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Natural Strategies to Improve Quality in Food Protection

Special Issue Editor in Chief: Flora V. Romeo
Guest Editors: Antonino Malacrinò and Juliana A. Macedo

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Editorial Board

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Editorial

Natural Strategies to Improve Quality in Food Protection

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In a world of climatic and social challenges and related changing eating habits, the health and environmental consequences of the persevered use of synthetic chemicals and overprocessed foods are triggering radical changes in the way we approach food protection. Therefore, new food processing methods based on natural products could represent an alternative way to guarantee both food security and safety while preserving health and environment. Postharvest losses are mainly caused by microorganisms and arthropod pests; however, the wide use of synthetic chemicals is being limited by an increasing public concern regarding residues in food and by the development resistance to pesticides. Also, extensive use of preservatives and overprocessing methods are often required to extend the shelf life of processed food, negatively impacting on nutritional quality.

Plant secondary metabolites represent an invaluable source of molecules with antimicrobial activity against a wide range of bacteria, yeasts, molds, and viruses. Indeed, the antimicrobial effect exhibited by many herbs and spices extracts is due to their phytochemical constituents [1]. The concentration of these bioactive compounds is usually variable depending on plant variety, seasonal factors, and geographical factors, but biotic and abiotic stresses may increase the concentration of the secondary metabolites that plants produce as defense factors. Among these phytochemicals, polyphenolic extracts and essential oils are the most studied and used antimicrobials in food and are often applied as antioxidants and flavor- and color-enhancing agents [2, 3]. The development of nano- and microstructures able to stabilize and/or deliver bioactive compounds of natural origin has recently attracted the attention of researchers and industry. Since phytochemicals are degraded during processing and storage and they are often characterized by a strong odor, these new encapsulation techniques are useful to stabilize and deliver bioactive compounds, micronutrients, fibers, prebiotics, and probiotics in food and to mask undesired flavors [4]. These approaches can also help replace the traditional processes with new mild and ecofriendly techniques. Heat treatments, for example, can reduce the bioactivity of phytochemical compounds; even though in some cases, this process induces the formation of novel compounds with increased antioxidant potential [1, 5].

The use of natural products in food protection against arthropods is attracting particular interest, thanks to a combination of efficiency in pest control with a reduction of typical issues associated to chemical pesticides [6, 7]. The natural availability of these products can have economic benefits especially in developing countries [8, 9]. Although an increasing number of laboratory studies is accounting for their efficacy, the published evidence on the real implementation of natural products in pest management programs is somewhat thin, especially outside of R&D projects [10]. Although the biopesticide market is quickly increasing, according to Chandler et al. [11], it needs a major boost in order to override the prevalence of synthetic chemical pesticides. However, as pointed out by the same authors, investments in this direction can be constrained by failure in reducing fixed costs, farmers' risk aversion to unknown products, integration costs into current pest management programs, and lack of profit from a niche market. Indeed,

companies have to go through a strict procedure to be able to commercialize pesticides based on natural products, which has a cost. Furthermore, the authorization process is conceived for conventional synthetic pesticides, with regulatory barriers that can severely hinder, or even prevent, products to enter the market.

The current special issue addresses research and review articles related to new developments on the use of natural extracts or natural compounds to improve food quality, safety, and security.

The possibility of using different doses of cornelian cherry juice as a functional additive in the production of beef burgers was investigated in this special issue by A. M. Salejda et al. The cornelian cherry juice effectively reduced lipid oxidation and allowed maintaining the sensory characteristics of the beef products. This indicates the feasibility of using the active compounds of cornelian cherry juice (iridoids and polyphenols) to prolong the shelf-life of meat products and, at the same time, to offer novel nutraceutical products. The obtained results can be applied in the meat industry to develop novel products.

Another study demonstrates that extracts from different herbs, which are specific to spontaneous flora in Romania, can be incorporated successfully into a fermented dairy product, in particular into yoghurt. A. Dabija et al. examined the effects of aqueous extracts prepared from four herbs (thistle, hawthorn, sage, and marjoram) on the yoghurt's qualitative characteristics. The results showed that the physicochemical and rheological properties of the yoghurt with herbs extracts addition were improved compared to the control sample after 28 days of storage. Marjoram extract exhibited the best antioxidant properties and nutritional values, while the thistle extract showed the best physicochemical and rheological properties. Since yoghurt is a source of bioactive peptides, these obtained results certainly encourage the production of yogurts fortified with natural antioxidants from natural sources.

In another study about fermented milkless beverages, U. T. Jasińska et al. focused on probiotics immobilization to prolong their survival, a current challenge for food industries. The immobilization of probiotic bacteria in alginate, or low-methoxylated pectin hydrogel particles, significantly increased the survival rate of these strains in fermented milkless beverages compared with free bacteria cells. The authors also evaluated the impact of the immobilizing method on the sensory properties of the beverages. The results confirmed that the supplementation of fermented beverages with microencapsulated bacteria does not affect the overall sensory quality of beverages during the storage period.

Another approach to improve food quality is based on mathematical and statistical methods, which can help to optimize processes and workflows or to help in estimating risk levels. R. H. Abiyev et al. constructed a Z-number-based fuzzy system to predict the food security risk level using data about cereal production in Turkey, demonstrating the applicability of the designed system in real life.

Since the use of insecticidal molecules extracted from plants is attracting considerable interest from both researchers and consumers, O. Campolo et al. focused on reviewing the literature about the use of plant essential oils (EOs) in stored products protection carried out in last 15 years. Although the efficacy of EOs as repellents and/or insecticides has been already recognized, this is the first critical review on their use in foodstuff protection, a challenging sector where pest control techniques are quite limited. The authors presented the major findings about the insecticidal activity and the impact on insect life-history traits (e.g., fertility and fecundity) of EOs on stored products' pests. Furthermore, the authors reviewed the available information about their putative modes of action, suggesting that EO-based insecticides may act on insect-specific metabolic pathways, different from those of vertebrates, making them safe towards nontarget organisms.

The studies reported in the present issue offer the opportunity to deepen the possibilities of using natural extracts to improve quality in food protection. Moreover, the use of mathematical models can further help companies and researchers to raise the level of food quality and safety. We are assisting to increase efforts in testing the efficacy of natural products in food protection. Researchers are improving formulates and their integration into production chains, while policy-makers are putting efforts to ease their regulation, with the joint aim to help the transition from conventional approaches. Therefore, now it is time to climb through the looking glass, search beyond the lab bench, and to push our research efforts into real-world situations boosting the transition from conventional strategies to a more environmental-friendly food protection.

Conflicts of Interest

The Lead Guest Editor and the other Guest Editors do not have any possible conflicts of interest or private agreements with companies.

Flora V. Romeo
Juliana Alves Macedo
Antonino Malacrinò

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Review Article

Essential Oils in Stored Product Insect Pest Control

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Among botanical extracts used as insecticides, essential oils (EOs) are promising alternatives to chemical insecticides. EOs are synthesized by plants, and they play a key role in plant signaling processes including also attractiveness toward pollinators and beneficial insects. Plant species producing essential oils (over 17,000 species) are called aromatic plants and are distributed worldwide. Our review aims to evaluate research studies published in the last 15 years concerning the use of EOs in stored product protection. More than 50% of the retrieved manuscripts have been published by authors from Eastern countries (Iran, China, India, and Pakistan), investigating different aspects related to insect pest management (exposure route, effect on the target pest, and mode of action). Coleoptera was the most studied insect order (85.41%) followed by Lepidoptera (11.49%), whereas few studies targeted new emerging pests (e.g., Psocoptera). Almost all the trials were carried out under laboratory conditions, while no experiments were conducted under real operating conditions. Future research studies concerning the use of EOs as insecticides should focus on the development of insecticide formulations which could be successfully applied to different production realities.

1. Introduction

The ecotoxicological, environmental, and social consequences of the widespread use of chemical insecticides in agriculture have led researchers to find viable alternatives that are more environmentally friendly than synthetic chemicals. In this context, the use of insecticides based on botanical extracts is attracting considerable interest both among researchers and consumers. Among botanical extracts used as insecticides, essential oils (EOs) are a promising alternative because of their worldwide availability and relative cost-effectiveness.

Essential oils are secondary metabolites synthesized by plants, and they play very important roles in plant defense (both against biotic and abiotic stresses) and signaling processes, including also the attraction of pollinators and beneficial insects [1–4].

EOs are synthesized by plants both internally (secretory glands allocated inside the plants) as well as externally (secretory glands placed on the plant surface) [5]. They are produced by different plant organs such as flowers, herbs, buds, leaves, fruit, twigs, bark, seeds, wood, rhizomes, and roots and can be accumulated in specific histological structures (glandular trichomes, secretory cavities, and resin ducts) [6, 7]. Plant species that produce essential oils are called aromatic plants and are distributed worldwide; these plants (over 17,000 species) belong to a limited number of families: Asteraceae, Cupressaceae, Lamiaceae, Lauraceae, Rutaceae, Myrtaceae, Piperaceae, and Poaceae [5, 8].

Essential oils are mainly constituted by monoterpenes and sesquiterpenes synthesized in the cytoplasm and plastids. All terpenes are synthetized via either the methylerythritol 4-phosphate (MEP) pathway or the mevalonate-dependent (MVA) pathway. Two (C_5) isoprene precursors, isopentenyl pyrophosphate (IPP) and dimethylallyl pyrophosphate (DMAPP), are involved in the terpene synthesis and the isoprene units determine their class (monoterpenes, C_{10}; sesquiterpenes, C_{15}) [9]. Sesquiterpenes contain 15 carbon atoms, and they are less volatile and have a higher boiling point than monoterpenes. As a consequence, fewer of them contribute to the fragrance of EOs [10].

EOs are constituted by a blend of 20 to 70 organic compounds, some of which represent more than 80% of the constituents as append, e.g., in Sweet Orange EO, the main compound, limonene reaches 88–97% of the whole oil [11, 12]. Generally, the main components characterize the biological activity of the EOs.

EOs are hydrophobic and generally lipophilic, their density is often lower than that of water, and they are soluble in organic solvents.

Despite the numerous extraction methods used to obtain EOs from natural raw plant material, only four methods, such as (i) hydrodistillation, (ii) steam distillation, (iii) dry distillation, and (iv) mechanical processes, are considered in the European Pharmacopoeia and the International Standard Organization on Essential oils (ISO 9235:2013) [4]. The EO can undergo physical treatments, which do not result in any significant change in its composition (e.g., filtration, decantation, and centrifugation). The resulted products consist of a blend of volatile compounds with a strong odor [13].

1.1. Hydrodistillation. This method is considered the simplest one to obtain EOs from the plant material by immersion of biomass in boiling water [10]. The oil contained in the oil cells diffuses by means of osmosis in the hot water; then the steam, produced by boiling water, carries the oil vapors in a condenser. The condensed EOs are separated from water by decantation.

1.2. Steam Distillation. In the steam distillation, the vapor is supplied in such a way that liquid water does not come into contact with the vegetal raw material. In the simplest version, steam is generated by water added in the lower part of the distiller; the plant raw material is separated from the liquid water by a perforated grid. The steam that passes through the plant material carries the oil vapors, and after passing through a condenser, EO is separated from water by decantation.

1.3. Dry Distillation. This technique involves heating the plant material in the absence of oxygen, which would promote combustion, and without adding water or steam. This method is not commonly used. The EOs produced using dry distillation are cade and birch. Rectification is often necessary to remove undesirable molecules that may have formed.

1.4. Mechanical Process. The mechanical process, also known as cold-pressing method, consists in extracting EOs at ambient temperature without involving heat [10]. This method is used for the production of *Citrus* spp. and *Fortunella* spp. peel oils.

In addition to those previously described, other extraction methods are developed with the aim to improve the quality, the yield, and to decrease the energy consumption (i.e., solvent extraction, microwave-assisted extraction, ultrasonic extraction, Soxhlet extraction, subcritical or superheated water extraction (SCWE), and supercritical fluid extraction).

2. EOs against Stored Product Insects

Although some reviews on the potential of essential oils as repellents and/or insecticides have been published, there is no critical review about their use in stored products protection. One of the most important characteristics of essential oils, their phytotoxicity, may favor their use as herbicides, but at the same time limit their use in crop protection [14, 15]. Stored product sector seems to be a perfect candidate for the development of new EO-based alternative pest control strategies.

The aim of this review was to analyze research studies on the use of essential oils in stored product protection (sensu lato) as carried out in the last 15 years. The scientometric analysis of publications on EOs against stored product pests was based on documents retrieved from the Scopus database (see Supplementary Materials for Supplementary Method 1).

In the last 15 years, 210 documents were published (Figure 1). Among the 210 publications, 197 are articles, 9 book chapters, 3 reviews, and 1 conference paper. The three retrieved reviews deal with general aspects of the use of essential oils such as green pesticides against a range of insects, including few stored product pests [16], related to few plant families [17], or applied only as fumigants [18].

These studies were published by researchers of 47 countries distributed worldwide, but almost the 50% of the retrieved studies were published by researchers from Iran (21.63%), China (17.14%), India (5.30%), and Turkey (5.30%) (Figure 2).

The EOs used in the various experiments were extracted mainly from aerial parts (71.88%), with leaves (28.51%) as the major EO source material. The other plant materials used for the EO extraction were resin, gum, rhizomes, and roots. In 22.49% of the trials, the EO sources were not reported. Hydrodistillation was the most widely used extraction method (52.91%) followed by steam distillation (8.25%). Many analysed papers (29.12%) report neither the extraction method used for the EOs production nor the part of the plant processed, probably due to the use of commercial oils. In the various experiments carried out, 65.18% of the EOs were chemically characterized, whereas in 30% of the trials, this information was missing. Furthermore, book chapters and review papers, as well as papers aimed to evaluate just the activity of single components of some EOs, provide no information about the chemical characterization of the mentioned EOs.

The retrieved studies investigate several topics related to the control of stored product insects (Figure 3). Since many retrieved papers address different insecticidal activity (contact toxicity, fumigation, mode of action, etc.) and/or different essential oils, our data analysis, unless otherwise specified, refers to single trials (EO—target species—effect).

Among those aimed at killing the insect pests, 44.21% of the trials regard the use of essential oils, or EO-based insecticide formulations, applied as fumigants; 21.66% evaluated the contact toxicity, and less than 1% of the studies

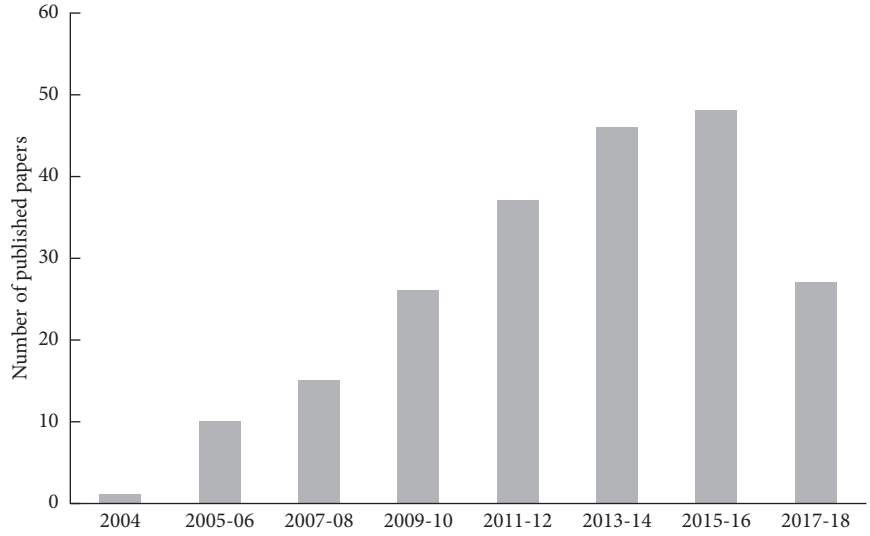

Figure 1: Number of papers published in the last 15 years on essential oils.

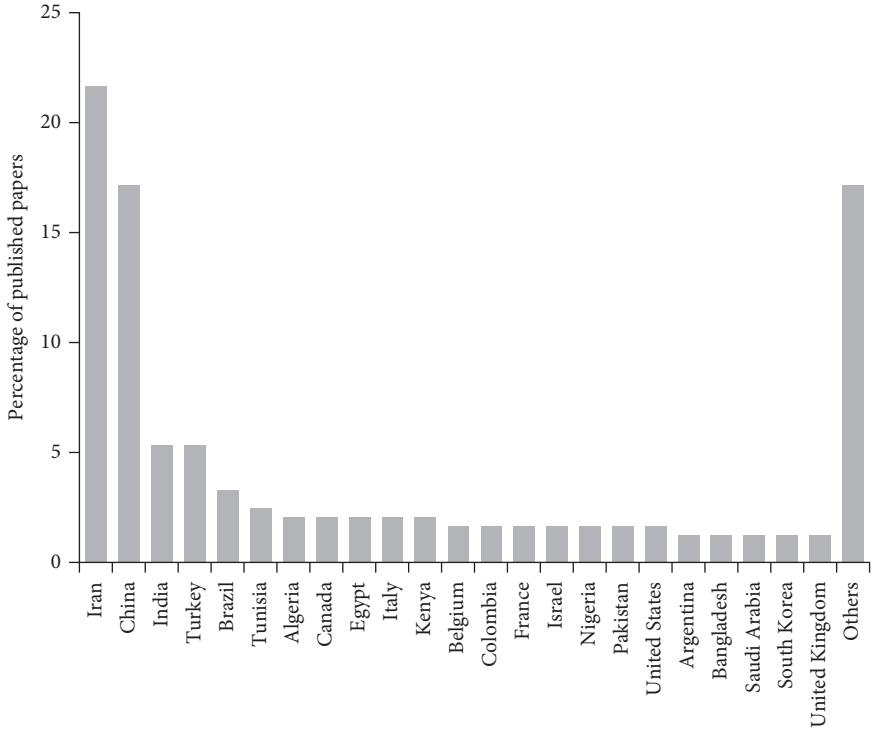

Figure 2: Distribution by country of papers published in the last 15 years.

tested the insecticidal efficacy by ingestion route. Other investigated aspects were the mode of action of EOs (2.48%) or the effects of these natural compounds on the life history traits of insects (6.72%).

Coleoptera were the most studied insect order (85.41%) with the two key species *Tribolium castaneum* (Herbst) (Coleoptera: Tenebrionidae) and *Sitophilus oryzae* (L) (Coleoptera: Curculionidae) which accounted for almost 50% of the coleopteran studies. Lepidoptera were used in 11.49% percent of the trials, in which *Plodia interpunctella* (Hübner) (Lepidoptera: Pyralidae) and *Ephestia kuehniella* (Zeller) (Lepidoptera: Pyralidae) represented almost all the studies carried out against this insect order. The remaining studies concerned aspects related to Psocoptera control, and *Liposcelis bostrychophila* (Badonnel) (Psocoptera: Liposcelididae) was used in almost all studies involving this insect order (29 out of 35 trials) (Figure 4).

3. Insecticidal Activity

A huge number of research studies aimed at assessing the insecticidal activity of EOs against crop pests as well as

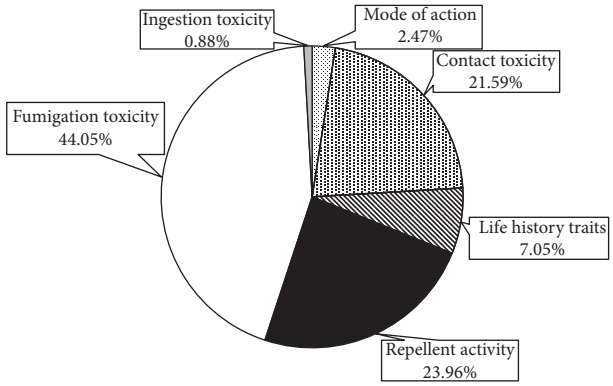

Figure 3: Percentage of different research topics accounted in the analysed literature.

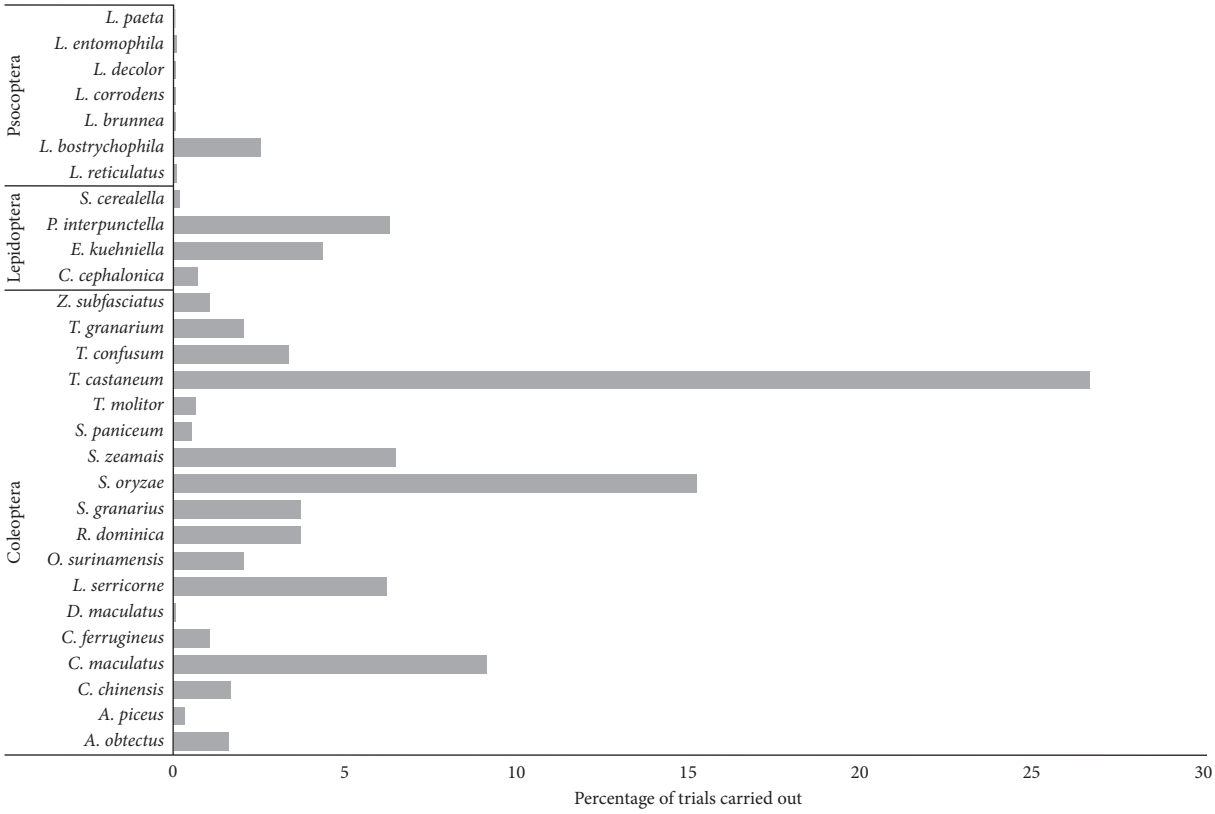

Figure 4: Percentage of trials carried out for every insect species accounted in the reviewed literature.

against disease vectors [1, 19], but less attention has been paid to stored product pests. Here, we briefly review the results achieved using EO treatments against stored product pests according to their application method (i.e., contact, fumigation, and ingestion) and insecticidal activity (i.e., toxicity and repellence). In detail, we empathize the most promising results for every insect family, highlighting, when possible, similarities or divergences between pest and/or EO plant species. Furthermore, studies aimed at evaluating modifications of EOs activity attributable to geographic origin, EO-based formulations, synergism with other insecticidal compounds, and research studies reporting characteristic results were reported.

4. Contact and Ingestion Toxicity

In the last decade, several research studies focused on the insecticidal activity of EOs through contact and ingestion routes (see Supplementary Materials for Table S1). Many research studies did not discriminate between these two kinds of administration, since EOs were used to treat the food matrix on which pests moved and fed. However, we documented 74 papers claiming to evaluate contact toxicity, investigating the contact or the ingestion toxicity in 254 trials, each one involving a different combination of tested EO (or EO-based formulation) and target insect species. According to EO plant families, Lamiaceae (68 combinations), Asteraceae (33 combinations), Rutaceae (33

combinations), and Myrtaceae (20 combinations) were predominant. Most research studies focused on Coleoptera species (232 combinations), followed by Lepidoptera (13 combinations) and Psocoptera (10 combinations).

Regarding Coleoptera, many insect families were evaluated, although most efforts were directed toward Curculionidae (88 combinations) and Tenebrionidae (78 combinations). However, the effects of EOs on the mortality of stored product coleopteran species are highly variable. Abdelgaleil et al. [20] evaluated the toxic impact of 20 plant EOs against the curculionid *S. oryzae,* highlighting that only few plants exerted strong insecticidal contact activity. In detail, *Artemisia judaica* (Asteraceae), *Callistemon viminalis* (Myrtaceae), and *Origanum vulgare* (Lamiaceae) had LD_{50} values (i.e., the EO dose lethal for 50% of tested insects) of 0.08, 0.09, and 0.11 mg/cm^2, respectively. Promising results against *S. oryzae* were recorded also for EOs extracted from *Syzygium aromaticum* (Myrtaceae) and *Lavandula officinalis* (Lamiaceae) (LD_{50} values 0.04 and 0.07 mg/cm^2, respectively) [21], from *Acorus calamus* (Araceae) (LD_{50} value 54.46 μg/cm^2) [22], and from *Coriandrum sativum* (Apiaceae), *Eucalyptus obliqua* L'Hér. (Myrtaceae), and *Pinus longifolia* (Pinaceae) (LD_{50} values 36.68, 52.77 and 77.30 μg/cm^2, respectively) [23]. However, the lowest LD_{50} values were recorded for *Aster ageratoides* (Asteraceae) (LD_{50} = 27.16 μg/cm^2 [24]), *Dracocephalum moldavica* (Lamiaceae) (LD_{50} = 22.10 μg/cm^2 [25]), and *Litsea salicifolia* (Lauraceae) (LD_{50} = 0.079 μL/insect [26]) against the close-related species *Sitophilus zeamais* (Motschulsky) (Coleoptera: Curculionidae), suggesting that these EOs may be reliable insecticidal sources also at very low dosages for curculionid stored product pests.

Overall, Tenebrionidae species were reported to be less susceptible to EOs compared with Curculionidae beetle [21, 22, 26]. Moreover, some plants showed important insecticidal activity against tenebrionid pests. In detail, *Atalantia guillauminii* (Rutaceae) presented an LD_{50} value of 17.11 μg/cm^2 [27] and *Eucalyptus procera* (Myrtaceae) an LD_{50} of 0.129 μL/cm^2 [28] against *T. castaneum*. For tenebrionid species, which are external feeders, also the ovicidal activity of EOs has been deemed. External pests (also known as secondary pests) develop for their whole life outside the grains, in contrast to internal feeders (or primary pests). Curculionid weevils are internal feeders, and their larval stages develop inside the kernels until the adult emergence, keeping them protected during the preimaginal stages.

Among Anobiidae species, research studies mainly involved *Lasioderma serricorne* F (Coleoptera: Anobiidae) and *Callosobruchus* spp. Among the tested EOs, *L. serricorne* showed higher susceptibility to *Perilla frutescens* EO (LD_{50} = 1.46 μg/adult) [29], while the bruchids *Callosobruchus chinensis* L. and *Callosobruchus maculatus* F. (Coleoptera: Bruchidae) were more susceptible to *A. calamus* (LD_{50} = 13.30 μg/cm^2) and *E. procera* (LD_{50} = 0.124 μL/cm^2), respectively [22, 28].

