) inhibits the secretion of both VacA and CagA, two key virulence factors of *H. pylori* [131, 132]. C3G downregulates VacA secretion in *H. pylori* via inhibition of SecA expression (a protein involved in translocation of bacterial proteins out of the bacterial plasma membrane), causing a decrease in apoptosis in *H. pylori*-infected cells [132].

Cyanidin-3-sambubioside, a natural anthocyanin derived from black elderberry extract, binds to influenza virus neuraminidase within the 430-cavity, acting as a potent inhibitor of sialidase activity. This natural anthocyanin binds in the vicinity of neuraminidase residues 356–364 and 395–432, shielding proteases from releasing these peptide segments from the active site. This binding mode has not been seen with other influenza neuraminidase inhibitors so that the

compound and its derivatives definitely offer the potential for the development of a new class of antivirals against influenza [133].

2.5. Isoflavones. Almost all isoflavones (daidzein, genistein, and formononetin) exist as glucosides and therefore are not absorbed across enterocytes due to their high polarity and molecular weight. These flavonoids are present almost exclusively in plants from the *Fabaceae* family (soy, lentils, beans, and chickpeas). Their bioavailability requires therefore conversion of glucosides into the bioactive aglycones via the action of intestinal β-glucosidases from small intestine bacteria (*Lactobacillus*, *Bifidobacterium*). Then, these aglycones are uptaken to the peripheral circulation [134].

One of the most active isoflavones, daidzein, is metabolized in two different ways depending on subjects and their gut microbiota. Some subjects produce (*S*)-equol via dihydrodaidzein and tetrahydrodaidzein (resulting from the activities of *Streptococcus intermedius*, *B. ovatus*, *Ruminococcus productus*, *Lactobacillus mucosae* EPI2, *E. faecium* EPI1, *Veillonella* spp., *Eggerthella* sp. Julong732, and *Finegoldia magna* EPI3) [31–33] (Figure 8). However others produce O-desmethylangolensin (O-DMA) via 2′-dehydro-O-demethylangolensin (generated by *Clostridium* spp.) [34] (Figure 8) (Table 1). Therefore, there are two groups of subjects, (*S*)-equol producers and nonproducers. The inability to produce (*S*)-equol is a consequence of the lack of specific components in the intestinal microbiota, as the species described before. (*S*)-equol shows high antioxidant activity due to its nonplanar structure, which enables it to penetrate more easily into the interior of the cell membrane, preventing oxidative damage *in situ*. Also its estrogenic activity on mammal cells is higher in comparison with other phytoestrogens. This compound binds to estrogen receptor in mammal cells, downregulating its activity. This may have potential application in breast and prostate cancer therapy and prevention [135–137]. In addition to (*S*)-equol and O-DMA, other less active microbial metabolites of daidzein have been reported [138].

Microbial metabolism of isoflavone genistein is different from that of daidzein. Genistein is reduced to dihydrogenistein, which is further metabolized to 6′-hydroxy-O-desmethylangolensin [35].

Other less common isoflavones found in red clover are formononetin and biochanin A, which are converted in a similar way to microbial metabolites. Formononetin is rapidly converted via daidzein to O-DMA and (*S*)-equol. Biochanin A is metabolized via genistein to 6′-hydroxy-O-desmethylangolensin [36]. Then, all these isoflavone aglycones are further transformed by C-ring cleavage and dehydroxylation reactions in the colon.

Apart from their estrogenic activity, studies with respect to the antimicrobial activity of isoflavones have been described, as, for example, inhibition of *S. aureus* MRSA strains at concentrations over 128 μg/mL [139–141]. These activities are thought to be due to inhibition of bacterial topoisomerase IV [142].

2.6. Flavones. These flavonoids (luteolin, apigenin) share the 2-phenylchromen-4-one (2-phenyl-1-benzopyran-4-one) backbone. They are present in food as cereals, parsley, thyme, celery, and citrus fruits. Once the corresponding glucosides have been hydrolyzed at intestinal level, unabsorbed aglycons are further metabolized by colon microbiota (*C. orbiscindens*, *Enterococcus avium*), breaking down their C-ring towards phloretin chalcone, 3-(3,4-dihydroxyphenyl)-propionic acid, 3-(4-hydroxyphenyl)-propionic acid, 3-(3-hydroxyphenyl)-propionic acid, and 4-hydroxycinnamic acid, which are absorbed and excreted by urine [37] (Table 1).

Luteolin and its glycosides have been isolated from plants used in traditional medicine to treat a wide range of diseases. Tests for antiherpetic substances from crude methanol leaf extract of *Avicenna marina* have shown that the most active fraction isolated and analyzed contained luteolin 7-O-methylether-3′-O-beta-D-glucoside (LMEG). LMEG exerts an inhibitory effect on the early stage of herpes simplex virus 2 (HSV-2) infection probably inhibiting HSV attachment to the cell membrane and its entry into the cell [143]. Among several compounds isolated from *Swertia macrosperma*, luteolin was the most active compound in inhibiting the secretion of hepatitis B virus surface antigen (HBsAg) and hepatitis B virus e-antigen (HBeAg) with IC50 values of 0.02 and 0.02 mM, respectively [144].

Regarding the antibacterial effects, luteolin is active against *B. subtilis*, *S. aureus*, *P. fluorescens* and *E. coli* [145, 146]. The major constituents isolated from the methanol extract of *Daucus carota* (carrot) seeds are luteolin, luteolin-3′-O-beta-D-glucopyranoside, and luteolin-4′-O-beta-D-glucopyranoside. Both luteolin and its 4′-O-glucoside demonstrated bactericidal activity against *S. aureus* and *E. coli* (MIC = 5.0×10^{-2} and 1.0×10^{-1} mg/mL, resp.) [147]. Luteolin shows antibacterial and synergistic activity against amoxicillin-resistant *E. coli*, acting via three mechanisms: inhibition of proteins and peptidoglycan synthesis, inhibition of extended-spectrum β-lactamases, and alteration of outer and inner membrane permeability [148].

Luteolin and its glycosides also show antiparasitic activity. Luteolin present in extract from *Melampyrum arvense* was the most active compound against *Trypanosoma brucei* ssp. *rhodesiense* and *L. donovani* (IC(50) values 3.8 and 3.0 μg/mL) [149]. Luteolin-7-O-β-glucopyranoside displayed the best antiplasmodial activity against *P. falciparum* (IC(50) value 2.9 μg/mL) [149].

2.7. Hydrolyzable Tannins. Hydrolyzable tannins are a class of polyphenols that include gallotannins and ellagitannins (ETs) (Figure 4). These compounds are present in fruits like raspberry, cranberries, strawberries, walnuts, grapes, and pomegranate. A main difference between these two groups is that, upon gut microbial hydrolysis, gallotannins yield glucose and gallic acid, whereas ellagitannins undergo lactonization producing ellagic acid (Figure 4).

Ellagic acid is largely metabolized by the colon microbiota, giving rise to urolithin A (3,8-dihydroxy-6H-dibenzopyran-6-one) and its monohydroxylated analog known as urolithin B [150, 151]. There is a large interindividual variation

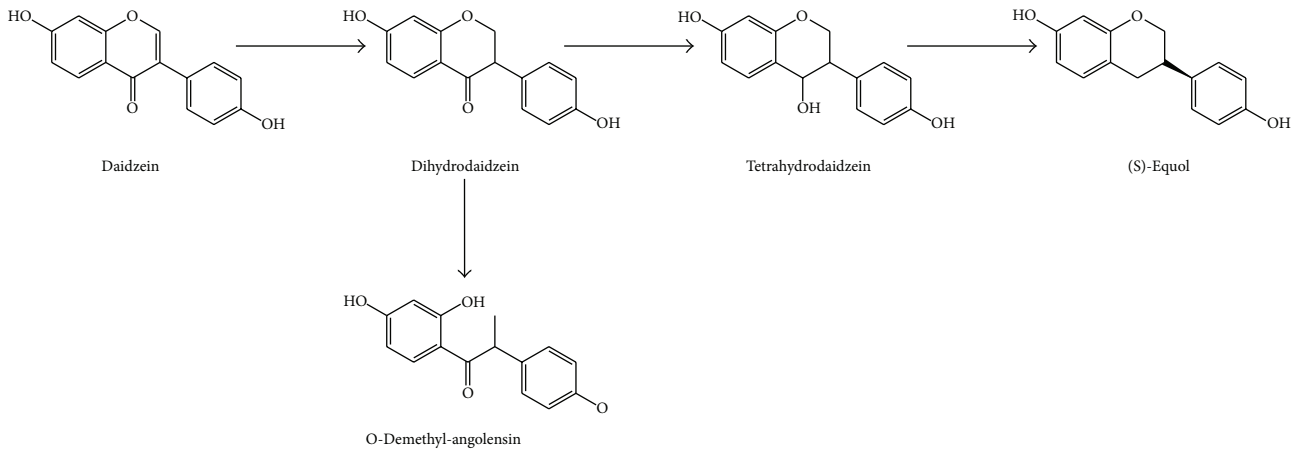

Figure 8: Colonic formation of (*S*)-equol and O-demethylangolensin from the isoflavone daidzein.

in the timing, quantity, and types of urolithins excreted in urine by humans. These variations are due to the variations in colonic microbiota composition [152, 153]. Despite all the data indicating the microbial origin of urolithins, no specific bacteria for urolithin biosynthesis have been yet identified. One bacterium (*Butyrivibrio* spp.), responsible for ellagitannins modification, has been identified in rumen fluids [38] (Table 2).

Ellagic acid from *Phyllanthus urinaria*, a domestic plant grown in Korea, shows specific antiviral activity against hepatitis B virus (HBV), by inhibiting HBeAg secretion, in HBV-infected cells [154]. ETs are potent antiviral agents against herpes simplex virus, specially eugenin extracted from *Geum japonicum* and *Syzygium aromaticum* [155]. Pomegranate (*Punica granatum*) polyphenols suppress the replicative ability of influenza A virus in host cells. Punicalagin is the most effective anti-influenza component in this extract, blocking replication of influenza virus RNA and inhibiting agglutination of chicken red blood cells by the virus [156, 157]. Geranin and corilagin are two ETs extracted from *Phyllanthus amarus* restrained by 50% the interaction of glycoprotein 120 of HIV-1 at concentrations from 2.65 to 0.48 μg/mL on the primary cellular receptor CD4 [158].

Plant extracts from *Pteleopsis hylodendron,* containing mainly ellagic acid, are active against *Klebsiella pneumoniae*, *Bacillus cereus*, *E. coli*, and *S. typhi* [159]. ETs present in pomegranate peel are effective also in inhibiting *S. aureus*, *Salmonella*, *L. monocytogenes*, and *E. coli* [160–164]. Ellagic acid extract from pomegranate inhibits formation of biofilms by *S. aureus*, methicillin resistant *S. aureus* (MRSA), and *E. coli*. [79].

Punicalagin, punicalin, gallagic, and ellagic acids show antifungal properties against *Candida albicans*, *C. neoformans*, and *Aspergillus fumigatus* [165]. Apart from inhibiting biofilm formation, pomegranate extracts disrupt preformed biofilms and inhibited germ tube formation in *C. albicans* [164].

In vitro antimalarial activity of ellagic acid has been reported, with high *in vitro* activity against all *P. falciparum* strains regardless of their levels of chloroquine and mefloquine resistance (50% inhibitory concentrations ranging from 105 to 330 nM) [166]. This antimalarial activity takes place at the mature trophozoite and young schizont stages, corresponding to protein and nucleic acid synthesis. Ellagic acid potentiates also the activity of current antimalarial drugs such as chloroquine, mefloquine, artesunate, and atovaquone [167].

2.8. Lignans. Lignans include a number of diphenolic compounds with a 1,4-diarylbutane structure such as secoisolariciresinol, matairesinol, pinoresinol, lariciresinol, isolariciresinol, and syringaresinol. They are common in seeds as flax and cereals and in fruits as strawberries and apricots.

Lignan metabolism involves both mammalian (glucuronidation and to a lesser degree sulfation) and gut microbial processes [39]. Biological activity of lignans is related to the activation of these compounds by *Bacteroides and Clostridium* species (in the gut microbiota) to enterolactone and enterodiol (Figure 9), which are phytoestrogens in mammals [40]. This transformation of lignans into phytoestrogens is carried out after demethylation and dehydroxylation reactions (carried out by *Peptostreptococcus* and *Eubacterium* species), which increase the structural diversity of enterolignan derivatives in blood circulation (Table 2) [41] (Figure 9). Thus, enterolactone conversion from enterodiol is a complex phenomenon, involving several precursors, different intermediary metabolites, and diverse conjugation patterns. Production of enterolactone was compared to that of enterodiol and a ratio of enterolactone- and enterodiol-converting bacteria of 1 : 2000 was observed, indicating that enterodiol-producing bacteria are dominant in human gut [42] (Table 2).

2.9. Chlorogenic Acids. Chlorogenic acids are a group of compounds comprising hydroxycinnamates (such as caffeic acid, ferulic acid, and *p*-coumaric acid) (Figure 1), linked to a quinic acid to form a range of conjugated structures

Table 2: Main metabolites derived from nonflavonoids and identified bacteria involved in their transformation.

Precursors	Main identified metabolites	Bacteria	References
Ellagitannins	Urolithins	*Butyrivibrio* spp.	[38]
Lignans	Enterodiol Enterolactone	*Bacteroides distasonis*, *Bacteroides fragilis*, *Bacteroides ovatus*, *Clostridium cocleatum*, *Butyribacterium methylotrophicum*, *Eubacterium callanderi*, *Eubacterium limosum*, *Peptostreptococcus productus*, *Clostridium scindens*, *Eggerthella lenta*	[39–42]
Hydroxycinnamates	3-Hydroxyphenylpropionic acid Benzoic acid 3-(4-Hydroxyphenyl)propionic acid Vanillin	*Escherichia coli*, *Bifidobacterium lactis*, *Lactobacillus gasseri*	[43–45]

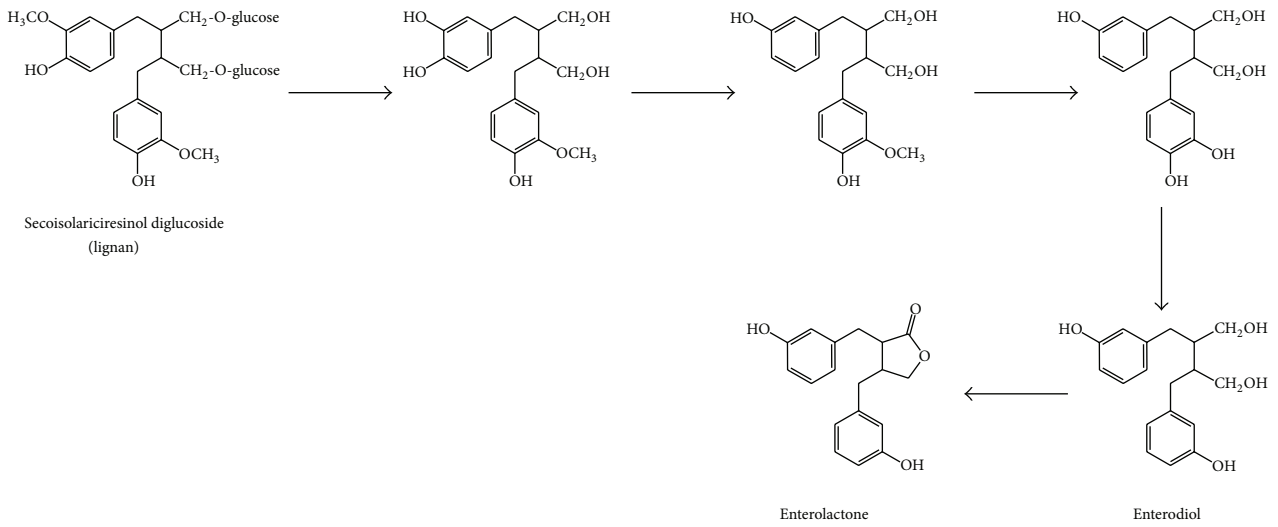

Figure 9: Colonic formation of enterodiol and enterolactone from the lignan secoisolariciresinol diglucoside.

known, respectively, as caffeoylquinic acids, feruloylquinic acids, and *p*-coumaroylquinic acids. They are abundant in fruits as peaches and plums and in some seeds, like coffee.

Literature describing the bioavailability of chlorogenic acids is scarce and contradictory. However, several microbial metabolites have been identified. The main microbial metabolites of caffeic acid are 3-hydroxyphenylpropionic acid and benzoic acid, generated by the action of *E. coli*, *B. lactis*, and *Lactobacillus gasseri* (Table 2). The first one is formed by de-esterification, reduction of a double bond, and dehydroxylation. Furthermore, β-oxidation shortens the side-chain and forms benzoic acid in small degree. Both metabolites are also obtained from chlorogenic acid [43]. The most frequent metabolites from ferulic acid produced by colonic microbiota are vanillin and 3-(4-hydroxyphenyl)-propionic acid [44, 45].

The antimicrobial activity of 22 polyphenols, including gallic acid, was investigated against 26 bacterial species. It was found that a structure-activity relationship between the strongest antibacterial activity for those polyphenols and a higher number of pyrogallol rings in their structure [168]. As gallic acid has one of those rings, its antibacterial activity was classified by these authors as moderate. The role of gallic acid is also of practical interest in the prevention of formation of biofilms by different bacteria. When biofilms formed by *E. coli*, *P. aeruginosa*, *S. aureus*, and *L. monocytogenes* were studied, a reduction in biofilm activity >70% for all the biofilms tested was found [169]. Gallic acid also inhibits bacterial growth of *Streptococcus* mutants and the biofilm formation *in vitro* and also influences the adhesion properties of *S. aureus* [170].

Experimental evidences regarding the antiviral activity of gallic acid have been published as the inhibition in human rhinoviruses (HRVs), replication and reduction of HRV-induced cytopathic effect *in vitro*, and antienterovirus 71 activity [171] were found. The same positive results of gallic acid against herpes simplex virus type-2 were previously mentioned [172].

Gallic acid purified from *Terminalia nigrovenulosa* bark has shown strong antifungal activity against *Fusarium solani* and strong nematicidal activity against *Meloidogyne incognita* [173, 174].

3. Conclusions

Most polyphenol nutraceuticals from plant origin must undergo intestinal transformations, by microbiota and enterocyte enzymes, in order to be absorbed at enterocyte and

colonocyte levels. This gives rise to diverse beneficial effects in the consumer, including a vast array of protective effects against viruses, bacteria, and protozoan parasites. These enzymatic transformations include elimination of glycosidic tailoring by gut microbiota of diverse genera (*Lactobacillus*, *Eubacterium*, and *Bifidobacterium*), as well as further transformations in these aglycones' level, giving rise to more stable bioactive compounds that are incorporated into the blood stream, as a vast array of benzoic acids, phenolic acids, urolithins, and the phytoestrogens (*S*)-equol, enterodiol, and enterolactone. In most cases, a complex network of different intestinal microbiota species is necessary for full biotransformation, whereas earlier and simple reactions as deglycosylation can be carried out individually by specific gut strains. The individual variability, at consumer level, with respect to richness, and biodiversity of own intestinal microbiota taxa are key determinants regarding the ability of a person to get the most fully bioactive derivatives from ingested polyphenols. Final absorbed bioactive derivatives have shown antimicrobial properties against viruses (as HBV), Gram-positive bacteria (as *S. aureus*, *L. monocytogenes)*, and Gram-negative bacteria *(S. enterica*, *P. aeruginosa*), but also against eukaryote species as fungi (*Candida* spp., *T. mentagrophytes*) or protozoans (*T. cruzi*, *P. falciparum*). Therefore, consumption of food with high levels of polyphenols, together with having appropriate gut microbiota diversity, is extremely important, in order to help in the fight against infectious diseases. Fermented dairy foods, as well as other ones with high levels of beneficial microorganisms, can therefore contribute to maintaining this appropriate gut microbiota diversity, facilitating intestinal production of bioactive metabolites from dietary polyphenols, as well as their absorption and bioavailability.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Review Article

The Role of Probiotic Lactic Acid Bacteria and Bifidobacteria in the Prevention and Treatment of Inflammatory Bowel Disease and Other Related Diseases: A Systematic Review of Randomized Human Clinical Trials

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Inflammatory bowel disease (IBD), which includes Crohn's disease (CD) and ulcerative colitis (UC), is a chronic inflammation of the small intestine and colon caused by a dysregulated immune response to host intestinal microbiota in genetically susceptible subjects. A number of fermented dairy products contain lactic acid bacteria (LAB) and bifidobacteria, some of which have been characterized as probiotics that can modify the gut microbiota and may be beneficial for the treatment and the prevention of IBD. The objective of this review was to carry out a systematic search of LAB and bifidobacteria probiotics and IBD, using the PubMed and Scopus databases, defined by a specific equation using MeSH terms and limited to human clinical trials. The use of probiotics and/or synbiotics has positive effects in the treatment and maintenance of UC, whereas in CD clear effectiveness has only been shown for synbiotics. Furthermore, in other associated IBD pathologies, such as pouchitis and cholangitis, LAB and bifidobacteria probiotics can provide a benefit through the improvement of clinical symptoms. However, more studies are needed to understand their mechanisms of action and in this way to understand the effect of probiotics prior to their use as coadjuvants in the therapy and prevention of IBD conditions.

1. Introduction

Inflammatory bowel disease (IBD) can be defined as a disease of disrupted physiology, microbiology, immunology, and genetics [1]. IBD mainly includes Crohn's disease (CD) and ulcerative colitis (UC), which are characterized by chronic inflammation of the gastrointestinal tract. CD and UC differ by the intestinal localization and features of the inflammation. In this way, CD inflammation occurs anywhere in the gastrointestinal tract, whereas UC inflammation starts in the rectum and is restricted to the colon [1, 2].

Microorganisms in the human gut act in symbiosis to modulate different functions, such as the stimulation-regulation of epithelial innate immunity, the competitive exclusion of pathogens, and the production of important metabolites (i.e., carbohydrates, vitamins, and short chain fatty acids (SCFAs)) [3–5].

Traditional fermented products, breast milk, gastrointestinal tract content, and the feces of human subjects are the primary sources of LAB and bifidobacteria [6]. LAB and bifidobacteria produce lactic acid as a major metabolic end-product of carbohydrate fermentation and exhibit an increased tolerance to acidity. These bacteria contribute to the organoleptic and textural profile of many foods [7]. In addition to having important applications in the food industry, LAB and bifidobacteria can have beneficial health effects as an adjuvant to decrease the intestinal microbiota imbalance induced by the use of antibiotics or by pathological conditions, particularly IBD [5–11].

Probiotics are defined as "live microorganisms which, when administered in adequate amounts, confer a health benefit on the host" according to the consensus of a multinational expert group of scientists convened in 2001 by the Food and Agriculture Organization of the United Nations (FAO) [12]. The term synbiotic refers to a product that contains both probiotics and prebiotics. By understanding the mechanism of action of the bacterial strains that act as probiotics, it would be possible to define not only a specific and efficient therapy but rather an individual customized therapy to improve the specific disease symptoms and also restore the basic functioning of the gut. For this purpose, lactobacilli and bifidobacteria are the most widely used probiotics in humans.

The main aim of this work was to review the scientific evidence on the role of LAB and bifidobacteria, which are commonly used as probiotics, mainly in the prevention and treatment of IBD and other related IBD. In addition, we provide potential mechanisms of action of LAB and bifidobacteria in those conditions.

2. Methodology

In this paper, we performed a systematic review of the role of fermented dairy products and LAB and bifidobacteria probiotics in the prevention and treatment of IBD. PubMed and Scopus were searched for human randomized clinical trials articles that were published between 1990 and June 2014 in English using the MeSH terms "dairy products" and "probiotics" combined with "inflammatory bowel disease," "Crohn's disease," and "ulcerative colitis." Here, we evaluate results obtained using the following equation search ("dairy products" [MeSH Terms] OR ("dairy" [All Fields] AND "products" [All Fields]) OR "dairy products" [All Fields] OR ("dairy" [All Fields] AND "product" [All Fields]) OR "dairy product" [All Fields]) OR ("probiotics" [MeSH Terms] OR "probiotics" [All Fields]) OR ("microbiota" [MeSH Terms] OR "microbiota" [All Fields]) AND (("inflammatory bowel diseases" [MeSH Terms] OR ("inflammatory" [All Fields] AND "bowel" [All Fields] AND "diseases" [All Fields]) OR "inflammatory bowel diseases" [All Fields] OR ("inflammatory" [All Fields] AND "bowel" [All Fields] AND "disease" [All Fields]) OR "inflammatory bowel disease" [All Fields]) OR ("colitis, ulcerative" [MeSH Terms] OR ("colitis" [All Fields] AND "ulcerative" [All Fields]) OR "ulcerative colitis" [All Fields] OR ("colitis" [All Fields] AND "ulcerative" [All Fields]) OR "colitis, ulcerative" [All Fields]) OR ("crohn disease" [MeSH Terms] OR ("crohn" [All Fields] AND "disease" [All Fields]) OR "crohn disease" [All Fields]) AND Clinical Trial [ptyp]). One hundred and thirteen original articles matching these criteria were initially selected, although only those articles that included specific LAB and bifidobacteria results (sixty) were later considered for the review and separated into four major topics: general aspects of probiotics in inflammatory bowel diseases, LAB, and bifidobacteria in Crohn's disease, in UC and on other inflammatory bowel diseases. In addition, we focused on the possible probiotic mechanism of action in IBD. Figure 1 shows the flow diagram of searched articles [13] and Table 1 shows the summary of randomized clinical intervention trials of probiotics in IBD.

3. General Aspects of Probiotics in Inflammatory Bowel Disease

Nutrition seems to play a causal role in both UC and CD [14–17]. In this sense, in the past, IBD patients usually avoided dairy products to decrease disease symptoms [18]. However, currently, the recommendation is to have a complete and varied diet to prevent malnutrition, since a restrictive diet can lead to potential deficiencies in calcium, vitamin D, iron, vitamin B12, and ω-3 fatty acids, among other nutrients [19]. No specific diet has been shown to prevent or treat IBD. Only rather general statements have been done, and it seems that in genetically predisposed individuals, a high consumption of milk and other dairy products, as well as refined sugar and processed fat, may trigger the onset of IBD [16–21]. On the other hand, a diet rich in dietary fiber and fruits seems to be protective [20].

The efficacy of some probiotics to improve IBD patients' quality of life has been recently reported [22–28]. The human intestinal microbiota confers a multitude of important functions to the host, such as aiding in digestion or protecting from penetration by pathogenic microbes [29]. Moreover, microbial imbalance or dysbiosis, which is characterized by an increase in the harmful bacteria and a reduction in the levels of beneficial bacteria, is commonly associated with diseases such as IBD [30]. Both CD and UC are pathologies located in areas where there are high bacterial concentrations [10].

There is evidence that commensal enteric bacteria and their products create a local environment that affects the course of IBD [10]. These high bacterial concentrations in IBD patients are characterized by decreased numbers of LAB and bifidobacteria and increased numbers of *E. coli*, coliforms, and bacteroides in the colon [11]. In this sense, probiotics might increase intestinal biodiversity and improve the symptoms of IBD patients. Probiotics that may suppress inflammation and/or activate innate immunity could be used within therapeutic strategies to restore the host gut microbiota [31–33].

An individualized diet together with the use of a suitable probiotic may be the best strategy for improving IBD patients' quality of life. The specific knowledge of the mechanisms of action of probiotics would be a helpful tool to design an efficient and specific therapy to improve the specific disease symptoms in IBD.

Some of the proposed mechanisms by which probiotics may exert beneficial effects are (1) the production of SCFAs and lactate, which inhibit the growth of potentially pathogenic organisms and have an anti-inflammatory effect on the gut; (2) the increased transit time by the net flow of water from the blood to the intestinal lumen, which influences the adherence of bacteria to the intestinal wall; and (3) the reduced production of noxious substances that may contribute to the pathogenesis of IBD [34].

An altered epithelial barrier function contributes to intestinal inflammation. Moreover the gut microbiota plays a fundamental role in the maturation of the host's innate and adaptive immune responses [35]. The regulation of the

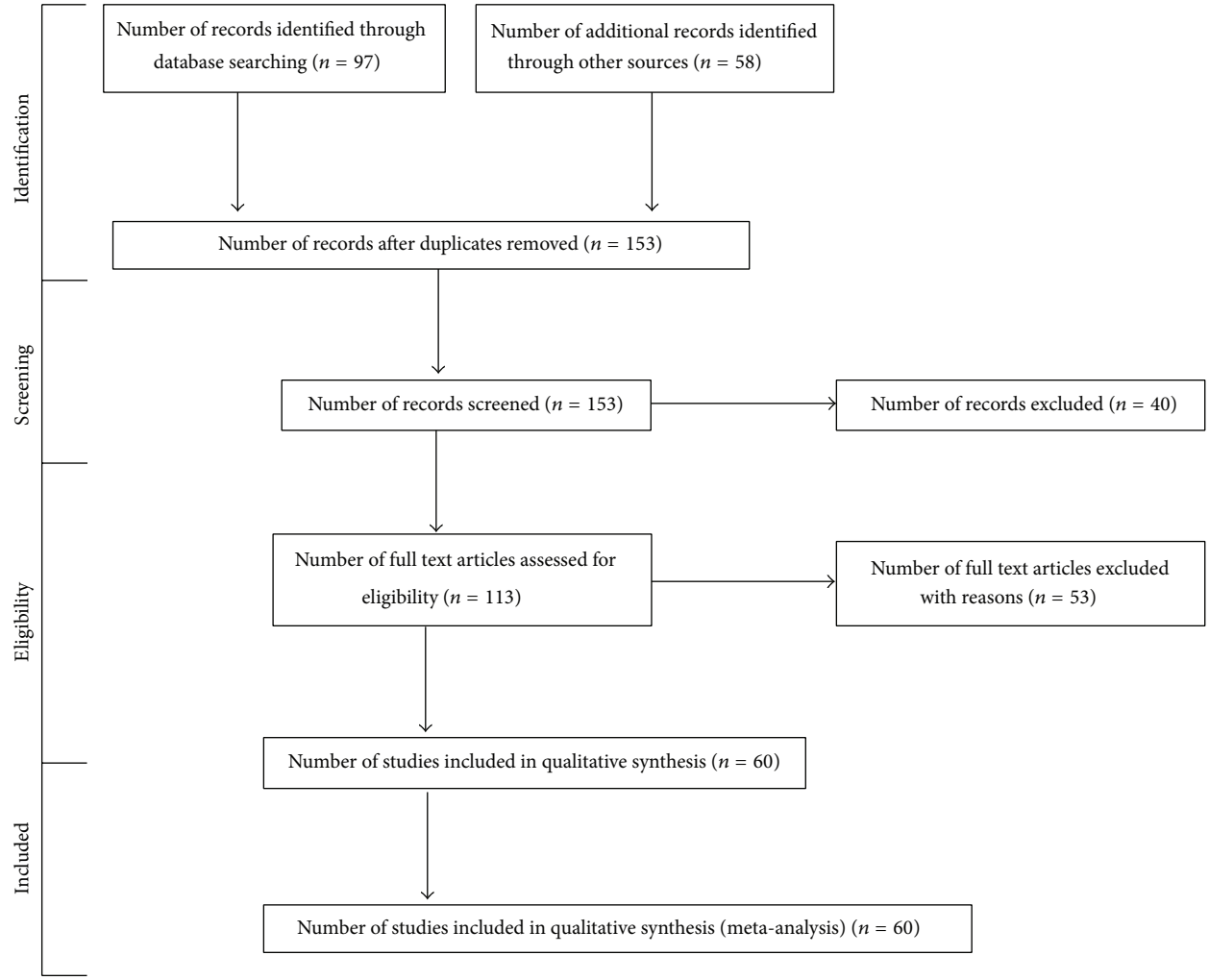

Figure 1

host immune response by microbiota could involve toll-like receptors (TLR), since these receptors have also been shown to be an important link between innate and adaptive immunity through their presence in dendritic cells (DCs) and intestinal epithelial cells (IECs) [5, 36–38].

The induction of tolerance or intestinal inflammation depends on a host's ability to distinguish between pathogenic invaders and harmless resident organisms [36]. In IBD, patients seem to lose the normal human tolerance to commensal bacteria and their immune response is upregulated. Thus, TLRs recognize antigens from the microbiota as pathogens that are expressed by a variety of cells, including IEC and DCs [35]. TLR2 and TLR4 are involved in the maintenance of intestinal epithelial homeostasis [37]. In fact, a high expression of TLR2 and TLR4 is associated with IBD [5]. Pathogenic bacteria activate TLR4, enhancing barrier disruption, subsequently facilitating allergen translocation in the gut mucosa and the production of proinflammatory cytokines, such as tumor necrosis alpha (TNF-α), interleukin (IL)-1, and IL-6 [5, 35, 37, 38].

On the other hand, apical TLR9 activation in intestinal epithelial cells by *Lactobacillus rhamnosus GG* (LGG) prevents the degradation of I$\kappa\beta$-α, consequently suppressing nuclear factor kappa B (NF-κB) activation and, in this way, preventing the production of proinflammatory cytokines [36, 38]. However, it is more complicated than that: genomic DNAs from *Bifidobacterium* and *Lactobacillus* strains interact with TLR2 and/or TLR9 to enhance the intestinal epithelial barrier function and to facilitate T_{reg} cell conversion via CD103+ DC [36, 37]. The interplay between microbiota and the gut immune system is complex.

Thus, Zeuthen et al. [37] reported that the combination of *L. acidophilus* X37, *L. paracasei* Z11, *L. casei* CRL431, LGG, *B. longum* Q46, *B. bifidum* Z9, *B. breve* 20091, and *B. bifidum* 20082a decreased IL-12 and TNF-α concentrations in culture supernatants and inhibited the Th1 skewing effect induced by strong stimulatory lactobacilli. This immunoinhibitory effect of bifidobacteria is TLR2-dependent and NOD2-independent [37]. Furthermore, a cell-free culture supernatant (CFS) from *Bifidobacterium breve* CNCM I-4035 also

Table 1: Summary of randomized clinical intervention trials of probiotics in IBD.

	Reference	Type of study	Number of patients	Age of patients (years)	Characteristics of patients	Probiotic strain	Medication	Intervention time/dose	Form of administration	Main outcome
Crohn's disease (CD)	Prantera et al., 2002 [50]	RDBPCT	45	22–71	Patients with a complete resection of all diseased intestine	*Lactobacillus rhamnosus* GG	No	365 d/6 × 10^{10} CFU twice daily	Oral	No effects compared with placebo group
	Schultz et al., 2004 [51]	RDBPCT	11	—	Patients with moderate to active CD	*Lactobacillus rhamnosus* GG	Yes	183 d/2 × 10^{9} CFU per day	Oral	No effects compared with placebo group
	Bousvaros et al., 2005 [52]	RDBPCT	75	5–21	Patients on CD remission	*Lactobacillus rhamnosus* GG	Yes	730 d/1 × 10^{10} CFU twice daily	Oral	No effects compared with placebo group
	Marteau et al., 2006 [53]	RDBPCT	98	27–42	Patients that had undergone surgical resection	*Lactobacillus. johnsonii* LA1	Yes	183 d/2 × 10^{9} CFU twice daily	Oral	No effects compared with placebo group
	van Gossum et al., 2007 [54]	RDBPCT	70	18–65	Patients with an elective ileocaecal resection	*Lactobacillus johnsonii* LA1	No	84 d/1 × 10^{10} CFU per day	Oral	No effects compared with placebo group
	Chermesh et al., 2007 [56]	RDBPCT	30	25 (mean age)	Patients that had undergone surgery treatment	*Synbiotic 2000	Yes	730 d/1 × 10^{10} CFU per day	Oral	No effects compared with placebo group
	Fujimori et al., 2007 [55]	CS	10	27 (mean age)	Patients with active CD	*Synbiotic therapy	Yes	395 d/7.5 × 10^{10} CFU per day and 3.3 g of psyllium thrice daily	Oral	Synbiotic therapy was safely and effectively used to treat active CD
	Steed et al., 2010 [57]	RDBPCT	35	18–79	Patients with active CD	*Bifidobacterium longum* plus *Synergy 1	Yes	183 d/2 × 10^{11} viable CFU and 6 g Synergy I twice daily	Oral	Synbiotic improved clinical symptoms in patients with active CD

Table 1: Continued.

	Reference	Type of study	Number of patients	Age of patients (years)	Characteristics of patients	Probiotic strain	Medication	Intervention time/dose	Form of administration	Main outcome
Ulcerative colitis (UC)	Ishikawa et al., 2003 [60]	RCT	21	39–60	Patients on UC remission	*BFM	Yes	365 d/1 × 10^{10} CFU per day	Oral	BFM supplementation successfully maintained remission
	Kato et al., 2004 [62]	RPCT	20	32 (mean age)	Patients with active UC	*BFM	Yes	84 d/1 × 10^{10} CFU per day	Oral	BFM supplementation was more effective than conventional treatment alone
	Tursi et al., 2004 [75]	RCT	90	19–69	Newly diagnosed or recently relapsed mild to moderate UC	*VSL#3	Yes	56 d/3 × 10^{11} CFU per day	Oral	Balsalazide/VSL#3 was significantly superior to balsalazide alone and to mesalazine in obtaining remission
	Cui et al., 2004 [65]	RCT	30	—	Patients with active UC	*BIFICO	Yes	56 d/1.26 g per day	Oral	BIFICO administration impeded the activation of NF-κB and elevated the expression of *IL-10*
	Furrie et al., 2005 [71]	RCT	18	24–67	Patients with active UC	*Bifidobacterium longum* plus *Synergy 1	Yes	28 d/2 × 10^{11} CFU and 6 g of Synergy 1 twice daily	Oral	Short-term treatment improved the full clinical appearance of chronic inflammation in patients with active UC
	Zocco et al., 2006 [67]	ROLT	187	33 (mean age)	Patients on UC clinical remission	*Lactobacillus*. GG	Yes	365 d/6 × 10^{9} CFU twice daily	Oral	*Lactobacillus* GG was not inferior to mesalazine and was significantly better at delaying relapses
	Fujimori et al., 2009 [72]	RCT	120		Patients on remission or with mildly active UC without a history of operation for UC	*Bifidobacterium longum* plus psyllium	Yes	28 d/2 × 10^{9} CFU per day and 4 g of psyllium twice daily	Oral	Synbiotic treatment improved the quality of life better than probiotic or prebiotic treatment
	Miele et al., 2009 [23]	RDBPCT	29	1.7–16.1	Children newly diagnosed with UC	*VSL#3	Yes	365 d/4.5 × 10^{11}–1.8 × 10^{12} CFU per day	Oral	VSL#3 was safe and effective in children treated for active UC**
	Hegazy and El-Bedewy, 2010 [68]	RCT	45	47 (mean age)	Mild to moderate UC patients with chronic diarrhea	*Lacteol	Yes	56 d/1 ×10^{10} CFU per day	Oral	Supplementation with probiotics could be advantageous in preventing relapse of UC and maintaining remission

Table 1: Continued.

Reference	Type of study	Number of patients	Age of patients (years)	Characteristics of patients	Probiotic strain	Medication	Intervention time/dose	Form of administration	Main outcome
Tursi et al., 2010 [61]	RDBPCT	131	47 (mean age)	Patients with mild to moderate relapsing UC	*VSL#3	Yes	56 d/1.8 × 10^{12} CFU twice daily	Oral	VSL#3 administration reduced the UCDAI scores in patients affected by relapsing mild-to-moderate UC
D'Incà et al., 2011 [70]	RCT	26	—	Patients with mild left-side UC	*Lactobacillus casei* DG	Yes	56 d/8 × 10^{8} CFU twice daily	Oral and rectal	5-ASA plus rectally administered probiotic modified the colonic microbiota, reduced the expression of *TLR-4, IL-1β*, and increased *IL-10* mRNA
Wildt et al., 2011 [64]	RDBPCT	32	≥18	Patients with UC in remission	*Probio-Tec AB-25	No	364 d/2.5 × 10^{10} CFU per day	Oral	Probio-Tec AB-25 was well tolerated
Ishikawa et al., 2011 [73]	RCT	41	45.5 (mean age)	Patients with mild to moderate UC	*Bifidobacterium breve* strain Yakult plus GOS	Yes	365 d/1 × 10^{9} CFU thrice a day and 5.5 g of GOS once a day	Oral	Synbiotic administration can improve the clinical condition
Oliva et al., 2012 [69]	RCT	31	7–18	Patients with mild to moderate UC	*Lactobacillus reuteris* ATCC 55730	Yes	61 d/1 × 10^{10} CFU per day	Rectal enema	Rectal infusion decreased the expression of proinflammatory cytokines and increased the expression of IL-10 in children

5-ASA: 5-aminosalicylic acid; BFM: bifidobacteria-fermented milk; CFU: colonic forming unit; CS: clinical study; d: days; GOS: galactooligosaccharide; IBS: irritable bowel syndrome; IL: interleukin; NF-κB: nuclear factor kappa B; OPUM: open-label prospective uncontrolled multicenter study; RCT: randomized clinical trial; RDBPCT: randomized double-blind placebo-controlled trial; ROLT: randomized open-label trial; RPCT: randomized placebo-controlled trial, TLR: toll-like receptor; UCDAI: ulcerative colitis disease activity index.

