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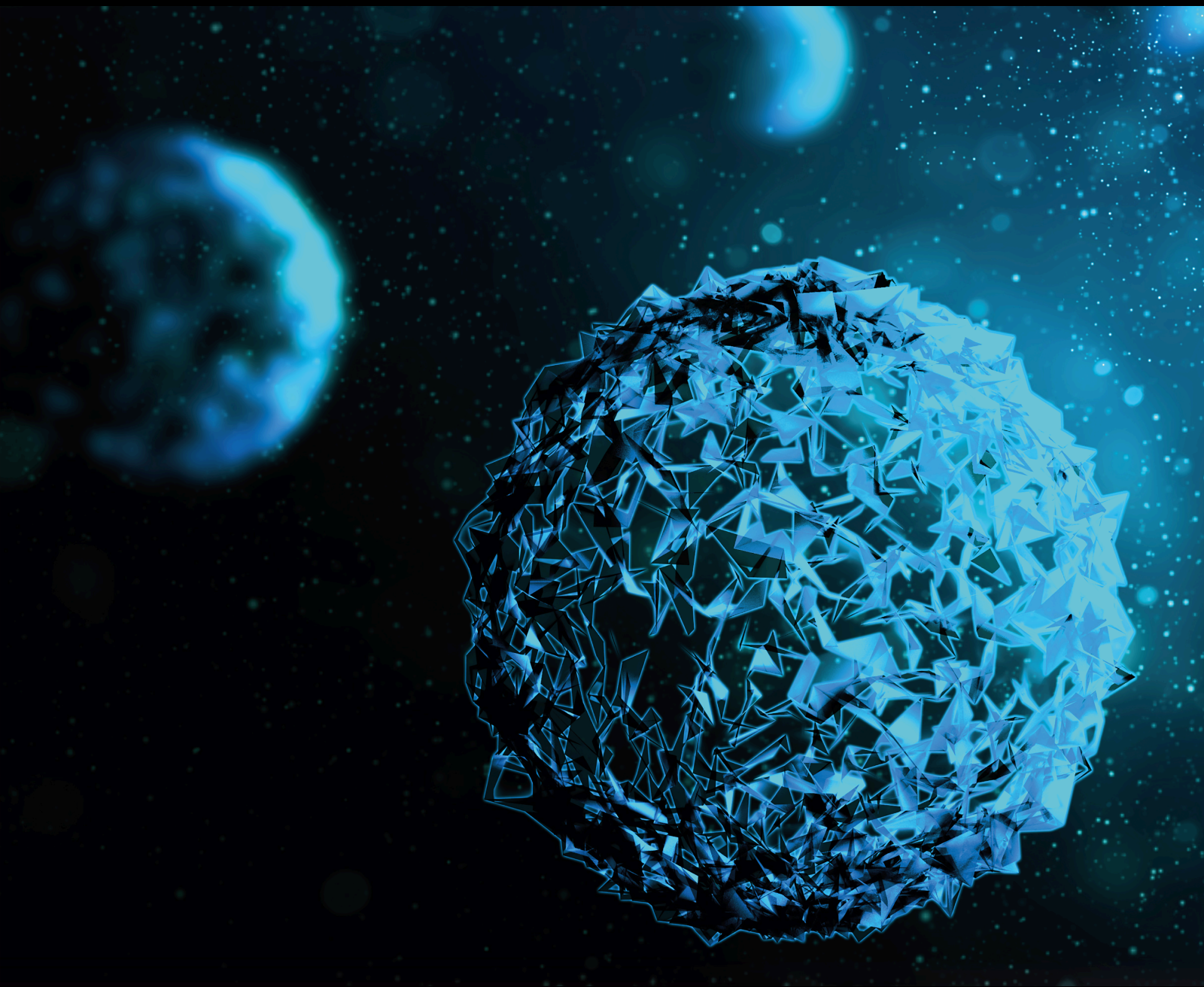
Guest Editors: Moreno Bondi, Andrea Laukova, Simona de Niederhausern, Patrizia Messi, Chrissanthy Papadopoulou, and Vangelis Economou



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Editorial Board

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Editorial

Controversial Aspects Displayed by Enterococci: Probiotics or Pathogens?

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Enterococci are facultative anaerobic, nonspore-forming Gram-positive bacteria belonging to the Lactic Acid Bacteria (LAB) from the phylum Firmicutes. They are tolerant of a wide range of environmental conditions, surviving in extreme temperature, pH, and sodium chloride concentrations, being found in soil, aquatic environment, plants, sewage, foods, and water, and are one of the standard bacterial indicators for the drinking and recreational water quality [1, 2]. Enterococci are colonizing the gastrointestinal tract of humans and animals (including insects and invertebrates) being part of the gut commensal microbiota. Their name comes from the Greek words "entero" ("*έντερο*") meaning "intestine" and "coccus" ("*κόκκος*") meaning "spherical particle," perfectly describing their origin and morphology together [3].

For centuries, selected enterococcal species have been widely used in the production of a variety of fermented and nonfermented food products ranging from dairy and meat products to vegetable and sea foods [4]. Enterococcal strains can produce bacteriocins, some of which are heat-stable peptides with low molecular weight exhibiting remarkable antibacterial activities. Enterococci also have properties that are of technological interest in the food industry, and some strains have been used as probiotics for the maintenance of normal intestinal microbiota, stimulation of the immune system, and improvement of the nutritional value of foods and feeds in humans and animals [5, 6].

However, following the emergence of antibiotic-resistant (AMR) enterococci and particularly of the vancomycin-resistant enterococci (VRE), these microorganisms have turned from generally recognized as safe (GRAS) for human consumption to significant pathogens threatening human health and thriving in the hospital environment. According to World Health Organization (WHO), VREs are pathogens of high priority in the list of microorganisms for which the development of new antimicrobials is an urgent demand [7]. Two species (*E. faecalis* and *E. faecium*) which are widely used as probiotics have been implicated in severe infections of the central nervous system, urinary tract, intra-abdominal and pelvic infections, endocarditis, and bacteremia [8]. Enterococci display important biological traits including the presence of drug-resistant genes, the production of cytolysin, adhesins, invasins, and gelatinase, which contribute to their virulence and ability to colonize tissues [9, 10].

Thus, recently the trend of using enterococci as probiotics for human consumption is in debate due to the controversial aspects of these bacteria which appear to be "friends and foes" [11]. There are published studies reporting that GRAS probiotics are causing infections, but there are no published reports that enterococcal probiotics cause human infections. Hence, taking into consideration the diversity of strains within each bacterial species and the impressive potential of the microorganisms to reorganize their genomes

in their eternal effort for survival, the question whether enterococci are beneficial probiotics or dangerous pathogens is very intriguing and may take long to be answered justifiably.

This special issue contains six articles reporting enterococci isolation from foods and sewage sludge, the presence of virulence genes, the antimicrobial resistance of *E. faecalis* and *E. faecium* isolates from humans and food, and the safety aspects and probiotic properties of *E. faecium* FL31 producing enterocin BacFL31, as well as a review summarizing the pros and cons of enterococci as probiotics and emerging pathogens.

The study by Maasjost et al. reports the presence of virulence genes detected in enterococcal species isolated from meat of turkeys. The isolates belonged to three species (*E. faecalis*, *E. faecium*, and *E. gallinarum*) and were examined for common virulence genes and their phenotypic expression. All isolates were analyzed for five selected putative virulence traits to explore their potential role in the pathogenicity using the chicken embryo lethality assay. The results differ markedly between the three *Enterococcus* species, with *E. faecalis* harboring the majority of the investigated genes and virulence traits. From the results of this study, it is clear that the presence or absence of virulence genes or corresponding phenotypes does not entirely correlate with the isolates' virulence potential and pathogenicity for chicken embryos.

Golob et al. in their study determine and compare the antimicrobial susceptibility and virulence traits of *E. faecalis* and *E. faecium* isolates from human clinical specimens and retail meat (fresh beef and pork). All isolates were investigated for susceptibility to 12 antimicrobials using a broth microdilution method and for the presence of seven common virulence genes using PCR. The results are quite favorable as all isolates were susceptible to daptomycin, linezolid, teicoplanin, and vancomycin with a considerably higher proportion of susceptible isolates from meat compared to clinical isolates (only 1.7% of meat isolates were multidrug resistant compared to 42.6% of the clinical isolates). The findings of this study show that *E. faecalis* and *E. faecium* from red meat most likely do not represent an important source of resistant strains to human consumers.

Laukova et al. report the isolation of four different enterococcal species from trouts in Slovakian water sources. The four species (*E. durans*, *E. faecium*, *E. mundtii*, and *E. thailandicus*) were identified using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS). The hemolytic, gelatinase, and nuclease activity determined by cultural techniques was found negative, while the enzymatic activity tested by biochemical and spectrophotometric methods was acceptable. All strains possessed gene for enterocin A production, and all strains were susceptible to antibiotics which is a very positive finding. This study reports in detail the properties of enterococci isolated from trout shedding more light into species isolated from wild sources.

Another study by Laukova et al. concerns the incidence of virulence factor genes among enterococci isolated from sewage sludge (cow's dung water). Species identification of 24 enterococcal strains by MALDI-TOF-MS allotted 23 strains to the species *E. faecium* with highly probable species identification and *E. faecalis* EEV20 with a score value meaning secure genus identification/probable species identification. Enterococci were absent of *cytolysin A* gene, *hyaluronidase* gene, and *element IS* gene. It is concluded that they were not invasive which is very important from the safety side. According to the results of this study, the most frequently detected gene was adhesin *E. faecium* (*efaAfm*, in 22 *E. faecium* strains and in one *E. faecalis*).

The safety aspects and probiotic properties of *E. faecium* FL31 strain producing enterocin BacFL31 in combination with the aqueous peel onion (*Allium cepa*) extract (APOE) in ground beef meat storage are explored in the study by Mtibaa et al. The biopreservative effect of two natural compounds (bacteriocin BacFL31 and APOE) added alone or in combination was evaluated by microbiological, physicochemical, and sensory analyses during 14 days at 4°C. The results show that the combination of APOE and BacFL31 was significantly more effective than the use of each active compound alone, limiting the microbial deterioration, decreasing thiobarbituric acid-reactive substances, slowing down metmyoglobin (MetMb) and carbonyl group accumulation, delaying the disappearance of sulfhydryl proteins, inhibiting efficiently the microflora proliferation, and indicating that enterocin BacFL31 derived from a safe *Enterococcus faecium* and combined with APOE is a promising natural preservative for ground beef.

Braïek and Smaoui review the pros and cons of enterococci in view of their future use as probiotics and discuss their dual and controversial features between opportunistic pathogens and promising probiotics providing a useful overview of the existing knowledge on their taxonomy, physiological and biochemical traits, habitats, occurrence in different foods, enterocin classification, spectrum and mode of action, pathogenicity, virulence factors, antimicrobial resistance (AR), transfer of virulence factors, and AR genes and finally discuss enterococci as probiotics.

We would like to thank all the authors for their contributions in this special issue and acknowledge all the reviewers for their time spent in assessing the submitted manuscripts. Also, we thank the editorial office of the *BioMed Research* journal for their assistance throughout the completion of this special issue.

Conflicts of Interest

The guest editors declare that they do not have conflicts of interest regarding the publication of the special issue entitled "Controversial Aspects Displayed by Enterococci: Probiotics or Pathogens?"

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Research Article

Enterococci Isolated from Trout in the Bukovec Water Reservoir and Čierny Váh River in Slovakia and Their Safety Aspect

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The aim of this study was to investigate enterococci as lactic acid bacteria and as part of Firmicutes phylum. We focused on the virulence factor, biofilm formation, and antibiotic resistance and also on lactic acid production and enterocin gene detection. Intestinal samples were taken from 50 healthy trout (3 *Salmo trutta* and 47 *Salmo gairdneri*) collected in April 2007, 2010, and 2015 from different locations at the Bukovec water reservoir and the Čierny Váh River in Slovakia. Twenty pure colonies were identified using the matrix-assisted laser desorption/ionization time-of-flight mass spectrometry identification system based on protein fingerprints, and then seven identified strains were also phenotyped. Based on the identification methods used, the identified enterococci (7) belong taxonomically to four different enterococcal species: *Enterococcus durans*, *E. faecium*, *E. mundtii*, and *E. thailandicus*. They were hemolysis, DNase, and gelatinase negative with acceptable enzymatic activity. They did not form biofilm and were mostly susceptible to antibiotics. All strains produced lactic acid amounting to 1.78 ± 0.33 mmol/l on average and possessed the gene for enterocin A production. This is the first study reporting more detailed properties of enterococci from trout in Slovakian wild water sources, and it produces new possibilities for studying microbiota in trout.

1. Introduction

Aquatic sources and/or aquaculture are increasingly used to produce aquatic food all over the world. Fish are mostly reared in two fish farming facilities with a capacity of 140.503 m^3 and in 485 fish pools covering an area of about 2000 Ha [1, 2]. In Slovakia, trout is the most popular food fish, and aquaculture can be classified into two groups: fish farm and lowland wild fish species [2]. In general, the microbiota in trout from fish farms is more studied. There is for instance new information regarding the lactic acid bacteria (LAB) in trout from a commercial fish farm [2, 3], but limited data are available regarding trout from wild sources. Different LAB have adapted to grow under widely different environmental conditions, and they are widespread in nature. Fish are exposed to a wide range of microorganisms present in the environment. Ringø and Gatesoupe [4] demonstrated that the genera *Streptococcus, Leuconostoc*, *Lactobacillus*, *Enterococcus*, and *Carnobacterium* belong to the normal microbiota of the gastrointestinal tract (GIT) in healthy fish. Didinen et al. [3] identified the species *Lactobacillus sakei* and *Lactococcus lactis* subsp. *cremoris* or subsp. *lactis* in rainbow trout from farms in Turkey using 16S rRNA gene sequence analysis. It has also been reported that some LAB isolated from the GIT of fish can act as probiotics [3, 5, 6]. These candidates are able to colonise the gut and act as antagonists against Gram-negative fish pathogens [3, 5]. Some of these bacteria can also produce bacteriocins, i.e., antimicrobial proteinaceous substances. Araújo et al. [7] evaluated enterococci from rainbow trout, their feed, and

rearing environment with inhibition potential against fish pathogens. Enterococci can produce enterocins. Known enterocins are produced mostly by strains representing the species *Enterococcus faecium* [8]. However, also other enterococcal species were detected to produce enterocins [9]. We were focused on enterococci in trout from wild sources. The aim of this study was to check for enterococcal strain benefits in fish such as lactic acid production or *enterocin* genes; we tested for properties of enterococci to contribute to basic microbiology but also to select a potential candidate for inhibiting undesirable bacterial agents in trout. Beneficial strains for use in aquaculture should be regarded as safe, not only for the aquatic hosts but also for their surrounding environment and for humans (consumers) [2, 5]. Finally, our intention was preliminary studying enterococci on virulence factor parameter, biofilm formation, and antibiotic resistance regarding the safety aspect.

2. Materials and Methods

2.1. Sample Collection. Intestines were taken from 50 healthy trout (3 *Salmo trutta* and 47 *Salmo gairdneri*) collected in April 2007, 2010, and 2015 from different locations at the Bukovec water reservoir near Košice in eastern Slovakia and the Čierny Váh River in central Slovakia. They were sampled at the point of collection of the trout, stored at 4°C for approximately 4 h, and transported to the laboratory. After delivery, the samples were treated using the standard microbial dilution method (International Organization for Standardization, ISO); they were stirred (1 : 9) in Ringer solution (pH 7.0, Merck, Germany); appropriate dilutions were plated onto cultivation medium M-Enterococcus agar (Difco, Detroit USA) to count colonies of enterococci. Plates were cultivated at 37°C for 48 h. Grown colonies (those from the highest dilution) on M-Enterococcus agar were randomly picked up and checked for purity by plating on Brain Heart Agar enriched with blood (BHA, Difco, USA) to check their growth—Gram stain morphology; then they were plated for further tests. The Microbank system (Pro-Lab Diagnostic, Richmond, Canada) was used to store identified strains.

2.2. Strain Identification. Twenty pure colonies were identified using the matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) identification system based on protein fingerprints (Bruker Daltonics) [10] and performed using a Microflex MALDI-TOF MS mass spectrometer as described in the previous study by Lauková et al. [11]. A pure single colony from BHA enriched with blood was mixed with matrix (α-cyano-4-hydroxycinnamic acid and trifluoroacetic acid), and the suspension was spotted onto a MALDI plate and ionized with a nitrogen laser (wavelength of 337 nm and frequency of 20 Hz). Results were evaluated using the MALDI BIOTYPER 3.0 (Bruker Daltonics USA) identification database. Taxonomic classification was evaluated on the basis of highly probable species identification (value score 2.300–3.000) and/or secure genus identification/probable species identification (2.000–2.299). Positive controls were those provided in the identification system. Identical colonies evaluated on the basis of MALDI-TOF MS score values were excluded. Finally, seven strains were submitted for further testing.

The strains were also phenotyped using commercial BBL Crystal Gram-positive ID System kit (Becton and Dickinson, Cockeysville, USA); the control strains were those included and recommended in the kit. This kit includes hydrolysis of urea, esculin, and arginine, hydrolysis of enzymes, and utilization of carbohydrates (trehalose, lactose, sucrose, mannitol, fructose, arabinose, etc.). In addition, fermentation of melibiose and galactose was tested.

2.3. Enzymatic Activity (API ZYM) and Lactic Acid Production. To evaluate the functionality/safety of strains, enzymatic activity was tested. The API ZYM tests (BioMériux, France) containing 19 different substrates were used. Suspensions of strains with a turbidity of one McFarland were prepared in 200 μl of sterile distilled water; 65 μl of suspensions was dispensed into each well with substrate. After inoculation, strains were incubated for 4 h at 37°C in incubation boxes provided by the test supplier. Then a drop of ZYM A and ZYM B reagent was added to each well, and reactions were read after exposure to light for a few seconds. The color intensity of the reaction was estimated in the range from 0 to 5 corresponding to the activity from 0 to 40 nmol.

Enterococci belong to lactic acid bacteria of the Firmicutes phylum; for this reason, production of lactic acid (LA) was analysed using the validated spectrophotometric method and measured at 565 nm (Specol 11, Carl Zeiss, Jena, Germany) as previously described by Lauková et al. [12]. This method is based on the conversion of lactic acid to acetaldehyde by heating with sulfuric acid. Acetaldehyde reacts with 4-hydroxybiphenyl, forming a color complex. The LA amount is expressed in millimole per liter (mmol/l).

2.4. Enterocin Gene Detection Using PCR. Some enterococci are known to produce antimicrobial proteinaceous substances possessing genes for their production. In this study, *enterocin* genes for six enterocins were checked: ent A, ent P, ent B, ent L50A, L50B, and ent 31. They were selected based on our previous studies [13]. Primer sequences for PCR amplification of *ents* genes were used according to Aymerich et al. [14], for ent A according to Cintas et al. [15, 16], and for ent P, L50A and L50B, and ent 31 according to De Vuyst et al. [17]. PCRs were carried out using a C1000™ thermal cycler (Bio-Rad Laboratories, Hercules, USA). PCR product was visualized by means of electrophoresis in 2% agarose gels (Sigma-Aldrich) buffered with 1x Tris-acetate-EDTA buffer (Merck) and 1 μg ethidium bromide. Positive control strains were *E. faecium* EK13/CCM7419 [18] for ent A and P and *E. faecium* L50 [15–17] for ent L50B, L50A, and ent 31. A template was added to the reagent mixture (25 μl) containing 1x reagent buffer, 0.2 mmol/l dNTPs (deoxynucleotide triphosphate) (Invitrogen), 1 μmol/l of each primer, 1 U Taq polymerase, template, and water. DNA (template) was

extracted by applying the rapid alkaline lysis method [19]. The cycle for ent A and ent 31 was as follows: denaturation at 95°C for 5 minutes, followed by 30 cycles at 95°C for 30 sec, then 58°C for 30 sec, 72°C for 30 sec, and finally 72°C for 5 min. The cycle for ent P, L50A, B, and L50B differed with the temperature used 56°C instead of 58°C.

2.5. Determination of Hemolytic, Gelatinase, and Nuclease Activity. To exclude virulence of strains, some parameters such as hemolysis, gelatinase, and nuclease activity were tested. Hemolysis activity was tested by streaking the cultures on De Man–Sharp–Rogosa (MRS) agar (Difco, Detroit USA) supplemented with 5% defibrinated sheep blood. Plates were incubated at 37°C for 24–48 h under semi-anaerobic conditions. The presence and absence of clearing zones around the colonies were interpreted as α, β-hemolysis and negative γ-hemolysis, respectively [20].

Gelatinase activity was tested with a 3% gelatin medium (Todd Hewitt agar, Becton and Dickinson, Cockeysville, Maryland, USA) according to Semedo-Lemsaddek et al. [20]. The loss of turbidity halos around colonies of tested strains was checked at 4°C.

Each identified strain was inoculated on the surface of deoxyribonuclease agar (DNase agar, Oxoid, USA). The production of DNase was evaluated after 24 h incubation at 37°C. Colonies producing DNase hydrolysed the deoxyribonucleic acid contained in the medium. After agar flooding and acidifying with 1N HCl (hydrochloric acid), the DNA precipitated, and the medium became turbid with clearing zone formation around DNase-positive colonies.

2.6. Biofilm Formation. The ability of enterococci to form biofilm is a parameter belonging to the group of virulence factors. Biofilm formation was assessed with a quantitative plate assay according to Chaieb et al. [21]. In brief, one colony of the tested strain grown overnight at 37°C on Trypticase soy agar (Difco, Michigan, USA) was transferred into 5 ml of Ringer solution (pH 7.0, 0.75% w/v) to obtain concentration cells in suspension corresponding to 1×10^8 CFU/ml. A volume of 100 μl from that culture was then transferred into 10 ml of Trypticase soy broth (TSY). That standardized culture (200 μl) was inoculated in a well on a polystyrene microtiter plate (Greiner ELISA 12 Well Strips, 350 μl, flat bottom, Frickenhausen GmbH, Germany) and incubated for 24 h at 37°C. The biofilm which was formed in the microtiter plate well was washed twice with 200 μl of deionized water and dried at 25°C for 30 min in an inverted position. The remaining attached bacteria were stained for 30 min at 25°C with 200 μl of 0.1% (m/v) crystal violet in deionized water. The dye solution was aspirated away, and the wells were washed twice with 200 μl of deionized water. After water removal and drying for 30 min at 25°C, the dye bound to the adherent biofilm was extracted with 200 μl of 95% ethanol and stirred. A 150 μl aliquot was transferred from each well and placed on a new microtiter plate for optical density-absorbance (OD-A) determination at 570 nm using a Synergy TM4 Multimode Microplate reader (Biotek, USA). Each strain and condition was tested in two independent tests with 12 replicates. Moreover, a sterile culture medium was included in each analysis as a negative control. *Streptococcus equi* subsp. *zooepidemicus* CCM 7316 was used as a positive control in each method (kindly provided by Dr. Eva Styková, University of Veterinary Medicine and Pharmacy, Košice, Slovakia, [22]). Biofilm formation was then classified as highly positive ($A_{570} \geq 1$), low-grade positive ($0.1 \leq A_{570} < 1$), and negative ($A_{570} < 0.1$).

2.7. Antibiotic Susceptibility or Resistance Testing. Knowing the reaction of strains to antibiotics is one of the diagnostic parameters as well as a factor regarding the safety of strains because of resistance elements. Antibiotic susceptibility/resistance testing in identified enterococci (100 μl of an 18 h culture of each strain) was tested using the qualitative agar disc diffusion method on Columbia agar (Becton and Dickinson) enriched with 10% of defibrinated sheep blood and on Mueller-Hinton agar (Difco) according to Clinical and Laboratory Standards Institute method-CLSI 2016 [23]. Thirteen antibiotic discs (Oxoid, Basingstoke, United Kingdom) were applied: clindamycin (DA-2 μg), novobiocin (Nb-5 μg), ampicillin (AMP-10 μg), penicillin (P-10IU), erythromycin (E-15 μg), azithromycin (AZM-15 μg), streptomycin (STR-25 μg), chloramphenicol (C-30 μg), rifampicin (RA-30 μg), tetracycline (TC-30 μg), vancomycin (VAN-30 μg), kanamycin (KAN-30 μg), and gentamicin (GN-120 μg). After incubation at 35 (37)°C overnight, the strains were evaluated as resistant or susceptible according to the manufacturers' instruction; the inhibition zone was expressed in millimeter. Antimicrobial free agar plates were included as a control for obligatory strain growth. The use of the antimicrobial agents was decided according to the manufacturers' recommendation (Oxoid) and the most relevant antibiotics for enterococci from clinical view. *Enterococcus faecium* CCM 4231 was used as a positive control [24].

3. Results and Discussion

The total enterococcal count from the GIT of 50 trout was 1.40 ± 0.71 CFU/g ($\log_{10}$) on average. Twenty colonies grown on agar (one sample from each) were picked up, and among those 20 colonies, seven strains were finally identified as belonging taxonomically to four different enterococcal species, namely, *Enterococcus durans*, *E. faecium*, *E. mundtii*, and *E. thailandicus*. The rest of the strains were not identified; they represented identical colonies, respectively (they were excluded from further testing). Two strains (both *E. faecium*) were evaluated as reaching a score which corresponded with highly probable species identification (2.300–3.000, Table 1). Five strains were evaluated with scores related to secure genus identification/probable species identification (2.000–2.299, Table 1). Phenotypic properties were compared with those for reference strains in Bergey's Manual of Determinative Bacteriology [25] and according to Tanasupawat and Sukontasing [26], respectively. They showed for instance

Table 1: Species identification, hemolysis, nuclease activity, gelatinase, and lactic acid production in enterococci from trout.

Strain	Species	MALDI score	Hem	DNase	Gel	LA
R36/1	*E. durans*	2.201	ng	ng	ng	1.43 (0.69)
R38/1	*E. durans*	2.148	ng	ng	ng	1.44 (0.69)
R35/1	*E. faecium*	2.409	ng	ng	ng	1.50 (0.66)
R38/2	*E. faecium*	2.400	ng	ng	ng	1.63 (0.62)
R39/2	*E. faecium*	2.182	ng	ng	ng	1.53 (0.65)
R39/1	*E. mundtii*	2.201	ng	ng	ng	1.70 (0.59)
R29/1	*E. thailandicus*	2.273	ng	ng	ng	1.45 (0.69)

MALDI-TOF score: highly probable species identification (value score 2.300–3.000) and/or secure genus identification/probable species identification (2.000–2.299). Hem: hemolysis negative; ng: negative; DNase: deoxyribonuclease, Gel: gelatinase; LA: lactic acid expressed in mmol/l ± SD.

positive reaction for galactose in *E. thailandicus*, *E. faecium*, *E. mundtii*, and *E. durans*. Similarly, positive reaction for xylose was found in *E. mundtii* and negative in *E. faecium* and *E. durans*. Fructose, lactose, and trehalose tests were evaluated as positive. Melibiose was fermented in *E. thailandicus* and *E. mundtii*; on the other hand, melibiose was not fermented in *E. durans*, and a dubious reaction was evaluated in *E. faecium*. Fermentation testing with sorbitol was mostly negative or dubious. Mannitol testing was positive in *E. durans*, *E. thailandicus*, and *E. mundtii* and dubious in *E. faecium*. Maltose was fermented (positive).

Regarding enzymatic activity, our strains showed low or 0 values in relation to trypsin and α-chymotrypsin, but also in relation to β-glucuronidase and other enzymes. All strains reached 10 mmol in the case of naphthol-AS-BI-phosphohydrolase (Table 2). Higher values appeared in the case of β-glucosidase, and the highest value measured, 20 mmol, was in strains EFR39/2 and ETR29/1.