EO toxicity in Lepidoptera and Psocoptera was only evaluated toward Pyralidae and Liposcelididae species. *E. kuehniella* and *Plodia interpunctella* (Hübner) (Lepidoptera: Pyralidae) are strongly susceptible to *Satureja hortensis* (Lamiaceae) (LD_{50} = 0.27 and 0.19 μL/cm^2, respectively) at the late larval stage [30]. The insecticidal activity of formulations against lepidopteran pests is usually evaluated toward the larvae, since the immature stages are responsible for direct food damage. Nevertheless, some studies also focused on the insecticidal toxicity of tested EOs toward adult moths, which are considered spreading agents and thus an important target for an appropriate pest-control program. However, contact toxicity against adult Lepidoptera is little investigated, since adult moths are generally less susceptible to EOs compared with Coleoptera species [23, 31]. Although this assumption is generally recognized, some EOs presented remarkable toxic activity against *P. interpunctella* adults; good mortality rates were reported for contact toxicity with *Cymbopogon martinii* (Poaceae) EO (LD_{50} = 22.8 μg/cm^2) [31], as well as for treatments with *Coriandrum sativum* (Apiaceae), which exerted an LD_{50} value of 47.93 μg/cm^2 [23]. Among Psocoptera, *L. bostrychophila* is the only psocid species studied for EO contact toxicity. Promising insecticidal activities were recorded for EOs extracted from *Laggera pterodonta* (Asteraceae) (LD_{50} = 28.53 μg/adult) [32], *Liriope muscari* (Asparagaceae) (LD_{50} = 21.37 μg/cm^2) [33], and *Dictamnus dasycarpus* (Rutaceae) (LD_{50} = 27.2 μg/cm^2) [34] used as contact insecticide.

When evaluating contact toxicity of EOs, it is not always possible to distinguish between the mere contact activity and the synergistic effect of ingestion and contact toxicity. For instance, several studies evaluate the toxicity by putting insect specimens on food grains treated with EOs. In this scenario, it is possible to hypothesize that EOs can act as contact insecticides, as well as they can exert ingestion toxicity when pests feed on the grains [35–40]. Few studies claimed to evaluate the ingestion toxicity of EOs. Popović et al. [41] evaluated the ingestion toxicity of 9 different EOs against *T. castaneum,* highlighting that at 1.14% EO concentration, only *Calamintha glandulosa* (Req.) Benth. (Lamiaceae) showed good insecticidal outcome (i.e., over 96% mortality). In contrast, all the other tested EOs presented mortality rates lower than 15%. For instance, it is acknowledged that the presence of foodstuff, and thus the direct treatment of food, usually limits the toxicity of EOs toward stored product pests, suggesting that ingestion toxicity plays a minor role on pest mortality [42].

EOs are botanicals extracted from cultivated and wild plants, and their composition is strongly subjected to variations according to their geographic origin. Thus, it is not surprising that EOs from different geographic areas may cause different responses in the same insect species. As an example, *Citrus sinensis* L. (Rutaceae) EOs from geographically different origin presented highly variable LD_{50} value against *S. oryzae*, ranging between 0.29 and 0.43 mg/cm^2 [20, 21]. Furthermore, also the physiological status (i.e., flowering, vegetative, etc.) of the plants, as well as the part of the plant from which EOs were extracted, can significantly alter the quality and the quantity of plant-borne compounds and thus the toxicity of the EOs [43, 44].

The contact toxicity of EOs toward stored product pests may also be enhanced or reduced when EOs are combined

with other control tools. A major criticism relative to EO employment as insecticide is their high volatility and thus low persistency. These characteristics force the operators to continuous and repeated applications. Several researches aimed at improving the stability of EOs through the combination with powders, which can be applied directly on foodstuffs. Indeed, some good results have been reported for montmorillonite clay, which could extend the effectiveness of *Ocimum gratissimum* (Lamiaceae) EO from 7 to 30 days against *Sitophilus zeamais* (Coleoptera:Curculionidae) [45]. Furthermore, also the employment of diatomaceous earths showed promising improvement of EO toxicity and consequently a strong reduction of employed EO dosages. Against curculionid and tenebrionid pests, the addition of diatomaceous earths may result in a 5- and 10-fold reduction, respectively, of the EO doses employed for the treatment [46]. On the contrary, Campolo et al. [47] demonstrated that diatomaceous earths could have antagonistic effect with *C. sinensis* EO in controlling *Rhyzopertha dominica* (F.) (Coleoptera: Bostrichidae). In contrast, substituting diatomaceous earths with kaolin to treat wheat grain, EO-kaolin mixture exhibited synergistic toxic activity against the bostrichid pest. For instance, the particle size of diatomaceous earths, as well as of other clays and dusts, could strongly affect their effectiveness when combined with EOs. Ziaee et al. [48] investigated the combination of diatomaceous earths and *Carum copticum* (L.) (Apiaceae) EO toward *T. confusum* and *Sitophilus granarius* (L.) (Coleoptera: Curculionidae) adults, comparing specifically the particle sizes. This study revealed that while particles with dimensions >37 μm presented synergistic activity against both pests, bigger particles (>149 μm) had antagonistic effect toward *T. confusum* [48]. On this basis, the development of nanoformulations and nano-sized particles may be helpful to improve EO toxicity. Among these techniques, oil-loaded nanocapsules can improve EOs insecticidal activity, as reported for polycaprolactone nanocapsules loaded with *Rosmarinus officinalis* (Lamiaceae) EO against *T. castaneum* [49].

5. Fumigant Toxicity

According to the revised literature, 125 papers accounted the insecticidal activity of EOs through fumigation (see Supplementary Materials for Table S2), investigating 499 different trials, each one involving a different combination of tested EO (or EO-based formulation) and target insect species. The most studied families were Lamiaceae (167 combinations), followed by Asteraceae (56 combinations), Myrtaceae (49 combinations), Apiaceae (47 combinations), and Rutaceae (42 combinations). Similar to contact toxicity tests, the insect order most studied was Coleoptera (428 combinations), followed by Lepidoptera (68 combinations) and Psocoptera (4 combinations). Among Coleoptera, research studies mainly focused on Tenebrionidae (161 combinations), Curculionidae (139 combinations), and Bruchidae (49 combinations), while among Lepidoptera and Psocoptera, only Pyralidae (68 combinations) and Liposcelididae (4 combinations) were evaluated.

Some EOs showed to be highly effective against many Coleoptera species, according to their LC_{50} values (i.e., the EO concentration which caused 50% of mortality). Among Lamiaceae, *Ocimum gratissimum*-EO fumigation showed interesting insecticidal activity against *R. dominica* (LC_{50} = 0.20 μL/L), *S. oryzae* (LC_{50} = 0.50 μL/L), *C. chinensis* (LC_{50} = 0.20 μL/L), and *Oryzaephilus surinamensis* L. (Coleoptera: Silvanidae) (LC_{50} = 0.19 μL/L), although this EO was less active toward *T. castaneum* (LC_{50} = 24.9 μL/L) [50]. Similarly, fumigation with *Artemisia scoparia*-EO exerted LC_{50} values of 2.05 μL/L for *T. castaneum,* LC_{50} of 1.87 μL/L for *S. oryzae* and LC_{50} of 1.46 μL/L for *C. maculatus* [51].

Sitophilus oryzae is the most studied curculionid species and reveals to be particularly susceptible to *C. copticum* (Apiaceae) (LC_{50} = 0.91 μL/L) [52]. Lamiaceae were the most effective plant family. For instance, EOs from *O. vulgare, Salvia fruticosa, S. officinalis, S. pomifera, Thymbra capitata,* and *Thymus persicus* showed high fumigation toxicity toward *S. oryzae*, with LC_{50} values ranging between 1.5 and 9 μL/L [20, 53, 54]. Among the other plant families, *L. nobilis* (Lauraceae) (LC_{50} = 8.0 μL/L) [53], *Eucalyptus* spp. (Myrtaceae) (LC_{50} between 7 and 8.5 μL/L) [55, 56], and *Citrus limon* (Rutaceae) (LC_{50} = 9.89 μL/L) [20] were particularly effective against *S. oryzae* adults applied as fumigant. Regarding other curculionid weevils, again Lamiaceae revealed to be very effective as fumigant (i.e., *Origanum acutidens* and *Mentha pulegium* against *S. granarius* [57, 58] and *D. moldavica* against *S. zeamais* [25]). Notably, also the EO from fruits of a plant belonging to the Lauraceae family, *L. salicifolia,* showed high insecticidal properties against *S. zeamais* when employed in fumigation trials (LC_{50} = 4.4 μL/L) [26]. Fumigation toxicity toward Curculionidae species mainly refers to adults. Indeed, as curculionid weevils are internal feeders, the toxicity of EOs toward larvae, pupae, and eggs has been little investigated and still inconclusive, although adults seemed to be more susceptible than the immature stages [59, 60].

Tribolium castaneum is an insect model to study fumigant toxicity of EOs. Among the reviewed papers, the most effective EO against *T. castaneum* was *Allium sativum* (Amaryllidaceae) with LC_{50} value of 1.52 μL/L [46]. As reported for Curculionidae, Lamiaceae EOs reveal strong toxicity also against *T. castaneum.* In detail, *Rosmarinus officinalis* (LC_{50} = 1.17 μg/mL) [61] and *Mentha* spp. (LC_{50} values between 12 and 13 μL/L after 24h) [62, 63] showed the highest insecticidal efficacy as fumigant agents. Furthermore, also the fumigations with EOs extracted from plants belonging to other plant families could have good knockdown abilities. Indeed, *Achillea wilhelmsii* (Asteraceae) gave good results against *T. castaneum* (LC_{50} = 10. 02 μL/L) [62], as well as *Eucalyptus* spp. (Myrtaceae) (LC_{50} values ranging between 11 and 14 μL/L) [28, 55, 56], *Citrus reticulata* (Rutaceae) (LC_{50} = 3.49 10^{-3}%) [11] and *Pistacia lentiscus* (Anacardiaceae) (LC_{50} = 8.44 μL/L) [64].

Conversely to Tenebrionidae and Curculionidae, Asteraceae plants were generally more toxic against Bruchidae species [56, 62, 65, 66]. Nevertheless, the lowest recorded LC_{50} values were noted for *Ocimum americanum*, belonging to Lamiaceae, and *Lippia multiflora,* from

Verbenaceae, which were able to halve bruchid population at 0.23 and 0.47 μL/L, respectively [67]. Similar to Bruchidae, the EO from *Artemisia herba-alba*, (Asteraceae) was the most effective fumigant against *O. surinamensis*, with an LC_{50} value of 3.50 μL/L [68]. Furthermore, good knock-down outcomes were also obtained in fumigation trials applying EO from Myrtaceae toward *R. dominica* adults (*Eucalyptus globules* LC_{50} = 3.5 μL/L) as well as against *L. serricorne* adult insects treated with Lamiaceae EO (*Lavandula stoechas* LC_{50} = 3.8 μL/L) [69]. In contrast with previously described results, the bruchid *Acanthoscelides obtectus* (Say) (Coleoptera: Bruchidae) and the dermestid *Trogoderma granarium* (Everts) (Coleoptera: Dermestidae) seemed to be slightly influenced by EO fumigations [70–73]. Although the majority of EOs could exert good insecticidal activity against target insects as fumigants, some of them caused low or no toxicity against stored product pests if applied through fumigation [60, 70, 74, 75]. Nevertheless, research studies on the fumigant toxicity of EOs against adult insects of Coleoptera other than Curculionidae and Tenebrionidae were limited, and results may be less reliable and conclusive. Furthermore, it should be accounted that under real operative conditions, one of the factors which mainly affects EO-fumigation outcomes is the presence/absence of grain and food that can impair the effectiveness of the treatments [42].

As reported for contact toxicity, EO fumigant activity was just evaluated toward Pyralidae and Liposcelididae species among Lepidoptera and Psocoptera. EOs extracted from plants of the Lamiaceae family caused the highest mortality to larvae of the moth *Plodia interpunctella.* In detail, *R. officinalis* (LC_{50} = 0.93 μL/L), *Zataria multiflora* (LC_{50} = 1.75 μL/L), *S. thymbra* (LC_{50} = 3.43 μL/L), and *Origanum onites* (LC_{50} =4.06 μL/L) were the most effective fumigants [58, 76]. With regard to *E. kuehniella*, the most toxic EOs as fumigant generally belonged to Lamiaceae too. Indeed, *Origanum onites*-EO presented an LC_{50} value against *E. kuehniella* larvae of 7.52 μL/L [76], similar to the closely related species *Origanum majorana* (LC_{50} = 3.27 μL/L) and to the Rutaceae species *C. limon* (LC_{50} = 4.05 μL/L) [77]. Lastly, few research studies aimed at investigating fumigant toxicity toward Psocoptera. Nevertheless, quite remarkable outcomes were reported for the fumigation treatments with the EOs from *Artemisia dubia* (Asteraceae) and *Litsea cubeba* (Lauraceae) against *L. bostrychophila*, reporting LC_{50} values of 0.74 and 0.73 mg/L [78, 79].

Since most Coleoptera and Lepidoptera species are external feeders, the insecticidal activity of EOs may be also assessed against preimaginal stages, as reported for Pyralidae moths. Among Tenebrionidae beetles, it was not possible to identify at which stage insects were more susceptible to EO treatment since susceptibility mainly depended on the used oil. While *Piper nigrum* (Piperaceae), *Laurus nobilis* (Lauraceae), *Cuminum cyminum,* and *Foeniculum vulgare* (Apiaceae) were less toxic to adults than to larvae in *T. castaneum*; *Alpinia conchigera* (Zingiberaceae) and *Myrtus communis* (Myrtaceae) acted in the opposite way [60, 80, 81]. Mondal and Khalequzzaman [82] investigated the ovicidal activity of 5 EOs on *T. castaneum* eggs, highlighting that the strongest effect was recorded for *Elettaria cardamomum* (Zingiberaceae), while, unexpectedly, *Azadirachta indica* (Meliaceae) presented the lowest impact on pest survival. However, it has been claimed that tenebrionid eggs and pupae are generally less susceptible to EO fumigations than adults [60]. In contrast, among Lepidoptera, the toxicity of fumigated EOs has been recognized to be higher against larvae than adults and, among larvae, younger ones were more affected than older ones [83–85]. The ovicidal activity of EOs toward moth eggs was also investigated by Ayvaz et al. [86], reporting 100 % mortality for both *E. kuehniella* and *P. interpunctella* eggs when treated with *S. thymbra* (Lamiaceae) EO. Furthermore, this EO, when fumigated at the concentration of 50 μL/L, determined LT_{99} values (i.e., time occurring to have 99% of mortality) of 158.50 h and 81.88 h for the eggs of *E. kuehniella* and *P. interpunctella*, respectively [86]. Generally, higher concentration of EOs can reduce the lethal time and thus treatment duration [87]. Zapata and Smagghe [88] demonstrated that the LC_{50} after 24h of *Laurelia sempervirens* (Monimiaceae) and *Drimys winteri* (Winteraceae) EOs against *T. castaneum* adults were 1.6–1.7 μL/L and 9.0–10.5 μL/L, respectively, but when the concentration was higher (>100 μL/L), 50% of the tested beetles were killed within 3.0–4.4 h for *L. semprevirens* and within 6.1–7.4 h for *D. winteri*.

As reported for contact toxicity, EO composition may vary according to its geographic origin, as well as to the plant part used for the extraction or to the extraction method, thus modifying its activity against stored product pests. Jemâa et al. [89] highlighted significant differences on *T. castaneum* and *R. dominica* mortality, attributable to geographic origin of *L. nobilis* leaves used for EOs extraction. Similarly, variable results may also be highlighted by research studies involving the same pest-plant but with different geographic origin [64, 89] or different EO extraction method [77, 90]. Furthermore, investigating the fumigant toxicity of EOs extracted from different plant parts has demonstrated that their toxicity may be deeply altered. As an example, the EOs from *Cinnamomum camphora* (Lauraceae) and *Platycladus orientalis* (Cupressaceae) fruits presented an insecticidal activity almost close to zero, compared with that recorded for EOs extracted from leaves and barks of the same plants [91, 92].

The synergistic effect of EOs with other compounds may enhance their fumigant toxicity. Thus, the formulation of EOs with other components, as well as the combination of EO fumigation with other treatments, may enhance plant-borne compounds insecticidal activity. Similar to contact toxicity trials, the combined effect of diatomaceous earths and fumigation with EOs was investigated, highlighting a synergistic effect of *C. reticulata* EO [93]. Remarkably, also the combination of gamma radiation and EOs was evaluated. Irradiation is used as a control tool against *T. castaneum,* but generally gamma radiations are used at high dosages to obtain good results. Thus, the combined effect of radiation and EOs may help reduce the doses of both "ingredients" by exploiting their synergistic effect. Ahmadi et al. [94] revealed that gamma radiations (230 Gy) may increase the insecticidal activity of *R. officinalis* and *Perovskia atriplicifolia*

(Lamiaceae) EOs at very low dosages (LD_5). When used as fumigants, EOs could be also combined and enhanced by other gaseous treatments, as CO_2 injections. Ye et al. [95] demonstrated that the EO extracted from *Perilla frutescens* (Lamiaceae), which caused high fumigation mortality against either adults (LC_{50} = 0.06), larvae (LC_{50} = 0.09), pupae (LC_{50} = 0.16), and eggs (LC_{50} = 0.10) of *Dermestes maculatus* (De Geer) (Coleoptera: Dermestidae), increased from 3 to 6 times its effectiveness when combined with 25% or 60% of CO_2, respectively.

However, some of the major criticism of EO fumigations is the low persistency and the cost related to extended treatments. On this basis, many research efforts have been routed to develop microencapsulation and other controlled release formulations. Polycaprolactone nanocapsules were proposed with good results in order to increase insecticidal efficiency and persistence of EOs, guaranteeing a slow and controlled release of the active substances [49]. Furthermore, other nanoformulations as nanogels of myristic acid-chitosan loaded by EOs were tested. Nanogels of *C. copticum* or *C. cyminum* revealed to be more toxic than the pure EOs, improving the persistency of *C. copticum* from 2–3 days to 21 days and maintaining *C. cyminum* toxicity around 60% after 12 days [96, 97].

6. Repellent Activity

Repellent activity of EOs toward stored product pests was investigated by 79 papers according to our research parameters (see Supplementary Materials for Table S3), 66 namely regarding repellence (224 pest-EO combinations), 8 feeding deterrence (41 combinations), and 5 oviposition deterrence (7 combinations). Combinations were defined as trials involving different combination of tested EO (or EO-based formulation) and target insect species. The plant families most evaluated were Lamiaceae (53 combinations), Rutaceae (44 combinations), Myrtaceae (28 combinations), Asteraceae (23 combinations), and Apiaceae (20 combinations), while the insect orders targeted were Coleoptera (221 combinations), Lepidoptera (30 combinations), and Psocoptera (21 combinations).

Among Coleoptera, the EOs from *Zanthoxylum* spp. (Rutaceae) were broad-spectrum repellents, since they were reported to repel both *T. castaneum,* and *L. serricorne* at 15.73 nL/cm^2 [98]. Tenebrionidae are the most studied Coleoptera family relative to repellence (77 combinations). Among the investigated EOs, *Evodia* spp. (Rutaceae) and *P. frutescens* (Lamiaceae) were repellent of class V (80.1–100% of repellency) at 7.86 nL/cm^2 after 4h [29, 99], while EOs from several *Murraya* spp. (Rutaceae), *L. muscari* (Asparagaceae), and *Artemisia anethoides* (Asteraceae) showed similar repellent results at higher concentration (15.73 nL/cm^2) [33, 100, 101]. Apart from Tenebrionidae, Curculionidae (34 combinations) was also a widely studied insect family for EO repellence. In detail, *S. zeamais* was significantly repelled (class V) by *Mentha longifolia* subsp. *capensis* (Lamiaceae) and *L. salicifolia* (Lauraceae) [26, 102], while the closely related species *S. oryzae* was more repelled by *Prangos acaulis* (Apiaceae) [103]. Furthermore, when used to treat directly the food grain, *O. gratissimum* (Lamiaceae) EO was able to fully repel *S. oryzae* adults at 0.2 μL/g grain, as well as to completely deter the bruchid *C. chinensis* [50]. Among Bruchidae, treatments with the EOs from *Chenopodium ambrosioides* (Chenopodiaceae) and *Adhatoda vasica* (Lamiaceae) caused high repellent activity against both *C. chinensis* and *C. maculatus* [104]. However, EOs able to repel stored product pests at reasonable dosage and for prolonged times are very limited. As an example, the only EO able to highly repel *Cryptolestes ferrugineus* (Stephens) (Coleoptera: Cucujidae) adults was *Citrus bergamia* (Rutaceae), while EOs extracted from other Rutaceae, as well as from Lamiaceae species, were unable to cause significant repellence [105].

To assess the repellent activity of EOs toward stored product Coleoptera, Y-tube and wind tunnel have been also used [106–108]. Nevertheless, these approaches provided less reliable results, since they appeared to more properly evaluate the attractiveness of the tested compounds, as reported by Wang et al. [108]. However, these approaches may be helpful to highlight the behavioral responses of each single individual and to evaluate differences between sexes. As an example, in Y-tube trials, Pimienta-Ramírez et al. [109] demonstrated that *S. zeamais* females were repelled by *Eupatorium glabratum* (Asteraceae) EO, while the conspecific males were attracted by the same EO. Indeed, EOs are rich of plant secondary metabolites, some of which may be recognized by insects as food attractant or allomones.

Unfortunately, when testing the repellence activity, authors commonly did not calculate RD_{50} values (i.e., the EO dose which determines 50% repellence of the tested insect), thus making comparisons among the outcomes of different EOs almost impossible. However, some interesting results have been reported for *Pistacia lentiscus* (Anacardiaceae) EO against several stored product pests, with low RD_{50} value for all the tested insects. For instance, this EO showed RD_{50} values of 0.015, 0.037, and 0.01 μL/cm^2 for *T. confusum, S. zeamais,* and *R. dominica,* respectively [110]. Nevertheless, regarding *T. confusum,* the most interesting result was obtained with *M. pulegium* (Lamiaceae) with RD_{50} value of 0.025 [63]. In contrast, *L. nobilis* (Lauraceae) EO tested for repellence against *T. confusum* adults determined inconstant results (RD_{50} = 0.045–0.139 μL/cm^2), which were accountable to the geographic origin of the tested EOs [111]. Moreover, promising repellence of *L. nobilis* EO was documented toward *R. dominica* (RD_{50} values ranging between 0.013 and 0.036 μL/cm^2 depending on the geographic origin) [111], while slight repellence was reported against *L. serricorne* adults (RD_{50} = 37.84 μL/cm^2) [112]. On the contrary, *L. serricorne* was more repelled by *M. pulegium* (Lamiaceae) and *C. sativum* (Apiaceae) EOs, noting RD_{50} values of 0.01 and 0.049 μL/cm^2, respectively [63, 113]. Overall, less research studies addressed RD_{50} of EOs against Curculionidae species. Furthermore, curculionid weevils are usually slightly less repelled than Tenebrionidae and Anobiidae species. Indeed, the best values of repellence for *Sitophilus* spp. were caused by EOs extracted from *Cymbopogon* spp.

(Poaceae) (RD_{50} = 0.03 μL/cm^2) and *C. sativum* (Apiaceae) (RD_{50} = 0.084 μL/cm^2) [113, 114].

Repellence activity of EOs toward Lepidoptera targeted mainly Pyralidae species (27 combinations). Furthermore, Allahvaisi et al. [115] demonstrated that several EOs had similar repellency against the pyralid *E. kuehniella* and *Sitotroga cerealella* Olivier (Lepidoptera: Gelechiidae). Overall, among pyralid moths, *E. kuehniella* adults were generally less repelled by EOs than *P. interpunctella* ones [30, 116]. Indeed, the most effective EOs evaluated toward *P. interpunctella* were *Anethum graveolens* (Apiaceae) and *R. officinalis* (Lamiaceae), which presented 100% of repellency at the concentration of 2 μL/L of air [117], while regarding *E. kuehniella,* the highest repellence (84.2%) was recorded for *L. nobilis* (Lauraceae) at the same concentration [90].

Among Psocoptera, *L. bostrychophila* was the most studied species. This species was highly repelled (over 90%) by EOs extracted from *L. muscari* (Asparagaceae) and *D. dasycarpus* (Rutaceae) at 6.32 nL/cm^2, and good results (88% repellency) were also noted when psocids were exposed to *A. guillauminii* (Rutaceae) EO at 15.73 nL/cm^2 (V class repellent) [27, 33, 34].

Beside the evaluation of short-term persistence relative to the repellent ability of the EOs, it is essential to improve the effectiveness of this kind of treatment under operative conditions. However, few information is available on the persistence of EO repellence toward stored product pests. Nevertheless, the repellence of *Cymbopogon nardus* (Poaceae) EO toward *T. castaneum* was demonstrated to last at least 16 weeks, with repellency rates of 50% at 0.2 g/m^2 [118]. In this scenario, testing more stable and durable EO-based formulations may be helpful to increase the use of EOs as repellents under real conditions. Furthermore, formulation of EOs with other compounds, as well as formulations of different EOs, may enhance repellent ability by synergistic activity of the components. For instance, Ngassoum et al. [119] proved that *Ocimum canum* (Lamiaceae) could improve the repellence of *Hyptis spicigera* (Lamiaceae) EO against *S. oryzae*, conversely to *Vepris heterophylla* (Rutaceae) which caused an antagonistic effect.

The characteristic of EOs to act as antifeedant compounds is a key repellent mechanism, with a number of perspectives under operative conditions. Nevertheless, the repellence efficacy of EOs is usually reduced when they are applied directly on pest food. Furthermore, consumers may arise some concerns and perplexities about the quality and safety of food products after treatments with EOs. However, some research studies addressed this kind of repellent mechanism and tried to determine the effectiveness of food treatments. Promising feeding deterrence activity was recorded for Rutaceae and Chenopodiaceae EOs against bruchid beetles, with feeding deterrence index (FDI = % reduction feeding activity) reaching even 100% [104]. Good outcomes have been already demonstrated also for *L. salicifolia* (Lauraceae) against *T. castaneum,* for *Eucalyptus floribunda* (Myrtaceae) against *R. dominica,* and for *Datura stramonium* (Solanaceae) against *C. ferrugineus* [26, 120, 121]. On the contrary, the deterrent activity of EOs on feeding behavior is mainly species dependant. As an example, research studies, aimed at evaluating the antifeedant ability of EOs against *T. granarium,* highlighted that this pest was not particularly affected by EO administration, since the EOs of numerous different plant species did not alter significantly its feeding activity [121, 122]. As reported for repellence trials, the formulations of EOs with different materials could improve also their antifeedant efficacy. Thus, also the scale of the employed insecticide may influence its efficacy, and the development of nanoparticle or nanoformulation can alter the antifeedant ability of plant-borne extracts. Werdin González et al. [39] demonstrated that, using polyethylene glycol (PEG) nanoparticles loaded with EOs, the nutritional physiology of both *T. castaneum* and *R. dominica* was specifically altered. While *R. dominica* adults were deterred more by the pure EOs than by PEG-EO nanoparticles, for *T. confusum* PEG formulation improved the antifeedant activity [39].

Another insecticidal mechanism which can be compared with repellence is oviposition deterrence. Although few studies addressed this interesting topic, remarkable oviposition deterrence was reported against *E. kuehniella* when the oviposition substrate was treated with *Ziziphora clinopodioides* (Lamiaceae) EO at 8000 ppm, reporting a reduction in the number of eggs laid of almost 90% [84]. In contrast, considering Coleoptera species, oviposition deterrence could usually appear at the highest tested dosages of EOs. Indeed, *T. castaneum* adult females were significantly repelled at the highest tested dose (70000 ppm) of *Tagetes* spp. (Asteraceae) EOs [65]. Similarly, the EO from *Cinnamomum aromaticum* (Lauraceae) showed good oviposition deterrence outcomes toward *C. maculatus* females at high dosages (62.85 μg/cm^2) [123].