*Description of the bacterial and prebiotic contents of each product Synbiotic 2000: *Pediococcus pentosaceus*, *L. raffinolactis*, *L. paracasei* subsp. *paracasei* 19, and *L. plantarum* 2362 (10^{10} CFU of each bacteria) and *β*-glucans, inulin, pectin, and resistant starch (2.5 g of each fermentable fiber). Synbiotic therapy: *Bifidobacterium breve* and *Lactobacillus casei* (3 × 10^{10} CFU/daily of each bacteria) and 1.5 × 10^{10} CFU/daily of *Bifidobacterium longum* plus 3.3 g of psyllium twice daily. Synergy I: Orafti, Tienen, Belgium. BFM: live Yakult strains of *Bifidobacterium breve*, *Bifidobacterium bifidum,* and *Lactobacillus acidophilus* YIT 0168 in at least 10^{9} per 100 mL bottle. VSL#3: *Lactobacillus casei*, *Lactobacillus plantarum*, *Lactobacillus acidophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus*, *Bifidobacterium longum*, *Bifidobacterium breve*, *Bifidobacterium infantis*, *Streptococcus salivarius* subsp. *thermophilus,* and cornstarch. BIFICO: bifid triple viable capsule (oral capsules of live enterococci, bifidobacteria, and lactobacilli). Lacteol: 1 × 10^{10} CFU of *Lactobacillus delbrueckii* and *Lactobacillus fermentum.* Probio-Tec AB 25: *Lactobacillus acidophilus* strain LA-5 and *Bifidobacterium animalis* subsp. *lactis* strain BB-12 (1.25 × 10^{10} of each bacteria). Synbiotic zir fos: *Bifidobacterium longum* W11 (5 × 10^{9} CFU) and Fos-Actilight (2.5 g). **Relative Risk of relapse within 1 year of follow-up (RR: 0.32 CI: 0.025–0.773).

provides immunomodulatory effects on human intestinal DCs, mediated by cytokines [39, 40].

Bacteroides supports T helper (Th) and regulatory T (T_{reg}) cell polarization in a TLR2-dependent manner through the recognition of polysaccharide A by DCs [36]. The short-term consumption of yogurt supplemented with *Lactobacillus strains* GR-1 and RC-14 promotes a desirable anti-inflammatory environment in patients that are consistent with the putative immunosuppressive role of the expanded $CD4+CD25^{high}$ T cell population in humans [41]. Similarly, one study in mice described that probiotic bacteria (a mix of specific lactobacilli and bifidobacteria) may confer protection against chemically induced intestinal inflammation by T_{reg} cells through an immunoregulatory response involving IL-10 and transforming growth factor beta (TGF-β) [42]. Via both IL-10 production (which induces the differentiation of T_{reg}) and direct interaction with IgA, probiotics attenuate the immune response against commensal bacteria [38]. More recently, Longhi et al. [43] described a human subpopulation of Th17 (supTh17) cells that are reduced in patients with IBD. This population of human supTh17 cells (in contrast to prototypic Th17) exhibits immune suppressive properties because it expresses high levels of both CD39 and FOXP3 and consequently produces extracellular adenosine. These differences suggest that supTh17 cells might be recruited as suppressor-type cells in the later steps on the immune response where these cells may help to resolve injury at specific sites [43].

In summary, a specific probiotic bacterial strain could improve the state of the intestine by facilitating epithelial barrier functions, inhibiting T_{reg} cell-mediated mucosal inflammation and increasing production of IL-10 and TGF-β. This inflammation reduction may prevent colitis. However, further research should be performed with new LAB strains in experimental models of IBD and humans with either CD or UC. Also, the use of combinations of different probiotics should be studied.

4. Role of Lactic Acid Bacteria and Bifidobacteria in Crohn's Disease

CD is a chronic inflammatory condition of the gastrointestinal tract driven by abnormal T cell responses to the intestinal microbiota [44]. Therapy often involves the induction of remission with corticosteroids and maintenance therapy with a combination of aminosalicylates and immunomodulators [45, 46]. Nevertheless, the importance of the intestinal microbiota in the etiology of mucosal inflammation provides a rationale for therapeutic strategies using probiotics and prebiotics in patients with CD [32].

Most of the published controlled trials showed that 5-aminosalicylic acid (5-ASA) is significantly more effective than placebo in preventing relapses of the disease. However, negative results have also been reported [47, 48]. Therefore, the prevention of relapses remains a major issue in the treatment of CD. The experimental and clinical data suggest that the intestinal bacteria may play a role in the postsurgical recurrence of CD. Consequently, the operated patient offers the best in vivo opportunity for assessing the influence of luminal microbiota on the occurrence of new lesions [49].

Prantera et al. [50] conducted a randomized, double-blind, controlled trial with LGG given immediately after all of the diseased gut was surgically removed. The basic idea of the study was that counterbalancing the harmful gut microbiota (a possible cause of recurrent lesions in CD) with a beneficial bacterium would prevent the appearance of lesions or reduce their severity. Forty-five patients were randomized to receive LGG or a placebo for 12 months. The results revealed no differences in endoscopic and clinical remission between the two groups [50]. In another similar study with fewer patients, Schultz et al. [51] also could not demonstrate a benefit of LGG in inducing or maintaining medically induced remission in CD [51].

The use of LGG is not restricted only to adult studies. Bousvaros et al. [52] conducted a randomized, double-blind, placebo-controlled trial to see if the addition of LGG to standard therapy prolonged remission in children with CD. Concomitant medications allowed in the study included aminosalicylates, 6-mercaptopurine, azathioprine, and low-dose alternate day corticosteroids. Seventy-five children with CD in remission were randomized to either LGG or placebo and followed for up to 2 years. The median time to relapse was 9.8 months in the LGG group and 11.0 months in the placebo group; 31% of the patients in the LGG group developed a relapse compared with 17% of the placebo group. However, these values were not significantly different [52]. The proposed explanation for these negative results was that patients with CD may be more resistant to colonization with this organism and thus might require a different dosage. Early endoscopic recurrence is frequent after intestinal resection for CD. Marteau et al. [53] tested *Lactobacillus johnsonii* LA1 in this setting with a randomized, double-blind, placebo-controlled study. Patients were randomized to receive two packets per day of lyophilized *L. johnsonii* LA1 or a placebo for 6 months, and no other treatment was allowed. The primary endpoint was endoscopic recurrence at six months, with a grade >1 in Rutgeerts' classification or an adapted classification for colonic lesions. Ninety-eight patients were enrolled (48 in the *L. johnsonii* LA1 group). At 6 months, endoscopic recurrence was observed in 64% of the placebo group and in 49% in the *L. johnsonii* LA1 group. The endoscopic score distribution did not differ significantly between the *L. johnsonii* LA1 and placebo groups. The *L. johnsonii* LA1 did not have a sufficient effect, if any, to prevent the endoscopic recurrence of CD [53].

Additionally, van Gossum et al. [54] evaluated the efficacy of oral administration of *L. johnsonii* LA1 on early postoperative endoscopic recurrence of CD. The oral administration of *L. johnsonii* LA1 in patients with CD failed to prevent early endoscopic recurrence at 12 weeks after ileocecal resection [54]. The use of individual LAB does not appear to produce clinical improvements in CD patients.

Probiotics differ strongly and it is not possible to extrapolate a positive or negative result from one strain to another strain. Therefore, the ineffectiveness of LGG in the study of Prantera et al. [50] cannot predict the inefficacy of *L. johnsonii* LA1 and cannot predict the inefficacy of other single strains

in future trials [50]. Extrapolation of doses between various strains or products is also not possible. Mixtures of various strains could theoretically have additional or synergistic effects but they may also have antagonistic properties. Further studies of the microbiological, immunological, and clinical effects of lactic acid bacteria in maintaining disease remission are necessary.

Prebiotics have been associated with increased SCFA, mainly acetate, propionate, and butyrate [55]. Short-chain fatty acids, important nutrients for epithelial cells, are produced in the large bowel by the anaerobic bacterial fermentation of undigested dietary carbohydrates and fiber polysaccharides. Additionally, SCFA may actively contribute to the maintenance of colonic homeostasis [55].

A synbiotic is a regimen whereby probiotics are combined with prebiotics. Chermesh et al. [56] evaluated the use of Synbiotic 2000 in a clinical study to determine the efficacy in preventing the postsurgical recurrence of CD. Thirty patients were enrolled. No differences in either the endoscopic or the clinical relapse rate were found between patients treated with a once-daily dose of Synbiotic 2000 or a placebo. The Synbiotic 2000 had no effect on the postoperative recurrence of CD. The authors conclude that larger studies will be required because the number of patients may be too small to account for the individual differences in disease state, insufficient dosage, or negative interactions between specific probiotics and prebiotics. Additionally, using higher doses of a probiotic cocktail might prove effective [56].

Ten outpatients with active CD without a history of operation for CD were enrolled in a clinical study to evaluate the effects of synbiotics. Probiotics mainly comprised *Bifidobacterium* and *Lactobacillus*. Prebiotics, such as psyllium, are dietary substances that stimulate the growth and metabolism of protective commensal enteric bacteria. Patients were free to adjust their intake of probiotics or prebiotics throughout the trial. The Crohn's disease activity index (CDAI), International Organization for the Study of Inflammatory Bowel Disease (IOIBD) score, and blood sample variables were evaluated and compared before and after the trial. By the end of therapy, each patient had taken a $4.5 \pm 2.4 \times 10^{10}$ colonic forming-unit (CFU) daily probiotic dose, with six patients taking an additional 7.9 ± 3.6 g daily psyllium dose. Seven patients had improved clinical symptoms following combined probiotic and prebiotic therapy. Both CDAI and IOIBD scores were significantly reduced after therapy. There were no adverse events [55]. This study confirmed that high-dose probiotic and prebiotic cotherapy can be safely and effectively used for the treatment of active CD.

Finally, Steed et al. [57] evaluated synbiotic consumption in active CD. Thirty-five patients with active CD were enrolled in a randomized, double-blind, placebo-controlled trial, using a synbiotic comprising *Bifidobacterium longum* and Synergy 1. Their clinical status was scored and rectal biopsies were collected at the start, then again at 3- and 6-month intervals. The transcription levels of immune markers and mucosal bacterial 16S rRNA gene copy numbers were quantified using real-time PCR. Significant improvements in clinical outcomes occurred with synbiotic consumption, with reductions in both CDAI and histological scores. The synbiotic had little effect on mucosal IL-18, interferon γ, and IL-1β. However, significant reductions occurred in TNF-α expression in synbiotic patients at 3 months, but not at 6 months [57]. The synbiotic consumption was effective in introducing beneficial bacteria into the gastrointestinal tract in Crohn's patients, thereby modulating the species composition of the mucosal biofilm in the large bowel.

In conclusion, the investigation presented provides evidence that synbiotics (pre- and probiotics) have the potential to be developed into acceptable therapies for acute and active CD. More studies are needed to determine whether the synbiotic modulates other anti-inflammatory components of the mucosal microbiota [58, 59], or whether other synbiotic combinations can be as effective in CD [57].

5. Role of Lactic Acid Bacteria and Bifidobacteria in Ulcerative Colitis

UC is a nonspecific colorectal erosive inflammatory condition characterized by inflammation of the mucosa, erosion, and ulceration [60]. Patients with UC have periods of exacerbations and periods of remission. The treatment consists of inducing remission periods and maintaining those conditions using anti-inflammatory molecules (i.e., 5-ASA compounds); systemic and topic corticosteroids, immunosuppression drugs such as 6-mercaptopurine, and TNF-α antibodies have been used. However, these treatments present side effects that mean that a significant proportion of patients do not tolerate the existing treatments [23].

Numerous studies, in both IBD patients and gnotobiotic animals, have noted the influence of the intestinal bacteria on the development and/or exacerbation of UC [60]. Moreover, a lower number of bifidobacteria have been observed in the feces of UC patients than in healthy subjects [60]. Modulation of the intestinal microbiota can be performed either by antibiotics or by probiotics, but the former are not good candidates for chronic disease because of antibiotic resistance, potential side effects, and ecological concerns [61]. The modification of the intestinal microbiota through direct supplementation with protective bacteria could play a protective role in the inflammatory process [62].

Bifidobacteria-fermented milk (BFM) supplementation may reduce exacerbations of UC through the normalization of the intestinal microbiota [61]. Ishikawa et al. [60] reported that BFM supplementation reduced the luminal butyrate concentration, a key molecule in the remission of colitis. This reduction reflected the increased uptake or oxidation of SCFAs by the improved colorectal mucosa [60]. Similarly, Kato et al. [62] found increased levels of fecal butyrate, propionate, and SCFA acid concentrations in patients with active UC (mild to moderate), who received BFM together with conventional treatment [62]. In this pilot study, patients supplemented with BFM showed a significantly lower clinical activity index than the placebo group. Likewise, the posttreatment endoscopic index and histological score were reduced in the BFM group [62].

TNF-α exerts a pivotal role in the pathogenesis of active UC; therefore, inhibiting its secretion in inflamed

UC mucosa is a major target for treating the disease and preventing relapse [63]. Coculturing colonic biopsies from active UC with *B. longum* reduced the release of TNF-α and IL-8 compared with the inflamed colonic tissue alone. It is well known that the activation of NF-κB can regulate inflammatory cytokines such as TNF-α, IL-8, and IL-6. Immunohistochemical staining of NF-κB p65 in colonic biopsies from active UC showed many cells with positive nuclear staining, whereas fewer NF-κB-positive cells were found in the lamina propria after the tissues were cocultured with either *B. longum* or dexamethasone, which indicates that *B. longum* can inhibit NF-κB activation in lamina propria cells [63].

Probio-Tec AB-25, a mixture of *L. acidophilus* strain La-5 and *B. animalis* subsp. *lactis* strain Bb-12, was tested for the maintenance of remission in patients with left-sided UC, in a 1-year, prospective, randomized, double-blind and placebo-controlled trial [64]. The safety and tolerance of Probio-Tec AB-25 and the placebo were good. Gastrointestinal symptoms were reported equally in both treatment groups and a relationship between Probio-Tec 25 and gastrointestinal side effects could not be established. At weeks 4 and 28, Bb-12 or La-5 were detected in 11 patients receiving probiotics. Five patients in the probiotic group (25%) and one patient in the placebo group (8%) maintained remission after 1 year of treatment. In the probiotic group, the median time to relapse was 125.5 days, versus 104 days in the placebo group. It is possible that in larger studies a significant difference could be achieved, but whether this would be of clinical significance is debatable [64].

The use of BIFICO (oral capsules of live enterococci, bifidobacteria, and lactobacilli) in combination with sulphasalazine (SASP) and glucocorticoid exerts some beneficial effects in preventing the relapse of UC [65]. The administration of BIFICO plus SASP and glucocorticoid to UC patients enlarged the number of bifidobacteria and lactobacilli and reduced the number of enterococci, bacteroides, and bifidobacteria present in the feces compared with the control group [65]. Moreover, Cui et al. [65] suggested that probiotics might block the activation of NF-κB, decrease the expression of the proinflammatory cytokines TNF-α and IL-1β, and increase the expression of the anti-inflammatory cytokine IL-10 [64].

In the same way, the administration of *B. infantis* 35624 (1×10^{10} CFU) for six weeks to patients with mild- to moderate-active UC, during concurrent treatment with 5-ASA, significantly reduced plasma C-reactive protein (CRP) levels versus the placebo-treated controls [66]. However, when comparing pre- and posttreatment levels, there were no significant differences in the UC patients. Although CRP levels in the placebo group increased posttreatment, this result was likely because these patients did not receive steroid treatment during the trial period. In the case of plasma TNF-α levels, no significant differences were observed between the group that received the probiotic strain and the placebo group, or in the UC patients before treatment and after treatment. Regarding plasma IL-6, Groeger et al. [66] found a lower plasma level in UC patients compared with placebo controls; however, the authors did not find any change in the IL-6 levels in the UC patients between the pre- and posttreatment [66].

The most studied probiotic in clinical trials is *L. rhamnosus*, which is represented in the bowel of healthy individuals [67]. In agreement with this, Zocco et al. [67] studied the efficacy of LGG supplementation versus standard mesalazine for maintaining disease remission in UC patients. After 6 and 12 months of treatment the percentage of patients maintaining clinical remission was, respectively, 91% and 85% for the LGG group (1.8×10^{10} viable bacteria/day), 87% and 80% for the mesalazine group (2.400 mg/day), and 94% and 84% for the combined treatment (LGG plus mesalazine) [67].

The oral administration of Lacteol (Lacteol Fort, Rameda, Egypt), a probiotic preparation that contains 1×10^{10} CFU of *L. delbrueckii* and *L. fermentum*, together with 2,400 mg/day of sulfasalazine, during 8 weeks, to UC patients with chronic diarrhea, inhibited the extent of inflammation, prevented mucosal injury, and alleviated colitis [68]. One inflammatory cascade within the gut tissues during UC is characterized by the recruitment of circulating leukocytes and the release of proinflammatory mediators [68]. Lacteol administration not only reduced myeloperoxidase (MPO) activity, an index of leukocyte infiltration, but also reduced the colonic concentration of IL-6 and *TNF-α*. Regarding NF-κB p65 levels, the UC patients showed the more activated NF-κB p65 protein, whereas the lowest level was observed in the probiotic group [68].

In children with distal active UC, rectal administration of *L. reuteri* ATCC 55730 (as an enema solution containing 1×10^{10} CFU) for 8 weeks in addition to standard oral mesalazine resulted in a significant decrease in the Mayo DAI score (Mayo Disease Activity Index-DAI) compared with the children that received the corresponding placebo. In addition, all of the children on *L. reuteri* had a clinical response, whereas only 53% of the children on the placebo responded. Clinical remission was achieved in 31% of the *L. reuteri* group and in no children of the placebo group. At the posttrial the rectal mucosal expression levels (determined by RT-PCR in biopsy samples) of *IL-10* were significantly increased, whereas a significant decrease was found in the levels of *IL-1β*, *TNF-α*, and *IL-8*, only in the *L. reuteri* group [69].

Additionally, D'Incà et al. [70] evaluated the effect of an 8-week oral and/or rectal administration of *L. casei* DG on colonic-associated microbiota, mucosal cytokine balance, and TLR expression in patients with mild left-sided UC. The patients were divided into three groups: the first group received oral 5-ASA alone, the second group received oral 5-ASA plus oral *L. casei* DG (8×10^{8} CFU), and the third group received oral 5-ASA and rectal *L. casei* DG (8×10^{8} CFU). A significant improvement of the histological disease severity scores was found in patients receiving the probiotic strain by the oral or rectal route of administration. Nevertheless, oral supplementation with *L. casei* DG did not have a significant effect on the counts of *Enterobacteriaceae* or *Lactobacillus*. However, the occurrence of *Lactobacillus* and *Enterobacteriaceae* cultured from biopsy specimens was

increased and decreased, respectively, in the group that took the probiotic rectally. Moreover, the rectal administration of *L. casei* DG significantly reduced *TLR-4* and *IL-1β* levels and significantly increased mucosal IL-10 [70].

Probiotic therapy can be improved through combination with a prebiotic (a nondigestible oligosaccharide that is absorbed in the upper gut). This combination is known as a synbiotic [71]. In a double-blinded randomized controlled trial, Furrie et al. [71] demonstrated that the administration of a synbiotic (*B. longum* plus Synergy 1), for a period of one month to patients with active UC, improved the full clinical appearance of chronic inflammation [71]. In this sense, the proinflammatory cytokines TNF-α and IL-1α were significantly reduced after treatment. In addition, the levels of bifidobacteria, determined by quantitative PCR, increased 42-fold in the synbiotic group but only 4.6-fold in the placebo group [71].

From this study, it is clear that synbiotic positively affects the chronic inflammation associated with UC. The comparison of the effectiveness of probiotics or prebiotics with that of synbiotic therapy was conducted by Fujimori et al. [72]. They designed a randomized trial to evaluate the effects of a 4-week treatment with probiotics, prebiotics, or synbiotics in patients with UC in remission. The probiotic group received 2×10^9 CFU of *B. longum* (Bificolon, Nisshin Kyorin Pharmaceutical Co., Ltd., Tokyo) once daily; the prebiotic group was prescribed 4.0 g of psyllium to be taken twice daily. The synbiotic group simultaneously underwent probiotic and prebiotic therapies. The doses of aminosalicylates and prednisolone for UC treatment remained the same throughout the trial in all groups [72]. At the end of the trial, the authors found a statistically significant improvement of the Inflammatory Bowel Disease Questionnaire (IBDQ) scores in the synbiotic group. However, in this open-label trial, the authors did not perform a standard evaluation of the disease activity (endoscopic or histological evaluation) [72].

The beneficial effects of live *Bifidobacterium breve* strain Yakult (BbY) and galactooligosaccharide (GOS), as a synbiotic, were evaluated by Ishikawa et al. [73]. Patients diagnosed with UC received 1 g of the freeze-dried powder containing BbY (1×10^9 CFU/g) and 5.5 g of GOS once/day. The control group comprised patients treated as usual (salazosulfapyridine, mesalazine, and steroids). After one year of intervention, the endoscopic scores of the synbiotic group were significantly lower than in the control group. In addition, the amounts of MPO in the lavage solution significantly decreased in patients with active UC after synbiotic treatment. Fecal bacteria analyses showed significant differences in the number of *Bacteroidaceae* before and after the synbiotic treatment in UC. Moreover, fecal pH was significantly lower after the synbiotic treatment [73].

The probiotic preparation VSL#3 has been extensively used. VSL#3 contains four strains of *Lactobacillus* (*L. casei*, *L. plantarum*, *L. acidophilus*, and *L. delbrueckii* subsp. *bulgaricus*), three strains of *Bifidobacterium* (*B. longum*, *B. breve*, and *B. infantis*), one strain of *Streptococcus salivarius* subsp. *thermophilus*, and cornstarch. VSL#3 is capable of colonizing the gut and significantly decreases fecal pH in UC patients that are intolerant or allergic to 5-ASA [74]. Furthermore, the intake of the probiotic mixture maintained remission in the great majority of UC patients that were intolerant or allergic to 5-ASA [74]. Additionally, it has been reported that balsalazide provides a more rapid relief of UC symptoms and induces complete remission in a greater percentage of patients than mesalamine, but these results were obtained using a high dose of balsalazide [75]. Balsalazide is converted into 5-ASA and 4-aminobenzoyl-β-alanine by the colonic bacteria. The use of 2.25 g of balsalazide (containing 750 mg of balsalazide disodium) plus 3 g of VSL#3 achieved remission faster than balsalazide or mesalazine. Moreover, balsalazide plus VSL#3 showed significant superiority in improving well-being and bowel frequency and endoscopic and histological scores were significantly better in the group of patients who received balsalazide/VSL#3 compared with the patients who received mesalazine at the end of the treatment [75]. Tursi et al. [75] showed that the combination of low-dose balsalazide plus VSL#3 resolved the problem of taking several capsules of balsalazide in comparison with mesalazine capsules to achieve remission in UC patients [75]. Therefore, the combination of low-dose balsalazide and VSL#3 may be a good choice in the treatment of active mild-to-moderate left-side- or distal-ulcerative colitis versus balsalazide or mesalazine alone [75]. This combination acts in two different ways to cease inflammation: 5-ASA inhibits some key enzymes of the inflammatory cascade, such as cyclooxygenase, thromboxane-synthetase, and platelet associated factor-synthetase and also inhibits the production of IL-1 and free radicals, whereas the action of probiotics includes the production of antimicrobials, competitive metabolic interactions with proinflammatory organisms and the inhibition of the adherence and translocation of pathogens [75].

In addition to this study, Tursi et al. [61] conducted a multicenter, double-blind, randomized, placebo-controlled, parallel study in patients affected by relapsing mild-to-moderate UC being treated with 5-ASA and/or immunosuppressants at stable doses to assess the effects of VSL#3 supplementation. They showed that VSL#3 supplementation (3.6×10^{12} bacteria per day) for 8 weeks was safe and able to reduce the UCDAI (Ulcerative Colitis Disease Activity Index) scores. Moreover, VSL#3 improved rectal bleeding and seemed to reinduce remission in relapsing UC patients, although these parameters did not reach statistical significance [61].

Bibiloni et al. [76] described that treatment of patients with active (mild to moderate) UC, not responding to conventional therapy, and receiving VSL#3 3.6×10^{12} bacteria daily in two divided doses for 6 weeks, resulted in a combined induction of remission/response rate of 94% in patients who completed the study. It is important to highlight that the authors reported no adverse events other than mild bloating [76]. In addition, *S. salivarius* subsp. *thermophilus* and *B. infantis* were detected by PCR/denaturing gradient gel electrophoresis, in association with biopsies collected after (but not before) treatment with VSL#3 in the case of 3 patients in remission [76].

In addition, the efficacy of VSL#3 in the induction and maintenance of remission and their safety and tolerability

in children has been evaluated in a prospective, 1-year (or until relapse), placebo-controlled, double-blind study conducted by Miele et al. [23]. Patients (age range: 1.7–16.1 years) with newly diagnosed UC received either VSL#3 (weight-based dose, range: 0.45–1.8 $\times$ 10^{12} bacteria/day) or an identical placebo associated with concomitant steroid induction treatment. Remission was achieved in 92.8% of the children treated with VSL#3 and IBD conventional therapy and in 36.4% of the patients treated with placebo and IBD conventional therapy. Furthermore, 21.4% of the patients receiving VSL#3 treatment and 73.3% receiving the corresponding placebo (both groups also received IBD conventional therapy) relapsed within 1 year of follow-up. Regarding the endoscopic and histological scores, at 6 months and 12 months, they were significant lower in the VSL#3 group. It is important to emphasize that no side effects or significant changes from baseline values in any of the laboratory parameters examined were reported that could be attributed to treatment with either VSL#3 or placebo [23].

In conclusion, the use of probiotics and/or synbiotics has a positive effect in the treatment of UC and in the maintenance of remission periods. Probiotics and/or synbiotics reduced the expression of proinflammatory cytokines such us TNF-α and enhanced the expression of anti-inflammatory cytokine such us IL-10, likely through the inhibition of NF-κB activation.

6. Role of Lactic Acid Bacteria and Bifidobacteria in Other Related Inflammatory Bowel Diseases

6.1. Pouchitis. Pouchitis is a common troublesome condition in surgical patients with ileal-pouch-anal-anastomosis (IPAA) [24] and is a nonspecific idiopathic inflammation of the ileal reservoir [77]. The daily administration of 500 mL of a fermented milk product (Cultura) containing live *L. acidophilus* (La-5) and *B. lactis* (Bb-12) for 4 weeks increased the number of lactobacilli and bifidobacteria in the UC/IPAA patients and remained significantly increased one week after the intervention. Moreover, involuntary defecation, leakage, abdominal cramps the need for napkins, fecal number, fecal consistency, fecal mucus, and urge to evacuate stools were significantly decreased/improved during the intervention period in the UC/IPAA patients [24].

The effects of the administration of VSL#3 (6 g/day) on patients with antibiotic therapy-induced pouchitis in remission have been studied by Kühbacher et al. [78]. The authors conducted a double-blind, randomized, placebo-controlled clinical trial. They took biopsies before and two months after the initiation of VSL#3 or placebo treatment. The patients who received the probiotic mixture were in remission at the time of the second biopsy, while the patients who received a placebo exhibited clinical and endoscopic signs of recurrent inflammation. Furthermore, there was an increase in the bacterial richness and diversity of the pouch mucosal microbiota in the VSL#3 patients compared with both patients in remission before therapy and patients developing pouchitis while receiving the placebo. The authors also described an increase in *Enterobacteriaceae* within the mucosa during the VSL#3 treatment. This fact indicates that remission maintenance during probiotic therapy is associated with the restoration of parts of the normal pouch biota [78].

Similarly, oral administration of high doses of VSL#3 was effective in the treatment of active mild pouchitis. The authors reported that treatment with VSL#3 significantly improved clinical, endoscopic, and histologic parameters on the PDAI (Pouchitis Disease Activity Index), with complete remission in almost 70% of the patients [77]. The microbiologic study showed a significant increase in the fecal concentration of bifidobacteria, lactobacilli, and *S. thermophilus*; however, no modification of *Bacteroides*, clostridia, coliforms, and enterococci was found, suggesting that the beneficial effect was not mediated by the suppression of the endogenous microbiota. These data indicate that the efficacy of VSL#3 may be related to increased concentrations of protective bacteria and further support the potential role for probiotics in IBD therapy [77].

In addition to these studies, Pronio et al. [79] carried out an open-label study with IPAA performed for UC; the patients received VSL#3 (0.45 $\times$ 10^{12} bacteria/day) or no treatment (control group) for 12 months. The patients treated with the probiotic showed a slight but significant reduction in PDAI scores after 3 months of treatment compared with baseline. This difference was maintained at 6 and 12 months of follow-up. Moreover, the data obtained by Pronio et al. [79] showed that probiotic administration in patients with IPAA expanded regulatory cells in the pouch mucosa. This finding was associated with an increased expression of *Foxp3* mRNA, a transcription factor needed for the generation and function of regulatory CD4+CD12+T cells and CD4+CD25+T cells that control the immune response to self and foreign antigens and are involved in oral tolerance. Furthermore, tissue samples showed a significant reduction in *IL-1β* mRNA expression. The authors concluded that the administration of probiotics after IPAA in patients without signs or symptoms of acute pouchitis induces an expansion of the associated regulatory cells [79].

6.2. Irritable Bowel Syndrome. IBD and irritable bowel syndrome (IBS) can be considered as different pathologies. IBD is recognized as an organic bowel disorder while IBS is a functional bowel disorder, although some particular cases in both disorders may display similar symptoms. Therefore, distinguishing clinical manifestations may be sometimes difficult [80, 81]. IBS, or spastic colon, is a symptom-based diagnosis characterized by chronic abdominal pain, discomfort, bloating, and altered bowel habits where the diarrhea or constipation may be predominate, or they may alternate. Indeed, the onset of IBS is more likely to occur after an infection [82, 83]. For that reason, favoring appropriate environmental intestinal conditions could delay or even avoid the onset of IBS. Thus, although considered as different pathologies, some authors recognized an association between IBD and IBS.

Hong et al. [84] evaluated the effects of probiotic LAB and bifidobacteria by-fermented milk (specifically *Lactobacillus*

sp. HY7801, *Lactobacillus brevis* HY7401, and *Bifidobacterium longum* HY8004) on seventy-four IBS patients through clinical parameters and ^{1}H nuclear magnetic resonance- (NMR-) based metabolomics from peripheral blood. This study reported decreased glucose and tyrosine levels and increased lactate in sera of patients but not in healthy volunteers. They argued that this increase in lactate in blood might be caused by intestinal microbiota that produce lactate through fermentation because of increased populations of intestinal LAB after probiotic administration. They further related the low serum glucose levels to elevated glycolysis in the body's attempt to accommodate the higher energy demand caused by small nutrient absorption [84]. They also suggested that decreased tyrosine is related to hepatobiliary disease, one of the most common extraintestinal manifestations of IBD, because tyrosine metabolism occurs mainly in the liver [84].

Furthermore, Dughera et al. [85] confirmed that the administration of a synbiotic agent in patients with constipation-variant IBS improved intestinal function and ameliorated the disease clinical manifestations. The synbiotic preparation included strains of *Bifidobacterium longum* W11, one of the most representative species of gut microbiota, and oligosaccharides, which exert a positive effect on intestinal motility and favor the development of bifidobacteria in the gut lumen [85]. Although these two works suggest that probiotics combined with prebiotics exert beneficial effects on IBS symptoms, more studies are needed to clearly demonstrate a positive effect [84, 85].

6.3. Cholangitis. Cholangitis is an infection of the common bile duct, the tube that carries bile from the liver to the gallbladder and intestines. It is usually caused by a bacterial infection, which can occur when the duct is blocked, such as a gallstone or tumor. The infection causing this condition may also spread to the liver [86].

The effects of a probiotic mixture (specifically *L. acidophilus*, *L. casei*, *L. salivarius*, *L. lactis*, *B. bifidum*, and *B. lactis*) have been evaluated on the liver biochemistry or function and symptoms in primary sclerosing cholangitis (PSC) patients with IBD that were receiving ursodeoxycholic acid (UDCA) maintenance therapy [87]. The absence of any significant positive effects was attributed to the concurrent use of UCDA, the relatively small number of patients studied, or the relatively short duration of treatment [87]. Nevertheless, Shimizu et al. [88] found that the combination of immunosuppressive therapy and a probiotic (*L. casei* Shirota, 3 g/day) provided benefits for both IBD and PSC. They suggested that bacterial microbiota and gut inflammation are closely associated with the pathogenesis of IBD-related PSC. This suppression of bowel inflammation and maintenance of bacterial homeostasis may be important for treating PSC [88] and other pathologies in which the host's relationship with the intestinal microbiota is relevant.

These contradictory effects described in the literature suggest that additional studies are needed to determine the effects of probiotics as adjunctive therapy for those inflammatory conditions of the gut.

7. Conclusions and Further Directions

This review focused on the clinical evidences that support the use of LAB and bifidobacteria probiotics as a valuable coadjuvant therapeutic strategy for the prevention and treatment of diseases such as IBD. The current scientific evidences are more significant in UC than in CD. However, more detailed mechanistic studies on the effectiveness of probiotics in IBD are necessary to determine their potential beneficial effects. Therefore, more clinical trials with the use of appropriate molecular tools are necessary to determine which main outcomes and additional immune- and inflammation-associated variables are clearly influenced, and particularly the cause of these changes in the development of IBD.

For this reason, more randomized double-blind placebo-controlled multicenter trials with appropriate doses and LAB are needed. However, well before this stage, preliminary studies confirming the potential probiotics' mechanisms of action need to be done in cell and animal models.

The investigation of the interactions between the environment, the diet, and the host constitutes one of the major issues in the development of IBD. The incidence of chronic disease in the adult state is related to epigenetic changes that happen earlier in life. Major clinical trials should also study the mechanisms of action of probiotics using new molecular tools such as the study of the microbiota changes using massive parallel sequencing (MPS), metabolomics, transcriptomics, and proteomics analyses of biopsies.

Beyond understanding the molecular mechanisms, further studies to evaluate the best dose-response-effect of probiotics are recommended, including following up with patients after the probiotic intervention to evaluate the persistence of beneficial effects.

Finally, determining the effect of fermented dairy products on the development and maintenance of the disease will also require specific clinical trials.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Review Article

A Survey of Modulation of Gut Microbiota by Dietary Polyphenols

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Dietary polyphenols present in a broad range of plant foods have been related to beneficial health effects. This review aims to update the current information about the modulation of the gut microbiota by dietary phenolic compounds, from a perspective based on the experimental approaches used. After referring to general aspects of gut microbiota and dietary polyphenols, studies related to this topic are presented according to their experimental design: batch culture fermentations, gastrointestinal simulators, animal model studies, and human intervention studies. In general, studies evidence that dietary polyphenols may contribute to the maintenance of intestinal health by preserving the gut microbial balance through the stimulation of the growth of beneficial bacteria (i.e., lactobacilli and bifidobacteria) and the inhibition of pathogenic bacteria, exerting prebiotic-like effects. Combination of *in vitro* and *in vivo* models could help to understand the underlying mechanisms in the polyphenols-microbiota-host triangle and elucidate the implications of polyphenols on human health. From a technological point of view, supplementation with rich-polyphenolic stuffs (phenolic extracts, phenolic-enriched fractions, etc.) could be an effective option to improve health benefits of functional foods such as the case of dairy fermented foods.

1. Introduction

More and more studies confirm the importance of the gut microbiota in host health, including mental health. Gut bacteria not only help us to maximize the absorption of nutrients and energy, but also are essential in the body health status [1]. In particular, microbial infections and imbalances in the composition of the gut microbiota are associated with intestinal disorders such as chronic inflammatory bowel diseases and with other immune related disorders [2, 3]. Although genetic and environmental factors are main determinants of gut microbiota composition, it is well established that diet influences microbial fermentation and total bacteria in the intestine. In fact, interindividual variation in gut microbiota may, in part, reflect differences in dietary intake, although the response of the gut microbiota to dietary change can also differ among individuals [4].

Phenolic compounds or polyphenols are secondary metabolites with a widespread occurrence in the plant kingdom. In nature, polyphenols can be classified into two major groups: flavonoids and nonflavonoids. Among flavonoids, various groups can be distinguished according to the C-heterocycle structure: flavonols, flavones, flavan-3-ols, isoflavones, flavanones, dihydroflavonols, anthocyanidins, and chalcones (Figure 1). Nonflavonoid phenolics include phenolic acids, hydrolysable tannins, and stilbenes, among others. Polyphenols also form part of the human diet, being present in a broad range of commonly consumed fruits, vegetables, and plant-derived products such as cocoa, tea, or wine. A number

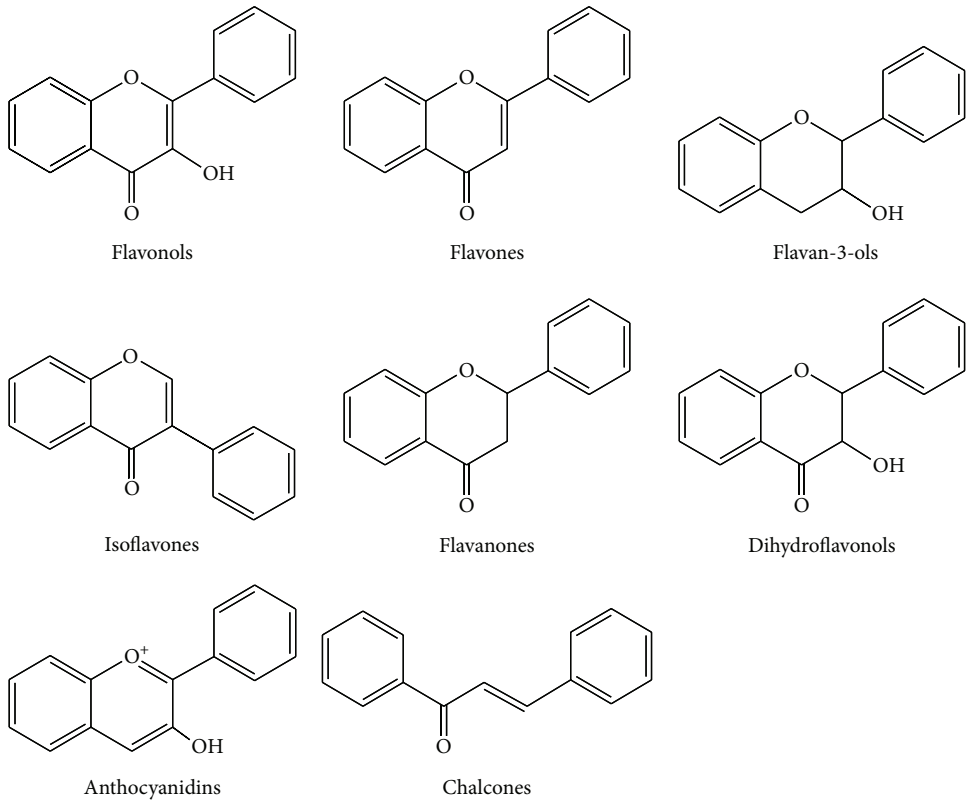

Figure 1: Common phenolic compounds in food.

of epidemiological studies have shown that the intake of diets rich in fruits and vegetables is inversely associated with the risk of various chronic diseases, such as coronary heart disease, specific cancers, and neurodegenerative disorders [5–7]. Indeed, a range of pharmacological effects have been demonstrated for different phenolic compounds—especially flavonoids—through *in vitro*, *ex vivo* and animal assays [8, 9]. However, health effects of these compounds depend on their bioavailability and, therefore, it is important to understand how they are absorbed, metabolized, and eliminated from the body, in order to ascertain their *in vivo* actions.

Modulation of gut microbiota by polyphenols has been a topic of increasing attention by the scientific community in the last years. Several studies have been carried out by different authors ranging from the simplest experimental approaches on the effect of polyphenols on the growth of isolated intestinal bacteria to complex approximations implying the whole fecal microbiota, either in fermentation experiments (batch cultures and continuous simulators) or through compositional analysis of animal and human fecal samples. The existing knowledge about relationships between polyphenols and gut microbiota has been object of many reviews from different perspectives. Thus, some authors have put their attention on the impact of food constituents (polyphenols included) in the gut microbiome [10, 11], while others have focused on the effects of dietary polyphenols on microbial modulation and their potential implications in human health [12–15]. Selma et al. [16] wrote probably the first review trying to put together the concepts of microbial degradation of polyphenols and modulation of gut microbiota by polyphenols and phenolic metabolites. This two-way interaction between phenolics and intestinal bacteria has been also reviewed focusing on wine [17] and tea polyphenols [18]. The development of improved biology and microbial techniques has allowed notable advances in the knowledge of the gut microbiota and their modulation by dietary components and hence polyphenols. The potential of the novel metabolomic approaches in the study of the impact of polyphenols on gut microbiome has been recently reviewed [19].

Being aware of all this previous reviewing work, we have aimed to update the available information about modulation of gut microbiota by dietary polyphenols with a perspective based on the experimental approaches used. After two general sections covering relevant aspects about gut microbiota (Section 2) and dietary polyphenols (Section 3), studies are presented according to their experimental design: batch culture fermentations (Section 4), gastrointestinal simulators (Section 5), animal model studies (Section 6), and human intervention studies (Section 7). Main findings and general conclusions generated from the different types of studies are finally discussed (Section 8).