LA production was high (Table 1), 1.53 ± 0.66 mmol/l on average. It was also well balanced, not depending on the species.

Among the six *ent* genes tested, only one-*ent* A gene was confirmed in all enterococcal species (Table 3). Enterococci were free of the other *enterocin* genes tested.

The enterococci detected were free of virulence factor phenotype such as hemolysis; they were DNase negative and gelatinase negative as well (Table 1). Moreover, four strains tested for biofilm formation did not form biofilm (Table 3). The values of absorbance (A_{570}) measured were less than 0.1.

The enterococci were mostly susceptible to the tested antibiotics (ATB) except kanamycin and gentamicin, which are chromosomally encoded in enterococci. This means that the strains EMR39/1, EFR38/2 EF35R/1, and ETR29/1 were monoresistant (Table 4), while *E. durans* EDR38/1was resistant to four ATBs and *E. durans* EDR36/1 was resistant to three ATBs, as well as strain EFR39/2. Strains were also resistant to streptomycin, and two were resistant to TC, Nb, and AZM, one in each strain. All strains were susceptible to VAN, P, AMP, E, C, and RA.

Enterococci are lactic acid bacteria comprising both pathogenic and commensal microorganisms ubiquitous in the environment, even as gut symbionts [27]. Although in fish detected enterococci did not participate in high amount, they are a part of the lactic acid bacteria (LAB); LAB such as lactococci or lactobacilli and also *E. faecium* were previously detected in fish [7, 28]. Detection of four different species among the seven identified enterococci from trout indicated their high species variability. It is interesting that the species detected belong to the same group (*E. faecium*) based on 16S rRNA gene similarity [29]. The MALDI-TOF identification system was successfully applied to the identified bacteria especially for research [30]. In this study, enterococci were detected with high identification score. *E. faecium* or *E. faecalis* are usually the most frequently detected enterococcal species either in the GIT or in the faeces of animals [31, 32]. *E. mundtii*, *E. durans*, or *E. thailandicus* are rarely isolated from animals' GIT. However, Lauková et al. [33, 34] isolated *E. mundtii* from pheasants and ostriches and *E. thailandicus* from the

Table 2: Enzymatic activity in identified enterococcal species (in nmol).

	R36/1	R38/1	R35/1	R38/2	R39/2	R39/1	R29/1
1	ng	ng	ng	ng	ng	ng	ng
2	5	5	5	5	5	5	0
3	10	10	10	5	10	5	10
4	10	10	10	5	10	5	5
5	0	0	0	0	0	5	0
6	5	10	10	5	20	5	0
7	5	0	0	0	0	0	0
8	5	5	0	0	5	5	0
9	0	0	0	0	0	0	0
10	0	0	0	0	0	0	0
11	0	0	5	5	0	0	0
12	10	10	10	10	10	10	10
13	5	5	5	0	5	0	0
14	5	0	0	0	0	5	5
15	0	0	0	0	0	5	0
16	5	0	5	0	0	0	5
17	5	5	10	10	20	10	20
18	0	5	5	5	5	0	5
19	5	0	0	0	0	0	5
20	0	0	0	0	0	5	0

(1) Control, (2) alkaline phosphatase, (3) esterase, (4) esterase-lipase, (5) lipase, (6) leucine, (7) valine, (8) cysteine, (9) trypsin, α-chymotrypsin, (11) acid phosphatase, (12) naphthol-AS-BI-phosphohydrolase, (13) α-galactosidase, (14) β-galactosidase, (15) β-glucuronidase, (16) α-glucosidase, (17) β-glucosidase, (18) N-acetyl-β-glucosaminidase, (19) α-mannosidase, and (20) α-fucosidase.

Table 3: Detection of *enterocin* genes and biofilm formation in enterococci from trout.

Strain	*Ent* gene	Biofilm
EDR36/1	+	0.051 ± 0.03
ED38/1	+	Nt
EFR35/1	+	0.001 ± 0.00
EFR38/2	+	0.020 ± 0.03
EFR39/2	+	0.007 ± 0.02
EMR39/1	+	Nt
ETR29/1	+	Nt

+ refers to presence of *enterocin* A gene. Genes for ent P, B, L50A, L50B, and Ent 31 were not present in tested strains. Nt: not tested.

Table 4: Testing for antibiotic resistance among enterococci from trout.

GM	DA	Nb	AMP	P	E	AZM	STR	KAN	C	VAN	RA	TC
7/7	7/1	7/1	7/0	7/0	7/0	7/2	7/3	7/7	7/0	7/0	7/0	7/2

x/*x*: number of tested strains/number of resistant strains; 0: tested strains were susceptible to antibiotic used; 7/1: seven tested strains and 1 strain was resistant to tested antibiotic; 7/2: out of 7 tested strains, 2 strains were resistant to tested antibiotic; 7/3: out of 7 tested strains, 3 were resistant to tested antibiotic; DA (2 μg): clindamycin; Nb (5 μg): novobiocin; AMP (10 μg): ampicillin; P: penicillin (10 International unit, IA); E (15 μg): erythromycin; AZM (15 μg): azithromycin; STR (25 μg): streptomycin; KAN (30 μg): kanamycin; C (30 μg): chloramphenicol; VAN (30 μg): vancomycin; RA (30 μg): rifampicin; TC (30 μg): tetracycline; GM (120 μg): gentamicin.

faeces of beavers. Moreover, as mentioned above, all detected species belong to the same group (*E. faecium*) based on 16S rRNA gene similarity [29].

Regarding enzymatic activity, the enterococci detected showed zero value in the case of enzymes trypsin and α-chymotrypsin which are usually associated with intestinal disease for example. They did not show protease activity. Moreover, the values of β-glucuronidase were 0 or 5 nmol (in strain EFR39/1), which is beneficial. For example, in humans, β-glucuronidase is an enzyme which can serve as a cancer marker [35]. Following the benefit of enzymatic testing, the values of β-glucosidase were not so high either. However, they were sufficiently high to have beneficial effect, for instance in lactose fermentation. Compared with the enterococci from aquaculture presented by Araújo et al. [7], the enzymatic activity values in this study were lower.

Lactic acid is a metabolic product of enterococci. Similar amounts of LA as in enterococci tested (1.78 ± 0.33 mmol/l, on average) were measured in enterococci isolated from the faeces of pheasants [33]. LA could also contribute to antimicrobial activity in the tested strains.

Among enterocins, only the gene for *ent* A was confirmed in all enterococcal strains. The gene most frequently detected in enterococci is *ent* P followed by e*nt* A [13]. The reason why enterococci have developed ability to produce these antimicrobial peptides (bacteriocins-enterocins) is unknown. It is possible that bacteriocin production is a beneficial probiotic trait in some environments [36]. One surprising point in this study was the detection of *ent* A gene in various species of enterococci, although the one described in the first instance was produced by *E. faecium* [8].

The strains were DNase negative. Similarly, in rabbit faeces, *E. faecalis* and *E. faecium* strains were detected, which were also DNase negative [37]. If enterococci are found to be hemolytic, then α-hemolysis is more typical. However, enterococci in this study did not form hemolysis. In addition, they were also gelatinase negative (but we did not test for the presence of any gene). It could be necessary to test for the occurrence of the *gelE* gene as well because in some cases the gelatinase-positive phenotype does not mean the presence of that gene, as reported for example in enterococci of Pannon White breed rabbits faeces by Lauková et al. [37]. On the other hand, the gelatinase-negative phenotype in a strain can harbour the gene. However, Araújo et al. [7] for instance confirmed the *gelE* gene presence in 46.9% of enterococci (64 strains) isolated from rainbow trout, their feed, and the rearing environment. Biofilm formation is assumed to be a factor of pathogenicity because it can serve as a protective barrier against host defences and the action of antimicrobials; thus, it could be a possible source for persistent infection [38]. However, the enterococci we tested did not form biofilm. On the other hand, it is known that *E. hirae* isolated from various animals produced biofilm [38].

As previously mentioned, enterococci are chromosomally resistant to KAN, which was also confirmed in this study. They were mostly susceptible to ATBs. All were susceptible to VAN, as also previously reported by Migaw et al. [39] in enterococci isolated from Mediterranean fish viscera.

As in every part of present-day life, there is interest in probiotic strains or their products in aquaculture as well. It has been found that beneficial (probiotic) organisms can also improve water quality in aquaculture ponds because probiotic bacteria are able to participate in the metabolizing of organic nutrients in the water [40]. In the next, these enterococci will be tested for their bacteriocin (antimicrobial) activity because they have *ent* A gene. They did not have any of the virulence factors we tested for (using phenotype), which is promising in terms of their possible application. In future, we plan to test the bacteriocin activity of those strains against fish pathogens. However, to confirm their safety, genes for virulence factors will be tested and resistance genes as well. In each case, whether probiotic or bacteriocin activity, this study is original as it provides basic knowledge for subsequent, more detailed studies of individual strains and their antimicrobial substances. These are its contributions to aquatic and aquaculture microbiology.

4. Conclusion

Based on the identification methods used, enterococci belong taxonomically to four different enterococcal species, namely, *Enterococcus durans*, *E. faecium*, *E. mundtii*, and *E. thailandicus*. They were hemolysis, DNase, and gelatinase negative; they did not form biofilm and were mostly susceptible to antibiotics. All strains possessed the gene for enterocin A production, and they produced lactic acid amounting to 1.78 ± 0.33 mmol/l on average with acceptable enzymatic activity. This is the first study reporting in more detail the properties of enterococci from trout, from "wild sources" in Slovakia, and it produces new possibilities for studying microbiota in trout.

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

Conflicts of Interest

The authors declare that there are no conflicts of interest regarding the publication of this paper.

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Research Article

Presence of Virulence Genes in *Enterococcus* Species Isolated from Meat Turkeys in Germany Does Not Correlate with Chicken Embryo Lethality

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Virulence-associated traits have frequently been studied in enterococci and are considered to contribute towards the pathogenicity of infections. In the present study, *Enterococcus* isolates were collected during diagnostic investigations from meat turkeys in Germany. Twenty-eight isolates of three different *Enterococcus* species were analyzed for five selected putative virulence traits to understand their potential role in the pathogenicity using the chicken embryo lethality assay. Ten *E. faecalis*, ten *E. faecium*, and eight *E. gallinarum* isolates were examined for the presence of common virulence genes and their phenotypic expression, namely, the cytolysin operon, five individual *cyl* genes ($cylL_L$, $cylL_S$, *cylM*, *cylB*, and *cylA*), gelatinase (*gelE*), hyaluronidase (hyl_{Efm}), aggregation substance (*asa1*), and enterococcal surface protein (*esp*). The *Enterococcus* isolates showed significant species-dependent differences in the presence of genotypic traits ($p < 0.001$ by Fisher's exact test; Cramer's $V = 0.68$). At least one gene and up to three virulence traits were found in *E. faecalis*, while six *E. faecium* isolates and one *E. gallinarum* isolate did not display any virulence-associated pheno- or genotype. More than half of the *Enterococcus* isolates ($n = 15$) harbored the *gelE* gene, but only *E. faecalis* ($n = 10$) expressed the gelatinase activity *in vitro*. The hyl_{Efm} gene was found in five *E. gallinarum* isolates only, while seven isolates showed the hyaluronidase activity in the phenotypic assay. In Cramer's V statistic, a moderate association was indicated for species ($V \leq 0.35$) or genotype ($V < 0.43$) and the results from the embryo lethality assay, but the differences were not significant. All *E. gallinarum* isolates were less virulent with mortality rates ranging between 0 and 30%. Two *E. faecalis* isolates were highly virulent, harboring the whole *cyl*-operon as well as *gelE* and *asa1* genes. Likewise, one *E. faecium* isolate caused high embryo mortality but did not harbor any of the investigated virulence genes. For the first time, *Enterococcus* isolates of three different species collected from diseased turkeys were investigated for their virulence properties in comparison. The results differed markedly between the *Enterococcus* species, with *E. faecalis* harboring the majority of investigated genes and virulence traits. However, the genotype did not entirely correlate with the phenotype or the isolates' virulence potential and pathogenicity for chicken embryos.

1. Introduction

Enterococci are opportunistic bacterial pathogens that belong to the gastrointestinal flora of mammals and birds. *Enterococcus faecalis* and *E. cecorum* are responsible for the majority of enterococcal infections in poultry. *E. faecalis* is known to provoke amyloid arthropathy in layers [1] and the pulmonary hypertension syndrome in broilers [2]. This species is also a common bacterial cause of increased first-week mortality in chicks [3] and can cause hepatic granulomas in turkey poults [4]. Clinical infections with *E. cecorum* became more prevalent over the past years and are responsible for severe inflammatory lesions of bones, joints, and internal organs in various poultry species including turkeys [5]. Besides animals, enterococci are a common cause of nosocomial infections in humans, and

therapeutic options are impaired by multiresistant strains [6, 7]. The role of livestock in contributing to antimicrobial and multidrug resistance by antibiotic treatment of large numbers of animals raise public health concerns and lead to a particular interest in virulence characteristics of circulating *Enterococcus* strains.

Resistance genes as well as virulence-associated genes are located on plasmids or transposons and can be transferred between different *Enterococcus* species and to other bacteria [8]. Several putative virulence traits have been described in enterococci. Cytolysin is a bacterial toxin with hemolytic activity, encoded by the cytolysin operon consisting of eight genes (*cylR1*, *cylR2*, $cylL_L$, $cylL_S$, *cylM*, *cylB*, *cylA*, and *cylI*) [9]. Gelatinase, encoded by the chromosomal *gelE* gene, is an extracellular zinc endoprotease that enables enterococci to hydrolyze gelatin, collagen, and other small peptides [10]. Enterococcal colonization of host tissues is also facilitated by degradation of hyaluronic acids [11], encoded by the hyaluronidase gene hyl_{Efm} [12], as well as by different adhesins. Aggregation substance, encoded by *asa1* [13], is a group of surface proteins that promotes bacterial adherence to renal tubular cells [14] and internalization by intestinal cells [15], while the enterococcal surface protein, encoded by *esp*, is associated with bacterial biofilm formation [16].

Animal infection experiments showed that some of these virulence traits may increase the pathogenicity of *Enterococcus* strains. In a rabbit model of *E. faecalis* endocarditis, mortality increased significantly in animals infected with bacterial strains that exhibited aggregation substance and cytolysin [17]. Another study indicated that strains with gelatinase activity seem to be more virulent in mice suffering from peritonitis than gelatinase-defective strains [18]. In humans, however, a study showed no association in enterococcal bacteremia cases between 14-day mortality and the presence of gelatinase, hemolysin, and the *esp* gene [19].

The presence of putative virulence genes and their expression *in vitro* do not allow definite conclusions about the virulence potential of bacterial strains under natural conditions [20, 21]. An alternative way to determine the virulence of a microorganism is the embryo lethality assay, which allows correlations with genotypic and phenotypic characteristics. Wooley et al. [22] used this laboratory-based assay as an alternative to chicken challenge models for differentiation of virulent and avirulent *E. coli* strains. A subsequent study of Gibbs et al. [23] aimed to determine whether different virulence traits of *E. coli*, isolated from healthy broilers and from broilers with colibacillosis, were suitable for the prediction of chicken embryo lethality results. Some traits correlated significantly with high embryo lethality; however, this correlation was not 100% based on a single trait. Sturzenhecker [24] used the embryo lethality assay to compare the presence of virulence traits with embryo test results of *Campylobacter jejuni* and *C. coli* isolates from poultry. She found no virulence-associated correlation for toxin- or flagella-producing isolates, whereas low-molecular-weight outer membrane proteins seemed to be characteristic for highly virulent isolates. Two recent studies by Borst et al. [25] and Jung et al. [26] investigated pathogenic and commensal *E. cecorum* isolates to compare the results from the chicken embryo lethality assay. The authors found significantly higher mortality in embryos infected with pathogenic isolates from poultry species and production systems where *E. cecorum* infections cause serious disease outbreaks.

The aim of the present investigation was to characterize 28 isolates of three different *Enterococcus* species collected from diseased turkeys in Germany based on their virulence properties and correlations between their genotype, phenotype, and embryo lethality.

2. Materials and Methods

The present study belonged to a doctoral project (Dr. med. vet.) that aimed to investigate the prevalence, antimicrobial resistance, and virulence of enterococci isolated in 2010 and 2011 from commercial poultry flocks in North Rhine-Westphalia and Lower Saxony, Germany [27, 28]. In the present investigation, twenty-eight isolates from meat turkeys belonging to three different *Enterococcus* species, namely, ten *E. faecalis*, ten *E. faecium*, and eight *E. gallinarum* isolates, were selected for phenotypic and genotypic characterization and determination of the embryo lethality index. The isolates were cultured during disease diagnostics from turkey poults with yolk sacculitis as well as from internal organs of subadult birds (Table 1) and were kindly provided by Poultry Clinics and Laboratory Dr. Pöppel (Delbrück, Germany). The initial bacterial identification was based on the multiplex PCR protocol from the study of Jackson et al. [29] with specific primers targeting the *sodA* gene [30] to differentiate between *E. faecalis*, *E. faecium*, and other *Enterococcus* species. Subsequently, a 16S rRNA gene analysis [31] was used for confirmation and to identify all isolates at the species level.

2.1. Detection of Virulence Genes. The following enterococcal virulence genes were investigated, namely, five cytolysin genes ($cylL_L$, $cylL_S$, *cylM*, *cylB*, and *cylA*), gelatinase (*gelE*), hyaluronidase (hyl_{Efm}), aggregation substance (*asa1*), and enterococcal surface protein (*esp*). All target genes, primer sequences, and the corresponding references are listed in detail in Tables S1 and S2 in Supplementary Materials. Bacterial DNA extraction and multiplex PCR analyses were performed according to the protocols from the study of Vankerckhoven et al. [32]. Two additional PCR assays were conducted for separate detection of the $cylL_S$ gene and the *cyl*-operon according to Camargo et al. [33] and Gaspar et al. [34], respectively. *Enterococcus faecalis* MMH594 (kindly provided by M. Gilmore, *Enterococcus* II initiative, Broad Institute (broadinstitute.org)) was used as a positive control in different PCR assays. Gene-specific products were confirmed by single PCRs and Sanger sequencing at LGC Genomics GmbH, Berlin, Germany.

2.2. Phenotypic Expression of Virulence Traits. All isolates were tested for their hemolytic (cytolysin) activity and gelatinase and hyaluronidase production *in vitro*. The expression of the *cytolysin activity* was tested on agar plates

Table 1: Inoculum concentrations (cfu), virulence genotype, phenotype and results from the chicken embryo lethality assay of 28 *Enterococcus* isolates from meat turkeys in Germany.

ID	Species	Age	Organ	cfu	Detected genes	Virulence traits by genotype	Virulence traits by phenotype	†/*n*	7-day EMR		7-day ESI	
2	*E. faecalis*	Subad	Heart	456	*cyl*-operon, *cylL$_L$L$_S$MBA*, *gelE*, *asa1*	CYL, GEL, ASA	GEL	8/20	40%	+	103	+
5	*E. faecalis*	Poult	Yolk sac	436	*cylL$_S$*, *gelE*, *asa1*	GEL, ASA	GEL	13/20	65%	++	74	++
30	*E. faecalis*	Poult	Yolk sac	500	*cyl*-operon, *cylL$_L$L$_S$MBA*, *gelE*, *asa1*	CYL, GEL, ASA	GEL	19/20	95%	+++	39	+++
50	*E. faecalis*	Subad	Heart	852	*gelE*, *asa1*	GEL, ASA	GEL	7/20	35%	+	111	+
68	*E. faecalis*	Poult	Yolk sac	856	*gelE*	GEL	GEL	15/20	75%	++	53	++
87	*E. faecalis*	Poult	Yolk sac	428	*cylL$_L$L$_S$MA*, *gelE*, *asa1*	GEL, ASA	GEL	2/20	10%	+	127	+
137	*E. faecalis*	Poult	Yolk sac	588	*gelE*	GEL	GEL	6/20	30%	+	103	+
159	*E. faecalis*	Subad	Heart	484	*cylA*, *gelE*, *asa1*	GEL, ASA	GEL	7/20	35%	+	108	+
162	*E. faecalis*	Subad	Heart	684	*cyl*-operon, *cylL$_L$L$_S$MBA*, *gelE*, *asa1*	CYL, GEL, ASA	GEL	16/20	80%	++	50	+++
165	*E. faecalis*	Subad	Heart	772	*cyl*-operon, *cylL$_L$L$_S$MBA*, *gelE*, *asa1*	CYL, GEL, ASA	GEL	16/20	80%	++	52	++
6-I	*E. faecium*	Subad	Air sac	340	none	none	none	2/20	10%	+	127	+
44	*E. faecium*	Subad	Lung	304	none	none	none	0/20	0%	+	140	+
60	*E. faecium*	Subad	Air sac	648	*cylL$_L$L$_S$MA*, *gelE*, *asa1*	GEL, ASA	none	0/20	0%	+	140	+
69	*E. faecium*	Subad	Lung	212	*gelE*, *asa1*	GEL, ASA	none	1/20	5%	+	133	+
73	*E. faecium*	Subad	Air sac	304	*gelE*, *asa1*	GEL, ASA	none	12/20	60%	++	61	++
101	*E. faecium*	Poult	Yolk sac	376	*cylL$_S$A*, *gelE*, *asa1*	GEL, ASA	none	10/20	50%	++	76	++
151	*E. faecium*	Poult	Yolk sac	416	none	none	none	9/20	45%	++	82	++
153-II	*E. faecium*	Poult	Yolk sac	332	none	none	none	14/20	70%	++	48	+++
157-II	*E. faecium*	Subad	Lung	476	none	none	none	6/20	30%	+	103	+
160	*E. faecium*	Subad	Lung	176	none	none	none	7/20	35%	+	95	++
58	*E. gallinarum*	Poult	Yolk sac	288	*hyl$_{Efm}$*	HYL	H, HYL	0/20	0%	+	140	+
77	*E. gallinarum*	Poult	Yolk sac	352	none	none	H, HYL	2/20	10%	+	135	+
79	*E. gallinarum*	Poult	Yolk sac	304	*gelE*	GEL	HYL	4/20	20%	+	122	+
94	*E. gallinarum*	Subad	Lung	288	none	none	none	2/20	10%	+	130	+
118	*E. gallinarum*	Poult	Yolk sac	280	*hyl$_{Efm}$*	HYL	H, HYL	6/20	30%	+	122	+
127	*E. gallinarum*	Poult	Yolk sac	224	*hyl$_{Efm}$*	HYL	H, HYL	3/20	15%	+	134	+
146	*E. gallinarum*	Poult	Yolk sac	404	*hyl$_{Efm}$*	HYL	H, HYL	2/20	10%	+	135	+
156	*E. gallinarum*	Poult	Yolk sac	296	*hyl$_{Efm}$*	HYL	H, HYL	4/20	20%	+	130	+

EMR, embryo mortality rate; ESI, embryo survival index; Subad, subadult; †/*n*, dead/tested embryos; CYL, cytolysin; H, beta-hemolysin; GEL, gelatinase; HYL, hyaluronidase; ASA, aggregation substance; virulence classification: +, less, ++, moderately, +++, highly virulent.

with 5% defibrinated horse blood (Oxoid, Wesel, Germany), incubated at 37°C for 24 to 48 hours. Only beta-hemolysis was assessed as positive reaction. The ability to *hydrolyze gelatin* was tested on nutrient gelatin plates (nutrient agar from Sifin Diagnostics, Berlin, Germany; nutrient gelatin from Oxoid) incubated at 37°C for 24 hours. After incubation, agar plates were stored at 4°C for 12 hours. Gelatin hydrolysis was indicated by clear zones around bacterial colonies [35]. *Hyaluronidase production* was tested on 7% Columbia sheep blood agar (Oxoid) incubated at 37°C for 24 to 48 hours using the *Streptococcus equi* decapsulation test [36]. *Staphylococcus aureus* ATCC 25923 was used as a positive control for phenotypic tests.

2.3. Embryo Lethality Assay. Specific pathogen-free (SPF) hatching eggs from VALO BioMedia GmbH (Osterholz-Scharmbeck, Germany) were used for the experiments. The chicken embryo lethality assay was conducted in 2014 as described by Maasjost [28], following established protocols for the inoculation of the allantoic cavity of ten-day-old chicken embryos [22, 37]. Bacterial growth curve experiments were performed in advance for the three *Enterococcus* species (strains K923/96, K808/97, and ATCC 49573) to adjust the inoculum by standardized optical density (OD) measurements as described by Maasjost [28]. Compared to Rudolph's [37] experiments, an inoculum between 250 and 500 colony-forming units (cfu) per egg was aimed and verified by viable bacterial cell counts. Twenty embryos were infected per isolate.

Ten embryos served as a negative control in each experiment and were inoculated with 200 μl sterile PBS instead to evaluate the potential influence of the inoculation. In four infection experiments, uninoculated eggs at the same stage of incubation were placed into the same egg incubator to control for potential independent errors such as egg quality and environmental conditions.

Eggs were incubated at 37.7°C and 60–70% relative humidity and candled daily for seven days post infection (p.i.) to detect dead embryos. The death of embryos was defined as loss of blood vessels and absence of spontaneous movement [38]. Seven days p.i., all surviving embryos were sacrificed at −20°C for two hours. Dead and killed embryos were randomly examined for gross pathology and enterococcal growth. Samples from the yolk sac and, starting on

day three, from the liver were plated on 7% Columbia sheep blood agar (Oxoid), incubated at 37°C for 24 hours, and evaluated.

Two well-characterized control strains, *E. faecalis* K923/96 (highly virulent) and *E. faecium* K808/97 (less virulent) as described by Rudolph [37], were kindly provided by Lohmann Tierzucht GmbH (Cuxhaven, Germany) and used as positive controls together with the reference strain *E. gallinarum* ATCC 49573 (isolated from the chicken intestine).

The embryo mortality rate (EMR) [22] and the embryo survival index (ESI) [37] were determined and used for virulence classifications (Table 2). The EMR was calculated for 20 infected embryos per isolate as the percentage of embryonic death after seven days. The ESI was determined adding the surviving embryos from 20 inoculated eggs from the first to the seventh day p.i. reaching a maximum value of 140 (20 embryos × 7 days). The classification according to the ESI was not always applied for the control strains because absolute numbers of embryos differed partly because of variable viability of the SPF eggs.

2.4. Statistical Analysis. The data analysis was performed in IBM SPSS Statistics version 25 by using descriptive statistics for nominal data. Fisher's exact test for small sample sizes was applied to assess significant differences ($p < 0.05$) in combination with Cramer's V correlation coefficient to estimate the strength of the association (0 = no association and 1 = perfect association). The categorical variables were as follows: *Enterococcus* species (*E. faecalis*, *E. faecium*, or *E. gallinarum*), presence of virulence traits by genotype (none, one, two, or more traits), age of the turkeys (poults or subadults), EMR (less, moderately, or highly virulent), and ESI (less, moderately, or highly virulent) corresponding to the contents in Table 1.

2.5. Ethical Statement. All experiments were performed in compliance with the animal protection laws of Germany. Experiments utilizing chicken embryos were terminated on day 17 of incubation that means four days prior to hatching [38].