7. Sublethal Physiological Effects

Many life-history traits of stored product pests may be slightly affected or deeply altered by EO treatments (see Supplementary Materials for Table S4). Indeed, plant-borne compounds may even not directly kill insects but could cause relevant reduction of the reproductive performances, as well as several developmental impairments. In the present review, we highlighted 20 papers addressing the impact of EOs on insect biological parameters. In detail, the majority of papers addressed the effect of EO-based treatment on the fecundity and the fertility, followed by research studies on other developmental parameters. As usual, most studies focused on Coleoptera species, while just few studies investigated the impact of EOs on Lepidoptera.

Insect reproductive ability mainly depends on fertility and fecundity of the populations. Here, the effects of EO-based treatments on (i) potential fecundity (i.e., number of eggs laid) (ii) fertility (i.e., the natural ability to produce offspring), and (iii) and lifetime fecundity (i.e., the actual reproductive rate) of stored product pests are reviewed.

When EOs were directly applied to foodstuffs, plant-borne compounds could act as oviposition deterrent, as well as ovicidal insecticides, reducing either the number of eggs laid and/or the percentage of hatched eggs. However, when the adult insects came in contact with and fed on EO-treated food, the presence of insecticidal molecules could also

influence the innate ability of female pests to lay viable eggs. As an example, *Salvia* and *Eucalyptus* spp. could strongly reduce, and even nullify, the number of eggs laid by bruchid females, with a reduction of oviposition inversely proportional to the increase in the EO dose employed [124, 125]. The reduction of potential fecundity in *C. chinensis* was not only related to the reduction of the egg-laying period (i.e., depending on the reduction of the lifespan of females), but it could also be attributed to disturbances during the vitellogenesis process. Interactions with the insect oocyte development were reported for the EOs extracted from *Artemisia herba-alba* (Asteraceae), *Salvia verbenaca* (Lamiaceae), and *Scilla maritima* (Amaryllidaceae), proving that EOs with high flavonoid content could significantly inhibit the egg-laying process and even the fertility of *C. chinensis* [124]. Apart from Bruchidae, different EOs were also able to reduce the lifetime fecundity and the number of eggs laid in *P. interpunctella,* when adult females were exposed to the botanicals for sublethal periods [31]. However, it is unclear if the potential fecundity reduction was attributable to a direct effect of the EOs on the gametogenesis or to an indirect disruption of the courtship and mating patterns of this species. Indeed, it has been demonstrated that residues of the EO could be adsorbed by *P. interpunctella* specimens and could modify male and female locomotion, thus restricting mating possibilities [31]. This kind of disruption of locomotion activity was also noted in some coleopteran species after EO application [48], impeding a clear understanding about the origins of fecundity reduction.

During fumigation with EOs, even short exposures to sublethal doses may affect pest fecundity and fertility. To determine the sublethal effects of the *Eucalyptus camaldulensis* (Myrtaceae) and *Heracleum persicum* (Apiaceae) EOs on *C. maculatus* females, a sublethal dose (i.e., LC_{20}) was tested for 24h as fumigation. After the treatment, the number of total and daily eggs laid for *C. maculatus* females was significantly reduced for both EOs, even if it was slightly higher (39.58% reduction) for *H. persicum* than for *E. camaldulensis* (27.58%) [126]. Fumigation with low doses of EOs could also alter fecundity in Lepidoptera. Adult females of the moth *P. interpunctella* were exposed to fumigation with LC_{30} of various EOs for just 6 h. Results revealed that *Artemisia khorassanica* (Asteraceae) and *Vitex pseudo-negundo* (Lamiaceae) EOs were able to reduce the potential fecundity of *P. interpunctella* by 17.71% and 12.11%, respectively. Similar to bruchid beetles, the reduction of potential fecundity was mainly attributable to the inferior daily egg production than a reduction in adult longevity [127]. Indeed, although adult moths presented shorter lifespans when exposed to EOs, egg laying was generally concentrated in the two days after mating, while later females produced few eggs per day [127]. Apart from potential fecundity, the exposure to *A. khorassanica* and *V. pseudo-negundo* EOs also reduced the fertility by 9.7% and 7.94%, respectively, as well as caused a decrease in larval weight [127]. For instance, fertility, and thus egg hatchability, could be prejudiced if the parental generation experienced EO treatments. As an example, egg viability could be impaired when adults come in contact with and/or feed on EO-treated grains or flour, as reported for *T. castaneum* [65]. In contrast to these results, poor effects were noted for the pyralid *E. kuehniella,* whose females after direct contact with *Ziziphora clinopodioides* (Lamiaceae) EO slightly modified their fecundity or fertility [84].

As previously reported, the longevity of pests after EO exposure may be significantly shortened, but it could not even strongly influence lifetime fecundity of stored product pests [126, 127]. Indeed, the literature reviewed here suggested that the reduction of lifetime fecundity was mainly attributable to a decrease in daily fecundity of insect females rather than to the reduction of female lifespan. Thus, the decrease in daily fecundity and in viability of laid eggs were the main factors related to the lower number of emerging adult offspring. Coleoptera species, as *T. castaneum* and *T. granarium,* showed great reductions of progeny production after parental exposure to the tested botanicals [72]. Particularly, *T. granarium* was more susceptible to plant products than *T. castaneum*. In detail, a complete reduction (100% inhibition) in F1 progeny of *T. granarium* was achieved with a concentration of 1.5% for the EOs of *Cinnamomum camphora* (Lauraceae) and *Ocimum basilicum* (Lamiaceae), while for *T. castaneum,* only the EO from *Pimpinella anisum* (Apiaceae) could completely nullify progeny production [72].

Residual insecticide activity of EOs could prevent adult emergence from pupae or impair the complete development of the larval stages [38]. Indeed, residues of EOs could remain in contact with pupae or larvae for a prolonged time when EOs are applied to the growing media, thus interfering with insect metabolism. For instance, Yang et al. [46] demonstrated that the reduction of progeny production of *S. oryzae* and *T. castaneum* after exposure to *Allium sativum* (Amaryllidaceae) EO was mainly attributable to the residual toxicity of the EOs on egg viability, as well as to its residual toxicity toward young larvae. Furthermore, when EO was used in combination with diatomaceous earths to prolong its persistence, F1 progeny was even greater inhibited [46]. Similar results on residual effect of EOs were reported for *R. dominica,* highlighting synergism between diatomaceous earths or kaolin with EOs. Since *R. dominica* females laid eggs over the grain surface and then larvae penetrate inside the kernels, the application of inert dusts increased progeny suppression, causing higher mortality rates at the stage of egg or young larva [47]. Lifetime progeny production could also be affected by the alteration and dilation of the developmental times (i.e., from egg to adult) of pests, which might consequently alter their doubling and generation times [127].

Essential oils are also known to act both as ingestion and antifeedant insecticides. The alteration of feeding activity might influence adult and larval performances, with particular reference to growth rate, food consumption, and food utilization. Nevertheless, Germinara et al. [42] suggested that the increased mortality of *S. granarius* adults exposed to sublethal concentrations of *L. angustifolia* (Lamiaceae) EO was not attributable to the ingestion toxicity, but to inhalation and contact toxicity of the plant-borne extract. Moreover, a direct effect of ingested EOs could not be

excluded for other insect species, since EO impact is species specific. Furthermore, reduction of insect-feeding activity could cause serious damage. Indeed, *Eucalyptus floribundi* (Myrtaceae) EO caused dose-dependent reduction of consumption rate toward both adult *R. dominica* and *O. surinamensis* and consequently caused a severe reduction of their growth rates [120].

8. Mode of Action

The intrinsic properties of EOs interfere with basic metabolic, biochemical, and physiological functions of insect pests (see Supplementary Materials for Table S5). In the lepidopteran species *P. interpunctella,* adults exposed to sublethal dosages of EOs extracted from *Artemisia khorassanica* (Asteraceae) and *Vitex pseudo-negundo* (Lamiaceae) produced larvae with significantly reduced energy content, by decreasing protein, lipid, and glycogen contents [127]. Thus, alterations attributable to EOs may be transferred by treated adults to the progeny. Furthermore, energy reservoirs are fundamental for lepidopteran attacking stored products, since as adults they generally limitedly feed or do not feed at all, exploiting the energy resources accumulated during preimaginal stages. Thus, a decrease of these kinds of resources at the larval stages may critically endanger insect survival and reproduction. Specifically, protein and lipid reservoirs are considered fundamental for reproductive parameters (i.e., egg production, fertility, and fecundity), while glycogen is generally linked to locomotion and flight ability.

Besides metabolic and physiological alteration, the ingestion of EOs may also produce histological modifications. Osman et al. [128] demonstrated that *T. granarium* larvae presented severe histological changes in their midguts concerning mainly the regenerative cells, thus causing the disruption of the epithelium and impairing the replacement of the functional epithelial cells. Moreover, the cells of hypodermis were necrotic and blackened, with no differentiation between exocuticle and endocuticle. Adults resulting from larvae treated with EOs presented fewer regenerative cells in the midguts, which were elongated with a narrower lumen and females presented germarium and follicular epithelium of the ovarioles with faint nuclei, prejudicing reproduction [128].

Several research studies showed neurotoxic actions of EOs, causing insect paralysis followed by death (reviewed by [129]). Among mechanisms of action, the inhibition of acetylcholinesterase (AChE) is one of the most investigated in stored product pests. AChE is one of the most important enzymes in neuronal and neuromuscular communication in insects and differs from mammalian enzyme by a single residue, making AChE an insect-selective target for newly developed insecticides. Essential oils were estimated to be a potential source of insecticides due to their ability to modifying the AChE activity of some stored product pests [20, 130, 131]. Studies on AChE-inhibitor activity of EOs were carried out on Coleoptera species, testing curculionid (8 insect-plant combinations) and bruchid (1 insect-plant combination) species. EOs from the following plants showed inhibition of AChE activity based on I_{50} values (i.e., the concentrations of the tested essential oil that inhibited the *in vitro*-hydrolysis of substrate by 50%): Asteraceae (*Artemisia judaica; Artemisia monosperma*), Lamiaceae (*Origanum vulgare*), Myrtaceae (*Callistemon viminalis; Melaleuca alternifolia*), Rutaceae (*Atalantia monophylla; Citrus aurantifolia; Citrus limon*). However, Abdelgaleil et al. [20] showed that *S. oryzae* adults may be differently affected by different EOs administration. Indeed, some EOs may present weak (*A. monosperma:* I_{50} = 120 mg/L) or moderate (*O. vulgare:* I_{50} = 61.3 mg/L) AChE inhibition, while EOs from other plants can cause significant inhibition. For instance, *A. judaica* showed the highest efficacy as AChE inhibitor (I_{50} = 16.1 mg/L), followed by *C. limon* (I50 = 20.2 mg/L), *C. viminalis* (I_{50} = 28.5 mg/L), and *C. aurantifolia* (I_{50} = 29.4 mg/L).

Some EOs seem to be rather weak inhibitors of AChE, as also reported by Nattudurai et al. [131]. In this study, the effectiveness of *Atalantia monophylla* EO was evaluated against *C. maculatus* and *S. oryzae*, highlighting that insects of both species exposed to sublethal EO doses presented weak (i.e., less than 50% of inhibition at the highest tested dose) AChE-inhibitor responses. Indeed, AChE activity was decreased in the range of 10.96–45.21% at LC_{10} and LC_{30} doses in *C. maculatus*, as for *S. oryzae* a decrease of 9.18–44.90% was recorded at LC_{10} and LC_{30}, respectively. However, *A. monophylla* EO affected the total esterase activity in insects since the authors registered a decrease of total esterases for both tested insects [131]. So far, esterases are known to be involved in the detoxification of foreign compounds and allelochemical volatiles. Similar to esterases, glutathione S-transferases (GSTs) are known to play a key role for insect detoxification mechanisms, with particular reference to their involvement in the neutralization and resistance mechanisms toward synthetic and natural insecticides [132, 133]. As already described for total esterase and AChE, the EO extracted from *A. monophylla* was also able to decrease GST activity. For instance, either *C. maculatus* or *S. oryzae* presented a reduction of GSTs of about 43% when the adult insects were treated with LC_{30} [131]. The ability of EOs to reduce and suppress the activity of detoxifying enzymes may improve the insecticidal efficacy of EO-based formulations, as well as be exploited as synergistic ingredient to enhance the efficacy of other insecticides.

In contrast to these results on *C. maculatus* and *S. oryzae,* Shojaei et al. [134] reported that the esterase activity in two Tenebrionidae species, *T. castaneum* and *T. confusum,* was not affected by the administration of *Artemisia dracunculus* (Asteraceae) EO, even at high dosages (LC_{70}). Similarly, the production of mixed function oxidases (MFOs) was not significantly altered with respect to the untreated control, even at the highest EO dosage (LC_{70}), in both *T. castaneum* and *T. confusum.* MFOs are considered as GSTs and esterases responsible of detoxifying ability in insects. On the contrary, species-specific responses were reported for GSTs. Treatment with EO slightly altered the GST production in *T. confusum,* by raising the GST activity according to concentration increase. Conversely, *T. castaneum* showed a decrease of GST production when EO concentration

increased. Nevertheless, control insects of both species showed the lowest enzyme activity, suggesting that EO administration enhanced the production of detoxifying enzymes as GST [134]. In this scenario, these results may shed light on the detoxification mechanism of some EO substances by tenebrionid insects, but not the mode of action for this EO. Furthermore, even if no modification of esterase and MFO activity was clearly reported, the results might be impaired by the tested dosages chosen for these trials (LC_{30}, LC_{50}, and LC_{70}), as lower concentrations could better detect alterations related to insect metabolism.

Metabolomic alterations caused by EO administration were also investigated for the curculionid *S. zeamais* using *Melaleuca alternifolia* (Myrtaceae) [130]. Indeed, *M. alternifolia* EO was shown to possess fumigant toxicity against *S. zeamais* along with the capacity to significantly inhibit the activity of 3 enzymes: two detoxifying enzymes, GST and carboxylesterase (CarE), as well as the nerve conduction enzyme AChE. In vivo enzyme inhibition was reported also for insects treated with EO dosages lower than LC_{50} (8.42, 7.70, and 6.78 mg/L air after 24, 48, and 72h, respectively). For instance, *M. alternifolia* EO induced a moderate enzyme inhibition at the dose of 5.39 mg/L air after 12 h and 24 h for every tested enzyme (AChE, GST, and CarE), even if a certain restoration of enzyme activity could be noted after 24 h [130]. These results highlighted a pattern of significant dose- and time-dependent inhibitory effect of *M. alternifolia* EO on the enzyme activity in *S. zeamais*. The significant inhibition of the hydrolytic enzyme AChE caused by EO fumigation suggested that the EO might interfere with the nervous system of *S. zeamais*. Furthermore, since generally insects activate detoxifying enzymes to prevent and counterattack oxidative damage, the reduced activity of GST and CarE might improve the insecticidal activity of *M. alternifolia* EO.

To gain a better understanding of the mechanisms associated with the mode of action of EOs, Liao et al. [130] performed, for the first time, a comparative transcriptome analysis of *S. zeamais* in response to EO fumigation. The results from comparative transcriptome analysis on *S. zeamais* through RNA-Seq identified a total of 3,562 differentially expressed genes (DEGs), of which 2,836 and 726 were upregulated and downregulated, respectively, in response to *M. alternifolia* EO treatment. Interestingly, the majority of DEGs were involved in insecticide detoxification and mitochondrial function, followed by genes associated with respiration and metabolism of xenobiotics, including cytochrome P450s, CarEs, GSTs, and ATP-binding cassette transporters (ABC transporters). In detail, in the first phase of xenobiotic metabolism, which results in the alteration of xenobiotic compounds in more reactive molecules, CarEs and cytochrome P450 play an indispensable role. In *S. zeamais*, the transcription of genes encoding P450 was significantly upregulated, indicating that these genes might be involved in detoxification of *M. alternifolia* EO. CarEs unigenes were also upregulated upon oil exposure, in contrast to results from in vivo enzyme inhibition analyses after 12h from exposure. In the second phase, the detoxifying enzymes further increase the water solubility of the metabolites, and GST is known to play an important role here. After exposure to *M. alternifolia* EO, *S. zeamais* adults presented 19 genes encoding GSTs upregulated, while 2 were downregulated, suggesting that insects try to recover from enzyme activity inhibition by increasing the production of different GSTs. Lastly, during the third phase, to transport conjugates of xenobiotic compounds out of the cell, ABC transporters were activated (30 genes upregulated). These results suggested the pathway used by *S. zeamais* to detoxify EO compounds [130].

However, over xenobiotic biodegradation, the alteration of mitochondrial functions, as the inhibition of respiratory enzymes or the alteration of regulation of oxygen/carbon dioxide ratio, may be a mode of action of plant EOs. Liao et al. [130] found that many genes associated with mitochondrial functions were differentially expressed, and some enzymes from the mitochondrial respiratory chain were downregulated by *M. alternifolia* EO treatment, causing the block of the electron flow by the hydrogen carrier and interfering with energy synthesis in the mitochondrial respiratory chain.

According to this hypothesis, adenosine triphosphatases (ATPases), a class of enzymes that catalyze the decomposition of ATP into ADP releasing energy, may be a target for EOs to impair chemical reactions, as well as respiration that would not otherwise occur. For the first time in insects, ATPases were found to be inhibited in *S. oryzae* adults exposed to different EOs (i.e., *Artemisia judaica, Artemisia monosperma, Origanum vulgare, Callistemon viminalis, Melaleuca alternifolia, Atalantia monophylla, Citrus aurantifolia,* and *Citrus limon*) [20]. In detail, the oils of *C. viminalis*, *O. vulgare*, and *C. limon* caused the highest enzyme inhibition with I_{50} values of 4.69, 6.07, and 9.69 mg/L, respectively, while EO from *C. aurantifolia* showed a slightly lower enzyme inhibition (I_{50} = 11.4 mg/L). In contrast, EOs from *A. judaica* (I_{50} = 21.4 mg/L) and *A. monosperma* (I_{50} = 24.6 mg/L) caused the weakest enzyme inhibition. Based on I_{50} values, in *S. oryzae*, EOs were more likely to inhibit the activity of ATPases than AChE one, suggesting that active compounds of EOs mainly affected energy chain reactions.

Overall, EOs are generally supposed to act as neuroinsecticides, and their insecticidal activity is considered species-dependent [129]. For this reason, in insects, other proposed mechanisms of EO action include the inhibition of GABA receptors (GABArs) and the alteration of the octopaminergic system. To the best of our knowledge, the ability of EOs to alter GABArs has never been proved for insects. On the contrary, modifications of the insect octopaminergic system following EO exposure have been already reported [135]. For instance, some EO components may compete with octopamine in binding to its receptor, causing an increase in the level of cAMP and calcium in nervous cells and modifying the neuron activity in *Periplaneta americana* L. (Blattodea: Blattidae) [135].

On this basis, it is possible to suggest that the broad-spectrum insecticidal activity of EOs could be attributable to the characteristics of these plant extracts, which are composed by numerous different compounds operating via several modes of action toward insect species.

9. Conclusions

Although an impressive increase in the number of publications involving botanical insecticides was recorded from 1980, as highlighted by Isman et al. [19], with over half of the papers on EOs (1,111) published in the last six years (2007/2012) of their survey period, the use of essential oils as insect-control tool in stored products still represents a niche compared with other sectors (i.e., crop protection, veterinary entomology, and mosquito control). Nevertheless, more than 200 papers have been published in the last 15 years. The increasing interest about essential oils derives from a number of factors such as their widespread availability, relatively low cost, and the belief that plant-borne extracts are nontoxic to humans and pets.

In the examined papers, EOs usually showed a noticeable acute toxicity (i.e., mortality), toward the target insects. This seems to be a foregone conclusion, given that these substances are synthesized by plants to defend themselves also from insects. Therefore, the question arises whether the feature is dose-dependent. And so it seems. Since many plants used for the EOs extraction often grow spontaneously in different natural habitats, their large-scale use should consider the cultivation of these essences to avoid negative impact in the ecosystems. Furthermore, many factors can influence the composition of essential oils. For example, the phenological stage and/or the part of the plant, the annual climatic variations, and the exposure can affect the relative amount of bioactive compounds constituting EOs. Thus, to validate the insecticidal activity of EOs and their potential as active ingredients for commercial pesticides, several trials should be carried out testing essential oils produced in different years and geographical areas.

Despite the promising results, there are few authorised commercial EO-based insecticide formulations available on the market. Future research studies about the mechanisms of action of the EOs against insects are needed to develop effective EO-based insecticides. Indeed, deeper knowledge on this topic may be helpful to estimate the impact of EOs toward nontarget species and their safety for consumers. In addition, the effect on the sensory analysis of food treated with these compounds should be evaluated since, although this aspect is a main concern for costumers, it has been often disregarded. Therefore, a multidisciplinary approach, involving also chemists and food technologists, could be a route to develop new EO-based insecticide formulations, which could be successfully applied to different productive sectors.

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

The file contains 1 Supplementary Method and 5 Supplementary Tables: Supplementary Method 1: string used on Scopus database to retrieve the overwide literature for scientometric analyses (Scopus database search: February 2, 2018). Table S1: overview of reviewed studies on EO contact (CT) and ingestion (IT) toxicity toward stored product pests. Table S2: overview of reviewed studies on EO fumigant toxicity toward stored product pests. Table S3: overview of reviewed studies on EO repellence toward stored product pests. Table S4: overview of reviewed studies on EO sublethal physiological effects toward stored product pests. Table S5: overview of reviewed studies on EO mode of action toward stored product pests. (*Supplementary Materials*)

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Research Article

Effect of Cornelian Cherry (*Cornus mas* L.) Juice on Selected Quality Properties of Beef Burgers

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Bioactive compounds of plant origin are becoming increasingly popular as food ingredients with a beneficial impact on human health. Therefore, the present study aimed to investigate the possibility of using different doses (0.5 g–1.5 g) of cornelian cherry juice (CCJ) as a functional additive in the production of beef burgers. Results of the experiment showed CCJ addition to cause high acidification of the meat emulsion and to decrease meat production yield was unbeneficial from the economic point of view. In contrast, the CCJ was highly effective in retarding lipid oxidation in beef burgers during storage wherein even a dose of 0.5 g CCJ resulted in beneficial inhibition of oxidative changes and at the same time had no negative effect on the sensory characteristics of beef burgers. CCJ can be applied in the meat industry to develop novel products; however, future research is needed regarding its acidifying properties.

1. Introduction

Food legislation (e.g., Regulation EC no 1129/2011 [1]) regulates the health safety issues of food additives used in food processing. However, many consumers still call for the negative impact of food additives of chemical origin on their health. The social anxiety about the use of synthetic additives in food production has prompted researchers and food manufacturers to explore natural substances with functional properties, for example, chemical compounds of plant origin [2] Fruits are a rich source of phytochemicals, for example, vitamins, minerals, and polyphenols, essential for human health [3]. These bioactive compounds can be used in food processing and thereby affect human health. Recent studies have demonstrated the potential of fruit-derived substances as functional additives to meat products. For example, Tyburcy et al. [4] proved that cranberry fruit juice can be applied in the production of pork burgers as an additive with antioxidant properties. A black currant fruit extract added to pork chops [5] or chokeberry juice used in the production of pork sausages [6] were also reported to inhibit the fat oxidation process. Despite their invaluable impact on human health, fruits are rarely used as additives in meat production, because they may induce changes in the sensory attributes (color and taste) of the finished products [7].

Among many wild fruits, an interesting raw plant material from the viewpoint of chemical composition is fruits of cornelian cherry (*Cornus mas* L.). They are a rich source of biologically-active compounds, such as various essential mineral elements, vitamin C, organic acids, pectin, phenolic acids, flavonoids, iridoids, and triterpenoids [8–15]. These phytochemicals elicit protective effects on atherosclerosis and display antioxidant and anti-inflammatory properties, as well express cardiotonic, antidiabetic, and antiobesity activities [16, 17]. Consumption of fruits is also recommended due to their astringent properties and their potency

for recovery and regeneration of damaged epidemic tissues, as well as for alleviating diarrhea and dysentery, sore throat, digestion problems, measles, and chicken pox [18, 19]. The cornelian cherry fruits are very convenient for processing to produce syrups, liqueurs, juices, and jams [12], while their use in the meat industry has not been investigated so far. Therefore, the aim of this study was to determine the effect of cornelian cherry juice on the quality properties of beef burgers. The authors assumed the feasibility of using cornelian cherry juice as a food additive, which could be applied instead of chemical additives to improve the quality of meat products.

2. Materials and Methods

2.1. Ingredients. The postrigor beef cuts (I class) were delivered 48 h postmortem from the meat processing plant "Edward and Grzegorz Dworeccy" (Golejewo, Poland) to the Department of Animal Products Technology and Quality Management, Wrocław University of Environmental and Life Sciences. Raw materials were cleaned, cut into strips, and ground (W-82AN Spomasz, Zary, Poland; 0.5 cm plate diameter). Then, the material was portioned, vacuum packaged, frozen, and stored at −18°C until use.

2.2. Preparation of CCJ. Juice of cornelian cherry (*C. mas* L.) fruits (CJ) was produced as per the following procedure: 1 kg of frozen ripe fruits of cornelian cherry were shredded and heated for 5 min at 95°C (Thermomix, Vorwerk, Wuppertal, Germany), and after heating, the pulp was subsequently cooled down to 40°C and depectinized (0.5 mL/kg of Panzym Be XXL, Begerow GmbH & Co., Darmstadt, Germany) at 50°C for 2 h. Next, the pulp was cleaned of stones and pressed in a laboratory hydraulic press (Zodiak SRSE, Warsaw, Poland). Then, ready juice (CJ) was lyophilized (FreeZone18L, Labconco, USA), vacuum packaged, frozen, and stored at −18°C until use (approx. 1 month). Before application to beef burgers, the juice was reconstituted by dilution in distilled water (1 : 1 w/w). Samples of diluted CJ (CCJ) had an intensive deep red color and bitter-sour taste. The acidity (pH) of CCJ was measured at 2.71 (pH meter inoLab pH 720, WTW, Weilheim, Germany, in accordance with the Polish Standard PN-EN 1132:1999 [20]).

2.3. Identification and Quantification of CJ Active Compounds by HPLC. Contents of iridoids and polyphenols in the samples were determined according to Kucharska et al. [21] by high-performance liquid chromatography HPLC on a Dionex chromatogram (USA). The chromatograph was equipped with an Ultimate 3000 diode detector, an LPG pump-3400A, an EWPS-3000SI autosampler, a TCC-3000SD column thermostat, and Chromeleon v. 6.8 software. The analysis was carried out on a C5–C18 Cadenza Imtakt column (75 × 4.6 mm, 5 μm). The solution of 4.5% v/v aq. formic acid (reagent C) and 100% acetonitrile (reagent D) was used as the eluent at the flow rate of 1 mL/min and split of 20 μL. Separation was carried out using the following gradient: 0-1 min 5% D in C, 20 min 25% D in C, 21 min 100% D, 26 min 100% D, and 27 min 5% D in C. The column was thermostated at 30°C. Anthocyanins were monitored at 520 nm, flavones at 360 nm, and ellagic acid and iridoids at 245 nm. Their contents were expressed as mg/100 mL of CJ.

Table 1: Formulation (g) of beef burgers.

Treatments	Beef	Salt	CCJ
CCJ0	100	1.6	0
CCJ0.5	100	1.6	0.5
CCJ1.0	100	1.6	1.0
CCJ1.5	100	1.6	1.5

CCJ: cornelian cherry juice.