2. Gut Microbiota Composition and Analysis

The human gut is the natural habitat of a large, diverse population and dynamics of microorganisms, mainly anaerobic bacteria, which have adapted to life on mucosal surfaces

in the gut lumen. The acquisition of gut microbiota begins at birth and is strongly influenced by a range of factors that include host genetics, immunological factors, antibiotic usage, and also dietary habits [20]. The microbial content of the gastrointestinal tract changes along its length, ranging from a narrow diversity and low numbers of microbes in the stomach to a wide diversity and high numbers in the large intestine, which can reach 10^{12} CFU/mL [21]. Most of intestinal bacteria belong to phylum Firmicutes (including *Clostridium*, *Enterococcus*, *Lactobacillus*, and *Ruminococcus* genera) and Bacteroidetes (including *Prevotella* and *Bacteroides* genera) which constitute over 90% of known phylogenetic categories and dominate the distal gut microbiota [22]. Recently, a novel classification of microbiota into three predominant "enterotypes," dominated by three different genera, *Bacteroides*, *Prevotella*, and *Ruminococcus*, has been suggested [23]. In this line, Wu et al. [24] demonstrated that long-term diet high in animal proteins and fats versus simple carbohydrates clustered the human subjects into the previously described enterotypes *Bacteroides* and *Prevotella*. However, there is a current debate if the enterotypes should be seen discontinuous or as a gradient [25]. But in any case, a common observation is that homeostasis and resilience are coupled to a highly diverse gut microbiota in healthy people, whereas inflammatory and metabolic disorders are linked to perturbations in the composition and/or functions of the gut microbiota [26].

Culture-based techniques employed to bacteria identification are fairly cheap, laborious, and time-consuming and gives a limited view of the diversity and dynamics of the gastrointestinal microbiota, with less than 30% of gut microbiota members having been cultured to date [27]. Since 1990s, the introduction of novel molecular biological procedures has made it possible to overcome some of these limitations with the use of culture-independent methods [28]. These procedures are based on sequence divergences of the small subunit ribosomal RNA (16S rRNA) and include techniques such as denaturing gradient gel electrophoresis (DGGE), terminal restriction fragment length polymorphism (T-RFLP), fluorescence *in situ* hybridization (FISH), quantitative polymerase chain reaction (qPCR), DNA microarrays, and next-generation sequencing (NGS) of the 16S rRNA gene or its amplicons [29]. NGS techniques have promoted the emergence of new, high-throughput technologies, such as genomics, metagenomics, transcriptomics, and metatranscriptomics. Metagenomics gives a more in-depth, unbiased microbial analysis beyond the group level and involves multiple species, besides showing shorter sequencing speed, extended read length, and lower costs [30]. However, the enormous amount of data generated becomes cumbersome to analyze and requires lots of dedicated time as well as expertise to manage [29].

In the context of polyphenol-microbiota interactions, the emerging high-throughput meta-genomic, transcriptomic, and proteomic approaches can be adopted to identify genes and micro-organisms involved in polyphenol (in)activation and conversion, to reconstruct metabolic pathways, and to monitor how microbial communities adjust their metabolic activities upon polyphenol exposure [30]. Application of these technologies to human fecal samples requires further investigation to determine how these samples reflect metabolism inside the gut and, ultimately, to improve the understanding of the impact of polyphenols on host health [12, 31].

3. Dietary Polyphenols

It has been estimated that 90–95% of dietary polyphenols are not absorbed in the small intestine and therefore reach the colon [32], although absorption and metabolism are largely influenced by their chemical structure. Most flavonoids are poorly absorbed from the small intestine and are highly metabolized in the large intestine. Isoflavones seem to be the best absorbed dietary flavonoids; catechins, flavanones, and flavonol glycosides are intermediate, whereas proanthocyanidins, flavan-3-ol gallates, and anthocyanins would be the worst absorbed [33].

The first step in the metabolism of flavonoids, with the exception of flavan-3-ols (i.e., catechins and proanthocyanidins), is likely to be deglycosylation before absorption in the small intestine. Hydrolysis of some flavonoid glycoside might already occur in the oral cavity, as both saliva and oral microbiota show β-glucosidase activity. But the mechanism most usually assumed for flavonoid deglycosylation is hydrolysis by lactase phlorizin hydrolase (LPH) in the brush-border of the small intestine epithelial cells [34, 35], so that the resulting aglycones would enter the enterocyte by passive diffusion. The resulting aglycone is rapidly biotransformed by phase II enzymes within the enterocyte and further in the liver, so that conjugated metabolites (i.e., glucuronides, *O*-mehtylethers, and/or sulphates) through the respective action of UDP-glucuronosyltransferase (UGT), catechol-*O*-methyltransferase (COMT), and sulphotransferases would be the circulating forms in the human body [36, 37].

Generally, a relevant fraction of dietary flavonoids is not absorbed in the small intestine and, together with the conjugated metabolites that returned to the intestinal lumen via enterohepatic circulation, reaches the large intestine where compounds are subjected to the action of the colonic microbiota. Intestinal bacteria show diverse deglycosylating activities, thus releasing aglycones that might be absorbed in a lesser extent and, more probably, degraded to simpler phenolic derivatives [38, 39]. Degradation of flavonoid aglycones by colonic microbiota involves ring-C cleavage and reactions affecting functional groups, such as dehydroxylation, demethylation, or decarboxylation [39]. Various hydroxylated aromatic compounds derived from the A-ring (e.g., phloroglucinol, 3,4-dihydroxybenzaldehyde, or 3,4-dihydroxytoluene) and phenolic acids derived from the B-ring have been reported as relevant products of the colonic transformation of flavonoids [40]. It has become evident that the beneficial effects attributed to dietary polyphenols appear to be due more to phenolic metabolites formed in the gastrointestinal tract, mainly derived from the action of gut bacteria, rather than to the original forms found in food [41].

In subsequent sections, main findings related to the modulation of gut microbiota by polyphenols are presented

as obtained from different methodological approaches and microbial analysis techniques.

4. Studies Using Batch Culture Fermentations

Although *in vivo* human or animal intervention trials are physiologically most relevant to study both polyphenol metabolism and microbial modulation, *in vitro* tools have been designed to simulate intestinal conditions. In combination with *in vivo* trials, *in vitro* experiments may help to elucidate the extent bioconversion processes mediated by the host itself [42, 43]. The complexity of *in vitro* gut models is diverse, ranging from simple static models (batch culture fermentation) to advanced continuous models (gastrointestinal simulators).

Simple, static gut models, also known as batch-type cultures, are generally closed systems using sealed bottles or reactors containing suspensions of fecal material that are maintained under anaerobic conditions. They are relatively easy to operate and cost-effective, have a fair throughput, and allow for parallel screening. This model approach is primarily used to assess the stability of polyphenols in the presence of human-derived gut microbiota and to evaluate which environmental conditions favor or limit polyphenol bioconversion. However, these static gut models are only adequate for simulating short-term conditions in the gut; for assessment of long-term adaptations of the gut microbial community, more complex dynamic models are needed [12].

Table 1 reports different studies of modulation of gut microbiota by dietary polyphenols using batch-type cultures. Details about fermentation conditions (fecal concentration, polyphenol origin and dose, and incubation time) and microbial techniques used, and main effects on bacteria groups (growth enhancement, growth inhibition, or no effect) have been included. As general characteristics, fecal fermentations employed feces concentration ≤10% (w/v) and lasted 48 h maximum. Both pure phenolic compounds and phenolic-rich extracts were added to the fecal medium at a final concentration <10% (w/v), and changes in specific bacterial groups were mainly assessed by FISH analysis. A first relevant experiment using batch culture fermentation was carried out by Tzounis et al. [44] who found that the flavan-3-ol monomers [(−)-epicatechin and (+)-catechin] promoted the growth of *Clostridium coccoides-Eubacterium rectale* group, which is known to produce large amounts of butyrate, a short-chain fatty acid (SCFA) with anti-inflammatory, and anti-neoplasic properties; (+)-catechin also increased the growth of *Lactobacillus-Enterococcus* spp., *Bifidobacterium* spp., and *Escherichia coli* but decreased the growth of *Clostridium histolyticum*. Also using standard compounds, Hidalgo et al. [45] found that anthocyanins (i.e., malvidin-3-glucoside and a mixture of anthocyanins) significantly enhanced the growth of *Lactobacillus-Enterococcus* spp. and *Bifidobacterium* spp. In addition, malvidin-3-glucoside showed a tendency to promote the growth of the *C. coccoides-E. rectale* group.

Similar results have been observed in batch culture fermentations with phenolic-rich extracts from different sources. Molan et al. [46] found that the addition of blueberry extracts to a mixture of fecal bacterial populations significantly increased the number of lactobacilli and bifidobacteria (Table 1). In the same line, Bialonska et al. [47] reported enhancement of the growth of total bacteria, *Bifidobacterium* spp., and *Lactobacillus-Enterococcus* spp. in response to a commercial extract of pomegranate, without influencing the *C. coccoides-E. rectale* and *C. histolyticum* groups (Table 1). Mandalari et al. [48] suggested a potential prebiotic effect for natural and blanched almond skins as these foodstuffs, in fermentations with fecal microbiota, significantly increased the populations of bifidobacteria and *C. coccoides-E. rectale* group and decreased the number of *C. hystolyticum* group. These authors related the possible prebiotic effect by almond skins not only to a high amount of dietary fibre, but also to some phenolic compounds such as ferulic acid, flavan-3-ols, and flavonols present in the almond skins [48]. Fogliano et al. [49] carried out an *in vitro* fermentation with a water-insoluble cocoa fraction in a three-stage continuous culture colonic model system. It was observed that this cocoa fraction presented prebiotic activity producing a significant increase in lactobacilli and bifidobacteria, as well as an increase in butyrate production. They concluded that the coexistence of fermentable polysaccharides and free flavanol monomers in cocoa, such as catechins, might be very effective in the modification of gut microbiota. Similar conclusions were drawn by Pozuelo et al. [50], who found a significant increase of the growth of *Lactobacillus reuteri* and *Lactobacillus acidophilus* in the presence of a grape antioxidant dietary fiber naturally obtained from red grapes. Our research group carried out several batch culture fermentations of two flavan-3-ol fractions with different degree of polymerisation and wine polyphenols, with fecal microbiota from different healthy volunteers [51, 52]. Both flavan-3-ol fractions promoted the growth of *Lactobacillus/Enterococcus* spp. and inhibited the *C. histolyticum* group during fermentation, although the effects were only statistically significant with the less polymerized fraction. Wine polyphenols only showed a slight inhibition in the *C. histolyticum* group, probably due to their lower content in flavan-3-ols.

Additionally, this type of fermentations has also been used to assess the contribution of certain probiotic strains to the colonic metabolism of polyphenols. In this sense, Barroso et al. [53] carried out fermentations of a red wine extract inoculated with human microbiota obtained from the colonic compartments of a dynamic simulator, in the presence and absence of the probiotic strain *L. plantarum* IFPL935. Microbial analysis by qPCR indicated that red wine polyphenols induced greater variations among *in vitro* batches harboring different colon-region (ascending colon, descending colon, and effluent) microbiota than those found when *L. plantarum* IFPL935 was added. Batches inoculated with microbiota from the ascending colon were shown to harbor the major proportion of saccharolytic bacteria (*Bacteroides*, *Bifidobacterium*, and *Prevotella*) whereas *Clostridium* groups were found in major numbers in the batches inoculated with microbiota simulating the distal regions [53] (Table 1).

Table 1: Studies using batch culture fermentation.

Reference	Fecal concentration	Phenolic compound/food	Dose	Time of incubation	Microbial technique	Growth enhancement	Growth inhibition	No effect
Tzounis et al. (2008) [44]	10%, w/v	(+)-catechin	150 mg/L, 1000 mg/L	<48 h	FISH	*Lactobacillus-Enterococcus* spp. *Bifidobacterium* spp. *C. coccoides-E. rectale* group *E. coli*	*C. histolyticum* group	
Molan et al. (2009) [46]	0.1%, v/v	Blueberry extracts	5, 10 and 25%	48 h	FISH	Lactobacilli Bifidobacteria		
Bialonska et al. (2010) [47]	10%, w/v	Pomegranate extract and punicalagin	10%	48 h	FISH	Total bacteria *Bifidobacterium* spp. *Lactobacillus-Enterococcus* spp.		*C. coccoides-E. rectale* group *C. histolyticum* group
Mandalari et al. (2010) [48]	10%, w/v	Almond skins	1%, w/v predigested almond skins	<24 h	FISH	Bifidobacteria *C. coccoides-E. rectale* group	*C. histolyticum* group	
Fogliano et al. (2011) [49]	5%, w/v	Water-insoluble cocoa fraction	1%, w/v	36 h	FISH	Bifidobacteria Lactobacilli		
Cueva et al. (2013) [51]	10%, w/v	Grape seed extract fractions	300–450 mg/L	<48 h	FISH	*Lactobacillus-Enterococcus* spp.	*C. histolyticum* group	
Hidalgo et al. (2012) [45]	10%, w/v	Malvidin-3-*O*-glucoside Anthocyanidins mixture	20 mg/L and 200 mg/L 4850 mg/L and 48500 mg/L	<24 h	FISH	*Lactobacillus-Enterococcus* spp. *Bifidobacterium* spp. *C. coccoides-E. rectale* group		
Sánchez-Patán et al. (2012) [52]	1% w/v	Red wine extract	600 mg/L	48 h	FISH		*C. histolyticum* group	*Lactobacillus-Enterococcus* spp.
Barroso et al. (2013) [53]		Red wine extract	500 mg/L	48 h	qPCR	*Lactobacillus* spp. *Bifidobacterium* spp. *Bacteroides* spp. *Ruminococcus* spp.		

Table 2: Studies using the gastrointestinal simulators (i.e., SHIME).

Reference	Simulator	Phenolic compound/food	Dose	Time	Microbial technique	Population increase	Population decrease
De Boever et al. (2000) [57]	SHIME	Soy germ powder	2.5 g/day	2 weeks	Plate count	Enterobacteriaceae Coliforms *Lactobacillus* spp. *Staphylococcus* spp. *Clostridium* spp.	
Kemperman et al. (2013) [31]	Twin-SHIME	Black tea extract	3 × daily dosing (1000 mg polyphenols as total daily dose)	2 weeks	Plate count qPCR PCR-DGGE pyrosequencing	*Klebsiella* spp. Enterococci *Akkermansia* spp.	Bifidobacteria *Blautia coccoides* *Anaeroglobus* spp. *Victivallis* spp.
Kemperman et al. (2013) [31]	Twin-SHIME	Red wine-grape extract	3 × daily dosing (1000 mg polyphenols as total daily dose)	2 weeks	Plate count qPCR PCR-DGGE pyrosequencing	*Klebsiella* spp. *Alistipes* spp. *Cloacibacillus* spp. *Victivallis* spp. *Akkermansia* spp.	Bifidobacteria *Blautia coccoides* group *Anaeroglobus* spp. *Subdoligranulum* spp. *Bacteroides*

5. Studies Using Human Gastrointestinal Simulators

In contrast to short-duration experiments with static gut models, longer-term experiments are required when the adaptation of the gut microbial community to dietary polyphenols needs to be assessed. To this end, dynamic *in vitro* gut models such as the "Reading" model [54], the Simulator of the Human Intestinal Microbial Ecosystem (SHIME), the TNO Intestinal Model 2 (TIM2) [55, 56], and the recent gastrointestinal simulator set up in our Institute (SIMGI) (unpublished work) have been developed where gut microbiota are cultured over a longer time frame (days to weeks) in one or multiple connected, pH controlled vessels representing different parts of the gastrointestinal tract.

As an example of the versatility and potential of human gastrointestinal simulators, Table 2 reports a series of studies about modulation of gut microbiota by polyphenols using the SHIME [57, 58]. This validated model comprises stomach and small intestinal sections for predigestion of food as well as vessels stimulating the ascending, transcending, and descending parts of the human colon, allowing assessment of changes in the different colonic areas that are very challenging to access in a human intervention. However, it should be underlined that this approach takes for granted that the extracts reach intact the colonic region, and no nutrient absorption is considered. The use of the SHIME to investigate the effects of a soy germ powder on the fermentative capacity of the simulated microbiota of the colon was the aim of a study carried out by De Boever et al. [57]. They observed that the addition of the soy germ powder in a 2-week treatment resulted into an overall increase of bacterial marker populations (Enterobacteriaceae, coliforms, *Lactobacillus* spp., *Staphylococcus* spp., and *Clostridium* spp.), with a significant increase of 2 log10 units in the *Lactobacillus* spp. population. More recently, Kemperman et al. [31], using the twin-SHIME model, studied the influence of a bolus dose and a 2-week continuous administration of complex dietary polyphenols from black tea or red wine grape extracts on the colonic microbiota. The Twin-SHIME system, involving two models that run in parallel, was inoculated with the same fecal sample for direct comparison of the effect of the two polyphenol types. A combination of analyses including cultivation, PCR-denaturing gradient gel electrophoresis (DGGE), quantitative PCR, and high throughput pyrosequencing of the 16S ribosomal RNA gene was applied to characterize microbial community changes. This study showed that complex polyphenols in the context of a model system can modulate select members of the human gut microbiota, revealing novel targets potentially involved in polyphenol metabolism and/or resistant microbes to be further investigated for polyphenol metabolism or resistance mechanisms [31].

6. Animal Models Studies

It is widely assumed that preliminary evidence should be warranted in animal models before human intervention trials. Animal models contribute to better understanding the mechanisms and biological effects that could be likely to happen in the human body. The metabolism of polyphenols has been object of numerous animal studies (mostly in rodents), especially for their impact on metabolic disorders [58], but only a few of these studies have followed the dynamics and composition of the intestinal microbiota in association with polyphenol metabolites retrieved from the host. Caution is required in extrapolating results to humans because culture-independent comparisons have revealed that most bacterial genera and species found in mice are not seen in humans, although the distal gut microbiota of mice and humans harbors the same bacterial phyla [59]. In this section, studies performed in animals in order to assess the effects of polyphenols on the modulation of intestinal microbiota are

Table 3: Animal model studies.

Reference	Animal	Phenolic compound/food	Dose	Treatment duration	Microbial technique	Population increase	Population decrease
Hara et al. (1995) [60]	Pigs	Tea polyphenols	0.2% (free access)	2 weeks	Plate count	Lactobacilli	Total bacteria *Bacteroidaceae* *C. perfringens*
Ishihara et al. (2001) [61]	Calves	Green tea extracts	1.5 g/day	4 weeks	Plate count		*Bifidobacterium* spp. *Lactobacillus* spp. *C. perfringens*
Smith and Mackie (2004) [66]	Rats	Proantocyanidins extracted from *Acacia angustissima*	0.7% (low tannin diet) and 2.0% (high tannin diet)	3.5 weeks treatment + 3.5 weeks washout	PCR-DGGE Dot blot hybridization	*Bacteroides fragilis* group *Bacteroides-Prevotella-Porphyromonas* group Enterobacteriaceae	*C. leptum* group
Dolara et al. (2005) [62]	Rats	Red wine polyphenols powder	50 mg/kg	16 weeks	Plate count	Lactobacilli Bifidobacteria	Propionibacteria *Bacteroides* Clostridia
Sembries et al. (2006) [63]	Rats	Apple juice	free access	4 weeks	Plate count	Lactobacilli Bifidobacteria	
Sembries et al. (2003) [64]	Rats	Apple pomace juice colloid	5% suppl. diet (free access)	6 weeks	Plate count FISH	*Bacteroidaceae*	
Larrosa et al. (2009) [68]	Rats	Resveratrol	1 mg/kg/day	25 days	Plate count	Lactobacilli Bifidobacteria	
Molan et al. (2010) [69]	Rats	Blackcurrant extracts (leaf or berry)	3 times/week: (i) 30 mg/kg (leaf) (ii) 13.4 mg/kg (berry)	4 weeks	FISH	Lactobacilli (berry extract) Bifidobacteria (leaf and berry extracts)	
Viveros et al. (2011) [65]	Broiler chicks	Grape pomace concentrate (GPC) Grape seed extract (GSE)	60 g/kg diet (GPC) 7.2 g/kg diet (GSE) (free access)	21 days	Plate count T-RFLP	*E. coli* *Enterococcus* spp. *Lactobacillus* spp.	
Lacombe et al. (2013) [70]	Rats	Lowbush wild blueberries	20 g feed/day (eq. 24 ± 5.2 mg anthocyanin/day)	6 weeks	Metagenomic sequencing	Thermonospora spp. *Corynebacteria* spp. *Slackia* spp.	*Lactobacillus* spp. *Enterococcus* spp.

summarized (Table 3). Experiments were mainly performed in rats, although other larger animals such as chicks, calves, or pigs have also been used. Gut microbial communities were evaluated by diverse methodologies including culture-based methods (plate count), DGGE, FISH, T-RFLP, qPCR, and metagenomic sequencing.

Animal studies performed in pigs [60] and in calves [61] demonstrated that tea polyphenols administration contributed to the improvement in the composition of the intestinal microbiota. Thus, the administration of tea polyphenols in pigs significantly increased the levels of lactobacilli whilst it diminished the levels of total bacteria and *Bacteroidaceae,* and a tendency to decrease in lecithinase positive clostridia including *C. perfringens* was also observed [60]. However, the reduction rate of *Bifidobacterium* spp. and *Lactobacillus* spp. was slow, while that of *C. perfringens* decreased faster in calves supplemented with the green tea extract [61].

Dolara et al. [62] showed that treatment with wine polyphenols in carcinogen-treated F344 rats was associated with a strong variation in the colonic microbiota, compared to the control-fed rats. Although the total bacterial counts and anaerobe/aerobe ratio of microorganisms in the feces from polyphenol-treated rats were similar to that from control rats, propionibacteria, *Bacteroides,* and Clostridia decreased while lactobacilli and bifidobacteria increased. Based on additional experiments, these authors concluded that reduction of oxidative damage, modulation of colonic flora, and variation in gene expression may be all connected in the action of wine polyphenols on the intestinal function and carcinogenesis.

In other study, rats fed with apple juice instead of drinking water showed more lactobacilli and bifidobacteria in fresh feces that differed from the controls by one-log10 colony forming units [63]. The same research group studied the effect of colloids isolated from apple pomace extraction juices on the intestinal microbiota in Wistar rats. An increase of *Bacteroidaceae* in almost one-log10 higher counts was observed in feces of rats fed with apple juice colloid than control rats [64]. Another animal experiment conducted to

study the effect on intestinal microbiota, of the inclusion of grape pomace extracts in the diet of broiler chicks [65], found that, for the cecum, birds fed grape extracts had higher populations of *E. coli*, *Lactobacillus,* and *Enterococcus* species than birds in any other treatment group. These authors concluded that grape polyphenol-rich products modified the gut morphology and intestinal microbiota and increased the biodiversity degree of intestinal bacteria in broiler chicks.

Inclusion of condensed tannins (proanthocyanidins) extracted from *Acacia angustissima* on rat diet resulted in a shift in the predominant bacteria towards tannin-resistant Gram-negative Enterobacteriaceaeand *Bacteroides* species and reduced the number of Gram-positive *C. leptum* group [66]. Compatible results were obtained in an experiment with rats fed a proanthocyanidin-rich cocoa preparation [67], where the authors found a significant decrease in the proportion of *Bacteroides*, *Clostridium,* and *Staphylococcus* genera in the feces of cocoa-fed animals. Interestingly, reductions in *Clostridium* species were found to correlate with weight loss and decrease in body mass index.

Larrosa et al. [68] observed an increase in lactobacilli and bifidobacteria when resveratrol (3,5,4′-trihydroxy-*trans*-stilbene), which naturally occurs in grapes and grape-derived foodstuffs such as red wine, was administered to rats. After induction of colitis by dextran sulphate sodium, proliferation of both *E. coli* and enterobacteria was lower in rats treated with resveratrol than in control rats. This could be the result of an indirect effect of resveratrol-supplemented diet, which increased bifidobacteria and lactobacilli counts preventing the colonization and invasion of tissues by enterobacteria including *E. coli*.

Prebiotic activity of wild blackcurrant extracts observed in *in vitro* experiments was further confirmed in rats by Molan et al. [69]. A significant increase in the population size of lactobacilli and bifidobacteria was observed after daily administration of those extracts to rats. Similarly, a grape antioxidant dietary fibre preparation was found to increase the population of *Lactobacillus* spp. when fed to rats, whereas populations of *Bifidobacterium* spp. decreased and changes in *E. coli* and *Bacteroides vulgatus* counts were not significant [50].

Recently, Lacombe et al. [70] studied the composition and functional potential of the colon microbiota from rats fed a diet enriched in lowbush wild blueberries. Application of novel metagenomic techniques (Illumina shotgun sequencing) revealed a significant reduction in the relative abundance of the genera *Lactobacillus* and *Enterococcus* associated with wild blueberries intake. In addition, hierarchical analysis showed a significant increase in the relative abundance of the phylum Actinobacteria, the order Actinomycetales, and several novel genera under the family Bifidobacteriaceae and Coriobacteriaceae in the blueberries group. The authors indicated that although the microbiome of rats differs from humans, the applied model was a powerful tool to study population dynamics and related metabolic functions. Metagenomic studies can determine microbial community profiles, gene presence/absence and abundance, and functional repertoire; however, they can only infer an observed phenotype since a gene presence does not imply its expression or functionality [71].

7. Human Intervention Studies

Investigations involving the use of humans potentially provide the best models for studying the interactions of food components (e.g., polyphenols) with microbiota, although *in vivo* intervention trials hold inevitable practical and ethical limitations [12]. The use of cross-over designs where volunteers serve as their own control permits multilevel analysis schemes that increase power but requires a relevant number of volunteers to allow for statistically significant multivariate models [72]. Up to now, only a few studies have examined the *in vivo* impact of dietary polyphenols on the human gut microbiota, and most of them were focused on single polyphenol molecules and selected bacterial populations. A summary of human intervention studies about effects of polyphenols in the modulation of the intestinal microbiota is collected in Table 4. In these studies, the polyphenol dose used was much dependent on the type of food preparation and its concentration, normally ranging from 0.1 to 4%; the treatment time was also variable, from 10 days to 2 months, and the applied microbial techniques were diverse (plate count, DGGE, FISH, T-RFLP, and qPCR).

In a study with a reduced number of subjects ($n = 8$), Okubo et al. [73] reported a notably increase in the percentages of *Bifidobacterium* spp. in total fecal counts after an intervention with a product containing 70% of tea polyphenols. A significant decrease of *C. perfringens* and other *Clostridium* spp. was also observed during the intake period. However, in a crossover feeding study (number of volunteers not reported) that investigated the effects of black tea drinking on hypercholesterolemic volunteers, Mai et al. [74] found that although specific bacterial groups were not affected, the total amount of bacteria significantly decreased, highlighting large interindividual variations. More recently, an intervention study ($n = 10$) by Jin et al. [75] confirmed an overall tendency for the proportion of bifidobacteria to increase because of green tea consumption, even though there were interindividual differences in the *Bifidobacterium* species.

Yamakoshi et al. [76] showed that administration of a proanthocyanidin-rich extract from grape seeds to healthy volunteers ($n = 9$) significantly increased the fecal number of *Bifidobacterium*, whereas the number of putrefactive bacteria such as enterobacteria tended to decrease. The interaction between proanthocyanidins and intestinal bacteria was also confirmed in a randomized, double-blind, crossover, and controlled intervention study ($n = 22$) ingesting two cocoa drinks exhibiting low and high polyphenol content [77]. Compared with the consumption of the low-flavan-3-ol cocoa drink, the daily consumption of the high-flavan-3-ol cocoa drink significantly increased the bifidobacteria and lactobacilli populations but significantly decreased clostridia counts.

Queipo-Ortuño et al. [78] performed a randomized, crossover, and controlled trial ($n = 10$) consisting of the intake of red wine, dealcoholized red wine, and gin over

Table 4: Human intervention studies.

Reference	Volunteer number	Phenolic compound/food	Dose	Treatment duration	Microbial technique	Population increase	Population decrease	No effect
Okubo et al. (1994) [73]	8	Green tea (Sunphenon)	0.4 g/3 times per day	4 weeks	Plate count		*C. perfringens* *Clostridium* spp.	
Yamakoshi et al. (2001) [76]	9	Proantocyanidin-rich extract from grape seeds	0.5 g/day	6 weeks	Plate count	*Bifidobacterium* spp.	Enterobacteriaceae	
Mai et al. (2004) [74]	15	Black tea	700 mg tea solids/5 times per day	21 days	FISH DGGE		Total bacteria	No changes
Clavel et al. (2005) [83]	39	Isoflavones	100 mg/day	2 months	TTGE FISH	*C. coccoides-E. rectale* group *Bifidobacterium* spp. *Lactobacillus-Enterococcus* spp. *Faecalibacterium prausnitzii* subgroup		
Costabile et al. (2008) [84]	31	Whole grain wheat cereals	48 g/day	3 weeks	FISH	Bifidobacteria Lactobacilli		Total bacteria *Bacteroides* spp. *C. histolyticum/perfringens* group *Acetobacterium* spp.
Jaquet et al. (2009) [87]	16	Coffee	3 cups/day	3 weeks	FISH DGGE	*Bifidobacterium* spp.		
Carvalho-Wells et al. (2010) [85]	32	Whole grain maize cereals	48 g/day	3 weeks	FISH	Bifidobacteria		Total bacteria *Bacteroides* spp. *C. histolyticum/perfringens* group *Acetobacterium* spp.
Gill et al. (2010) [80]	10	Raspberry puree	20 g/day	4 days	PCR-DGGE			No changes in the profile of colonic bacteria
Shinohara et al. (2010) [79]	8	Apples	2 apples/day	2 weeks	Plate count	*Lactobacillus* spp. *Streptococcus* spp. *Enterococcus* spp.	Enterobacteriaceae lecithinase-positive clostridia including *C. perfringens*, *Pseudomonas* spp.	

Table 4: Continued.

Reference	Volunteer number	Phenolic compound/food	Dose	Treatment duration	Microbial technique	Population increase	Population decrease	No effect
Tzounis et al. (2011) [77]	22	Cocoa flavanol	494 mg/day 29 mg/day	4 weeks	FISH	*Bifidobacterium* spp. *Lactobacillus* spp.	*C. histolyticum/perfringens* group	
Vendrame et al. (2011) [81]	15	Wild blueberry drink	25 g wild blueberries/day	6 weeks	qPCR	*Bifidobacterium* spp. *L. acidophilus*		*Bacteroides* spp. *Prevotella* spp. *Enterococcus* spp. *C. coccoides*
Queipo-Ortuño et al. (2012) [78]	10	Red wine	272 mL/day	20 days	qPCR	*Enterococcus* spp. *Prevotella* spp. *Bacteroides* *Bifidobacterium* spp. *Bacteroides uniformis* *Eggerthella lenta* *Blautia coccoides-E. rectale* group	*Clostridium* spp. *C. histolyticum group*	Actinobacteria
Jin et al. (2012) [75]	10	Green tea	1000 mL/day	10 days	T-RFLP qPCR	*Bifidobacterium* spp.		
Guglielmetti et al. (2013) [82]	15	Wild blueberries drink	25 g wild blueberries/day	6 weeks	qPCR	*B. longum* subsp. *infantis*		
Cuervo et al. (2014) [86]	38	Dairy products Fruits Vegetables Cereals		Food intake was recorded using an annual food frequency questionnaire	qPCR	*Lactobacillus*	*B. coccoides* *C. leptum*	

three consecutive periods. After the red wine period, the bacterial concentrations of proteobacteria, fusobacteria, Firmicutes, and Bacteroidetes markedly increased compared with the washout period; significant increases in the number of *Bifidobacterium* spp. and *Prevotella* spp. were also observed. However, *Lactobacillus* spp., *Clostridium* spp., and *C. histolyticum* group concentrations remained unchanged throughout the study.

In a small-scale observational study ($n = 8$), Shinohara et al. [79] found that the number of bifidobacteria in feces significantly increased during apple intake and the numbers of *Lactobacillus* spp., *Streptococcus* spp., and *Enterococcus* spp. tended to increase. On the contrary, enterobacteria and lecithinase-positive clostridia, including *C. perfringens* and *Pseudomonas* species, tended to decrease. However, that study did not use culture-independent microbiology techniques and suffered from the lack of a control group. Also in relation to fruits, another small human intervention study ($n = 10$) with raspberry puree [80] did not observe statistically significant alterations in the profile of colonic bacteria, probably due to high interindividual variation in fecal bacteria, although the profiles of microbial metabolites of raspberry polyphenols varied greatly between individuals, indicating that the type of gut microbiota affects catabolite profiles released by bacteria in the colon. This lack of effect on the intestinal microbiota after the intake of raspberry puree might also be due to the short duration of the treatment, as well as the techniques employed to quantify the intestinal microbiota.

Vendrame et al. [81] studied the potential prebiotic activity of a drink elaborated from wild blueberries especially rich in anthocyanins, in a small intervention trial ($n = 15$). A significant increase in *Bifidobacterium* spp. and *L. acidophilus* group was detected, while no significant differences were observed for *Bacteroides* spp., *Prevotella* spp., *Enterococcus* spp., and *C. coccoides*. In a further paper of the same group [82], seven different intragenus bifidobacteria taxonomic clusters that were among the most common and abundant bifidobacteria species inhabiting the human gut were targeted in the same samples. It was found that *B. adolescentis*, *B. breve*, *B. catenulatum/pseudocatenulatum,* and *B. longum* subsp. *longum* were always present in the group of subjects enrolled, whereas *B. bifidum* and *B. longum* subsp. *infantis* were not. In spite of the large interindividual variability, a significant increase of *B. longum* subsp. *infantis* cell concentration was observed in the feces of volunteers after the wild blueberry drink treatment, which was attributed to the presence of prebiotic (bifidogenic) molecules from blueberries, possibly fibers and glycosylated anthocyanins.

In a study with postmenopausal women ($n = 39$), Clavel et al. [83] found that isoflavone supplementation stimulated dominant microorganisms of the *C. coccoides-E. rectale* cluster, *Lactobacillus-Enterococcus* group, *Faecalibacterium prausnitzii* subgroup, and *Bifidobacterium* genus. It was also suggested that the concentration of *C. coccoides-E. rectale* cluster was related to women capacity to excrete equol, an intestinal metabolite from daidzein. In two intervention studies with whole grain breakfast cereals from wheat ($n = 31$) and maize ($n = 32$) [84, 85], the ingestion of both products resulted in significant increases in fecal bifidobacteria and/or lactobacilli without changing the relative abundance of other dominant members of the gut microbiota. Little or no changes were observed in the numbers of total bacteria, *Bacteroides* spp., *C. histolyticum/perfringens* group, and *Acetobacterium* spp. present in the feces. However, as whole grains are good sources of dietary fiber, it is difficult to ascribe the observed effects only to the phenolic compounds present in these foods. In this respect, Cuervo et al. [86] have recently studied the correlations between the intake of fiber and polyphenols from diet and fecal microbiota composition in a cohort of apparently healthy subjects. Results showed that the intake of soluble pectins and flavanones from oranges presented a negative correlation with the levels of *B. coccoides* and *C. leptum*. By contrast, the intake of white bread, providing hemicellulose and resistant starch, was directly correlated with *Lactobacillus*.

Finally, another human trial ($n = 16$) carried out by Jaquet et al. [87] assessed the impact of a moderate consumption of instant coffee on the general composition of the human intestinal bacterial population. Coffee beverages contain significant amounts of soluble fibre (mainly galactomannans and arabinogalactan-proteins) and phenolic compounds (chlorogenic acids), which are well utilised by the human fecal microbiota. It was observed that although fecal profiles of the dominant microbiota were not significantly affected after the consumption of the coffee, the population of *Bifidobacterium* spp. increased, being the largest increase observed for those volunteers showing the lowest initial bifidobacteria levels.

8. Conclusions

This review has tried to summarize the current knowledge in relation to the phenolic metabolism by gut microbiota and the modulation of the gut microbiota by phenolic compounds and polyphenol-rich dietary sources. There are evidences that the beneficial effects attribute to dietary polyphenols depend on their biotransformation by the gut microbiota. Therefore, it is important to investigate the bacterial species implicated in the metabolism of dietary polyphenols, and further research is still needed in relation to the resultant microbial metabolites to ascertain their mechanisms of action. On the other hand, a great number of *in vitro* and *in vivo* (in animals and humans) studies showing the influence of dietary polyphenols on gut-inhabiting bacteria have been published in recent years. Although *in vitro* assays facilitate experimentation, caution must be taken in extrapolating results to *in vivo* situation, as many factors are acting upon this process. In general, in both *in vitro* and *in vivo* studies, polyphenols or polyphenol-rich sources have shown to influence the relative abundance of different bacterial groups within the gut microbiota, reducing numbers of potential pathogens, including *C. perfringens* and *C. histolyticum*, and certain Gram-negative *Bacteroides* spp. and enhancing mainly beneficial Clostridia, bifidobacteria and lactobacilli. A better understanding of the interaction between dietary polyphenols and gut microbiota through the emerging advances in high-throughput metagenomic, transcriptomic, and proteomic approaches, would

be essential in order to identify genes and micro-organisms involved in polyphenol (in)activation and conversion and thus, to elucidate the implications of diet on the modulation of microbiota for delivering health benefits.

Functional foods are considered to enhance the protective effects against diseases derived from some food components. In the last decades, dairy fermented foods have probably been one of the most-developed functional products and have deserved intensive research. In this expansion, dairy fermented foods have been supplemented with fruits, cereals, and other stuffs of plant origin, all of which represent a high percentage of the current market of the dairy industry. These products have a healthy appeal, which attracts consumers. Thus, fruit juices/concentrates, and prepared fruits (in the form of pieces, pulp, and even flour) have been successfully incorporated in dairy fermented foods as sources of prebiotic fibers and phytochemicals. Among these phytochemicals present in plant-derived foods, polyphenols have gained much interest due to their diverse potential beneficial effects in human health. The supplementation of dairy fermented products with rich-polyphenolic stuffs (phenolic extracts, phenolic-enriched fractions, etc.) seems to be an effective technological option to improve the benefits of these products in the balance of the intestinal microbiota, due not only to the action of the probiotics but also to the potential modulation effects exerted by polyphenols, as it has been described in this review. Further research in this area will aim to accomplish the benefits of both probiotic strains and polyphenols in relation to gut health.

Conflict of Interests

The authors declare no conflict of interests.

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Research Article

The Effects of *Bifidobacterium breve* on Immune Mediators and Proteome of HT29 Cells Monolayers

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The use of beneficial microorganisms, the so-called probiotics, to improve human health is gaining popularity. However, not all of the probiotic strains trigger the same responses and they differ in their interaction with the host. In spite of the limited knowledge on mechanisms of action some of the probiotic effects seem to be exerted through maintenance of the gastrointestinal barrier function and modulation of the immune system. In the present work, we have addressed *in vitro* the response of the intestinal epithelial cell line HT29 to the strain *Bifidobacterium breve* IPLA20004. In the array of 84 genes involved in inflammation tested, the expression of 12 was modified by the bifidobacteria. The genes of chemokine CXCL6, the chemokine receptor CCR7, and, specially, the complement component C3 were upregulated. Indeed, HT29 cells cocultivated with *B. breve* produced significantly higher levels of protein C3a. The proteome of HT29 cells showed increased levels of cytokeratin-8 in the presence of *B. breve*. Altogether, it seems that *B. breve* IPLA20004 could favor the recruitment of innate immune cells to the mucosa reinforcing, as well as the physical barrier of the intestinal epithelium.

1. Introduction

Probiotics are live microorganisms which when administered in adequate amounts confer a health benefit on the host [1], the genus *Bifidobacterium* being among the most widely used. These microorganisms are common members of the human gut microbiota and they predominate in breast-fed infants [2]. Several beneficial health effects have been attributed to specific probiotic strains [3]. Although the knowledge on probiotic mechanisms of action is still limited some of these beneficial effects are exerted through their role in the maintenance of the gastrointestinal barrier function and by modulating the immune system [4, 5].

The interest in the immunomodulatory properties of probiotic bacteria derives from the observations that intestinal microbiota plays a critical role in the development and regulation of the immune system [6]. It is known that different probiotic bacteria present different effects upon the immune system [7, 8], making necessary the characterization of the effects of each specific potentially probiotic strain. Some strains promote Th1 responses, characterized by the production of IFNγ and TNFα, whereas other strains induce anti-inflammatory cytokines generating a Th2 profile [7, 8]. To determine these properties, the direct effect of the interaction of probiotic bifidobacteria with immune cells, either total peripheral blood mononuclear cells (PBMCs) or isolated immune cell types, is often studied. However, the potential effect of the cross talk between bifidobacteria and epithelial cells upon the immune system has received less attention. The intestinal epithelium separates microorganisms from the underlying immune cells. It consists of a layer of cells, mainly enterocytes, and a mucus layer that coats the epithelium [9]. Moreover, different immune cells are localized in the gut associated lymphoid tissue, which constitute the first contact point between gut commensals and the immune system [10]. Consequently, assessing the effect of the interaction

of potentially probiotic *Bifidobacterium* strains with the gut mucosa constitutes an important task for both probiotics selection and understanding of their mechanisms of action. This understanding would allow selection of specific strains with the desired properties for a specific application.

Previous studies carried out on the breast-milk isolate *Bifidobacterium breve* IPLA20004 [11] by our group indicated the ability of this strain to induce Th1 polarization of lymphocytes and to increase the physical resistance of the intestinal mucosa [12, 13]. These results suggest that this strain may be of interest for increasing the intestinal barrier against pathogens, firstly by strengthening the physical resistance of the epithelial layer and secondly by modulating the immune system towards a preactivated steady state. Moreover, some effects of the strain on the expression of chemokines and their receptors have been previously suggested [13]. To this regard an effect on the production of chemokines by intestinal epithelial cells may have a direct impact on the immune system by affecting the recruitment of immune cells to the mucosa.