3. Results

3.1. Detection of Virulence Genes. Table 1 lists the virulence genes detected in the 28 *Enterococcus* isolates. The PCR amplicons had the expected size in the gel electrophoreses and were confirmed by Sanger sequencing of short fragments and BLAST analyses (results not shown).

gelE was the most common gene detected in 15 out of 28 isolates, followed by *asa1* (12/28). Between one and up to five different *cyl* genes were found in nine isolates, while the *cyl*-operon was confirmed only in four *E. faecalis* isolates and the control strain K923/96 too. Predominant combinations were *cyl*-operon, $cylL_LL_SMBA$, *gelE*, *asa1* ($n = 3$; K923/96) and $cylAL_LL_SM$, *gelE*, *asa1* ($n = 2$). The hyl_{Efm} gene was exclusively found in *E. gallinarum* ($n = 5$). At least one of the virulence genes was found in *E. faecalis*, while six *E. faecium* isolates, two *E. gallinarum* isolates, and the corresponding control strains (K808/97 and ATCC 49573) were negative in the PCR analyses (Tables 1 and 3).

Table 2: Virulence classification according to the embryo mortality rate (EMR) [22] and embryo survival index (ESI) [37].

EMR (%)	ESI	Virulence
>80	0–50	Highly virulent
41–80	51–100	Moderately virulent
≤40	101–140	Less virulent

3.2. Phenotypic Expression of Virulence Traits. Ten *E. faecalis* isolates and the control strain K923/96 harbored the *gelE* gene and showed gelatinase activity. Four *E. faecium* isolates and one *E. gallinarum* isolate harbored the *gelE* gene too, but phenotypic tests were negative. Similarly, none of the *E. faecalis* or *E. faecium* isolates harboring *cyl* genes or the whole *cyl*-operon showed hemolytic activity (Table 1). Seven *E. gallinarum* isolates and the strain ATCC 49573 showed hyaluronidase activity. Five of the isolates and ATCC 49573 harbored the hyl_{Efm} gene; one isolate had only *gelE*, and one had none of the tested genes. Six *E. gallinarum* isolates and the strain ATCC 49573 were beta-hemolytic too, while none of the *cyl* genes or the *cyl*-operon was detected.

3.3. Embryo Lethality Assay. Concentrations of the inocula ranged between 136 and 856 cfu per egg for the 28 *Enterococcus* isolates under study with a mean of 431 cfu (Table 1). Inocula from the three control strains were within this range except for K923/96 that reached 10^3 cfu in one experiment (Table 3). Dead embryos showed ecchymotic hemorrhages and subcutaneous edema characteristic of sepsis. Bacteriological investigations recovered *Enterococcus* in pure bacterial cultures from all infected dead and killed embryos tested. No embryo mortality was observed for negative controls during the experiments.

The observed EMR of the isolates (vs. the control strain) at day 7 p.i. ranged from 10 to 95% (80–100%) for *E. faecalis* (highly virulent K923/96), 0 to 70% (10–53%) for *E. faecium* (less virulent K808/97), and 0 to 30% (15–60%) for *E. gallinarum* (unclassified ATCC 49573). More than half of the isolates ($n = 18$) were found to be less virulent in both classification schemes, including five *E. faecalis*, five *E. faecium*, and all *E. gallinarum* isolates. Three isolates, two *E. faecalis*, and one *E. faecium* isolates were classified as highly virulent based on their ESI. Inconsistencies between both classification schemes were observed for two of them (*E. faecalis* and *E. faecium*), classified as moderately virulent by their EMR, and for one *E. faecium* that was classified as moderately virulent by its ESI and less virulent by its EMR (Table 1). The control strains showed discrepancies in their virulence classifications in the six repetitions (Table 3).

3.4. Statistical Analysis. The data from this study were analyzed to identify potential correlations between the *Enterococcus* species ("species"), the age of infected turkeys ("age"), and the genotypic presence of virulence traits

Table 3: Inoculum concentrations (cfu), virulence genotype, phenotype and results from the chicken embryo lethality assay (performed in six repetitions) of three control strains.

ID	Species	cfu	Genotype	Phenotype	†/*n*	7-day EMR		7-day ESI	
K923/96	*E. faecalis*	760	*cyl*-operon, $cylL_LL_SMBA$, *gelE*, *asa1*	GEL	20/20	100%	+++	15	+++
K923/96	*E. faecalis*	660			19/20	95%	+++	35	+++
K923/96	*E. faecalis*	376			18/20	90%	+++	45	+++
K923/96	*E. faecalis*	1000			18/20	90%	+++	40	+++
K923/96	*E. faecalis*	384			16/20	80%	++	52	++
K923/96	*E. faecalis*	548			8/10	80%	++	n.d.	n.d.
K808/97	*E. faecium*	352	None	None	10/20	50%	++	73	++
K808/97	*E. faecium*	224			6/20	30%	+	104	+
K808/97	*E. faecium*	144			4/20	20%	+	120	+
K808/97	*E. faecium*	152			2/20	10%	+	130	+
K808/97	*E. faecium*	248			8/15	53%	++	n.d.	n.d.
K808/97	*E. faecium*	168			1/10	10%	+	n.d.	n.d.
ATCC 49573	*E. gallinarum*	544	None	H, HYL	12/20	60%	++	72	++
ATCC 49573	*E. gallinarum*	324			11/20	55%	++	77	++
ATCC 49573	*E. gallinarum*	256			7/20	35%	+	99	++
ATCC 49573	*E. gallinarum*	136			6/20	30%	+	117	+
ATCC 49573	*E. gallinarum*	216			4/20	20%	+	124	+
ATCC 49573	*E. gallinarum*	232			3/20	15%	+	125	+

EMR, embryo mortality rate; ESI, embryo survival index; †/*n*, dead/tested embryos; H, beta-hemolysis; GEL, gelatinase; HYL, hyaluronidase; virulence classification: +, less, ++, moderately, +++, highly virulent; n.d., not determined because of differing absolute numbers of available viable embryonated SPF eggs.

("genotype") or results from the embryo lethality assay ("EMR" or "ESI"). Statistically significant, strong associations were found between the variables "species" ($p < 0.001$ by Fisher's exact test; Cramer's $V = 0.68$) or "age" ($p = 0.011$ by Fisher's exact test; Cramer's $V = 0.595$) and "genotype." Other tested relationships were not significant and revealed moderate or weak associations in Cramer's V statistic. Different analyses are summarized in Table 4; results from the chi-squared test were included for comparison.

4. Discussion

Putative virulence traits have frequently been studied in different enterococci of animal and human origin. They are considered to contribute to the pathogenicity of infections [39–41], but the underlying mechanisms often remain unclear. In the present study, 28 *Enterococcus* isolates from poults and subadult turkeys were investigated for five common virulence traits, namely, the cytolysin toxin, the lytic enzymes gelatinase and hyaluronidase, and the aggregation substance and enterococcal surface proteins. These traits have been selected because they seem to be more prevalent in the clinical course of *Enterococcus* isolates [17, 18, 40]. The aim was to understand genotype-phenotype correlations and their potential role in pathogenicity using the chicken embryo lethality assay.

The *Enterococcus* isolates revealed significant species-dependent differences in the presence of the genotypic traits ($p < 0.001$ by Fisher's exact test; Cramer's $V = 0.68$). The *E. faecalis* isolates harbored the majority of virulence genes investigated in this study. Six of ten *E. faecium* isolates and the corresponding control strain K808/97, however, did not show a virulence genotype or phenotype. Most *E. gallinarum* harbored the hyl_{Efm} gene only but showed beta-hemolytic and hyaluronidase activity. The latter two *Enterococcus* species are mainly concomitant bacteria in poultry diagnostics [42] but important nosocomial pathogens of humans and potential food contaminants [43, 44].

Phenotypic assays were performed for three out of five virulence traits investigating beta-hemolytic (cytolysin) and enzymatic (gelatinase and hyaluronidase) properties. Genotype-phenotype discrepancies were observed for some isolates and are described for the individual traits below. In general, the lack of phenotypic expression despite genetic evidence indicates the presence of variant ("loss-of-function") or silent genes that can be activated under *in vivo* conditions [40, 45, 46]. Certain environmental factors such as temperature, ion concentration, or osmolality of the medium can downregulate the genetic expression and negatively affect the gene product [21, 40, 47]. Moreover, structural changes and divergent or newly acquired genes may account for hemolytic or enzymatic properties of isolates that are negative in PCR analyses [40].

The bacteriocin *cytolysin* has cytotoxic and hemolytic activity [48] and is one of the best-investigated virulence factors in enterococci [34, 49]. Between one and up to five different *cyl* genes were found in seven *E. faecalis* and two *E. faecium* isolates from turkeys, which is in good agreement with results from other studies in poultry [50–52]. Four *E. faecalis* isolates and the control strain K923/96 harbored the *cyl*-operon but did not show beta-hemolysis. In contrast, the majority of the *E. gallinarum* isolates and the strain ATCC 49573 were beta-hemolytic, but the corresponding *cyl* genes could not be confirmed.

The enzyme *gelatinase* catalyses the hydrolysis of proteins from the extracellular matrix [10]. Gelatinase activity

Table 4: Results from the statistical analyses.

Tested variables	Fisher's exact test	Pearson chi-square test	Cramer's V^*
"Species" × "genotype"	$p < 0.001$	25.90, $df = 6$, $p < 0.001$	0.680
"Age" × "genotype"	$p = 0.011$	9.91, $df = 3$, $p = 0.019$	0.595
"Species" × "age"	$p = 0.052$	5.99, $df = 2$, $p = 0.050$	0.462
"Species" × "EMR/ESI"	$p \geq 0.091$	6.78/6.89, $df = 4$, $p \geq 0.142$	0.348/0.351
"Genotype" × "EMR/ESI"	$p \geq 0.202$	9.26/10.14, $df = 6$, $p \geq 0.119$	0.407/0.426
"Age" × "EMR/ESI"	$p \geq 0.686$	1.42/0.34, $df = 2$, $p \geq 0.492$	0.225/0.109

*0 = no association, 1 = perfect association; EMR, embryo mortality rate; ESI, embryo survival index.

may favor bacterial colonization and damage host cells [51, 53]. Although the *gelE* gene is very common in enterococci, its phenotypic expression can be impaired *in vitro* but seems to be predominant in clinical isolates from humans and animals [40]. In poultry species, concurrent gelatinase geno- and phenotypes have frequently been detected in *E. faecalis* from broilers and partridges, and more often than other virulence traits [51, 54–56]. These results are consistent with those from isolates of turkey origin under study. All *E. faecalis* isolates (including K923/96) harbored the *gelE* gene and showed gelatinase activity, while four *E. faecium* and one *E. gallinarum* isolates had silent genes only.

The enzyme *hyaluronidase* is responsible for the breakdown of hyaluronic acids, which facilitates bacterial colonization by decreasing the viscosity of the extracellular matrix [11]. In the present study, the hyl_{Efm} gene was detected neither in *E. faecalis* nor in *E. faecium*, but in five of eight *E. gallinarum* isolates. Interestingly, the strain ATCC 49573 and seven *E. gallinarum* isolates showed hyaluronidase activity, including those five isolates harboring the hyl_{Efm} gene. One of the two remaining isolates was negative in all PCR assays, and the other harbored the *gelE* gene only. The knowledge about *E. gallinarum* and its pathogenic potential is still very limited, and virulence genes have only rarely been investigated so far [57, 58].

Aggregation substance and *enterococcal surface proteins* are likely to facilitate bacterial colonization and persistence in the host by promoting adhesion to surfaces and close bacteria-cell contacts [59]. In poultry, the *asa1* gene has been found in *E. faecalis* and in *E. faecium* isolates [56, 60, 61], whereas the esp gene could not be detected in several studies [51, 52, 62, 63]. In the present study, the *asa1* gene was present in eight *E. faecalis* and in four *E. faecium* isolates isolated from turkeys, while the *esp* gene could not be detected. The *E. gallinarum* isolates did not harbor the adhesin genes.

The 28 *Enterococcus* isolates (Table 1) and three control strains (Table 3) were investigated using the chicken embryo lethality assay to determine their virulence potential based on the embryo mortality rate (EMR) [22] and the embryo survival index (ESI) [37]. In both classifications, half of the investigated *E. faecalis* and *E. faecium* isolates and all eight *E. gallinarum* isolates were of low virulence ($n = 18$). The mean EMR was 55% in *E. faecalis* ($n = 10$), 31% in *E. faecium* ($n = 10$), and 14% in *E. gallinarum* ($n = 8$). A moderate association was indicated in Cramer's V statistic between species ($V \leq 0.35$) or genotype ($V < 0.43$) and the results from the embryo lethality assay, but the differences were not significant (Table 4). These results seem surprising since the isolates were collected from clinically affected birds. Limitations in the availability of embryonated eggs from turkeys only allowed experimental infection of chicken embryos. Because *Enterococcus* isolates were collected from diseased turkeys, this restriction could affect the actual virulence potential and associated EMRs. Enterococci, however, are opportunistic pathogens that require certain conditions to cause disease and may also benefit from coinfections or underlying diseases [20, 64]. In a former study, Rudolph [37] tested 27 *Enterococcus* isolates that originated from joint swabs from brown egg-laying hens suffering from amyloid arthropathy, where *E. faecalis* is the primary pathogen [1]. Eighteen were identified as highly pathogenic, while the remaining isolates varied in their EMRs from 5 to 90% and were of low ($n = 4$) or moderate ($n = 5$) virulence. Another study investigated *E. cecorum* isolates from different sources by inoculation of embryonated SPF eggs from chicken and found animal-specific variations. Pathogenic isolates from poultry species and production systems, where disease outbreaks occur, had higher mean EMRs compared to isolates from birds like turkeys, where disease symptoms only appear sporadically [26].

Several factors (e.g., infection route, embryo quality, and age at inoculation) are known to influence embryo lethality results and impair comparability (summarized in [65]). Previous studies, however, were often not able to draw definite conclusions about whether the infective dose influences the response. These experiments included enterococci [37, 66] but also a variety of other bacteria (e.g., [38, 67, 68]). A very recent study by Blanco et al. [65] used *E. faecalis* K923/96 only and with different inoculum concentrations to define the median lethality dose in chicken embryos. The authors found strong positive correlations between the infective dose, the EMR, and the embryo survival time that should be considered for future experiments. The strain K923/96 originated from a chicken with amyloid arthropathy and was highly virulent in seven repetitions in the study by Rudolph [37]. This strain served as the control in the present experiments too, which were conducted in 2014 with equivalent inoculum concentrations. In two out of six repetitions, the strain K923/96 reached the upper classification limit for moderately virulent strains with an EMR of 80% (Table 3). Likewise, the second control strain K808/97 showed moderate virulence in two out of six repetitions with an increased EMR of about 50% but was defined as less virulent in Rudolph's experiments [37]. The inocula were with 248 and 352 cfu slightly higher than those in the other four repetitions but reached the intended range

of 250 to 500 cfu in this study and were similar or less concentrated as those from the study of Rudolph [37]. The ATCC 49573 reference strain also varied in six repetitions between less and moderate in its virulence classifications. An inoculum-based tendency became apparent for this strain too, but not for K923/96 or the 28 isolates under study.

Differences between the two classification results were noticed for three *Enterococcus* isolates. They were classified more virulent by the ESI than by the EMR by taking the days p.i. into account when embryos died (Table 2). Isolates classified as highly virulent based on their ESI killed more than half (11–14) of 20 inoculated embryos within two days p.i. This initial high mortality is most probably caused by isolate-dependent abilities to grow and invade embryonic tissues, favoring early systemic infections [67]. Ten-day-old chicken embryos do not have a fully developed adaptive immune system to respond to invading pathogens [69].

The isolates from the present study originated from turkey poults with yolk sacculitis ($n = 15$) and from the internal organs of subadult birds ($n = 13$). Commercially raised poults experience different stressors during the first week of life and might be more prone to enterococcal infections compared to subadult birds [70]. Consequently, one would expect that *Enterococcus* infections in subadult turkeys are caused by virulent strains and by predominant species in poultry disease such as *E. faecalis*. The *Enterococcus* isolates from subadult birds belonged mainly to *E. faecalis* ($n = 5$) and *E. faecium* ($n = 7$). Significant age-dependent differences were identified in the presence of genotypic traits ($p = 0.011$ by Fisher's exact test), while Cramer's V statistic also showed age-dependent, moderate to strong associations for the *Enterococcus* species ($V = 0.46$) and the virulence genotype ($V = 0.595$). Eight of 13 isolates from subadult turkeys harbored two or three of the investigated virulence traits, but the identified age differences did not correlate with the results from the embryo lethality assay.

A potential correlation between the isolates' virulence genotype and the pathogenicity for chicken embryos was further investigated independently of the age. Considering the theory of virulence traits conferring pathogenicity, one might expect that a higher virulence potential correlates with the presence of essential genes. Two highly virulent and a moderately virulent *E. faecalis* isolates indeed harbored the whole *cyl*-operon as well as *gelE* and *asa1* just as the control strain K923/96, which could enhance their virulence potential. Another *E. faecalis* isolate, however, had an identical genotype but was less virulent in the embryo lethality assay with comparable inoculum concentrations. This inconsistency became more evident with the highly virulent *E. faecium* isolate that did not show a corresponding geno- and phenotype (Table 1). Similar conclusions have been reached in virulence comparisons of pathogenic and commensal *E. cecorum* isolates assuming species-specific mechanisms [26].

5. Conclusion

Studies on enterococci often search for genes conferring virulence and antimicrobial resistance to identify potential threats to public health. Comparisons, however, reaching beyond and challenging the concept of virulence traits as intrinsic bacterial properties and determinants of pathogenicity [20] are rare but important for studying opportunistic or secondary pathogens. The three *Enterococcus* species under study belong to the intestinal microbiota of poultry but were isolated during disease diagnostics from clinically affected birds. As opportunists, their ability to cause disease in turkeys might rather be influenced by the host and its defense mechanisms than by isolate-specific virulence traits. Indeed, half of the *E. faecalis*, half of the *E. faecium*, and all *E. gallinarum* isolates under study were of low virulence in the chicken embryo lethality assay. The presence of virulence traits differed markedly between the three *Enterococcus* species, with *E. faecalis* harboring the majority of investigated genes. By comparing the results from this study, it became clear that the presence or absence of virulence genes or corresponding phenotypes did not entirely correlate with the isolates' virulence potential and pathogenicity for chicken embryos.

Data Availability

Three partial 16S rRNA gene sequences (765 bp) from three isolates (IDs 2, 6-I, and 58), representing different *Enterococcus* species, have been submitted to the NCBI GenBank and are available under accession nos. MN387238–MN387240, respectively.

Conflicts of Interest

The authors declare that there are no conflicts of interest related to this article.

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Supplementary Materials

Table S1: details on multiplex PCR primers used for virulence gene detection. Table S2: details on primers used for individual PCRs and Sanger sequencing. (*Supplementary Materials*)

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Research Article

Virulence Factor Genes Incidence among Enterococci from Sewage Sludge in Eastern Slovakia following Safety Aspect

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The sewage sludges represent a potential health hazard because of the quantity of different microbiota detected in sewages. Among microbiota detected in sewages, also belong representatives of the phylum *Firmicutes*. In the past, environmental enterococci in addition to coliforms were widely used as indicators of faecal contamination. Regarding the enterococcal strains as potential pathogenic bacteria, their pathogenicity is mainly caused by production of virulence factors. Therefore, the aim of the study was to analyse incidence of virulence factors in enterococci from cows' dung water. Species identification of 24 enterococci using MALDI-TOF MS system allotted 23 strains to the species *Enterococcus faecium* with highly probable species identification and *E. faecalis* EEV20 with a score value meaning secure genus identification/probable species identification. Enterococci were absent of cytolysin A gene, hyaluronidase gene, and element IS gene. It can be concluded that they are not invasive which is very important from safety aspect. The most frequently detected gene was adhesin *E. faecium* (*efaAfm,* in 22 *E. faecium* strains and in one *E. faecalis*). Adhesin *efaAfs* gene was detected in *E. faecalis* EEV20 and in two *E. faecium*. *GelE* gene was present in three strains. *E. faecium* EF/EC31 was absent of virulence factor genes.

1. Introduction

Stabilized animal sewage sludge, including cow's dung water is frequently used for agricultural purposes to farmland application due to its high organic matter content serving, e.g., as a source of nutrients for plants [1]. However, it has brought the hygienic and/or safety aspect into focus. That is, the sewage sludges represent a potential health hazard. The hazards are mainly associated with the amount of different microbiota detected in sewages, especially pathogenic species [2]. Stiborová et al. [1] assessed sewage sludges in Czech Republic through both taxonomic and phylogenetic approaches. There, the bacterial community dominated was affiliated with *Proteobacteria* including the phyla *Deinococcus-Thermus* and *Thermotogae*. The most frequently detected genera in the sludge in Czech Republic were *Mycobacterium* and *Streptomyces* [1]. However, they also detected the phylum *Firmicutes*. In the framework of the phylum *Firmicutes*, Lauková et al. [3] reported representatives of the genus *Enterococcus*. Previously, environmental enterococci in addition to coliforms were widely used as indicators of faecal contamination [4]. Regarding the enterococcal strains as potential pathogenic bacteria, their pathogenicity is mainly caused by production of virulence factors and/or resistance to antibiotics [5]. Therefore, the aim of this study was to analyse incidence of virulence factors in enterococci isolated from cow's dung water (in Eastern Slovakia), which is useful to know from at least two aspects—characteristic and properties of enterococci from different niches in the framework of the basic research and from safety aspect. That is, in sewage sludge could perform possible conjugative transfer or another way of virulence factor gene transfer among strains which can threaten human population.

2. Materials and Methods

2.1. Sampling, Strain Management, and Identification. Bacterial strains tested (24) were isolated from cow's dung water (sewage sludge) as previously described by Lauková et al. [3]. They were

Table 1: Oligonucleotides used for amplification of virulence genes in enterococci isolated form sewage sludge.

Primer	Locus	Sequence (5′-3′)	bp
(1) Cytolysin	*cylA*	F:TAGCGAGTTATATCGTTCACTGTA R:CTCACCTCTTTGTATTTAAGCATG	1282
(2) ESP[a]	*esp*	F: TTGCTAATGCTAGTCCACGACC R: GCGGTCAACACTTGCATTGCCGAA	933
(3) Adhesin EE[b]	*efa*Afs	F:GACAGACCCTCACGAATA R:AGTTCATCATGCTGTAGTA	705
(4) Adhesin EF[c]	*efa*Afm	F: AACAGATCCGCATGAATA R:CATTTCATCATCTGATAGTA	735
(5) Gelatinase	*gelE*	F:ACCCCGTATCATTGGTTT R:ACGCATTGCTTTTCCATC	419
(6) Hyaluronidase	*hyl*Efm	F: GAGTAGAGGAATATCTTAGC R: AGGCTCCAATTCTGT	661
(7) Element IS16	*IS16*	F:CATGTTCCACGAACCAGAG R:TCAAAAAGTGGGCTTGGC	547

(1) Semedo et al. (2003), (2) [a]enterococcal surface protein, Eaton and Gasson (2001), (3) [b]adhesin *Enterococcus faecalis*, Eaton and Gasson (2001), (4) [c]adhesin *Enterococcus faecium*, Eaton and Gasson (2001), (5) Eaton and Gasson (2001), (6) Klare et al. (2005), (7) Werner et al. (2011).

collected from the basins of 25 cattle farms in 15 North-Eastern Slovakian districts. Forty-five samples were transported in bottles and analysed. They were treated according to ISO (International Organization for Standardization), diluted in Ringers solution (Oxoid), and appropriate dilutions were spread plated onto M-Enterococcus agar (Becton and Dickinson, Cockeysville, USA). Incubation was performed in a CO_2 atmosphere at 37°C for 24–48 h. Isolated strains were phenotyped with the API 20 Strep system (API, Biomerieux, L'Etoile, France). Then, they were stored using a freeze dryer (MicroModulyo,Thermo corp., Asheville, Nebraska, USA). However, before testing of virulence factor genes, strains were re-covered in MRS broth (De Man-Rogosa-Sharpe, Merck, Germany) by cultivating at 37°C for 24 h following their plating on Brain heart agar enriched with sheep's blood (5%), and then plated on M-Enterococcus agar (Difco, Detroit, Michigan, USA). Pure strains were stored for next analyses with the Microbank system (Pro-Lab Diagnostic, Richmond, Canada).

Besides phenotypization, species identification was performed using matrix–assisted laser desorption ionisation time-of-flight mass spectrometry (MALDI-TOF MS, Bruker Daltonics, Billerica, Maryland, USA); [6], meaning Biotyper™ identification system (Bruker Daltonics, USA [7]). This system is used especially for research microbiology. The method is based on analysis of bacterial proteins (fingerprints) using a Microflex MALDI-TOF mass spectrometer. Briefly, a single colony from M-Enterococcus agar was mixed with the matrix (α-cyano-4-hydroxycinnamic acid and trifluoroacetic acid) and the suspension was spotted onto a MALDI plate and ionized with a nitrogen laser (wave-length 337 nm, frequency 20 Hz). Lysates of bacterial cells were prepared according to the producer's instructions (Bruker Daltonics). The results were evaluated using the MALDI Biotyper 3.0 (Bruker Daltonics) identification database. Taxonomic allocation was evaluated on the basis of highly probable species identification-score 2.300–3.000, then secure genus identification/probable species identification (value score 2.000–2.299) and probable genus identification associated with score value of 1.700–1.999. Positive controls were *Enterococcus faecium* CCM4231 [8] and *Enterococcus faecalis* DSM 20478 (Bruker Daltonics database 2008).

2.2. Detection of Virulence Factor Genes. Genes for seven virulence factors were screened using PCR amplification with the primers and conditions reported by Kubašová et al. [9]. Genes for the following virulence factors were controlled: *gelE* (gelatinase), *esp* (enterococcal surface protein), *efaAfm* (adhesin *E. faecium*), *efaAfs* (adhesin *E. faealis), cylA (*cytolysin A*), hylEfm* (hyaluronidase*), IS16* (element IS, Table 1). The PCR products were separated by agarose gel electrophoresis (1.2% w/v, Sigma-Aldrich, Saint Louis, USA) containing 1μl/ml ethidium bromide (Sigma-Aldrich) using 0.5x TAE buffer (Merck, Darmstadt, Germany PCR fragments were visualized by UV light. The positive controls were the strains *E. faecalis* 9Tr1 (our strain), *E. faecium* P36 (Dr. Semedo-Lemsaddek, University Lisbon, Portugal) and *E. faecium* UW 9086 provided by Dr. Klare from Robert Koch University, Germany). Briefly, the PCRs were carried out in 25 μl volume, the mix consisted of 1x reaction buffer, 0.2 mmol/l of deoxynucleoside triphosphate, 3 mmol/l $MgCl_2$, 1 μmol/l of each primer, 1 U of Taq DNA polymerase, and 1.5 μl of DNA template with the cycling conditions previously reported by Kubašová et al. [9].

3. Results and Discussion

Species identification of 24 enterococcal strains, done using MALDI-TOF MS system alloted, 23 strains to the species *E. faecium* and one strain to the species *E. faecalis*. Thirteen strains of 23 *E. faecium* (56.5%) were identified with a score value ranging from 2.300 to 3.000, meaning highly probable species identification. Ten strains (43.5%) were alloted to the species *E. faecium* with a score value ranging from 2.000 to 2.299, indicating secure genus identification/probable species

Table 2: Score value and virulence factor genes detected in enterococci isolated from sewage sludge.