2.4. Preparation of Beef Burgers. All burger batches were manufactured with 100 g of meat and 1.6 g of salt. Four different batters were prepared (Table 1): Treatment CCCJ0: control samples without CCJ addition; treatments CCJ0.5, CCJ1, and CCJ1.5: samples supplemented, respectively, with 0.5 g, 1.0 g, and 1.5 g of CCJ. After thawing, meat was minced through a 0.3 cm plate, mixed with salt or salt and CCJ and formed into discs of 10 cm diameter (weighing approx. 94 g). Next, the burgers were heat-treated (gas and electric oven, Amica 51GE, 180 ± 5°C) until an internal temperature of 72°C has been reached (bayonet thermometer, Amarell TH-101). The finished products were cooled down, vacuum-packed, and stored for 5 months at −15 ± 1°C. The production was replicated in two independent series. All analyses were conducted immediately after the production process (Day 0) in cooled down meat products and in products after 5 months of storage (5 Mths) at −15 ± 1°C.

2.5. pH Measurement of Burger Batters. The pH value was measured directly in burger batters with an inoLab pH 720 pH meter (WTW, Weilheim, Germany) in accordance with the Polish Standard PN-EN PN-ISO 2917:2001 [22] at room temperature.

2.6. Yield of Burger Production Process. The yield of the production process was calculated from the following equation:

$$\%\ \text{production yield} = \frac{\text{weight after heat treatment} \times 100}{\text{weight of raw materials}}. \quad (1)$$

2.7. Color Parameters of Beef Burgers. Measurements of color parameters of beef burgers were conducted using a Minolta CR-400 reflectance colorimeter, and results were expressed as L^* = lightness, a^* = redness, and b^* = yellowness parameters in the CIE Lab system. The analysis was carried out at room temperature (22 ± 1°C), directly after the production process and after 5-month storage of burgers.

2.8. Sensory Evaluation. The sensory evaluation of ready-to-eat burgers was performed in accordance with the Polish

Standard PN-ISO 4121:1998 [23]. The evaluation was conducted directly after the production process on 1 cm × 1 cm slices of encoded samples at room temperature (22 ± 1°C) under white light. A professional team of 10 evaluators determined the degree of acceptance of the overall appearance, smell, taste, color, hardness, and juiciness of burgers, using a nine-point hedonic rating scale of acceptance (1, dislike extremely, to 9, like extremely [24]).

2.9. Texture Profile Analysis. The Zwick/Roell Z010 testing machine (Zwick Testing Machines Ltd., Leominster Herefordshire, UK) was used for the texture profile analysis of ready-to-eat burgers. Cylindrical samples of burgers were pressed to 50% of deformation (TPA 50 test, 60 mm/min head speed, 40 s relaxation time). The texture was determined by the following parameters: hardness, gumminess, and chewiness. The measurements were conducted at room temperature (22 ± 1°C), directly after the production process and after 5-month storage of burgers.

2.10. TBA Test. The intensity of oxidative processes in the ready-to-eat beef burgers was evaluated using a spectrophotometric 2-thiobarbituric acid (TBA) filtration method described by Luciano et al. [25] with slight modifications. Briefly, 1 g of a ground sample was homogenized with 10 mL of 10% trichloroacetic acid (Chempur, Piekary Slaskie, Poland) and centrifuged (1000 rpm, 10 min, Sigma 3K30; Sigma Laborzentrifugen GmbH, Osterode am Harz, Germany) at room temperature. Then, homogenates were filtered (Whatman no. 1 filter paper), and the supernatant was collected. Next, 2 mL of clear filtrate was mixed with 2 mL of 0.02 M aqueous thiobarbituric acid (Sigma-Aldrich, St. Louis, MO, USA), and the samples were incubated in a water bath at 100°C for 40 min (Julabo TW12, Julabo Inc., Allentown, USA). The absorbance of the supernatant was measured at 532 nm (spectrophotometer Rayleigh UV-1800, Beijing Rayleigh Analytical Instrument Corp., China). The TBARS values were expressed as equivalents of malondialdehyde (MDA) in mg per kg of sample calculated using 1,1,3,3-tetraethoxypropane (Sigma-Aldrich) as the standard. The determination was conducted immediately after the production process and after 5-month storage of burgers.

2.11. Data Analysis. Data were analyzed using Statistica software v. 13.1. (StatSoft Inc., Poland). Significant differences among groups were identified using Duncan's multiple range test ($P \leq 0.05$). The results were presented as mean values and standard error of the mean (mean ± standard error).

3. Results and Discussion

Results of quantification of CCJ active compounds by HPLC are presented in Table 2. They indicate that 100 mL of CCJ contained 203 mg of iridoids (of which 85% accounted for loganic acid), 8.9 mg of anthocyanins, 2.8 mg of ellagic acid, and 4.1 mg of flavonols. Iridoids are a large group of secondary metabolites; they belong to the group of monoterpenoids in the form of cyclopentanopyran [26, 27]. Iridoids have different pharmacological properties, including antibiotic, anti-inflammatory, or hypotensive ones [28]. Sozański et al. [29] proved protective effects of iridoids extracted from cornelian cherry fruit on diet-induced hypertriglyceridemia and atherosclerosis through enhanced PPARα protein expression and *via* regulating oxidative stress and inflammation. Also the presence of dietary polyphenolic compounds, such as anthocyanins and flavonols, can have a beneficial effect on human health [30]. Anthocyanins with their reactivity have the ability to neutralize free radicals and hence may be used as natural antioxidants [31]. Anthocyanins can replace chemical dyes, but their application in food processing depends on their stability [32].

Table 2: Data of active compounds of cornelian cherry juice, $n = 2$.

Group of compounds	Compound	mg/100 mL
Iridoids	Loganic acid	172.4 ± 8.6
	Cornuside	30.6 ± 1.6
	Total iridoids	**203.00**
Anthocyanins	Cyanidin 3-O-galactoside	1.84 ± 0.3
	Cyanidin 3-O-robinobioside	0.65 ± 0.1
	Pelargonidin 3-O-galactoside	5.44 ± 1.1
	Pelargonidin 3-O-robinobioside	0.90 ± 0.1
	Total anthocyanins	**8.83**
Ellagotanins	Ellagic acid	2.83 ± 0.5
Flavonols	Quercetin 3-glucuronide	2.38 ± 0.5
	Kaempferol galactoside	1.49 ± 0.4
	Kaempferol glucoside	0.29 ± 0.1
	Total flavonols	**4.16**

The addition of cornelian cherry juice to burger batters caused changes in their pH value. The highly acidic CCJ (pH = 2.71) decreased the pH of burger batters along with its growing content (5.87 CCJ0, 5.80 CCJ0.5, 5.78 CCJ1.0, and 5.48 CCJ1.5). The pH values of the batters were close to the isoelectric point (pI) of beef proteins and negatively affected the yield of the production process. The highest ($P \leq 0.05$) yield of the production processes was determined for the samples produced without CCJ addition (72.7%). The highest dose of the plant additive applied (1.5 g) caused the greatest cooking losses and the same caused the lowest production yield (65.3%). The isoelectric point for beef proteins is at 5.1-5.2 and is associated with a decrease in the ability of the protein to bind water [7]. The addition of CCJ to experimental batters caused a reduction in the capacity of meat proteins to retain water during heat treatment and hence in meat production yield. This dependency was also confirmed by Tyburcy et al. [4], who applied cranberry (*Oxycoccus palustris*) and rose (*Rosa rugosa*) juices in pork burgers. Also Zając et al. [33] observed that the addition of 0.05 and 0.1 g/kg of hyaluronic acid to smoked homogenized sausages reduced the stability of meat emulsion and decreased the yield of production process.

Color is one of the most important determinants of the quality of meat products. The color of meat products is

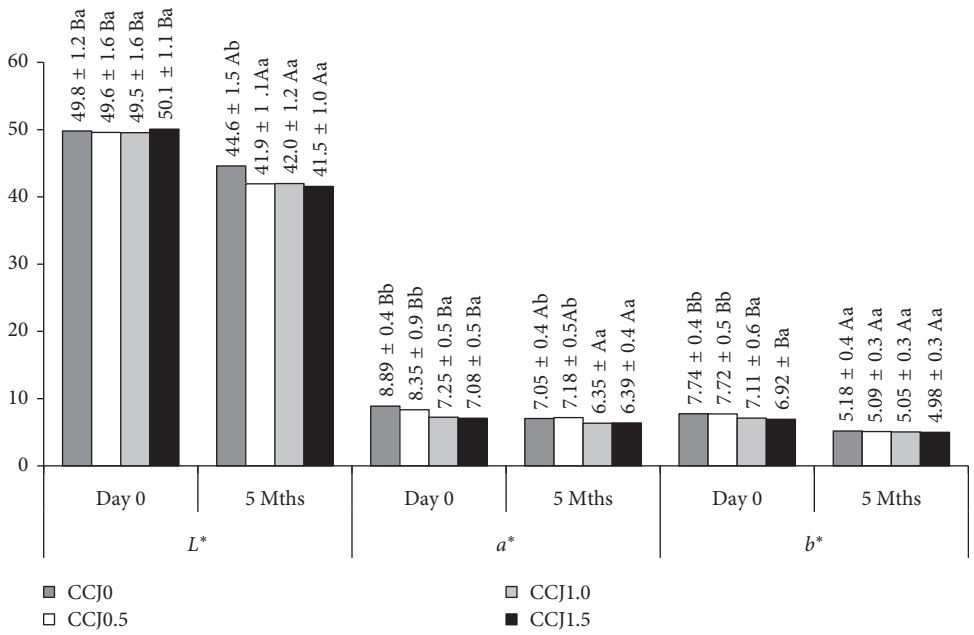

Figure 1: CIE L^*, a^*, and b^* color values of beef burgers, $n = 6$. Means followed by the different lowercase letters between the samples at the same storage time and capital letters between the same samples at different storage times indicate significant differences ($P \leq 0.05$) between values. L^* = color lightness, a^* = redness, and b^* = yellowness.

affected by many factors, including pigmentation of raw meat and fat, functional additives, and technological treatments applied [34]. Raw meat color is significantly affected by the content of myoglobin and the degree of its oxygenation. The content of myoglobin in beef ranges from 6 to 20 mg/kg and is determined by many factors such as age, breed, or kind of animal muscle tissue [35]. The increase in cornelian cherry juice content burger batter caused no change in the values of the L^* parameter (brightness) in burgers (Figure 1). However, all formulations darkened during storage, while the samples of beef burgers manufactured without CCJ were significantly ($P \leq 0.05$) brightest ($L^* = 44.6$). Beef burgers with CCJ added at doses of 1.0 and 1.5 g had lower ($P \leq 0.05$) values of parameter a^* than the samples with 0 g and 0.5 g addition, and these values were much lower after 5-month storage. Immediately after the production process, the highest values of parameter b^* were found in the CCJ0 and CCJ0.5 samples. The use of 1.0 g and 1.5 g of the plant additive resulted in a statistically significant ($P \leq 0.05$) decrease in the values of this parameter. In the own studies, the color of the finished products was mainly attributed to the denaturated form of myoglobin—metmyoglobin, imparting the gray color to meat after the heat treatment [36]. Freeze-storage caused the darkening of beef burgers, which could be due to chemical reactions involving oxygen. The same observation was made by Tril et al. [6] who analyzed meat products with the addition of chokeberry juice. The decrease in the values of parameters a^* and b^* with the growing addition of cornelian cherry juice probably result from the Maillard reaction, which involved reducing sugars present in the CCJ. Kucharska [10] has shown that the content of reducing sugars in fruits of cornelian cherry is 9–14.7%. A similar conclusion was reached by Tyburcy et al. [4] who added cranberry and wild rose juices to pork burgers. Although the CCJ had an intense red color, owing to the presence of anthocyanins (Table 2), this had no direct impact on the color of the experimental beef burgers measured in the CIE $L^*a^*b^*$ scale. The authors attributed this effect to its low level in relation to the raw meat or to the heat treatment which could cause transformation of anthocyanins into colorless chalcones, which as a result of oxidation produce compounds of brown color [37, 38].

Different doses of the cornelian cherry juice added to burger batters influenced results of the sensory evaluation of beef burgers because of its physicochemical properties (Figure 2). The increasing CCJ addition to burger batters resulted in a greater acceptability of the aroma of the finished products; however, the most preferable ones in this respect turned out to be burgers with the highest CCJ addition (5.7 CCJ1.5, 5.4 CCJ1.0, 5.0 CCJ0.5, and 4.4 CCJ0). Better acceptability of this sensory attribute may be associated with a higher content of reducing sugars (ubiquitous in CCJ) in the samples which entered into the Maillard reaction and thereby produced compounds with a desirable, attractive aroma [4, 39]. In assessing the acceptability of the overall appearance of experimental products only, burgers manufactured with the highest, that is, 1.5 g of addition, were rated significantly ($P \leq 0.05$) lower than the others. The taste and the color of experimental beef burgers was rated as high in the case of samples produced with the 0.5 g addition of CCJ and in those produced without the plant additive. Results of the color assessment made by panelists corresponded with those obtained in color measurement in the CIE $L^*a^*b^*$ scale and are probably related to the presence of Maillard reaction products and changes in the stability of

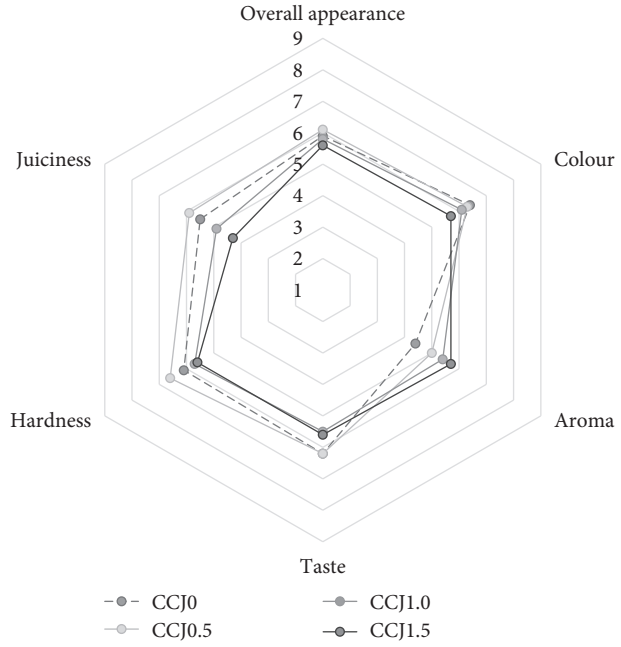

Figure 2: Sensory evaluation of beef burgers, $n = 10$.

Table 3: TPA (texture profile analysis) test of beef burgers directly after production process (Day 0) and after 5 months (5 Mths) of storage, $n = 5$.

	Time of storage	Hardness (N)	Gumminess (Nm)	Chewiness (N)
CCJ0	Day 0	65.7 ± 2.5 Ba	44.5 ± 6.7 Ba	38.7 ± 5.4 Ba
	5 Mths	36.9 ± 2.5 Aa	26.1 ± 1.7 Aa	24.1 ± 1.5 Aa
CCJ0.5	Day 0	73.3 ± 3.9 Bb	42.8 ± 5.9 Ba	37.3 ± 5.4 Ba
	5 Mths	35.9 ± 3.6 Aa	25.5 ± 2.1 Aa	23.4 ± 2.0 Aa
CCJ1.0	Day 0	73.9 ± 5.2 Bb	43.7 ± 3.8 Ba	38.4 ± 3.7 Ba
	5 Mths	40.7 ± 6.0 Ab	27.2 ± 4.4 Aa	24.9 ± 3.7 Aa
CCJ1.5	Day 0	72.0 ± 5.1 Bb	42.3 ± 3.5 Ba	37.8 ± 1.3 Ba
	5 Mths	39.0 ± 3.2 Ab	28.1 ± 3.4 Aa	25.7 ± 2.9 Aa

anthocyanins. Adverse changes in taste caused by the increasing CCJ content in the model meat products could be attributed to the increase in the salty taste of beef burgers, which was highlighted by the evaluation team. The immediate reason for this was the decrease of production yield, associated with an increase in the acidity of the batters, and hence larger water losses and increased salt content in the finished products [4]. Increased cooking losses with the growing addition of CCJ were also the cause of lesser acceptability of juiciness and hardness of the burgers in the sensory assessment. The burgers with 0.5 g of CCJ received the highest acceptability scores in the assessment of juiciness (5.9) and hardness (6.6), while the lowest scores (5.6) were given to CCJ1.5 samples.

Textural properties of meat products are dependent on the type and amount of used meat and fat and on the addition of water and functional substances [40]. The cornelian cherry juice used in our study had a significant effect on the selected texture parameters of model beef burgers (Table 3). CCJ addition to burger batters increased hardness of the ready-to-eat burgers. The most pronounced effect on burger hardness had CCJ addition of 1.0 g (73.9 N) whereas the smallest changes were recorded using 1.5 g of CCJ (72.9 N) compared to the control sample (65.7 N). In the formulations with CCJ, these changes were not statistically significant. Increase in hardness due to plant additive addition to the recipe was also reported by Tril et al. [6] and Salejda et al. [41] who applied chokeberry juice and tea extract, respectively, in model pork products. In our study, the freeze-storage of beef burgers caused a significant ($P \leq 0.05$) decrease in their hardness, with the highest values measured in CCJ1.0 and CCJ1.5 samples. In the case of such texture parameters such as gumminess and chewiness measured directly after the production process, the highest values (44.5 N and 38.7 Nm, resp.) were determined for the samples manufactured without CCJ. The addition of cornelian cherry juice resulted in the decrease in values of those parameters, but these differences were not statistically significant. In all analyzed formulations, freeze-storage caused a significant ($P \leq 0.05$) decrease in the values of gumminess and chewiness. The observed decrease in values of all measured textural parameters during storage might be associated with water and fat separation from the meat protein matrix and, resultantly, with destabilization of the emulsion [42].

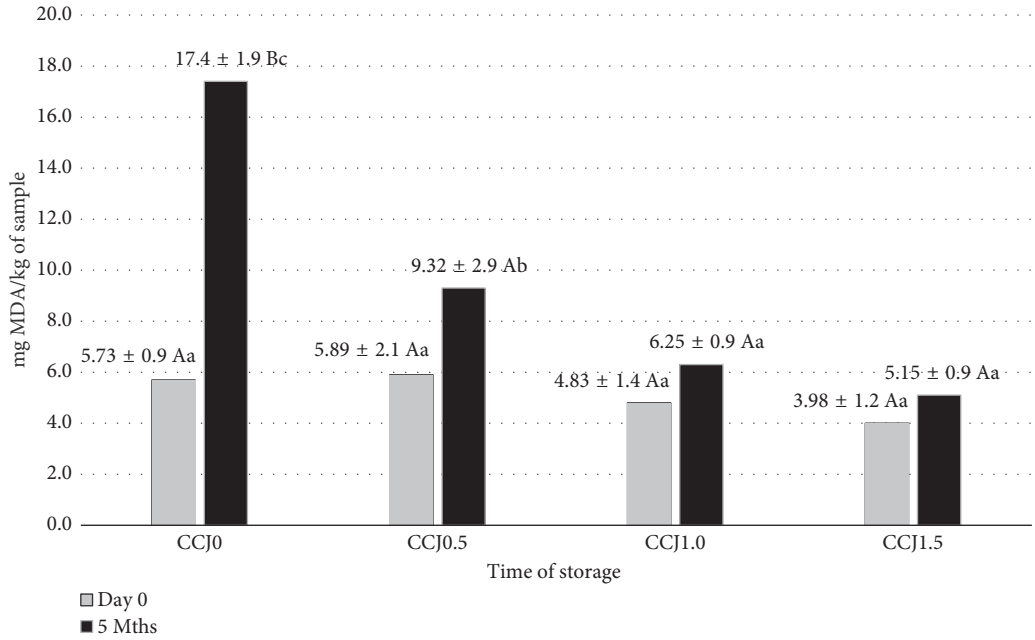

Figure 3: Thiobarbituric acid (TBA) values of beef burgers during storage, $n = 3$. Means followed by the different lowercase letters between the samples at the same storage time and capital letters between the same samples at different storage times indicate significant differences ($P \leq 0.05$) between values.

Results of the present study indicate that lipid oxidation processes occurred in the lipid fraction of experimental beef burgers during 5-month storage in freezing conditions (Figure 3). However, the plant preparation applied effectively limited the intensity of this process. After freeze-storage, the beef burgers with cornelian cherry juice had a lower content of thiobarbituric acid reacting substances (5.15–9.32 mg·MDA/kg) compared with the samples manufactured without the plant additive (17.4 mg·MDA/kg). The highest CCJ dose applied in the study (1.5 g) was the most effective in lipid oxidation inhibition, contrary to the control sample where MDA content increased almost twofold. Inhibition of oxidation processes might be associated with a high content of compounds with antioxidant properties, that is, monoterpenoids (iridoids), phenolic acids, flavonols, and anthocyanins, in the applied cornelian cherry juice. Tang et al. [43] have also demonstrated effective antioxidant action already at the lowest dose of the plant preparation applied. Greater effectiveness in preventing oxidation of lipids with an increasing dose of the plant preparation was also confirmed by Yu et al. [44] and Jia et al. [5], where higher doses of extracts of rosemary and black currant in the recipe were the most effective ones during cold storage (4°C) of meat products.

4. Conclusion

The use of the cornelian cherry juice in the production of beef burgers resulted in a reduction of meat production yield, which suggests the need of introducing functional substances which will neutralize acidifying properties of CCJ to their recipe. Even the lowest dose of the cornelian cherry juice effectively reduced lipid oxidation and allowed maintaining the sensory characteristics of products. This indicates the feasibility of using CCJ to prolong the shelf-life of meat products, and at the same time, of offering novel products enriched with active components of cornelian cherry to consumers.

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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Research Article

Assessment of the Antioxidant Activity and Quality Attributes of Yogurt Enhanced with Wild Herbs Extracts

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The milk and yogurt products assortment has expanded by their enhancement with herb extracts, fibres extracted from by-products of the fruit processing industry and also fresh spices. The aim of the present study was to test to what extent the addition of different herb extracts in yogurt will improve its qualitative characteristics and antioxidant activity. The aqueous extracts obtained from the four plants are considered in this study, respectively, thistle (*Silybum marianum* L.), hawthorn (*Crataegus monogyna*), sage (*Salvia officinalis* L.), and marjoram (*Origanum vulgare* L.). It was examined the effect of aqueous extracts prepared from four herbs (0.25/1%) (w/w) on yogurt's qualitative characteristics (pH, titratable acidity, syneresis, water holding capacity, antioxidant activity, colour parameters, and rheological parameters) on both one day and 28 days after preparation. The final results show that the physicochemical and rheological properties of the yoghurt with herb extracts addition were improved compared to the control sample after 28 days of storage. The best results in terms of antioxidant properties were obtained when marjoram extract (*Origanum vulgare* L.) was incorporated. According to the data obtained, the best quality in terms of the physicochemical and rheological properties were in the case of the sample with 0.5% thistle extract (*Silybum marianum* L.) addition, while from point of view of the nutritional value, the best quality was in the case of the sample with 1% marjoram extract (*Origanum vulgare* L.) addition. The present study leads to the conclusion that yogurts enhanced with natural extracts may serve as functional food products, with significant health benefits.

1. Introduction

Yogurt is a fermented dairy product, and it is obtained and consumed massively in many countries and also highly appreciated, being considered important in human diet [1]. Yogurt is a source of bioactive peptides which are formed during fermentation, but generally has a limited content of antioxidant activity. For this reason, several attempts to produce yogurts fortified with natural antioxidants from natural sources have a considerable interest and present a novel approach for product development [2, 3].

In this regard, extracts of many plants, herbs, fruits, and mushrooms rich in bioactive compounds are increasingly used as additive in yogurt for better nutritional and functional improvement. For example, extracts have been used for preparation of yogurt by many researchers: crude extracts from artichoke [4], grape and grape callus extracts [5, 6], tea infusions [7], *Lycium barbarum* water extract [8], seaweed extracts [9], spirulina [10], *Pleurotus ostreatus* aqueous extract [11], black tea extracts [12], and mangosteen rind (*Garcinia mangostana* Lin.) extract [13].

Herbal nutraceuticals are commonly used by people who seek alternative health care for prevention and treatment of disease. Therefore, in the recent past, there has been rapid growth in demand for herbal medicines in food products like yogurt.

Thistle (*Silybum marianum* L.), in family *Asteraceae*, is one of the important hepatoprotective crops. The active ingredient in milk thistle is silymarin, which is composed of flavonolignans that include silydianin, silychristin, and

silybin [14]. Silymarin is a strong antioxidant, which can promote liver cell regeneration, reduce blood cholesterol, and help in the prevention of cancer [15, 16]. Recently, silymarin has been widely used in commercial preparations, herbal teas, and as a biologically active ingredient in food supplements and medical products. These products are currently among the most rapidly growing sectors in the food product industry [17]. In addition, thistle fruit extracts have antiviral and antitumor activities, and their constituents are under intense research in the clinical therapy of cancer for chemoprevention, treatment, and amelioration of chemotherapy associated side effects [18, 19].

Hawthorn (*Crataegus monogyna*), a member of the *Rosaceae* family, is native to northern temperate zones, including those of North America, East Asia, Central Asia, and Europe [20, 21]. Hawthorns grow as large shrubs or small trees and are usually armed with thorns. Many studies have demonstrated the beneficial effect of extracts of hawthorn fruits on the heart, blood circulation system, and they prevent myocardial dysfunction, improve coronary circulation, and possess hypolipidemic effects. Hawthorn contains a variety of biological active substances, among which polyphenols are a class of the most important and effective components [22, 23].

Sage (*Salvia officinalis* L.) is the most widespread species of the *Lamiaceae* family and encompasses about 900 species distributed throughout the world, which has been recognized for many medicinal plants with designated radical scavenger activity [24, 25]. Its biomass before flowering has been extensively used not only in food processing as a spice but also in pharmaceutical preparations showing a broad range of biological and medicinal activities [26]. Traditionally, it has been widely used as herbal tea, spice, and food flavouring agent, while industrially it found application as fragrance agent in cosmetics, perfumery, and pharmaceutical industry. Different sage species are reported to show many biological activities and medicinal properties, such as antimicrobial, antioxidant, antibacterial, anti-inflammatory, antitumoral, anxiolytic, antidiabetic, antifungal, antiplasmodial, hypoglycaemic, and anticarcinogenic effects [27, 28].

Marjoram (*Origanum vulgare* L.), popularly known as oregano, is a very versatile plant and although it has been used in folk medicine as diaphoretic, carminative, anti-inflammatory and tonic, only now it has been recognized for its antimicrobial property. Marjoram has been traditionally used for the treatment of gastrointestinal disturbances, cough, and bronchial diseases [29]. Marjoram is used in mouthwashes for oral hygiene and also applied topically to relieve symptoms of the common cold, such as nasal congestion. Several studies reported that extracts of marjoram had high antioxidant capacity mostly due to the polyphenolic compounds present in them. This herb has aroused interest among researchers in recent times because it shows biological activities including antimicrobial, antifungal, and antioxidant, and it may have the greatest potential for use in industrial food applications [30, 31].

This study proposed the use of aqueous extracts of some herbs in manufacture of yogurt, plants which are specific to spontaneous flora in Romania. The objective of this study was to investigate the effect of adding natural ingredients, such as herbs aqueous extracts (ranging concentration of 0.25/1%), on the functionality and the structural properties of yogurt in comparison with a yogurt sample with no additions. The production of yogurt with antioxidant properties has a promising potential for utilization as functional product. The novelty of this study is the choice of these plants to be used as an addition in yogurts, the choice that has been made taking into account the health benefits of the bioactive compounds present in these plants.

2. Materials and Methods

2.1. Materials. The yogurt samples were obtained in laboratory conditions, using the following raw materials: cow's fresh milk with 3.5% fats, 4.5% carbohydrates, 3% proteins; lactic bacteria cultures (*Lactobacillus bulgaricus* and *Streptococcus thermophillus*) supplied by Danisco Romania S.R.L. The herbs used in the experiments were supplied by Fares Bio Vital Laboratories, Romania: thistle (*Silybum marianum* L.)-Y_{thistle}, hawthorn (*Crataegus monogyna*)-Y_{hawthorn}, sage (*Salvia officinalis* L.)-Y_{sage}, and marjoram (*Origanum vulgare* L.)-Y_{marjoram}. These herbs were milled and made into a fine powder. These herbs were stirred in 200 mL distilled water for 30 minutes at 70°C, stored at 4 ± 0.5°C for 12 h, and filtered before use in yogurt production.