For the above-mentioned reasons we decided to evaluate the effect of *B. breve* IPLA20004 on the expression of genes related to the inflammatory response and on the production of cytokines, by the human intestinal epithelial cell line HT29. Moreover, the effect of the strain on HT29 cells was also assessed by proteomic analyses.

2. Materials and Methods

2.1. Bacteria Culture Conditions. To evaluate the effects of the *B. breve* IPLA20004 on HT29 cells, cultures were freshly prepared by growing the microorganisms in MRS medium (Difco, Becton, Dickinson and Company, Le Pont de Claix, France) supplemented with a 0.25% L-cysteine (Sigma Chemical Co., St. Louis, MO, USA) (MRSc) at 37°C under anaerobic conditions (10% H_2, 10% CO_2, and 80% N_2) in a chamber Mac 500 (Don Whitley Scientific, West Yorkshire, UK).

2.2. HT29 Cell Line Culture Conditions. The epithelial intestinal cell line HT29 (ECACC number 91072201), derived from human colon adenocarcinoma, was purchased from the European Collection of Cell Cultures (Salisbury, UK). HT29 cell culture passages 146-147 were used for the experiments. The cell line was maintained in McCoy's medium supplemented with 3 mM L-glutamine, 10% (v/v) heat-inactivated bovine fetal serum, and a mixture of antibiotics to give a final concentration of 50 μg/mL penicillin, 50 μg/mL streptomycin, 50 μg/mL gentamicin, and 1.25 μg/mL amphotericin B. All media and supplements were obtained from Sigma. The incubations took place at 37°C, 5% CO_2 in an SL water-jacketed CO_2 incubator (Sheldon Mfg. Inc., Cornelius, Oregon, USA). Culture media were changed every two days and the cell line was trypsinized with 0.25% trypsin-EDTA solution (Sigma) following standard procedures. For gene expression experiments and protein profile determinations, 10^5 cells/mL were seeded in 24-well plates and incubated to reach a confluent and differential state (reaching about 10^7 HT29 cells/mL) after 13 ± 1 days.

2.3. Gene Expression Analysis. *B. breve* IPLA20004 was grown overnight in MRSc, harvested by centrifugation, washed twice with Dulbecco's PBS buffer (Sigma), and resuspended in McCoy's medium without antibiotics. Five hundred μL of a bacterial suspension containing 10^8 cfu/mL (as determined by plate counting) in McCoy's medium or McCoy's medium without bacteria (control) was added to each well containing HT29 monolayers (bacteria/HT29 cell ratio 10 : 1) previously washed twice with Dulbecco's PBS to remove the antibiotics. Plates were then incubated for 6 h at 37°C, 5% CO_2 in a Heracell 240 incubator (Thermo Electron LDD GmbH, Langenselbold, Germany). After incubation the culture media were removed and stored at −80°C, the monolayers were resuspended in 500 μL of RNA Protect Cell Reagent (Qiagen GmbH, Hilden, Germany), and the cells were kept frozen at −80°C until RNA extraction. At least three independent experiments were carried out.

RNA from HT29 cells was extracted by using the RNeasy Plus Mini Kit (Qiagen) and QIAshredder homogenizer columns (Qiagen) following manufacturer instructions. Quality of RNA was monitored by gel electrophoresis and it was quantified by using an Epoch apparatus (BioTek Instruments, Inc., Winooski, VT, USA). For reverse-transcriptase PCR analyses 1 μg of RNA was reverse-transcribed to cDNA by using the RT2 First Strand Kit (SABiosciences, Qiagen, Frederick, MD, USA), and gene expression was quantified by using the 96-well RT2 Profiler PCR Array for human inflammatory cytokines and receptors (SABiosciences) following manufacturer's instructions. The array comprises 84 key genes involved in the inflammatory response including chemokine and cytokine genes (CCL1 [I-309], CCL11 [eotaxin], CCL13 [mcp-4], CCL15 [MIP-1d], CCL16 [HCC-4], CCL17 [TARC], CCL18 [PARC], CCL19, CCL2 [mcp-1], CCL20 [MIP-3a], CCL21 [MIP-2], CCL23 [MPIF-1], CCL24 [MPIF-2/eotaxin-2], CCL25 [TECK], CCL26, CCL3 [MIP-1a], CCL4 [MIP-1b], CCL5 [RANTES], CCL7 [mcp-3], CCL8 [mcp-2], CXCL1, CXCL10 [IP-10], CXCL11 [I-TAC/IP-9], CXCL12 [SDF1], CXCL13, CXCL14, CXCL2, CXCL3, CXCL5 [ENA-78/LIX], CXCL6 [GCP-2], CXCL9, IL13, IL8, IFNA2, IL10, IL13, IL17C, IL1A, IL1B, IL1F10, IL1F5, IL1F6, IL1F7, IL1F8, IL1F9, IL22, IL5, IL8, IL9, LTA, LTB, MIF, SCYE1, SPP1, and TNF), chemokine and cytokine receptor genes (CCR1, CCR2, CCR3, CCR4, CCR5, CCR6, CCR7, CCR8, CCR9, CX3CR1, XCR1 [CCXCR1], IL1R1, IL1RN, IL5RA, IL8RA, IL8RB, IL9R, IL10RA, IL10RB, and IL13RA1), other genes involved in the inflammatory response (ABCF1, BCL6, C3, C4A, C5, CEBPB, CRP, ICEBERG, LTB4R, and TOLLIP), and five housekeeping genes (B2 M, HPRT1, RPL13A, GAPDH, and ACTB) for normalization of data.

2.4. Cytokines and C3a Determination. Cytokine and C3a levels in the cell culture supernatants of HT29 cells cultured with or without *B. breve* as indicated above were quantified by using the High Sensitivity ELISA Kits for human IL10, IL12p70, IL1β, and TNFα and the Platinum ELISA Kits for human IL8 and C3a (eBioscience Inc., San Diego, CA, USA). Colour development after ELISA was measured in a Modulus Microplate Photometer (Turner Biosystems, Sunnyvale, CA, USA). All the results were expressed as pg/mL. Detection

limits for the ELISA kits used were 0.05, 0.1, 0.05, 0.13, 2, and 70 pg/mL for IL10, IL12p70, IL1β, TNFα, IL8, and C3a, respectively.

2.5. Determination of the Proteomic Profiles. *B. breve* IPLA20004 was grown and added to the wells containing HT29 as previously indicated. Plates were then incubated for 3 h at 37°C, 5% CO_2, gently washed three times with Dulbecco's PBS buffer to remove the nonadhered bacteria, and the HT29 monolayers were kept for further proteomic analysis.

For protein extraction and two-dimensional electrophoresis analysis, HT29 monolayers were disaggregated with 440 μL of lysis buffer (30 mM Tris, 7 M urea, 2 M thiourea, 4% (w/v) CHAPS, and 100 mM DTT; all reagents were purchased by GE Healthcare Life Sciences) containing complete protease inhibitors (Roche Diagnostics, Mannheim, Germany). Total protein from the cell suspensions was obtained by sonication for one min in ice-chilled water (two cycles), with one min of delay between the two cycles. After adding 2 mg of RNase A (Sigma-Aldrich) and 100 U of DNase I (Sigma-Aldrich), the cell lysates were incubated for 30 min at RT. Finally, the pellet was centrifuged for 10 min at 16,000 g and 4°C to precipitate insoluble components and cell debris. Protein concentration was estimated using the BCA Protein Assay Kit (Pierce, Rockford, IL).

Isoelectric focusing (IEF) was performed in immobilized pH gradient (IPG) strips containing a nonlinear pH range of 3–10 (GE Healthcare Life Sciences), using 500 μg of protein. When needed, lysis buffer was added up to 450 μL. In all the cases, the IPG-buffer corresponding to pHs 3–10 was added to a final concentration of 0.5% (v/v). IEF was conducted at 20°C for 60,000 Vhrs in an IPGphor system (GE Healthcare Life Sciences). Proteins were resolved by SDS-PAGE (12.5% w/v polyacrylamide gel) and stained with GelCode Blue Safe Protein Stain (Pierce). Gels were scanned using ImageScanner (GE Healthcare Life Sciences), and spot detection and volume quantification were performed with ImageMaster Platinum software (version 5.00, GE Healthcare). The relative volume of each spot was obtained by determining the spot intensity in pixel units and normalizing that value to the sum of the intensities of all the spots of the gel. Each experiment was performed independently four times, and the differences in normalized volumes were analyzed statistically using paired Student's t-tests (control condition versus presence of the bifidobacteria strain).

2.6. Statistical Analyses. Differences in the measured variables, between the control HT29 cells and those exposed to the *B. breve* strain, were evaluated by one-way ANOVA test. Results were represented by mean ± standard deviation. The SPSS 18.0 statistical software package (SPSS Inc., Chicago, IL, USA) was used for all determinations and a value of $P < 0.05$ was considered significant.

3. Results

3.1. Effect of B. breve IPLA20004 on the Expression of Genes Mediating the Inflammatory Response in HT29 Cells. When using the human inflammatory cytokines and receptors pathway focused RT-PCR array, comprising 84 key genes involved in the inflammatory response, we observed some statistically significant changes in gene expression in HT29 cells after coincubation with *B. breve* IPLA20004. These changes were in general modest and most of the studied genes were expressed at low basal levels (Ct values around 30, data not shown), with the exception of CCL25 (Ct value of 19 in the control HT29 cells) and CCR1 (Ct value 26 in the control). The genes whose expression was significantly modified by the strain are shown in Table 1. The expression of chemokine genes CCL2, CCL25, and CXCL14 and the cytokines genes IL10 and IL13 genes was significantly downregulated. On the contrary the gene for CXCL6 chemokine was found to be upregulated. With regard to chemokine receptor genes, a statistically significant downregulation of CCR1, CCR4, CCR5, and XCR1 and induction of CCR7 were observed. Interestingly, *B. breve* IPLA20004 upregulated very significantly (17-fold) the expression of the complement component C3 (Table 1). No statistically significant differences were observed for any of the other genes analyzed in the RT-PCR array (data not shown).

Table 1: Changes in cytokines and receptors gene expression in HT29 cells after exposition to bifidobacteria when compared to exposition to culture medium without bifidobacteria (control), as determined by RT-PCR.

Gene	Up- or downregulation (compared to control)	
	B. breve IPLA20004	
	Fold regulation	*P*
C3	17.71	*0.001*
CCL2	−3.29	*0.026*
CCL25	−5.32	*0.039*
CCR1	−2.60	*0.012*
CCR4	−6.07	*0.006*
CCR5	−10.36	*0.004*
CCR7	3.19	*0.011*
CXCL6	2.12	*0.028*
CXCL14	−2.43	*0.015*
IL10	−1.64	*0.021*
IL13	−2.75	*0.009*
XCR1	−3.65	*0.001*

3.2. Effect of B. breve IPLA20004 on Cytokines and C3 Production by HT29 Cells. The levels of the different cytokines measured, as well as those of C3a, in supernatants of HT29 cells are shown in Table 2. In general the levels detected were low, in some cases being barely over the detection limits of the ELISA kits used. No statistically significant differences between control and *B. breve*-exposed HT29 cells were observed for IL10, IL1β, TNFα, or IL8 levels. On the contrary coculture of HT29 cells with *B. breve* IPLA20004 significantly increased the production of IL12p70 and C3a, although for the former cytokine the detected levels (0.19 and 0.3 pg/mL for control and *B. breve*-exposed HT29 cells, resp.)

Table 2: Effect of *B. breve* IPLA20004 on cytokines and C3a levels in HT29 cells supernatants. All the results are expressed as pg/mL. Control cells were exposed to culture medium without bifidobacteria.

	Concentration (pg/mL)		
	Control	*B. breve* IPLA20004	*P*
IL10	0.43 ± 0.44	0.83 ± 0.38	0.302
IL12p70	0.19 ± 0.03	0.30 ± 0.02	*0.007*
IL1β	0.17 ± 0.17	0.26 ± 0.20	0.604
TNFα	2.19 ± 1.45	3.35 ± 0.18	0.242
IL8	264.82 ± 38.41	423.51 ± 200.29	0.249
C3a	217.33 ± 38.37	311 ± 42.14	*0.045*

were only slightly above the detection limit of the technique used and, therefore, the relevance of this observation is unclear.

3.3. Effect of B. breve IPLA20004 on the Proteome of HT29 Cells. The comparison of the proteomes of HT29 cells cocultured with or without *B. breve* revealed that two proteins were significantly ($P < 0.05$) upregulated in the HT29 cells by the strain *B. breve* IPLA20004 (see Figure 1 in the Supplementary Material available online at http://dx.doi.org/10.1155/2014/479140). These proteins were excised from the gels and identified as cytokeratin-8 (2.8 times fold induction) and the chain A of the tapasin-ERp57 (4.7 times fold induction).

4. Discussion

The interaction of bacteria with intestinal epithelial cells may play a role in immune modulation by modifying gene expression and local immune environment through, for instance, production of chemokines and other immune active molecules. Chemokines are chemotactic cytokines that guide the migration of cells regulating leukocyte traffic and exert their effects by interacting with their specific receptors that are selectively found on the surfaces of their target cells.

We studied the interaction of *B. breve* IPLA20004 with colonic epithelial cells HT29 and found changes in the expression of genes related to the inflammatory response and immune cell chemotaxis in the HT29 cell line. The strain was observed to significantly induce the expression of some immunoactive molecules, such as C3 and CXCL6, and to downregulate the expression of others including CCL2, CCL25, CXCL14, IL10, or IL13. It should be noted, however, that in some cases such as IL10 the magnitude of the change in gene expression, although significant, was small (less than 2) and perhaps of limited biological relevance. The effect of probiotics, mainly *Lactobacillus* strains, on transcriptional responses of human epithelial cells has been previously assessed both *in vitro* [14–16] and *in vivo* [17–19]. Although the studies on bifidobacteria are scarcer there are also some examples [12, 13, 20, 21]. These studies show a limited response of human intestinal epithelia cells lines to stimulation with bifidobacteria. Nevertheless, it is still interesting to see that our results, although *in vitro*, suggest an effect of the strain *B. breve* IPLA20004 in a number of genes coding for cytokines, chemokines, and receptors, which is in agreement with some *in vivo* studies on the effect of probiotic lactobacilli upon gene expression patterns in the human small bowel [17] and supports a link between the interaction of bacteria with epithelial cells and the immune system. Interestingly, in spite of the different models used, some of the genes found to be modulated in this study have been previously reported to be modulated by probiotic *Lactobacillus* strains both *in vitro* using epithelial cells [22] and *in vivo* in the human small bowel mucosa [17, 19]. To this regard, the colonic epithelial cell line used in our study (HT29) may better resemble the small bowel, where the mucus layer is thin, than the colon where a thick mucus layer is known to be present which prevents the close contact of bacteria with the epithelial cell [9]. Moreover, coculture of mice primary colonic epithelial cells with *L. rhamnosus* GG induced the expression of IL1β, TNFα, CXCL5 (ENA-78), CXCL10 (IP10), CCL20 (MIP3α), CCL2 (MCP1), CCL7 (MCP3), CXCL2 (MIP2α), and CCL5 (RANTES) [22], and our results indicated a significant downregulation of CCL2 without affecting the other *L. rhamnosus* GG-induced genes. This may suggest a differential response to our bifidobacteria with regard to *L. rhamnosus* GG, although the influence of the different colonocyte models used cannot be overruled. Administration of *L. rhamnosus* GG to human volunteers induced the expression of some of these genes (CCL24, CCL2, CXCL3, CXCL13, CXCL12, CCR3, CCL19, CCL21, or lymphotoxin-β [LTB], among others) on the small bowel mucosa, whilst other *Lactobacillus* strains (*L. acidophilus* Lafti L10) resulted in a different expression profile (inducing CXCL10 and CXCL11, among others) [19]. On the contrary, generalizing, in our *in vitro* model *B. breve* IPLA20004 tended either to downregulate or not to affect these genes which suggest a limited stimulatory activity of this strain when compared with the immune-stimulatory ability of lactobacilli. It should be noted, however, that the differences existing between the *in vivo* studies and our *in vitro* results with HT29 cells may be partly related to the different experimental conditions used; for instance, we performed the incubations under a 5% CO_2 atmosphere in comparison with the anaerobic intestinal environment which may have an effect on an anaerobic microorganism such as *B. breve*.

As indicated above, chemokines function mainly as chemoattractants for leukocytes, recruiting monocytes, neutrophils, and other effectors cells from the blood to sites of infection or tissue damage [23]. Chemokines such as CCL2 or CCL25 attract immune cells, such as macrophages and T-lymphocytes expressing their receptors (CCR2 and CCR9, resp.) to the tissue [23]. Actually, expression of CCR9 has been found to be involved in the homing to the intestine of thymic T-cells [24]. On the other hand, the only chemokine gene found to be upregulated in our study was that of CXCL6 (human granulocyte chemotactic protein-2, GCP-2). This chemokine attracts and activates neutrophils [25] being, together with IL8, the only CXC-family chemokine recognized by both CXCR1 and CXCR2 receptors. IL8 is the most active protein chemoattracting neutrophil, although in

our study its gene was not found to be significantly upregulated and IL8 determination in the supernatants showed higher, but not statistically significant, values. Moreover, a recent study demonstrated an increased production of the chemokine CXCL16 (not studied in this work) in germ-free animals, which resulted in an increased recruitment of immune cells to the intestinal mucosa [26]. This underlines the importance of chemokines in immune cells recruitment and the modulation of their production by the intestinal microbiota.

Interestingly, the complement component C3 was among the most strongly upregulated genes in the small bowel mucosa after administration of *L. rhamnosus* GG to healthy volunteers [17]. Similarly, the expression of this gene in HT29 cells was the most clearly upregulated by our *B. breve* strain and a significantly higher production of C3 by the epithelial cell line was confirmed by means of ELISA tests. C3 is the most abundant complement protein in serum, it enhances phagocytosis promoting innate immunity, and it is also important for an effective antibody response, thus constituting a link between the complement system and the acquired immune response [27]. In our study the downregulation of the expression of genes such as CCL2 or CCL25 together with the upregulation of the expression of CXCL6 and C3 by colonic cells suggests a local effect by suppressing the recruitment to the mucosa of lymphocytes and by increasing that of the innate immunity cells such as neutrophils and mastocytes. However, the limitations of our study design do not allow the establishment of firm conclusions on whether the differences obtained with regard to the reports by other authors are due to the different strains used or to the models' responsiveness.

Finally, in order to complement the data on the interaction between *B. breve* IPLA20004 and HT29 cells we performed a proteomic approach. This analysis allowed us to detect the overproduction of cytokeratin-8 (CK-8) or type I cytoskeletal 8, a keratin protein encoded by the krt8 gene; this protein is located in the nucleoplasm and the cytoplasm where, as a part of the cytoskeleton, it is known to help to link the contractile machinery to dystrophin at the costamere in striated muscle cells [28]. Interestingly, this strain has been previously found to increase the transepithelial resistance of the HT29 cell monolayer [13] which may be correlated with this induction of changes in the cytoskeleton. Moreover, the chain A of the tapasin-ERp57 was also overproduced. The heterodimer formed by tapasin-ERp57, linked by a stable disulfide bond, is part of the major histocompatibility complex (MHC) class I peptide-loading complex [29]. This heterodimer has been shown as the functional unit for loading MHC class I molecules with high-affinity peptides [30]. It has been shown that upregulation of tapasin may facilitate optimal peptide loading on the MHC class I molecule [31], although the putative functions in enterocytes have passed unnoticed until now.

In this study we have determined the effects of *B. breve* IPLA20004 on intestinal epithelial cells, observing a potential improvement of the epithelial barrier. This, together with previous studies carried out on the interaction of the strain with immune cells indicating a Th1 profile [8, 12] or showing an increase of the transepithelial resistance of the colonic epithelial cells monolayer [13], suggests the interest in conducting experiments in which both polarized epithelial cells and immune cells are cocultured.

In summary, our results suggest that this strain offers possibilities for increasing the intestinal barrier against pathogens in populations in which the barrier may be compromised. This could be achieved by two independent mechanisms: firstly by strengthening the cell cytoskeleton and, therefore, the physical resistance of the epithelial layer and secondly by modulating the immune environment at local mucosal level towards a "prestimulated" innate immune response by recruiting immune cells.

Conflict of Interests

All the authors have declared no conflict of interests.

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Review Article

Bifidobacteria-Host Interactions—An Update on Colonisation Factors

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Bifidobacteria are one of the predominant bacterial groups of the human intestinal microbiota and have important functional properties making them interesting for the food and dairy industries. Numerous *in vitro* and preclinical studies have shown beneficial effects of particular bifidobacterial strains or strain combinations on various health parameters of their hosts. This indicates the potential of bifidobacteria in alternative or supplementary therapeutic approaches in a number of diseased states. Based on these observations, bifidobacteria have attracted considerable interest by the food, dairy, and pharmaceutical industries and they are widely used as so-called probiotics. As a consequence of the rapidly increasing number of available bifidobacterial genome sequences and their analysis, there has been substantial progress in the identification of bifidobacterial structures involved in colonisation of and interaction with the host. With the present review, we aim to provide an update on the current knowledge on the mechanisms by which bifidobacteria colonise their hosts and exert health promoting effects.

1. Introduction

1.1. Host Colonisation by Bifidobacteria. On a cellular basis, humans can be regarded as superorganisms. As a rough approximation, these super-organisms consist of 90% microbial cells with the vast majority of the microbial diversity being located in the human gastrointestinal tract (GIT) [1]. The development and composition of a normal GIT microbiota is crucial for establishing and maintaining human health and well-being [2–4]. It is generally accepted that, before birth, the intrauterine environment and thus the GIT of the unborn foetus are sterile [4]. During delivery, newborns acquire microorganisms from their mothers faecal, vaginal, and skin microbiota. Interestingly, considerable numbers of bifidobacteria and other components of the infant intestinal microbiota were also isolated from human breast milk [5, 6]. Some of the strains recovered in the mother's milk were identical to those detected in the faecal samples of the infant [7] suggesting that human milk might contribute to the establishment and development of the intestinal microbiota of children.

The succession of colonisation follows more or less a classical pattern with facultative anaerobes such as *Escherichia coli* or *Enterococcus sp*. dominating for the first hours or days. Once these organisms have consumed the residual oxygen in the GIT, strictly anaerobic bacteria including *Bifidobacterium sp.*, *Clostridium sp.*, and *Bacteroides sp.* rapidly become predominant [4]. In naturally delivered, breast-fed children up to 95% of all bacteria are bifidobacteria [8–10] making them by far the predominant bacterial component of the faecal microbiota in this group. The bifidobacteria most frequently isolated from healthy breast-fed infants belong to the species *B. longum*, *B. bifidum*, and *B. breve* [10, 11].

Following the period of exclusive breast-feeding, the composition of the faecal microbiota rapidly changes due to the introduction of solid foods, constant exposure to food-derived and environmental microorganisms, and other factors such as hygiene, antibiotic treatment, and so forth [4, 12]. During the first three years of life, the faecal microbiota then gradually develops into the microbiota of adults [9]. The adult colonic and faecal microbiota is dominated by obligate anaerobes with *Firmicutes* and *Bacteroidetes* together representing more than 80% followed by *Actinobacteria*, which contribute up to 10% to the total bacterial flora. The vast majority (up to 100%) of *Actinobacteria* in faecal samples are representatives of the genus *Bifidobacterium* [12].

Table 1: Factors and medical conditions associated with changes in the composition of the faecal microbiota.

Factor/disease	Effect/observation	References
Caesarean section	Higher numbers of the *Clostridium difficile* group 1 Delayed/reduced colonisation with *Bifidobacterium sp.*, *Lactobacillus sp.* and *Bacteroides sp.*	[14–16]
Infant feeding	Formula-fed infants with lower levels and diversity in *Bifidobacterium sp.*	[11, 15, 17]
Ageing	Increase in *Enterobacteriaceae* and *Bacteroidetes* Reduced levels of *Bifidobacterium sp.*	[18, 19]
Antibiotic-associated diarrhea and chronic *C. difficile* infections	Reduced diversity Increase in *Enterobacteriaceae* and *Firmicutes* Reduced levels of *Bifidobacterium sp.* and *Bacteroidetes*	[18, 20–22]
Irritable bowel syndrome	Increase in *Firmicutes* Reduced levels of *Bacteroidetes* and *Bifidobacterium sp.*	[23–25]
Inflammatory bowel disease	Reduced diversity Lower levels of *Faecalibacterium sp.* Increase in *Enterobacteriaceae* and *Bifidobacterium sp.* Reduced levels of *Bifidobacterium sp.* in pediatric IBD	[26–29]
Atopic disease/Allergy	Increase in *Clostridium sp.* Reduced levels of *Bifidobacterium sp.*	[30–32]
Autism	Increase in *Clostridium sp.* Reduced levels of *Bifidobacterium sp.*	[33–35]

Members of this genus are nonmotile, non-spore-forming, strictly anaerobic, gram-positive bacteria characterised by genomes with a high G + C content, an unusual pathway for sugar fermentation termed bifidus shunt, and an unusual V- or Y-shaped morphology formed by most strains under specific culture conditions [13].

1.2. Effects of Bifidobacteria on Host Health. In healthy individuals, the composition of the intestinal microbiota is relatively stable throughout adulthood with minor day-to-day variations [36, 37]. However, a number of factors have profound impact on the composition of the microbiota and more substantial and persistent changes in the microbiota, a state also termed dysbiosis, are associated with various diseases [2, 38]. A common feature of most diseases with changes in the (intestinal) microbiota is a reduction or change in the relative abundance of bifidobacteria along with an increase in other bacterial groups, such as *Enterobacteriaceae* or clostridia (Table 1). These alterations might be implicated in onset, perpetuation, and/or progression of disease [12]. However, in most cases, it is not clear whether the altered community profiles of the microbiota are a cause or consequence of the disease.

Besides the implication in various diseases, the intestinal microbiota in general and bifidobacteria in particular are important to establish and maintain health of the host. Studies in germ-free animals nicely illustrate that the presence of a normal microbiota is required for proper development and function of the immune and digestive systems (reviewed in [38, 39]). Their predominance during neonatal development suggests that bifidobacteria play a major role in this process [4].

Various beneficial effects have been claimed to be related to presence or administration of bifidobacteria including cholesterol reduction, improvement of lactose intolerance, alleviation of constipation, and immunomodulation [13, 40, 41]. Different strains of bifidobacteria were shown to have profound effects on dendritic cells, macrophage, and T cells of healthy humans and in animals models of allergy or intestinal inflammation [42–47]. One class of molecules that seems to be of particular relevance for the immunomodulatory properties of bifidobacteria is exopolysaccharides (EPS). Mutants of *B. breve* UCC2003 that lack EPS production induce higher numbers of neutrophils, macrophages, NK, T and B cells in mice compared to the wild type strain indicating that EPS production renders this strain less immunogenic by an unknown mechanism [48].

A promising target for bifidobacterial treatments are amelioration of chronic inflammatory disorders of the GIT [42, 49, 50]. Different strains of bifidobacteria were shown to dampen NF-κB activation and expression and secretion of proinflammatory cytokines by IECs or immune cells in response to challenge with LPS, TNF-α, or IL-1β [51–56]. Also, various strains of bifidobacteria or mixes of probiotics containing bifidobacteria were able to counteract intestinal inflammation in different models of chronic intestinal inflammation [49, 53, 55–60]. In murine models, different strains of bifidobacteria have been shown to be able to counteract chronic intestinal inflammation by reducing proinflammatory Th1 and inducing regulatory T-cell populations and lowering of colitogenic bacteria [42, 45, 46, 50, 60].

Experiments in mice indicate that some strains of bifidobacteria confer resistance against infections with *Salmonella enterica* serovar Typhimurium [61], enteropathogenic *E. coli* [62, 63], or *Yersinia enterocolitica* [64]. Interestingly,

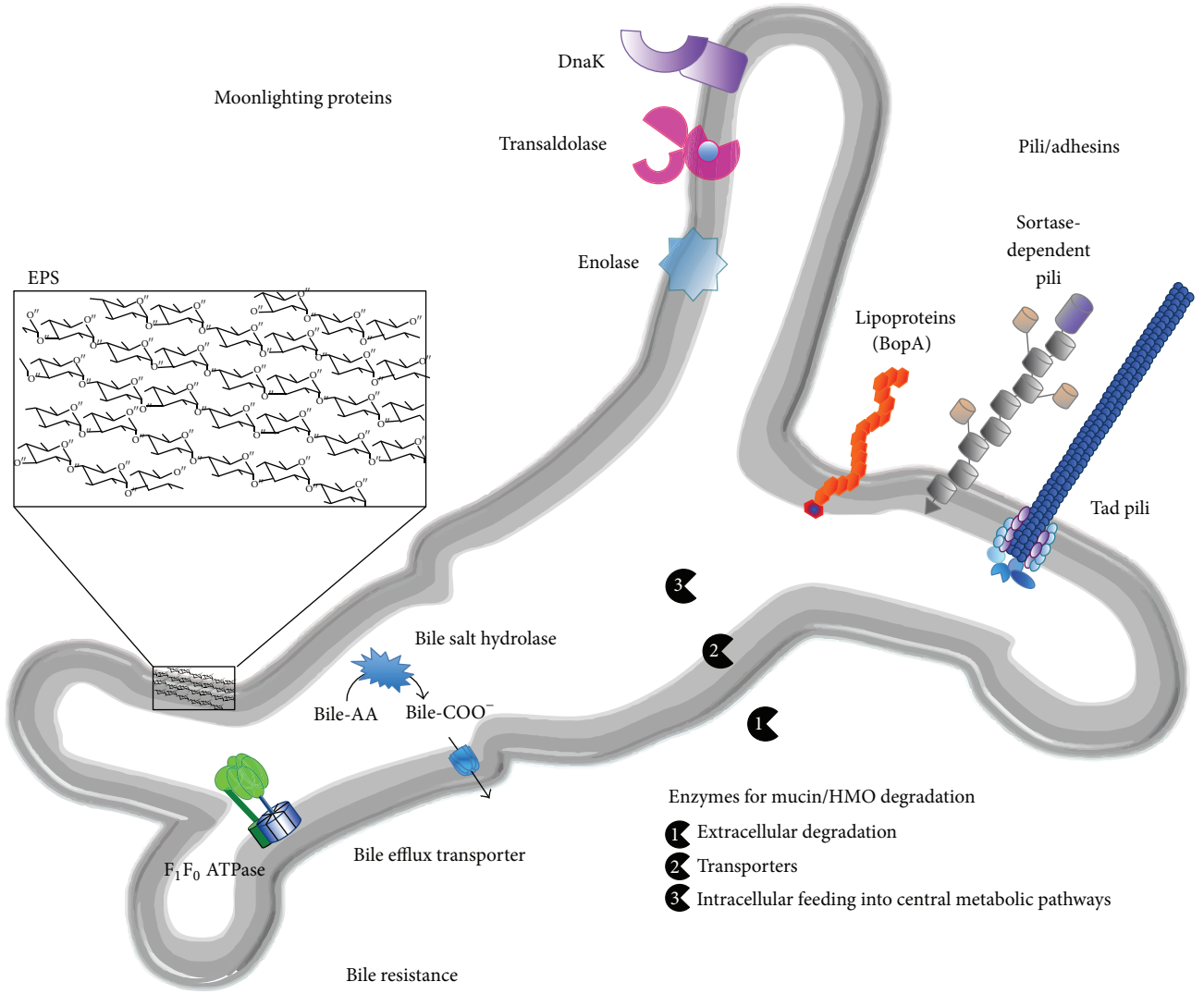

Figure 1: Host colonisation factors of bifidobacteria identified by genome analysis and supported by experimental evidence obtained in *in vitro* experiments and/or murine model systems (bile-AA: conjugated bile acids; bile-COO^-: deconjugated bile acids; Tad: tight adherence; EPS: exopolysaccharides; HMO: human milk oligosaccharides).

B. breve UCC2003 is able to protect mice against infections with *C. rodentium* and this ability depends on EPS production [48, 65]. The protective effect of other bifidobacteria towards enteric infections and intestinal inflammation was shown to be mediated by the production of short chain fatty acids, that is, the end products of bifidobacterial sugar fermentation [50, 63]. It is thus likely that the contribution of EPS production by *B. breve* UCC2003 to protection against *C. rodentium* is related to the improved colonisation [48].

2. Colonisation Factors of Bifidobacteria

Due to the aforementioned effects of bifidobacteria, genomic approaches were pursued to understand the genetic and physiological traits involved in colonisation of and interaction with the host. The first genome sequence of a *Bifidobacterium sp.* strain was published in 2002 [66]. Since then, the genomes of over 200 strains of bifidobacteria belonging to 25 species and 5 subspecies have been sequenced (http://www.genomesonline.org/). Of these bifidobacterial genomes, 37 are complete and published and 42 are available as permanent drafts. Analysis of these genome sequences has provided insights into the very intimate association of bifidobacteria with their hosts and the adaptation to their gastrointestinal habitat and has led to the identification of a large number of genes with a potential role in these processes [67]. Some of these factors have been analysed in more detail (summarized in Figure 1).

2.1. Resistance to Bile. Bile salts are detergents that are synthesized in the liver from cholesterol and secreted via the gall bladder into the GIT lumen [68]. They exert various physiological functions including lipid absorption and cholesterol homeostasis [69]. Since bile salts have considerable

antimicrobial activity at physiological concentrations [70], resistance to bile is important for colonisation and persistence of gastrointestinal microorganisms and is thus one of the criteria for the selection of novel probiotic strains [71]. In a number of bifidobacteria, several genes and proteins conferring bile resistance including bile salt hydrolases and bile efflux transporters were identified and characterised *in vitro* [72–82]. Interestingly, the F_1F_0-type ATPase of *B. animalis* IPLA4549 was also shown to be involved in bile resistance [83]. The only example for *in vivo* functionality, however, is a recombinant strain of *B. breve* UCC2003 expressing the bile salt hydrolase BilE of *Listeria monocytogenes* [84]. Compared to the wild type, this strain showed improved bile resistance *in vitro* and prolonged gastrointestinal persistence and protection against *L. monocytogenes* infections in mice.

2.2. Carbohydrate Utilisation. The genome sequences of bifidobacteria of human origin display a remarkable enrichment in genes involved in breakdown, uptake, and utilisation of a wide variety of complex polysaccharides of dietary and host origin [13, 85–92]. Since most of the simple carbohydrates are absorbed by the host or metabolised by bacteria in the upper gastrointestinal tract, this can be regarded as a specific adaptation of bifidobacteria to their colonic habitat. The ability of bifidobacteria to ferment these complex carbohydrates is the rationale for the use of prebiotics, that is, nondigestible oligosaccharides, to boost bifidobacterial populations in the GIT [93].

The ability to utilise human milk oligosaccharides (HMOs) is thought to provide a selective advantage to bifidobacteria over other microorganisms during initial colonisation of breast-fed newborns and to be, at least partially, responsible for the dominance of bifidobacteria in these children [85, 91]. The genomes of bifidobacteria particularly abundant in breast-fed infants, especially *B. longum* subsp. *infantis*, reflect their adaptation to the utilisation of HMOs [89, 90, 94] and some of the enzymes involved have been characterised [95–97].

Another nutritional adaptation of bifidobacteria to the intestinal niche is the ability to degrade and ferment host-derived mucins. Mucins are high molecular weight glycoproteins secreted by goblet cells as a protective coating for the intestinal epithelium [98]. Similar to the HMO-degradation pathways of *B. longum* subsp. *infantis*, *B. bifidum* strains were shown to grow on mucin as sole carbon source and harbour the respective genes for mucin degradation [85, 92].

2.3. Adhesins. Another property frequently associated with host colonisation of commensal and probiotic bacteria is adhesion to intestinal epithelial cells, mucus, or components of the extracellular matrix [99, 100]. Although definite proof for a role of adhesion of bifidobacteria to host-structures in colonisation is missing, these properties are thought to contribute to prolonged persistence and pathogen exclusion. Moreover, the presence of various receptors on the host surface for molecules of probiotic bacteria suggests direct interactions at least at some stage [101].

Strain-dependent adhesion of bifidobacteria to cultured intestinal epithelial cells has been shown in a number of studies [56, 102–115]. However, there are only very few reports investigating adhesion of bifidobacteria from a mechanistic point of view. For example, enolase was shown to mediate binding to human plasminogen by different bifidobacteria [104]. DnaK is another plasminogen-binding protein of *B. animalis* subsp. *lactis* Bl07 [105] and transaldolase is involved in mucus binding of four *B. bifidum* strains [116]. Using a proteomic approach, some of these proteins were shown to be induced in *B. longum* NCC2705 upon cocultivation with intestinal epithelial cells *in vitro* [117]. This indicates that bifidobacteria might be able to sense the presence of intestinal epithelial cells and react by expressing adhesive molecules that mediate interaction with these cells. Interestingly, the role of all these proteins as adhesins seems to be rather a moonlighting function, since they are cytoplasmic proteins with a primary role in bacterial metabolism. Similar moonlighting proteins have been shown to be involved in virulence of different pathogenic bacteria [118].

Bbif_0636, also termed BopA, is a lipoprotein with a cell wall anchor and was previously shown to be involved in adhesion of *B. bifidum* MIMBb75 to IECs [109]. A more detailed analysis performed by our group found the corresponding *bopA* gene to be specifically present in the genomes of *B. bifidum* strains. A purified BopA fusion protein with an N-terminal His_6-tag inhibited adhesion of *B. bifidum* S17 to IECs. Moreover, expression of this His-tagged protein enhanced adhesion of *B. bifidum* S17 and *B. longum* E18 to IECs. The *bopA* gene is part of an operon encoding a putative oligopeptide ABC transporter and BopA contains an ABC transporter solute-binding domain [109, 112]. This indicates that its primary role might be uptake of nutrients and suggests a moonlighting function in adhesion. A recent study questioned the role of BopA as an adhesin [119]. The authors could show that neither BopA antiserum nor C-terminal His_6-BopA fusion protein had an effect on adhesion of two *B. bifidum* strains to IECs. However, the His_6-BopA fusion protein used in this study lacked both the signal sequence and the cell wall anchor motif. Thus, further experiments have to be performed to clarify the role of the position of the His_6-tag, the contribution of the signal sequence and cell wall anchor, and BopA as an adhesin in general.

A recent bioinformatic analysis of the genome sequence of *B. bifidum* S17 for genetic traits potentially involved in interactions with host tissues revealed that the genome of *B. bifidum* S17 contains at least 10 genes that encode for proteins with domains that have been described or suspected to interact with host tissue components and may thus serve as potential surface-displayed adhesins [120]. Most of the genes for the putative adhesins of *B. bifidum* S17 are expressed *in vitro*, with higher expression during exponential growth phase [120]. Increased expression of the putative adhesins in exponential growth phase was associated with higher adhesion of *B. bifidum* S17 to Caco-2 cells [120].

2.4. Pili. All bifidobacterial genomes sequences analysed so far harbour clusters of genes encoding for Tad and/or sortase

dependent pili [120–123]. For example, *B. bifidum* S17, *B. breve* S27, and *B. longum* E18 all harbour a complete gene locus for Tad pili. By contrast, *B. longum* E18 genome only contains an incomplete gene cluster for sortase-dependent pili suggesting absence of such structures and *B. breve* S27 encodes one gene cluster and *B. bifidum* S17 encodes three complete gene clusters for sortase-dependent pili. For a range of bifidobacteria, expression of the genes of these pili operons under *in vitro* conditions and in the mouse gastrointestinal tract could be demonstrated [120, 121, 123]. Several studies have also shown presence of pili on the surface of bifidobacteria under these conditions using immunogold labelling and transmission electron microscopy [122] or atomic force microscopy [121, 123]. For one strain of *B. breve* it was demonstrated that Tad pili are indeed important for host colonisation in a murine model [122].

2.5. EPS. Genes for EPS production were identified in most genome sequences of *Bifidobacterium sp.* strains [124]. The genetic organisation of EPS gene clusters is not well conserved in bifidobacteria and this is reflected by a high structural variability in the EPS of different bifidobacteria [124]. A recent study has indicated that production of EPS by *B. breve* UCC2003 is important for host colonisation [48]. Mutants of *B. breve* UCC2003 that lack EPS production are significantly less resistant to acidic pH and bile. Moreover, these mutants less efficiently colonize the gastrointestinal tract of mice compared to the wild type strain. Also, EPS-deficient mutants were considerably less immunogenic as the wild type in mice as reflected by lower numbers of immune cells in spleens and lower serum titres of specific antibodies.

Hidalgo-Cantabrana and colleagues characterized the EPS of *B. animalis* subsp. *lactis* A1 and isogenic derivatives, which were obtained by exposure of strain A1 to bile salts (strain A1dOx) followed by cultivation for several generations in the absence of bile (strain A1dOxR). The strain A1dOxR displays a ropy phenotype and shows higher expression of a protein involved in rhamnose biosynthesis along with higher rhamnose content in its EPS [125]. Interestingly, these strains elicited different responses by peripheral blood mononuclear cells and isolated lamina propria immune cells of rats [126].

Despite the presence of EPS gene clusters in most bifidobacteria, it remains to be determined experimentally whether all bifidobacteria actually do produce EPS, if this EPS has a role in host colonisation, and how different EPS structures impact the immune response of the host.