Strains	Score value	Virulence factor genes		
		gelE	*efaAfm*	*efaAfs*
EF/KK1	2.339	−	+	−
EF/EC2	2.280	−	+	−
EF/EE3	2.170	−	+	−
EF3A	2.369	−	+	−
EF/EC5	2.366	−	+	−
EF/EEV6	2.291	−	+	−
EFP7	2.356	−	+	−
EF9	2.418	−	+	−
EFV10	2.299	+	+	−
EF/EE11	2.399	−	+	−
EF20	2.329	+	+	+
EF/EA21	2.310	−	+	−
EF/ED21	2.077	−	+	+
EF/SA25	2.239	−	+	−
EF/EC31	2.349	−	−	−
WF/EC32	2.300	−	+	−
EF/EC45	2.250	−	+	−
EF/EC46	2.112	−	+	−
EF/EC47	2.202	−	+	−
EF/EC48	2.200	−	+	−
EF11697	2.353	−	+	−
EF1421198	2.367	−	+	−
EF34697	2.379	−	+	−
EEV20	2.281	+	+	+

EF-*Enterococcus faecium*; EE-*E. faecalis*; gelE-gelatinase; efaAfm−; efaAfs−, − it means no gene detected; +, it means, gene was detected; score value in MALDI-TOF mass spectrometry results evaluation.

identification. The strain *E. faecalis* EEV20 was alloted taxonomically with a score value ranging from 2.000 to 2.299 (Table 2).

All tested enterococci from sewage sludge were absent of cytolysin A-*cylA* gene, hyaluronidase—*hylEfm* gene and element IS (*IS16*) gene. The most frequently detected virulence factor gene was adhesin *E. faecium (efaAfm)*. This *efaAfm* adhesin gene was present in 22 *E. faecium* strains and even in one *E. faecalis* strain (EEV20, Table 2); altogether in 95.8% strains out of all tested. Only *E. faecium* EF/EC31 strain was absent of *efaAfm* gene. On the other hand, adhesin *efaAfs* gene was detected not only in *E. faecalis* strain EEV20 but also in two *E. faecium* strains EF20 and EF/ED21. *GelE* gene was present in three strains, two *E. faecium* (EFV10 and EF20) as well as in the strain *E. faecalis* EEV20 (Table 2). Regarding the strains, only one strain-*E. faecium* EF/EC31 was absent of virulence factor genes. *E. faecium* EFV10 had *gelE* gene and *efaAfm* gene; *E. faecium* EF/ED21 had also two virulence factor genes: *efaAfm* and *efaA*fs. Three genes (*gelE, efaAfm, efaAfs*) were detected in the strains *E. faecium* EF20 and *E. faecalis* EEV20.

Mass spectrometry is an analytical technique in which chemical compounds are ionized into charged molecules and the ratio of their mass to charge (m/z) is measured. The development of electron spray ionization (ESI) and matrix assisted laser desorption ionization (MALDI) in 1980s increased the applicability of MS to large biological molecules like proteins. In both spectrometries, peptides are converted into ions by either addition or loss of one or more than one proton [10]. MALDI-TOF is now considered to be a real alternative for bacterial identification due to the provision of rapid and specific determination analogous to molecular sequencing techniques, with benefit of significant time and cost savings. Comparing previously identified species using phenotypization, *E. faecium* was predominant species [3] as was also confirmed using MALDI-TOF system here. In addition, the species *E. faecalis* was also identified among those strains previously. However, phenotypization also indicated the representatives of the other species such as *E. casseliflavus* and *E. durans* among those strains; this was not confirmed using MALDI-TOF MS but a higher percentage of strains identified belonged to the species *E. faecium*. Our experience confirmed a high identification score using MALDI-TOF MS technology associated with phenotypization not only in species *E. faecium* [11] but also *E. faecalis* [12]. High quality MALDI-TOF mass spectra were also obtained in identification of environmental bacteria [13]. E.g., Cherkaoui et al. [14] compared MALDI-TOF technology with conventional phenotypic identification of clinical bacterial strains and they found high confidence identifications for 680 isolates, of which 674 (99.1%) were correct with phenotypization. So, implementation of MS as an identification strategy would improve its efficacy in further analyses to study additional properties of strains e.g., in the framework of basic research. Only one limitation of MALDI-TOF MS technology is that the spectral database containing peptide mass fingerprints of the type strains has to be upgraded to involve new species [10, 13]. As formerly indicated, potential pathogenicity of enterococci is associated with the presence of virulence factor determinants/genes or virulence factor production. Gene *IS16* is a marker specific for clinical *E. faecium*/*E. faecalis* strains associated with nosocomial infection. None of our environmental enterococci contained *IS16* gene. Similarly, enterococci tested were absent of *hyl* (hyaluronidase) gene. Hyaluronidase acts on hyaluronic acid and increases bacterial invasion [15]; it can play a role in different inflammations in host organism, e.g., ear inflammation. It was described to be a part of a genomic island located on a plasmid and this was shown to be enriched in hospital-associated, polyclonal subpopulation of *E. faecium* strains [16]. Enterococcal surface protein (esp) supports adhesion of bacteria; enterococci tested were also absent of *esp* gene. Similarly, Kubašová et al. [9] described no occurrence or only rare occurrence of those genes in faecal canine enterococci. In addition, faecal rabbits' enterococci from Pannon White breed of rabbits were free of *hylefm* and *IS16* genes [12]. On the other hand, the most frequently detected determinants were those encoding adhesin *efaAfm* (95.8%) which is again similar as reported in canine enterococci by Kubašová et al. [9]. This is also in association with the most frequently detected species *E. faecium* in our study because adhesin *efaAfm* is typical for *E. faecium* species [17]. Cyl A, the cytolysin activator (bacterial toxin with hemolytic activity against eukaryotic cells encoded by *cylA* gene [18]; it can even induce tissue damage. Also this *cylA* gene was not present in tested enterococci. Although our strains were not tested to form a biofilm, we would be interested in further

parameters because these factors not only participate in invasion and colonization of host but may also contribute to biofilm formation [19]. Gelatinase, extracellular metalloprotease is able to hydrolyze gelatin, collagen and hemoglobin, which has contributed to bacterial adherence and biofilm formation [20]. Occurrence of virulence factor determinants can be influenced with a source of tested strain or also with a species; that is, Eaton and Gasson [17] reported that *E. faecium* strains and *E. faecalis* showed significantly different patterns in the incidence of virulence determinants. Abouelnaga et al. [21] found three strains out of 88 from fermented food free from virulence determinants, and 16% strains from unfermented food were free of virulence factor determinants. Because enterococci tested were mostly absent of virulence factor determinants such as *cylA* gene, IS16 element, *hylefm* gene *esp* gene and rare in *efaAfs, gelE* gene detection; it can be concluded that they are not invasive which is very important from safety aspect. It can be supposed that their occurrence in environment did not represent health risk. However, here no antibiotic profile was shown; but resulting from previous studies, they were mostly susceptible to antimicrobials (bacteriocins) [22].

4. Conclusion

To conclude from our results, prevalence of the species *E. faecium* was detected in cow's dung water (sewage sludge from samples in Eastern Slovakia) with few species of *E. faecalis*. Detected strains were, however, mostly absent virulence factor determinants. This indicates that they did not present invasive character and pathogenicity in environment and host regarding safety aspect. Of course, other studies are underway. Moreover, this study is also a contribution to the basic knowledge regarding the environmental enterococci.

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

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Review Article

Enterococci: Between Emerging Pathogens and Potential Probiotics

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Enterococci are ubiquitous microorganisms that could be found everywhere; in water, plant, soil, foods, and gastrointestinal tract of humans and animals. They were previously used as starters in food fermentation due to their biotechnological traits (enzymatic and proteolytic activities) or protective cultures in food biopreservation due to their produced antimicrobial bacteriocins called enterocins or as probiotics, live cells with different beneficial characteristics such as stimulation of immunity, anti-inflammatory activity, hypocholesterolemic effect, and prevention/treatment of some diseases. However, in the last years, the use of enterococci in foods or as probiotics caused an important debate because of their opportunistic pathogenicity implicated in several nosocomial infections due to virulence factors and antibiotic resistance, particularly the emergence of vancomycin-resistant enterococci. These virulence traits of some enterococci are associated with genetic transfer mechanisms. Therefore, the development of new enterococcal probiotics needs a strict assessment with regard to safety aspects for selecting the truly harmless enterococcal strains for safe applications. This review tries to give some data of the different points of view about this question.

1. Introduction

In recent years, probiotics are being consumed increasingly. Several studies have shown that probiotics, viable microorganisms, are known for their beneficial health effects in human and animal such as immune system strengthening, metabolic disorder reduction, and feed digestibility improvement [1].

In order to screen and select microbial strains with probiotic abilities, the Food and Agriculture Organisation (FAO) and World Health Organisation (WHO) have established some basic criteria, such as the examination of tolerance to the orogastrointestinal transit, production of antimicrobial substances and antibiotic susceptibility, adherence to human intestinal mucosa, and desired immunomodulation activity [1]. Previously, only lactic acid bacteria (LAB) isolated from human gastrointestinal tract were recommended by FAO and WHO for human use [2]. However, many research studies showed that some strains isolated from animals, fermented or nonfermented food products, could be potential candidates to be used as promising probiotics for humans and animals [2]. Among several microorganisms, LAB are popular as probiotic candidates due to their being generally recognised as safe status (GRAS). Bacteria belonging to the genera *Bifidobacterium* and *Lactobacillus* are more commonly used in the fermented food production. Nevertheless, probiotic potential of several other genera of LAB, such as *Aerococcus, Carnobacterium*, and *Enterococcus*, were also explored, due to their technological advantage in the food industry and their health-promoting properties [3]. *Enterococcus*, one of the main genera belonging to the LAB group with nearly 50 species, could include strains that are known to be opportunistic microorganisms causing several diseases in humans [4].

In addition, many recent studies have demonstrated an alarming increase in multidrug resistant enterococci, particularly vancomycin-resistant strains and their ability to acquire and transfer antibioresistance genes and virulence factors [5].

Hence, based on these findings, the use of enterococci as probiotics generates serious concern leading to the need of deep research studies to better understand the pathogenicity of these versatile microorganisms and elaborate urgent and accurate measures to distinguish safe strains and select them as efficient probiotics.

The main aims of this review are to summarise the pros and cons of enterococci in view of their future use as probiotics and discuss their dual and controversial features between opportunistic pathogens or promising probiotics.

2. General Characteristics of Enterococci

2.1. Taxonomy. Enterococci are Gram-positive cocci that occur in pairs or short chains, nonspore forming, catalase and oxidase-negative, and facultative anaerobic [6, 7]. The genus *Enterococcus* belongs to lactic acid bacteria (LAB) and represents the third-largest LAB genus after *Lactobacillus* and *Streptococcus* with 37 species classified based on phylogenetic assessment using 16S rRNA sequencing and DNA-DNA hybridisation [3]. Indeed, new species have been recently discovered such as *E. thailandicus*, *E. ureasiticus*, *E. pallens*, *E. caccae*, *E. cammelliae, E. lactis*, etc. [8–12]; however, *E. faecium* and *E. faecalis* remain the most important enterococcal species. Taxonomically, enterococci were classified separately in 1984 [13] after being described as streptococci. Some authors recommend revising the classification of some taxa because of insufficient differences between them to be described as separate species such as *E. flavescens* and *E. casseliflavus* or to regroup species due to similar characteristics such as the case for *E. avillorum* and *E. porcinus* [14].

2.2. Physiological and Biochemical Traits. Enterococci are mesophilic bacteria that could grow from 10°C to 45°C with optimal temperature comprised between 30°C and 35°C [15, 16]. Also, they are able to grow in a huge range of pH from 4.4 and 9.6 and in hyper salty media with 6.5% NaCl. Traits that differentiate enterococci from streptococci are their abilities to survive after 30 min of heating at 60°C, to grow in broth supplemented with 40% of bile salts and to hydrolyse esculin [17, 18].

2.3. Habitat. Enterococci are ubiquitous microorganisms that could be present in different environments such soil, water, sewage and plants. Furthermore, they are known to belong to the commensal microbiota of human and animals [19]. Currently, *E. faecalis* predominates the *Entercoccus* species of the gastrointestinal tract followed by *E. faecium*, then *E. durans*, and *E. hirae* [20–22].

2.4. Occurrence in Foods. Enterococci occur in different foods; dairy products (cheeses, raw milk) [23–26], fermented vegetables (olives, fermented sorghum) [27–33], meats, fish, and sea foods [34–38].

2.4.1. Enterococci in Dairy Products. The prevalence of enterococci in milk has been traditionally considered as a result of faecal contamination, but many studies have reported that this occurrence is not always related to faecal contamination [7, 23, 24]. In fact, *Enterococcus* spp. has the capacity of adaptation to diverse substrates and growth conditions. Indeed, enterococci could be present in both raw and pasteurised milk of cow, sheep, goat, or camel [7, 39, 40]. Enterococcal strains examples that have been isolated from raw milk are *E. faecalis* and *E. casseliflavus* [41], *E. lactis* [42], *E. italicus*, and *E. faecium* [43].

Enterococci could also occur in cheeses made from raw or pasteurised milk and were commonly *E. faecium*, *E. faecalis*, *E. durans*, *E. casseliflavus,* and *E. lactis* [41, 44–46]. This prevalence is different among cheeses resulting in cheese type, milk used in the manufacture, production season, and conditions of production, and ripening [47, 48]. Moreover, it is important to denote that *Enterococcus spp*. play a beneficial role in cheese fermentation as well as in cheese ripening and development of specific flavour, texture, and taste probably through proteolytic, esterolytic and lipolytic activities, citrate breakdown and production of diacetyl, and other important volatile compounds [47–51].

2.4.2. Enterococci in Fermented Vegetables. Enterococci can be present in fermented vegetables due to the fermentation reaction with the predominance of *E. faecium* and *E. faecalis* in fermented soya, sorghum, and olives [18, 52–55].

2.4.3. Enterococci in Meat. Since enterococci are part of the commensal microflora of animal gastrointestinal tract, they could thus occur in meat when slaughtering. The common species are *E. faecium*, *E. faecalis*, *E. mundtii*, *E. durans*, *E. casseliflavus*, *E. gilvus,* and *E. hirae* [56–58]. Fermented salamis and sausages could also host enterococci [59, 60].

2.4.4. Enterococci in Fish and Sea Food. Several enterococcal species have been isolated from fish (viscera and skin): *E. mundtii*, *E. faecium,* and *E. durans* [61–66]. Regarding sea food, the prevalence of enterococci is lower than that in fermented or raw fish [67]. The common isolated strains were *E. faecium*, *E. faecalis, E. casseliflavus*, and *E. hirae* [68]. In regard to fresh shrimps, strains of *E. faecium*, *E. faecalis*, *E. lactis*, *E. casseliflavus*, and *E. gallinarum* have been isolated and reported in many studies [69–72].

3. Enterocins

3.1. Classification. Enterocins are the bacteriocins produced by *Enterococcus* spp. They are ribosomally synthesised, cationic, hydrophobic, and heat stable peptides with small molecular weight containing about 20-60 amino acids [19, 37, 66, 74–77]. They are insensitive to rennet and stable over a wide range of pH values [78, 79]. They are classified into four classes: lantibiotic enterocins (class I) such as cytolysin, nonlantibiotic enterocins (class II) with three subclasses (1, 2, and 3) such as enterocin A (class II-1), enterocin Q (class II-2), and enterocin B (class II-3), followed by cyclic enterocins (class III) such as enterocin AS-48 and enterocins with high molecular weights (class IV) such as enterolysin A [73].

Table 1: Classification of enterocins [73].

Class	Sub-class	Sub-group/ Characteristic	Examples
Class I	Lantibiotic enterocins	Heamolytic bacteriocins Formed by two peptides cylLs and cylLL Their action needs the presence of the two peptides	Cytolisin
Class II, small nonlantibiotic peptides	*II.1* possesses a cationic and hydrophile region with consensus sequence YGNGV in the N-terminal extremity and a disulphide bridge formed by two cysteins in the N-terminal extremity	*Sub-group 1* possessesan ABC transporter for the secretion of enterocins	Enterocin A, Enterocin CRL35
		Sub-group 2 The production is realised via a mature pre-protein	Enterocin P, Enterocin SEK4, Bacteriocin 31, Bacteriocin T8
	II.2 synthesised without leader peptide, did not possess the consensus sequence, nor the system of secretion ABC transporter	*Sub-group 1* Monomeric proteins	Enterocin RJ-11, Enterocin Q, Enterocin EJ97
		Sub-group 2 Need for the formation of an heterodimeric complex	Enterocin L50, Enterocin MR10
	II.3	Linear enterocins with leader peptide	Enterocin B, Bacteriocin 32 Enterocins1071 A and B
Class III, cyclic enterocins		Cyclic peptides	Enterocin AS-48 Enterocin AS-48 RJ
Class IV, proteins of high molecular weight		Peptides of high molecular weight (34.5 kDa) and heat-labiles	Enterolysin A

Table 1 represents with details the enterocins' classification. Most of the characterised enterocins belong to the class II.

The hemolytic bacteriocin (cytolysin) and the circular AS-48 were known as *E. faecalis* bacteriocins and were genetically and biochemically well characterised [80–84].

The subclass II.1 represents the largest enterocin subclass which includes the most abundant enterocins of enterococci. These enterocins share the consensus sequence YGNGV in their N-terminal part which is a prerequisite for their antimicrobial activity and particularly antilisterial activity. It is important to note in this context that enterocin A is among the most potent antimicrobial bacteriocin in this subclass [85–89]. Interestingly, enterocin A is known to be coproduced with other bacteriocins, often in combination with enterocin B [90] and occasionally with enterocin P, enterocins L50, or enterocin Q [91, 92]. Hence, enterococci seem to have the genetic capacity to produce more than one enterocin, as commonly observed among some other multiple-producing bacteriocin lactic acid bacteria (LAB) [93–95].

3.2. Spectrum of Action. Enterocins produced by enterococci are small antimicrobial peptides known to display broad-spectrum of inhibitory activity against spoilage bacteria and foodborne pathogens [96–99]. Remarkable antimicrobial inhibitions were observed towards *Listeria monocytogenes*, *Bacillus cereus*, *Staphylococcus* spp., and *Clostridium* spp. [71, 78, 79, 83, 97, 100]. Antagonistic activities against Gram-negative bacteria such as *Pseudomonas aeruginosa*, *Escherichia coli*, and *Vibrio cholera*, against fungi and yeasts, as well as against virus, were also observed with enterocins [66, 101, 102].

3.3. Mode of Action. Enterocins, as most bacteriocins, have the cytoplasmic membrane as their primary target [103–106]. They form pores in the cell membrane, thus depleting the transmembrane potential and/ or the pH gradient which result in the leakage of indispensable intracellular molecules [107–109]. The mode of action enterolysin A is quite different from the other enterocins because it attacks susceptible bacteria by degrading the cell wall structure, which eventually leads to lysis of the cells of target strains [110].

4. Pathogenicity of Enterococci

Enterococci are among the most common nosocomial pathogens that could cause important infections and diseases such as endocarditis, bacteremia, urinary, intra-abdominal and pelvic infections, central nervous system infections, etc. [4]. Among these infections, approximately 80% were associated with *E. faecalis* [111]. Enterococci, previously viewed as microorganisms of minimal clinical impact, have emerged now as common opportunistic pathogens of humans [112].

Traits implicated in their pathogenicity are virulence factors and the increase of antibiotic resistant strains, especially vancomycin-resistant enterococci (VRE) [5, 113, 114]. As a result, *Enterococcus* spp. represent a main challenge to health

staff when identified as the principal cause of infection or illness, particularly in immunocompromised patients [115]. Infections caused by enterococcal strains are originated from the intestinal microbiota of the patient and can be transferred from one person to another or can be acquired by the consumption of contaminated food and water [116]. *Enterococcus* spp. is capable of transferring the antibiotic resistant genes (ARG) to produce β-haemolysis, gelatinase and aggregation substance that are common enterococcal virulent traits [117].

4.1. Virulence Factors. A virulence factor is an effector molecule that enhances the capacity of a microorganism to cause illness. Virulence factors of enterococci play a significant role in the pathogenicity of enterococcal strains. These factors have been intensively investigated in the last few years. The most common and well described virulence determinants in enterococci are aggregation substances (*agg*, *asa1*), cytolysin (*cyl*), gelatinase (*gelE*), extracellular surface protein (*esp*), adhesion to collagen (*ace*, *acm*), and adhesion-like endocarditis antigens (*efaAfs* and *efaAfm*) [118].

Aggregation substances (*agg* and *asa1*) are virulence factors inducing surface protein of *Enterococcus* spp. strains which promote aggregate formation during bacterial conjugation and mediate the specific binding to epithelial cells for colonisation and exchange of plasmids carrying virulence traits and antibiotic resistance genes as well [119, 120]. In addition, the aggregation substances could bind to extracellular matrix proteins such as collagen type I, fibronectin, and thrombospondin [3]. Regarding *agg* gene increases the hydrophobicity of the enterococcal surface inducing localisation of cholesterol to phagosomes and delaying fusion with lysosomal vesicules [121]. Up to date, *agg* determinant is exclusively found in *E. faecalis* strains [122, 123].

Cytolysin (or β-haemolysin) is known as protein bacteriocin/heamolysin bifunctionality and is the most studied virulence factor in enterococci. It constitutes a peptidic toxin able to lyse cells by forming pores in the cytoplasmic membrane of bacterial target cells [124]. The frequency of death caused by infection due to a cytolysin-producing *Enterococcus* is five times higher than that observed in a noncytolysin-producing enterococcal infection [125]. Studies on endocarditis have shown that there is a synergism between *cyl* and *agg* genes.

Gelatinase is an extracellular Zn-metallo-endopeptidase (EC 3.4.24.30) implicated in the hydrolysis of gelatin, collagen, β-insulin, haemoglobin, casein, and other bioactive peptides [126]. Gelatinase is able to cleave fibrin and damage host tissue allowing thus bacterial migration and spread which raise its implication in virulence of enterococci particularly *E. faecalis* [3]. Furthermore, this protease plays an important role in the formation of biofilm which allows enterococci to colonise tissues and persist in some infection sites [126]. It is necessary to mention that some researchers reported that even when the *gelE* determinant gene is detected, a negative phenotype could be found [127, 128].

Extracellular surface protein (*esp*) is a virulent gene determinant associated with the cell-cell adhesion, particularly adhesion to eukaryotic cells and evasion of the immune response of the host [129, 130]. This gene, which promotes colonisation, is located in a highly conserved chromosome region within the genus and is mostly common in *E. faecium* [129, 130].

The adhesion genes to collagen, *ace,* and *acm*, of *E. faecalis* and *E. faecium*, respectively, bind to collagen types I and IV enhancing virulence strains, while *acm* could also bind to laminin [3]. Also, the adhesion *acm* is known to be part of the subfamily of bacterial adhesions surface called Microbial Surface Components Recognising Adhesive Matrix Molecules (MSCRAMM) that adhere specifically to the protein layer of the extracellular matrix of the host [129, 130].

The *efaA* virulence gene is strongly involved in endocarditis [3]. The most known are *efaAfs* and *efaAfm* for *E. faecalis* and *E. faecium*, respectively [131].

Other virulence determinants are less identified in enterococci and not well described that are also implicated in enterococcal infections. Among these virulence factors is *sag* gene secreted by *E. faecium* which was able of broad-spectrum binding to extracellular matrix proteins [132]. Another *E. faecium* adhesion called *scm* could efficiently bind to collagen type IV [133]. Furthermore, the *ebp* gene encoding endocarditis and biofilm-associated pili were observed to enhance biofilm formation in *E. faecalis* [134]. Also, the *bee* gene (biofilm enhancer in *Enterococcus*) was shown to confer a high biofilm-forming phenotype to *E. faecalis* [135]. Finally, a further virulence factor nominated *hyl*, encoding a hyaluronidase, was shown to hydrolyse hyaluronic acid with a possible role in translocation [136]. This virulence factor was shown to be associated with antibiotic resistance genes and pilin genes on the plasmid [137].

In general, the incidence of all of these virulence factors was lower in *E. faecium* strains than in *E. faecalis* strains, and the virulence of enterococci could not be explained only by the presence of virulence determinants; antibiotic resistance genes play an imminent role in the pathogenicity of enterococcal strains [3, 138].

4.2. Antibiotic Resistance. Resistance of some enterococci to commonly used antibiotics is another important virulence trait which strongly enhances the pathogenicity of *Enterococcus* spp. by making them effective opportunistic microorganisms in nosocomial infections [139–141]. In fact, continuous exposure to antibiotics and their intensive use in human and veterinary medicines as prophylactic agents or growth promoters, respectively, have provoked increase in the incidence of enterococcal strains resistant to multiple different classes of antibiotics and may be through genetic mutations conferring this antibioresistance of enterococci and enabling their survival. Hence, this drug resistance becomes an important public health concern. Antibiotic resistance in enterococci could be generally produced by target modification, alterations that affect access of the drug to the target or enzymatic drug inactivation [142].

Intrinsic antibiotic resistance of enterococci includes resistance to cephalosporins, sulphonamides, lincosamides, β-lactams, and aminoglycosides, located in the chromosomes [130, 143]. Acquired resistances in enterococci from

other microorganisms, via plasmids or transposons, could be observed toward chloramphenicol, erythromycin, fluoroquinolones, tetracycline, penicillin, ampicillin, aminoglycosides (gentamicin, kanamycin, and streptomycin) and glycopeptides especially vancomycin [142, 144]. In fact, vancomycin resistance is of special concern because VRE were known to cause serious infections and diseases that could not be treated with conventional antibiotic therapy [63, 145]. So, VRE posed a real challenge to clinicians since this antibiotic has traditionally considered the "drug of last resort" in the treatment of enterococcal infections as it is often used to replace penicillin, ampicillin, and aminoglycosides in patients with allergies [146]. For this reason, new drugs were evaluated as alternative candidates to vancomycin such as quinupristin-dalfopristin, oxazolidinones, everninomycins, and daptomycin [143].

At present, there are six known genes of glycopeptide resistance in enterococci: *vanA*, *vanB*, *vanC*, *vanD*, *vanE*, and *vanG*. The *vanA* type is the most important operon characterised by strains with high levels of resistance to vancomycin and teicoplanin and its main reservoir is *E. faecium* [130]. The *vanB* operon induces several levels of vancomycin resistance but not teicoplanin resistance. Only *vanA* and *vanB* genes have the ability to transfer vertically and horizontally and to confer high levels of resistance [130]. The *vanC* determinant induces low level of vancomycin resistance and intrinsic sensitivity to teicoplanin. The *vanD*, *vanE*, and *vanG* operons encode low to moderate resistance to vancomycin [130]. In general, it is interesting to know that *vanA*, *vanB*, *vanD*, *vanE,* and *vanG* genes are considered to be acquired properties, while *vanC* gene is an intrinsic trait of motile enterococci [130].

On the other hand, several studies performed in European and American countries reported that VRE colonisation occurs in the community besides human reservoir; animal, environmental, and food reservoirs could act as community sources for VRE outside the health care setting [143]. In this context, VRE were detected with *vanA* gene cluster in animal husbandry due to the use of avoparcin as a feed additive [143]. Effectively, in 1975 avoparcin was used as growth promoter in Europe, Australia, and several other countries, but was not allowed in the USA and Canada [145]. Interestingly, high level occurrence of VRE was observed in European animal farms; however, no VRE were detected in animal farms in the US [147]. Thus, the use of the glycopeptide avoparcin for animal growth promotion was prohibited in Europe and as a likely result, there was a rapid decline of VRE in European farms but no a total disappear [145]. Many hypotheses were suggested to explain this VRE persistence; the first one reports the fact that the use of macrolide tylosin could coselect for VR since both the resistance determinants are located on the same plasmid or that plasmid addiction systems could be implicated in the retention of the resistance [145].

Furthermore, VRE could also occur in human outside hospitals confirming that a transfer of resistance genes between animal and human or a clonal spread of resistant strains could explain this prevalence. In addition, VRE could reach foods via environmental contamination from different sources; waste water from sewage treatment, livestock faeces, and manure from poultry farms [143, 148].