2.2. Yogurt Preparation. The production of yogurt with different formulations has been carried out by following a traditional process on cow's milk, including pasteurization at 90°C for 15 minutes, cooling to 41°C, inoculation with 0.02% (w/v) starter culture, dosing in packages (yogurt jars), adding herb extracts (0%, 0.25%, 0.5%, 0.75% and 1.0%) (v/v), and incubation at 41°C, until the samples reached a pH of 4.6 [32]. The finished yogurt samples were stored at 4°C ± 0.5°C for 24 h, and then the analyses were performed in order to determine the physicochemical and rheological properties of samples.

2.3. Physicochemical Analysis

2.3.1. Titratable Acidity and pH of Yogurt Samples. Titratable acidity of yogurt samples was determined by titration with NaOH 0.1N and expressed in Thörner degrees. pH was measured with a pH-meter (Mettler Toledo, Germany) in different stages of samples preparation: during fermentation, in finished product, and during storage.

2.3.2. Colour Evaluation. Colour parameters were determined using a CR400 Chroma Meter (Konica Minolta, Japan), with illuminate D65 as a reference: L^* (100 = white; 0 = black), a^* (+, red; – green), b^* (+ yellow; – blue). Samples were analyzed in triplicate.

2.3.3. Susceptibility to Syneresis (S) and Water Holding Capacity (WHC). Syneresis of the different yogurt samples was determined according to the methodology proposed by

Barkallah et al. [10]. Therefore, 100 mL of each sample was placed in a funnel lined with Whatman filter paper number 1. After 6 h of drainage, the volume of whey was measured and the following formula was used to calculate susceptibility of syneresis:

$$S = \left(\frac{V1}{V2}\right) \times 100, \quad (1)$$

where $V1$ is the volume of whey collected after drainage and $V2$ is the volume of yogurt sample.

Water holding capacity of yogurt samples was determined by the centrifugation of 5 g of yogurt at 4500 ×g for 15 minutes at 4°C (Spin MPW 223E Centrifuge, MPW Med. Instruments, Warsaw, Poland). The WHC was calculated according to the following equation:

$$\text{WHC}(\%) = \left(1 - \frac{W1}{W2}\right) \times 100, \quad (2)$$

where $W1$ is the weight of whey after centrifugation and $W2$ is the weight of yogurt [10].

2.4. Determination of Antioxidant Activity

2.4.1. Determination of the Total Polyphenol Content (TPC). Total polyphenol content was determined by an assay applied by Maksimović et al. [33]. TPC was expressed as mg gallic acid equivalent (GAE)/g.

2.4.2. Radical Scavenging Activity (RSA %) Assay. Free radical scavenging activity (RSA) of the samples was measured using the method of Brand-Williams et al. [34]. 100 μm of the sample solution was mixed with 2.9 mL of DPP (2,2-diphenyl-1-picrylhydrazyl) 60 μM in methanol solution. The reaction mixture was left in the dark for 30 minutes, after which the absorbance was measured at 517 nm using a spectrophotometer (Spectrophotometer UV-3600). Methanol was used as blank. Antioxidant activity was expressed as percentage inhibition of the DPPH radical and was determined by the following equation:

$$\text{RSA } \% = \left[\frac{\left(A_{\text{control}} - A_{\text{sample}}\right)}{A_{\text{control}}}\right] \times 100. \quad (3)$$

2.5. Rheological Analysis. The Modular Advanced Rheometer System (Thermo Haake Mars, Germany) was used for studying the rheological properties of yogurt samples. The samples were allowed to rest for 10 minutes at 4°C on the Ti 40 mm geometry plate, before conducting the analyses. The samples were subjected to frequency dependency experiments from 0.1 to 10.0 Hz, at 4°C. The storage module (G') and the loss module (G'') at 1 Hz frequency were monitored. Also, there were conducted viscosity tests depending on time (10 minutes at a constant shear rate of 100 s^{-1}). Three determinations of the two tests were conducted for each sample [32]. The Haake RheoWin Data Manager software was used for all the analyses to obtain the graphical representation of viscosity curves and dynamic tests [35, 36].

2.6. Statistical Analysis. The statistical analysis was done using XLSTAT (free trial version 2016, Addinsoft, Inc., Brooklyn, NY, USA) at a significance level at $p < 0.05$. The graphical representation of the principal component analysis (PCA) allows the data to be analyzed on a two-dimensional $F1/F2$ map and to identify the trends between variables at a significance level $p < 0.05$. When two variables are far from the centre and close together, they correlate significantly. If they are on the opposite side of the centre, then they are significantly negatively correlated.

3. Results and Discussion

3.1. Physicochemical Characteristics and Antioxidant Activity. The yogurt samples were analyzed after two storage periods (1 and 28 days) at 4°C. The results are shown in Table 1 and are expressed as the mean value of each analysed parameter of the samples, conducted in triplicate for the two storage periods considered.

An overall decrease of pH values of yogurt samples occurred during storage in refrigeration conditions. The pH for all the yogurt samples decreased during storage period, for example, in the case of $Y_{\text{thistle-1\%}}$ sample, the pH decreases from 4.62 in the first day to 4.25 in the last day of storage. The addition of herbs aqueous extracts did not seem to significantly affect the pH of the analysed samples compared to the control sample. The results presented above are in agreement with those obtained by Pelaes Vital et al., who reported that pH for control yogurt was approximately the same as pH of yogurts with *Pleurotus ostreatus* aqueous extract [11]. According to Caleja et al., the analysis of this parameter in yogurts is very important in terms of product safety [3].

Titratable acidity of all yogurt samples was between 88 and 111°T on the first day of storage, and between 101 and 116°T, after 28 days of storage in refrigeration conditions for all the analyzed samples. Similarly, Barkallah et al. concluded that additional *Spirulina platens* increased the buffering capacity that required additional acid development by starter cultures during the whole storage period (28 days) at 4°C [10]. The results obtained shows all yoghurt samples with herb extracts have lower pH values ($p < 0.05$) and a higher acidity ($p < 0.001$) than the control sample. One explanation for this aspect is that the herb extracts improve the growth of bacteria contained in the yogurt samples.

Syneresis is considered by many researchers as one of the most important parameters indicating the quality of yogurt during storage [10, 36–39]. The control yogurt samples showed a higher syneresis compared to yogurt samples with different levels of herbs aqueous extracts addition, after the 28 days of the storage. The lowest syneresis after 28 days of storage was observed for yogurt with 0.50% thistle (*Silybum marianum* L.). In the same time, an improvement of the WHC parameter after 28 days of storage was observed for all yogurt samples containing extracts compared to the control

Table 1: Physicochemical characteristics and antioxidant activity of yogurt samples.

Storage day	Sample	Characteristics					
		pH	Acidity (T)	Syneresis (%)	WHC (%)	TPC (mg·GAE/g)	RSA (%)
Day 1	Y_{Control}	4.41 ± 0.01	105 ± 0.2	54.4 ± 0.1	35.77 ± 0.1	0.99 ± 0.02	10.56 ± 0.1
	$Y_{\text{thistle-0.25\%}}$	4.50 ± 0.01	101 ± 0.1	53.6 ± 0.2	32.44 ± 0.1	0.98 ± 0.02	10.14 ± 0.1
	$Y_{\text{thistle-0.50\%}}$	4.38 ± 0.02	107 ± 0.2	48.0 ± 0.1	34.11 ± 0.3	0.88 ± 0.01	12.80 ± 0.1
	$Y_{\text{thistle-0.75\%}}$	4.56 ± 0.01	97 ± 0.1	49.8 ± 0.3	34.56 ± 0.1	0.90 ± 0.01	11.02 ± 0.2
	$Y_{\text{thistle-1\%}}$	4.62 ± 0.01	88 ± 0.1	54.2 ± 0.1	30.78 ± 0.1	1.27 ± 0.01	12.67 ± 0.1
	$Y_{\text{hawthorn-0.25\%}}$	4.52 ± 0.02	100 ± 0.1	51.6 ± 0.1	34.86 ± 0.1	3.46 ± 0.01	19.23 ± 0.1
	$Y_{\text{hawthorn-0.50\%}}$	4.36 ± 0.03	111 ± 0.1	49.8 ± 0.1	36.04 ± 0.3	3.88 ± 0.01	21.60 ± 0.1
	$Y_{\text{hawthorn-0.75\%}}$	4.44 ± 0.01	106 ± 0.3	48.0 ± 0.2	35.60 ± 0.1	4.02 ± 0.02	32.02 ± 0.2
	$Y_{\text{hawthorn-1\%}}$	4.50 ± 0.01	104 ± 0.1	53.6 ± 0.2	31.28 ± 0.1	4.34 ± 0.01	33.38 ± 0.1
	$Y_{\text{sage-0.25\%}}$	4.49 ± 0.01	110 ± 0.1	55.0 ± 0.1	37.61 ± 0.2	2.18 ± 0.01	23.14 ± 0.1
	$Y_{\text{sage-0.50\%}}$	4.52 ± 0.03	102 ± 0.3	54.2 ± 0.1	35.02 ± 0.1	3.88 ± 0.03	22.30 ± 0.1
	$Y_{\text{sage-0.75\%}}$	4.51 ± 0.01	105 ± 0.1	54.8 ± 0.1	35.72 ± 0.1	4.17 ± 0.01	20.23 ± 0.3
	$Y_{\text{sage-1\%}}$	4.54 ± 0.02	101 ± 0.1	54.8 ± 0.1	33.92 ± 0.1	4.08 ± 0.01	21.21 ± 0.1
	$Y_{\text{marjoram-0.25\%}}$	4.45 ± 0.3	107 ± 0.2	52.4 ± 0.1	33.21 ± 0.3	3.21 ± 0.03	33.14 ± 0.1
	$Y_{\text{marjoram-0.50\%}}$	4.51 ± 0.01	97 ± 0.1	53.6 ± 0.3	30.90 ± 0.1	3.88 ± 0.01	42.30 ± 0.1
	$Y_{\text{marjoram-0.75\%}}$	4.48 ± 0.01	108 ± 0.1	52.0 ± 0.1	30.38 ± 0.1	5.11 ± 0.01	34.23 ± 0.1
	$Y_{\text{marjoram-1\%}}$	4.41 ± 0.01	106 ± 0.2	48.8 ± 0.3	31.67 ± 0.1	5.28 ± 0.01	41.21 ± 0.1
Day 28	Y_{Control}	4.20 ± 0.01	119 ± 0.1	58.4 ± 0.1	33.72 ± 0.1	0.36 ± 0.01	8.12 ± 0.3
	$Y_{\text{thistle-0.25\%}}$	4.28 ± 0.02	116 ± 0.1	48.3 ± 0.1	45.17 ± 0.1	1.46 ± 0.02	12.67 ± 0.1
	$Y_{\text{thistle-0.50\%}}$	4.26 ± 0.03	114 ± 0.1	45.6 ± 0.1	45.41 ± 0.3	1.12 ± 0.01	19.03 ± 0.2
	$Y_{\text{thistle-0.75\%}}$	4.29 ± 0.03	110 ± 0.1	48.4 ± 0.1	45.24 ± 0.1	0.96 ± 0.01	10.52 ± 0.1
	$Y_{\text{thistle-1\%}}$	4.25 ± 0.02	102 ± 0.3	50.6 ± 0.1	41.35 ± 0.1	1.92 ± 0.02	12.04 ± 0.2
	$Y_{\text{hawthorn-0.25\%}}$	4.32 ± 0.01	105 ± 0.3	53.6 ± 0.1	37.77 ± 0.2	3.82 ± 0.01	19.03 ± 0.2
	$Y_{\text{hawthorn-0.50\%}}$	4.31 ± 0.01	101 ± 0.1	55.6 ± 0.1	37.73 ± 0.1	4.46 ± 0.01	23.74 ± 0.1
	$Y_{\text{hawthorn-0.75\%}}$	4.30 ± 0.01	107 ± 0.2	56.0 ± 0.1	38.15 ± 0.1	4.68 ± 0.01	30.76 ± 0.1
	$Y_{\text{hawthorn-1\%}}$	4.29 ± 0.02	107 ± 0.2	54.0 ± 0.1	35.43 ± 0.2	5.12 ± 0.01	33.49 ± 0.2
	$Y_{\text{sage-0.25\%}}$	4.35 ± 0.02	105 ± 0.1	55.8 ± 0.2	36.80 ± 0.2	2.72 ± 0.01	29.03 ± 0.1
	$Y_{\text{sage-0.50\%}}$	4.33 ± 0.02	108 ± 0.1	54.0 ± 0.1	37.56 ± 0.3	3.36 ± 0.03	22.84 ± 0.1
	$Y_{\text{sage-0.75\%}}$	4.34 ± 0.01	110 ± 0.2	54.4 ± 0.3	35.47 ± 0.2	3.40 ± 0.01	20.52 ± 0.1
	$Y_{\text{sage-1\%}}$	4.28 ± 0.03	116 ± 0.2	55.0 ± 0.1	39.32 ± 0.1	4.17 ± 0.02	20.32 ± 0.3
	$Y_{\text{marjoram-0.25\%}}$	4.30 ± 0.03	107 ± 0.1	51.8 ± 0.1	38.33 ± 0.1	3.42 ± 0.01	33.12 ± 0.1
	$Y_{\text{marjoram-0.50\%}}$	4.29 ± 0.02	110 ± 0.1	54.0 ± 0.2	39.04 ± 0.1	4.16 ± 0.02	40.50 ± 0.1
	$Y_{\text{marjoram-0.75\%}}$	4.30 ± 0.01	105 ± 0.1	55.0 ± 0.1	35.74 ± 0.2	5.40 ± 0.01	38.53 ± 0.1
	$Y_{\text{marjoram-1\%}}$	4.30 ± 0.02	106 ± 0.2	56.8 ± 0.1	35.74 ± 0.1	6.30 ± 0.01	42.09 ± 0.2

samples. The best results were obtained for the 0.5% thistle (*Silybum marianum* L.) addition.

The factor that influencing these parameters, as have been shown by Barkallah et al. and other researchers, is the ability of proteins to retain water and milk fat cells in the structure of yogurt [10, 36–39].

The results for total polyphenol content in the analyzed period (0–28 days) varied in yogurt depending on the type of herb extract that was used. The best results were obtained for the yogurt with marjoram extracts addition; in this case, the TPC level reaching up to 6.30 mg GAE/g during storage.

The yogurt samples with extracts of hawthorn (*Crataegus monogyna*) and sage (*Salvia officinalis* L.) were also rich in polyphenols, and the obtained results show values of 5.12 mg GAE/g and 4.17 mg GAE/g. The total polyphenol content in yogurt samples increases during storage. The total content of polyphenols in the analyzed samples is variable, and the correlation ratio ($r^2 = 0.139$ and $r^2 = 0.091$) indicates a scattered distribution of the data. The best results were obtained for samples with marjoram (*Origanum vulgare* L.) extracts, which is also noted in the samples with sage (*Salvia officinalis* L.) extracts.

The results of the antioxidant activity correlate with the total content of polyphenols. High antioxidant activity is noted in yogurts with the addition of marjoram extracts both on the first day and on the last day of storage. Antioxidant activity was preserved during storage and even increased in some samples. It can be appreciated that lactic acid and herbal extracts affect the stability of the product over time. According by Muniandy et al., the inclusion of herb extracts prior to bacterial fermentation significantly increased the ($p < 0.05$) antioxidant activity compared to milk alone [12]. This could be attributed to the high content of flavonoids in herb extracts. Similar studies describe that the antioxidant activity of yogurts was enhanced by the presence of natural extracts [3, 14, 16, 22, 23, 25, 29].

The obtained values of colour parameters according to the CIE colour scale are presented in Table 2, for all analysed yogurt samples. The incorporation of the herbs aqueous extracts did not change the colour parameters of the samples. In a similar study, performed by Chouchouli et al., it was shown that the fortification of yogurt with grape seed extract did not affect the colour of the yogurts, and changes were not visually detected [6]. The same aspect was observed

Table 2: Colour parameters of the yogurt with different herb extracts.

Storage day	Sample	Colour parameters					
		L^*	a^*	b^*	C^*_{ab}	h^*_{ab}	ΔE^*
Day 1	$Y_{Control}$	84.91	−6.89	12.61	14.37	118.65	—
	$Y_{thistle-0.25\%}$	83.85	−6.67	11.55	13.34	120.01	1.52
	$Y_{thistle-0.50\%}$	78.8	−6.29	10.87	12.56	120.06	6.38
	$Y_{thistle-0.75\%}$	78.74	−6.21	10.8	12.46	119.90	6.47
	$Y_{thistle-1\%}$	74.27	−5.83	9.97	11.55	120.32	11.01
	$Y_{hawthorn-0.25\%}$	78.59	−6.08	10.71	12.32	119.58	6.65
	$Y_{hawthorn-0.50\%}$	77.99	−6.09	10.88	12.47	119.24	7.18
	$Y_{hawthorn-0.75\%}$	77.47	−5.99	10.84	12.3849	118.9243	7.70
	$Y_{hawthorn-1\%}$	75.23	−5.65	10.61	12.02059	118.036	9.96
	$Y_{sage-0.25\%}$	85.1	−6.77	12.3	14.04005	118.8287	0.38
	$Y_{sage-0.50\%}$	84.56	−6.27	13.01	14.44206	115.7311	0.82
	$Y_{sage-0.75\%}$	80.99	−5.82	12.78	14.04282	114.4845	4.07
	$Y_{sage-1\%}$	84.62	−6	13.57	14.83728	113.8527	1.34
	$Y_{marjoram-0.25\%}$	75.43	−5.47	10.46	11.80392	117.6071	9.82
	$Y_{marjoram-0.50\%}$	75.32	−5.14	10.77	11.93367	115.5129	9.92
	$Y_{marjoram-0.75\%}$	74.78	−4.86	10.99	12.01664	113.856	10.46
	$Y_{marjoram-1\%}$	76.06	−4.92	11.34	12.36131	113.4542	9.16
Day 28	$Y_{Control}$	97.97	−6.04	9.6	11.34	122.18	—
	$Y_{thistle-0.25\%}$	86.02	−7.26	11.92	13.96	121.34	12.23
	$Y_{thistle-0.50\%}$	85.34	−7.21	11.81	13.84	121.40	12.88
	$Y_{thistle-0.75\%}$	82.52	−6.75	11.68	13.49	120.02	15.61
	$Y_{thistle-1\%}$	86.35	−7.25	12.19	14.18	120.74	11.97
	$Y_{hawthorn-0.25\%}$	81.09	−6.58	11.25	13.03	120.32	16.97
	$Y_{hawthorn-0.50\%}$	78.71	−6.27	11.23	12.86	119.18	19.33
	$Y_{hawthorn-0.75\%}$	83.87	−6.79	12.28	14.0322	118.9396	14.37
	$Y_{hawthorn-1\%}$	83.66	−6.67	12.33	14.01848	118.4115	14.58
	$Y_{sage-0.25\%}$	85.52	−6.95	12.23	14.06682	119.6085	12.76
	$Y_{sage-0.50\%}$	82.49	−6.44	12.08	13.68941	118.0626	15.68
	$Y_{sage-0.75\%}$	84.13	−6.3	12.62	14.10512	116.5287	14.17
	$Y_{sage-1\%}$	85.17	−6.43	13.09	14.584	116.1609	13.27
	$Y_{marjoram-0.25\%}$	86.21	−6.61	12.48	14.12241	117.9078	12.12
	$Y_{marjoram-0.50\%}$	85.52	−6.3	12.68	14.15883	116.4203	12.83
	$Y_{marjoram-0.75\%}$	83.88	−6.03	12.78	14.13115	115.2594	14.44
	$Y_{marjoram-1\%}$	80.44	−5.43	12.45	13.58261	113.5642	17.77

by Caleja et al. in the case of chamomile decoction addition in yogurt [3].

3.2. Evaluation of the Rheological Properties. Figures 1–4 show the evolution of samples' viscosity as a function of time at a constant shear rate of $100\,s^{-1}$, for each storage period. This analysis showed the thixotropic characteristics of yogurt samples, which showed a reduction of viscosity in time, in accordance with the data presented in the literature. Several studies confirm that the yogurt is a thixotropic fluid, for example, Mathias et al. [40], Sidor et al. [35], and Sanz et al. [41]. In day 1 of storage, all fortified samples, independent of the amount of herb extract added, showed lower viscosity values than the control sample. However, the graphical representation showed higher viscosity values of fortified samples in the 28th day of storage comparing with the control sample. Sidor et al., evaluating the effect of sea buckthorn powder addition in yogurt, also observed that increasing the dose promoted the formation of a more viscous product [35].

The mechanical spectra obtained for the analyzed samples are shown in Figures 5–8. It can be observed that the form of the spectra was typical for weak gels, in both monitored storage times, with consistency module values (G') slightly higher than those of firmness module values (G''). Same trend regarding the viscoelastic properties of yogurt was observed by Sanz et al., when studying the effect of yogurt enrichment with fibers obtained from the non-edible part of asparagus shoots [41]. Also, the incorporation of all types of herb extracts produced an increase in the value of both modules in comparison with the control sample, which can be observed, especially, in the 28th day of storage.

3.3. Statistical Analysis. The principal component analysis (PCA) was used to illustrate the relationship between the physicochemical characteristics, the polyphenols content, and the antioxidant activity in yogurt samples. The obtained results for PCA are shown in Figures 9 and 10.

The addition of 1% hawthorn extract, 0.75% marjoram extract, and 0.75% sage has a significant positive influence on

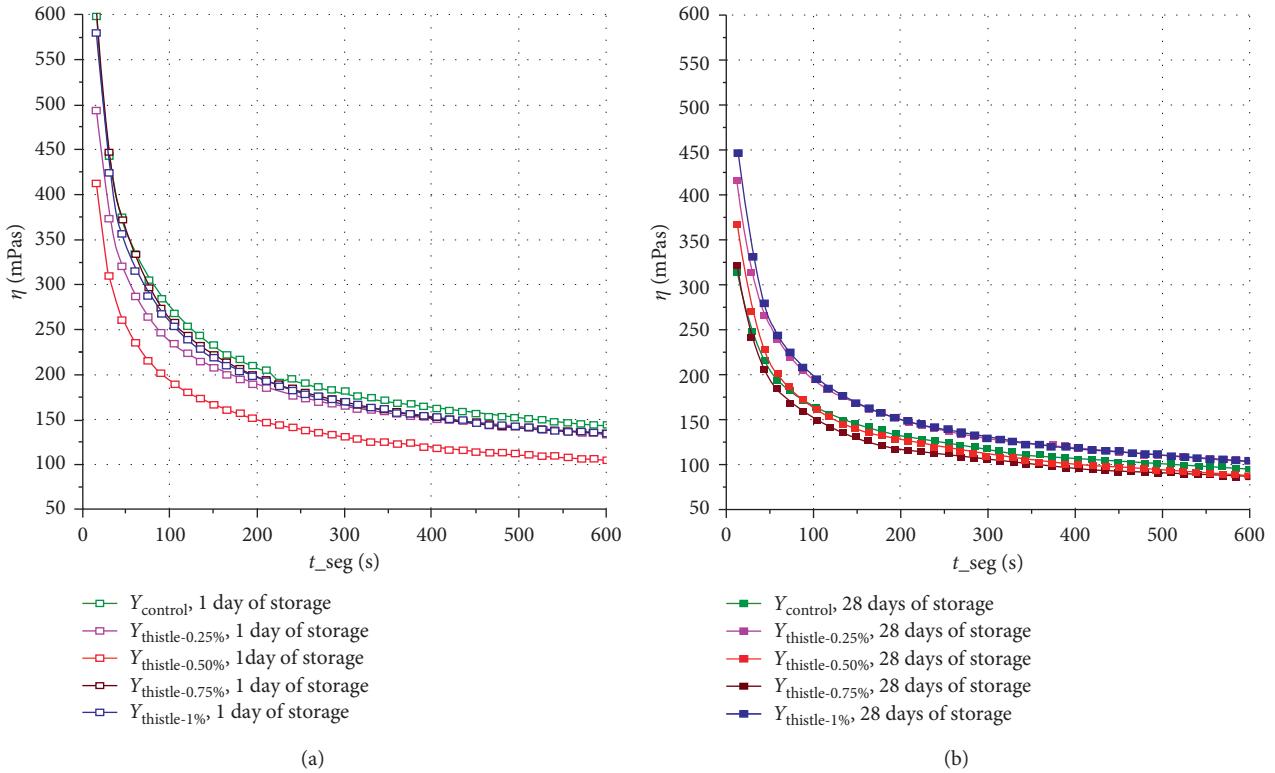

Figure 1: Curve of viscosity versus time of testing thixotropy of yogurt with thistle (*Silybum marianum* L.) extract: (a) 1 day of storage; (b) after 28 days of storage.

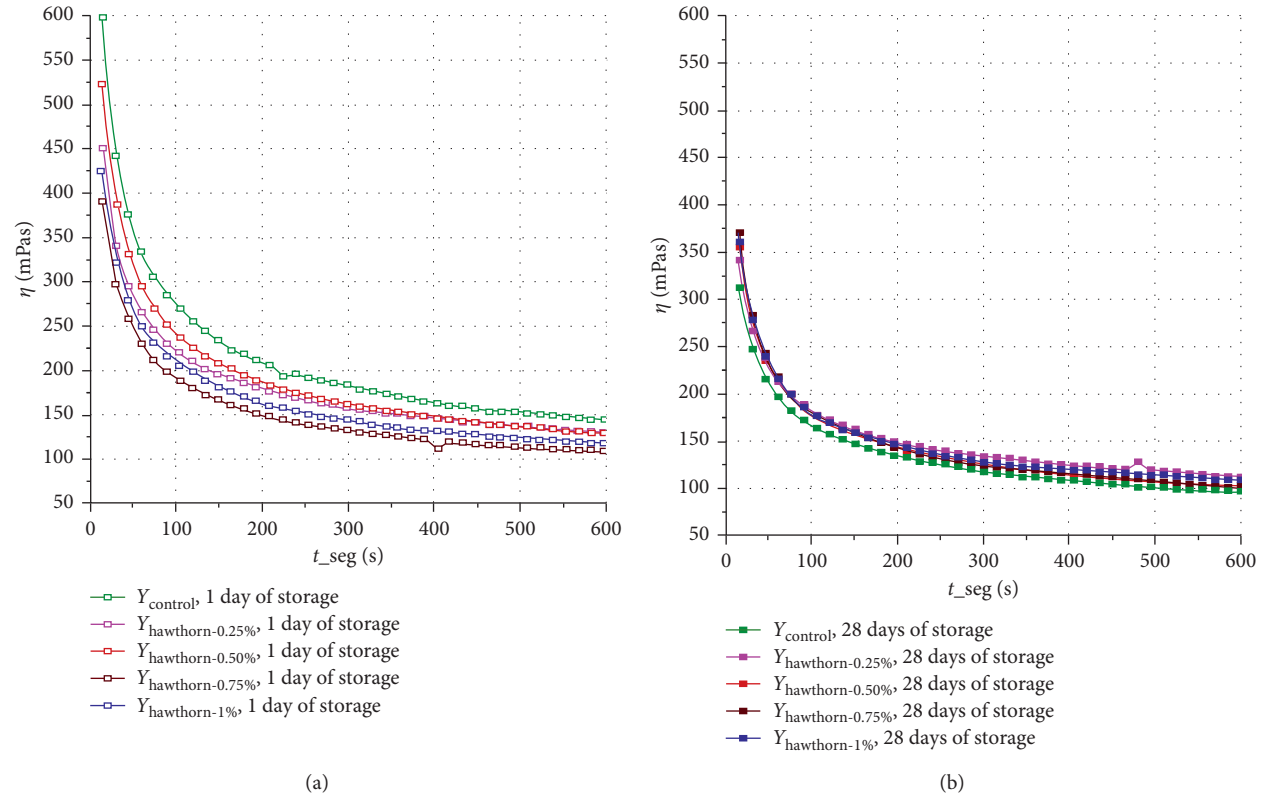

Figure 2: Curve of viscosity versus time of testing thixotropy of yogurt with hawthorn (*Crataegus monogyna*) extract: (a) 1 day of storage; (b) after 28 days of storage.