2.6. Other Factors Involved in Host Colonisation. Besides bile, another important stress encountered by bifidobacteria during gastrointestinal transit and colonisation is acidic pH in the stomach and small intestine. A number of *B. animalis* subsp. *animalis* and *lactis* strains were shown to survive acidic pH in the physiological range (pH 3–5) in a strain-specific manner and tolerant strains exhibited higher ATPase activity at pH 4 than at pH 5 [127]. Ventura et al. identified the *atp* operon encoding the F_1F_0-type ATPase of *B. lactis* DSM10140 and were able to show that its expression was markedly increased upon exposure to acidic pH [128]. Similarly, various ATPase subunits were upregulated in *B. longum* subsp. *longum* NCIMB 8809 in response to acid stress (pH 4.8) as shown by a proteomic approach [129]. This suggests that pH resistance of this strain is inducible and might help to cope with the conditions of the gastrointestinal tract thereby supporting host colonisation. Interestingly, resistance to bile and low pH somehow seems to be connected in the closely related *B. animalis* subsp. *lactis* ILPA 4549. In this strain, expression of the F_1F_0-type ATPase and ATPase activity in the membrane was increased in the presence of bile [83]. Moreover, the spontaneous mutant *B. lactis* 4549dOx, which shows increased bile resistance, was also able to better tolerate exposure to acidic pH [83].

More recently, one of the mechanisms by which bifidobacteria might be able to sense their environment and regulate expression of factors important for host colonisation and adaptation to the intestinal niche has been investigated in more detail. A proteomic analysis in *B. longum* NCC2705 identified LuxS as one of the proteins with the most prominent host-induced changes in expression compared to *in vitro* growth [130]. LuxS is an enzyme of the activated methyl cycle of bacteria for recycling of S-adenosylmethionine [131]. By-products of this pathway are autoinducer-2 (AI-2)-like molecules, which are also used by bacteria as signaling molecules and were shown to be involved in biofilm formation, virulence, production of antimicrobials, motility, and genetic competence in a number of gram-positive and gram-negative bacteria [132, 133]. All publicly available genome sequences of bifidobacteria harbour *luxS* homologues, which are functional in the production of AI-2 [134]. Moreover, homologous overexpression of *luxS* in *B. longum* NCC2705 increased AI-2 levels in the supernatant and enhanced biofilm formation [134]. For *B. breve* UCC2003, *luxS* was shown to be important for colonisation of the murine gastrointestinal tract [135].

3. Concluding Remarks

Collectively, the available data suggests that individual strains of bifidobacteria exert health-promoting effects on their hosts. An important prerequisite for these effects, is resistance to the conditions of the GIT and, at least, transient colonisation of the host. In recent years, there has been considerable progress in the identification of bifidobacterial structures that play a role in host colonisation and health-promoting effects. However, the vast majority of studies have been performed *in vitro* or in animal models. Based on the fact that they have not been substantiated sufficiently by clinical studies in humans, the European Food Safety Authority has rejected all of the health claims submitted for probiotics. This highlights the need for well-performed clinical trials with a clear definition of target groups and relevant biomarkers and a more detailed analysis of the molecular mechanisms responsible for host colonisation and the positive effects of probiotic bifidobacteria.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Research Article

Functional Screening of Antibiotic Resistance Genes from a Representative Metagenomic Library of Food Fermenting Microbiota

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Lactic acid bacteria (LAB) represent the predominant microbiota in fermented foods. Foodborne LAB have received increasing attention as potential reservoir of antibiotic resistance (AR) determinants, which may be horizontally transferred to opportunistic pathogens. We have previously reported isolation of AR LAB from the raw ingredients of a fermented cheese, while AR genes could be detected in the final, marketed product only by PCR amplification, thus pointing at the need for more sensitive microbial isolation techniques. We turned therefore to construction of a metagenomic library containing microbial DNA extracted directly from the food matrix. To maximize yield and purity and to ensure that genomic complexity of the library was representative of the original bacterial population, we defined a suitable protocol for total DNA extraction from cheese which can also be applied to other lipid-rich foods. Functional library screening on different antibiotics allowed recovery of ampicillin and kanamycin resistant clones originating from *Streptococcus salivarius* subsp. *thermophilus* and *Lactobacillus helveticus* genomes. We report molecular characterization of the cloned inserts, which were fully sequenced and shown to confer AR phenotype to recipient bacteria. We also show that metagenomics can be applied to food microbiota to identify underrepresented species carrying specific genes of interest.

1. Introduction

Bacterial fermentation products provide specific sensory properties which characterize a wide variety of foods. Foodborne fermenting microorganisms can either be added to sterilized matrices as commercial starter mixtures composed of specific strains [1] or they can originate from the environment as in the case of the raw ingredients employed for artisanal food production. This latter condition is the most frequent in traditional cheese manufacturing, which does not employ selected industrial starters as it relies on the microflora naturally present in raw material, often represented by complex microbial consortia whose species profile reflects local microenvironments. Lactic acid bacteria (LAB) are prevalent microorganisms within the fermenting food microbiota. Complex environmental bacterial communities have been extremely difficult to characterize, mostly due to the limitations imposed by culture-dependent approaches [2]. The proportion of bacteria from natural environments that are not readily culturable was estimated to about 99% [3]. Therefore, the majority of environmental strains have never been described and cannot be exploited for research and for biotechnological applications. Metagenomics represents, at the moment, the most promising culture-independent, DNA-based molecular method to overcome such difficulties [4, 5]. Food microbiology has taken advantage of the application of such innovative strategies, which were applied to study the composition and the evolution, as well as the spatial distribution of fermenting microbial ecosystems [6, 7].

Metagenomic libraries can be constructed from a variety of sources and through several methods, depending on the objective to be pursued. Taxonomic analysis requires comparison of conserved genome stretches, and therefore total DNA

extracted from environmental microbiota is mostly PCR-amplified prior to cloning into the appropriate vectors, resulting in gene-specific metagenomic libraries (most frequently 16S-ribosomal DNA libraries) that are easily analyzed using bacterial genome databases and tools. However, the PCR step introduces a bias in DNA complexity, by altering the relative species proportions with respect to their relative abundance within the original microbiota. On the other hand, direct cloning of total DNA extracted from complex microbial communities, although quantitatively more reliable, requires very high cloning efficiencies to avoid selection against the least represented genomes. The choice of methodological approach is therefore strictly dependent on the purpose of the study, although the majority of metagenomic libraries described in the literature employ PCR-amplified DNA as starting material.

Our laboratory has focused on studying antibiotic resistance (AR) genes from microbial food sources and their corresponding genomic context which represents the main driver of horizontal transfer to human opportunistic pathogens [8, 9]. AR genes are widely distributed in several different environments, including food production systems [10]. Recent findings suggest the possibility of horizontal gene transfer among bacteria within food matrices, since fermented and minimally processed foods contain high titers of live microbial cells [11]. We have chosen a specific water buffalo fermented cheese as a model; that is, Mozzarella di Bufala Campana (MBC), which is produced in restricted geographical regions of Italy, is consumed fresh and therefore supplies high titers of live bacteria [12, 13]. Fermentation in this product is performed by specific thermophilic microbial communities provided by natural whey starter cultures (NWSC) [14], which, together with the microbiota of raw milk, contribute a wide variety of uncharacterized, environmental strains to the final cheese ready for consumption. Although PCR amplification with gene-specific primers of total DNA extracted from MBC had shown the presence of several AR genes, when applying culture-dependent approaches to isolate the corresponding AR strains, we were able to identify AR colonies only from the raw materials employed for cheese production, in which microbial titers are higher than in the final product [13]. Previous studies by other laboratories demonstrated the efficacy of culture-independent approaches in the identification of AR clones from oral metagenome libraries [15]. We turned therefore to metagenomics, with the aim of constructing a representative library of the entire cheese microbiome that could allow detection and analysis of AR genes carried by nonculturable or underrepresented species within the microbiota of fermented food products. Our experimental design involved construction of a fosmid metagenomic library containing large fragments of total DNA extracted from MBC, followed by functional screening of recombinant clones on representative antibiotics belonging to different pharmacological classes and employed in the past in animal farming and/or presently used in human therapy, namely, ampicillin, kanamycin, gentamycin, and tetracycline. To best reflect the complexity of the fermenting microbiota, the metagenomic library needed to be quantitatively representative of the different species present in the starting material, and we thus had to confront with several technical aspects representing crucial steps towards our goal. We describe in this paper the choices deriving from such a challenge, which resulted in the construction and screening of a cheese metagenomic library leading to the identification of fosmidborne, LAB derived genes expressing an AR phenotype in the *E. coli* host. To the best of our knowledge, this is the first report of direct, nonamplified metagenomic cloning of microbial genes from a complex fermented food matrix.

2. Materials and Methods

2.1. Mozzarella Processing and Sampling. Samples of MBC were received on the day of production from four dairy factories located in different provinces of central and southern Italy (Latina-LT; Salerno-SA; Caserta-CE; Foggia-FG). We exclusively selected dairy plants with associated animal farming, which guarantees reproducible sources of milk and associated microbiota profiles for cheese production. Samples were stored at 4°C and processed within 12 h. Pooled or single samples of MBC were homogenized with a BagMixer400 (Interscience, France) in sodium citrate solution (2% w/v) at a concentration of 0.5 g/mL. In order to test the titer of mesophilic cultivable LAB, serial dilutions were made in Quarter Strength Ringer's solution and plated on MRS agar medium (Oxoid Ltd, Basingstoke, Hampshire, England), as previously reported [13]. Plates were incubated at 30°C for 48 h, under aerobic and anaerobic conditions (Anaerocult A, Merck, Germany).

2.2. DNA Extraction. Total DNA extraction from MBC was performed by a modified version of a published method [16]. Relevant methodological modifications are described in Section 3. Microscopic observation of sample aliquots during the lysis procedure was carried out to monitor the progressive disappearance of intact microbial cells. The yield of total DNA obtained from MBC samples was about 0.5 μg/g.

2.3. Library Construction. Metagenomic library construction was performed using the EpiFos Library Production Kit (Epicentre Technologies, Madison, Wisconsin, USA), following manufacturer's indications with the following modifications: ligation reaction was carried out with ligase enzyme from Stratagene, and incubation time of *E. coli* host cells with phage particles during the infection process was extended to 40 min. Such modifications resulted in increased packaging efficiency as well as in improved titer of packaged fosmid clones by about 4-fold.

2.4. Antibiotics and Reagents. Antibiotics (ampicillin, chloramphenicol, erythromycin, gentamycin, kanamycin, tetracycline, and vancomycin) were purchased from Sigma (Italy). Restriction enzymes were provided by Takara (Italy). PCR reagents were obtained from Invitrogen (Italy).

2.5. Bacterial Strains and Growth Conditions. *E. coli* EPI100-T1^R [F$^-$ mcrA Δ(*mrr-hsd*RMS-*mcr*BC) ϕ80d*lac*ZΔM15 Δ*lac*X74 *rec*A1 *end*A1 *ara*D139 Δ (*ara*, *leu*)7697 *gal*U *gal*K λ^-

Table 1: Primers used for PCR experiments.

Primer pair	Sequence	Target gene	Reference
P0	GAGAGTTTGATCCTGGCT	*Bacterial 16S rDNA*	[46]
P6	CTACGGCTACCTTGTTAC		
SINE-F	GGATCCGGCATTGCCGTTAG	*Swine short interspersed nuclear elements*	[47]
SINE-R	GTCTTTTTTTGCCATTTCTTGG		
ITS1	TCCGTAGGTGAACCTGCG	*Yeast 5.8S rDNA*	[48]
ITS4	TCCTCCGCTTATTGATATGC		
BufGH-F	TTGGGCCCCTGCAGTTC	*Buffalo growth hormone*	[49]
BufGH-R	GGTCCGAGGTGCCAAACAC		

*rps*L *nup*G *ton*A] was grown in LB medium (Difco) overnight at 37°C with shaking. Recombinant libraries were stored at −80°C in LB-Cm (LB medium added with chloramphenicol at a final concentration of 12.5 mg/L) containing glycerol (15% v/v). For screening purposes, libraries were plated on LB-Cm agar plates and a total of 20.000 recombinant *E. coli* clones were picked and stored in 96-multiwell plates containing 10 clones/well. This plates were then replica-plated on LB-agar added with the appropriate antibiotic, with the aid of a metallic replica plater for 96-multiwell (Sigma, Italy), and grown overnight at 37°C.

2.6. DNA Amplification and Molecular Analysis. Microbial DNA was amplified by PCR as previously described [9]. Fosmid DNA was isolated with FosmidPrep kit (Epicentre Technologies, Madison, Wisconsin, USA) according to manufacturer's instructions. Primers used are listed in Table 1. Restriction analysis and southern hybridization were performed by standard protocols, using probes labelled with digoxigenin-11-dUTP (Roche Diagnostics, Milan, Italy).

The two-step gene walking method consisted of a walking-PCR (step 1) followed by direct sequencing of the PCR product (step 2) [17]. Walking-PCRs were performed as described [18], with the specific primer Epifos-FW (Epicentre). PCR products were purified using a NucleoSpin Extract II kit according to the manufacturer's instructions and sequenced with T7 primer.

2.7. Full Sequencing of Recombinant AR Fosmids. Sequencing was performed at the DNA sequencing facility of GenProbio s.r.l., Italy (http://www.genprobio.com/).

3. Results and Discussion

3.1. Microbial Representativeness for Metagenomic Analysis. The first step towards library construction concerned sampling of MBC from different sources to ensure metagenome representativeness of the entire microbiota that characterizes this specific food product. To this aim, MBC cheeses were collected from four different dairy plants located in Italian provinces where the majority of producers are present (see Section 2). Some of the selected geographical areas are over 300 Km apart from each other and represent different pedoclimatic microenvironments leading to diverse milk microbial profiles [13]. We reasoned that pooling these samples should lead us to obtain genomic DNA representing the great majority of microbial genera/species entering the human GI tract through MBC consumption. Moreover, the titer of the mesophilic LAB component of the MBC microbiota resulted in about 10^6 Cfu/g (data not shown), in accordance with our previous findings [13]. The four MBC samples were therefore pooled in equal proportions and microbial DNA for library construction was extracted from the resulting homogenate.

3.2. Food-Derived DNA as a Source of Bacterial Genomes. A strategic aspect that we had to confront with in order to achieve representative, nonamplified metagenomic DNA of good quality was the optimization of qualitative/quantitative steps in the DNA extraction procedure. Fat represents a major component in dairy products, and its presence can impair bacterial recovery and lysis, which in turn greatly affects DNA yields. In order to obtain high molecular weight genomic DNA required for fosmid library construction, we therefore modified a previously published protocol [16], improving fat removal and DNA extraction efficiencies by introducing serial washes of dairy homogenates in Na-citrate buffer, followed by a combination of freeze-thaw cycles and mechanical as well as enzymatic lysis.

The presence of contaminating DNA from eukaryotic cells is another crucial aspect affecting representativeness of microbial genomes within the library, which is usually overcome by PCR amplification. Unlike meat fermentation products, dairy foods should contain almost exclusively microbial DNA, with very low contamination from higher eukaryotic cell DNA [8], but this aspect needed to be assayed before proceeding with our approach of direct cloning unselected high molecular weight DNA extracted from food. To this aim, a PCR approach was carried out including DNA extracted from fermented swine meat sausages for comparison, with primers specific for either microbial or eukaryotic species-specific genes, namely, bacterial 16S rDNA, yeast 5.8S rDNA, buffalo growth hormone gene, and swine SINE (short interspersed nuclear element). Primer sequences are reported in Table 1. The results shown in Figure 1 confirm that total DNA extracted from MBC is almost exclusively of microbial origin. Bacterial DNA represented the major component, while yeast DNA accounted for about 10% of the amplicons (Figure 1(b)). On the other hand, eukaryotic DNA was almost undetectable in MBC samples, while representing a great proportion of the total DNA extracted from fermented sausages. These results

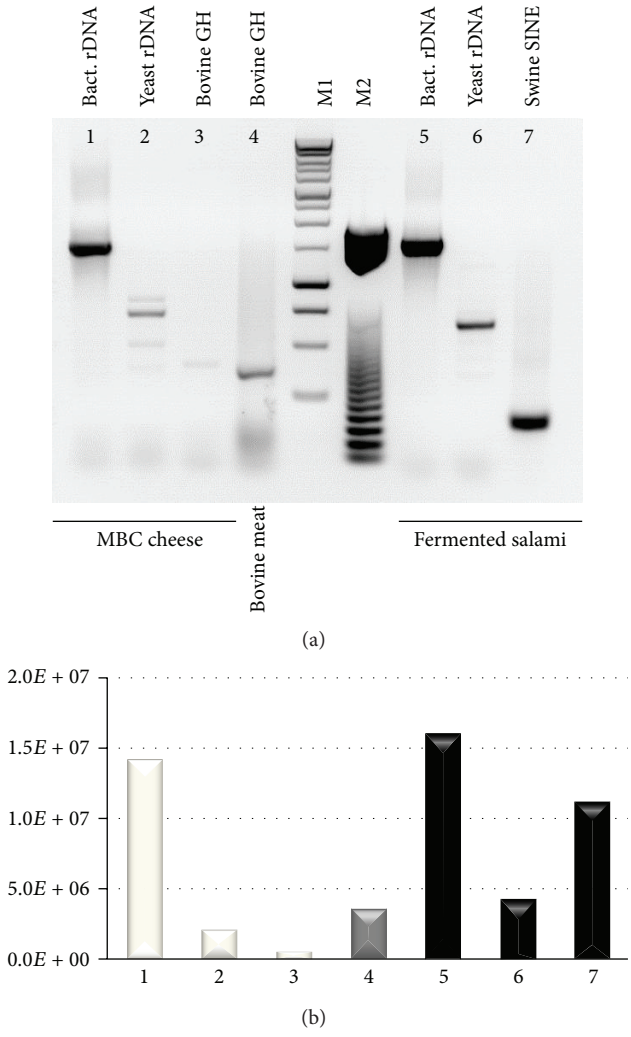

Figure 1: Total DNA extracted from dairy products contains almost exclusively microbial DNA with undetectable contamination from higher eukaryotic cell DNA. (a) PCR amplifications of DNA extracted from cheese (left) or meat (right) matrices using the species-specific primers listed in Table 1. M1: 1 Kb DNA ladder. M2: 50 bp DNA ladder. (b) Amplicon quantification obtained with the freely available ImageJ densitometry software [45]. Numbers indicate the corresponding PCR amplicons in (a).

unequivocally show that microbial genomes constitute the great majority of unamplified DNA extracted from a dairy food matrix such as MBC, which could then be used directly for metagenomic library construction.

3.3. MBC Metagenomic Library. The fosmid vector that we chose for library construction is suitable for cloning genomic inserts of approximately 40 kilobases in size. This feature allows us to characterize also the genomic context surrounding specific genes, thus increasing the chances of identifying the bacterial species of origin through sequencing of flanking regions. In the case of AR genes, analysis of the genomic context can also reveal association with mobile elements, indicative of a potentiality for horizontal inter- and intraspecies transfer [19]. The EpiFOS vector was also chosen because it utilizes a novel strategy for cloning randomly sheared, end-repaired DNA, leading to generation of highly random DNA fragments, in contrast to DNA fragmentation by partial restriction digestion that leads to more biased libraries. Fosmid clones containing high molecular weight fragments ranging between 35 and 45 Kb were used to infect the recipient *E. coli* EPI-100T1^{R} strain, resulting in a 4×10^6 CFU/mL library titre. We estimated the minimum required representativeness of the library using the formula $N = \ln(1 - P)/\ln(1 - f)$, where P is the desired probability (expressed as a fraction) of a given sequence being present in the library, f is the proportion of the metagenome within a single clone, and N is the number of clones required. Metagenomic samples introduce additional constraints, due to the unpredictable number of different species/strains that constitute the original microbiota; thus, only a rough estimate can be derived on the relative abundance of different populations within the complex bacterial community. For example, assuming an average genome size of 4 Mb, a library with 40 kb average inserts would require at least 100 clones to provide coverage of the entire genome, provided all clone inserts contain distinct sequences. If the genome of this reference organism represents about 10% of the total metagenome, screening 1.000 clones would likely provide a reasonable chance of detecting a specific sequence of interest. Basing on these calculations and considering an average fragment length of 25–30 Kbp, we estimated a total number of 20.000 clones to account for a well-represented MBC fermenting microbiome, as the overall size encompasses 1 Gbp which corresponds to approximately 250 times the size of the *E. coli* genome (4×10^6 bp).

To ensure that the library reflected the original DNA complexity, total DNA extracted from the pooled MBC samples was compared to pooled library DNA through PCR amplification of bacterial 16S rDNA and yeast 18S-28S intergenic sequences. The results in Figure 2 show that the DNA was qualitatively similar before and after library construction, thus proving that our cloning strategy can preserve the DNA complexity of foodborne microbial genomes.

Moreover, sequence analysis of randomly selected clone inserts followed by sequence similarity searches in public genome databases (Blast, http://blast.ncbi.nlm.nih.gov/Blast.cgi) confirmed the presence of both bacterial and yeast genomes in the original proportions within the MBC metagenomic library (data not shown). As a control for the presence of specific AR genes, we also confirmed that *tet*(M) and *tet*(S), which are among the best characterized tetracycline resistance determinants in LAB, are well represented in both total MBC DNA and library clones.

3.4. Functional Screening for Antibiotic Resistance Genes. Functional metagenomics requires heterologous expression of exogenous genes, coupled with activity-based assays that can be easily performed on plates to select specific protein functions. This approach is more efficient than other two-step molecular methods based on detection of specific gene sequences and subsequent demonstration of their functionality, but it can be hampered by potential incompatibility

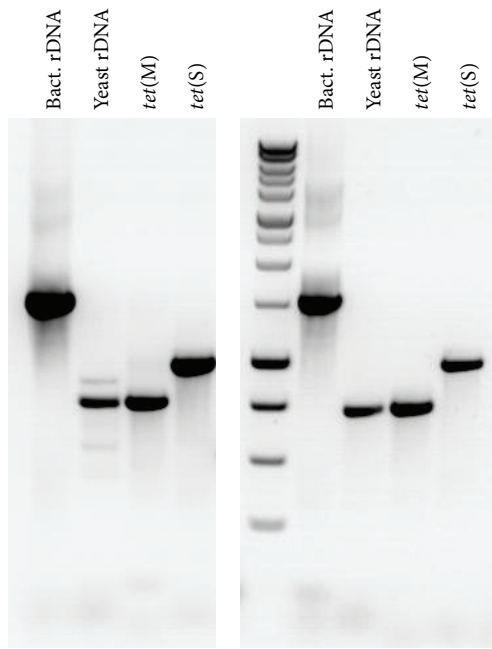

Figure 2: Fosmid cloning of total MBC DNA does not alter complexity PCR amplification of total DNA extracted from pooled MBC samples (a) or from pooled recombinant fosmids following metagenomic library construction (b). Primer pairs: bacterial rDNA, yeast rDNA, *tet*(M), and *tet*(S) (Table 1). M: 1 Kb DNA ladder.

between donor and host expression machineries [20–22]. The emergence and spread of antibiotic resistance determinants in the fermenting microbiota from different foods has been increasingly reported and reviewed by several groups worldwide, including ours [23–25], pointing at the need for deeper understanding of the mechanisms for horizontal transfer of AR genes, which are still partially unknown. AR genes can be easily selected on antibiotic containing media and were therefore chosen in this work to test the efficiency of recovery of LAB genes, which are the most represented species in the MBC microbiome under study. Moreover, AR genes for some of the most common antibiotics are not as well characterized in Gram-positives as they are in Gram-negative pathogens, and functional screening could therefore lead to the possible identification of novel proteins conferring AR in LAB. We therefore sought to test the MBC metagenomic library through functional screening with antibiotics belonging to five different pharmacological classes (tetracycline, aminoglycosides, beta-lactams, macrolides, and glycopeptides), which were chosen on the basis of their relevance in animal and human therapy and/or due to their widespread use in the past as growth promoters. Tetracyclines have been widely used in livestock farming and several tetR determinants were later identified in foodborne LAB from different fermented food sources [8, 26–28]. Along with tetracycline, the macrolide antibiotic erythromycin has also been intensively used in the past as growth promoter, and erythromycin-resistance genes represent, together with the TetR genes, the most widespread resistance determinants in foodborne bacteria [8, 27, 29]. Aminoglycosides and beta-lactams, on the other hand, have never been used as growth promoters, but they represent clinically relevant antibiotics whose corresponding resistance genes have also been described in foodborne LAB strains (lactobacilli and lactococci) [23].

As a first step towards functional screening for AR clones within the MBC library and to avoid interference from AR potentially present in the *E. coli* host, minimum inhibitory concentrations (MIC) were determined for the *E. coli* Epi100T1^R strain on each antibiotic to be tested. Streptomycin was not considered as the corresponding resistance gene *rpsL* is known to be carried by the Epi100T1^R strain. The resulting MIC values are reported in Table 2, showing that the *E. coli* host strain is phenotypically resistant to erythromycin and vancomycin, while displaying susceptibility to tetracycline, kanamycin, gentamycin, and ampicillin, with MIC values of 5, 25, 12, and 25 mg/L, respectively (Table 2). These latter four antibiotics were therefore chosen for functional screening of the MBC library at concentrations corresponding to their respective MIC values for *E. coli*. As positive control, clones were replicated on LB agar containing chloramphenicol, whose resistance determinant represents a selective marker (chloramphenicol acetyl transferase) encoded by the fosmid vector. Functional screening by replica plating of 20.000 independent library clones on antibiotic containing plates led to the selection of 4 TetR, 2 KanR, and 6 AmpR colonies. No colonies were rescued on gentamycin containing plates.

To confirm that phenotypic resistance in the surviving colonies was conferred by resistance determinants encoded by cloned inserts, fosmid DNA was extracted from each AR clone, packaged into phage particles, and used to infect the *E. coli* host Epi100T1^R. Secondary screening of the resulting clones was performed on LB agar plates containing the appropriate antibiotic. All kanamycin and ampicillin resistant bacteria confirmed their ability to grow on the corresponding antibiotic-containing medium following this secondary screening (Table 2). Unexpectedly, the tetracycline resistant colonies identified by primary screening resulted in false positives. A possible explanation is that they arose by spontaneous mutations in the *E. coli* genome induced by the mutagenic effect of chloramphenicol [30]. Unlike kanamycin and ampicillin resistant clones, TetR colonies had indeed been selected on plates containing both antibiotics (tetracycline and chloramphenicol) in the growth medium to increase the selective pressure. Several antibiotics, among which chloramphenicol, are known to induce mutagenesis and recombination within bacterial genomes, which may facilitate bacterial adaptation to different types of stress, including antibiotic pressure [30]. For this reason chloramphenicol was excluded from screening plates used for selection of ampicillin and kanamycin resistant colonies.

3.5. Molecular Characterization of AR Recombinant Clones. To further characterize the genomic features of the AR clones, fosmid DNA was extracted from both AmpR (clones Amp1-6) and KanR (clones Kan1 and 10) colonies and subjected to restriction analysis with the HindIII endonuclease, which

Table 2: Summary of screening procedure and resulting AR recombinant library clones.

Pharmacological class	Antibiotic	Target	*E. coli* MIC (mg/L)	Library clones identified	Verified by secondary screening
Tetracyclines	Tetracycline	Ribosome	5	4	0
Aminoglycosides	Kanamycin , gentamycin	Ribosome	25 12	2 0	2 —
Macrolides	Erythromycin	Ribosome	Resistant	—	—
Beta-lactams	Ampicillin	Cell wall	25	6	6
Glycopeptides	Vancomycin	Cell wall	Resistant	—	—

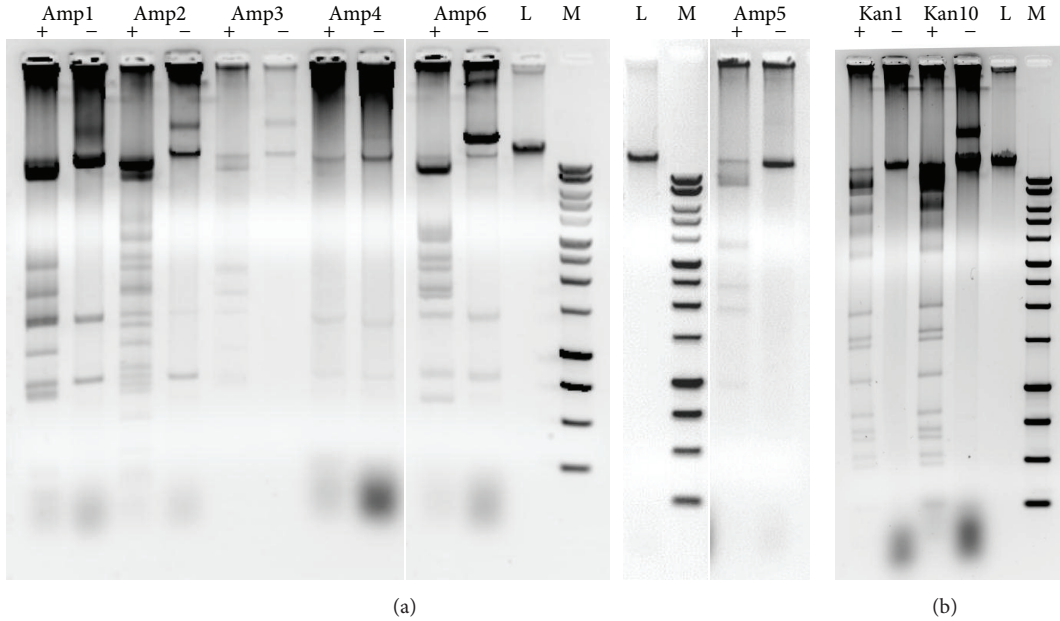

Figure 3: Restriction analysis of AR recombinant fosmids. Fosmid DNA extracted from each clone was digested (+) or not (−) with Hind III and fractionated by agarose gel electrophoresis. (a) AmpR clones. (b) KanR clones. L: undigested phage lambda DNA. M: 1 Kb DNA ladder.

cuts the pEpiFOS-5 vector at a unique site. The results are reported in Figures 3(a) and 3(b) for the AmpR and KanR clones, respectively. With the exception of clone Amp4, which remained undigested by HindIII, the remaining AmpR clones displayed different restriction patterns with almost no overlapping bands, suggesting that the cloned inserts likely originate from distinct genomes within the metagenomic DNA. On the other hand, restriction of the two kanamycin resistant clones yielded fully overlapping restriction bands, indicating identity of the inserts. We therefore considered them as a single resistant clone in our subsequent analysis.

The presence of the AR gene within a large genomic fragment allows species identification even before the full sequence of the cloned fragment is obtained. Preliminary analysis in this direction, performed by two-step gene walking [17], led to associate *Streptococcus salivarius* subsp. *thermophilus* genomic sequences to clones Amp1, 2, 3, and 6 and *Lactobacillus helveticus* genomic sequences to clones Amp4 and 5 (data not shown).

Full sequencing of the clone inserts, performed for 3 AmpR clones and for the KanR clone, confirmed species identification. *S. thermophilus* is expected to be a very abundant species in MBC, especially within the first few days of cheese production, as the last processing step for this specific product includes heating at 95°C for a few minutes. The full sequences, whose deposition in public databases is in progress, are provided as supplementary data (See Supplementary Material available online at http://dx.doi.org/10.1155/2014/290967), while the most relevant features for each clone are summarized in Table 3. Fragment size in the four sequenced clones ranged between 14 and 38 Kbp with correspondingly increasing number of predicted ORFs (14–43). Sequence analysis revealed the presence of two genes encoding penicillin-binding proteins (PBP) in clone Amp3 and of RNA methyltransferase genes in clones Amp3 and Kan10. Synthesis of low-affinity PBPs represents an important mechanism of resistance in some Gram-positive bacteria. Several PBPs have been described in resistant strains, including PBP2a from methicillin resistant *Staphylococcus aureus* (MRSA), PBP2x from penicillin resistant *Streptococcus pneumoniae*, and PBP5fm from drug resistant *Enterococcus faecium* [31, 32]. Blast similarity searches revealed that ORFs

Table 3: Summary of insert sequencing results of AR fosmids. ORFs with a possible function in AR, as well as transposase genes, are listed.

	Amp1	Amp3	Amp6	Kan10
Insert length (bp)	14.380	38.386	21.644	33.491
Predicted ORFs (*n*)	14	43	21	35
Species	*S. thermophilus*	*S. thermophilus*	*S. thermophilus*	*S. thermophilus*
Relevant ORFs for AR	Serine endopeptidase	Penicillin-binding protein 2A Penicillin-binding protein 1A RNA methyltransferase GNAT family acetyltransferase	Phosphoglucomutase	23S rRNA methyltransferase
Transposase sequences (*n*)	2	5	10	1
MIC of the corresponding antibiotic (mg/L)	50	50	50	25

PBP1A and PBP2A from clone Amp3 were homologous to *S. thermophilus* penicillin-binding proteins. Two distinct ORFs encode rRNA methyltransferases in clones Amp3 and Kan10, and only the gene present in the Kan10 clone can be specifically identified as 23S rRNA methyltransferase on the basis of sequence similarity searches, while the Amp3 clone cannot be specifically attributed to 16S or 23S. Ribosomal RNA methylation is a frequent mechanism for macrolide and aminoglycoside resistance. RNA methyltransferases were shown to specifically target 16S rRNA in the case of resistance to aminoglycosides such as kanamycin [33]. However, the 23S rRNA methyltransferase encoded by *Cfr* gene of *S. aureus* and *E. coli*, which confers a wide spectrum of resistance to five chemically distinct classes of antimicrobials, was not tested with aminoglycosides [34]. We therefore need to confirm this gene as a possible basis for AR in the Kan10 clone with more detailed genotypic/phenotypic associations. As for the Amp3 clone, it also contains an acetyl transferase sequence belonging to the GCN5-related N-acetyltransferase (GNAT) superfamily of previously characterized gentamicin and kanamycin resistant bacteria [35]. Noteworthy, the remaining two sequenced clones (Amp1 and Amp6) do not appear to contain ORFs encoding protein functions commonly described in AR bacteria. They do, however, contain at least one ORF with the capacity to mediate bacterial antimicrobial resistance (Table 3).

In particular, clone Amp1 encodes a serine protease whose function includes serine beta-lactamase activities, which deactivate beta-lactam antibiotics by hydrolyzing the beta-lactam ring [36, 37]. The Amp6 clone, on the other hand, contains a phosphoglucomutase (PGM) ORF encoding the key enzyme catalyzing interconversion between glucose-1-phosphate (G1P) and glucose-6-phosphate (G6P) [38]. PGM plays a role in the biosynthesis of several bacterial exoproducts. Increased susceptibility to several antimicrobial agents was observed in *pgm* deletion mutants, suggesting a possible role in AR [39, 40]. Noteworthy, almost all clones also contain ORFs annotated as encoding "hypothetical proteins," whose function might be related to AR. Although each clone contains a variable number of genes that could be related to AR, all of them display identical MIC values, suggesting that no additive effects due to the activity of multiple AR genes should be in place in any of the clones. Functional characterization of the putative AR gene sequences within the cloned fragments requires therefore further investigation. Another important feature deserving deeper analysis is the genomic context, as the sequencing output identifies a variable number of transposase genes within all sequenced inserts, usually clustered at a single site that likely represents an insertional hotspot. Transposases are integral parts of IS elements which mediate insertion/excision events known to promote lateral gene transfer events [41] and are especially important in horizontal gene transfer of AR genes.

4. Conclusions

We have reported in this work a novel metagenomic approach to identify AR genes within a complex, foodborne microbiome derived from a traditional fermented dairy product and constituted mainly by environmental strains of commensal bacteria. To increase the probability of identifying genes carried by underrepresented species, as well as to enhance representativeness of the library, we adopted a strategy based on direct cloning of total, unamplified DNA extracted from the food matrix, into a fosmid vector that can bear up to 40 Kbp inserts. Functional screening of the resulting metagenomic library, which we have calculated as representative of the entire microbiome, was carried out on antibiotics belonging to different pharmacological classes allowing recovery of ampicillin and kanamycin resistant clones. AmpR and KanR resistance genes are poorly characterized in LAB, although an important role for these bacterial genera as reservoir of transmissible AR genes is increasingly recognized [42]. Molecular characterization of the cloned inserts identified them as distinct regions of the *S. thermophilus* and *L. helveticus* genomes, hosting several ORFs which could confer AR phenotypes. The presence of several transposase sequences also emerged from full sequencing of the clone inserts, suggesting potential for lateral gene transfer of the surrounding genomic regions. This aspect is of special relevance, as IS mediated lateral gene transfer events represent the mechanistic basis for AR spreading from the reservoir of nonpathogenic, commensal bacteria to opportunistic pathogens [43]. From the food safety viewpoint, gene transfer events are particularly important as they might also occur through consumption of fermented foods and subsequent gene exchanges, which are known to occur

between the food and the gut microbiota of the host [8, 44]. However, the low frequency of recovery of AR clones from our metagenomic library likely reflects a correspondingly low occurrence of AR bacteria in the food product, thus indicating its safe use for human consumption. Our results further support the evidence that metagenomic approaches can overcome the limitations of culture-dependent methods, representing an efficient and sensitive tool to detect genes occurring at low frequencies. Noteworthy, sequence analysis of the cloned inserts, which we had shown to retain the specific AR phenotype following transfer to new *E. coli* host cells, highlighted a number of genes whose involvement in AR might be novel. This observation points at the power of a screening strategy employing phenotypic selection, as, unlike primer-based methods that require known sequences as starting point, it can uncover novel genes performing similar functions. This work can therefore be considered a pioneer example of the application of metagenomics to food microbiota, and we hope it will pave the way to extend the strategy to other fermented foods, towards a deeper understanding of bacterial metabolic functions which could be beneficial to human health or of technological interest.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Acknowledgments

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Research Article

Production of Conjugated Linoleic and Conjugated α-Linolenic Acid in a Reconstituted Skim Milk-Based Medium by Bifidobacterial Strains Isolated from Human Breast Milk

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Eight bifidobacterial strains isolated from human breast milk have been tested for their abilities to convert linoleic acid (LA) and α-linolenic acid (LNA) to conjugated linoleic acid (CLA) and conjugated α-linolenic acid (CLNA), respectively. These bioactive lipids display important properties that may contribute to the maintenance and improvement human health. Three selected *Bifidobacterium breve* strains produced CLA from LA and CLNA from LNA in MRS (160–170 and 210–230 μg mL^{-1}, resp.) and, also, in reconstituted skim milk (75–95 and 210–244 μg mL^{-1}, resp.). These bifidobacterial strains were also able to simultaneously produce both CLA (90–105 μg mL^{-1}) and CLNA (290–320 μg mL^{-1}) in reconstituted skim milk. Globally, our findings suggest that these bifidobacterial strains are potential candidates for the design of new fermented dairy products naturally containing very high concentrations of these bioactive lipids. To our knowledge, this is the first study describing CLNA production and coproduction of CLA and CLNA by *Bifidobacterium breve* strains isolated from human milk in reconstituted skim milk.

1. Introduction

Conjugated linoleic acid (CLA) and conjugated α-linolenic acid (CLNA) are bioactive lipids with potentially relevant benefits to human health. They have been shown to have *in vitro* and *in vivo* anticarcinogenic, antiatherogenic, anti-inflammatory, and antidiabetic activities and ability to reduce body fat [1, 2].

In relation to CLA, the bioactive isomer *cis 9, trans 11* CLA is the most abundant in the diet, constituting more than 90% of the total CLA content in milk fat [2, 3]. On the other hand, the CLNA isomers resulting from the metabolism of intestinal and rumen bacteria are *cis 9, trans 11, cis 15* CLNA and *trans 9, trans 11, cis 15* CLNA [1]. The predominant isomer is *cis 9, trans 11, cis 15* CLNA, which has been detected at low concentrations in milk fat [4, 5]. Presence of CLA and CLNA isomers in ruminant milk fat are the result of microbial partial biohydrogenation of dietary linoleic (LA) or α-linolenic (LNA) acid to stearic acid metabolism pathway in the rumen by the action of the linoleic acid isomerase [6].

CLA may also be formed through endogenous conversion of trans-vaccenic acid by the enzyme Δ^9-desaturase in the mammary gland [7–9].

Nevertheless, as the current nutritional recommendations for whole fat dairy products are that their consumption should be limited, the CLA and CLNA content of human diet is too low for obtaining health beneficial effects. Therefore, a promising strategy to increase human intake of these bioactive lipids would be to include CLA and/or CLNA-producer bacteria in fermented dairy products. In the last years, several studies have reported that some lactic acid bacteria and bifidobacterial strains are able to efficiently convert LA to CLA in milk, milk-based media, and dairy products [10–12]. Moreover, other study demonstrated that a CLA-producing *Bifidobacterium breve* strain can be applied for the development of functional dairy products when used as a started culture [13]. In contrast, at present, we have not found studies showing CLNA production by bacteria in milk and dairy products. In this context, the aim of the present work was to evaluate the ability of some bifidobacterial strains isolated from human breast milk to produce CLA and/or CLNA when growing not only in MRS broth but also in reconstituted skim milk. In this study, we demonstrated that some *Bifidobacterium breve* strains are able to (co)produce CLA and CLNA in both media.

2. Material and Methods

2.1. Analytical Reagents. All reagents used in the lab procedures were of HPLC grade: hexane and sulphuric acid were obtained from Labscan (Dublin, Ireland), linoleic acid (C18:2 *cis 9 cis 12*) from Sigma-Aldrich (St. Louis, MO, USA), linolenic acid (C18:3 *cis 9 cis 12 cis 15*) from Nu-Chek Prep, Inc. (Elysian, USA), and high CLA content oil (Tonalin) from Cognis (Illertissen, Germany). LA and LNA were prepared as a 30000 μg mL^{-1} stock solution containing 2% (w/v) Tween 80 (Scharlau, Sentmenat, Barcelona, Spain) and filter-sterilized through a 0.45 μm-pore size membrane (Sarstedt, Nümbrecht, Germany).