Other antibiotic resistant enterococci have been found among food animals and environment worldwide. In fact, high gentamicin-, kanamycin-, streptomycin-, tetracycline- and glycopeptides-resistances have been observed among enterococci (*E. faecalis*, *E. faecium*, *E. casseliflavus*, and *E. gallinarum*) isolated from bovine mastitis (80%), chickens (62-64%), pigs (57%), food of animal origin (e.g., white and red meats), uncooked food (e.g., lettuce), sewage, and water [145, 149–151].

In general, the emergence of this high antibiotic resistance in all of these various reservoirs and environments suggests interstrain transmission of resistance genes.

4.3. Transfer of Virulence Factors and AR Genes. Enterococci are known for their genome plasticity [142]. Indeed, they are able to integrate and use some mobile genetic elements like plasmids, transposons, prophages, and insertions sequences allowing them to easily transfer acquired determinants among strains of the same species, or species of the same genus or other pathogenic and nonpathogenic bacteria as well. In this context, enterococcal virulence factors and AR genes are renowned to be associated with some highly transmissible plasmids [127]. Virulence traits and antibioresistance in enterococci were previously reported to be caused by gene horizontal or vertical transfer mechanisms and by ability to receive genetic material [143]. In this context, Coburn et al. [152] demonstrated the horizontal transfer of a 150 kb cluster called "pathogenicity island" (PAI), previously described in *E. faecalis* by Shankar et al. [153] that contain about 100 operons some of which code for virulence genes (toxins, cytolysin, surface proteins, and aggregation). This horizontal transfer of the pathogenicity island was carried by a plasmid in response to pheromones. Regarding resistance to macrolide antibiotics, lincosamides, and streptogramins (MLS), De Leener et al. [154] have demonstrated, through a genetic marker (*ermB*), the horizontal transfer of these AR genes from an *E. faecium* strain of animal origin to a strain of human origin. This mechanism of propagation via the transfer of genetic elements (plasmids and/or transposons) is more important than clonal dispersal of antibiotic resistant strains [155]. These experiments were conducted on animal models and did not take into account the natural environment that strongly influences the transfer of moving elements.

Of concern, transconjugation in which enterococci acquired virulence and AR determinants could represents a real risk to a safe enterococcal strain that is free of these virulent determinants could unfortunately acquire such genes in both of human or nonhuman reservoirs which raises serious worry regarding their safety for use as probiotics.

5. Enterococci as Probiotics

Probiotics are defined as "live microorganisms which when consumed in sufficient amounts, affect beneficially the health of the host." Health benefits that confer probiotic

microorganisms include modulating immunity, enhancing intestinal barrier function, or altering pain perception [1].

Most probiotics are of intestinal origins and belong to the lactic acid bacteria (LAB) particularly to genera of *Bifidobacterium* and *Lactobacillus*, while enterococcal strains are occasionally used [3]. In this context, many studies have been conducted to evaluate the probiotic characteristics of *Enterococcus* strains and clear beneficial and significant health-promoting effects of enterococci were reported [3, 156–160]. Indeed, enterococci were used as probiotics for several purposes and these different applications include pharmaceutical industry, human and veterinary medicines and food industry since some probiotic enterococci could be used in the production of functional foods [1].

In fact, some enterococcal strains such as *E. faecium* M74 and *E. faecium* SF-68 are included as food supplements in several probiotic preparations that have been proved to be effective and safe, such as FortiFlora® and Cernivet® (containing *E. faecium* SF68®, Cerbios-Pharma SA, Switzerland), and Symbioflor® 1 with *E. faecalis* (Symbiopharm, Herborn, Germany) [142, 161, 162].

Enterococcal probiotics can be used in treatment and/or prevention of certain human and animal diseases such as alleviation of irritable bowel syndrome symptoms and antibiotic-induced diarrhea and prevention of different functional and chronic intestinal diseases [163]. Moreover, some enterococci exhibit antimutagenic, anticarcinogenic, hypocholesterolemic, and immune regulation effects [17].

E. durans M4-5 has been found to generate butyrate, short chain fatty acids (SCFAs), that induce significant anti-inflammatory effects and contribute to the integrity of the intestinal epithelium [164, 165].

E. mundtii ST4SA was recently presented as another potential probiotic strain [166] and *E. durans* KLDS 6.0930 has been postulated as a probiotic candidate through lowering human serum cholesterol levels [167].

More recently, the strain *E. durans* LAB18s was recommended useful for use as a source of dietary selenium supplementation [168], while *E. faecium* LCW 44 and *E. durans* 6HL were shown highly potent against Gram-positive [169] and Gram-negative bacteria [169, 170], respectively.

In feed regulation, the European Food Standards Agency (EFSA) authorised certain strains of enterococci for use as silage additive and dietary supplements. For instance, some enterococcal probiotics were included in the group of feed additives for stabilising the microbial communities of the digestive tract in both monogastric and ruminant animals [171]. Strains of *E. faecium* NCIMB 11181 and *E. faecium* DSM 7134 were approved as feed additives for calves and piglets by EFSA. The probiotics *E. faecium* SF68® and *E. faecalis* Symbioflor 1 are also used to prevent or treat diarrhea in pigs, poultry, livestock, and pets [3]. Furthermore, among the claimed advantages of probiotic enterococci is its positive effects on the performance characteristics of the growth and health of farm animals. In this context, feeding pigs with a probiotic *Enterococcus* spp. was found to reduce intestinal pathogens [172]. Likewise, oral administration of *E. faecium* NHRD IHARA by postweaning piglets has increased serum and fecal IgA levels and improved piglets growth [173]. In chickens, *E. faecium* was demonstrated to improve growth, intestinal morphology, and the caecal microbiota homeostasis [174]. *E. faecium* was also reported to enhance the metabolic efficiency and decrease inflammatory responses in broilers [175].

On the other hand, numerous studies have shown the beneficial effects of enterococci in aquaculture. In fact, several works reported a wide spectrum of inhibition by *E. faecium* toward aquatic pathogens including *Yersinia ruckeri, Vibrio harveyi, Streptococcus agalactiae,* and *Aeromonas veronii* [176]. In addition, many trials have investigated the efficacy of *E. faecium* incorporated in feed to improve fish growth and stimulate immune response [177].

Due to safety concerns, lack of safety information, and legislation, only a limited number of enterococcal probiotics are commercialised. *Enterococcus* has not yet obtained the status GRAS [3]. However, some well characterised enterococcal strains are used as starter cultures, cocultures, or protective cultures in food industry and/or probiotics due to their positive attributes. The dual trait of being good candidates as probiotics and opportunistic pathogens of enterococci remains a controversial issue which turns about the question whether enterococci are safe for probiotic use that also remains difficult to answer. The main concern for *Enterococcus* spp. as probiotics is their pathogenicity based on horizontal transfer of virulence factors and AR genes, as explained above, and the increasing number of enterococcal infections in recent decades [1, 178]. Nevertheless, the most important and interesting evidence is that enterococci are not suggested as foodborne pathogens [179]. Indeed, after being suspected of causative agents of foodborne illness in 1926, many studies on enterococci, particularly *E. faecalis* and *E. faecium*, including experiments on animals and volunteer humans were carried out to prove that enterococci cause foodborne illness, but investigations yielded negative results because these bacteria are generally identified in mixed presence with other pathogens such as staphylococci or others [180]. Subsequently, enterococci have emerged as nosocomial- and community-acquired pathogens rather than foodborne pathogens [181, 182]. Still, the safety of enterococci before their use in foods or in probiotic preparations should be carefully assessed. Effectively, when selecting an enterococcal probiotic strain, a number of properties should be considered involving safety aspect and functional and beneficial traits. Since probiotic effect is strain dependent, it should be thus well characterised (phenotypically and genotypically) and must be safe and free of any pathogenicity such as the absence of virulence factors and acquired AR genes [183, 184]. Desirable characteristics for probiotic strain include also the ability to survive and retain viability at harsh gastrointestinal tract conditions of a healthy human (low pH, pepsin, pancreatin, bile salts), their unability to translocate the intestinal mucosa, their susceptibility to phagocytic killing, and the ability to produce antimicrobial substances such as enterocins [1, 183, 184]. Further considerable trait for potential enterococcal probiotics is that they should have limited ability to exchange DNA *in vivo* [1].

6. Conclusion

Enterococci are ubiquitous microorganisms that could be naturally present in several food products. Many studies have reported the beneficial effects of enterocin-producing *Enterococcus* strains as starters, adjunct starters, protective cultures, or probiotics. However, very few enterococci have been used as probiotics or feed additives because of the safety concern associated with their pathogenic trait as opportunistic microorganisms capable of causing severe infections and diseases due to their potential virulence factors and antibiotic resistance genes. To date, there have been no reports of disease caused by probiotic enterococci that are currently on the market such the case of *E. faecium* SF68 and *E. faecalis* Symbioflor, which is a great indication of the safety of these enterococcal probiotic strains.

Hence, enterococcal strains in view of future use as probiotics must be well characterised and perfectly assessed regarding safety aspects. For this, modern scientific techniques, up-to-date knowledge of enterococci and their properties, implementation of adequate guidance, and appropriate legislation are strongly recommended to differentiate between pathogenic and safe enterococcal strains and thus could help industrials, health staff, and consumers to accept these strains as potential candidates for useful and beneficial applications as probiotics, like other LAB strains. These measures should be complemented by a more prudent use of antibiotics in human and veterinary medicines and a strict control regarding the presence of enterococci in environmental and food sources to prevent or limit the spread of pathogenic enterococcal strains. Finally, a specific assessment of community transmission is also needed.

Therefore, until now, the debate remains open. In fact, as a coin with two sides, for enterococci, despite their health-promoting properties, they may possess detrimental traits which make it difficult to establish a clear decision within enterococcal strains between emerging pathogens and potential probiotics.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

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Research Article

Antimicrobial Resistance and Virulence Genes in *Enterococcus faecium* and *Enterococcus faecalis* from Humans and Retail Red Meat

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The emergence of antimicrobial-resistant and virulent enterococci is a major public health concern. While enterococci are commonly found in food of animal origin, the knowledge on their zoonotic potential is limited. The aim of this study was to determine and compare the antimicrobial susceptibility and virulence traits of *Enterococcus faecalis* and *Enterococcus faecium* isolates from human clinical specimens and retail red meat in Slovenia. A total of 242 isolates were investigated: 101 from humans (71 *E. faecalis*, 30 *E. faecium*) and 141 from fresh beef and pork (120 *E. faecalis*, 21 *E. faecium*). The susceptibility to 12 antimicrobials was tested using a broth microdilution method, and the presence of seven common virulence genes was investigated using PCR. In both species, the distribution of several resistance phenotypes and virulence genes was disparate for isolates of different origin. All isolates were susceptible to daptomycin, linezolid, teicoplanin, and vancomycin. In both species, the susceptibility to antimicrobials was strongly associated with a food origin and the multidrug resistance, observed in 29.6% of *E. faecalis* and 73.3% *E. faecium* clinical isolates, with a clinical origin (Fisher's exact test). Among meat isolates, in total 66.0% of *E. faecalis* and *E. faecium* isolates were susceptible to all antimicrobials tested and 32.6% were resistant to either one or two antimicrobials. In *E. faecalis*, several virulence genes were significantly associated with a clinical origin; the most common (31.0%) gene pattern included all the tested genes except *hyl*. In meat isolates, the virulence genes were detected in *E. faecalis* only and the most common pattern included *ace*, *efaA*, and *gelE* (32.5%), of which *gelE* showed a statistically significant association with a clinical origin. These results emphasize the importance of *E. faecalis* in red meat as a reservoir of virulence genes involved in its persistence and human infections with reported severe outcomes.

1. Introduction

Enterococci are ubiquitous bacteria that primarily inhabit the intestinal tract of humans and warm-blooded animals, where they are part of the normal microbiota [1]. In addition, they are found in many foods of animal and plant origin as they are able to survive many adverse environmental conditions and play an important beneficial role in the production of various traditional fermented foods with unique organoleptic properties [1–4]. They are also employed in the biopreservation of foodstuffs as they produce several bactericidal substances like lactic acid and bacteriocins (enterocins) [5, 6]. The latter exert antimicrobial activity against several important Gram-positive foodborne pathogens including *Listeria monocytogenes*, *Staphylococcus aureus*, and *Clostridium botulinum* [7–12]. Enterococci are also used as probiotics for humans and animals, but their ability to acquire virulence and antibiotic resistance genes through horizontal gene transfer should be considered as a significant obstacle to their use as probiotics or as starter/adjunct cultures in foods [6, 8, 13].

Enterococci are among the leading nosocomial pathogens; they can be transmitted person-to-person, and also through contaminated food or environment, causing soft tissue or wound infections, bacteraemia, endocarditis, and especially infections of the urinary tract [3, 8, 14–16].

Due to their ability to invade the extraintestinal regions by translocation across an intact intestinal epithelium, they can shift from commensals to pathogens [17]. *Enterococcus faecalis* and *Enterococcus faecium* are the most common enterococcal species detected in clinical and food samples. In the first wave of nosocomial enterococcal infections, *E. faecalis* was responsible for approximately 90% of the human infections and *E. faecium* for the remaining 10% [1, 18, 19]. However, over the past two decades, the second wave has commenced with *E. faecium*, which is much more frequently resistant to vancomycin (VRE), ampicillin (ARE), and high levels of aminoglycosides (HLAR) than *E. faecalis* [20, 21].

Enterococci are intrinsically resistant or tolerant to many antimicrobials and easily acquire the high-level drug resistance via horizontal gene transfer. The natural resistance of *E. faecalis* and *E. faecium* includes cephalosporins, aminoglycosides (low-level resistance), macrolides, and sulphonamides, also clindamycin and quinupristin/dalfopristin in *E. faecalis* [22]. Moreover, enterococci are showing the potential for resistance to virtually all antimicrobials used in human infections [23]. Some strains are multidrug-resistant (MDR), i.e., resistant to three or more groups of antimicrobial agents [1, 23, 24]. The resistance to vancomycin or teicoplanin is of special concern due to the important therapeutic use of these agents against the MDR enterococci and other Gram-positive bacteria [23, 24]. Ampicillin, vancomycin, and gentamicin are the most relevant antimicrobials for the treatment of MDR enterococcal infections, but the extensive use of vancomycin generated a raise in the number of VRE that constitute a serious risk group [4, 25]. Enterococci resistant to antimicrobials, including VRE, play an important role in the inter- and intraspecies transfer of antimicrobial resistance genes [26].

Since enterococci are present in the intestine of animals, contamination of meat during slaughter is common. Enterococci should be screened for specific genetic traits that determine their virulence potential, aiming also to confirm their zoonotic transmission, which represents a serious health concern [27, 28]. Many factors determine the virulence of *Enterococcus* species, for example, the ability to colonize the gastrointestinal tract or to adhere to a range of extracellular matrix proteins or to the epithelial cells [29]. Several enterococcal virulence genes that may be involved in the onset of a disease in humans or exacerbation of the disease symptoms have been described [30]. Importantly, many of these determinants are also found in the strains isolated from foods [3, 31]. The aggregation substance (*asa1*), gelatinase (*gelE*), cytolysin (*cylA*), enterococcal surface protein (*esp*), hyaluronidase (*hyl*), collagen-binding-protein (*ace*), endocarditis antigen (*efaA*), and extracellular superoxide are among the most important enterococcal virulence determinants [4, 32–34].

The aim of the present study was to determine and compare for the first time in Slovenia the antimicrobial resistance and the presence of virulence genes in *E. faecalis* and *E. faecium* isolates recovered from human patients in 2016–2018 and from fresh beef and pork in 2017.

2. Materials and Methods

2.1. Enterococci from Human Clinical Specimens. Enterococci were isolated in the National Laboratory of Health, Environment and Food, Slovenia. From January 2016 to June 2018, a total of 14 *E. faecium* and 16 *E. faecalis* isolates were retrieved from blood cultures. From March to June 2018, 16 *E. faecium* isolates were obtained from other clinical specimens, i.e., urine (n=11), tracheal aspirate (n=2), abdominal drainage aspirate (n=1), central venous catheter (n=1), and wound (n=1). In the same period, 55 *E. faecalis* isolates from urine (n=38), vagina (n=8), wound (n=4), primary sterile sites (n=3), ear (n=1), and ejaculate (n=1) were obtained. In total, 101 isolates (71 *E. faecalis* and 30 *E. faecium*) were retrieved from clinical samples collected from the patients during 2016–2018 in Slovenia. All specimens were cultivated in different selective and nonselective media according to the standard protocols [35]. The suspect colonies were identified by the matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry (Microflex LT system; Bruker Daltonics, Germany) according to the manufacturer's instructions.

2.2. Enterococci from Red Meat. From January to December 2017, 141 *E. faecalis* and *E. faecium* isolates were retrieved from unpacked and packed chilled fresh pork and beef of Slovenian and foreign origin. A total of 70 isolates (60 *E. faecalis*, 10 *E. faecium*) were collected from fresh pork and 71 isolates (60 *E. faecalis*, 11 *E. faecium*) from fresh beef samples. Sampling was performed throughout the territory of Slovenia as a part of the national monitoring within the framework of the European baseline study on antimicrobial resistance. Sampling took place in the retail establishments that directly supply the final consumer (trade). Original packages of prepacked meat were randomly selected from the sales display. In the butcher's shops, the sampler randomly selected a piece of meat in the total weight of at least 100 g. Fresh beef and pork samples were collected throughout the year and transported in the cooling boxes to the laboratory of the Veterinary Faculty, Slovenia. Twenty-five grams of each meat sample was supplemented with 225 ml of buffered peptone water (Biolife, Italy), homogenized in a stomacher, and incubated at 37°C for 16–20 h. Subsequently, the liquid enrichment culture was spread with a 10-μl loop onto the selective Slanetz Bartley agar (Biolife, Italy) and ChromID VRE chromogenic selective agar (bioMerieux, France) for the detection and differentiation of *E. faecium* and *E. faecalis* showing acquired vancomycin resistance. The agar plates were incubated at 37°C for 24–48 h. Isolates with typical morphology were selected and pure subcultures from single colonies on the blood agar plates were obtained. The suspect colonies were identified by MALDI-TOF mass spectrometry (see above).

2.3. Antimicrobial Susceptibility Testing. Isolates were phenotypically tested for their susceptibility to 12 different antimicrobials using a broth microdilution method to determine the minimum inhibitory concentration (MIC). A total of 242 isolates were tested with a commercially available 96-well broth microdilution plate (EUVENC, Sensititre,

Trek Diagnostic Systems; Thermo Scientific, USA) following the manufacturer's instructions and including the following antimicrobials: ampicillin (AMP), chloramphenicol (CHL), ciprofloxacin (CIP), daptomycin (DAP), erythromycin (ERY), gentamicin (GEN), linezolid (LZD), quinupristin/dalfopristin (Synercid, SYN), teicoplanin (TEI), tetracycline (TET), tigecycline (TGC), and vancomycin (VAN). MICs were determined after 24 h of incubation at 35°C in aerobic conditions using the Sensititre cation adjusted Mueller-Hinton broth with TES (CAMHBT, Sensititre, Trek Diagnostic Systems; Thermo Scientific, USA) according to the manufacturer's instructions. The MIC endpoint was determined as the next dilution above the last dilution where growth was observed. Reference strain *E. faecalis* ATCC 29212 was used as a control.

E. faecalis and *E. faecium* isolates were classified as susceptible or resistant based on the epidemiological cut-off values (ECOFFs) according to the European Committee on Antibiotic Susceptibility Testing (EUCAST) [36] and the recommendations of the EU Reference Laboratory for Antimicrobial Resistance (EURL-AR) [37]. The evaluation was based on the interpretation of MIC values obtained in concordance with the Decision 2013/652/EU of the European Commission. Because *E. faecalis* exhibited the intrinsic resistance to quinupristin/dalfopristin, MIC data for SYN were not included in Table S1 and Table S2. The intrinsic resistances were adopted from the EUCAST expert rules [38] and were excluded from the result tables except for gentamicin, to which enterococci exert only a low level of intrinsic resistance. MIC_{50} and MIC_{90} were also determined, both for human and meat isolates, describing MICs of the tested antimicrobials required to inhibit the growth of 50% and 90% of the obtained *E. faecalis* or *E. faecium* isolates, respectively (Table S1 and Table S2).

2.4. Molecular Detection of Virulence Factors. Genes encoding the enterococcal virulence factors *ace*, *asa1*, *cylA*, *efaA*, *esp*, *gelE*, and *hyl* were detected using PCR. DNA was extracted from the bacterial cultures grown on the sheep blood agar plates with a simple cell lysis (boiling at 95°C for 15 min, centrifugation at 14,000×*g* for 2 min). The supernatant was used as a template for PCR without further purification. Virulence genes were detected using two multiplex PCR tests: PCR 1 for the detection of *asa1*, *cylA*, *esp*, *gelE*, and *hyl* [32] and PCR 2 for the detection of *ace* and *efaA* [39]. Briefly, a 25-μl reaction mixture for both PCR assays contained 12.5 μl of 2× Multiplex PCR Master Mix (Qiagen, Germany), 2.5 μl of 10× primer mix (containing 2 μM of each primer for *asa1*, *gelE*, and *hyl*; 1 μM of each primer for *cylA* and *esp* for PCR 1; and 2 μM of each primer for *ace* and *efaA* for PCR 2), and 2.5 μl of DNA template. An initial activation step at 95°C for 15 min was followed by 30 cycles of denaturation at 94°C for 30 sec, annealing for 90 sec (PCR 1: 56°C, PCR 2: 55°C), and extension at 72°C (PCR 1: 60 sec, PCR 2: 90 sec), followed by one cycle at 72°C for 10 min. Amplicons were detected using the QIAxcel capillary electrophoresis system (Qiagen, Germany).

2.5. Statistical Analysis. The Fisher's exact test implemented in the GraphPad Prism v6.01 (GraphPad Software, USA) was used to assess the association between different traits (origin of isolation, virulence gene, resistance phenotype). To compare the isolates according to the origin of isolation, isolates from beef and pork samples were joined into a single group and compared to human isolates. To assess the association between the antimicrobial resistance phenotypes and virulence genes, isolates of different origin were joined into a single group and compared according to the resistance pattern. Each species was analyzed independently and only groups with an expected frequency of >5 were compared. For the analysis of correlation between the antimicrobial resistance and virulence, the number of cooccurring resistant phenotypes and virulence genes was considered. The p value of ≤ 0.05 was considered as statistically significant.

3. Results and Discussion

Several studies demonstrated *E. faecalis* and/or *E. faecium* to be the most common enterococcal species found in food of animal origin [40–44]. This is in accordance with the present study, as *E. faecalis* and *E. faecium* were the predominant species isolated from red meat, found in 69.5% and 11.3% of the samples, respectively. In addition, *Enterococcus* species *E. hirae*, *E. casseliflavus*, *E. durans, E. devriesei*, *E. gilvus, E. mundtii*, and *E. thailandicus* were also isolated from beef and pork samples (data not shown). *E. faecium* and *E. faecalis* were also the predominant enterococcal species isolated from healthy cattle, pigs, and chicken in nine EU countries, detected in 30.6% and 25.7% of the investigated samples, respectively [45]. The presence of enterococci in food is considered as an indicator of faecal or environmental contamination and represents a potential risk to human health [26]. Enterococcal endocarditis remains one of the most difficult enterococcal infections to treat due to the high level of antimicrobial resistance observed [35, 46]. Enterococcal bacteraemia, associated with high mortality rates [35, 46], represented 5.4–8.1% of human bloodstream infections in 2006–2011 in Slovenia [47].

3.1. Antimicrobial Resistance. The antimicrobial susceptibility of *E. faecalis* and *E. faecium* isolates from humans and red meat differed significantly for both species, as shown in Table 1, Table S1, and Table S2. In both species, susceptible isolates were strongly associated with a food origin ($p<0.0001$); 65.9% of meat isolates were classified as susceptible compared to only 11.9% of human clinical isolates. All *E. faecalis* and *E. faecium* isolates from both origins were susceptible to daptomycin, linezolid, teicoplanin, and vancomycin. The MDR phenotype was significantly overrepresented among the human clinical isolates in comparison with the meat isolates for both species ($p<0.0001$; Table 1). Among the clinical isolates, mostly from blood cultures, 30.5% showed the MDR phenotype. Almost one-third (29.6%) of *E. faecalis* isolates from different clinical samples (blood cultures, urine, wound, vagina, and ejaculate) were classified as MDR and the most common resistance pattern was ERY-GEN-TET. In *E. faecalis*,

Table 1: Overview of the susceptibility testing results for 191 *Enterococcus faecalis* and 51 *Enterococcus faecium* isolates from human clinical specimens and red meat.

Resistance to no. of antimicrobials	Human clinical specimens No. of isolates [%]		Red meat No. of isolates [%]	
	E. faecalis (*n*=71)	*E. faecium* (*n*=30)	*E. faecalis* (*n*=120)	*E. faecium* (*n*=21)
6	1 [1.4]	3 [10.0]	0 [0.0]	0 [0.0]
5	4 [5.6]	***13 [43.3]	0 [0.0]	0 [0.0]
4	4 [5.6]	3 [10.0]	0 [0.0]	0 [0.0]
3	**12 [16.9]	3 [10.0]	2 [1.7]	0 [0.0]
MDR	****21 [29.6]	****22 [73.3]	**2** [**1.7**]	**0** [**0.0**]
2	***14 [19.7]	3 [10.0]	4 [3.3]	1 [4.8]
1	27 [38.0]	2 [6.7]	39 [32.5]	2 [9.5]
Susceptible	9 [12.7]	3 [10.0]	****75 [62.5]	****18 [85.7]
Total	101		141	

Note: significant associations (the Fisher's exact test) of antimicrobial resistance/susceptibility and origin of isolation for each species are indicated with asterisks. Significance levels: ** p <0.005; *** p <0.0005, **** p <0.0001.
MDR denotes the multidrug resistance.

a significant association with a clinical origin was identified for the following antibiotics: CIP (p<0.0001), ERY (p<0.0001), GEN (p<0.0001), and TET (p<0.0001) (Figure 1(a)). Furthermore, 73.3% of clinical *E. faecium* isolates from blood cultures, urine, and tracheal aspirate were MDR with the most common resistance pattern AMP-CIP-ERY-GEN-TGC. In *E. faecium*, a significant association with a clinical origin was identified for the following antibiotics: AMP (p=0.0002), CIP (p<0.0001), ERY (p<0.0001), GEN (p<0.0001), SYN (p=0.0001), and TGC (p<0.0001) (Figure 1(b)). Among the meat isolates, only two (1.7%) *E. faecalis* isolates showed the MDR resistance pattern (ERY-GEN-TET). Distributions of MICs, MIC50, and MIC90 for the human clinical and meat isolates are shown in Table S1 and Table S2.

In 2017, the Slovenian National Antimicrobial Susceptibility Testing Committee reported a low resistance of human clinical *E. faecalis* isolates to ampicillin (0.6%), linezolid (0.4%), vancomycin (0.04%), and ciprofloxacin (0.5%) [48]. A higher resistance of *E. faecalis* was observed for nitrofurantoin (24.2%) and high level of gentamicin (19.2%) [48]. *E. faecalis* has acquired resistance to gentamicin, but the resistance to ampicillin and vancomycin is less common than in *E. faecium* [49]. In the present study, only 12.7% of clinical *E. faecalis* isolates from different clinical specimens were susceptible to all antimicrobials tested (Table 1). The majority of clinical *E. faecalis* isolates were resistant to tetracycline (78.9%), followed by the resistance to erythromycin (46.5%) (Table S1, Figure 1(a)). Twenty (28.2%) clinical *E. faecalis* isolates, originating from blood cultures, urine, vagina, operation wound, and ejaculate, showed the HLAR phenotype (Table S1); of these, 15 isolates also showed the resistance to tetracycline and erythromycin. In addition, two HLAR isolates from blood cultures were also resistant to ampicillin (MIC>64 μg/ml) and ciprofloxacin (MIC>16 μg/ml). A lower frequency of resistance in clinical *E. faecalis* isolates was noticed for ciprofloxacin (18.3%), tigecycline (7.0%), chloramphenicol (5.6%), and ampicillin (2.8%) (Table S1). These findings contrast a study which reported higher resistance rates of *E. faecalis* to tetracycline (88.0%), erythromycin (62.3%), and ciprofloxacin (39.4%) [50].