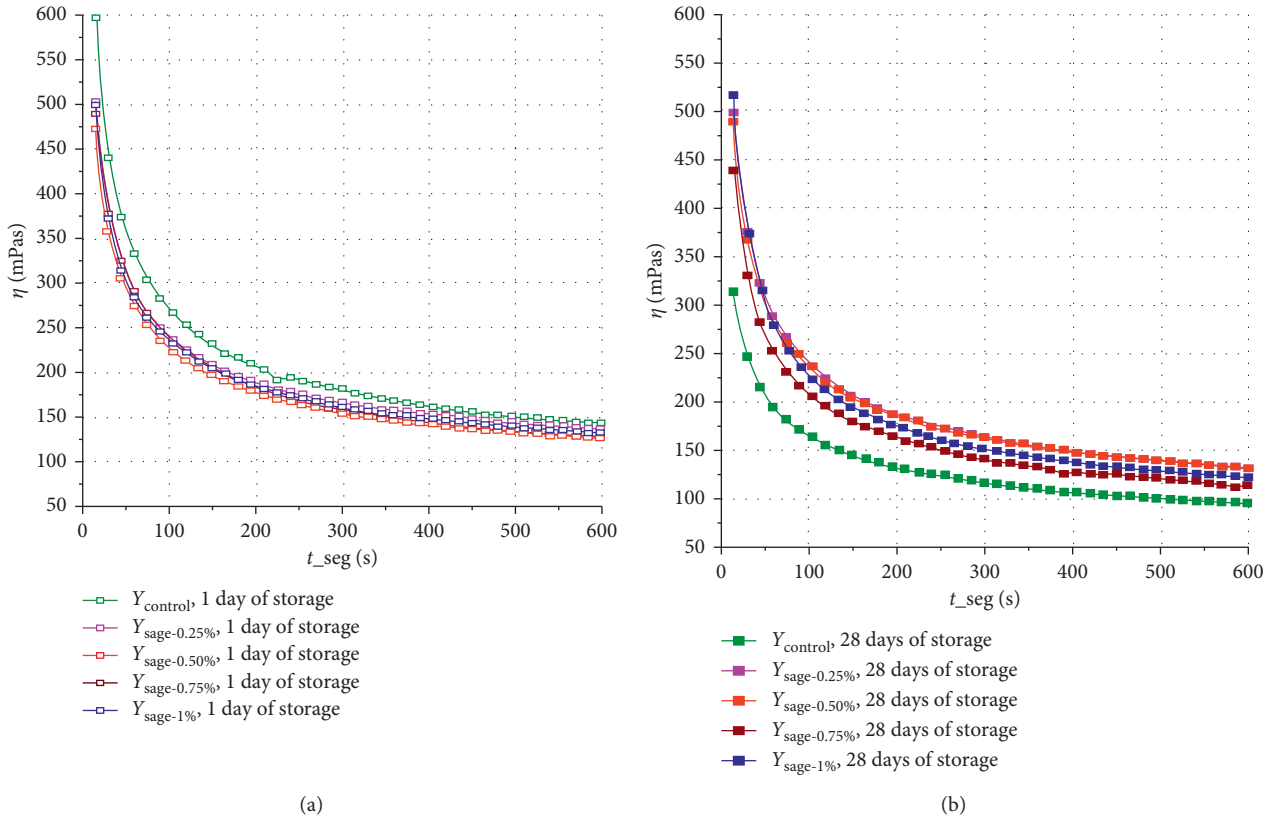

FIGURE 3: Curve of viscosity versus time of testing thixotropy of yogurt with sage (*Salvia officinalis* L.) extract: (a) 1 day of storage; (b) after 28 days of storage.

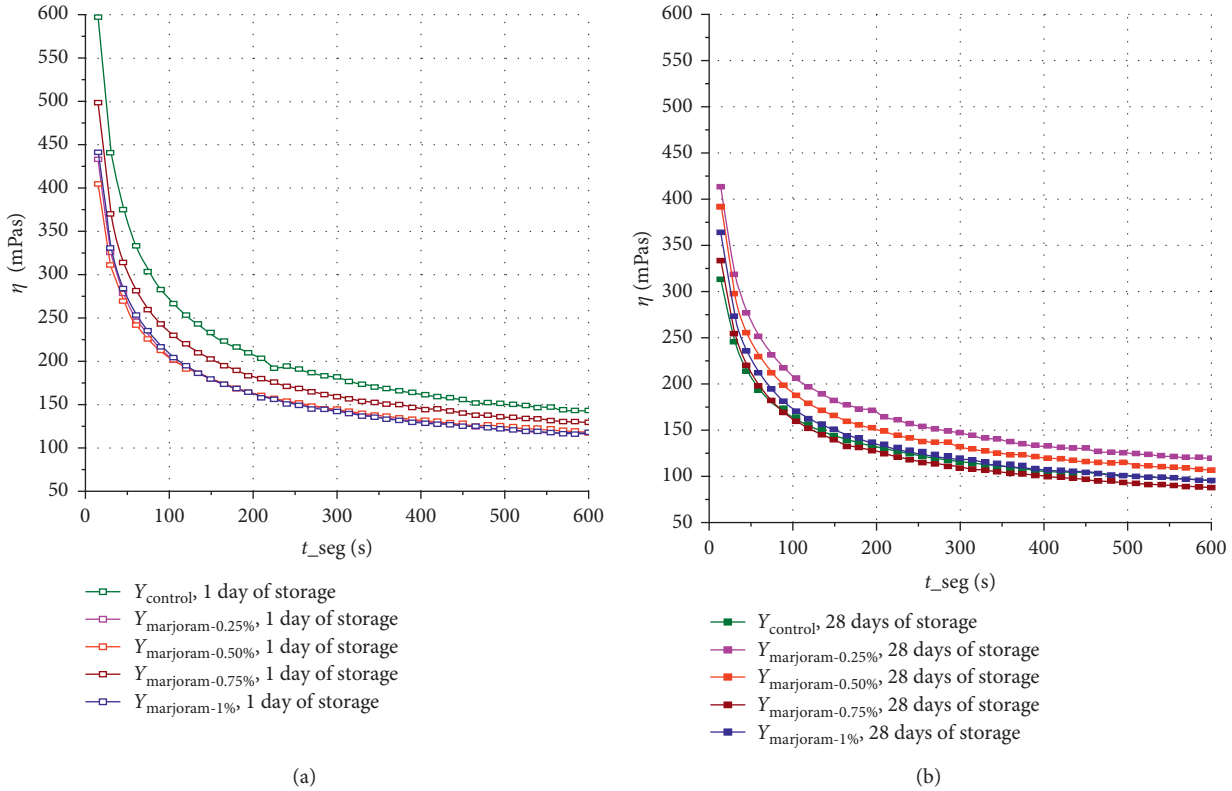

FIGURE 4: Curve of viscosity versus time of testing thixotropy of yogurt with marjoram (*Origanum vulgare* L.) extract: (a) 1 day of storage; (b) after 28 days of storage.

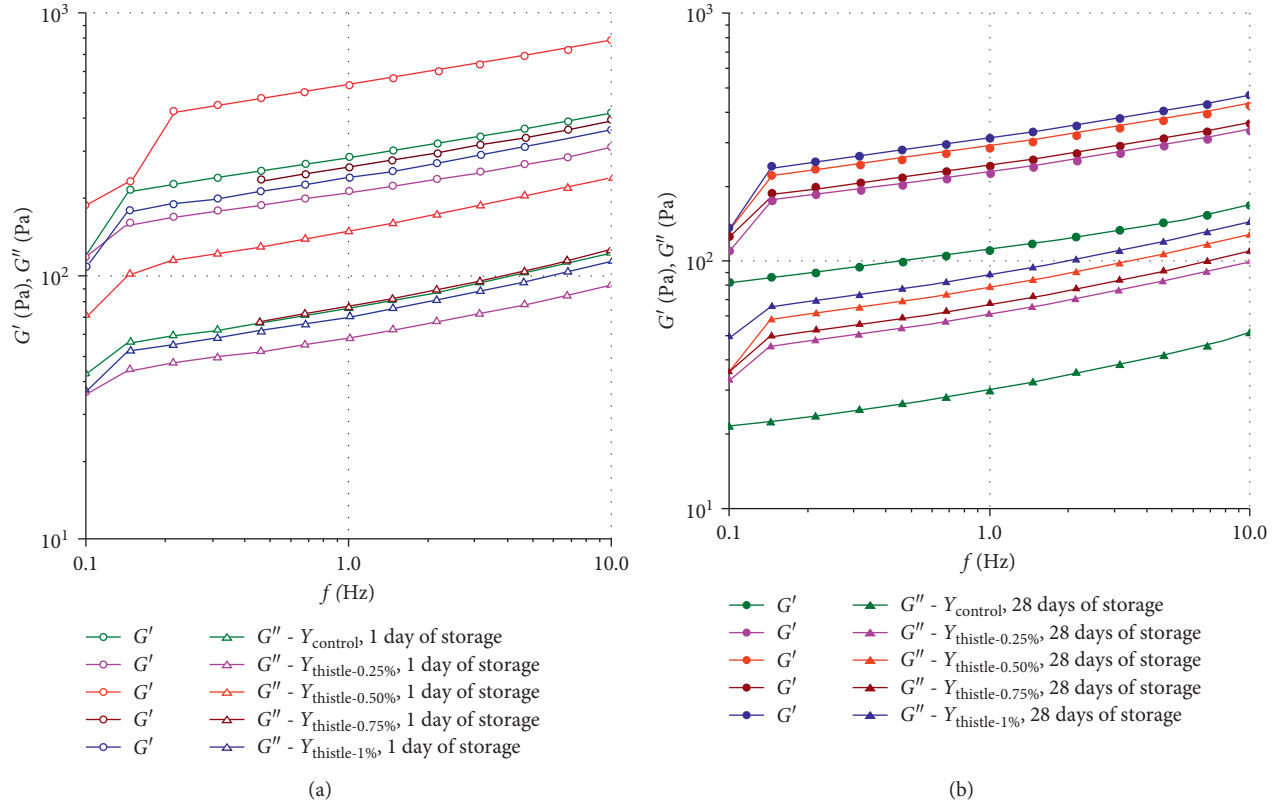

Figure 5: Viscoelastic properties of yogurt with thistle (*Silybum marianum* L.) extract: (a) 1 day of storage; (b) after 28 days of storage.

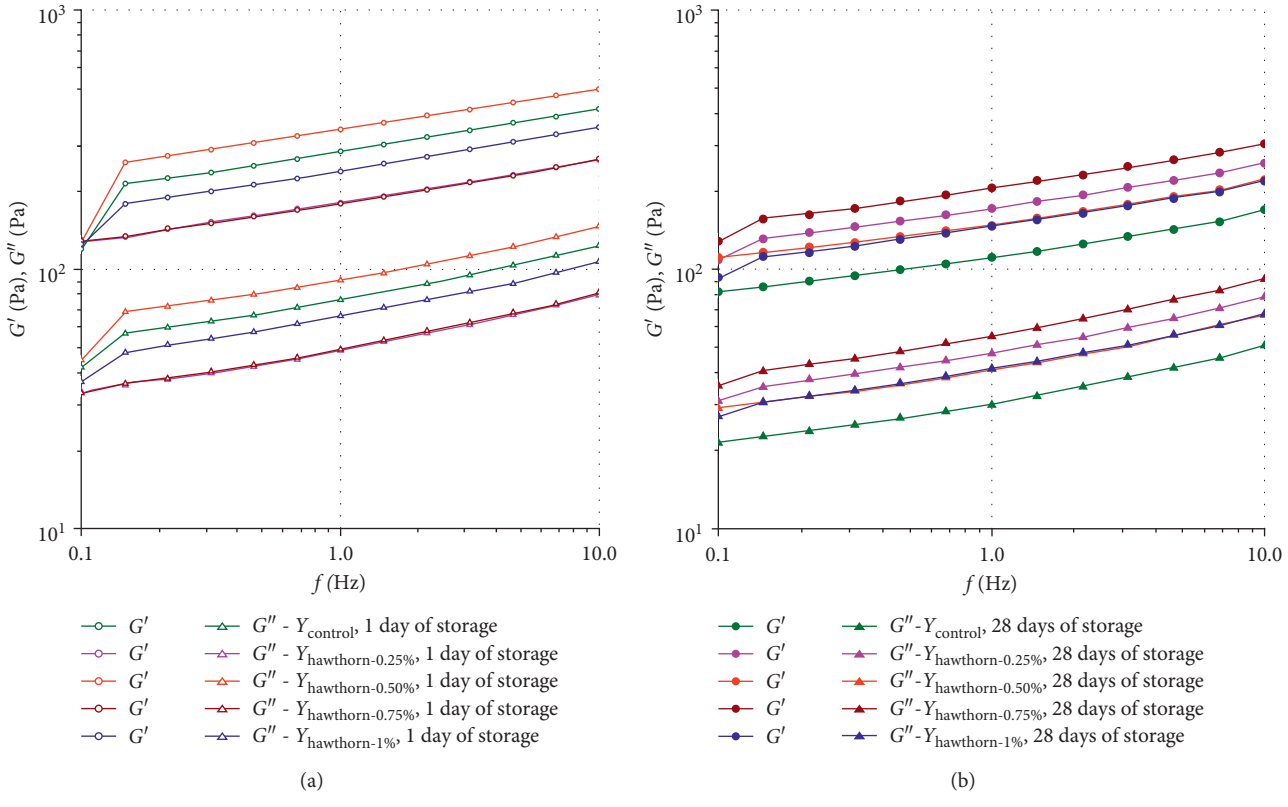

Figure 6: Viscoelastic properties of yogurt with hawthorn (*Crataegus monogyna*) extract: (a) 1 day of storage; (b) after 28 days of storage.

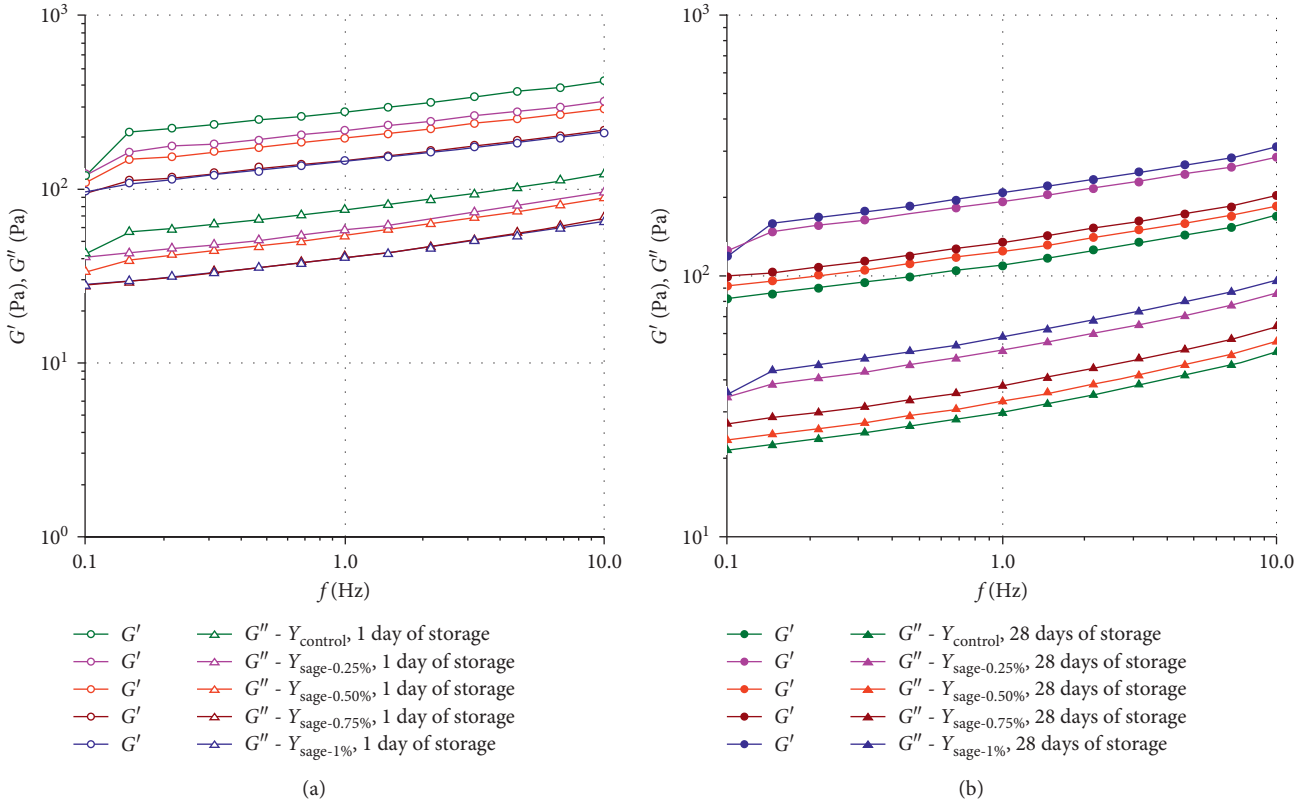

Figure 7: Viscoelastic properties of yogurt with sage (*Salvia officinalis* L.) extract: (a) 1 day of storage; (b) after 28 days of storage.

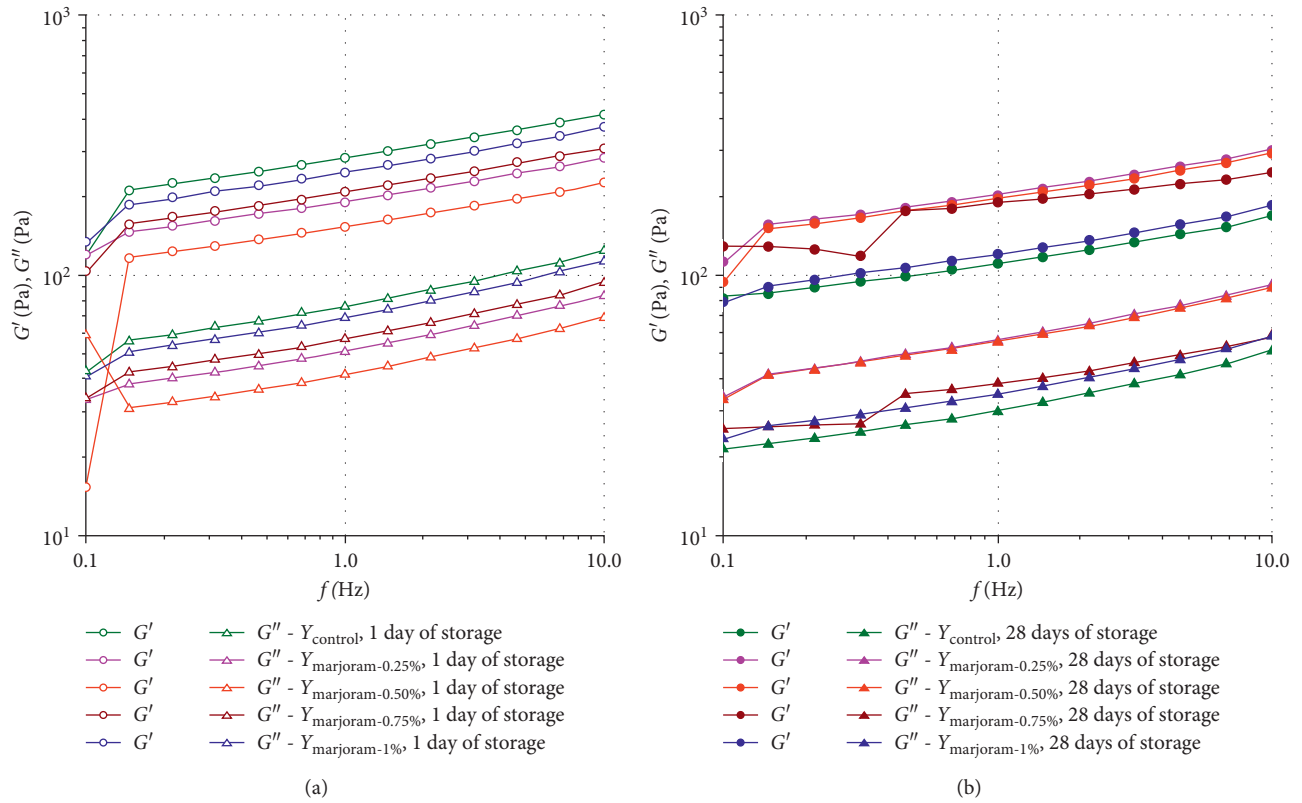

Figure 8: Viscoelastic properties of yogurt with marjoram (*Origanum vulgare* L.) extract: (a) 1 day of storage; (b) after 28 days of storage.

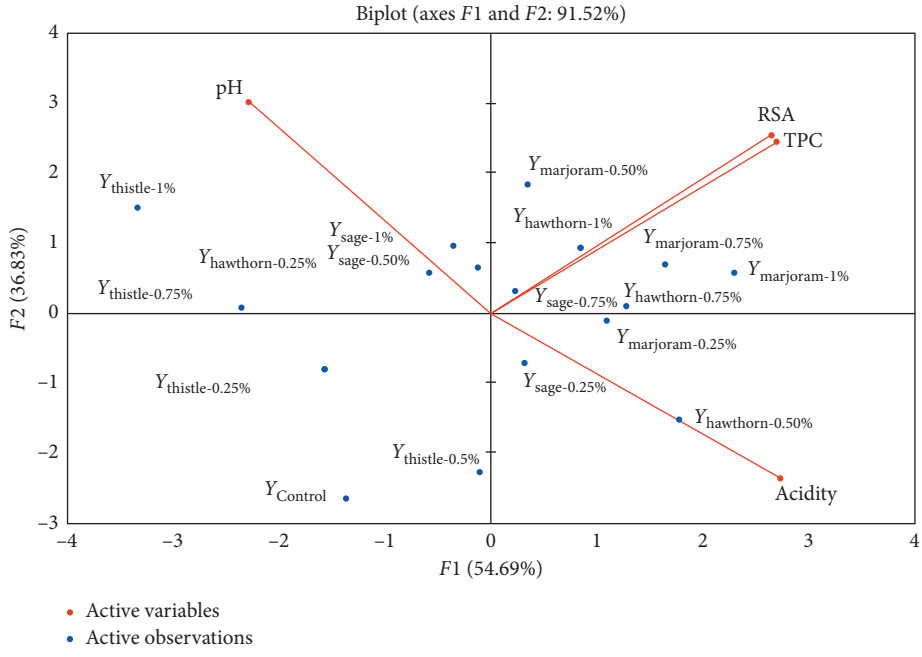

Figure 9: PCA loadings of the yogurt characteristics after 1 day of storage.

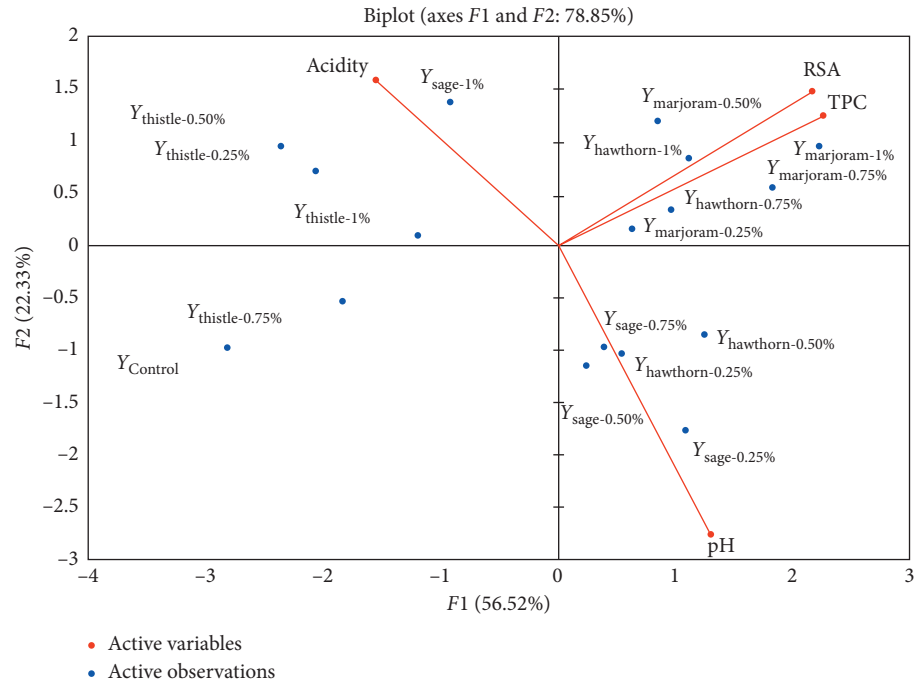

Figure 10: PCA loadings of the yogurt characteristics after 28 days of storage.

the antioxidant character of yogurt samples. The acidity of yogurt after a day of manufacture is lower compared to the pH value. The addition of 0.5% hawthorn extract influences the decrease of yogurt acidity.

The addition of sage extract in yogurt does not influence the analyzed physicochemical characteristics. This aspect is distinguished by the distribution of values in separate dials and compared to the control sample (without the addition of plant extracts).

The PCA chart at 28 days after yogurt production indicates an increase in acidity in yogurt samples and a decrease in pH value during storage. The values of the antioxidant activity and the polyphenols content remain high, especially, in the yogurt samples with the addition of marjoram extract. The addition of the hawthorn extract in high amount (0.75/1%) favors the growth of the antioxidant character. These results are in agreement with those obtained by Shyamala which obtained a stability in time of the yogurt

samples when different levels of coriander (*Coriandrum sativum*) and spinach (*Spinacia oleracea*) extracts were added [42].

4. Conclusions

This manuscript demonstrates that extracts from different herbs can be incorporated successfully into a fermented dairy product such as yogurt.

So far, the aqueous extracts obtained from the four plants which are considered in this study, respectively, thistle (*Silybum marianum* L.), hawthorn (*Crataegus monogyna*), sage (*Salvia officinalis* L.), marjoram (*Origanum vulgare* L.), have not been used in the manufacture of yogurt. The final results show that the physicochemical and rheological properties of the yoghurt with herb extracts addition were improved compared to the control sample after 28 days of storage.

Syneresis and water holding capacity was improved due to the thistle extract (*Silybum marianum* L.) addition. The best results in terms of antioxidant properties were obtained when marjoram extract (Origanum vulgare L.) was incorporated. The highest rheological values were obtained for the samples with thistle extract (*Silybum marianum* L.) addition. According to the data obtained, the best quality in terms of the physicochemical and rheological properties was in the case of the sample with 0.5% thistle extract (*Silybum marianum* L.) addition, while from the point of view of the nutritional value, the best quality was in the case of the sample with 1% marjoram extract (*Origanum vulgare* L.) addition.

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.

Acknowledgments

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Research Article

Estimation of Food Security Risk Level Using *Z*-Number-Based Fuzzy System

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Fuzzy logic systems based on If-Then rules are widely used for modelling of the systems characterizing imprecise and uncertain information. These systems are basically based on type-1 fuzzy sets and allow handling the uncertain and imprecise information to some degree in the developed models. Zadeh extended the concept of fuzzy sets and proposed *Z*-number characterized by two components, constraint and reliability parameters, which are an ordered pair of fuzzy numbers. Here, the first component is used to represent uncertain information, and the second component is used to evaluate the reliability or the confidence in truth. *Z*-number is an effective approach to solving uncertain problems. In this paper, *Z*-number-based fuzzy system is proposed for estimation of food security risk level. To construct fuzzy If-Then rules, the basic parameters cereal yield, cereal production, and economic growth affecting food security are selected, and the relationship between these input parameters and risk level are determined through If-Then fuzzy rules. The fuzzy interpolative reasoning is proposed for construction of inference mechanism of a *Z*-number-based fuzzy system. The designed system is tested using Turkey cereal data for assessing food security risk level and prediction periods of the food supply.