2.2. Bacterial Strains, Growth Media, and Conditions. Eight bifidobacterial strains previously isolated from human milk [14, 15] were used in the study (Table 1). The bacterial strains were grown overnight at 37°C in MRS broth supplemented with 0.05% (w/v) L-cysteine-HCL (Sigma) and 0.1% (w/v) Tween-80 (MRS-Cys broth) under anaerobic conditions in an anaerobic station (Bactron II, Shellab, Cornelius, Oregon, USA). Three percent (v/v) of these cultures were transferred to fresh MRS-Cys broth (10 mL) containing free LA (500 μg mL^{-1}) and/or free LNA (500 μg mL^{-1}) and incubated at 37°C for 24 h under anaerobic conditions. The samples were analyzed when the bifidobacterial strains reach the early stationary phase, obtaining concentrations of ~1×10^9 cfu mL^{-1}. Only the strains that showed CLA production in MRS-Cys broth after an initial qualitative screening were subsequently tested for CLA and/or CLNA production in 10% skim milk (Scharlau, Sentmenat, Barcelona, Spain) supplemented with 0.05% (w/v) L-cysteine and 0.8% (w/v) casamino acids (milk-based medium), as described above.

Table 1: CLA production level by the bifidobacterial strains screened in this study.

Strains	Production of CLA
Bifidobacterium breve ZL12-22	–*
Bifidobacterium breve ZL12-28	+++
Bifidobacterium breve 29M2	+++
Bifidobacterium breve M7-70	+++
Bifidobacterium infantis ZL50-25	–
Bifidobacterium longum ZL89-79	–
Bifidobacterium longum ZL114-24A	–
Bifidobacterium longum ZL114-24B	–

*(–) No production; (+) slight production; (++) moderate production; (+++) high production.

Since the production of CLA and CLNA by the three selected *Bifidobacterium* strains in MRS-Cys was similar, *B. breve* M7-70 was chosen as the model strain for the subsequent assays. First, the sensitivity of *B. breve* M7-70 to different concentrations of LA or LNA (0, 250, 500, 1000, 1500, and 2000 μg/mL) was evaluated in MRS-Cys broth since LA and LNA have antimicrobial properties. Then, this strain was submitted to a comparative analysis of CLA *versus* CLNA production at different times (0, 1, 2, 3, 4, 6, 8, 24, and 48 h) in MRS-Cys broth.

2.3. Qualitative Screening of CLA Producers by UV Spectroscopy Method. Lipid isolation from culture media was carried out using a chloroform/methanol (2 : 1, v/v) solution according to Folch method modified by [16]. The lipid residues obtained were subjected to a N_2 flow and remained dissolved in chloroform at −20°C until spectrophotometric analysis. For this analysis, lipid extracts (200 μL) from each sample were placed on a quartz 96-well plate (Hellma GmbH & Co. KG, Müllhein, Germany) and total CLA was quantified at a wavelength of 233 in a spectrophotometer (Varioskan Flash, Thermo Fisher Scientific, Waltham, MA, USA) according to [10]. Measurements were obtained in triplicate.

2.4. Quantitative Analysis of CLA and CLNA Production. The concentrations of CLA and CLNA in the culture media were determined using a direct transesterification method [17]. Heptadecanoic acid (C17:0; Sigma) was added to the samples as an internal standard. The fatty acid methyl esters (FAMEs) were dissolved in n-hexane and determined by gas liquid chromatography (GLC) in a chromatograph (Clarus 500, Pelkin Elmer, Beaconsfield, UK) equipped with a VF-23 column (30 m × 0.25 nm × 0.25 μm, Varian, Middelburg, Netherlands). For GLC analysis, the initial temperature was 80°C. Then, the temperature was increased to 170°C at 30°C min^{-1}, held at 170°C for 3 min, increased to 230°C at 30°C min^{-1} and, and finally held at 230°C for 7 min. Helium was used as the carrier gas at a pressure of 15 psig and with a split ratio of 1 : 50. The injection volume was 0.5 μL and the analysis time was 15 min. Peaks were identified by

comparing the retention times of CLA methylated standards (Nucheck, USA) and by gas chromatography-mass spectrometry (GC/MS). CLA and CLNA concentrations were expressed as $\mu g\,mL^{-1}$ and their conversion rates from LA and LNA were calculated using the formula [CLA/(CLA + LA)] × 100 and [CLNA/(CLNA + LNA)] × 100, respectively.

3. Results

The eight bifidobacterial strains assayed in this work were screened spectrophotometrically at A_{233} for CLA production from the LA added to the growth media, following the rapid method described by [10]. With this approach, a total of 3 strains (*Bifidobacterium breve* ZL12-28, *B. breve* 29M2, and *B. breve* M7-70) were identified as able to transform LA into CLA (Table 1).

Subsequently, each of the three selected CLA-producing strains was assayed for CLA and CLNA production by GLC determination after incubation for 24 h at 37°C in MRS-Cys and skim milk (Tables 2 and 4). The concentration of CLA produced by the selected *Bifidobacterium* strains reached values above 158.7 $\mu g\,mL^{-1}$, indicating that the minimal conversion rate from the added LA was approximately 74%. The bacterial strains were able to produce different CLA isomers, such as *cis 9, trans 11* CLA, *trans 10, cis 12* CLA and *trans 9, trans 11* CLA (Figure 1); among them, *cis 9, trans 11* CLA (rumenic acid) was the most abundant isomer, accounting for more than 80% of the total CLA in all cases (Table 2). As it has been reported, many bacteria are inhibited by free long-chain fatty acids in the media [18]; however, *B. breve* M7-70 was able to grow in the presence of LA and LNA at concentrations up to 1500 and 500 $\mu g\,mL^{-1}$, respectively (Table 3).

Production of CLNA in MRS-Cys by *B. breve* strains was higher than that of CLA since the concentrations found in the respective culture media were higher than 200 $\mu g\,mL^{-1}$, and the LNA to CLNA conversion rate was close to 100% (Table 2). Two CLNA isomers (*cis 9, trans 11, cis 15* CLNA and *trans 9, trans 11, cis 15* CLNA) could be detected in the chromatogram profiles (Figure 2), and *cis 9, trans 11, cis 15* CLNA (rumenic acid) accounted for approximately 80% of the total amount of CLNA in the cultures of the three strains (Table 2). The conversion of LNA to CLNA by *B. breve* M7-70 in MRS-Cys was faster than that of LA to CLA since CLNA production began after 2 h incubation at 37°C while CLA formation required at least 6 h of incubation to be detected (Figure 3).

Subsequently, the three selected bifidobacterial strains were assayed for CLA and/or CLNA production in the milk-based medium after 24 h incubation at 37°C. CLA production was above 75 $\mu g\,mL^{-1}$ for the three *B. breve* strains (Table 4). Both CLA production and LA conversion rate were lower in milk than in MRS-Cys. As expected, the predominant CLA isomer produced in reconstituted skim milk was *cis 9, trans 11* CLA, accounting for more than 80% of total CLA (Table 4).

All the selected strains were able to produce CLNA when growing in the milk-based medium. In this case, the concentration of CLNA produced by these bifidobacteria

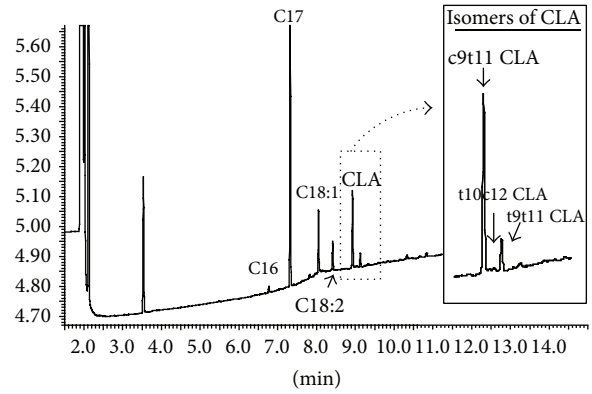

Figure 1: (a) Chromatogram profile assessed by gas chromatography of the fatty acid content present in the culture media obtained from *B. breve* M7-70 in MRS broth with 500 $\mu g\,mL^{-1}$ LA added as a substrate. (b) The insert shows a blow-up of the part of the chromatogram corresponding to the CLA isomers.

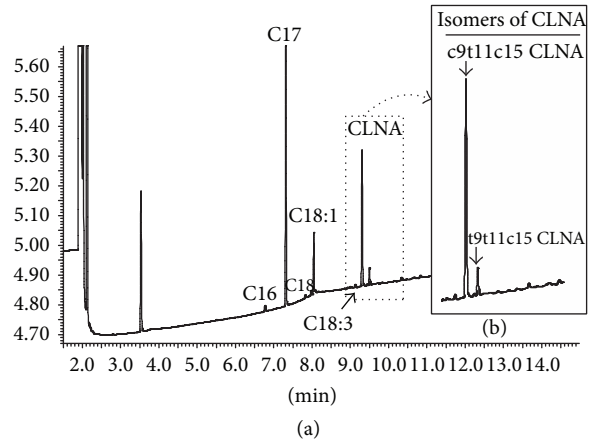

Figure 2: (a) Chromatogram profile assessed by gas chromatography of the fatty acid content present in the culture media obtained from *B. breve* M7-70 in MRS broth with 500 $\mu g\,mL^{-1}$ LNA as a substrate. (b) The insert shows a blow-up of the part of the chromatogram corresponding to the CLNA isomers.

(200 $\mu g\,mL^{-1}$) and the conversion rates (~100%) were similar to those observed in MRS-Cys broth (Table 4). Rumenic acid was also the dominant isomer, accounting for approximately 90% of the CLNA total amount.

Finally, the three selected bifidobacterial strains were tested for their ability to produce CLA and CLNA when both substrates (LA and LNA) were added to the milk-based medium. The three *B. breve* strains were able to simultaneously produce CLA and CLNA in these growth conditions (Figure 4). The CLA and CLNA concentrations produced were about 100 $\mu g\,mL^{-1}$ of CLA and 300 $\mu g\,mL^{-1}$ of CLNA (Figure 4). Interestingly, the production of both bioactive lipids was much higher when both substrates, LA and LNA, were present at the same time in the growing medium than when they were individually added (Table 4 and Figure 4).

Table 2: CLA and CLNA production ($\mu g\,mL^{-1}$) and conversion (%) of LA and LNA by the bifidobacterial strains when growing in MRS-Cys. Conversion calculated as $\sum CLA/(LA + \sum CLA) \times 100$ and $\sum CLNA/(LNA + \sum CLNA) \times 100$, respectively, based on the results of GC.

Strains	CLA	*cis 9 trans 11* (% CLA)	LA conversion (%)	CLNA	*cis 9 trans 11 cis 15* (% CLNA)	LNA conversion (%)
B. breve ZL12-28	170.6 ± 38.5 (a)	81.2 ± 0.8 (a)	74.6 ± 6.2 (a)	218.8 ± 39.0 (a)	82.7 ± 8.7 (a)	98.8 ± 0.6 (a)
B. breve 29M2	158.7 ± 48.3 (a)	85.6 ± 0.7 (b)	74.1 ± 7.0 (a)	211.6 ± 59.3 (a)	91.4 ± 3.2 (a)	95.7 ± 4.8 (a)
B. breve M7-70	170.3 ± 46.4 (a)	81.7 ± 1.0 (a)	77.8 ± 2.8 (a)	234.2 ± 81.5 (a)	85.7 ± 3.3 (a)	98.7 ± 0.9 (a)

Values are means of triplicate experiments and standard deviation (±SD).
Means in the same column with different lowercase letters are significant at $P < 0.05$.

Table 3: Growth of *B. breve* M7-70 in the presence of different concentrations of LA and LNA.

Concentration ($mg\,mL^{-1}$)	LA	LNA
0	+++*	+++
0.25	++	+
0.50	++	+
1.0	+	−
1.5	+	−
2.0	−	−

*(−) No growth; (+) slight growth; (++) moderate growth; (+++) optimal growth. All the bifidobacterial strains assayed showed the same level of inhibition.

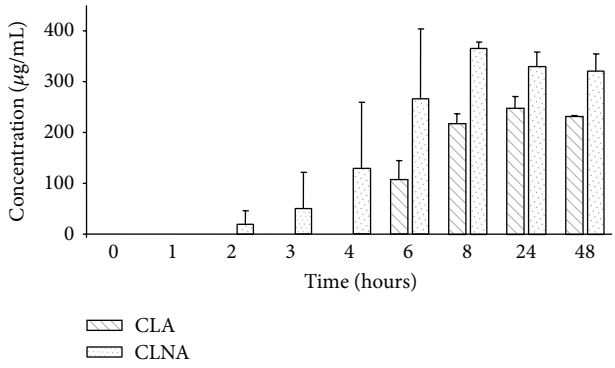

Figure 3: Comparative analysis of CLA versus CLNA production at different times. The *B. breve* M7-70 strain was incubated in MRS broth containing 500 $\mu g\,mL^{-1}$ free linoleic acid (LA) or 500 $\mu g\,mL^{-1}$ free linolenic acid (LNA) for 48 hours under anaerobic conditions. Samples were taken at the indicated times. Values are means ± SD of three independent experiments.

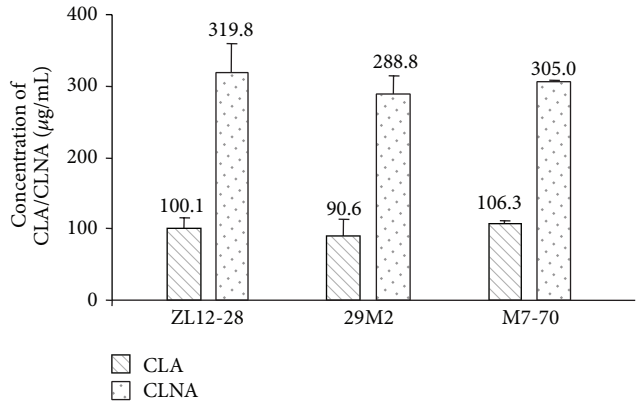

Figure 4: CLA/CLNA production by selected bacteria of screening. The cultures were incubated in reconstituted skim milk containing 500 $\mu g\,mL^{-1}$ free linoleic acid (LA) and 500 $\mu g\,mL^{-1}$ free linolenic acid (LNA) for 24 hours under anaerobic conditions. Values are means ± SD of three independent experiments.

4. Discussion

Bifidobacteria are numerically important members of the human gut microbiota and are believed to play a beneficial role in maintaining the health of the host. Some studies have suggested that infants with delayed bifidobacterial colonization and/or decreased bifidobacterial numbers may be more susceptible to a variety of gastrointestinal or allergic conditions [19, 20]. In these cases, the exogenous administration of selected bifidobacterial strains, alone or in combination with lactic acid bacteria, can reduce the incidence of such conditions [21–23]. Therefore, bifidobacteria are generally regarded as potentially probiotic microorganisms.

Recently, it has been shown that human milk is a source of live lactic acid bacteria and bifidobacteria to the infant gastrointestinal tract [24, 25]. If health benefits could be associated with bifidobacterial strains isolated from such biological fluid, then they would be immediately regarded as particularly attractive microorganisms since they would fulfil some of the main criteria generally recommended for human probiotics, such as human origin and adaptation to mucosal and dairy substrates [26, 27].

Since production of conjugated fatty acids by some bifidobacteria and lactic acid bacteria has been reported in the last years [1, 2, 12, 28], it is considered of interest to have human milk strains available with such capability. In this work, we describe the assayed conditions to select and characterize three *B. breve* strains with the ability to produce high amounts of CLA and CLNA during their growth in skim milk. It is not strange that the selection process ended with three strains belonging to the species *B. breve*, since this species seems to be particularly suited for production of these bioactive lipids [28–31]. The bioactive lipid production is a strain-specific attribute and, in fact, a non-CLA-producing *B. breve* strain (*B. breve* ZL12-22) was also found in this study.

It has been hypothesized that some bacteria can convert LA to CLA and LNA to CLNA as a detoxification mechanism

Table 4: CLA and CLNA production (μg mL^{-1}) and conversion (%) of LA and LNA by *B. breve* strains when growing in skim milk. Conversion calculated as $\sum$CLA/(LA + $\sum$CLA) × 100 and $\sum$CLNA/(LNA + $\sum$CLNA) × 100, respectively, based on the results of GC.

Strains	CLA	*cis 9 trans 11* (% CLA)	LA conversion (%)	CLNA	*cis 9 trans 11 cis 15* (% CLNA)	LNA conversion (%)
B. breve ZL12-28	75.0 ± 9.5 (a)	83.6 ± 5.3 (a)	31.3 ± 14 (a)	243.7 ± 39.8 (a)	93.6 ± 0.8 (a)	96.5 ± 1.2 (a)
B. breve 29M2	95.0 ± 12.4 (a)	87.0 ± 6.6 (a)	41.6 ± 11 (a)	219.8 ± 30 (a)	90.4 ± 4.2 (a)	94.0 ± 6.5 (a)
B. breve M7-70	75.9 ± 6.1 (a)	83.4 ± 4.1 (a)	29.6 ± 12 (a)	210.1 ± 23.8 (a)	90.9 ± 1.9 (a)	97.0 ± 1.4 (a)

Values are means of triplicate experiments and standard deviation (±SD).
Means in the same column with different lowercase letters are significant at $P < 0.05$.

[18, 28, 29, 32]. LA and LNA have antimicrobial activity and may alter the permeability of the plasmatic membrane of some Gram-positive bacteria [33]. It has been shown that both substrates have inhibitory effects on the growth of CLA and CLNA producing strains [28, 34]. Generally, free LNA is more toxic to bacteria than free LA, which is coincident with the results obtained in this work with *B. breve* M7-70. The hypothesis of the detoxification mechanism may explain why CLNA production by the three *B. breve* strains was more efficient than CLA production. It also could explain why the production of both CLA and CLNA was higher when both substrates were added together than when the strains were grown on each substrate separately (Table 4 and Figure 4).

At present, CLA is better characterised than CLNA. There are some research works that report the health-promoting properties of CLNA [1]. A recent study demonstrated the ability of a *B. breve* strain to produce CLNA and other conjugated fatty acids, such as conjugated γ-linolenic acid (CGLA) or conjugated stearidonic acid (CSA), from γ-linolenic acid and stearidonic acid, respectively [35]. Another work reported that *B. breve* NCIMB 702258 displayed a high conversion rate (79%) of α-linolenic acid into the *cis 9 trans 11 cis 15* CLNA isomer. This isomer can inhibit the growth of SW480 colon cancer cells [31]. All these mentioned works have described the CLNA production during growth of the strains in MRS broth. In contrast, the results of our study indicate that the selected *B. breve* strains were able to produce significant amounts of CLNA (200 μg mL^{-1}) in skim milk, with a conversion rate of LNA close to 100%. In addition, this study has demonstrated that *B. breve* strains were able to coproduce high levels of CLA and CLNA during growth in skim milk. Coproduction of CLA and CLNA in the described concentrations would be of relevance to increase the amount of these bioactive lipids in fermented milks. Globally, these results suggest that the three selected *B. breve* strains have a strong potential to be used as probiotics in fermented milks in order to increase human intake of CLA and CLNA. Further studies to optimize the culture conditions for increasing CLA and CLNA production at large-scale by fermentation processes would be needed. Additional studies will be also required to further elucidate the relevance of consumption of these bioactive lipids in human health.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Research Article

Development of a Potential Probiotic Fresh Cheese Using Two *Lactobacillus salivarius* Strains Isolated from Human Milk

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Cheeses have been proposed as a good alternative to other fermented milk products for the delivery of probiotic bacteria to the consumer. The objective of this study was to assess the survival of two *Lactobacillus salivarius* strains (CECT5713 and PS2) isolated from human milk during production and storage of fresh cheese for 28 days at 4°C. The effect of such strains on the volatile compounds profile, texture, and other sensorial properties, including an overall consumer acceptance, was also investigated. Both *L. salivarius* strains remained viable in the cheeses throughout the storage period and a significant reduction in their viable counts was only observed after 21 days. Globally, the addition of the *L. salivarius* strains did not change significantly neither the chemical composition of the cheese nor texture parameters after the storage period, although cheeses manufactured with *L. salivarius* CECT5713 presented significantly higher values of hardness. A total of 59 volatile compounds were identified in the headspace of experimental cheeses, and some *L. salivarius*-associated differences could be identified. All cheeses presented good results of acceptance after the sensory evaluation. Consequently, our results indicated that fresh cheese can be a good vehicle for the two *L. salivarius* strains analyzed in this study.

1. Introduction

Among all dairy products, cheese has the highest consumption rate worldwide because of its versatility. Fresh cheeses are usually not or minimally aged, have high moisture content, do not have a rind, and got very mild flavour and a soft and smooth texture. In this category, milk coagulation is due to rennet and/or acid produced from a bacterial culture or other sources such as lemon juice. When bacteria are involved in their manufacture, they also contribute to develop typical flavours, to improve quality, and/or to promote health benefits if they display probiotic properties [1].

Probiotics are defined as "live microorganisms which when administered in adequate amounts confer a health benefit on the host" [2], being *Lactobacillus* and *Bifidobacterium* the most frequently used genera [3]. Yogurt and fermented milks are the most common foods for delivery of probiotic bacteria, but some studies have found that their characteristics may compromise the viability of the probiotic strains [4–6]. Cheese may offer several advantages as a probiotic carrier due to its higher pH and fat content and harder consistency compared to fermented milks [7]. These features provide more protection to probiotics not only during cheese production, ripening, and storage, but also during the passage through the gastrointestinal tract, allowing bacteria to arrive in higher numbers at the target site after ingestion [7].

Several studies have confirmed that human milk is a source of live bacteria, mainly staphylococci and streptococci, but also contains lactic acid bacteria and bifidobacteria [8–11]. The lactobacilli species more frequently isolated from milk samples of healthy women are *Lactobacillus casei*, *Lactobacillus fermentum*, *Lactobacillus gasseri*, *Lactobacillus gastricus*, *Lactobacillus plantarum*, *Lactobacillus reuteri*, *Lactobacillus*

salivarius, and *Lactobacillus vaginalis* [12]. Some lactobacilli isolated from human milk have been characterized and shown to have probiotic potential [13–15]. Specifically, *L. salivarius* CECT5713 that was isolated from human milk and infant feces of a healthy mother-child pair has been shown to have remarkable probiotic potential because it had high rate of survival in simulated gastrointestinal tract conditions and strong adherence to mucus and intestinal cells *in vitro*, stimulated the expression of mucin-encoding genes, and produced antimicrobial compounds [14–17]. More recently, its complete genome has been sequenced [18], and its genetic features, such as proteins potentially involved in human molecular mimetism, may explain its immunomodulatory, anti-inflammatory, and anti-infectious properties [19, 20]. Moreover, its safety and health beneficial effects have been proved in animal models and in human clinical assays [20–23]. More recently, *L. salivarius* PS2 has also been isolated from human milk and preliminary assays have shown similar traits and probiotic potential.

The aim of this work was to evaluate the performance of these two human milk *L. salivarius* strains (CECT5713 and PS2) in fresh cheese in order to develop a probiotic cheese. The survival of these two *L. salivarius* strains in the cheese has been studied as well as their impact on chemical composition, volatile compounds, texture and other organoleptic properties, and overall consumer acceptance of the experimental cheeses.

2. Materials and Methods

2.1. Starter and Probiotic Organisms. *Lactococcus lactis* ESI153, originally isolated from artisanal raw milk cheese [24], was selected to be used as starter culture. *Lc. lactis* ESI153 cells were grown in M17 (Oxoid, Basinstoke, UK) broth supplemented with 0.5% (wt/vol) glucose (GM17) at 32°C. Before use, *Lc. lactis* ESI153 cells were subcultured (1%) into reconstituted at 11% (wt/vol) and heat-treated (121°C, 5 min) nonfat dry milk (HT-NFDM) and incubated overnight at 32°C.

Freeze-dried cultures of probiotic *L. salivarius* CECT5713 and PS2 were prepared as follows. A fully grown liquid culture on de Man, Rogosa, and Sharpe (MRS) (Oxoid) broth was centrifuged at 10000 ×g for 10 min at 4°C. The cell pellet was washed with 0.85% (wt/vol) NaCl and resuspended in HT-NFDM to one tenth of its original volume. The cell suspension was frozen at −80°C for 12 h in metal trays. Freeze drying was carried out at, first, 0°C for 24 h and, then, at 20°C for 24 h under 1.3 Pa in a Lyph-Lock Stoppering Tray Dryer model 77560 (Labconco Corporation, Kansas City, MO, USA). Freeze-dried cultures containing approximately 10.3 $\log_{10}$ colony forming units (cfu)/g were vacuum packaged and stored at 4°C before use.

2.2. Experimental Cheese Manufacture. Cheeses were made from commercial pasteurized (high temperature short time, HTST) cow's milk (Ganadería Priégola SA, Villanueva del Pardillo, Madrid, Spain) following a laboratory-scale procedure described previously by Rodríguez et al. [25] and Reviriego et al. [26] with some modifications (Figure 1). Briefly, pasteurized milk (1.5 L/vat) at 32°C with 0.01% (wt/vol) $CaCl_2$ was inoculated with *Lc. lactis* ESI153 (approximately 9 $\log_{10}$ cfu/mL) as starter culture. Rennet (Fromase, 44 IMCU/L; DSM Food Specialities, Seclin Cedex, France) was added to milk 30 min after the inoculation of *Lc. lactis* ESI153. Curds were cut 40 min after rennet addition and heated at 38°C for 40 min. Whey was drained off and freeze-dried *L. salivarius* CECT5713 or PS2 were added to the curd, to reach a final concentration of ~8 $\log_{10}$ cfu/g before the curds were distributed into the molds. Control cheese was manufactured at the same conditions with the addition of the equivalent amount of HT-NFDM used as the excipient for freeze drying the lactobacilli strains. Cheeses were pressed for 16 h at room temperature and salted in 15% brine (wt/vol) during 3 h. The resulting cheeses (~190 g) were cut into pieces, which were individually vacuum-packed in Cryovac plastic bags and kept refrigerated at 4°C during 28 days. All cheese manufacturing trials were made in triplicate.

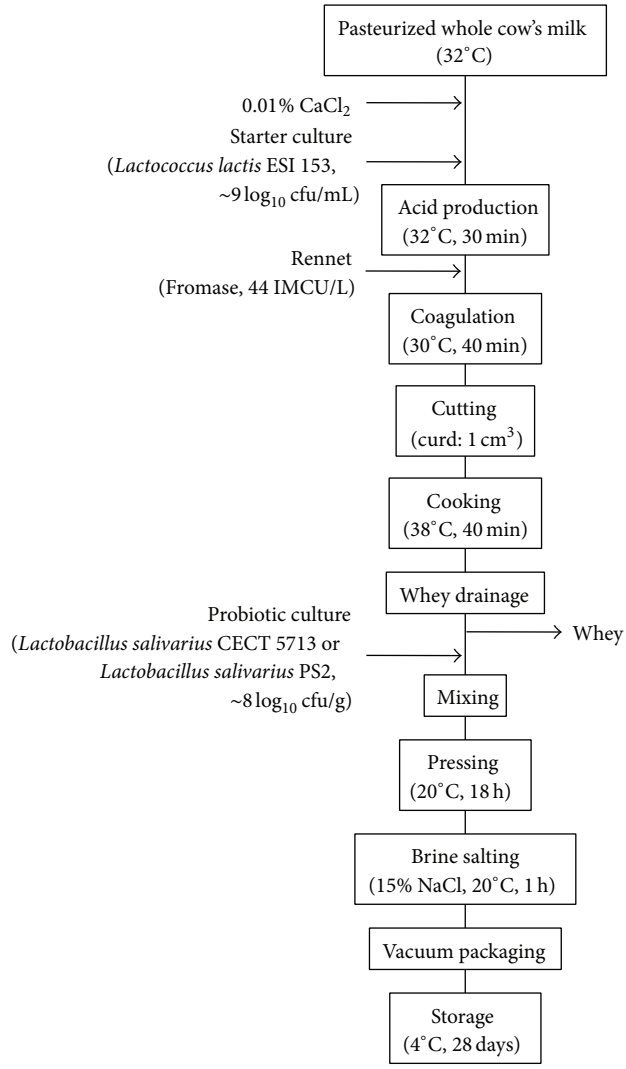

Figure 1: Flowchart of the cheese making process.

2.3. Gross Composition, pH, and Water Activity in Cheese. Cheese samples were analyzed for moisture (ISO 5534/IDF 004:2004), fat (ISO 1735/IDF 005:2004), protein (ISO 8968-1/IDF 020-1:2014), and ash content (AOAC 935.42). The pH value of a cheese slurry prepared by blending 20 g of grated cheese with 12 mL of water [27] was measured with a pH meter (Crison Digit-501). The water activity (a_w) was determined with a Decagon CX-1 hygrometer (Decagon, Pullman, Washington, USA). Determinations were made on triplicate samples.

2.4. Viable Bacterial Counts in Cheese. Viability of the *L. salivarius* strains was monitored in cheese samples at 0, 7, 14, 21, and 28 days at 4°C. For this purpose portions (10 g) of cheese were blended with 100 mL of 0.1% (wt/vol) sterile peptone water in a stomacher. Serial dilutions were made also in sterile peptone water and plated following the surface plate technique in appropriate media. *L. salivarius* strains were enumerated on de Man, Rogosa and Sharpe (MRS, Oxoid) agar containing 0.002% (wt/vol) of bromophenol blue after 24 h at 37°C under aerobic conditions. *Lc. lactis* ESI153 was enumerated on M17 (Oxoid) agar supplemented with 0.5% (wt/vol) glucose (GM17) after 24 h at 32°C under aerobic conditions. To confirm their identity, selected colonies were observed by optical microscopy to check their morphology and Gram staining and typed by Random Amplification of Polymorphic DNA (RAPD) using primer OPL5 (5′-ACG CAG GCA C-3′), as described by Ruiz-Barba et al. [28]. Ten randomly chosen isolates sharing the same RAPD profile (only two different RAPD profiles were obtained) were subjected to Pulse Field Gel Electrophoresis (PFGE) after digestion with *Sma*I following the procedure described by Martín et al. [12]. The absence of *Enterobacteriaceae* and *Bacillus cereus* in cheese samples was assessed by pouring onto MacConkey and PEMBA agar (BioMérieux, Marcy l'Etoile, France), respectively, and incubation in aerobically conditions at 37°C for up to 48 h. Bacterial counts were recorded as the cfu/g of cheese and transformed to $\log_{10}$ values before statistical analysis.

2.5. Isolation of Bacterial DNA and PCR-DGGE Analysis. Cheeses samples (5 g) were homogenized into sodium citrate (50 mL) using a stomacher (260 rpm × 1 min). Then, an aliquot of the mixture (10 mL) was centrifuged at 19,000 ×g during 5 min. The resulting pellet was used for the isolation of total bacterial DNA from each cheese sample following the protocol described previously by Moles et al. [29]. DNA yield was measured using a NanoDrop ND 1000 UV spectrophotometer (Nano-Drop Technologies, Wilmington, Delawere, USA) and was stored at −20°C until PCR DGGE analysis.

Primers U968-GC-f (5′-CGC CCG GGG CGC GCC CCG GGC GGG GCG GGG GCA CGG GGG GAA CGC GAA GAA CCT TAC-3′) and L1401-r (5′-CGG TGT GTA CAA GAC CC-3′) [30] and Lab159f (5′-GGA AAC AGG TGC TAA TAC CG-3′) and Uni-515-GCr (5′-CGC CCG GGG CGC GCC CCG GGC GGG GCG GGG GCA CGG GGG GAT CGT ATT ACC GCG CTG CTG GCA C-3′) [31] were used to amplify V6–V8 regions from 16S rRNA genes on bacterial DNA. The PCR reaction was performed in a total reaction volume of 50 μL containing 5× My Taq Red reaction buffer (Bioline, London, UK), My Taq Red DNA polymerase (Bioline), and 10 g/mL of the isolated DNA. The amplification program was as follows: 95°C for 2 min, 35 cycles of 95°C for 30 s, 56°C for 40 s, 72°C for 60 s, and then 72°C for 5 min. PCR products were stored at −20°C until use.

PCR fragments were separated by denaturing gradient gel electrophoresis (DGGE) using a DCode System (BioRad Laboratories, Inc., Hercules, California, USA) and gels with a linear denaturant gradient of 30 to 50% as described by Martín et al. [32]. A DNA mixture made with equal amounts of amplicons from *L. salivarius* CECT5713 or PS2 and *Lc. lactis* ESI153 was used as a marker.

2.6. Analysis of Cheese Texture. Texture profile analysis (TPA) of the cheeses was performed in a texturometer TA-XT2i (Stable Micro Systems Ltd., Surrey, UK). The texturometer was provided with a 0.2 N load cell and a 20 mm diameter probe at a crosshead speed of 5 mm/s to perform a uniaxial compression test in two consecutive compressions. Cheese samples were prepared by cutting 2 cm^3 cubes, which were kept during 1 h at 25°C before performing the assay. The cheese cube was placed between the two parallel plates and compressed to 50% of its original height sample. TPA parameters (hardness, cohesiveness, adhesiveness, chewiness, gumminess, and springiness) were determined from the TPA two-compression force-time curve with the aid of the Texture Expert for Windows software, version 1.20 (Stable Micro Systems). All measurements were made in triplicate.

2.7. Colour Analysis. The colour of cheese samples was determined with a tristimulus colour analyzer (Minolta Chroma Meter CR300, Minolta Corporation, Ramsey, NJ, USA) that measures reflective colours. The measurement was made both on the surface and the core of cheese samples, and the results were expressed using the CIE $L^*a^*b^*$ (CIELAB) space. This three-dimensional model describes all the colours visible to the human eye by means of three spatial coordinates: a central vertical axis that represents lightness (L^*) in which values run from 0 (black) to 100 (white); a second perpendicular axis (a^*) that represents the red-green channel, where positive values indicate red and negative values indicate green; and the third perpendicular axis (b^*) that represents the opponent yellow-blue channel where positive values indicate yellow and negative values indicate blue. Therefore, each colour can be represented as a point in a three-dimensional space defined by its three parameters L^*, a^*, and b^*. All measurements were made in triplicate.

2.8. Analysis of Volatile Compounds. Cheese samples were wrapped in aluminium foil, vacuum-packed in Cryovac plastic bags, and frozen at −80°C until analysis. Volatile compounds were extracted by headspace solid-phase microextraction (SPME) and, then, analysed by gas chromatography-mass spectrometry GC-MS (HP6890-MSD HP 5973, Agilent, Palo Alto, CA, USA) according to the procedure described by

Lee et al. [33]. Cheese samples (10 g) were homogenized with anhydrous sodium sulphate (20 g) and 20 μL of an aqueous solution containing cyclohexanone (1058 ppm) and camphor (1040 ppm) as internal standards using a mechanical grinder. Then, 5 g of this mixture was weighed in a 40 mL glass vial that was sealed with a polytetrafluoroethylene (PTFE) faced silicone septum. Volatile compounds were isolated using a SPME manual holder equipped with a 2 cm × 50/30 μm Stable Flex Divinylbenzene/Carboxen/Polydimethylsiloxane (DVB/CAR/PDMS) coated fibre (Supelco, Bellefonte, Pennsylvania, USA). Vials were equilibrated in a thermostatic bath at 37°C for 20 min before the fiber was inserted through the PTFE septum for headspace extraction. The fiber was exposed to the headspace for 30 min, and then it was inserted into the GC injection port for desorption (260°C/10 min in splitless mode).

Separation of volatile compounds was performed on a Zebron ZB-WAX plus (60 m × 0.25 mm × 0.50 μm) capillary column coated with 100% polyethylene glycol (Phenomenex, Torrance, California, USA). For chromatographic separation, the temperature was maintained at 40°C for 7 min, increased from 40°C to 90°C at a rate of 2°C/min, from 90°C to 150°C at a rate of 3°C/min, from 150°C to 240°C at a rate of 9°C/min and, finally, held at 240°C for 8 min. Detection was performed with the mass selective detector operating in the scan mode, collecting data at a rate of 5.16 scans/s over a range of 33–300 m/z at ionization energy of 70 eV. Identification of volatile compounds was based on comparison of spectra using the Wiley 275 Library (Wiley and Sons Inc., New York, USA). Relative abundances of compounds were expressed as percentages of their peak areas to the cyclohexanone peak area. Samples were tested in duplicate.

2.9. Sensory Evaluation. The sensory evaluation of cheeses was done by panellists (staff and students of the Department of Food Science and Nutrition, Universidad Complutense de Madrid) who were familiar with sensory evaluation techniques. The evaluation was performed in individual booths under controlled conditions of environment and light.

Representative cheese samples (~20 g) after 28 d of storage at 4°C were equilibrated at room temperature and presented to the tester in disposable plastic containers, except for odour assessment for which samples were presented in closed glass flasks. Samples were codified with random 3 digit numbers following a completely randomized block design. Cheese portions from replications of the same batch were mixed, so a representative sample was presented to the panellists.

Initially, 30 tester semitrained panellists participated in a triangle test to determine if potential probiotic cheeses containing lactobacilli differ in any aspect from the control cheese. Significant differences were determined using the method of Roessler et al. [34]. Later on, 18 selected trained panellists were asked to perform a descriptive test for a number of specific descriptors clustered in groups related to odour (buttery, cow, fermented milk, floral, fruity, lawn, rancid, and vinegar), flavour and taste (aftertaste, astringent, bitter, cow, fermented milk, fruity, salty, and vinegar), and texture and appearance (adhesive, bright, colour (white or yellow), creamy, friable, hardness, moist, springy, and smooth) and to score for the overall impression using a 10-point intensity scale.

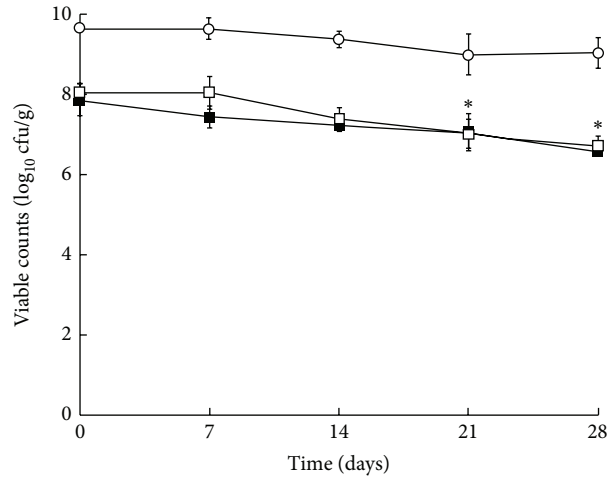

Figure 2: Viable counts ($\log_{10}$ cfu/g) of starter *Lc. lactis* ESI153 (○) and *L. salivarius* CECT5713 (□) and PS2 (■) in fresh cheese during storage at 4°C.

2.10. Statistical Analysis. The influence of the addition of *L. salivarius* CECT5713 and PS2 to cheeses was analyzed by one way analysis of variance (ANOVA). Tukey's multiple range tests were applied to determine differences among the cheeses. Differences were considered significant at $P < 0.05$. StatGraphics Centurion XVI version 16.1.15 (Statpoint Technologies Inc., Warrenton, Virginia, USA) was used to perform these analyses.

3. Results

3.1. Gross Composition, pH, and Water Activity. The mean composition of control cheeses without the addition of probiotic bacteria was 24.5% (wt/wt) fat, 17.8% (wt/wt) protein, and 3.1% (wt/wt) ash after 28 days of storage at 4°C (Table 1). Chemical composition of cheeses manufactured with *L. salivarius* CECT5713 or PS2 was similar; slightly higher moisture and lower fat and protein contents were found compared to control cheeses, although the difference was statistically significant only for the protein content. The presence of lactobacilli in cheeses was associated with a lower final pH compared to control cheeses, but it did not modify the a_w of the final product (0.96-0.97).

3.2. Viability of L. salivarius during Cheese Storage. The growth of *L. salivarius* CECT5713 and PS2 in MRS agar containing bromophenol produced large and blue colonies, while in the same conditions *Lc. lactis* ESI153 colonies were small and white. Initial viable counts in MRS agar of *L. salivarius* CECT5713 and PS2, according to the morphology of the colony, were 8.1 and 7.9 $\log_{10}$ cfu/g, respectively (Figure 2). Both *L. salivarius* strains remained viable in the cheeses after

Table 1: Chemical composition, pH, and water activity of control cheese and cheeses using *L. salivarius* CECT5713 or PS2 after 28 days of storage at 4°C*.

	Cheese			P value**
	Control	*L. salivarius* CECT5713	*L. salivarius* PS2	
Moisture (%, wt/wt)	52.48 ± 2.14	56.17 ± 1.95	56.62 ± 1.96	0.086
Fat (%, wt/wt)	24.47 ± 1.30	20.88 ± 1.84	22.37 ± 1.27	0.067
Protein (%, wt/wt)	17.75 ± 0.80[a]	14.94 ± 0.79[b]	15.43 ± 0.85[b]	0.012
Ash (%, wt/wt)	3.10 ± 0.11	3.28 ± 0.45	2.88 ± 0.10	0.278
pH	4.86 ± 0.13	4.71 ± 0.03	4.75 ± 0.11	0.287
a_w	0.96 ± 0.01	0.96 ± 0.01	0.97 ± 0.01	0.398

*Results are expressed as mean ± standard deviation values of triplicate samples; **one-way ANOVA to determine differences on chemical composition, pH, and a_w between cheeses. [a,b]Mean values within the same row followed by different letter were significantly different when compared using the Tukey's test.