As for human clinical *E. faecium* isolates, low resistance was observed in Slovenia in 2017 for vancomycin (0.6%) and linezolid (0.5%), while higher resistance rates were reported for ampicillin (89.9%), ciprofloxacin (94.3%), and high level of gentamicin (49.1%) [48]. In the present study, most of the clinical *E. faecium* isolates were resistant to erythromycin (76.7%), ampicillin (70.0%), and ciprofloxacin (70.0%) (Table S1, Figure 1(b)). More than half (56.7%) of the isolates, originating from blood cultures, urine, and tracheal aspirate, were highly resistant to gentamicin (HLAR *E. faecium*) (Table S1); among these, 15 were resistant also to ampicillin, ciprofloxacin, and erythromycin. Resistance to quinupristin/dalfopristin was observed in 56.7% of clinical *E. faecium* isolates. Previous studies reported a wide range (1–70%) of quinupristin/dalfopristin resistance in human isolates [50, 51]. Furthermore, 30.0% of isolates were also resistant to tigecycline, while the proportion of tetracycline-resistant isolates was lower (13.3%). A higher rate of ampicillin resistance in *E. faecium* compared to *E. faecalis* isolates from human clinical samples is in congruence with previous studies reporting that *E. faecium* isolates acquire ampicillin and vancomycin resistance more frequently than *E. faecalis*, which is less efficient in accumulating resistance, although *E. faecalis* is responsible for more human infections than *E. faecium* [25, 52]. On the other hand, *E. faecium* is an important nosocomial pathogen and has acquired resistance to different classes of antimicrobials. Moreover, MDR *E. faecium* is associated with an increased mortality rate in humans [49, 53].

Based on the ECOFFs, all red meat isolates were susceptible to ampicillin, chloramphenicol, daptomycin, linezolid, teicoplanin, and vancomycin (Table S2 and Figure 1). Susceptibility to the last four antimicrobials is of particular importance since they are categorized as critically important

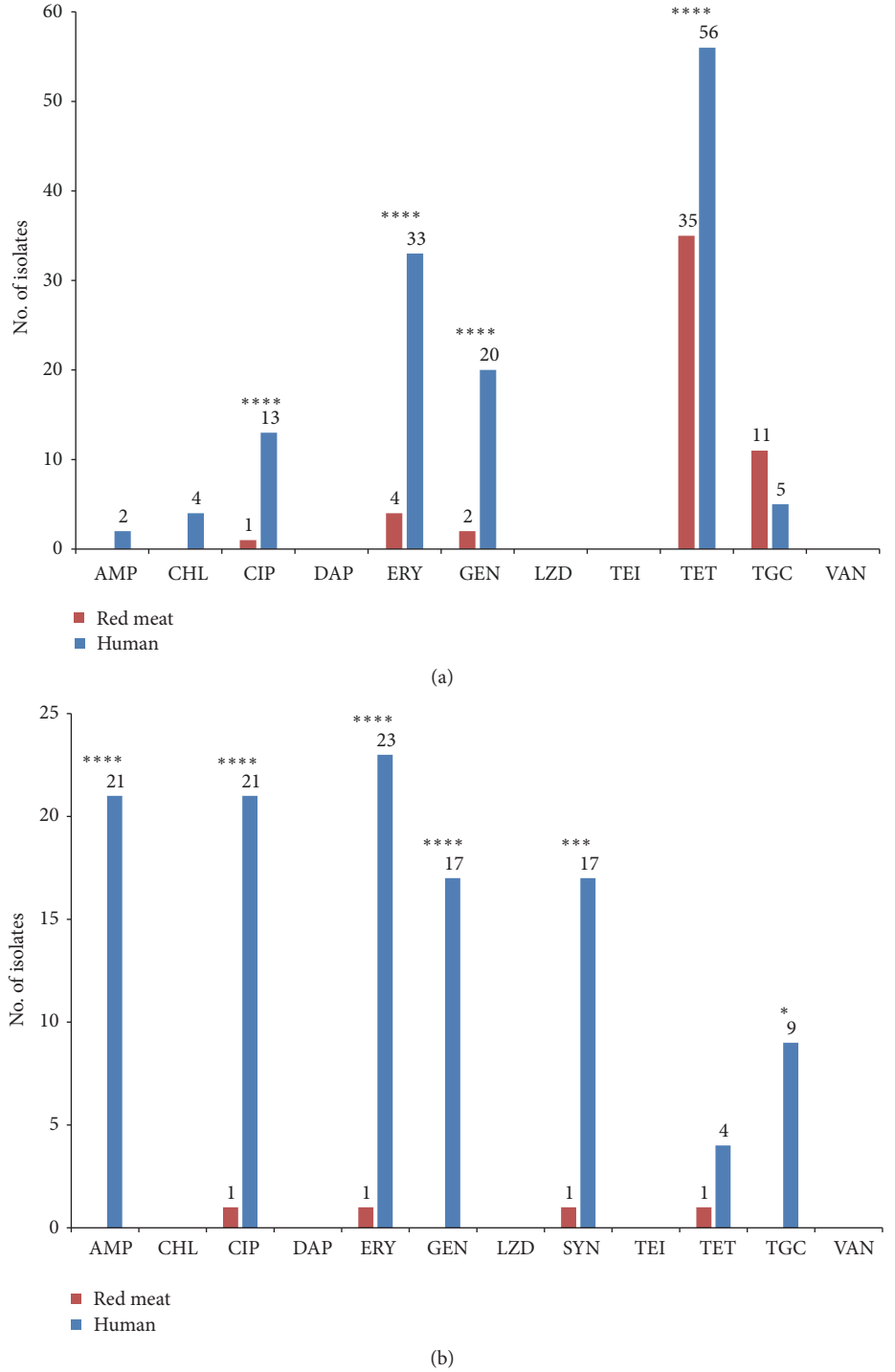

Figure 1: Antimicrobial resistance in *Enterococcus* isolates from human clinical specimens and red meat. (a) *Enterococcus faecalis* isolates (n = 191). (b) *Enterococcus faecium* isolates (n = 51). Numbers at the top of each column indicate the number of isolates; only numbers ≥1 are shown. Significant associations (the Fisher's exact test) of antimicrobial resistance and origin of isolation for each species are indicated with asterisks. Significance levels: $^{*}p$ <0.05; $^{***}p$ <0.0005; $^{****}p$ <0.0001.

antimicrobials (CIA) in human medicine. In the present study, VRE were not isolated from fresh pork and beef samples. This is in agreement with some studies reporting the absence of teicoplanin and vancomycin resistance in enterococci from red meat [41–43, 54], but in contrast with other studies describing the presence of VRE in raw meat [55, 56]. Almost one-third (32.6%) of red meat isolates were resistant to either one or two antimicrobials; two *E. faecalis* meat isolates were resistant to three (Table 1). In comparison, 63.0% of isolates resistant to at least one antimicrobial tested were

reported in Turkey [44] and only 3.4% of enterococci from retail meat in Canada were susceptible to all antimicrobials tested [42]. In the present study, *E. faecalis* meat isolates were most often resistant to tetracycline (29.2%) (Table S2, Figure 1(a)). Reduced susceptibility among *E. faecalis* isolates was observed for tigecycline (9.2%) and ciprofloxacin (0.8%). Two isolates (1.7%), one from pork and one from beef, were classified as HLAR with MIC value for gentamicin >1024 μg/ml. *E. faecium* meat isolates showed a very low frequency of antimicrobial resistance to ciprofloxacin, erythromycin, quinupristin/dalfopristin, and tetracycline (4.8% each) (Table S2, Figure 1(a)). Results of the present study were in congruence with previous studies on the resistance of enterococci in meat, with the exception of higher resistance to tetracyclines in previous reports [41, 42, 54].

In 2013, Slovenia and three other EU countries reported the antimicrobial resistance of enterococcal isolates from broiler, pig, and bovine meat [57]. Overall, *E. faecalis* isolates showed resistance to tetracyclines (42.2%), streptomycin (11.1%), and erythromycin (6.7%). *E. faecium* isolates were resistant to quinupristin/dalfopristin (50.0%) and tetracyclines (9.1%). In general, enterococci from pork and beef were less resistant in comparison with enterococci from broiler meat, except for chloramphenicol and linezolid. Slovenia reported resistance to the selected antimicrobials for 93 *Enterococcus* isolates from broiler meat and for 52 *E. faecalis* isolates from pork. Among the latter, 50.0% were resistant to tetracyclines, 21.2% to erythromycin, and 17.3% to streptomycin [57]. Interestingly, a higher rate of antimicrobial resistance in pork isolates was observed in 2013 than in the present study. According to the current EU legislation, the monitoring of antimicrobial resistance of enterococci (*E. faecalis* and *E. faecium*) from animals and derived meat is not mandatory. However, the surveillance of antimicrobial resistance in enterococci from meat, in particular if it is eaten raw and does not undergo the processing steps to eliminate live bacteria before consumption, is important for the assessment of possible zoonotic risks [58]. In the present study, the reduced susceptibility was also observed for some *E. hirae* and *E. durans* isolates from red meat with MIC value of 128 μg/ml for tetracycline (data not shown), which may indicate a possible interspecies transfer of resistance determinants. Enterococci are considered as reservoirs of antimicrobial resistance genes, which can be transferred to humans via the food chain. Identification of resistance genes, in addition to the phenotypic characterization of resistance, may provide additional information for the studied isolates. However, according to the whole-genome sequencing (WGS), *E. faecalis* and *E. faecium* resistance genotypes correlated with the resistance phenotypes in 96.5% of cases for the 11 investigated antimicrobials [59]. This suggests that the phenotypic susceptibility testing cannot yet be fully replaced by WGS.

3.2. Virulence Genes. The results on the presence of virulence genes are summarized in Tables 2 and 3. As expected and in congruence with previous studies [31, 60–62], the virulence traits were more commonly detected in clinical than in meat isolates. A significant association between the presence of a virulence gene and a clinical origin was identified among *E. faecalis* isolates for *asa1* ($p<0.0001$), *cylA* ($p<0.0001$), *esp* ($p<0.0001$), and *gelE* ($p=0.0005$) genes (Table 2). This is in congruence with previous studies, reporting the enterococcal surface protein (*esp*) as one of the most important factors for colonization and persistence of *E. faecalis* in human urinary tract infections [63] and biofilm production [64]. The production of gelatinase (*gelE*) was also confirmed previously in clinical *E. faecalis* strains, but its connection with biofilm formation remains unclear [64]. A higher frequency of adhesion genes (*esp* and *asa1*) and gelatinase (*gelE*) was described for clinical *E. faecalis* isolates in comparison with *E. faecium* [65].

In the present study, none of the isolates harbored all of the virulence genes simultaneously. All the tested virulence genes were found in human clinical isolates, whereas in the red meat isolates all but *hyl* were detected (Table 2). A simultaneous presence of more than two virulence genes was demonstrated in 94.4% of *E. faecalis* and 13.3% of *E. faecium* isolates from humans and in 68.3% of *E. faecalis* isolates from red meat (Table 3). In the clinical *E. faecalis* isolates, 14 virulence gene patterns were discovered, with *ace-asa1-cylA-efaA-esp-gelE* being the most common as it was detected in one-third ($n=22$) of the isolates (Table 3), mostly from the urine samples ($n=13$) but also from the blood culture ($n=3$), vagina ($n=3$), wound ($n=2$), and ejaculate ($n=1$). Moreover, 73.2% ($n=52$) of clinical *E. faecalis* isolates harbored four or more virulence genes at the same time, compared to only 10.0% ($n=2$) of clinical *E. faecium* isolates. These findings contrast with a study which reported that isolates linked with bacteraemia did not show any particular propensity for the carriage of virulence genes, whereas isolates from the urinary tract infections usually possessed two to four virulence traits [66]. Among the six distinct virulence gene patterns found in clinical *E. faecium* isolates, *esp-hyl* was the most frequent as it was detected in half ($n=16$) of the isolates (Table 3), mostly from the blood cultures ($n=10$) but also from the urine samples ($n=4$) and tracheal aspirates ($n=2$). Moreover, far less clinical *E. faecium* isolates harbored virulence genes than *E. faecalis*, with the exception of *esp* (Table 3). The *hyl* gene was found in 53.3% of *E. faecium* and in only 2.8% of *E. faecalis* clinical isolates (Table 2). This gene is widely distributed among the clinical *E. faecium* isolates and it was previously considered as restricted to this species [67]. However, it has been recently described also in *E. faecalis* [28, 65, 68], which supports the findings of the present study. In addition, herein we showed that *esp* and *hyl* were overrepresented among *E. faecium* isolates of a clinical origin in comparison with food isolates ($p<0.0001$) (Table 2). The incidence of *esp* in clinical *E. faecium* is reported to be increasing compared to clinical *E. faecalis* isolates [50]. The carriage of *esp*, coding for the enterococcal surface protein, in enterococci from foods and humans has also been described before [31, 69]. However, it was more frequently observed in clinical isolates than in commensal isolates [70] and it was found in a low proportion in the meat samples [71, 72]. In the present study, *esp* was detected in equally high proportions of clinical *E. faecalis* and *E. faecium*, while its occurrence in the red meat *E. faecalis* isolates was much lower.

Table 2: The presence of virulence genes in 191 *Enterococcus faecalis* and 51 *Enterococcus faecium* isolates originating from human clinical specimens and red meat.

Virulence gene	Human clinical specimens No. of isolates [%]		Red meat No. of isolates [%]	
	E. faecalis	*E. faecium*	*E. faecalis*	*E. faecium*
ace	54 [76.1]	4 [13.3]	92 [76.7]	0 [0.0]
asa1	****46 [64.8]	2 [6.7]	38 [31.7]	0 [0.0]
cylA	****32 [45.1]	2 [6.7]	6 [5.0]	0 [0.0]
efaA	69 [97.2]	4 [13.3]	115 [95.8]	0 [0.0]
esp	****51 [71.8]	****21[70.0]	13 [10.8]	0 [0.0]
gelE	***63 [88.7]	2 [6.7]	79 [65.8]	0 [0.0]
hyl	2 [2.8]	****16 [53.3]	0 [0.0]	0 [0.0]
Total	71	30	120	21

ace: collagen-binding protein; *asa1*: aggregation substance; *cylA*: cytolysin; *efaA*: endocarditis antigen; *esp*: enterococcal surface protein; *gelE*: gelatinase; *hyl*: hyaluronidase.
Note: significant associations (the Fisher's exact test) of the presence of virulence gene and origin of isolation for each species are indicated with asterisks. Significance levels: *** $p < 0.0005$; **** $p < 0.0001$.

Table 3: Virulence gene patterns observed in *Enterococcus faecalis* and *Enterococcus faecium* isolates from human clinical specimens and red meat.

Virulence gene pattern	Human clinical specimens No. of isolates		Red meat No. of isolates	
	E. faecalis	*E. faecium*	*E. faecalis*	*E. faecium*
ace-asa1-cylA-efaA-esp-gelE	22	1	0	0
ace-asa1-cylA-efaA-gelE	2	0	1	0
ace-asa1-efaA-esp-gelE	2	0	0	0
ace-efaA-asa1-esp-cylA	4	0	4	0
asa1-cylA-efaA-esp-gelE	4	0	1	0
ace-asa1-cylA-efaA	0	1	0	0
ace-asa1-efaA-gelE	9	0	17	0
ace-efaA-esp-gelE	7	1	3	0
asa1-efaA-esp-gelE	2	0	1	0
ace-asa1-efaA	0	0	9	0
ace-efaA-esp	1	1	0	0
ace-efaA-gelE	7	0	39	0
asa1-efaA-esp	1	0	0	0
asa1-efaA-gelE	0	0	3	0
efaA-esp-gelE	6	0	4	0
ace-efaA	0	0	19	0
asa1-efaA	0	0	1	0
asa1-gelE	0	0	1	0
efaA-gelE	2	0	8	0
esp-hyl	2	16	0	0
efaA	0	0	5	0
esp	0	2	0	0
gelE	0	0	1	0
None	0	8	3	21
Total	71	30	120	21

Genes coding for the virulence were not detected in 26.6% of clinical *E. faecium* isolates nor in any of *E. faecium* isolated from meat (Table 3). However, according to the literature, the presence of *asa1*, *cylA*, *efaA*, *esp*, *gelE*, and *hyl* genes has been confirmed in *E. faecium* isolates from meat and meat products [71, 73]. In general, *E. faecalis* isolated from food is showing more virulence traits than *E. faecium* [71]. *E. faecium* of animal origin is not highly important for human infections, but should be considered in the view of transferring the resistance genes to other pathogenic enterococci [74]. Furthermore, also *E. hirae* and *E. mundtii* could represent a reservoir of virulence genes, as in the present study *ace*, *asa1*, and *efaA* were found in *E. hirae* and *ace*, *efaA*, and *gelE* in *E. mundtii* (data not shown). On the other hand, *E. faecalis* of animal origin was reported as a human hazard *per se* as the same types of *E. faecalis* were found in animals, meat, human faecal samples, and patients with enterococcal bacteraemia [74].

Results of the present study showed a high prevalence of virulence genes in *E. faecalis* isolates from meat (Table 2). More than three virulence genes were detected in 68.3% of isolates (Table 3), which is a much higher percentage than previously reported [62]. Furthermore, 97.5% of isolates harbored at least one virulence gene (Table 3). This is in accordance with a previous study, in which a higher percentage of *E. faecalis* isolated from four meat types, including beef and pork, was reported [42]. In the present study, the *ace-efaA-gelE* virulence gene pattern was the most common for *E. faecalis* from meat. This is an important finding as *efaA* and *gelE* were shown to be associated with the exacerbation of infective endocarditis in humans [75, 76]. In addition, it has been shown before that the presence of *ace* is often confirmed in clinical and retail meat *E. faecalis* isolates [42, 66, 71]. This is in accordance with the results of the present study, showing that three-quarters of *E. faecalis* isolates from both origins harbored *ace* while it was found in only few clinical *E. faecium* isolates (Table 2).

Similarly, the concurrent expression of virulence factors cytolysin and aggregation substance was reported to result in the increased pathogenicity of *E. faecalis* isolates [77]; herein, the presence of both *cylA* and *asa1* genes was confirmed in a small number of *E. faecalis* isolates from red meat. Regardless of the gene patterns, the presence of *asa1* was demonstrated in one-third of *E. faecalis* meat isolates (Table 2), but its frequency in *E. faecalis* food isolates was previously reported to be high [31, 42, 60, 71]. In the present study, *cylA* was confirmed in low numbers of *E. faecalis* meat isolates and *E. faecium* clinical isolates, while it was more abundant in clinical *E. faecalis* (Table 2). Previously, this gene was also reported in low numbers of isolates originating from fermented dry sausages, beef, and pork [42, 71].

Gene encoding the endocarditis antigen was the most frequently detected virulence gene in both clinical and meat *E. faecalis* isolates in the present study (Table 2), which is in congruence with a study hypothesizing that *efaA* is important also for the persistence of enterococci in environments other than human tissues [78]. On the other hand, *efaA* was only found in few clinical and none of the meat *E. faecium* isolates (Table 2), which contrasts with the study reporting 63% *efaA*-positive *E. faecium* isolates from food of animal origin [69]. Similarly, the presence of gelatinase encoding gene (*gelE*) was also frequently demonstrated in *E. faecalis* clinical and meat isolates, while it was rarely seen in clinical *E. faecium* isolates (Table 2). This corresponds with previous reports on high proportions of *E. faecalis* and low proportions of *E. faecium* isolates from meat harboring *gelE* [42, 71, 72].

3.3. Association between Antimicrobial Resistance and Virulence Genes. In *E. faecalis*, a moderate positive correlation between the phenotypic resistance and virulence genes was observed (Spearman correlation coefficient r_S=51.4%, p<0.0001). Similarly, in *E. faecium*, a strong positive correlation was observed (r_S=71.9%, p<0.0001). Several significant associations between the antimicrobial resistance phenotype and virulence genes were identified (Table S3 and Table S4). In *E. faecalis*, the presence of *asa1*, *esp*, and *cylA* genes was significantly associated with the resistance to ERY, GEN, and TET (Table S3). In *E. faecium*, the presence of *esp* and *hyl* genes was significantly associated with the phenotypic resistance to AMP, CIP, ERY, GEN, and TGC (Table S4). In both species, a positive association between the presence of virulence genes and phenotypic resistance was more evident in clinical isolates in comparison with meat isolates. This suggests there is a cooccurrence of resistance and virulence determinants in clinical isolates belonging to both analyzed species, possibly due to the antimicrobial treatment favoring the coselection of both traits.

4. Conclusions

Herein, we revealed a relatively favorable situation regarding the resistance of *E. faecalis* and *E. faecium* isolates from human clinical specimens and red meat as all isolates were susceptible to daptomycin, linezolid, teicoplanin, and vancomycin, which is of particular importance since these agents are categorized as CIA for human infections. In addition, a considerably higher proportion of susceptible isolates from meat compared to clinical isolates was shown. Only 1.7% of meat isolates were MDR compared to 42.6% of clinical isolates. Therefore, *E. faecalis* and *E. faecium* from red meat most likely do not represent an important source of resistant strains for human colonization of infection. Clinical *E. faecalis* isolates showed an increased presence of virulence genes as 47.9% of isolates harbored more than five virulence genes simultaneously compared to 5% of meat *E. faecalis* isolates. However, the most common combinations of virulence genes in *E. faecalis* isolates from beef and pork, including *efaA*, *ace*, and *gelE*, revealed a similarity in virulence characteristics to human isolates. Even though the most frequent virulence gene patterns in the red meat isolates were less common in human isolates, beef and pork could be regarded as a source of virulent *E. faecalis* strains. In contrast, the red meat could not be assumed as an important vehicle for the transmission of virulent *E. faecium*.

Data Availability

The data used to support the findings of this study are included within the article and the supplementary information file. The raw data on isolation source, antibiotic susceptibility, and presence of virulence genes for each isolate are available from the corresponding author upon request.

Conflicts of Interest

The authors declare that there are no conflicts of interest regarding the publication of this paper.

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Supplementary Materials

Table S1. *Distribution of MICs and resistance [%] in E. faecalis (n=71) and E. faecium (n=30) from human clinical specimens*. Data on MIC [μg/ml] distribution, including MIC_{50} and MIC_{90}, and resistance [%] to the tested antimicrobials of *E. faecalis* and *E. faecium* isolates from human clinical cases is shown. Table S2. *Distribution of MICs and resistance [%] in E. faecalis (n=120) and E. faecium (n=21) from red meat*. Data on MIC [μg/ml] distribution, including MIC_{50} and MIC_{90}, and resistance [%] to the tested antimicrobials of *E. faecalis* and *E. faecium* isolates from beef and pork is shown. Table S3. *Association between phenotypic antimicrobial resistance and virulence genes for E. faecalis isolates from human clinical specimens and red meat (n=191)*. The numbers denote *p* values obtained using the Fisher's exact test; only groups with an expected frequency of >5 were compared. Table S4. *Association between phenotypic antimicrobial resistance and virulence genes for E. faecium isolates from human clinical specimens and red meat (n=51)*. The numbers denote *p* values obtained using the Fisher's exact test; only groups with an expected frequency of >5 were compared. *(Supplementary Materials)*

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Research Article

Enterocin BacFL31 from a Safety *Enterococcus faecium* FL31: Natural Preservative Agent Used Alone and in Combination with Aqueous Peel Onion (*Allium cepa*) Extract in Ground Beef Meat Storage

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Safety aspects and probiotic properties of *Enterococcus faecium* FL31 strain producing an enterocin, named BacFL31 were previously demonstrated. Taking into account its originality, the enterocin BacFL31 was added alone at 200 AU/g or in combination with the aqueous peel onion (*Allium cepa*) extract (APOE) at 1.56 ± 0.3 mg/mL to ground beef meat. Its biopreservative effect was evaluated by microbiological, physicochemical and sensory analyses during 14 days at 4°C. The APOE was characterized for its phytochemical content: total phenolic (TPC), flavonoids (TFC) and tannins contents (TAC), its antioxidant capacity using the *in vitro* 1,1-diphenyl-2-picrylhydrazyl (DPPH) and its antilisterial activity. APOE had a high TPC, TFC and TAC respectively with 140 ± 2.05 (mg GAE/g), 35 ± 0.5 (mg QE/g) and 20.6 ± 1.4 (mg CE/g). Equally, APOE showed a potential radical scavenging activity compared to the butylated hydroxytoluene (BHT), with an anti-radical power (ARP) of 46 ± 1.5. During 14 days of storage at 4°C, the combination between APOE and BacFL31 limited the microbial deterioration ($P < 0.05$), led to a decrease in thiobarbituric acid reactive substances (TBARS) values and slowed down the metmyoglobin (MetMb) and carbonyl group accumulation and delayed the disappearance of sulfphydryl proteins ($P < 0.05$). The combination was also efficient ($P < 0.05$) against microflora proliferation, decreased primary and secondary lipid oxidation ($P < 0.05$), reduced protein oxidation and enhanced significantly ($P < 0.05$) the sensory attributes. Thus, the enterocin BacFL31 use from a safe *Enterococcus faecium* combined with APOE as a potential natural preservative to biocontrol ground beef was promising as it was effective at low concentration. The data lay bases for new tests to be carried out in other food matrices.

1. Introduction

Due to its composition, meat and meat products are prone for growth of several microorganisms and pathogenic bacteria as well as oxidation reactions [1, 2]. These latter have been considered as one of the most significant causes of quality deterioration in meat and meat products during processing and storage [3–5]. The main targets of this type of redox reaction in meats are lipids and proteins. In this regard, lipid oxidation affects unsaturated lipids and leads to development of rancidity and degradation of sensory and nutritional value reducing their shelf-life time [6, 7]. In addition, during protein oxidation, reactive oxygen species may attack the side chain of amino acids and the peptide backbone, which leads to formation of carbonyl compounds, decrease in the sulfhydryl contents, loss of essential amino acids and water-holding capacity, reduction in protein solubility and eventually degradation of texture and color [8–10].

The use of additives with antioxidant properties and antimicrobial activities could be an adequate strategy to deal with the oxidation and the microbial proliferation in meat and meat products [11, 12]. However, consumer concerns about the relationship between health and nutrition, challenge food technologists to develop healthy meat products with improved characteristics. In order to answer the demand from consumers, many newly products with natural preservative have been developed in order to reduce the use of synthetic additives which have been linked to health risks is increasing.

Amongst others, the use of essentials oils, plant extracts or bacteriocins from lactic acid bacteria (LAB) constitute different ways to control lipid and protein oxidation and pathogenic bacteria proliferation in meat systems [3, 13–15]. In this context, natural antioxidants from plant extracts have been obtained from different sources such as fruits: grapes, pomegranate, date, kinnow, vegetables: broccoli, potato, drumstick, pumpkin, curry, nettle, herbs and spices, and investigated to decrease lipid oxidation and to preserve and improve the overall quality of meat and meat products [2, 11, 16].