1. Introduction

In the real world, the data are often imperfect because of their unreliability and nature. The complementary aspects of imperfect information are uncertainty and imprecision [1, 2]. Uncertainty characterizes the degree of truth, and imprecision characterizes the content of the data. Fuzzy set theory, introduced by Zadeh [1], can be used to deal with these factors in the modelling of the systems. Therefore, different industrial and nonindustrial problems that were characterized by uncertainty and imprecision were solved by means of the fuzzy set theory [2]. In this paper, the application of fuzzy sets to measurement of food security is considered.

The food security, as it was defined by United Nations' Committee on World Food Security, is a social, physical, and economic access to the sufficient nutritious food that meets the needs of the pupil and also food preferences for an active and healthy life. Food security indicators are built on four pillars: availability, access, utilization, and stability [3]. Food security is highly affected by various factors such as a growing global population, changing the climate, rising food prices, and environmental stressors [4–6]. The possibility of negative actions causes a hazard and increases risk level of food security. The analyses of different factors having influences on food security have been done in different research works. Wu.et al. [6] combined the social, economic, and biophysical factors for estimation of the potential risks of food security. The two indicators, per capita food availability and per capita gross domestic product, were used to estimate the food availability, stability, accessibility, and affordability and to assess the potential future risk of food insecurity. Ehrlich et al. [7] examined two views on the causes of global food insecurity: biophysical and demographic. They concluded that the achieving food-secure future for all humanity is positively influenced by the enhancement of equality, the reduction of the human fertility,

and improvement of the ecosystem health. Hubbard et al. [5] examined a number of factors that contribute to the food security. Wang et al. [8] designed a prediction model through the combination of the stepwise regression method with BP neural network for forecasting the total food yield. Xiao et al. [9] used BP neural network and considered the development of risk early warning system for food security under the supplied chain environment.

The impacts of factors affecting food security are imprecise, and it is difficult to describe them with certain numbers. For this reason, the fuzzy set theory is a very effective way of estimation of food security risk level. Uncertain availability of nutritious and safe foods is also called food insecurity that can be measured by risk level of food security. Nowadays, the fuzzy set theory is one of the important tools for determination of the risk level of food security and also for risk management [10–16]. Wang et al. [10] considered the combination of the fuzzy set theory and analytical hierarchy process for a risk assessment. Lee [11] evaluated the rate of aggregative risk by fuzzy set theory. Using fuzzy set theory and Dempster–Shafer evidence theory, Xu et al. [12] proposed a fuzzy evidential grain security warning method for food management. In the research works [10–14], using various factors the design of fuzzy rules is performed for management of the risk. Kadir et al. [15] designed a fuzzy system for assessment of food security risk level. The designed system is based on fuzzy If-Then rules that used three important factors cereal production, cereal yield, and economic growth for the determination of food security risk level. The values of these factors are estimated linguistically using fuzzy values. These fuzzy rules are developed using the knowledge of human experts. In the above research papers, the premise and the consequent parts of the fuzzy If-Then rules are basically designed from numeric data sets or linguistic information using type-1 membership functions. Abiyev et al. [16] designed the type-2 fuzzy system for assessment of food security risk level. Jiang et al. [17] presented the failure mode and effect analysis model based on a fuzzy evidential method for evaluation of the risk of failure modes. Ranking of the risk of the failure model is presented by fusing the occurrence, severity, and detection information and Dempster–Shafer theory.

The fuzzy knowledge-based systems used for decision-making are basically based on knowledge, experience, intuition, and assumption of humans. Sometimes, these qualities cannot completely cover all the complexity of the considered real-world problem. The modelling of fuzzy uncertainty inherent to the perception of human experts in evaluating the parameters of input and output variables of food security is very important in modelling the decision-making process. The fuzziness and reliability are associated with each other during evaluation of uncertainties related to the fuzzy values of input-output variables. The Z-number proposed by Zadeh allows modelling such kind of uncertainty [18]. As we know, the concepts in the human brain for evaluating natural events are vague and imprecise. The boundaries of the parameters low, average, or high level used for evaluating input-output variables cereal yield, cereal production, economic growth, and security level are not exactly defined. Therefore, the assertion that follows from them also becomes vague. The usage of Z-numbers allows estimation of input and output relationships using the concept of fuzzy information and partial reliability. Zadeh proposed Z-number to represent uncertain information using constraint and reliability information. Z-number as represented uses an ordered pair of fuzzy numbers (A, B). Here, A is the fuzzy restriction, and B is the reliability used for valuation of A [18]. Z-number allows us to use uncertainty measures to estimate the ambiguity associated with the estimating food risk security. The theoretical background of Z-number-based systems has been considered in [18, 19]. Z-number-based fuzzy set using fuzzy constraints and reliability factors describe the human knowledge. Based on the concept of Z-number, different research works have been done for solving different problems. Aliev et al. [20] and Kang et al. [21] used Z-number to solve multicriteria decision-making (MCDM). Kang et al. [21] solved the decision-making problem by converting Z-number to crisp numbers using the approach given in [22]. Xiao et al. [23] converted Z-number to type-2 fuzzy set to solve multicriteria decision-making. Here, by calculating the centroid type-2 fuzzy sets, it is converted to the crisp numbers for decision-making. Azadeh et al. [24] applied Z-number to solve the AHP. The research paper uses the approach described in [22] for problem-solving. Lorkowski et al. [25] considered a fair price approach for decision-making under interval, set-valued, fuzzy, and Z-number-based uncertainty. Abiyev et al. [26] proposed a Z-number-based interpolative reasoning for control of the dynamic plant. Z-numbers are used to solve the problems related to computing with words [27] and decision-making [28] problems. In [29–31], using discrete fuzzy numbers, a new vision of Z-numbers is described. An aggregation method is presented for group decision-making problems. In [32], a Z-number version of the data envelopment analysis (DEA) model is transformed into possible linear programming and then by applying an alternative α-cut approach, a crisp linear programming model is obtained. The proposed model is used for a portfolio selection problem. In [33], a Z-number-based risk-minimization negotiation model is designed for a transmission company and a power purchaser under incomplete information. Here, Z-number is used to estimate the uncertainty distribution of the annual electricity transmission, and the benefit and the loss measured by the conditional value at risk is analyzed. Aliev et al. [34] presented an approximate reasoning with Z-rules on a basis of linear interpolation for evaluation of the job satisfaction and educational achievement of the students. Wang et al. [35] represented experts' opinions by Z-numbers and presented a method based on the Choquet integral for MCDM problems using linguistic Z-numbers. Wu et al. [36] used experts' opinions for representing a method for ranking Z-numbers. Based on this ranking method, the transformation of Z-numbers into basic probability assignments is presented. Two experiments on risk analysis and medical diagnosis illustrate the efficiency of the proposed methodology. In [37], the total utility of Z-number is applied to determine the ordering of Z-numbers. The approach is used in the application of MCDM under uncertain environments, and it is implemented using Gaussian and triangular

Z-numbers. Jiang et al. [38] presented a method for ranking generalized fuzzy numbers. Here, the weight of centroid points, the spreads of fuzzy numbers, and degrees of fuzziness are taken into consideration. In this paper, the principles of ranking Z-numbers are considered. Using intuitive vectorial centroid, Ku Khalif et al. [39] presented a hybrid fuzzy MCDM model for Z-numbers. The presented model is applied to staff recruitment. Aliev et al. [40] presented the ranking of Z-numbers using a human-like fundamental approach. The considered approach is based on two main ideas: optimality degrees of Z-numbers and adjusting the obtained degrees using a degree of pessimism. Kang et al. [41] presented the stable strategies analysis based on the utility of Z-number to simulate the human's competition in the evolutionary games.

The use of Z-numbers in the solution of different problems needs to use efficient inference mechanism. The interpolative reasoning is proposed by Koczy and Hirota [42, 43] for the sparse fuzzy rule base. The method can provide the logical interpretation of modus ponens. The proposed method is based on distance measure and used for approximating fuzzy reasoning [42, 43]. Johanyák et al. [44] showed that different distance measures can be used in the fuzzy sets. But these distance measures do not give full information about the shape of the membership functions. Kozcy et al. [43] mentioned that the Kozcy measure based on α-cuts of two fuzzy sets can be used to solve this problem. Using α-cuts, Koczy and Hirota proposed a fuzzy interpolative approximate reasoning [43]. Kovács et al. [45] and Hsiao et al. [46] used the interpolative reasoning for the solution of different practical problems. The novelty of this paper is emphasized in the following stages: using α-cuts and Koczy and Hirota interpolative reasoning, the design of Z-number-based interpolative reasoning mechanism is presented; the development of the Z-number-based fuzzy inference system for estimation of food security risk level is proposed.

The paper is organized as follows: Section 2 presents the fuzzy rule interpolation. Section 3 describes the design Z-number-based fuzzy inference system. Section 4 presents the application of the developed Z-number-based interpolative reasoning mechanism for estimation of food security risk level. Section 5 presents the conclusions.

2. Fuzzy Rule Interpolation

Fuzzy sets are the extension of classical sets whose elements have degrees of membership. Let's give definition of fuzzy sets and fuzzy triangular membership functions that will be used in this paper.

Definition 1. Assume that $U = \{x_1, x_2, \ldots, x_n\}$ is the universe of discourse. A fuzzy set A in $U\,(A \subset U)$ is defined as a set of ordered pairs $\{(x_i, \mu_A(x_i))\}$. Here, $x_i \in U$, $\mu_A{:}U \to [0,1]$ is the membership function of A, and $\mu_A(x) \in [0,1]$ is the degree of membership of x in A [1].

Definition 2. A triangular fuzzy number A is a fuzzy subset defined by (a_1, a_2, a_3) triplet on R, where the membership function can be determined as

$$\mu_A(x) = \begin{cases} 0, & -\infty \le x \le a_1 \\ \dfrac{x - a_1}{a_2 - a_1}, & a_1 \le x \le a_2 \\ \dfrac{a_2 - x}{a_3 - a_2}, & a_2 \le x \le a_3 \\ 0, & a_3 \le x \le \infty. \end{cases} \tag{1}$$

The fuzzy triangular membership function is often used for the numeric representation of linguistic terms. In the paper, triangular membership functions are used for the representation of the constraint and reliability of Z-number.

Fuzzy rule-based systems include fuzzy sets in antecedent and consequent parts of the rules and define relationships between input and output of the system. Using fuzzy rule base and input data, the fuzzy inference is implemented for making output decision. Different fuzzy reasoning mechanisms are suggested to process uncertain information. These mechanisms are mainly based on the analogy and similarity, compositional inference rule, interpolation, and the concept of distance. The processing capabilities, speed, and complexity of these inference mechanisms are important issues.

This paper considers the inference mechanism based on fuzzy interpolative reasoning proposed by Koczy and Hirota [42, 43]. The fuzzy interpolative reasoning can efficiently be used for processing sparse rule base and requires the satisfaction of the following conditions: the used fuzzy sets should be continuous, convex, and normal, with bounded support.

The interpolative reasoning is based on distance measure between two fuzzy sets. In the paper, the α-cut is used to measure distance between fuzzy sets. Let's consider two fuzzy sets: A_1 and A_2. α-cut of fuzzy sets A_1 and A_2 will be denoted as A_1^α and A_2^α. We say that fuzzy sets A_1 is less than A_2, that is, $A_1 < A_2$, if

$$\inf\{A_1^\alpha\} < \inf\{A_2^\alpha\} \text{ and } \sup\{A_1^\alpha\} < \sup\{A_2^\alpha\}, \tag{2}$$

where $\inf\{A_1^\alpha\}$ and $\inf\{A_2^\alpha\}$ are infimum of A_1 and A_2, and $\sup\{A_1^\alpha\}$ and $\sup\{A_2^\alpha\}$ are supremum of A_1 and A_2 (Figure 1).

Let us consider interpolative reasoning mechanism. Consider the fuzzy controller based on single-input and single-output fuzzy rule base. Assume that, in the result of observation, the current input variable X is A^*. Let us determine the value of output Y of the rule base system corresponding to A^*. Assume that A^* is less than fuzzy sets A_1 and more than the fuzzy set A_2, that is, $A_1 < A^* < A_2$ and also $B_1 < B_2$. Let us determine the system output for the input A^*. The problem can be expressed as follows:

$$\begin{aligned} &\text{Given that } X \text{ is } A^* \\ &\text{Rules: if } X \text{ is } A_1, \text{ then } Y \text{ is } B_1 \\ &\text{If } X \text{ is } A_2, \text{ then } Y \text{ is } B_2 \\ &\text{Find conclusion } Y = B^*? \end{aligned} \tag{3}$$

In [42, 43] it was shown that

$$\frac{d(A^*, A_1)}{d(A^*, A_2)} = \frac{d(B^*, B_1)}{d(B^*, B_2)}, \tag{4}$$

where $d(*)$ is the distance between two fuzzy sets.

Koczy and Hirota [42, 43] determined the final fuzzy sets using distance based on α cuts. Using α, cut lower d^L and upper d^U distances between two fuzzy sets can be calculated as follows:

$$\begin{aligned}
d_{ij}^L &= d^L\left(A_{ij}^\alpha, X_j^\alpha\right) = d\left(\inf\left\{A_{ij}^\alpha\right\},\ \inf\left\{X_j^\alpha\right\}\right) \\
&= \inf\left\{A_{ij}^\alpha\right\} - \inf\left\{X_j^\alpha\right\}, \\
d_{ij}^U &= d^U\left(A_{ij}^\alpha, X_j^\alpha\right) = d\left(\sup\left\{A_{ij}^\alpha\right\},\ \sup\left\{X_j^\alpha\right\}\right) \\
&= \sup\left\{A_{ij}^\alpha\right\} - \sup\left\{X_j^\alpha\right\}, \\
d_j^L &= d^L\left(B_j^\alpha, Y_j^\alpha\right) = d\left(\inf\left\{B_j^\alpha\right\},\ \inf\left\{Y_j^\alpha\right\}\right) \\
&= \inf\left\{B_j^\alpha\right\} - \inf\left\{Y_j^\alpha\right\}, \\
d_j^U &= d^U\left(B_j^\alpha, Y_j^\alpha\right) = d\left(\sup\left\{B_j^\alpha\right\},\ \sup\left\{Y_j^\alpha\right\}\right) \\
&= \sup\left\{B_j^\alpha\right\} - \sup\left\{Y_j^\alpha\right\}.
\end{aligned} \tag{5}$$

In (5), the Hamming or Euclidean formula can be used to measure the distances. Using distance measure, Koczy and Hirota [42, 43] proposed interpolated conclusion for the output of 2k rules.

$$\begin{aligned}
\inf\left\{B_j^{*\alpha}\right\} &= \frac{\sum_{i=1}^{2k}\left(1/d^L\left(A_{ij}^\alpha, A_i^{*\alpha}\right)\right)\inf\left\{B_j^\alpha\right\}}{\sum_{i=1}^{2k} 1/d^L\left(A_{ij}^\alpha, A_i^{*\alpha}\right)}, \\
\sup\left\{B_j^{*\alpha}\right\} &= \frac{\sum_{i=1}^{2k}\left(1/d^U\left(A_{ij}^\alpha, A_i^{*\alpha}\right)\right)\sup\left\{B_j^\alpha\right\}}{\sum_{i=1}^{2k} 1/d^U\left(A_{ij}^\alpha, A_i^{*\alpha}\right)},
\end{aligned} \tag{6}$$

where $B_j^{*\alpha} = [\inf\{B_j^{*\alpha}\}, \sup\{B_j^{*\alpha}\}]$. In Section 3, we consider the use of interpolative reasoning for finding the output of the Z-number fuzzy system.

3. Z-Number-Based Fuzzy Inference System

Definition 3. A Z-number is an ordered pair of fuzzy numbers denoted as $Z = (A, B)$, where the first component A is a restriction on the values of fuzzy variable X, and the second component B is a measure of reliability for the A component [18].

In Figure 2, the triangular membership functions are used to represent the components of Z-number. Here, the A will represent the fuzzy value of the variable, and B will represent the degree of truth or reliability measure or probability of A; for example, X is A that is referred to as a possibilitic restriction, that is,

$$B(X)\colon X \text{ is } A \rightarrow \text{Poss}(X = u) = \mu_A(x), \tag{7}$$

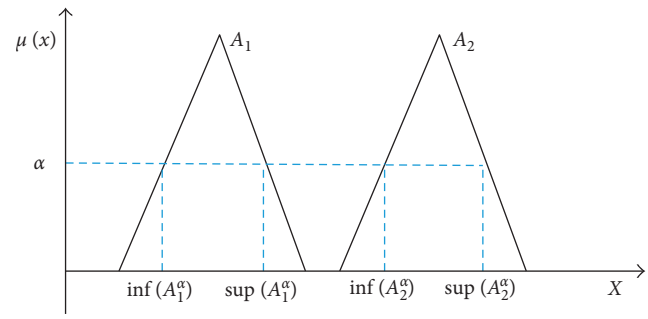

Figure 1: α-cut of membership function: infimum and supremum.

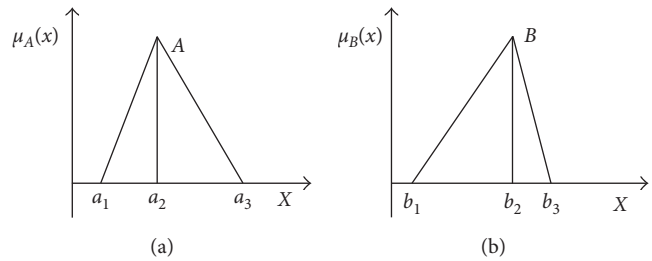

Figure 2: Z-number, $Z = (A, B)$. (a) Restriction; (b) reliability.

where $\mu_A(x)$ is membership function of A which may be viewed as constraint-associated with $B(X)$, and u is a generic value of X. $\mu_A(x)$ is membership degree to which u satisfies.

Z-number is one of an effective way for representation of uncertain information. For example, let us consider the prediction of cereal yield in Turkey. As it is known this parameter depends on number of factors. If we say "This year the cereal yield will be something higher" is considered as a more possible event with the reliability of 100%. This event can be expressed more exactly as "This year the cereal yield will be something higher, very likely."

As shown the event can be described by Z-number, that is, "X is $Z = (A, B)$." Here, the fuzzy variable X is "cereal yield." The values of the X variable is described by the pair of (A, B). A is the constraint which is "something higher," and B is the reliability of A which is "very likely."

Definition 4. Let $Z_1 = (A_1, R_1)$ and $Z_2 = (A_2, R_2)$ be the two Z-numbers. The α-level distance between Z-numbers Z_1 and Z_2 is defined as

$$\begin{aligned}
D\left(Z_1^\alpha, Z_2^\alpha\right) = &\left|a_1^{L\alpha} - a_2^{L\alpha}\right| + \left|a_1^{R\alpha} - a_2^{R\alpha}\right| \\
&+ \left|r_1^{L\alpha} - r_2^{L\alpha}\right| + \left|r_1^{R\alpha} - r_2^{R\alpha}\right|,
\end{aligned} \tag{8}$$

where $a_1^{L\alpha}, a_2^{L\alpha}, r_1^{L\alpha}, r_2^{L\alpha}, a_1^{R\alpha}, a_2^{R\alpha}, r_1^{R\alpha}$, and $r_2^{R\alpha}$ are α-levels of fuzzy sets of left and right sides of fuzzy triangular numbers A_1, R_1, A_2, and R_2 correspondingly [19].

Let us consider the development of Z-number-based interpolative reasoning. Assume that multi-input-single-output fuzzy If-Then rules have the following form:

$$\begin{aligned}
&\text{if } x_1 \text{ is } (A_{11}, R_{11}), \ldots, \text{ and } x_m \text{ is } (A_{1m}, R_{1m}),\\
&\text{then } y \text{ is } (B_1, R_1),\\
&\text{if } x_1 \text{ is } (A_{21}, R_{21}), \ldots, \text{ and } x_m \text{ is } (A_{2m}, R_{2m}),\\
&\text{then } y \text{ is } (B_2, R_2),\\
&\text{if } x_1 \text{ is } (A_{n1}, R_{n1}), \ldots, \text{ and } x_m \text{ is } (A_{nm}, \mathrm{R}_{nm}),\\
&\text{then } y \text{ is } (B_n, R_n),
\end{aligned} \tag{9}$$

where A_{ij} and R_{ij} are fuzzy constraint and reliability parameters, respectively, and x_i and y are inputs and output of the system. Here, $j = 1, \ldots, m$, and m is a number of input signals; $i = 1, \ldots, n$, and n is a number of rules.

Let us use fuzzy interpolation given in above Section 2 for the Z-number-based fuzzy rules of Figure 2. Assume those input signals are entered to the input of the fuzzy system. At first iteration, the distance between the incoming input signal and fuzzy values of corresponding variables in the antecedent part of the rules will be computed. Computation of distance is carried out using α-cut with the use of the Hamming or Euclidean distances. In the antecedent part, the distances will be calculated for each constraint and reliability variables separately.

Formula (5) is used to find lower and upper distances of membership function in the antecedent part of the rules. α cuts are applied to find the lower and upper distances:

$$\begin{aligned}
d_{ij}\left(A_{ij}^{\alpha}, X_j^{\alpha}\right) = d_{ij}^{\alpha} &= \left|A_{ij}^{\alpha} - X_j^{\alpha}\right|,\\
d_i^{\alpha} &= \sum_j^m d_{ij}\left(A_{ij}^{\alpha}, X_j^{\alpha}\right),
\end{aligned} \tag{10}$$

where $d_j(A_{ij}^{\alpha}, X_j^{\alpha})$ is a distance between two fuzzy sets defined for the ith rule; $j = 1, \ldots, m$, and m is a number of input signals; $i = 1, \ldots, n$, and n is a number of rules. The distances are represented by lower and upper distances, that is, $d_{ij}^{\alpha}(A_{ij}^{\alpha}, X_i^{\alpha}) = \{d_{ij}^{L}(A_{ij}^{\alpha}, X_i^{\alpha}),\ d_{ij}^{U}(A_{ij}^{\alpha}, X_i^{\alpha})\}$ and $d_i^{\alpha} = \{d_i^{L}, d_i^{U}\}$. For special case, $\alpha = \{0, 1\}$. Formula (10) is applied for constraint A and reliability R values separately. The total distance will be the sum of two distances:

$$d_i^{\alpha} = dc_i^{\alpha} + dr_i^{\alpha}, \tag{11}$$

where dc_i^{α} and dr_i^{α} are distances computed by (8) for the constraint and reliability parameters correspondingly. d_i^{α} is the distance computed for the ith rule. Each of these distances is characterized by two left and right values:

$$\begin{aligned}
dc_i^{\alpha} &= \left(dc_i^{L}, dc_i^{U}\right),\\
dr_i^{\alpha} &= \left(dr_i^{L}, dr_i^{U}\right),\\
d_i^{\alpha} &= \left(d_i^{L}, d_i^{U}\right).
\end{aligned} \tag{12}$$

The output fuzzy set in the output of the rule is calculated as

$$\begin{aligned}
Y^{\alpha} &= \frac{\sum_{i=1}^{n}\left(1/d_i^{\alpha}\right)B_{Y_i}^{\alpha}}{1/\sum_{i=1}^{n} 1/d_i^{\alpha}},\\
R_Y^{\alpha} &= \frac{\sum_{i=1}^{n}\left(1/d_i^{\alpha}\right)R_{Y_i}^{\alpha}}{1/\sum_{i=1}^{n} 1/d_i^{\alpha}}.
\end{aligned} \tag{13}$$

Formula (13) is applied for finding constraint and reliability values of output fuzzy sets. If we combine (10) and (13), then we can get the following equations:

$$\begin{aligned}
Y^{\alpha} &= \frac{\sum_{i=1}^{n}\left(1/\sum_j^m d_L\left(A_{X_{i,j}}^{\alpha}, X_j^{\alpha}\right)\right)B_{Y,i}^{\alpha}}{1/\left(\sum_{i=1}^{n}\left(1/\sum_j^m d_L\left(A_{X_{i,j}}^{\alpha}, X_j^{\alpha}\right)\right)\right)},\\
R_Y^{\alpha} &= \frac{\sum_{i=1}^{n}\left(1/\sum_j^m d_L\left(A_{X_{i,j}}^{\alpha}, X_j^{\alpha}\right)\right)R_{Y,i}^{\alpha}}{1/\left(\sum_{i=1}^{n}\left(1/\sum_j^m d_L\left(A_{X_{i,j}}^{\alpha}, X_j^{\alpha}\right)\right)\right)}.
\end{aligned} \tag{14}$$

Using (10)–(13), we can derive the output Z fuzzy signal of the system. It is needed to note that, for finding the output signal, we use B_{Yi}, for finding the reliability, we use R_{Yi} variables in the right side of (13) or (14). After getting output signals, conversion of Z-number to the crisp number is performed. The formula for calculating the mean of two fuzzy numbers is used for this purpose. The formula $Y = ((Y_l + 4 * Y_m + Y_r)/6) * ((R_{Y_l} + 4 * R_{Y_m} + R_{Y_r})/6)$ is used to derive the crisp value of the output signal [21, 22]. Here, Y is the fuzzy output value, and R_Y is the reliability. It is needed to mention that in the antecedent and consequent parts of the rule base, we are using the triangular type fuzzy sets for the input and output parameters. If we are considering $\alpha = 0$ and $\alpha = 1$ levels, then we can get left Y_l, middle Y_m, and right Y_r values of the output signal. Left (Y_l, R_{Y_l}) and right (Y_r, R_{Y_r}) values are corresponding to the $\alpha = 0$ level, the middle (Y_m, R_{Y_m}) value is corresponding to the $\alpha = 1$ level. At first, the left and right values corresponding to the $\alpha = 0$ level are determined [26], and then the highest value of the triangle corresponding to the $\alpha = 1$ level is determined [46]. These values are used to find output triangular membership function.

4. Simulation Studies

The solution of food security risk level starts with the design of appropriate knowledge base. The knowledge base consists of the If-Then rules demonstrating the association between input parameters and output signal. During designing KB, the input and output variables are described by linguistic values. In this paper, Z-number-based fuzzy If-Then rules described above are applied for the determination of the food security risk level in Turkey.

The inputs for the system are Turkey cereal yield, Turkey cereal production, and Turkey economic growth. The parameter cereal yield is defined as the monthly farm gate cereal output measured in metric tons, cereal production is defined as cereals that are processed into food products measured in kg per hectare, and economic growth is defined as the percentage of gross domestic product (GDP) in percentage. The values of the parameters are represented using Z-numbers and used for determination of the food security risk level. The decision-making process is conducted with the Z-number-based interpolative inference engine described in Section 3.