Table 2: Texture profile analysis of control and experimental cheeses using *L. salivarius* CECT5713 or PS2 after 28 days of storage at 4°C*.

Parameter	Cheese			P value**
	Control	*L. salivarius* CECT5713	*L. salivarius* PS2	
Hardness (N)	21.00 ± 0.71[a]	24.42 ± 0.97[b]	21.27 ± 0.65[a]	0.001
Cohesiveness	0.15 ± 0.03	0.15 ± 0.01	0.14 ± 0.01	0.662
Adhesiveness (N s)	−0.57 ± 0.30	−0.77 ± 0.09	−0.57 ± 0.04	0.236
Springiness (m)	0.0048 ± 0.0015	0.0043 ± 0.0017	0.0048 ± 0.0010	0.851
Gumminess (N)	3.22 ± 0.64	3.61 ± 0.23	2.99 ± 0.29	0.171
Chewiness (N m)	0.015 ± 0.004	0.014 ± 0.005	0.013 ± 0.001	0.927

*Texture parameters are expressed as mean ± standard deviation values of quadruplicate measurements in triplicate samples; **one-way ANOVA to determine differences on texture parameters between cheeses. [a,b]Mean values within the same row followed by different letter were significantly different when compared using the Tukey's test.

28 days at 4°C (Figure 2). The probiotic counts decreased during storage, but the reduction in viable counts was significant only after 21 days. Final concentrations of *L. salivarius* CECT5713 andPS2 were 6.7 and 6.6 $\log_{10}$ cfu/g, respectively, representing about a 1.3 $\log_{10}$-unit reduction at the end of 28-day storage. The identity of selected colonies isolated at the end of the storage period was confirmed by RAPD and PFGE (results not shown). On the other hand, initial viable counts of the starter culture *Lc. lactis* ESI153 were higher (about 9 $\log_{10}$ cfu/g in all cheese samples) and remained fairly constant along the storage period. Bacterial growth was not detected on PEMBA and MacConkey agar plates, confirming the absence of contamination with *B. cereus* and *Enterobacteriaceae*.

3.3. PCR DGGE Analysis. DGGE analysis was also performed in order to check the bacterial diversity and to confirm the presence of the probiotic strains' DNA in the cheese samples (Figure 3). The amplification of the V6–V8 variable region of the 16S rRNA gene of *L. salivarius* CECT5713 and *Lc. lactis* ESI153 using the primers U968-GC-f and L1401-r resulted in a single fragment differing in size for each bacterial species (Figure 3(a)). The same primer pair did not amplify any fragment when *L. salivarius* PS2 DNA was used as template. However, amplification of one fragment corresponding to the V6–V8 variable region of the 16S rRNA gene of *L. salivarius* PS2 was successful when *Lactobacillus*-specific primers (Lab159f and Uni-515-GCr) were used (Figure 3(b)). The DGGE profile obtained from *L. salivarius* PS2 using this pair of primers comprised 3 dominant bands and it was different from the one obtained for *Lc. lactis* ESI153.

The DGGE profiles of cheese samples analyzed and the ladders constructed in this study (with amplicons obtained from pure cultures of *Lc. lactis* ESI153 and the corresponding *L. salivarius* strain), using universal or *Lactobacillus*-specific primers, were identical (Figure 3). This result indicates that the inoculated strains were the predominant in the respective cheeses during storage.

3.4. Textural Analysis of Cheeses. Texture parameters at the end of the cheese storage were similar in control and *L. salivarius*-containing cheeses (Table 2). No significant differences were observed in cohesiveness, adhesiveness, springiness, gumminess, and chewiness values. Globally, cheeses had a crumbly and brittle texture requiring only a relatively small load to fracture. However, cheeses manufactured with *L. salivarius* CECT5713 presented significantly higher values of hardness compared to control cheeses and cheeses containing *L. salivarius* PS2.

3.5. Colour Analysis. The colour was measured both on the surface and the core of cheese samples by tristimulus reflectance measurement (Table 3). In general, all samples had high lightness (L^* ~ 92 to 94), indicating no differences in the mechanical openings exhibited by the three cheeses, low yellow (b^* ~ 11 to 12), and very low green (a^* ~ −1 to −2) colour. Globally, the lightness value was lower in the surface than in the interior, possibly reflecting a closer structure in

Table 3: Colour parameters (L^*, a^*, and b^*) of control and experimental cheeses manufactured with *L. salivarius* CECT5713 or PS2 after 28 days of storage at 4°C[†].

Parameter	Cheese			P value[††]
	Control	*L. salivarius* CECT5713	*L. salivarius* PS2	
Surface				
L^*	92.20 ± 0.90	92.67 ± 0.56	92.41 ± 0.12	0.664
a^*	−1.58 ± 0.02[a]	−1.16 ± 0.04[b]	−1.33 ± 0.11[c]	0.001
b^*	11.66 ± 0.41[a]	10.74 ± 0.16[b]	11.92 ± 0.26[a]	0.006
Core				
L^*	93.13 ± 0.57	93.29 ± 0.08	93.81 ± 0.46	0.202
a^*	−1.44 ± 0.09[a]	−1.48 ± 0.12[ab]	−1.70 ± 0.04[b]	0.024
b^*	10.75 ± 0.17	10.69 ± 0.21	10.92 ± 0.34	0.521

[†]Colour parameters are expressed as mean ± standard deviation values of quadruplicate measurements in triplicate samples; [††]one-way ANOVA to determine differences on colour parameters between cheeses. [a,b,c]Means values within the same row followed by different letter were significantly different when compared using the Tukey's test.

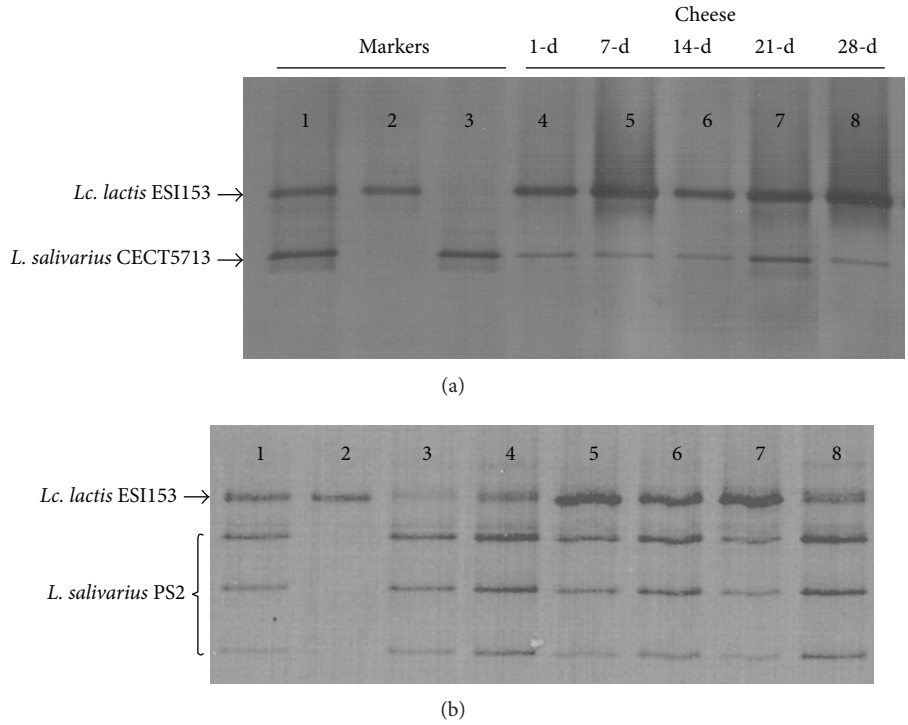

Figure 3: DGGE profiles of 16S rRNA gene V6–V8 regions obtained from samples of cheese manufactured with *L. salivarius* CECT5713 using universal primers U968-GC-f and L1401-r (a) and *L. salivarius* PS2 using *Lactobacillus*-specific primers Lab159f and Uni-515-GCr (b) during storage at 4°C. (a): lane 1: marker (*L. salivarius* CECT5713 and *Lc. lactis* ESI153); lane 2: *Lc. lactis* ESI153; lane 3: *L. salivarius* CECT5713; lane 4: 1-day cheese; lane 5: 7-day cheese; lane 6: 14-day cheese; lane 7; 21-day cheese; lane 8: 28-day cheese. (b) Lane 1: marker (*L. salivarius* PS2 and *Lc. lactis* ESI153); lane 2: *Lc. lactis* ESI153; lane 3: *L. salivarius* PS2; lane 4: 1-day cheese; lane 5: 7-day cheese; lane 6: 14-day cheese; lane 7; 21-day cheese; lane 8: 28-day cheese.

the surface and more open pores in the interior. The opposite was observed for b^* parameter, indicating more yellowness in the surface. The surface of cheeses elaborated with *L. salivarius* CECT5713 was whiter and had less intense green colour than the others (Table 3).

3.6. Volatile Analysis. A total of 59 volatile compounds were identified in the headspace of experimental cheeses, including aldehydes, ketones, alcohols, esters, alkanes, and carboxylic and fatty acids (Table 4). All cheese samples presented high relative abundance of the alcohols 3-methyl-1-butanol

Table 4: Volatile compounds in control cheese and cheeses containing *L. salivarius* CECT5713 or PS2 after 28 days at 4°C*.

Volatile compound	RT**	Cheese			*P* value***
		Control	*L. salivarius* CECT5713	*L. salivarius* PS2	
Aldehydes					
3-Methylbutanal	12.42	2.82 ± 0.15[a]	0.98 ± 0.14[b]	1.62 ± 0.25[c]	0.000
Hexanal	23.21	0.64 ± 0.24	0.50 ± 0.07	0.56 ± 0.06	0.552
Ketones					
2-Butanone	8.01	3.66 ± 0.28	2.25 ± 0.39	2.64 ± 0.86	0.101
2-Propanone	11.58	16.85 ± 1.99[a]	15.00 ± 1.36[a]	21.39 ± 1.08[b]	0.001
2-Heptanone	30.78	4.02 ± 0.16[a]	1.89 ± 0.29[b]	2.09 ± 0.09[b]	0.000
3-Hydroxy-2-butanone	38.08	2.14 ± 0.36	1.91 ± 1.46	0.98 ± 0.31	0.413
Alcohols					
Ethanol	13.76	45.05 ± 2.86	47.95 ± 3.96	50.79 ± 3.01	0.212
3-Methyl-1-butanol	32.89	348.48 ± 6.49	358.80 ± 12.98	368.84 ± 20.33	0.375
2-Furanmethanol	55.59	0.87 ± 0.06	0.11 ± 0.05	0.52 ± 0.49	0.217
3-Methyl-3-buten-1-ol	35.71	1.04 ± 0.03	1.04 ± 0.10	1.18 ± 0.14	0.138
Esters					
Ethyl acetate	10.93	0.71 ± 0.01	0.65 ± 0.20	0.69 ± 0.15	0.905
Ethyl butanoate	20.02	0.55 ± 0.19	1.00 ± 0.22	1.06 ± 0.21	0.110
Ethyl hexanoate	34.41	0.34 ± 0.03	0.51 ± 0.11	0.54 ± 0.15	0.221
Alkanes					
2-Methylpentane	4.33	0.31 ± 0.04	0.35 ± 0.06	0.50 ± 0.17	0.200
3-Methylpentane	4.44	0.25 ± 0.02	0.34 ± 0.06	0.47 ± 0.15	0.073
Hexane	4.51	1.82 ± 0.36	1.82 ± 0.46	1.80 ± 0.64	0.998
Heptane	5.40	0.72 ± 0.01	0.65 ± 0.19	0.40 ± 0.09	0.064
2,4-Dimethylheptane	7.55	1.32 ± 0.05[a]	0.40 ± 0.09[b]	0.40 ± 0.15[b]	0.001
4-Methyloctane	9.30	0.83 ± 0.04	0.20 ± 0.03	0.28 ± 0.18	0.113
Carboxylic and fatty acids					
Acetic acid	46.78	22.76 ± 1.02[a]	42.61 ± 2.37[b]	33.53 ± 8.62[ab]	0.016
Propanoic acid	50.93	0.07 ± 0.00	0.08 ± 0.00	0.08 ± 0.00	
Butanoic acid	54.47	20.36 ± 1.80	28.23 ± 2.99	26.15 ± 6.26	0.209
Hexanoic acid	59.58	14.82 ± 0.99	22.49 ± 3.51	20.04 ± 4.14	0.110
Octanoic acid	62.89	7.42 ± 2.30	13.80 ± 3.86	10.85 ± 2.04	0.133
Heptanoic acid	61.33	0.48 ± 0.01	0.62 ± 0.09	1.08 ± 0.44	0.083
Nonanoic acid	64.46	1.35 ± 0.19	0.73 ± 0.26	6.58 ± 8.90	0.362
Decanoic acid	66.21	3.15 ± 1.89	5.66 ± 3.18	3.29 ± 1.27	0.401
Others					
D-Limonene	31.78	0.74 ± 0.02	1.18 ± 0.13	0.72 ± 0.47	0.163

*Relative abundance of compounds was expressed as percentages of their peak areas to the cyclohexanone peak area; **RT: retention time; ***one-way ANOVA to determine differences on the relative abundance of volatile compounds between cheeses. [a,b,c]Mean values within the same row followed by different letter were significantly different when compared using the Tukey's test.

and ethanol as well as of acetic, butanoic, and hexanoic acids, although significant differences were observed only for acetic acid that had higher abundance in cheeses containing *L. salivarius* CECT5713. Aldehyde 3-methylbutanal, ketone 2-heptanone, and alkane 2,4-dimethylheptane were present at statistically significant lower levels in cheeses manufactured with *L. salivarius* than in control cheese. Level of 2-propanone was higher in cheese made with *L. salivarius* PS2 than in the other two cheeses.

3.7. Sensory Evaluation. The results of the triangle test to evaluate differences in sensory properties indicated that significant differences were detected at the end of the storage. The panellists could appreciate significant variations between control cheese and cheese manufactured with *L. salivarius* CECT5713 ($P = 0.018$) or PS2 ($P = 0.002$).

Trained panellists performed a quantitative descriptive analysis using attributes describing odour, flavour, taste, texture, and appearance of cheeses after 28 days of storage at 4°C

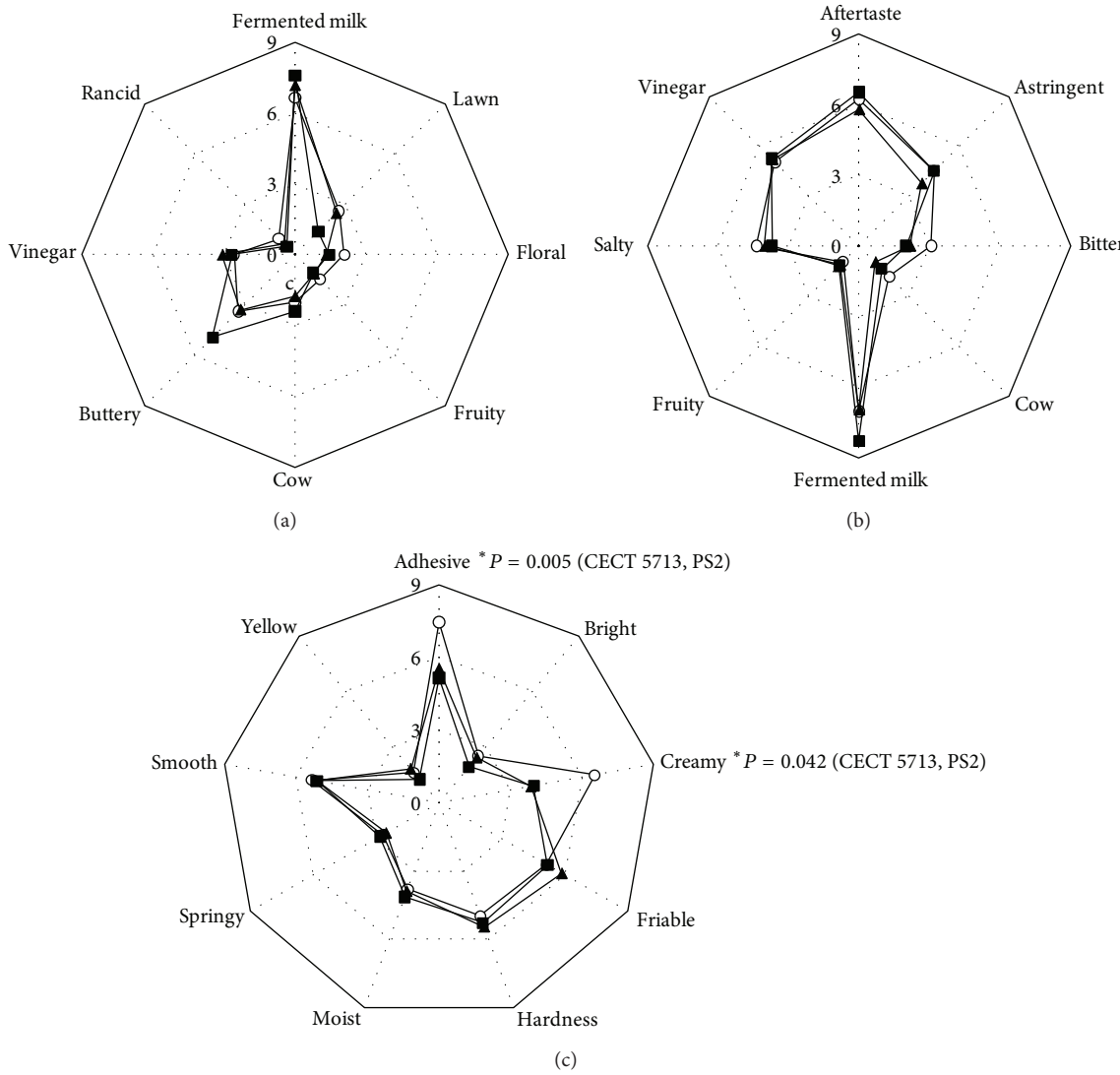

FIGURE 4: Graphical charts of the sensory profile of control cheese (○) and cheeses containing *L. salivarius* CECT5713 (■) and PS2 (▲) after 28 days at 4°C. (a) Odour related attributes. (b) Flavour and taste related attributes. (c) Texture and appearance descriptors.

(Figure 4). Globally, the descriptors that obtained the highest scores were fermented milk taste and smell and butter-like smell. Among all attributes, only the intensity of adhesiveness and creaminess in control cheese was statistically higher than that of the cheeses containing *L. salivarius* CECT5713 or PS2. However, these differences did not have any statistically significant effect on the overall quality of the cheeses ($P < 0.05$), and all cheese samples presented good results of acceptance after 28 days of storage at 4°C. In average, the acceptance level of odour, flavour, appearance, and texture, as well as the global score, was up to 6 (on a 0–10 numeric rating scale) in the three types of cheese.

4. Discussion

Cheese has been considered as an excellent alternative to fermented milk and yogurts as a food vehicle for probiotic delivery. Its buffering capacity is one of its advantages because it protects probiotics against the highly acidic stomach environment. The structure of the gel and its high fat content and solid consistency also add to the probiotic protection [35, 36]. Several studies have demonstrated that cheese is an excellent carrier for probiotic bacteria, including fresh and Cheddar cheese varieties [5, 27, 36–38]. However, variable results have been obtained with different probiotic strains and each strain should be tested individually. Therefore, the objective of this study was to check the viability of two lactobacilli strains isolated from human milk after their incorporation to cheese curds and to test their impact in the final product.

Theoretically, the probiotic bacteria could be added either directly to milk and/or incorporated at a later stage during the manufacture of cheese. Ong et al. [36] manufactured probiotic Cheddar cheese containing different combinations of six probiotic *Lactobacillus* and *Bifidobacterium* strains which were cocultured with the cheese starter culture. They reported some loss of probiotic lactobacilli and bifidobacteria in whey

(about 6-7 $\log_{10}$ cfu/g), but final counts in all fresh cheeses were high and acceptable (8-9 $\log_{10}$ cfu/g). In preliminary trials, following our procedure, *L. salivarius* CECT5713 and PS2 were also cocultured with the starter culture, but only a small amount of probiotic was retained in the curd, resulting in a high loss of lactobacilli in the cheese whey (results not shown). Notably, the addition of the probiotic lactobacilli to the curds after whey drainage, when most of the whey had been removed and before molding, resulted in improved retention of lactobacilli in the cheese.

In any probiotic food, in order to have industrial application and to exert its health benefit to the host, the incorporated probiotic strain must maintain its viability during the manufacture, through the shelf life of the product and up to the time of consumption. In the present study, both *L. salivarius* strains remained viable in the experimental cheeses after 28 days at 4°C. In addition, the hygienic quality of the final product was adequate and growth of any other bacteria was not detected in the culture media used. Antibacterial properties against pathogenic bacteria have been reported for *L. salivarius* CECT5713 due to the production of antimicrobial compounds such as lactate, acetate, and hydrogen peroxide [14]. This strain also harbors a bacteriocin cluster located in a megaplasmid that contains several genes that would allow the biosynthesis of several bacteriocins, but a deletion at the beginning of the regulatory system results in the absence of any bacteriocin production [19].

A minimum probiotic daily dose of 10^8-10^9 cfu has been recommended in processed foods in order to exert their beneficial effects [35, 39]. This would be equivalent to a daily intake of 100 g of product containing 10^6-10^7 cfu/g. The results obtained in this study show that the counts of *L. salivarius* CECT5713 and PS2 in cheeses were always in the range of this recommended level. Therefore, they would satisfy this criteria established for a probiotic food. Furthermore, the presence of these potentially probiotic bacteria did not interfere with the performance of starter lactococci, as it has been described by other authors [27].

Another challenge associated with the addition of probiotic bacteria during cheese manufacturing is to maintain the characteristics of the cheese. Actually, consumers demand the addition of probiotic cultures to many foods, including cheese, but a primary consideration is that the sensory properties, especially taste, of any probiotic food should be appealing [40]. The addition of certain levels of any viable bacteria, and their enzymes, to cheese most probably will contribute to glycolysis, proteolysis, and lipolysis processes that take place during manufacture and cheese ripening and contribute to the organoleptic properties of the final product [41]. In order to maintain an adequate organoleptic quality, probiotic bacteria must not adversely affect cheese composition, texture, flavour, and final acceptance. The addition of probiotic *L. salivarius* CECT5713 and PS2 did not result in a substantial change of the fresh cheese composition, which was within the gross chemical composition of this type of cheeses.

A slight (but not statistically significant) increase in moisture content of cheeses containing lactobacilli compared to control cheese was noted, although a_w values remained unchanged. These differences may be related, at least in part, to several factors during cheese manufacture that exert a great influence on moisture retention in the curd such as cutting intensity, final size of the curds, or curd manipulation [42]. On the other hand, exopolysaccharide- (EPS-) producing lactic acid bacteria have been reported to increase moisture retention in cheese [43–45] and could improve the texture of reduced-fat cheese that tends to be tough and rubbery. Two gene clusters for EPS biosynthesis have been described in *L. salivarius* strains, although it has been reported that the level of production of EPS does not correlate with the presence of these clusters, depends on the available carbohydrate, and is highly strain-dependent [46]. However, at present it is not known if *L. salivarius* CECT5713 and PS2 are EPS-producing strains. Another tentative explanation may involve microbial dynamics and metabolism. It has been reported that cheese microbiota and its metabolic activity may confound the effect of moisture on a_w [47]. Changes in the type and concentration of low molecular weight soluble compounds, such as an increase in lactate, free fatty acids, amino acids, and, even, very small peptides, might decrease the value of a_w, although this effect is usually more pronounced for ripened cheeses [47, 48].

Among the texture parameters analyzed in this work, instrumental methods only detected differences in hardness, which was higher in cheeses containing *L. salivarius* CECT5713 than in control cheeses and those manufactured with *L. salivarius* PS2. However, this difference was not perceived by panellists during the descriptive test, indicating that it did not have a relevant impact in the sensory quality and acceptance of the cheese. On the contrary, the panellists identified both cheeses containing probiotic lactobacilli as having lower adhesiveness than the control cheese, although the texture profile analysis did not reveal a statistically significant difference. Reduction in adhesiveness in cheeses made with an EPS-producing *Streptococcus thermophilus* culture has been related to the production and liberation of EPS [49, 50]. Also, a higher perception of creaminess in control cheese was reported in contrast to cheeses manufactured with *L. salivarius* CECT5713 and PS2. Creaminess is often related to a high fat level and the presence of fat globules, in agreement with the slightly higher fat content of control cheese, although sensorial discrimination of fat levels in solid foods is more difficult than in liquid products [51]. Regarding colour, small although statistically significant differences were also detected when using instrumental methods of analysis, but they were not perceived by the trained panellists. This indicates that the presence of probiotic lactobacilli did not disturb the distinctive white colour of fresh cheese.

Following the general component balance theory, cheese flavour is the result of a synergistic effect of the appropriate and balanced blend of various flavour compounds produced from proteins, lipids, and lactose through numerous biochemical reactions involving enzymes from milk, rennet, starter cultures, secondary cheese microbiota, and, even, spontaneous reactions [52]. The volatile composition of the cheese made with both *L. salivarius* strains was not qualitatively different from that of the control cheese, and only a

few quantitative differences were observed. The main change detected was a higher acetic acid concentration in the cheeses containing *L. salivarius* CECT5713 and PS2, probably related to higher lactose degradation during cheese storage which will explain a lower final pH in the probiotic cheese. However, these differences did not impact the sensory perception given by the panellists or the global acceptance of the cheeses manufactured with *L. salivarius* CECT5713 and PS2, as it has been described with other probiotic strains by other authors [36–38, 49, 50, 53].

5. Conclusion

The results of the present study demonstrate that *L. salivarius* CECT5713 and PS2 incorporated into fresh cheese survived at adequate levels during a 28-day storage at 4°C. The presence of the lactobacilli did not interfere with normal growth of starter culture and did not modify significantly the composition and organoleptic properties of the probiotic cheeses containing *L. salivarius* strains that had good acceptance by trained panellists.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Acknowledgments

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Research Article

Immune Modulating Capability of Two Exopolysaccharide-Producing *Bifidobacterium* Strains in a Wistar Rat Model

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Fermented dairy products are the usual carriers for the delivery of probiotics to humans, *Bifidobacterium* and *Lactobacillus* being the most frequently used bacteria. In this work, the strains *Bifidobacterium animalis* subsp. *lactis* IPLA R1 and *Bifidobacterium longum* IPLA E44 were tested for their capability to modulate immune response and the insulin-dependent glucose homeostasis using male Wistar rats fed with a standard diet. Three intervention groups were fed daily for 24 days with 10% skimmed milk, or with 10^9 cfu of the corresponding strain suspended in the same vehicle. A significant increase of the suppressor-regulatory TGF-β cytokine occurred with both strains in comparison with a control (no intervention) group of rats; the highest levels were reached in rats fed IPLA R1. This strain presented an immune protective profile, as it was able to reduce the production of the proinflammatory IL-6. Moreover, phosphorylated Akt kinase decreased in gastroctemius muscle of rats fed the strain IPLA R1, without affecting the glucose, insulin, and HOMA index in blood, or levels of Glut-4 located in the membrane of muscle and adipose tissue cells. Therefore, the strain *B. animalis* subsp. *lactis* IPLA R1 is a probiotic candidate to be tested in mild grade inflammation animal models.

1. Introduction

Probiotics, together with the prebiotic substrates that support the growth of the beneficial intestinal microbiota, constitute one of the largest segments of the worldwide functional food market. Fermented foods, and especially dairy products, are the most popular carriers for the delivery of these microorganisms in humans [1]. Probiotics are defined as "live microorganisms that, when administered in adequate amounts, confer a health benefit on the host" [2]. Strains from *Bifidobacterium* and *Lactobacillus* are frequently used as probiotics for humans; some of their species have the "Qualified Presumption of Safety" (QPS) status [3] because of their long history of safe consumption.

There are several reports supporting the fact that certain ingested probiotics are able to impact the human health by direct interaction with the host cells, or through the modulation of the intestinal microbiota [4, 5]. The relevance of this microbiota community is especially highlighted in some chronic disorders of the gut in which a dysbiosis of this microbial community has been detected [6]. In addition, scientific evidence suggests an intricate relationship between the intestinal microbiota and some extraintestinal disorders, such as obesity. The modulation of the gut microbiota by diet could be effective in improving the low-grade inflammation associated with obesity and related diseases [7, 8]. Prebiotic and probiotic supplements could modify the altered gut microbiota present in obesity-associated diseases by

influencing gut barrier function, insulin sensitivity, systemic inflammation, and host energy homeostasis [9, 10]. The mechanism(s) by which probiotics interact with the host remains to be completely understood, although some clues have been obtained from studies performed using different animal models [11–13].

Surface components of probiotic envelopes are claimed to be the molecules that establish the initial interaction with eukaryotic cells. In this scenario, exopolysaccharides (EPS) produced by members of the intestinal microbiota, or by beneficial microorganisms ingested with foods, can be active players. There are a few works studying *in vivo* the involvement of these polymers on bacteria-host interactions [14–16]. Most of the evidence of the immune modulation capability of EPS from probiotics has been obtained by *in vitro* approaches. It seems that the physicochemical characteristics, such as composition (mainly the presence of charged substituents) and molecular weight (size), of these polymers are the key parameters determining the capability to induce a mild response (acid and small polymers) or to reduce the production of cytokines (neutral and big polymers) [17]. In parallel to the direct interaction with immune cells of the host, the immunomodulation could also be achieved through intervention on the intestinal microbiota [18, 19]. Previously we have demonstrated that the administration of the EPS-producing strains *Bifidobacterium animalis* IPLA-R1 and *Bifidobacterium longum* IPLA-E44 to male Wistar rats modified their intestinal microbiota by influencing the short chain fatty acid (SCFA) profile and by increasing *Bifidobacterium* population levels in the gut [15]. Therefore, the aim of the current study was to check whether the oral intake of these two EPS-producing bifidobacteria could modify some health-related parameters, such as the systemic inflammatory profile and/or the insulin-dependent glucose homeostasis, in healthy rats fed with a standard diet. The final goal is to suggest target human population(s) for the potential application of these strains as probiotics.

2. Material and Methods

2.1. Experimental Design and Samples Collection. The animal study design was previously reported [15] and was conducted under the approval of the Animal Experimentation Ethical Committee of Oviedo University (Asturias, Spain). The EPS-producing strains *B. animalis* subsp. *lactis* IPLA-R1 and *B. longum* IPLA-E44 were tested in adult, male Wistar rats. Briefly, three groups of rats (8 per group) were fed daily, through an intragastric cannula, with the delivery vehicle (100 μL skimmed milk, group V) or with 10^9 cfu per day (in 100 μL skimmed milk) of the strains IPLA-R1 (group B1) or IPLA-E44 (group B2). After an intervention period of 24 days, animals were anaesthetized with halotone and killed by exsanguination. Additionally, a group of 8 rats was used as a basal reference control (no intervention, group C) and killed under the same conditions.

Blood samples (4 mL) were collected from the jugular vein into heparinized tubes and centrifuged at 1,000 ×g for 20 min at 4°C, and the plasma fraction was immediately collected and stored frozen at −20°C until it was assayed. The gastrocnemius muscle and retroperitoneal adipose tissue (100 mg) were dissected, frozen in liquid nitrogen, and kept at −80°C until the analyses.

2.2. Immunoglobulins and Cytokine Profile in Plasma. The cytokine levels in the plasma samples were quantified by a "cytometric bead array" (CBA) using the BD FascCanto II flow cytometer and the software FCAP (BD Biosciences, San Diego, CA, USA). The CBA flex set (BD Biossciences) included the cytokines IL-1a, IL-4, IL-6, IL-10, IFNγ, and TNFα, which were assayed under conditions recommended by the manufacturer. The TGFβ was measured by means of the eBioscience platinum ELISA test (eBioscience, Bender MedSystems GmbH, Vienna, Austria); the colorimetric reaction was measured at 450 nm in the modulus microplate photometer (Turner Biosystems, CA, USA). The limit of detection was 4.0 pg/mL for IL-1a, 3.4 pg/mL for IL-4, 1.6 pg/mL for IL-6, 19.4 pg/mL for IL-10, 6.8 pg/mL for IFNγ, 27.7 pg/mL for TNFα, and 8 pg/mL for TGFβ.

The levels of immunoglobulin (Ig) IgG and IgA were determined by means of ELISA tests (GenWay Biotech, Inc., San Diego, CA, USA) following the manufacturer's instructions. Additionally, IgA was measured in supernatants obtained after centrifugation from fecal samples homogenized (1/10) with PBS.

2.3. Determination of Insulin, Glucose, and Calculation of the HOMA-Index. The tail vein blood glucose levels were measured using a portable device (Accu-Chek Aviva Nano System, Roche Farma, S.A., Barcelona, Spain) while fasting plasma insulin was measured by ELISA assay (Millipore Ibérica, S.A., Madrid, Spain) following the manufacturer's recommendations. Homeostasis Assessment Model-(HOMA–) index was calculated using the following formula: [insulin (μU/mL) × glucose (mg/dL)]/2.43 [20].

2.4. Analysis of the Protein Kinase B (Akt) and the Glucose Transporter Type 4 (Glut4). The content of total and phosphorylated Ser473 Akt kinase, as well as that of the insulin-regulated glucose transporter type 4 (Glut4), was determined by means of western-blot analyses in samples of crude intracellular extracts and in cell-membrane fractions, obtained from the muscle and retroperitoneal adipose tissues of the rats as follows. To obtain the intracellular crude extracts, both tissue types were homogenized in lysis buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 1% Nonidet P40, 0.05% sodium deoxycholate, sodium orthovanadate, 5 mM EDTA, and 10% glycerol) at 4°C. The homogenized samples were centrifuged at 21,800 ×g at 4°C for 10 min to collect the supernatants (crude extracts) and its protein content was determined by the Bradford method. To obtain cell membrane fractions, a modification of the method described by Hirshman et al. [21] was used. Briefly, a total of 500 mg of tissues was homogenized with a Polytron operated at maximum speed for 30 s at 4°C in a buffer containing 100 mM Tris (pH 7.5), 20 mM EDTA (pH 8.0), and 255 mM sucrose (pH 7.6).

The homogenate was then centrifuged at 1,000 ×g for 5 min and the resulting supernatant was centrifuged again at 48,000 ×g for 20 min. The pellet from this centrifugation was used for the preparation of the membrane fraction that is enriched in the membrane marker Na^+-K^+-ATPase. The pellet was resuspended in 20 mM HEPES and 250 mM sucrose, pH 7.4 (buffer A). An equal volume of a solution containing 600 mM KCl and 50 mM sodium pyrophosphate was added and the mixture was vortexed, incubated for 30 min on ice, and then centrifuged for 1 h at 227,000 ×g over a 36% sucrose cushion in buffer A. The resulting interface and the entire buffer above it were collected, diluted in an equal amount of buffer A, and centrifuged for 1 h at 227,000 ×g. The resulting pellet was used as the cell membrane fraction and its protein content was determined by the Bradford method.

To carry out the western-blot analysis, proteins in the crude tissue extracts or in the cell membrane fractions were resolved by SDS-PAGE (10% Tris-Acrylamide-Bis) and electrotransferred from the gel to nitrocellulose membranes (Hybond-ECL, Amersham Pharmacia, Piscataway, NJ) as described by Towbin et al. [22]. Nonspecific protein binding to the nitrocellulose membrane was reduced by preincubating the filter with blocking buffer (TNT, 7% BSA); then, membranes were incubated overnight with the primary antibodies Glut4 (sc-7938, diluted 1 : 2,500), Akt (sc-7126, diluted 1 : 2,000), and phosphorylated-Ser473-Akt (sc-101629, diluted 1 : 2,500). All antibodies were obtained from Santa Cruz Biotechnologies (Santa Cruz, CA). After incubation with the primary antibody, the nitrocellulose membranes were washed and incubated with the corresponding anti-rabbit antibody coupled to horseradish peroxidase (HRP, sc-2004, diluted 1 : 20,000), or the anti-goat antibody coupled to HRP (sc-2768, diluted 1 : 20,000). Additionally, all membranes were stripped and probed with monoclonal antibodies used as reference controls: anti-β-actin antibody (sc-1615, diluted 1 : 2,500), anti-Na^+-K^+-ATPase α1-subunit antibody (sc-16041, diluted 1 : 5,000), or anti-GAPDH (sc-20356, diluted 1 : 1,000). Immunoreactive bands were detected using an enhanced chemiluminescence system (ECL, Amersham Pharmacia Biotech, Little Chalfont, Bucks, UK). Films were analyzed using a digital scanner Nikon AX-110 (Nikon, Madrid, Spain) and NIH Image 1.57 software (Scion Corp., MD, USA). The density of each band was normalized to its respective loading control (β-actin, ATPase, or GAPDH). In order to minimize interassay variations in each experiment, samples from all animal groups were processed in parallel.

2.5. Statistical Analysis. The SPSS/PC 19.0 software package (SPSS Inc., Chicago, IL, USA) was used for all statistical analyses. After checking the normal distribution of the parameters involved in the homeostasis of glucose, one-way ANOVA tests were used to determine the differences between the three groups of rats and the reference control. Moreover, differences among the three experimental groups, compared two by two, were also tested by means of one-way ANOVA tests. These parameters were represented by mean and standard deviation (SD).

Data of cytokines and Igs were not normally distributed; thus, the nonparametric Mann-Whitney test for two independent samples was used to assess differences. The same comparisons among samples previously described were carried out. Cytokine data were represented by median, interquartile range and maximum and minimum values (box and whiskers plot).

3. Results

3.1. Immune Parameters. Several proinflammatory and immune-suppressor cytokines were measured in the blood plasma obtained from the four groups of rats (Figure 1). Levels of most cytokines (IFNγ, IL-1α, IL4, IL-10, and TNFα) remained without significant variations in the four groups of rats; this indicates that the daily intake for 24 days of the two bifidobacteria, or the vehicle (milk), has not strongly modified the immune response, since most of the cytokine levels in the intervention groups (V, B1, and B2) were similar to those found in the control group (C). In spite of this, the oral intake of the two bifidobacteria significantly increased the production of the suppressor-regulatory TGF-β cytokine, the levels reached with the strain *B. animalis* subsp. *lactis* IPLA-R1 (group B1) being the highest ($P < 0.05$). In addition, this strain also induced the lowest ($P < 0.05$) production of IL-6 as compared with the other two intervention (V and B2) groups, although none of the three intervention groups significantly differed from the control group. Thus, it seems that the strain IPLA-R1 showed an *in vivo* immune suppressive profile by reducing the proinflammatory cytokine IL-6 and inducing the synthesis of the regulatory TGF-β.

The levels of IgA were determined in blood plasma and fecal homogenates and the amount of IgG was measured in plasma. The oral intake of skimmed milk, alone or used as vehicle for the bifidobacterial delivery, produced a significantly higher ($P < 0.05$) ratio IgG/IgA in the three groups, in comparison with the basal control group (Figure 2(a)). No variations in secretory IgA were detected in the fecal samples of the four groups of rats (Figure 2(b)), which is of special relevance since this antibody plays a critical role in maintaining the immune homeostasis in several mucosae, including the intestinal mucosa. Therefore, (cow's) milk induced a humoral systemic response; this immune reaction was not surprising since this food is not a current component of a rat's diet, and therefore these animals have not developed oral tolerance to it.

3.2. Biochemical Parameters. The current setup of data showed that the concentration of glucose and insulin in plasma collected after a fasting period, as well as the HOMA index, were not modified by the intervention study (Table 1). The concentrations in the groups of rats treated for 24 days with vehicle (skimmed milk), or with the two bifidobacteria, were similar among them and with respect to the control group.