Onions (*Allium cepa*) are utilized in various types of food, and they are one of the major sources of antioxidant content [17]. The major flavonoids found in onion dry peel, considered usually as waste, contain large amounts of phenolic compounds, such as quercetin, the major flavonoid, gallic acid, ferulic acid, and kaempferol which are effective antioxidants and have many pharmacological properties [18, 19]. The onion extracts had been widely studied on its antioxidant properties were largely evaluated in food preservation. For example, the brined onion extracts could enhance the quality of turkey breast rolls during seven days of refrigerated storage [20, 21]. Ground beef patties with added onion tissue showed decreased mutagenicity [22] and formation of heterocyclic aromatic amines during frying [23]. Equally, onion peel extract was demonstrated to be a very effective inhibitor of lipid oxidation and has potential as a natural antioxidant in raw ground pork [24].

On the other hand, bio-preservation by bacteriocins produced by LAB has gained increased attention as means of naturally controlling the safety and extending the shelf life of different meat matrix [15, 25]. The most common protective cultures belong to *Lactobacillus* and *Bifidobacterium* genera, while strains of *Enterococcus* spp. are occasionally used [26]. Most of these microorganisms are able to produce bacteriocins, named enterocins, active against pathogenic and spoilage bacteria. Therefore, enterocin produced by *Enterococcus* spp. are interesting candidates for guaranteeing the safety of meat and meat products [27, 28]. In this context, enterocins A and B have been extensively studied for their strong antibacterial properties especially in meat products [29]. Likewise, in our previous work, the addition of enterocin BacFL31 extended the shelf life and enhanced the sensory attributes of turkey meat samples stored at 4°C [15].

Despite that enterococci are considered as beneficial with technological properties; there has been increasing concern about the prevalence of virulence factors and antibiotic-resistance genes, which could compromise their foods application [26]. In this regard, enterocin-producing strains should be carefully assessed with regard to safety aspects before being used in food technology. Once their safety characterization and enterocin-mediated antagonism against foodborne pathogens and spoilage bacteria are confirmed, safe enterococci could be good candidates for potential use in bio-preservation.

In previous study, an *Enterococcus faecium* FL31 strain producing the enterocin BacFL31 was deeply studied for its antimicrobial activity and the probiotic properties and as well as safety aspects were characterized [15, 30, 31].

The present paper aimed to evaluate the potential bio preservative effect of BacFL31 alone or in combination with peel onion extract on ground beef meat during storage at 4°C. The microbial evaluation, the lipid and protein oxidation as well as sensory attributes were assessed. To our knowledge, combined addition of enterocin and plant extracts in meat products preservation has not been reported to date.

2. Materials and Methods

2.1. Bacterial Culture and Growth Conditions. The *E. faecium* FL31, enterocin BacFL31 producer strain, was characterized as described previously by Chakchouk-Mtibaa et al. (2014) [30]. This strain was grown in De Man, Rogosa and Sharp medium (MRS) broth at 37°C for 18 h [32]. *L. monocytogenes* ATCC 19117 was used as target strain in the determination of bacteriocin and APOE activities and was cultured and counted on Brain Heart Infusion (BHI) medium. Serial dilutions were prepared, then, 0.1 mL volumes of each dilution were spread in BHI agar plates and incubated at 35°C for 48 h. Presumptive colonies of *L. monocytogenes* were counted and values were measured as CFU/mL on agar plates. The data represent results from three replicates.

2.2. Bacteriocin BacFL31 Preparation. A partially purified enterocin BacFL31 was recovered from a 900 mL of an 18h-old culture of *E. faecium* FL31 using a two purification step as described elsewhere [30]. To eliminate organic acids effect produced by this strain, the obtained active solution was neutralized at pH 6.5, concentrated to one-tenth of the original volume in a Rotavapor at 70°C, sterilized through a 0.45 μm pore size filters (Millipore) and submitted to antimicrobial activity evaluation against *L. monocytogenes* ATCC 19117 using the agar well diffusion assay [33].

2.3. Aqueous Peel Onions Extract (APOE) Preparation. Onion peels extract was prepared with red onion peels provided by a local market in the region of Sfax - Tunisia. The collected onion peels were washed three times with distillated water and were shade-dried. The obtained dried onion peels was mechanically crushed with a food grinder (Moulinex Mixer Grinder LM2421). Then, the powders obtained were mixed with ultrapure water. The extract was filtered and then dried in a lyophilizer (Martin Christ, Alpha 1-2 LD plus Germany). The obtained extract was weighed and then mixed with water at a concentration of 20 mg/mL.

2.4. Quantitative Determination of Phenolic Compounds

2.4.1. Total Polyphenols Content. Total polyphenols content of APOE was calculated according to the Folin-Ciocalteau method described by Waterman and Mole (1994) with some modifications [34]. Ten microliters of diluted extract solution was shaken for 5 min with 50 μL of Folin-Ciocalteau reagent. Then 150 μL of 20 % Na_2CO_3 was added. The obtained mixture was shaken once again for 1 min. Finally, the solution was brought up to 790 μL by adding distilled water. After 2 hours, the absorbance at 760 nm was evaluated using a spectrophotometer. Gallic acid was used as a standard for the calibration curve. Total polyphenolics content (TPC) of the APOE was calculated according to the following equations:

$$Y = 0.012 \times x + 0.017 \quad \left(R^2 = 0.997\right) \tag{1}$$

TPC was expressed as μg gallic acid equivalent per milligram of powder peel extract (μg GA/mg) using the linear equation based on the calibration curve.

2.4.2. Flavonoids Content. Flavonoids content in APOE was determined using the method of Quettier-Deleu et al. (2000) [35]. Briefly, 1 mL of $AlCl_3$ was added to 1 mL diluted extract solution and vortexed and then incubated for 15 min in the dark. The absorbance at 430 nm was evaluated for the samples and the quercetin was used as standard for the calibration curve. Total flavonoids content (TFC) of the APOE was calculated according to the following equations:

$$y = 0.051 \times x + 0.0003 \quad \left(R^2 = 0.999\right) \tag{2}$$

TFC was expressed in μg of quercetin equivalent per milligram of powder peel extract (μg QE/mg).

2.4.3. Tannins Concentration. The determination of the tannins was carried out according to the method of Julkunen-Titto (1985) [36]. 0.5 mL of APOE were mixed vigorously with three milliliters of 4 % vanillin in methanol. Immediately 1.5 mL of concentrated HCl was added to the mixture. The absorbance was read at 500 nm after 20 min at room temperature. Catechin was used as the standard. The tannin concentration (TAC) is expressed as catechin equivalents in mg per gram of extract (CE/g extract) and the content is obtained from the catechin calibration curve following the equation:

$$Y = 0.5825 \times x \quad \left(R^2 = 0.918\right) \tag{3}$$

2.5. Antioxidant Activity. Antioxidant activity of APOE was estimated by the measurement of the DPPH radical scavenging activity. This assay determines the scavenging effect of stable radical species according to the method of Kirby and Schmidt (1997) with slight modifications [37]. Briefly, the extract was diluted with ultrapure water at different concentrations (25; 50; 100, 200 and 400 μg/mL). Then, 500 μL of a DPPH radical solution (6 10^5 M in HPLC grade methanol) was mixed with 500 μL of samples. The mixture was incubated for 30 min in the dark at room temperature. Then, the absorbance of the resulting solution was read at 517 nm against a blank. The percentage of antiradical activity (% ArA) had been calculated as follows:

$$\%\ \text{ArA} = \left[\frac{(\text{Absorbance of Control} - \text{Absorbance of test Sample})}{\text{Absorbance of Control}}\right] \times 100 \tag{4}$$

The efficient concentration EC_{50} which represent the antioxidant amount necessary to decrease the initial DPPH concentration by 50 % was calculated from a calibration curve by linear regression. EC_{50} was expressed in terms of the concentration of sample extract in relation to the amount of initial DPPH (mg/mg DPPH). The antiradical power ARP was determined as the reciprocal value of the EC_{50} (mg/mg DPPH) following the equation:

$$\text{ARP} = \frac{100}{\text{EC50}} \tag{5}$$

as described by kroyer (2004) [38].

2.6. Antibacterial Activity of the APOE and the Minimal Inhibitory Concentration (MIC) Determination. Minimal Inhibitory Concentration (MIC) of the APOE against *L. monocytogenes* ATCC 19117 was determined in BHI broth. The test was performed in sterile 96-well microplates with a final volume in each microplate well of 100 μL. A stock solution of 20 mg/mL of APOE was two-fold serially diluted in LB medium. Ten μL of *L. monocytogenes* ATCC 19117 cell suspension at 10^6 CFU/mL were seeded in each microplate well. Then, plates were incubated overnight at 37°C. The MIC was defined as the lowest APOE concentration at which the microorganism does not demonstrate visible growth after incubation. Positive growth control wells consisted of bacterium only in their adequate medium. Cells suspension at the same concentration supplemented with ampicillin was used as control. Then, twenty five μl of Thiazolyl Blue Tetrazolium Bromide (MTT) at 0.5 mg/mL were added to the wells and incubated at room temperature for 30 min. All experiments were performed in triplicate

2.7. Meat Samples Preparation. A fresh beef meat, purchased from a local supermarket (Sfax-Tunisia), was immediately transported to the laboratory at 4°C and was minced by grinding in a sterile grinder. Ground beef was divided into five equal lots: T_0 (negative control: meat without any addition), T_1 (positive control: meat added with 0.01% of the usual antioxidant BHT), T_2 (meat supplemented with 200 AU/g of partially purified BacFL31), T_3 (meat supplemented with active APOE at a concentration of 1 × MIC/g) and T_4 (meat added with 200 AU/g of the partially purified BacFL31 combined with active APOE at a concentration of 1 × MIC/g).

These ingredients were homogenized in a blender (Moulinex Mixer Grinder LM2421) for 10 min, then packed in sterile plastic bags to produce three replicates and stored in a refrigerator at 4°C. Samples were withdrawn at 0, 3, 7,

10 14 days and analysed for: (i) microbial counts, (ii) physicochemical analysis consisting of metmyoglobin (MetMb), protein carbonyls, sulfhydryls groups, peroxide value (PV), thiobarbituric acid reactive substances (TBARS) and conjugated dienes (CD), and finally (iii) sensory attributes (color, texture, odour and overall acceptability).

2.8. Microbiological Analysis. Microbiological assays on meat samples were performed using international standard methods. Twenty five grams of meat were placed into a sterile stomacher bag and added to 225 mL of sterile buffered peptone water solution (0.1 g/100 mL). A 100 μL of serial decimal dilutions were spread on the surface of agar plates. The International Organization for Standardization ISO 4833-2 [39], ISO 17410 [40] and ISO 21528-2 [41] were used respectively to enumerate aerobic plate counts (APC), aerobic psychrotrophic counts (PTC) and Enterobacteriaceae. Plates containing 25 - 250 colonies were selected and counted. The average number of CFU (colony forming units)/g was calculated and expressed as $\log_{10}$ CFU/g meat.

2.9. Physicochemical Analysis

2.9.1. Lipid Oxidation

(i) Peroxide Value (PV). Peroxide values of samples were performed according to the method of Folch et al. (1957) [42]. Five grams of each sample were placed in a glass vial containing 50 mL of chloroform: methanol, 2:1 (*v/v*) and mixed in an orbital shaker at room temperature for 24 h. Subsequently, the homogenate was filtered using filter paper and washed with 15 mL of NaCl at 0.9 %. After a few seconds of vortexing, 10 mL of sample were collected from the bottom layer and evaporated under a stream of nitrogen gas, leaving the extracted lipids for PV analysis. The lipid sample was treated with 35 mL of a solvent mixture (acetic acid: chloroform, 3:2) and shaken thoroughly, then 0.5 mL of saturated potassium iodide solution was added. The mixture was kept in the dark for 5 min and 75 mL of distilled water were added followed by vigorous mixing. Soluble starch solution in phosphate buffer (2.5 mL at 1 % *w/v*) was used as an indicator. The peroxide value was determined by titration of the iodine liberated from potassium iodide using standardized 0.005 N sodium thiosulfate solutions. The PV was calculated by the following equation:

$$\mathrm{PV}\,(\mathrm{mEq/Kg}) = \frac{[(\mathrm{S} - \mathrm{B}) \times \mathrm{F} \times 0.01]}{\mathrm{W}} \times 1000 \qquad (6)$$

Where S is the volume (mL) of sodium thiosulfate required to titrate the sample; B is the volume (mL) of sodium thiosulfate required for the control; F is the calculated normality of the standardized sodium thiosulfate solution and W is the weight of the sample (g). The results are expressed as milli-equivalents of peroxide O_2 per kg of meat.

(ii) Thiobarbituric Acid Reactive Substance Value (TBARS). Lipid oxidation was evaluated by thiobarbituric acid reactive substances (TBARS) according to the method described by Eymard et al. (2005) [43]. Two grams of sample were mixed with 100 μL of butylated hydroxytoluene in ethanol at 1 g/L and 16 mL of trichloroacetic acid (TCA) at 50 g/L, then homogenized for 10 min and filtered. Two millilitres of filtrate (or 2 mL of TCA for blank) were added to 2 mL of thiobarbituric acid solution at 20 mol/L of concentration. The tube content was immediately vortexed and heated at 100°C for 15 min and rapidly cooled in ice. Absorbance was read against the blank at 508 ($A_{508\ nm}$), 532 ($A_{532\ nm}$) and 600 ($A_{600\ nm}$) with a spectrophotometer (Thermo Scientific/Genesys 20 Germany). The absorbance measured at the maximum ($A_{532\ nm}$) was corrected for the baseline drift as follows:

$$\begin{aligned} &\mathrm{A532\ nm\ corrected} \\ &\quad = \mathrm{A532\ nm} \\ &\qquad - \left[\mathrm{A508\ nm} - \mathrm{A600\ nm} \times \frac{(600 - 532)}{600/508}\right] \\ &\qquad - \mathrm{A600\ nm} \end{aligned} \qquad (7)$$

The results were expressed as mg of malonaldehyde equivalent per kg of sample (mg/kg) using the molar extinction coefficient of the MDA - TBA adduct at 532 nm (1.56×10^5 M^{-1} cm^{-1}) according to Buege and Aust (1978) [44]. The malonaldehyde equivalent was determined using the following equation:

$$\begin{aligned} \mathrm{mg\ MDAeq/kg} = {} & \mathrm{A\ corrected} \times \mathrm{VTCA} \times 2 \\ & \times \mathrm{MMDA} \times \frac{0.01}{1.56} \times \mathrm{m} \end{aligned} \qquad (8)$$

(iii) Analysis of Conjugated Dienes. One gram of each sample of beef meat was suspended in 10 mL of distilled water and homogenized. A 0.5 mL aliquot of this suspension was mixed with 5 mL of extracting solution: hexane: isopropanol at 3:1 (*v/v*) for 1 min, then centrifuged at 2000 × *g* for 5 min. The absorbance of the supernatant was read at 233 nm. The concentration of conjugated dienes was calculated using the molar extinction coefficient of 25,200 M^{-1} cm^{-1} and the results were expressed as μmole per mg of ground beef meat sample [45].

2.9.2. Protein Oxidation

(i) Metmyoglobin Analysis. Metmyoglobin (MetMb) content was described by Krzywicki (1982) [46]. Briefly, 5 g of sample were placed into a 50 mL polypropylene centrifuge tube and homogenized with 25 mL of ice-cold phosphate buffer (40 mM at pH 6.80) for 1 min. The homogenized solution was kept at 4°C for 1 h and centrifuged at 4.500 × *g* for 30 min at 4°C. The supernatant was filtered through 0.45 μm pore size filters (Millipore), and absorbance was read at 572, 565, 545, and 525 nm using a spectrophotometer.

The MetMb percentages were then calculated based on those absorbance values using the following formula:

$$\text{MetMb}\,(\%) = \left[-2.51\left(\frac{A_{572\text{nm}}}{A_{525\text{nm}}}\right) + 0.777\left(\frac{A_{565\text{nm}}}{A_{525\text{nm}}}\right) + 0.8\left(\frac{A_{545\text{nm}}}{A_{525\text{nm}}}\right) + 1.098\right] \times 100 \quad (9)$$

A refers to the corresponding absorbance.

(ii) Determination of Carbonyls Contents. The classical approach to the detection of protein carbonyl groups involves their reaction with 2,4-dinitrophenylhydrazine (DNPH) according to the method of Oliver et al. (1987) [47]. Two procedures were used for the determination of protein oxidation in meat sample: carbonyl content and protein quantification. One gram of ground beef sample was homogenized in 10 mL of 0.15 M KCl buffer for 60 sec at the speed of 20980 × *g*. A 50 μL of the resulting blend was transferred into an Eppendorf vial containing 1 mL of TCA at 10 % (*w/v*). Samples were centrifuged for 5 min at 2880 × *g* and supernatant was removed. For carbonyl measurement, 1 mL of 2 M HCl containing 0.2 % 2,4- dinitrophenyl hydrazine (DNPH) and for proteins 1 mL of 2 M HCl was added to the Eppendorf vials. Samples were then incubated for 1 h at room temperature, with vortexing every 20 min. Following the incubation, 1 mL of 10 % TCA was added, vortexed and centrifuged again for 10 min at 2880 × *g*. The supernatant was removed, and the pellet was washed twice with 1.5 mL of ethanol/ethyl acetate (1:1; *v/v*), shaken, and centrifuged for 5 min at 12000 × *g*. After the complete removal of DNPH residues, the pellets were dried under N_2 gas and dissolved in 1.5 mL of 6 M guanidine hydrochloride in 20 mM sodium phosphate buffer (final pH of 6.5), shaken, and centrifuged for 5 min at 4000 × *g*.

(iii) Determination of Sulfhydryl Groups. Total free sulfhydryl groups (SH) content was determined by reacting with 5, 5′-dithiobis (2-nitrobenzoic acid: DTNB). According to Ellman (1959), a 0.5 g of meat sample was dissolved in 10 mL phosphate buffer (pH 7.2, 0.05 M) by shaking at room temperature for 1 hour [48]. Then, 1 mL of the homogenate was mixed with 9 mL phosphate containing 8 M urea, 0.6 M NaCl and 6 mM EDTA and the mixture was centrifuged for 20 min at 14000 × *g* at 4°C. Three mL of supernatant were incubated with 1 mL DTNB reagent (0.01 M DTNB in 0.05 M sodium acetate) at 40°C for 15 min. The absorbance was measured at 420 nm. Control sample was run with 1.0 mL phosphate buffer without DTNB; reagent blank was run with water only. The sulfhydryl content was calculated based on sample absorbance using a molar extinction coefficient of 13600 $M^{-1}cm^{-1}$ and the results were expressed as mmol sulfhydryl per g of ground beef sample.

2.10. Sensory Evaluation. Sensory evaluation of ground beef meat was performed by a panel of 25 researchers at the Centre of Biotechnology of Sfax - Tunisia. Each panellist performs five different assays for meat samples. For each analysis (0, 3, 7, 10 and 14 days of storage at 4°C), each sample was evaluated in three sessions. The panellists scored the sensory color, texture, odour and overall acceptability attributes by using a 9-point hedonic scale (9 = like extremely, 8 = like very much, 7 = like moderately, 6 = like slightly, 5 = neither like nor dislike, 4 = dislike slightly, 3 = dislike moderately, 2 = dislike very much, 1 = dislike extremely). A score of 5 was taken as the lower limit of acceptability.

2.11. Statistical Analysis. The experiments were done in triplicate. The results are given as mean standard deviation (SD).

Student's t-test was used for comparison between two treatments at ($P < 0.05$).

A one-way analysis of variance (ANOVA) with two factors (treatments and storage time), was applied for each parameter by using SPSS 19 statistical package (SPSS Ltd., Woking, UK). Means and standard deviation were calculated and a probability level of $P < 0.05$ was used in testing the statistical significance of all experimental data. Tukey's post hoc test was used to determine significance of mean values for multiple comparison at ($P < 0.05$).

3. Results and Discussion

3.1. Total Phenolic, Total Flavonoid and Tannin Contents. Total phenolic (TPC), total flavonoid (TFC) and tannin (TAC) contents of APOE were determined and expressed in gallic acid equivalents (mg GAE/g), quercetin equivalents (mg QE/g) and (mg CE/g) respectively. As presented in Table 1, APOE had a high TPC of 140 mg GAE/g. Other studies reported similar TPC of 125 mg GAE/g for aqueous extract of peel onion at 165°C [49]. Same observations have been reported by Lee et al. (2014) when proving that the onion peel extracted by heated water for 3 h at 60°C contained 120.60 mg GAE/g [50].

The TFC of APOE, established by $AlCl_3$ method, was about 35 mg QE/g (Table 1). Previous studies by Lee et al. (2014) showed that the hot water extract of onion peel contained 54.5 mg QE/mg of extract [50]. The quercetin compounds are major flavonoids in onions and are related to skin colors and disease in plant [50, 51]. Gorinstein et al. (2008) reported that red onions had twice higher quercetin levels than that of white onions [52]. By comparing different extraction methods, ethanol extraction showed greater concentrations of TPC and TFC, respectively, of 327.50 mg GAE/g and 183.95 mg QE/mg of extract [50].

The determination of TAC concentration reveals that the APOE contains 20.6 mg CE/g (Table 1). It should be noted that the phytochemical composition of onions is believed to vary according to species and cultivation technique. Among the species of onions, the red onion is known to be rich in polyphenols, flavonoids, flavonol, and tannin [53].

3.2. Evaluation of Antioxidant Activity. DPPH is a stable free radical, which has been widely used as a tool for estimating free radical-scavenging activities of antioxidants substances [54]. Plants with radical scavenging property and antioxidant capacity are useful for medicinal applications and as food

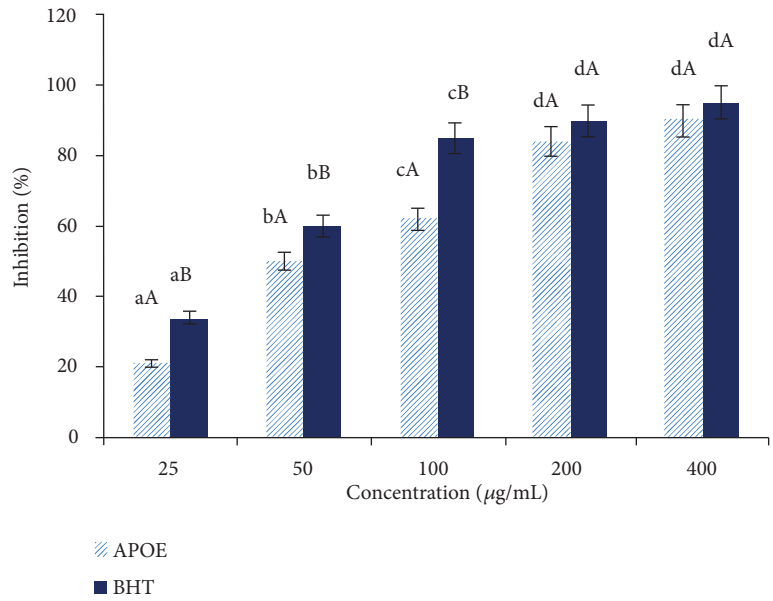

Figure 1: DPPH radical-scavenging activity of the APOE at different concentrations (25 - 400 μg/mL) compared to the BHT. ±: Standard deviation of three replicates. A - B: A *t*-Student test was applied to determine the significant differences between treatments at $P < 0.05$; a - d: Tukey's post-hoc test was used to compare the significant differences at each concentration at $P < 0.05$.

Table 1: Total phenolic, total flavonoid and tannins contents, antioxidant and antibacterial activities of the aqueous extracts of onion peel.

	Phytochemical contents				Antioxidant activity			Antibacterial activity
	TPC (mg GAE/g)	TFC (mg QE/g)	TAC (mg CE/g)		EC_{50} (mg/mL)	EC_{50DPPH} (mg/mg)	ARP	MIC (mg/mL)
APOE	140 ± 2.05	35 ± 0.5	20.6 ± 1.4	APOE	0.05 ± 0.00	2.17 ± 0.10	46 ± 1.51	1.56 ± 0.3
				BHT	0.033 ± 0.00	1.43 ± 0.07	69.69 ± 2.75	

TPC: total phenolic content. TFC: total flavonoid content. TAC: total tannin content. EC: efficient concentration. ARP: antiradical power. MIC: minimal inhibitory concentration.
±: standard deviation of three replicates.

additive. So, in the present study the antioxidant capacity of APOE was evaluated using DPPH radical scavenging method by comparing with the activity of the BHT as a conventionally applied antioxidant. The DPPH radical-scavenging activity of the APOE with varying concentrations from 25 to 400 μg/mL was determined and compared to the BHT activity (Figure 1). The antiradical activity assay of the APOE was dose-dependent. APOE at a concentration of 25 μg/mL, showed the lowest radical activity in comparison with the free radical activity of the BHT, while at 400 μg/mL, APOE revealed a very interesting DPPH activity in comparison with the BHT one (Figure 1).

In correlation with the high contents of TPC, TFC and TAC, APOE exerted effective radical scavenging activity with an efficient concentration EC_{50} of 0.05 mg/mL, respectively and 2.17 ± 0.10 mg/mg DPPH and an antiradical power (ARP) of 46 ± 1.51. In comparison of the study of Singh et al. (2009), ARP of aqueous fraction was 1.8 ± 0.3 [55]. The latter study demonstrated that ARPs of different fractions extracted by dichloromethane, diethyl ether, ethyl acetate, butanol and water were 1.2 ± 0.3, 4.9 ± 0.6, 75.3 ± 4.5, 13.4 ± 0.8 and 1.8 ± 0.3, respectively [55].

3.3. Antibacterial Activity of the APOE and MIC Determination. Minimal Inhibitory Concentration (MIC) of the APOE against *L. monocytogenes* has been determined and is equal to 1.56 ± 0.3 mg/mL as shown in Table 1.

3.4. Application of Enterocin BacFL31 Alone and in Combination with APOE during Conservation of Ground Beef Meat at 4°C

3.4.1. Microbiological Characteristics. The aerobic plate counts (APC), aerobic psychrotrophic counts (PTC) and Enterobacteriaceae counts of treated samples were significantly ($P < 0.05$) lower than those of control ones during storage (Figure 2).