To detect potential changes in the insulin-dependent glucose signaling route, the levels of the protein Akt and the

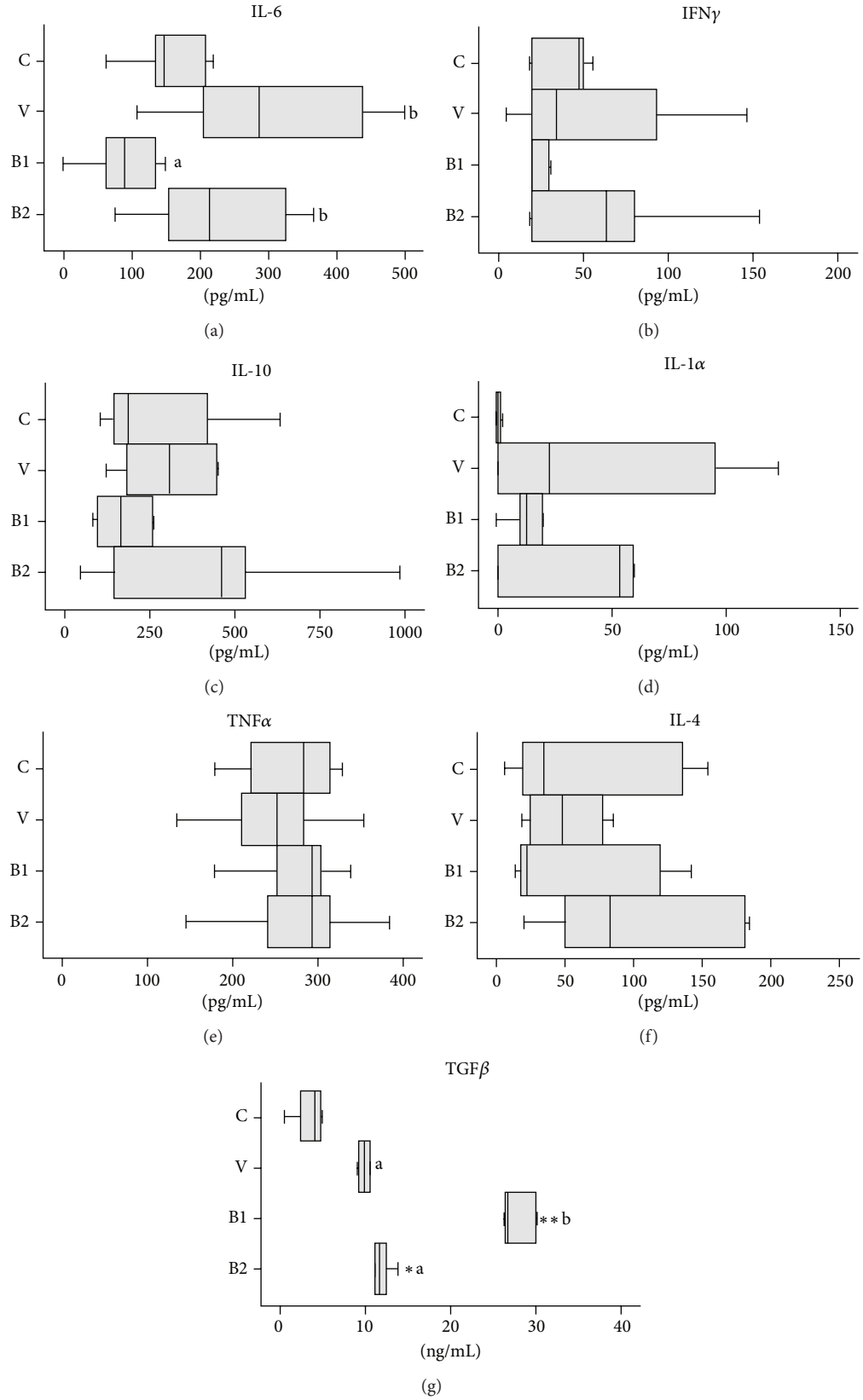

Figure 1: Cytokines measured in blood (plasma) samples of Wistar rats fed for 24 days with vehicle (100 μL of skimmed milk, V group) or 10^9 cfu per day of *B. animalis* subps. *lactis* IPLA-R1 (B1 group) or *B. longum* IPLA-E44 (B2 group). The control rats were not submitted to the intervention study (C group). For each cytokine, the box and whiskers plot represents median, interquartile range and minimum and maximum values obtained from 8 rats per group. The nonparametric Mann-Whitney test for two independent samples was used to compare each treatment group with the control, and differences are indicated with asterisks ($^{*}P < 0.05$, $^{**}P < 0.01$). Additionally, the same test was used to assess differences among the treatment groups compared two by two. In this case, treatment groups that do not share the same letter are statistically different ($P < 0.05$).

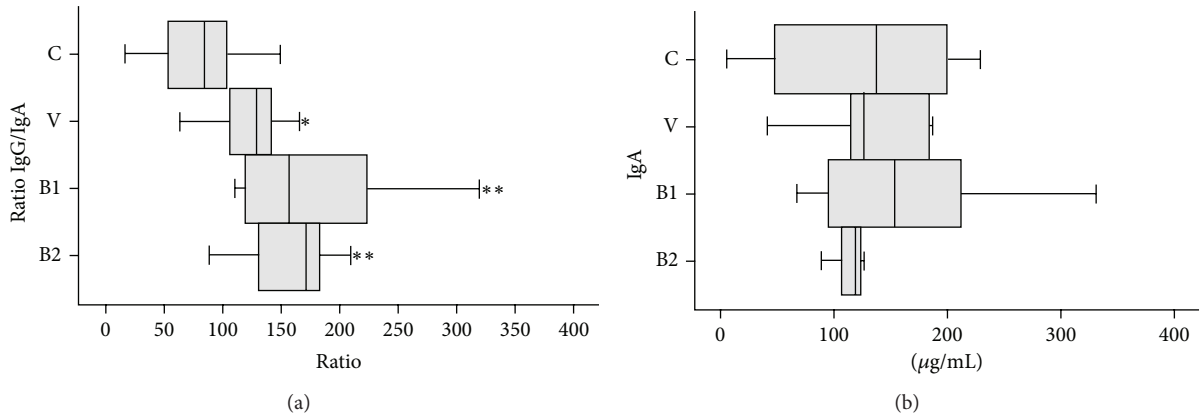

Figure 2: Ratio IgG/IgA in blood (plasma) samples (a) and amount of IgA (μg/mL) secreted in fecal samples (b) of Wistar rats fed for 24 days with vehicle (100 μL of skimmed milk, V group) or 10^9 cfu per day of *B. animalis* subps. *lactis* IPLA-R1 (B1 group) or *B. longum* IPLA-E44 (B2 group). The control rats were not submitted to the intervention study (0 days). The same statistical treatment indicated in Figure 2 was applied.

Table 1: Parameters related to the glucose homeostasis measured in the plasma of Wistar rats fed for 24 days with vehicle (100 μL of skimmed milk) or 10^9 cfu per day of *B. animalis* subps. *lactis* IPLA-R1 (B1 group) or *B. longum* IPLA-E44 (B2 group). Control rats were not submitted to the intervention study (0 days). The one-way ANOVA analyses did not show statistical differences.

Rat group	Mean ± SD		
	Glucose (mg/dL)	Insulin (μg/mL)	HOMA
Control (0 d)	76.2 ± 15.4	0.0060 ± 0.0045	0.20 ± 0.091
Vehicle (24 d)	74.3 ± 12.3	0.0061 ± 0.0052	0.21 ± 0.093
B1 (24 d)	69.6 ± 12.3	0.0063 ± 0.0049	0.19 ± 0.089
B2 (24 d)	82.4 ± 7.9	0.0063 ± 0.0051	0.17 ± 0.090

glucose transporter Glut4 were quantified by western blot (Figure 3). The levels of glucose transporter Glut4 located in the cellular membrane of both retroperitoneal adipose tissue and gastrocnemius muscle were similar in all groups of rats (Figure 3(a)). Similarly, no statistical differences were detected in the percentage of the intracellular kinase Akt, phosphorylated in the serine 473 residue, in adipose tissue (Figure 3(b)). However, the phosphorylated-Akt was significantly ($P < 0.05$) lower in the gastrocnemius muscle of rats fed for 24 days with *B. animalis* subsp. *lactis* IPLA-R1 (group B1) in comparison with the other two intervention groups (vehicle or *B. longum* IPLA E44 fed), as well as in comparison with the control group.

4. Discussion

In recent years, there is an increasing evidence that some specific probiotic strains are able to modulate the immune response. In the case of *Bifidobacterium* genus, most strains studied showed an anti-inflammatory profile in animal models genetically modified or challenged with different factors to induce an inflammatory process [23–25]. Our experimental model was performed with standard, naïve (not challenged) Wistar rats that simulate a healthy state. Thus, this could be the main reason why most cytokines tested were not significantly modified by the ingestion of the two bifidobacteria, in comparison with the placebo fed rats. However, it should also be taken into account that both bifidobacteria are producers of EPS; these are polymers that could mask other immune-reactive molecules present in the bacterial surface and therefore allow them to escape the immune system survey. In this regard, Fanning and coworkers [14] have demonstrated in a naïve murine model that the EPS-producing *Bifidobacterium breve* UCC2003 strain failed to elicit a strong immune response in comparison to its EPS-deficient variant strains; it seems that the EPS+ strain is able to evade the B-cell response. We have recently demonstrated that bifidobacterial EPS, differing in their physicochemical composition, *in vitro* induced a variable cytokine production pattern by human peripheral blood mononuclear cells [26]. In general, those EPS having high molecular weight were those eliciting the lowest production of any cytokine [27, 28]. Thus, it seems that not only the presence/absence of the polymer, but also the characteristics intrinsic to each EPS are relevant for their capability to induce immune response. In this regard the two bifidobacteria strains used in the current work produce polymers of different chemical composition [15]; only the group of rats receiving the strain *B. animalis* subsp. *lactis* IPLA R1 showed a significantly reduced production of IL-6 and increased synthesis of TGF-β. The differential immune response elicited by the two strains cannot be exclusively attributed to the production of different EPS, since other strain-associated traits could also be responsible. Nevertheless, it seems that IPLA R1 strain is able to elicit an imunosuppressive profile *in vivo* after oral intake for a prolonged period (24 days).

Regarding the glucose homeostasis, the levels of circulating glucose and insulin, as well as the HOMA index, were not modified by the consumption of the two bifidobacteria in the context of a standard (no high fat, no high carbohydrate) diet.

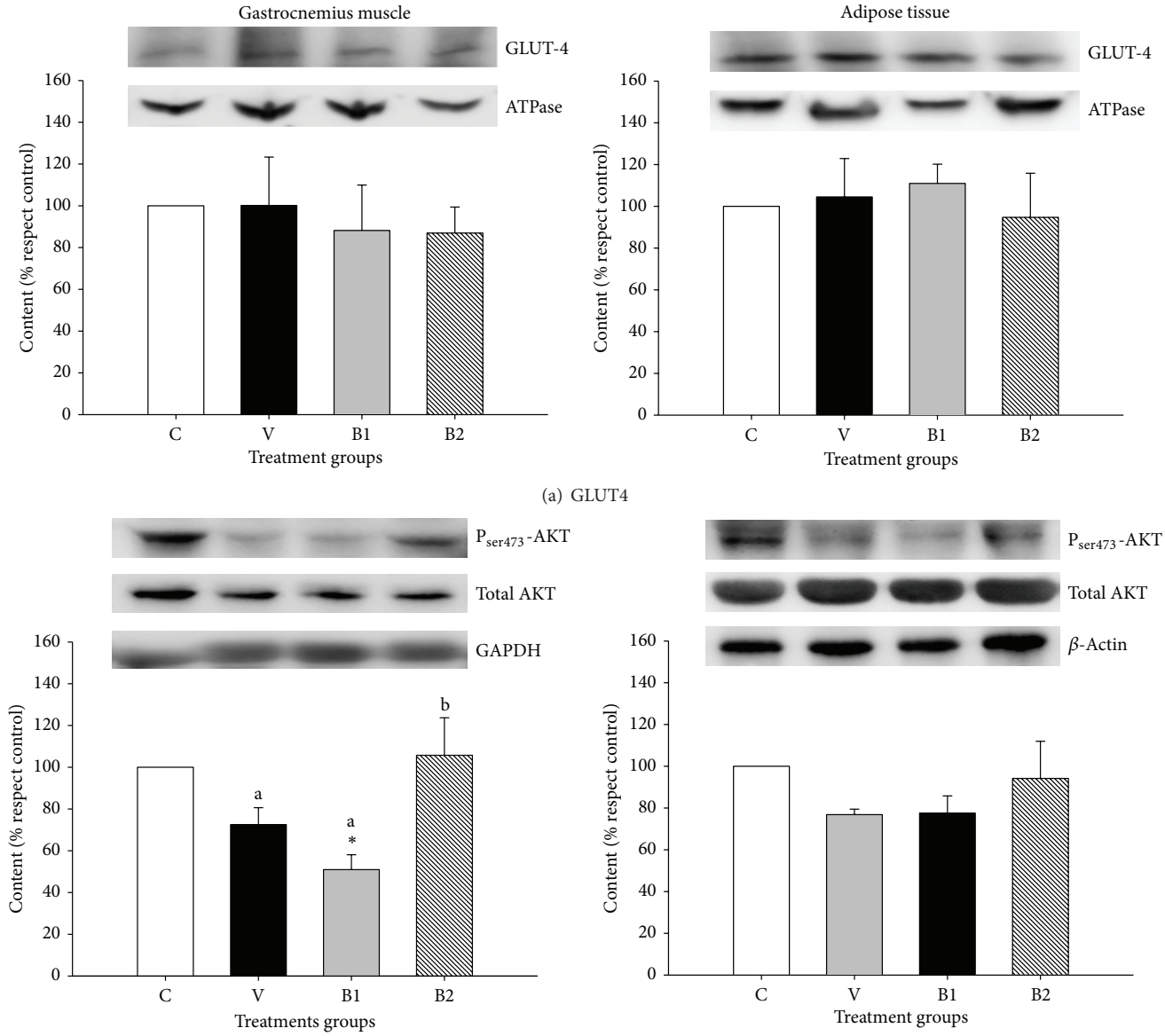

Figure 3: Content of the cell-membrane Glut4 (a) as well as the intracellular Akt and phosphorylated-Ser_{473}-Akt (b) in gastrocnemius muscle and adipose tissues from rats fed daily for 24 days with delivery vehicle (100 μL of skimmed milk, V group) or 10^9 cfu per day of *B. animalis* subps. *lactis* IPLA-R1 (B1 group) or *B. longum* IPLA-E44 (B2 group). Data were referred to those obtained in the control rats (C group) which were not submitted to the intervention study. Bars represent mean and standard deviations obtained from 8 rats per group. Independent one-way ANOVA tests were used to compare each treatment group with the control, and differences are indicated with asterisks ($^*P < 0.05$). Additionally, the same test was used to assess differences among the treatment groups compared two by two. In this case, treatment groups that do not share the same letter are statistically different ($P < 0.05$).

In this regard, it has been described that some probiotics can improve the resistance to insulin in different animal models of diet-induced diabetes or with different genetic backgrounds [29–32]. Additionally, a double-blind, randomized intervention study in humans showed that an intake of *Lactobacillus acidophilus* NCFM for 4 weeks improved the insulin sensitivity [33]. In most of these reports no mechanism of action is proposed or is a general one suggested, such as the modulation of the intestinal microbiota, or the modification of the inflammatory state. In our study, we checked some critical points in the cascade of the glucose uptake mediated by insulin, such as the location of the glucose transporter Glut4 and the levels of the active (phosphorylated) Akt kinase [34]. The two EPS-producing bifidobacteria strains tested did not modify the insulin-regulated trafficking of the glucose transporter Glut4 from intracellular vesicles (endosomes) to the cell membrane of either adipose or muscular tissues. The failure of this translocation in response to insulin is one of

the steps in the development of insulin resistance and type 2 diabetes. Therefore, the presence of similar Glut4 levels in the cell membrane of tissues obtained from the four groups of rats explains the absence of variations in the levels of circulating glucose and insulin. One of the proteins involved in the insulin-mediated Glut4 trafficking is the phosphatidylinositol 32-kinase (PI 3K)-dependent Ser473 kinase Akt. In response to insulin, Akt is activated by phosphorylation which directs the traffic of Glut4 from vesicles to the cell membrane; therefore, Akt acts as a regulator of glucose transport [35]. In our experimental model, the intracellular levels of phosphorylated-Akt in adipocytes were not significantly modified by the intake of the two bifidobacteria; this result is consistent with the absence of differences in the amount of Glut4 located in the cell membrane, as well as the lack of variation in circulating glucose, among the four groups of rats. However, the percentage of phosphorylated-Akt was significantly lower in the gastrocnemius muscle of rats fed with the strain *B. animalis* subsp. *lactis* IPLA R1. Since, in rats from this group, the glucose homeostasis parameters and the content of the Glut4 located in the cell membrane of muscle and adipose tissue remained without significant variations, differences in the phosphorylated-Akt could be explained by the participation of this kinase in other metabolic routes apart from the insulin-mediated glucose transport. In this regard, it has been indicated that the PI 3K-dependent Ser/Thr kinase Akt is a regulator that acts in many different metabolic routes and several events related with the cellular cycle [35].

Aiming to have a general picture of the differences detected in our experimental model, which were mainly driven by the strain *B. animalis* subsp. *lactis* IPLA R1, it should be pointed out that levels of circulating IL-6 and phosphorylated-Akt in muscle were directly related. In this regard, the skeleton muscle and the adipose tissue are important sources for systemic IL-6 [36]. In addition, during strong exercise muscular cells are also targets for the action of IL-6, where the insulin action is favored, among other events, by enhancing the phosphorylation of Akt [37]. However, IL-6 has adverse effects on other tissues that are targets for insulin action, such as the liver and adipose tissue [38]. At present, we cannot establish a hypothesis to explain the relationship between systemic IL-6 and phosphorylated-Akt in muscle found in rats fed *B. animalis* subsp. *lactis* IPLA R1. Nevertheless, recent articles show that Akt activity has a role in regulating immune response since it is involved in the differentiation and response of several cellular subsets, such as T cells and macrophages [39, 40]. The activity of Akt in signaling immune pathways is induced in some cases by the presence of bacterial components, such as the lipopolysaccharide from gram-negatives [41] or peptidoglycan from gram-positives [42]. This kinase also plays a role in the innate immunity signaling, since it participates in the modulation of mucin secretion by intestinal epithelial cells in response to pathogens [43]. Furthermore, the activity of Akt has been associated with dendritic cell differentiation and stimulation driven by Gram-positive probiotics, such as the strain *Bifidobacterium breve* C50 [44].

5. Conclusion

In this study, we found that the oral administration of the EPS-producing *B. animalis* subsp. *lactis* IPLA R1 in healthy rats is associated with an immune protective profile, since this EPS producing strain can suppress the proinflammatory cytokine IL-6 and promote the synthesis of the regulatory cytokine TGF-β. These results suggest that, in the future, this bifidobacteria could be tested in experimental models of low grade inflammation state, such as that linked to obesity. Additionally, the capability of strain IPLA R1 to reduce the systemic levels of IL-6, linked with a reduction in the phosphorylated state of Akt in the muscle, without affecting the glucose homeostasis, prompts us to propose the potential application of this strain for sportspeople undertaking strong exercise.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Research Article

Safety Characterization and Antimicrobial Properties of Kefir-Isolated *Lactobacillus kefiri*

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Lactobacilli are generally regarded as safe; however, certain strains have been associated with cases of infection. Our workgroup has already assessed many functional properties of *Lactobacillus kefiri*, but parameters regarding safety must be studied before calling them probiotics. In this work, safety aspects and antimicrobial activity of *L. kefiri* strains were studied. None of the *L. kefiri* strains tested caused α- or β-hemolysis. All the strains were susceptible to tetracycline, clindamycin, streptomycin, ampicillin, erythromycin, kanamycin, and gentamicin; meanwhile, two strains were resistant to chloramphenicol. On the other hand, all *L. kefiri* strains were able to inhibit both Gram(+) and Gram(−) pathogens. Regarding the *in vitro* results, *L. kefiri* CIDCA 8348 was selected to perform *in vivo* studies. Mice treated daily with an oral dose of 10^8 CFU during 21 days showed no signs of pain, lethargy, dehydration, or diarrhea, and the histological studies were consistent with those findings. Moreover, no differences in proinflammatory cytokines secretion were observed between treated and control mice. No translocation of microorganisms to blood, spleen, or liver was observed. Regarding these findings, *L. kefiri* CIDCA 8348 is a microorganism isolated from a dairy product with a great potential as probiotic for human or animal use.

1. Introduction

Kefir grains are composed of a complex community of yeasts, lactic acid, and acetic acid bacteria confined in a matrix of polysaccharides and proteins [1]. The product obtained by fermentation of milk using these grains is called "kefir" and several health-promoting properties have been associated to its consumption [2–5].

As it is known, probiotics are "live microorganisms which, administered in adequate amounts, exert a beneficial effect to the health of the host" [6]. Specific strains of lactic acid bacteria, in particular some of the genera *Lactobacillus*, are extensively used as probiotics [7, 8] since their ability to modulate the immune system has been demonstrated [9, 10] as well as their capacity to inhibit the growth or invasion of pathogenic bacteria and parasites [11–13].

The study of the beneficial properties attributed to isolated microorganisms constitutes a field of great interest for the development of functional foods. Lactobacilli are generally regarded as safe (GRAS) and most of them (as *Lactobacillus kefiri*) are included in the QPS list of the European Union [14] due to their long history of use in fermented dairy products and their presence in human intestinal tract. However, certain *Lactobacillus* strains have been associated with cases of sepsis, endocarditis, or bacteremia, mostly in association with a severe underlying disease [15–18]. On the other hand, the absence of the acquired antimicrobial resistance is a very important criterion for evaluating the safety of lactic acid bacteria (LAB) used as food started or probiotics [19]. The breakpoints for the antibiotic list were defined by the European Food Safety Authority (EFSA) in order to assess the bacterial resistance to antibiotics of human or veterinary importance [20, 21].

Our workgroup has isolated and characterized numerous species of LAB and yeasts from kefir, including several strains of *Lactobacillus kefiri* [22–24], one of the most predominant

Table 1: Minimum inhibitory concentrations (MIC) for antibiotic resistance.

Antibiotics	Breakpoints[a]	MIC ($mg\,L^{-1}$) CIDCA 8321	CIDCA 8345	CIDCA 8348	CIDCA 83115	CIDCA 83111	CIDCA 83113
Ampicillin	**2**	<0.032	<0.032	<0.032	<0.032	<0.032	<0.032
Clindamycin	**1**	<0.032	<0.032	<0.032	<0.032	<0.032	<0.032
Chloramphenicol	**4**	**8**	**16**	2	2	1	2
Erythromycin	**1**	<0.125	<0.125	<0.125	<0.125	<0.125	<0.125
Gentamicin	**16**	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5
Kanamycin	**32**	<2	<2	<2	<2	<2	<2
Streptomycin	**64**	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5
Tetracycline	**8**	<0.125	<0.125	4	2	4	<0.125

[a]These are the recommended breakpoints for heterofermentative lactobacilli EFSA Panel on Additives and Products or Substances used in Animal Feed (2012) [20].

species present in kefir-fermented milk (ranged from 2×10^8 to 1×10^9 *L. kefiri* cells mL^{-1}) [25].

We have already demonstrated the potential of *L. kefiri* as a probiotic microorganism *in vitro* after verifying that secretion products and surface proteins from these heterofermentative lactobacilli exert a protective action against the invasion of *Salmonella enterica* serovar Enteritidis [26] and that they are able to antagonize the cytotoxic effects of clostridial toxins on Vero cells [27]. On the other hand, it has been demonstrated that *L. kefiri* strains are able to preserve a high percentage of viability after both spray-drying [28, 29] and freeze-drying procedures [30]. However, no parameter regarding *L. kefiri's* safety was ever evaluated. Since it is known that both the beneficial properties such as harmful characteristic are dependent on the strain, the individual study of the safety of potential probiotic microorganisms should be considered.

Taking into account the potential of *L. kefiri* as a novel probiotic, we reported in this work some safety characteristics of *L. kefiri* strains, as well as the capacity of strains to produce antimicrobial compounds against some intestinal pathogens.

2. Materials and Methods

2.1. Bacterial Strains and Culture Conditions. Pure cultures used in this study comprised *Lactobacillus kefiri* strains CIDCA 8321, 8345, 8348, 83111, 83113, and 83115 [23, 31]. These bacteria were cultured in MRS (Difco, Detroit, USA) for 48 h at 37°C. The following pathogenic bacteria were also used, *Enterococcus faecalis* ATCC 29212, *Staphylococcus aureus* ATCC 6538, *Shigella flexneri* ATCC 9199, *Pseudomona aeruginosa* ATCC 15442, a clinical isolate of *Salmonella enterica* serovar Enteritidis CIDCA 101 (Hospital de Pediatría Professor Juan P. Garrahan, Buenos Aires, Argentina), enterohemorrhagic *Escherichia coli* EDL 933, *Listeria monocytogenes* ATCC 7644, and *Bacillus cereus* ATCC 10876. All mentioned strains, except *B. cereus*, were grown using brain heart infusion (BHI) broth (Biokar Diagnostics, Beauvais, France) in agitation at 37°C for 16 h. *B. cereus* was grown in BHI growth supplemented with dextrose (Anedra, Argentina) $1\,g\,L^{-1}$ (BHIg) in agitation at 37°C for 16 h.

2.2. Hemolysis. Hemolysis was tested by growth of the strains on LAPTg agar (peptone $15\,g\,L^{-1}$; tryptone $10\,g\,L^{-1}$; dextrose $10\,g\,L^{-1}$; yeast extract $10\,g\,L^{-1}$; Tween 80 $1\,g\,L^{-1}$; and bacteriological agar $15\,g\,L^{-1}$) supplemented with 5% human blood (group O) and incubated for 48 h at 37°C under aerobic conditions. The appearance of clear zones around the bacterial colonies indicated the presence of β-hemolysis whereas green zones around the colonies suggested α-hemolysis. *Enterococcus faecalis* ATCC 29212 was included as a positive hemolytic control.

2.3. Minimum Inhibitory Concentration (MIC) for Antibiotic Resistance. The minimum inhibitory concentrations (MICs) of the antimicrobial agents tested (Table 1) were determined by broth microdilution according to the ISO 10932/IDF 233 standard from 2010 [32]. All antibiotics (Sigma-Aldrich, USA) were dissolved for preparing stock solutions of 1280 $\mu g\,mL^{-1}$. Stock solutions were diluted in LSM broth (90% IST plus 10% MRS) to obtain solutions with preliminary concentrations in the range of 0.25–128 $\mu g\,mL^{-1}$. Bacterial inocula were prepared by suspending colonies from 48 h incubated in MRS medium to 5 mL 0.85% NaCl solution. Subsequently, inocula were adjusted to $OD_{625\,nm}$ 0.18–0.24 and diluted 1 : 500 in LSM broth for inoculation of microdilution plates by adding 50 μL of diluted inoculum to each well containing 50 μL of an antibiotic solution. In these conditions, the bacterial inoculum was around 2-3 $\times$ 10^5 CFU mL^{-1} in the wells. After incubating plates under anaerobic conditions at 37°C for 48 hours, the MICs value was read as the lowest concentration of an antimicrobial agent in which visible growth was inhibited.

MICs results were compared with the recommended breakpoints for heterofermentative lactobacilli by the EFSA Panel on Additives and Products or Substances used in Animal Feed [20].

2.4. PCR Detection of Chloramphenicol Resistance Gene. *Cat*, chloramphenicol acetyltransferase gene, was assessed using the primers and PCR conditions described by Hummel et al. [33]. A plasmid from *L. reuteri* G4 was used as a positive control.

2.5. Growth Inhibition of Bacterial Pathogens. The agar spot test described by Schillinger and Lücke [34] was used. Briefly, 5 μL of a suspension $OD_{625\,nm}$ 1 of *L. kefiri* strains was spotted into MRS agar and incubated for 24 h at 37°C. The following day, pathogens were seeded into soft BHI agar and plated over the spotted lactobacilli. After 18 h of incubation at 37°C, the inhibition halos were measured. The width of the clear zone (R) was calculated as follows: R = (dInhib − dSpot)/2, where dInhib is the diameter of the zone without pathogen growth and dSpot is the diameter of the spot. Inhibition scores are as follows: negative (−), $R < 2$ mm; low inhibition capacity (+), 2 mm $< R <$ 5 mm; and high inhibition capacity (++), $R > 6$ mm. At least three independent experiments were performed.

2.6. In Vivo Studies

2.6.1. Ethics Statement. All animal procedures were performed in strict accordance with the guidelines issued by the European Economic Community "86/609."

2.6.2. Experimental In Vivo Protocol. Male 6-week-old Swiss albino mice (Janvier, Le Genest Isle, France) were quarantined 2 weeks after arrival and then randomized by body weight into experimental and control groups of 5–7 animals each. Mice were housed under standard laboratory conditions with free access to food and water. The temperature was kept at 22°C and a 12-hour light/dark schedule was maintained. Mice received by gavage 10^8 CFU of *L. kefiri* CIDCA 8348 (Lk group) or PBS (control group) daily for 21 days.

2.6.3. Safety Evaluation. Mice were weighted every two days; behavior and signs of pain were analyzed daily [35]. At the end of the experimental protocol, ileum and colon were removed and histological studies were performed using hematoxylin-eosin staining [36].

2.6.4. Translocation Assay. Liver and spleen were removed and blood samples were collected aseptically. Liver and spleen were homogenized in 0.1% sterile PBS (0.1 g of organ per mL) and serially diluted. One hundred microliters of each organ homogenate or blood was plated on VRBG Agar (Biokar Diagnostics, Beauvais, France) for enterobacteria and MRS agar for LAB. Plates were incubated under aerobic conditions for 24 h at 37°C for VRBG and for 48 h at 37°C for MRS before examination.

2.6.5. Microorganism Counts in the Ileum. Ileum content was washed with 1 mL sterile PBS and then serial dilutions were plated as indicated above.

2.6.6. Cytokine Release by Intestine and Colon Explants. Explants were cultured in RPMI medium supplemented with 10% foetal bovine serum (Gibco-Invitrogen, Carlsbad, CA, USA), 10 mg/L streptomycin and 10 IU/mL penicillin G, and 100 mg/L gentamicin (all from Sigma Chemical Co., St. Louis, MO, USA) for 24 h at 37°C in a 5% (v/v) CO_2-95% (v/v) air atmosphere [37, 38]. Supernatants were collected, centrifuged, and frozen for cytokines (IL-6, IL-17A, TNF-α, IFN-γ, and GM-CSF) measurements (eBioscience Ready Set Go, France). All assays were performed according to the manufacturer's instructions.

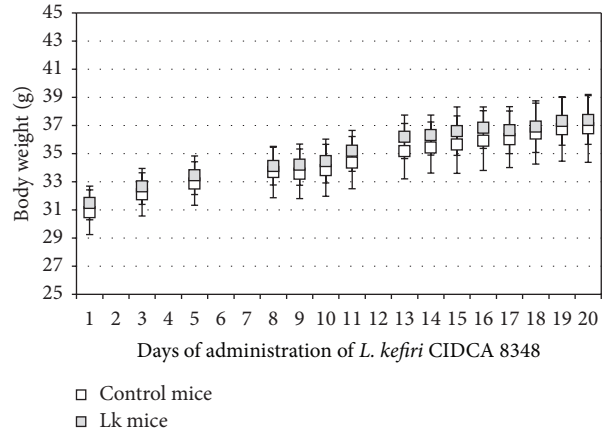


Figure 1: Body weight gain of treated (Lk) and control mice along 21 days of *L. kefir* CIDCA 8348 administration. No differences were observed between control mice and Lk mice ($P > 0.05$).

3. Results and Discussion

In the present work, six potentially probiotic *L. kefiri* strains isolated from kefir were studied in order to evaluate both their safety and antimicrobial properties.

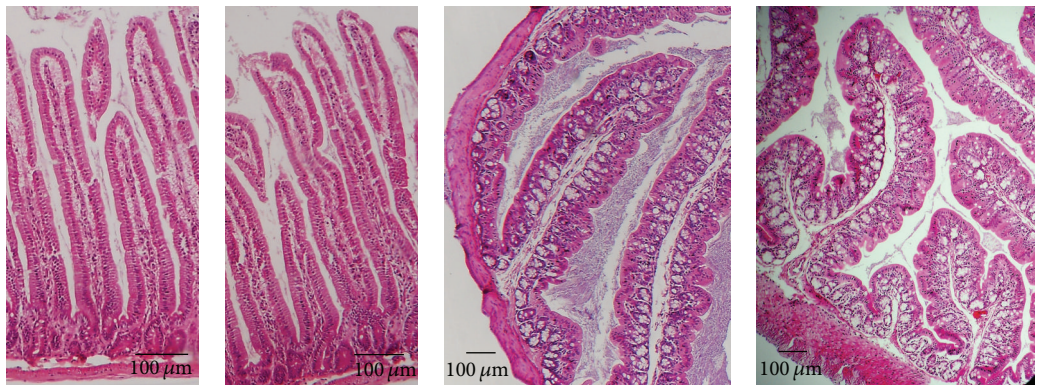
Since hemolysis is a common virulence factor among pathogens, the first safety parameter evaluated *in vitro* was bacterial hemolytic activity. In this study, none of the *L. kefiri* strains tested caused α- or β-hemolysis (data not shown). In this genus, hemolytic activity has a very low frequency and only α-hemolysis has been reported for lactobacilli isolated from foods and dairy products [39–41].

Another important feature regarding safety is the sensitivity to antibiotics. The results obtained for *L. kefiri* strains are shown in Table 1. All tested bacteria exhibited MIC values lower than the breakpoints recommended for heterofermentative lactobacilli [20] for tetracycline, clindamycin, streptomycin, ampicillin, erythromycin, kanamycin, and gentamicin. However, the strains CIDCA 8321 and 8345 were resistant to chloramphenicol although the amplification of CAT encoding gene was negative for all the *L. kefiri* strains (data not shown). In this regard, Hummel et al. [33] reported that some lactobacilli strains carrying *cat* genes were susceptible to chloramphenicol; meanwhile, in other resistant strains *cat* genes could not be amplified. Further research, such as the study of the distribution of chloramphenicol MICs, could contribute to determine whether resistance is acquired (not acceptable strain) or intrinsic (acceptable strain) according to EFSA [21].

To our knowledge, antibiotic sensitivity of *L. kefiri* was evaluated just in two publications. Nawaz et al. [42] studied

Table 2: Antimicrobial activity of *Lactobacillus kefiri* strains against pathogens by agar spot test.

Strain	Growth inhibition ability					
	CIDCA 8321	CIDCA 8345	CIDCA 8348	CIDCA 83115	CIDCA 83111	CIDCA 83113
	Gram negative bacilli					
Pseudomona aeruginosa	++	+	++	+	+	+
Salmonella Enteritidis	+	−	+	−	+	+
Shigella flexneri	+	−	+	−	+	−
EHEC	−	−	−	−	−	−
	Gram positive bacilli					
Listeria monocytogenes	+	−	+	−	+	−
Bacillus cereus	++	+	++	+	+	++
	Gram positive cocci					
Enterococcus faecalis	+	−	+	−	−	−
Staphylococcus aureus	++	+	++	+	++	+



(a) (b) (c) (d)

Figure 2: Hematoxylin-eosin staining of ileum and colon section. (a) Ileum of control mice; (b) ileum of mice receiving *L. kefiri* CIDCA 8348 for 21 days; (c) colon of control mice; (d) colon of mice receiving *L. kefiri* CIDCA 8348 for 21 days. No differences were observed among groups in any tissue.

one *L. kefiri* strain isolated from a dairy product, which was resistant to kanamycin and tetracycline but sensitive to other antimicrobial agents tested in LSM medium. Chang et al. [43] observed that all the *L. kefiri* strains, among other lactobacilli, isolated from swine intestines were resistant to tetracycline, with MIC values higher than 256 $\mu g\,mL^{-1}$, and that they possessed at least one resistance gene. Taking into account that tetracycline is the most widely used antimicrobial agent in swine production, its continuous administration might be selecting tetracycline resistant microorganisms on swine's microbiota. This feature and the different origin of our *L. kefiri* strains could contribute, at least in part, to the disagreement between our results and those from other authors.

The secretion of molecules able to inhibit the growth of pathogens is a desirable characteristic, among others, for a potentially probiotic bacteria [44], and it could also be a technological advantage in the food industry since they might be used as functional starter cultures [45, 46]. We evaluated the pathogen growth inhibition capacity of the six *L. kefiri* strains studied. As observed in Table 2, the inhibition profile was strain dependent, and Gram positive pathogens showed higher sensibility to *L. kefiri* strains than Gram negative bacteria. It is important to notice that the addition of MRS acidified with HCl or lactic acid to pH 4.3 (final pH reached by *L. kefiri* cultures) was not able to produce inhibition of pathogens in our tests (data not shown). All the strains inhibited growth of *Bacillus cereus* and *Staphylococcus aureus* but none of them inhibited enterohemorrhagic *Escherichia coli* (EHEC). The strains *L. kefiri* CIDCA 8321, CIDCA 8348, and CIDCA 83111 were able to inhibit growth of the rest of the tested pathogens. Many mechanisms associated with bacterial inhibition have been described for *Lactobacillus* species [47]. The production of antimicrobial molecules is usually strain dependent, which is in accordance with our results, and the introduction of probiotic bacteria able to inhibit other microorganisms could have a positive impact in animal and human health [48, 49].

Up to here, *L. kefiri* CIDCA 8321, 8348, and 83111 demonstrated to be the most active strains against pathogens; however, CIDCA 8321 showed resistance to chloramphenicol. In consequence, among the other two strains, we selected CIDCA 8348 to perform *in vivo* studies in Swiss mice.

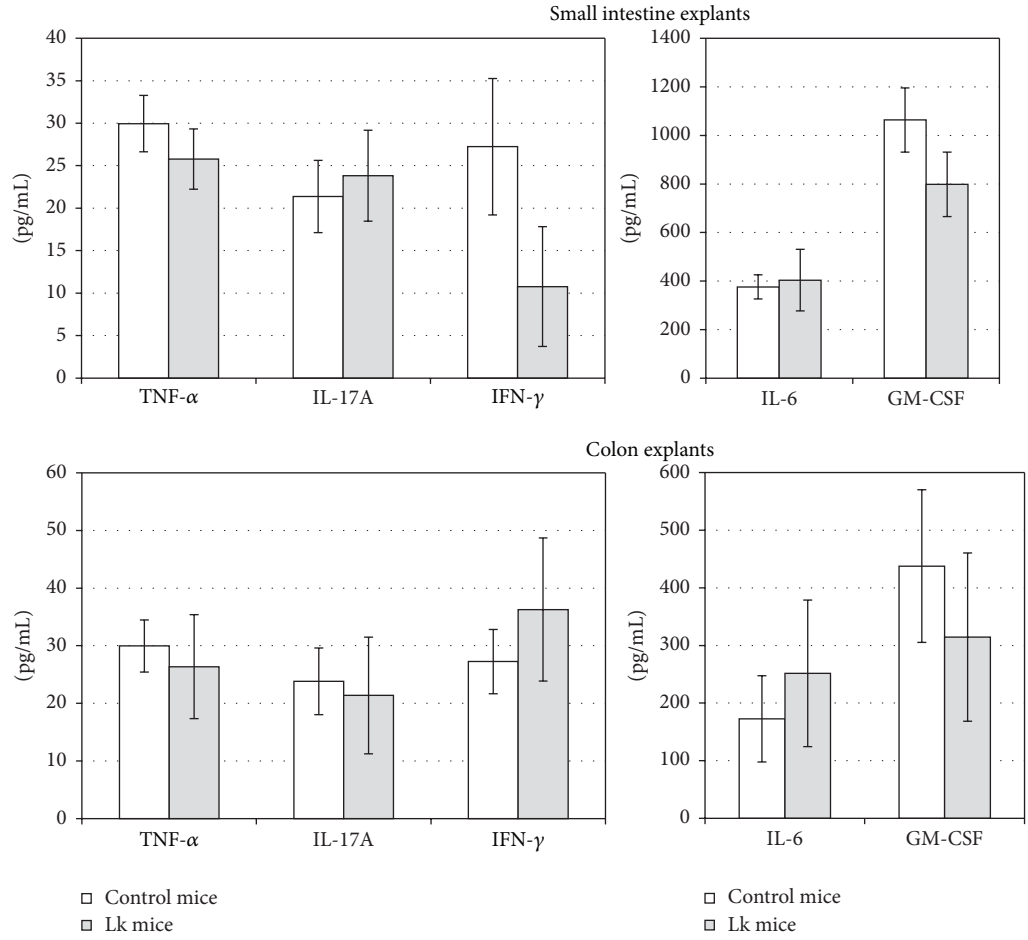


Figure 3: Secretion of proinflammatory cytokines by intestine and colon explants from mice receiving *L. kefiri* CIDCA 8348 for 21 days (Lk) and control mice determined by ELISA. Statistical analysis: one way ANOVA, posttest Bonferroni, $\alpha = 0.05$.

As observed in Figure 1, no differences in body weight were observed between mice that received 100 μL of a 10^9 CFU mL^{-1} suspension of *L. kefiri* CIDCA 8348 (Lk group) and mice receiving 100 μL of PBS (control group) daily for 21 days. Moreover, there were no differences in food and water intake between groups (data not shown). In accordance with these results, Lk group did not show any signs of pain, lethargy, dehydration, or diarrhea during treatment. No signs of inflammation or damage were observed in any organ during necropsy. Length of each mouse's colon was measured, since it has been reported that increasing levels of inflammation result in shortening of the colon [50]. No significant differences in colon's length of Lk mice and control mice were observed (12.4 ± 0.6 versus 12.6 ± 0.8). Moreover, the histological study of ileum and colon was consistent with the already described observations; no signs of inflammation, edema, erosion/ulceration, crypt loss, or infiltration of mono- and polymorphonuclear cells [51] were observed in Lk mice's tissues (Figure 2), in concordance with previous report by Bolla et al. [30] who administered this strain as a constituent of a mixture of five kefir-isolated microorganisms to BALB/c mice. Additionally, no differences in the secretion levels for proinflammatory cytokines such as IL-6, IL-17A, IFN-γ, TNF-α, and GM-CSF were observed in the small intestine and colon explants from Lk and control mice (Figure 3). On the other hand, no translocation of microorganisms was observed on blood, spleen, or liver (bacterial counts were negative), which means that the epithelial barrier was not disrupted since intestinal permeability was not affected by *L. kefiri* CIDCA 8348 administration [52]. Besides, the viable counts of enterobacteria ($3.5 \pm 0.8 \times 10^7$ versus $4.8 \pm 0.9 \times 10^7$) and LAB ($1.1 \pm 0.6 \times 10^7$ versus $2.6 \pm 0.8 \times 10^7$) in the ileum were comparable between control and treated mice.

4. Conclusion

Taking into account all these findings, we conclude that *L. kefiri* CIDCA 8348 isolated from a dairy product present a great potential as probiotic for human or animal use and can be used also for producing functional foods.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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