APC of different samples was above 3.0 CFU/g ($P > 0.05$) at the beginning of storage period. After seven days of storage for the negative control sample (T_0), APC value increased significantly ($P < 0.05$) with the increase of the storage time at 4°C and reached the minimal spoilage level at 7.0 $\log_{10}$ CFU/g [56]. During the storage period of 7 days, a gradual increase ($P < 0.05$) in the APC for all treated samples (T_1,

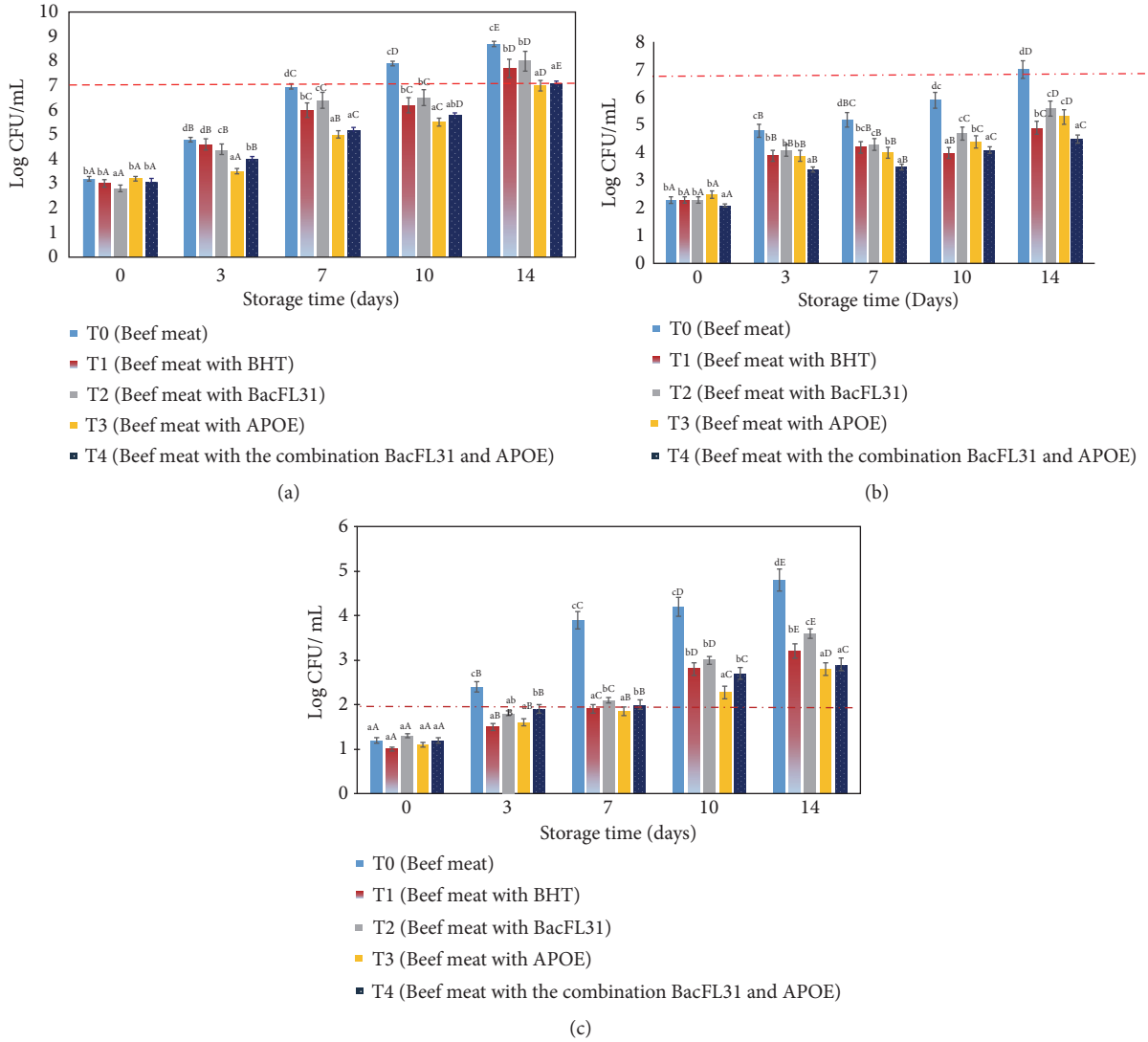

FIGURE 2: (a) Effect of the enterocin BacFL31 at 200AU/g, APOE at 1MIC/g, and the combination (BacFL31 + APOE) on the microbial load of APC of ground beef meat during storage at 4°C. ±: Standard deviation of three replicates. Values with a different letter (a - c) at the same storage day are significantly different ($P < 0.05$). Values with a different letter (A - D) of the same treatment are significantly different ($P < 0.05$) by using Tukey's post-hoc test. (b) Effect of the enterocin BacFL31 at 200AU/g, APOE at 1MIC/g, and the combination (BacFL31 + APOE) on the microbial load of PTC of ground beef meat during storage at 4°C. ±: Standard deviation of three replicates. Values with a different letter (a - c) at the same storage day are significantly different ($P < 0.05$). Values with a different letter (A - D) of the same treatment are significantly different ($P < 0.05$) by using Tukey's post-hoc test. (c) Effect of the enterocin BacFL31 at 200AU/g, APOE at 1MIC/g, and the combination (BacFL31 + APOE) on the microbial load of PTC of ground beef meat during storage at 4°C. ±: Standard deviation of three replicates. Values with a different letter (a - c) at the same storage day are significantly different ($P < 0.05$). Values with a different letter (A - D) of the same treatment are significantly different ($P < 0.05$) by using Tukey's post-hoc test.

T_2, T_3 and T_4) was observed and respectively reached 6.01, 6.4, 5.01 and 6.0 $\log_{10}$ CFU/g. For T_2 sample, the minimal spoilage level was reached after 12 days of storage, while the APC counts recorded for T_3 and T_4 were noted to remain under the detection limits (7.0 log $\log_{10}$ CFU/g) until days 14 of storage. In fact, as illustrated in the Figure 2(a), T_3 and T_4 samples were most effective ($P < 0.05$) and could extend the shelf life storage 2 days than the meat treated with BacFL31 alone at 200 AU/g (T_2).

As indicated in Figure 2(b), PTC of the treated samples by BacFL31 (T_2), APOE (T_3) and the combination Bac FL31 + APOE (T_4) was lower ($P < 0.05$) than the untreated sample (T_0). According to Speck (1984), a count of above 6.7 log CFU/g of psychrotrophic bacteria makes the product unsuitable for consumption for ground beef meat [57]. In our case, all treated samples never exceeded the maximal limit, while for the control samples (T_0 and T_1), 14 days are sufficient to attain this limit (Figure 2(b)). In a previous work, the PTC reduction on poultry meat has been reported by Chakchouk-Mtibaa et al. (2017) [15]. The authors proved that a treatment with 400 AU/g of enterocin BacFL31 could extend the shelf life of chicken breast to 15 days whereas the control

samples started to deteriorate after eight days of storage. For T_3 and T_4 samples, the increase in APC and PTC was comparatively lower ($P < 0.05$) than control products which might be attributed to the presence of phenolic compounds [55].

For the negative control sample, *the Enterobacteriaceae* counts reached rapidly the detection limit which is 2 log CFU/g according to AFNOR V01-003 (2004) [56]. For the treatments T_1, T_2, T_3 and T_4, a significantly ($P < 0.05$) reduction of the *Enterobacteriaceae* count was observed and the standard limit was reached after seven days of storage at 4°C for all treatments. In previous work, 200 AU/g of BacFL31 was demonstrated to be able to reduce the growth of *Enterobacteriaceae* and extend the shelf life of raw ground turkey escalope to 10 days, which reached 14 days with concentrations of 400 AU/g [15]. Interestingly, in this current study, both the addition of 200 AU/g of BacFL31 (T_2), APOE (T_3) and their combination (T_4) were able to reduce the growth of *Enterobacteriaceae* and extend the shelf life of raw ground beef meat to four days compared to the control samples (Figure 2(c)).

3.4.2. Physicochemical Analyses

(1) Development of Protein Oxidation Products

(i) Metmyoglobin (MetMb). Meat color, depending on the chemical state of myoglobin, is an important factor that influences product acceptability by consumers. In fact, the undesirable discoloration of meat during preservation is largely due to myoglobin oxidation and the MetMb formation [58]. The changes of MetMb content in the ground beef meat during storage at 4°C are presented in Table 2. MetMb % increased rapidly in the first seven days of storage and reached values above 40.9% in the negative control sample (T_0), whereas for treated samples (T1–T4) the MetMB percentage were ranged from 32.04 (T_4) to 34.93 (T_1). The treated samples T_2 and T_3 exceeded the limit of acceptability after ten days whereas, for the treated sample T_4, the limit was attained after fourteen days of storage. It is worth noting that consumer rejection of meat products occurred at 40% of MetMb [58]. We can explain our results by the strong antioxidant properties of APOE due to its phenolic components [24, 55]. In fact, free radical scavengers could inhibit the formation of MetMb [59]

(ii) Protein Carbonyls. Carbonylation is generally recognized as one of the most remarkable chemical modifications in oxidized proteins [5]. The formation of carbonyl compound (aldehydes and ketones) in meat proteins principally derives from the oxidation of threonine, proline, arginine and lysine residues [5]. The BacFL31 and the APOE addition had very significant effect ($P < 0.05$) on the carbonyls formation (Table 2). During storage time, control negative sample had significantly ($P < 0.05$) higher values of protein carbonyls than the treated ones. At the first day of storage, no significant difference ($P > 0.05$) between the carbonyl contents values of the control sample and all treated samples: T_1, T_2, T_3 and T_4. The carbonyl level of control sample increased ($P < 0.05$) during storage reach a maximum values of 6,41 nmol/mg protein after seven days then decreased to 4.51 nmol/mg protein at the end of the storage period (Table 2).

For T_2 sample, the amount of carbonyl groups reached its maximal value with a concentration of 5.45 nmol/mg protein lower ($P < 0.05$) than the control samples (T_0 and T_1). For T_3 sample, the maximum value was reached at the same time with a concentration of 4.15 nmol/mg protein. The T_4 sample was very efficient ($P < 0.05$) on preventing carbonyl formation. The maximum value of the carbonyl contents for the T_4 treatment was approximately twice lower than the control sample T_0 (Table 2). Similarly, the decrease in carbonyl groups under storage was reported for beef meat balls [60] and turkey meat sausage [14]. According to Estévez et al. (2011), the formation of protein carbonyls from particular amino acid side chains contribute to impair the conformation of myofibrillar proteins leading to denaturation and loss of functionality [61].

(iii) Sulfhydryl Content. Proteins may contain several actual or potential sulfhydryl groups. The measurement of thiol (sulfhydryl) content are an interesting way to evaluate free radical attack on proteins and to measure the degree of oxidative reactions in meat during refrigerated storage [61]. In fact, the determination of sulfhydryl groups concentration is an appropriate indicator of protein oxidation level [62]. During storage, concentration of sulfhydryl groups decreases ($P < 0.05$) with the progress of oxidative reaction. Treatments with BacFL31 (T_2) and APOE (T_3) were effective ($P < 0.05$) in the protection of SH groups against alteration by oxidation processes during refrigerated storage of the ground beef meat. As shown in Table 2, the maximum decrease was observed in control samples and the minimum decrease was observed in samples treated with the combination of the enterocin BacFL31 and the APOE (T_4) with final sulfhydryl concentrations of 29.14 and 42.19 nmol/mg protein, respectively, at the end of storage. On the other hand, as seen in Table 2, no significant difference ($P > 0.05$) was observed between the meat added with APOE (T_3) or added with the combination of APOE and BacFL31 (T_4). These results indicated that the addition of plant extract (T_3) inhibit the oxidation process and reduce the loss of sulfhydryl groups. Previous studies reported that the efficiency of plant extract was increased with the concentration of phenolic compounds [60, 62].

(2) Development of Lipid Oxidation Products

(i) Peroxide Value (PV). PV, an important characteristic of primary lipid oxidation, is the most used parameter for measuring the primary products of oxidative degradation in meat [14]. During the refrigerated storage at 4°C, as shown in the Table 2, treated samples had significantly ($P < 0.05$) lower PVs compared to the negative control sample (T_0). For treated samples, the lowest ($P < 0.05$) was observed in the meat treated with the APOE (T_3) alone or combined with the enterocin BacFL31 (T_4). The latter was the most effective ($P < 0.05$) treatment to retard the primary auto-oxidation up to 14 days. These results are in accordance with the study of Shim et al. (2012) [24], who reported that raw

Table 2: Effect of BacFL31, APOE, and their combination on MetMb (%), protein carbonyl (nmoles carbonyl/mg protein), sulfhydryls (nmoles sulfhydryl/mg protein), peroxide values (meq peroxide/Kg of meat), TBARS (mg MDA/kg meat) and the conjugated dienes (μmol/mg of meat) of the ground beef meat during storage at 4°C.

	Days of storage at 4°C				
	0	3	7	10	14
			Protein oxidation products		
MetMb					
T_0	23.98 ± 0.02^{aA}	35.13 ± 0.04^{aB}	40.9 ± 0.12^{aC}	51.33 ± 0.15^{aD}	58.23 ± 0.22^{aE}
T_1	24.03 ± 0.11^{aA}	31.15 ± 0.13^{bB}	$34.93 \pm 0.1b^{BC}$	$37.14 \pm 2.06c^{dC}$	43.06 ± 2.51^{bD}
T_2	23.85 ± 0.10^{aA}	31.12 ± 0.09^{bA}	36.09 ± 0.12^{cB}	42.94 ± 0.19^{bC}	45.03 ± 2.22^{bD}
T_3	23.93 ± 0.17^{aA}	$27.1\ 2\pm 0.11^{cB}$	34.26 ± 0.10^{dC}	39.54 ± 0.21^{cD}	42.2 ± 0.11^{bE}
T_4	23.61 ± 0.11^{aA}	26.34 ± 0.12^{dB}	32.04 ± 0.11^{eC}	35.14 ± 0.13^{dD}	40.23 ± 0.09^{bE}
Carbonyls contents					
T_0	4.02 ± 0.9^{aA}	5.11 ± 0.11^{aAB}	6.41 ± 0.78^{aB}	5.84 ± 0.74^{aAB}	4.51 ± 0.69^{aA}
T_1	3.58 ± 0.11^{aC}	4.3 ± 0.11^{bA}	4.45 ± 0.08^{bcA}	3.85 ± 0.11^{bBC}	4.01 ± 0.15^{aB}
T_2	3.88 ± 0.11^{aC}	4.5 ± 0.12^{bB}	5.45 ± 0.18^{abA}	4.1 ± 0.16^{bC}	3.88 ± 0.13^{aC}
T_3	3.04 ± 0.16^{aBC}	3.55 ± 0.10^{cAB}	4.15 ± 0.48^{cdA}	3.1 ± 0.17^{bBC}	2.67 ± 0.10^{bC}
T_4	3.11 ± 0.33^{aAB}	2.22 ± 0.20^{dB}	3.23 ± 0.37^{cA}	3.38 ± 0.41^{bA}	2.29 ± 0.33^{bB}
Sulfhydryls groups					
T_0	45.88 ± 0.20^{aA}	42.55 ± 0.19^{bB}	39.14 ± 0.03^{bC}	32.31 ± 1.11^{dD}	29.14 ± 0.81^{dE}
T_1	45.45 ± 022^{aA}	42.10 ± 2.10^{bAB}	40.6 ± 0.08^{abAB}	40.19 ± 0.81^{bBC}	37.60 ± 0.22^{bC}
T_2	46.03 ± 0.93^{aA}	42.45 ± 0.01^{bAB}	39.07 ± 1.67^{bAB}	36.68 ± 1.61^{cAB}	33.55 ± 1.09^{cC}
T_3	45.53 ± 0.77^{aA}	42.23 ± 1.01^{bB}	43.07 ± 1.23^{aC}	42.68 ± 1.22^{abD}	40.55 ± 1.04^{aE}
T_4	46.07 ± 0.33^{aA}	44.55 ± 1.06^{aAB}	43.27 ± 1.55^{aB}	43.08 ± 0.28^{aB}	42.19 ± 1.09^{aB}
			Lipid oxidation products		
Peroxide values					
T_0	2.21 ± 0.51^{aA}	6.32 ± 0.24^{aB}	11.06 ± 0.41^{aC}	14.22 ± 0.23^{aD}	11.85 ± 0.82^{aC}
T_1	1.89 ± 0.57^{aA}	4.74 ± 0.44^{bB}	6.48 ± 0.11^{bC}	7.9 ± 0.25^{cD}	8.16 ± 0.64^{abD}
T_2	2.05 ± 0.45^{aA}	5.05 ± 0.33^{bB}	7.11 ± 0.25^{bC}	9.48 ± 0.41^{bD}	10.75 ± 0.32^{abE}
T_3	1.89 ± 0.17^{aA}	3.95 ± 0.60^{bB}	5.21 ± 0.11^{cC}	7.58 ± 0.21^{cD}	7.91 ± 0.45^{abD}
T_4	2.05 ± 0.22^{aA}	4.11 ± 0.43^{bB}	4.74 ± 0.31^{cB}	7.58 ± 0.41^{cC}	8.16 ± 0.32^{cC}
TBARS value					
T_0	0.48 ± 0.12^{aA}	1.45 ± 0.07^{aB}	2.12 ± 0.09^{aC}	2.71 ± 0.11^{aD}	2.98 ± 0.12^{aD}
T_1	0.43 ± 0.01^{aA}	0.81 ± 0.14^{abB}	1.19 ± 0.13^{cdC}	1.58 ± 0.11^{cD}	2.01 ± 0.12^{cE}
T_2	0.44 ± 0.12^{aA}	1.09 ± 0.09^{abB}	1.59 ± 0.12^{bC}	2.01 ± 0.09^{bD}	2.48 ± 0.08^{bE}
T_3	0.40 ± 0.07^{aA}	0.8 ± 0.12^{abB}	1.31 ± 0.13^{bcC}	1.61 ± 0.11^{cC}	2.08 ± 0.13^{cD}
T_4	0.48 ± 0.11^{aA}	0.69 ± 0.12^{bAB}	$0.\ 9 \pm 0.11^{dB}$	1.22 ± 0.13^{dC}	1.88 ± 0.08^{cD}
CD					
T_0	0.717 ± 0.13^{aA}	0.752 ± 0.24^{aA}	0.685 ± 0.13^{aA}	0.629 ± 0.29^{aA}	0.626 ± 0.23^{aA}
T_1	0.667 ± 0.65^{aA}	0.689 ± 0.01^{aA}	0.642 ± 0.09^{aA}	0.616 ± 0.36^{aA}	0.585 ± 0.21^{aA}
T_2	0.663 ± 0.33^{aA}	0.681 ± 0.10^{aA}	0.655 ± 0.14^{aA}	0.613 ± 0.35^{aA}	0.555 ± 0.23^{aA}
T_3	0.633 ± 0.14^{aA}	0.686 ± 0.022^{aA}	0.613 ± 0.11^{aA}	0.525 ± 0.10^{aA}	0.435 ± 0.13^{aA}
T_4	$0.64\ 3\pm 0.22^{aA}$	0.681 ± 0.55^{aA}	0.603 ± 0.33^{aA}	0.505 ± 0.21^{aA}	0.412 ± 0.22^{bB}

±: standard deviation of three replicates. Values with a different letter (a - c) within a row of the same storage day of each treatment are significantly different ($P < 0.05$). Values with a different letter (A - E) within a column of the same treatment are significantly different ($P < 0.05$) by using Tukey's post-hoc test.

samples containing 0.2 % peel onion extract exhibited lower PV than negative control and treated samples with ascorbic acid. The negative control sample reached the maximum value (14.2 meq peroxide/Kg of meat) after ten days of storage and then a rapid decrease ($P < 0.05$) was observed. This decrease in PV was related to hydroperoxide degradation and secondary lipid formation [60]. For the treated samples (T_2, T_3 and T_4) and the positive control (T_1) a slight increase was observed ($P < 0.05$) during storage. The maximum PVs were reached in samples T_2, T_3 and T_4 and were respectively 10.75, 7.91, and 8.16 meq peroxide/Kg of meat. The slight significant ($P < 0.05$) increase observed indicated that the antibacterial effect of the enterocin BacFL31 and APOE delay the progression of initial oxidation step and the degradation of the formed peroxides. In accordance with our results, Mir et al. (2017) [63], reported that the addition of spices at level of

Table 3: Effect of BacFL31 and APOE and their combination on color, texture, odor, and overall acceptability of ground beef meat during storage at 4°C.

	Days of storage at 4°C				
	0	3	7	10	14
Color					
T_0	7.18 ± 0.24^{cD}	5.68 ± 0.14^{bC}	3.43 ± 0.31^{aB}	2.92 ± 0.10^{aA}	2.71± 0.14^{aA}
T_1	6.43 ± 0.17^{abD}	6.23 ± 0.16^{aC}	5.18 ± 0.15^{bB}	5.11 ± 0.21^{bB}	4. 91± 0.23^{aB}
T_2	6.91 ± 0.44^{bD}	6.81 ± 0.33^{cD}	6.11 ± 0.09^{dC}	5.75 ± 0.32^{dB}	5.19 ± 0.25^{dA}
T_3	6.51 ± 0.23^{aE}	6.35 ± 0.21^{aD}	5.93 ± 0.12^{cC}	5.5 5± 0.29^{cB}	5.0 6± 0.23^{cA}
T_4	6.71 ± 0.22^{abE}	6.31 ± 0.25^{aD}	6.11 ± 0.12^{dC}	5.79 ± 0.13^{eB}	5.29 ± 0.14^{dA}
Texture					
T_0	7.06 ± 0.17^{cD}	5.13 ± 0.27^{aC}	3.63 ± 0.12^{aB}	3.41 ± 0.15^{aB}	2.1 ± 0.15^{aA}
T_1	6.81 ± 0.42^{bE}	6.62 ± 0.55^{dD}	5.44 ± 0.33^{bC}	5.14 ± 0.22^{bcB}	4.51± 0.20^{bA}
T_2	6.55 ± 0.12^{aE}	6.25 ± 0.14^{cD}	6.00 ± 0.12^{dC}	5.19 ± 0.22^{cB}	4.81± 0.25^{dA}
T_3	6.56 ± 0.14^{aE}	6.06 ± 0.13^{bD}	5.44 ± 0.12^{bC}	5.09 ± 0.25^{bB}	4.73 ± 0.11^{dA}
T_4	6.88 ± 0.13^{bD}	6.18 ± 0.15^{cC}	5.81 ± 0.34^{cB}	5.13 ± 0.21^{bcA}	5.03 ± 0.23^{cA}
Odor					
T_0	7.06 ± 0.18^{cD}	4.55 ± 0.15^{aC}	3.25 ± 0.16^{aB}	2.12 ± 0.11^{aA}	2.03 ± 0.13^{aA}
T_1	6.73 ± 0.10^{aE}	6.12 ± 0.10^{dD}	5.17 ± 0.17^{bC}	3.88 ± 0.39^{bB}	3.31 ± 0.29^{bA}
T_2	6.63 ± 0.15^{aE}	5.93 ± 0.16^{cD}	5.24 ± 0.13^{bcC}	4.23 ± 0.29^{cB}	3.66 ± 0.17^{cA}
T_3	6.78 ± 0.11^{abD}	5.64 ± 1.24^{bC}	5.30 ± 0.11^{cB}	5.10 ± 0.10^{dA}	5.01± 0.12^{dA}
T_4	6.81 ± 0.17^{bD}	5.80 ± 0.27^{cC}	5.29 ± 0.13^{cB}	5.22 ± 0.21^{dB}	5.0 7± 0.15^{dA}
Overall acceptability					
T_0	6.93 ± 0.13^{cD}	4.87 ± 0.18^{aC}	4.15 ± 0.14^{aB}	3.9 ± 0.42^{aB}	2.93 ± 0.32^{aA}
T_1	6.75 ± 0.29^{bE}	6.2 5± 0.18^{cD}	5.53 ± 0.12^{cC}	5.31± 0.10^{cB}	3.88 ± 0.10^{bA}
T_2	6.77 ± 0.05^{bE}	6.0 6 ± 0.04^{bD}	5.31± 0.13^{bC}	5.04 ± 0.16^{bB}	4.77 ± 0.26^{cA}
T_3	6.52 ± 0.19^{aE}	5.93 ± 0.11^{bD}	5.33 ± 0.17^{bC}	5.14 ± 0.02^{bcB}	4.80 ± 1.12^{cA}
T_4	6.96 ± 0.24^{cE}	6.27 ± 0.20^{cD}	5.92 ± 1.11^{dC}	5.12 ± 0.27^{bB}	4.85 ± 0.23^{cA}

±: standard deviation of three replicates. Values with different letter (a - c) within a row of the same storage day of each treatment are significantly different ($P < 0.05$). Values with a different letter (A - E) within a column of the same treatment are significantly different ($P < 0.05$) by using Tukey's post-hoc test.

0.1 % caused decrement PV values in rista, a traditional meat product of India, compared to the control.

(ii) TBARS. TBARS is a reactive aldehyde produced by lipid peroxidation of meat polyunsaturated fatty acids [14]. TBARS values of ground beef meat are shown in Table 2. They were increased ($P < 0.05$) during storage in all samples. The TBARS values in the negative control sample (T_0) were higher ($P < 0.05$) than treated samples. The control sample (T_0) becomes unacceptable beyond 7 days of storage and a TBARS value of 2.12 mg MDA/kg of meat was recorded. According to Campo et al. (2006), an index of 2 mg MDA/kg of meat was considered the limiting threshold for the acceptability of oxidized beef meat [64]. For T_2 sample, the limit of acceptability was reached after ten days of storage whereas the samples treated with the BHT (T1), APOE (T_3), and the combination BacFL31+ APOE (T_4) remained acceptable at the end of storage (Table 2).

These results showed that the enterocin BacFL31 and the aqueous peel onion extract addition can protect the ground beef meat against lipid oxidation and extend the shelf life of meat. The use of APOE was very effective against the development of oxidative rancidity in beef meat. The phenolic compounds present in the peel onion extract could be an efficient electron donor capable to react with free radicals during the oxidation reaction.

(iii) Conjugated Dienes (CD). The CD values in control and treated samples during refrigerated storage are presented in Table 2. CD analysis revealed that the treatments and storage period significantly ($P < 0.05$) affected the lipid oxidation of beef meat samples. During storage period, the CD value of the negative control sample was higher ($P < 0.05$) than the treated ones. As shown in Table 2, we noticed that the concentration of CD increased significantly ($P < 0.05$) for all treatments at the beginning then decreased until the end of storage. This decrease in CD values proved that the conjugated hydroperoxides are expected to be transformed to secondary products as the TBARS formation occurs [65]. These findings were in accordance with previous studies of turkey meat sausage treated with bacteriocin BacTN635 [14].

3.4.3. Sensory Evaluation. The changes in attribute scores of sensory evaluation: color, texture, odor and overall acceptability of untreated (T_0) and treated (T_1, T_2, T_3 and T_4) ground beef meat during the fourteen days of refrigerated storage are shown in Table 3. It should be noted that sensory attributes scores of meat samples untreated and treated with

enterocin BacFL31 and APOE were assessed by the panellists with scores above the rejection limit set to 5. Furthermore, the addition of BacFL31 at 200 AU/g (T_2), APOE (T_3), and their combination (T_4) and storage time have a significant effect ($P < 0.05$) on the sensory parameters of ground beef meat (Table 3). The negative control sample displayed the lowest score at day 14, demonstrating unacceptable odor, texture and color as well as a very low overall acceptability. Equally, at the end of the storage period (14 days), T_4 sample showed the significant ($P < 0.05$) and highest color, texture, odor, and overall acceptability scores which were respectively 5.29 ± 0.14, 5.03 ± 0.23, 5.07 ± 0.15 and 4.80 ± 1.12 (Table 3). Whereas the negative control sample become unacceptable after 3 days of storage, the overall acceptability of ground beef meat treated with BacFL31 (T_2) remains acceptable until 10 days of storage. The meat treated with the APOE (T_3) and with combination (T_4) remains acceptable for two more days than the meat treated with BacFL31 (T3).

4. Conclusion

In this study, we used two natural compounds in the preservation of the ground beef meat at 4°C during 14 days of storage. The bacteriocin BacFL31 at 200 AU/g from the safe strain *E. faecium* FL31 and the aqueous peel onion extract (APOE) at 1 MIC/g were added alone or in combination for meat biopreservation. The impact of the different treatments as regards microbiological, physico-chemical and sensory properties was evaluated. The use of the combination between bacteriocin and plant extract was significantly more effective than the use of each active compound alone. To the best of our knowledge, this is the first report using such combination and may provide novel solutions for improved meat safety. These findings provide interesting information for meat preservation, delaying lipid and protein oxidation and preventing the pathogens proliferation.

Data Availability

The safety Enterococcus faecium FL31 strain, the enterocin BacFL31, the aqueous peel onion and their results of the treated ground beef meat data used to support the findings of this study are included within the article.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

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