The forms of these linguistic values are taken in a triangle form. Each of them is represented by triangle membership functions and has the range. During designing these triangle

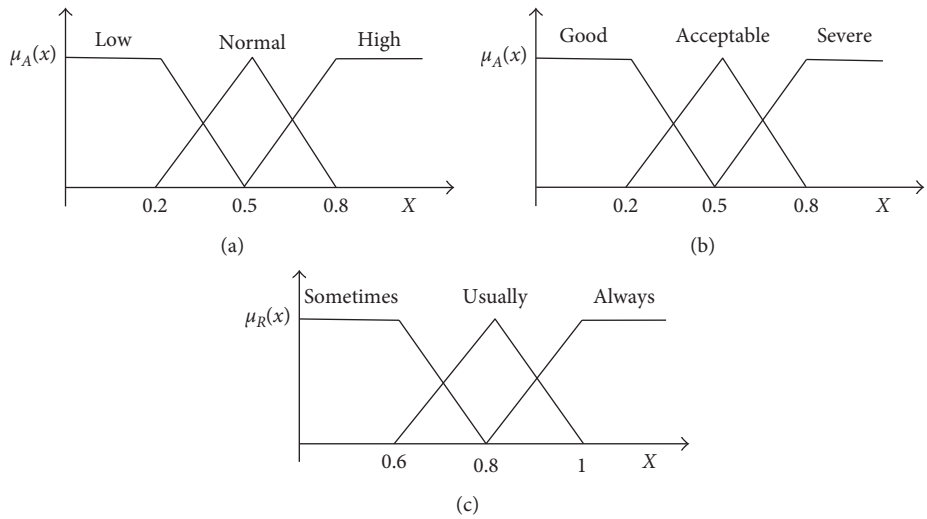

Figure 3: Membership functions used for (a) cereal yield, cereal production, economic growth, (b) risk level, and (c) reliability.

membership functions for the linguistic values, it is necessary to determine the universe of discourse and number of linguistic values for each input parameter. In this paper, the number of membership functions is taken equal to 5. The linguistic values for the input and output parameters are denoted as NL (negative large), NS (negative small), Z (zero), PS (positive small), and PL (positive large). For simplicity, we scale the range of variables between 0 and 1. The membership functions used to describe input and output variables are shown in Figure 3.

After defining membership functions for each parameter, the construction of fuzzy If-Then rules is performed. The antecedent part of rule base includes three parameters: cereal yield, cereal production, and economic growth, and the consequent part includes one parameter-security risk level. The construction of the If-Then rules is performed using experts' knowledge. At the same time, the statistical data from the World Bank online database for Turkey are used in construction of rule base. In the design of rule bases, the three linguistic terms are used for each input and output parameters. The values of these linguistic terms are represented using Z-numbers. Each fuzzy value is represented by two parameters: constraint and reliability parameters. The membership high yield, normal yield, and low yield functions of constraint and reliability parameters are accepted as triangle. The high yield, normal yield, and low yield linguistic terms are used to represent the cereal yield. The high, normal, and low linguistic terms are used to represent the economic growth parameter. The cereal production is also represented by high, normal, and low linguistic terms. Using the change interval (the ranges) of statistical data, the numeric values of linguistic terms of input parameters are determined. The statistical data are taken from the web page of "Global Food Security Index." Maximum and minimum values of statistical data for the given parameter are used to determine the ranges of input parameters. All the values of parameters are scaled in the interval 0–1.

The If-Then fuzzy rule base of security risk level is constructed using experts' opinions. The development of rule base is implemented using all possible combination of the values of the input parameters. In the rule base, the values of the parameters are represented using triangular membership functions. After determination of the input fuzzy values, using the opinion of the experts, the construction of the If-Then rules has been performed. The design of If-Then rules is carried out using all possible combination of the values of the input parameters. The input-output associations are defined using expert knowledge. In the rule base, for the input and output constraint and reliability parameters, the values of the

Table 1: Fuzzy knowledge base.

Cereal yield	Cereal production	Economic growth	Risk level
High	High	High	Good
High	High	Normal	Good
High	High	Low	Acceptable
High	Normal	High	Acceptable
High	Normal	Normal	Good
High	Normal	Low	Acceptable
High	Low	High	Severe
High	Low	Normal	Severe
High	Low	Low	Severe
Normal	High	High	Severe
Normal	High	Normal	Acceptable
Normal	High	Low	Acceptable
Normal	Normal	High	Acceptable
Normal	Normal	Normal	Good
Normal	Normal	Low	Good
Normal	Low	High	Severe
Normal	Low	Normal	Acceptable
Normal	Low	Low	Severe
Low	High	High	Severe
Low	High	Normal	Severe
Low	High	Low	Severe
Low	Normal	High	Severe
Low	Normal	Normal	Acceptable
Low	Normal	Low	Acceptable
Low	Low	High	Severe
Low	Low	Normal	Severe
Low	Low	Low	Acceptable

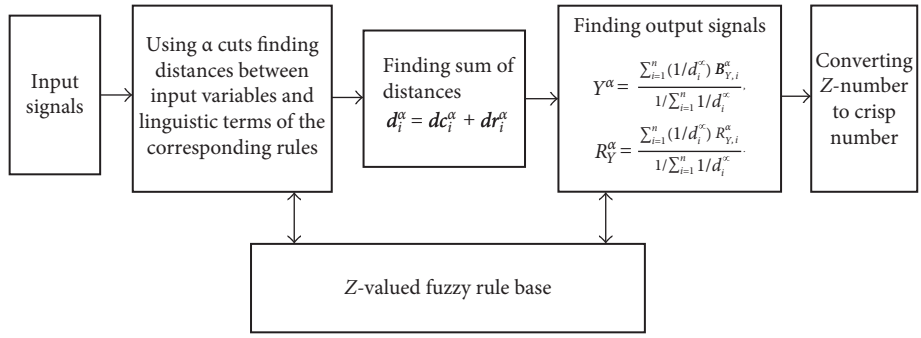

Figure 4: Architecture of the Z-number-based system.

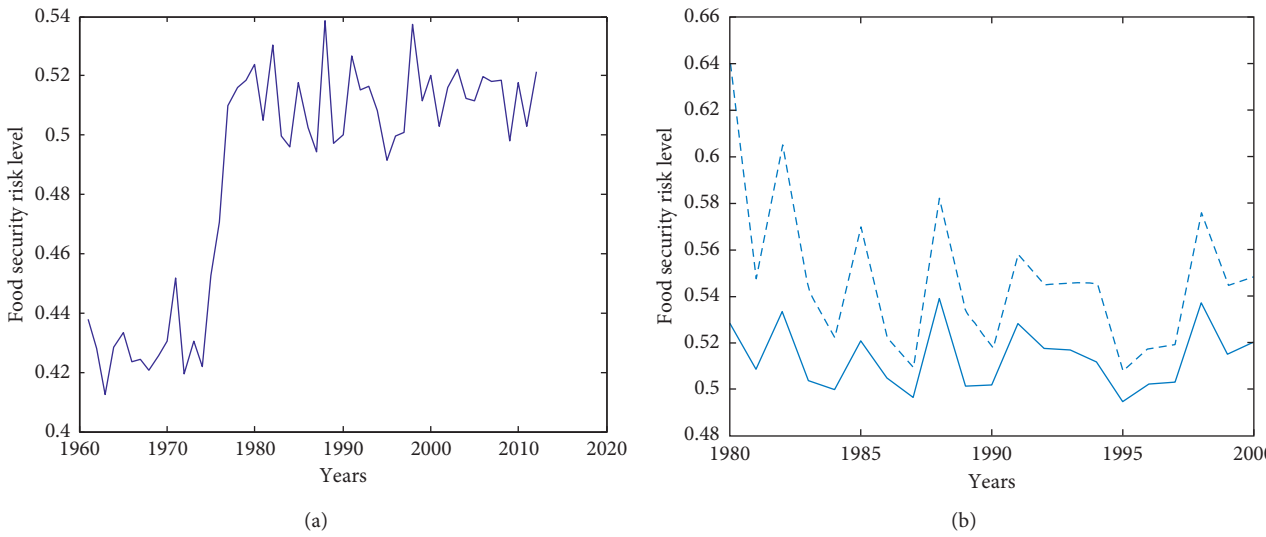

Figure 5: The plots of food security risk level: (a) Z-number-based fuzzy system for the years 1961–2013, (b) comparisons of simulation results of the conventional type-1 fuzzy system (dashed line), and Z-number-based fuzzy system (solid line) for the years 1980–2000.

parameters are represented using triangular interval membership functions. Figure 3 describes the membership functions used for input and output variables.

The output of food security risk level variable is represented using three fuzzy sets: good, acceptable, and severe. The knowledge base representing the input output association is given in Table 1.

The relationship between input and output variables is represented by fuzzy rule base. It was found that high cereal yield and high cereal production lead to the high level of food security. It can be observed that even economic growth is low. But if the quantities of cereal yield and cereal production are low, there may not be enough food.

After the development of fuzzy rule base, the design of the fuzzy inference system is implemented using interpolative reasoning mechanism. The designed system will use fuzzy rule base and statistical input data and make a conclusion about risk level. Using fuzzy rule interpolation, the structure of the inference system is designed (Figure 4). In the structure, the distances between incoming input signals and the membership functions of the antecedent part of If-Then rules are determined. Here, using α cuts distances between input variables, and corresponding linguistic terms of the rules are determined. The same procedure is repeated for reliability parameters also. After this operation, sum of distances are determined. Using sum of distances and active rules based on (13), the fuzzy outputs are determined. For each constraint and reliability parameters, the outputs are determined. Based on the mean formula of two fuzzy numbers, the crisp output of the system is determined.

Using real annual historical data between 1961 and 2013 years in Turkey [47] and the Z-number-based fuzzy system, the risk level of food security is determined. The real data are fed into the system input. Figure 5(a) depicts the changes of food security risk level obtained by the Z-number-based fuzzy system for the statistical data between 1961 and 2013 year. As shown every year, the assessment of risk level of food security is changed according to the change of the values of cereal yield, cereal production, and economic growth. The risk level of food security is acceptable between 0 and 0.8. As an example, if we consider 1988 and 1998 years, then we can observe that when Turkey cereal yield was 1742.108 kg/ha, cereal production was 23498600 tons, and economic growth was 0.29%, the value of food security risk level was 0.54 for 1988. When cereal yield was 2075.197 kg/ha, cereal production was 28885720 tons, and economic growth

was −3.36%, the value of food security risk level was 0.535 for 1998. Both of them are related to the food security risk level conditions "Acceptable" with the membership ≈0.87.

The system demonstrates that the high values of cereal yield and cereal production will lead to the high security level if economic growth is low or normal. In these cases, most people will pay low prices for their food, and consumer will expend fewer resources to get the best food; then, in this situation, the expensive high-quality food may be wasted. But when economic growth is high and cereal yield and cereal production are low, food security will be low and there may not be enough food for everyone.

In the second simulation, for comparison purpose, using the rule base given in Table 1, the design of a conventional type-1 fuzzy system with the interpolative reasoning is carried out. For clear comparison, we plotted the changes in the risk levels for the conventional type-1 fuzzy systems and Z-number-based system for the years 1980–2000 that is given in Figure 5(b). Here, the solid line is the security risk level obtained by the Z-number-based system, and the dashed line is the security risk level obtained by the conventional type-1 fuzzy system. As shown, there are some differences between the simulation results. If we consider for the years 1988 and 1998, the values of food security risk level for the conventional type-1 fuzzy system were obtained as 0.62 and 0.56 correspondingly. The results are between the conditions of "Acceptable" and "Severe." The first result with the membership 0.6 is related to the condition of "Acceptable," and the membership 0.4 is related to the condition of "Severe." The second one with the membership 0.8 is related to the condition of "Acceptable," and the membership 0.2 is related to the condition "Severe." As shown from the graphics, the reliability degrees for the linguistic values in rule base affect the food security risk level. If we try to get the Z-number-based system output using reliability degree of each linguistic value equal to one, then we can get the same results for the food security risk level as in the conventional type-1 fuzzy system. True assessment shows that the value of reliability degree allows getting more accurate value for the food security risk level.

From the simulation, it was obvious that the food security risk level is acceptable in all the time period for both systems. As shown from the figure, the results given by the Z-number-based fuzzy system is more reliable than another one.

5. Conclusions

In this paper, the design of Z-number-based fuzzy system is implemented for determining food security risk level. Based on Koczy–Hirota interpolative reasoning, the Z-number-based inference engine is designed. The designed system is applied for measuring the food security risk level in Turkey. Using input parameters cereal yield, cereal production, and economic growth, the output food security risk level is evaluated and the rule base is developed. The relationships between input and output variables are expressed using Z-valued numbers. The designed system is tested using statistical data taken from the Global Security Index database for 1961 and 2013. Based on the Z-number-based inference system, rule base and statistical data, the prediction of the risk level of food security of Turkey is performed. The obtained results demonstrate the applicability of the designed system in real life.

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

Immobilization of *Bifidobacterium infantis* Cells in Selected Hydrogels as a Method of Increasing Their Survival in Fermented Milkless Beverages

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The aim of the study was to examine whether immobilization of *Bifidobacterium infantis* inside hydrogels could prolong their survival in fermented milkless beverages. The starter culture *Streptococcus thermophilus* was used to obtain fermented nonmilk beverages: oat, oat-banana, and oat-peach. The biota of beverages were supplemented with *Bifidobacterium infantis* cells, free and immobilized, in three types of spherical hydrogel particles: microcapsules with a liquid and gelled core, microbeads of 0.5 mm diameter, and beads of 2.5 mm diameter. As a carrier material, low-methoxylated pectin and alginate were used. Microbeads and microcapsules were obtained using extrusion techniques: vibrating and electrostatic method, and beads were obtained using manual method with a syringe. A significantly lower decrease in the count of cells immobilized in hydrogels compared to free cells was observed during storage of fermented beverages at 4°C. Microcapsules were more effective compared to microbeads in terms of bacterial cells protection. The observed effect was better for higher biopolymer concentration. The highest survival of the strain was noted in cells immobilized in low-methoxylated pectin beads of 2.5 mm diameter. Supplementing the biota of fermented beverages with microencapsulated bacteria did not negatively affect the overall sensory quality of beverages during the entire storage period.

1. Introduction

Recently, a constant increase is being observed in the consumption of milk products, recognized as the best carriers of probiotic bacteria [1, 2]. At the same time, there is a growing population with allergies or intolerance to milk proteins, thus creating the need to introduce milkless beverages of plant origin, which contain bacterial strains beneficial for human body and can also be considered to be a functional food, onto the market. The most common probiotics introduced into food are *Bifidobacterium* and *Lactobacillus* spp. [3–5].

Keeping the probiotic bacterial cells, especially *Bifidobacterium*, alive in fermented products during storage is difficult and very challenging for the food industry, considering the unfavourable physicochemical characteristics of the environment, especially the low pH and the presence of oxygen [1, 6]. One of the possible solutions to this problem is the immobilization or encapsulation of bacterial cells [1, 7, 8] using appropriate carrier materials. However, the effectiveness of applying encapsulated bacteria depends on many factors, such as the type and concentration of the material used for immobilization, the encapsulation/immobilization method, the size of the capsules obtained, and the

physicochemical characteristics of the environment [9]. The material most commonly used to immobilize bacterial cells is naturally occurring polysaccharide sodium alginate, mainly because of its safety, its good gelling properties (temperature and pH), and biocompatibility [4, 10]. Low-methoxylated pectin is also a nontoxic polymer which, like alginate, forms gels with divalent metal ions, that is, calcium [11, 12]. Pectin is a cheap and easily available compound of plant origin. Some literature data suggest that pectin provides better protection for bacterial cells than alginate [13], which may result due to its lesser sensitivity to chemical interactions of the environment [14]. Moreover, also other research studies show that pectin from fruits may also be used as an effective prebiotic [15], thereby positively affecting human health.

Bacterial cells are immobilized in hydrogels using different methods and equipment, that is, the electrostatic and vibrating technique, which enables us to obtain, in a one-step procedure, uniform, spherical microcapsules or microbeads with a diameter of 0.15–2.0 mm (vibrating technique) or 0.2–3.0 mm (electrostatic technique), with a small size distribution [16–19]. Both methods belong to the extrusion techniques, which provide the most favourable conditions for the survival of encapsulated microorganisms, and they are relatively easy to apply [8, 20–22]. It should be noted that the size of the capsules or beads may affect not only the number of surviving bacteria but also the physicochemical and sensorial properties of food products [23].

There is a lack of reports on the supplementation of fermented milkless beverages with immobilized probiotic bacteria cell. Therefore, the aim of this work was to examine, if immobilization of *B. infantis* ATCC15697 KKP2029p cells in selected hydrogels can increase their survival in such environments during refrigerated storage. The impact of immobilized bacteria on sensory quality of beverages was also the scope of the work.

2. Materials and Methods

2.1. Biological Material. The biological material consisted of two strains from the IBPRS Culture Collection of Industrial Microorganisms: *Bifidobacterium infantis* ATCC15697 KKP2029p and *Streptococcus thermophilus* T_KM_3 KKP2030p. The strains were stored at −70°C using Viabank (Abtek Biologicals Ltd., Liverpool, GB), propagated on a suitable medium: *S. thermophilus* T_KM_3 KKP2030p on modified LAB (lactic acid bacteria) medium without agar [24] and *B. infantis* ATCC15697 KKP2029p on modified Garche medium [25], and incubated at 37°C for 24 h. The cells were harvested by centrifugation at 6000 rpm for 10 min at 4°C and then washed twice and suspended in a small volume of saline. The bacterial suspension was used for inoculation immediately after its preparation.

2.2. Preparation of Fermentation Matrices. The matrices for fermentation were prepared by mixing the dry components, introducing the mixture into the amount of water (Table 1) given in the recipe and gelatinization of the oat (*Avena sativa* L.) flour (Młyny Stoisław, Poland) suspension at 85°C for 5 min while stirring. Banana (*Musa paradisiaca* L.) puree (Maspex, Poland) was introduced into the cereal matrix. The matrices were sterilized for 10 min at 118°C, cooled to the fermentation temperature (37°C), and subsequently inoculated with *S. thermophilus* T_KM_3 KKP2030p at approximately 6 log cfu·g^{-1}.

Table 1: Composition of the fermentation matrices.

Ingredient (%)	Oat-banana	Oat	Oat-peach
Banana puree	10.0	—	—
Peach filling	—	—	30.0
Oat flour	4.0	4.0	4.0
Sucrose	4.5	4.5	4.5
Water		Up to 100	

The oat-banana and oat beverages were obtained as a result of the fermentation process. For further experiments, a portion of the oat beverage was combined with peach (*Prunus persica* (L.) Batsch) yoghurt filling with large fruit pieces (PRPH Kandy, Poland) in the amount of 30%. The biota of all three beverages was subsequently supplemented with free and immobilized cells of *B. infantis* ATCC15697 of approximately 9 log cfu·g^{-1}, and their survival during storage at 4°C was determined.

2.3. Immobilization of B. infantis ATCC15697 KKP2029p Cells in Biopolymers. The immobilization of *B. infantis* ATCC15697 KKP2029p cells was conducted in aseptic conditions, using sterile solutions and vibrating and electrostatic techniques in calcium alginate and low-methoxylated pectinate gel, using disposable syringes. The process conditions for each technique were chosen experimentally.

An encapsulator B-395 Pro (Büchi, Switzerland) was used in the vibrating technique following the Büchi [19] procedure to produce microbeads and microcapsules. Spherical microbeads were obtained with a freshly prepared suspension of bacterial cells in saline combined with a 1.5% sodium alginate solution (low viscosity; Büchi, Switzerland) with a viscosity of 98 mPa·s, in a 1 : 5 proportion. A 300 μm diameter single nozzle was used. The one-step procedure of microcapsule production with a liquid core was carried out using a set of concentric nozzles, comprising an inner nozzle of 150 μm diameter and an outer nozzle of 300 μm diameter. The liquid core of the microcapsules was a bacterial suspension in saline, and the shell was a 1.5% sodium alginate gel.

Cell encapsulation using the electrostatic technique [16, 17] was conducted with an electrostatic droplet generator—developed in the Nalecz Institute of Biocybernetics and Biomedical Engineering, PAS, Warsaw. This apparatus enables the formation of uniform microbeads as well as obtaining, in a one-step procedure, uniform microcapsules, in which the hydrogel core containing the bacterial cells is surrounded by an additional polysaccharide shell. A sterile 2% sodium alginate (medium viscosity; Sigma-Aldrich Co., USA) solution in saline was used with viscosity differing depending on the method of sterilization: viscosity of 170 mPa·s for sterilization of alginate solution at 121°C for 15 min or viscosity of 240 mPa·s for sterilization of alginate

powder at 121°C for 15 min. To produce microbeads, a freshly prepared bacterial suspension in saline was combined (1 : 10) with a sodium alginate solution (2%) with a viscosity of 240 mPa·s, and then the cell suspension in alginate was forced through a single nozzle with an outer/inner diameter of 330/600 μm. To produce microcapsules, a head consisting of two concentric nozzles with the following diameters: inner nozzle 330/600 μm and outer nozzle 680/1000 μm, was used. The core liquid (pressed through the inner nozzle) was sodium alginate solution (viscosity of 240 mPa·s) containing bacterial cells, and the shell was pure alginate solution with a viscosity of 170 mPa·s.

Alginate microbeads and microcapsules, obtained using the vibrating and electrostatic technique, were gently stirred in a bath with gellifying solution (0.1 M aqueous solution of $CaCl_2$; POCH, Polska) for 10 min, filtered through a stainless steel sieve (0.4 mm) and washed with sterile water and used immediately after preparation.

The immobilization of bacterial cells in a 5% aqueous solution of low-methoxylated pectin (Aglupectin LA-S20; Hortimex, Poland) and a viscosity of 180 mPa·s and in a 2% sodium alginate solution with a viscosity of 240 mPa·s was carried out by combining 1 g of freshly prepared bacterial suspension with 10 g of pectin solution or 10 g of alginate solution. The suspension thus prepared was manually pressed using a 20 mL syringe through a medical needle with a diameter of 0.2/0.4 mm (BD Microlance 27 G 3/6″; BD Dicardit I) into the gellifying solution, 0.1 M $CaCl_2$. The suspension was left for 1/2 h with gentle stirring until hardening of the pectin or alginate droplets, filtered through a stainless steel sieve (0.4 mm) and washed with water.

2.4. Examination of the Appearance and Size of Alginate Microbeads/Microcapsules and Pectin Beads. The appearance of the alginate microbeads and microcapsules obtained using the vibrating technique was assessed using an Olympus CX40 optical microscope, at 60x magnification. Photographs were taken with an Olympus C5060 digital camera. The size of the alginate microgels before storage in fermented beverages was determined by the laser diffraction method, using a Mastersizer 3000 (Malvern Instruments Ltd., GB) and was expressed as the average sphere diameter equal in terms of surface D (3,2) and in terms of volume D (4,3) [10, 23]. However, measurements of diameters of alginate microgels after 28 days of storage were made using the scale from the images.

The alginate microbeads and microcapsules produced using the electrostatic technique and pectin and alginate beads were characterized using an Olympus CKX 41 inverted optical microscope with CellSens software for making measurements and taking pictures. Magnifications of 20x, 40x, and 100x were used.

2.5. Determination of the Bacterial Count. Counts of *S. thermophilus* T_KM_3 KKP2030p in fermented beverages were determined using the plate count method according to ISO 7889:2003 [26], spread on selective M-17-agar (BTL, Łódź, Poland) medium. The count of free *B. infantis* ATCC15697 KKP2029p cells was determined according to ISO 29981:2010 [27], spread on TOS-MUP agar (Merck, Darmstadt, Germany).

To determine the count of immobilized *B. infantis* ATCC15697 KKP2029p, the hydrogels with bacterial cells were drained off in a sieve and washed out with sterile deionised water. Next, 1 g of capsules or beads was placed, in order to gel liquefaction, in 29 mL of 0.1 M sodium citrate (Sigma-Aldrich Co., St. Louis, MO, USA) and was vortexed for 5 min. The bacteria released from the hydrogels were serially diluted with Ringers liquid and then spread on agar medium, similarly to the free cells population. The cell count was expressed as cfu·g^{-1}.

2.6. Determination of pH. The pH value measurement was carried out using the potentiometric method and a Mettler Toledo MP235 pH meter (Switzerland).

2.7. QDA Sensory Profile of the Beverages. The sensory profile and overall sensory quality of the beverages were evaluated in a sensory laboratory, designed in accordance with PN-ISO 8589:1988 [28], by a trained [29] 6-person panel in one-week intervals, over a one-month period. The evaluation was carried out using quantitative descriptive analysis (QDA) [30], that is, setting the descriptors of the sensory notes perceived as the most important for texture, odour, and taste attributes. The intensity of particular notes, as well as the overall sensory quality (SQ) of the assessed samples, was quantified using a 10-unit nonstructured linear scale with defined border restraints denominated as "very weak/none" to "very strong" [31].

2.8. Statistical Analysis. A statistical analysis of the results was carried out using a one-way ANOVA and Duncan test ($P \leq 0.05$) (Statistica 7.1 StatSoft).

3. Results and Discussion

3.1. The Appearance and Size of Alginate Microbeads/Microcapsules and Pectin Beads Containing B. infantis ATCC15697 KKP2029p Cells. Producing microcapsules and microbeads of narrow size distribution is the greatest challenge of all the microencapsulation technologies [17]. When the process parameters are incorrectly set, the undesirable satellite fraction of very small beads may be formed, and there may also be a nonuniform distribution of the cell suspension inside the capsules. However, despite the immobilization method used, the authors managed to achieve alginate microbeads and microcapsules uniform in terms of their size and shape, preserving their size and shape during the whole storage period. Examples of the microgels obtained using the vibrating technique are shown in Figure 1 and using the electrostatic technique in Figures 2 and 3.

Laser diffraction measurements confirmed that the initial average diameters of the spherical alginate microgels obtained using the vibrating technique were uniform (Table 2) and slightly lower for microbeads: 499 μm (D (3,2)) and

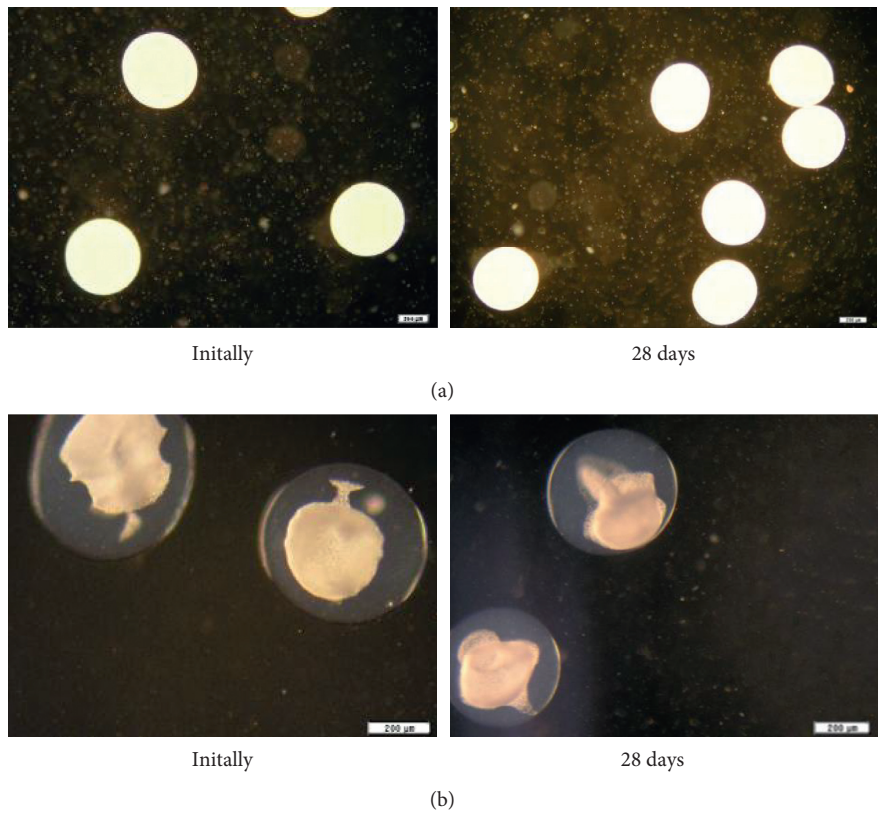

Figure 1: Optical microscopic images (reflected light) of the spherical microgels made with 2% calcium alginate and obtained using the vibrating technique (60x): microbeads (a) (initially and after 28 days) and microcapsules (b) with a liquid core (initially and after 28 days).

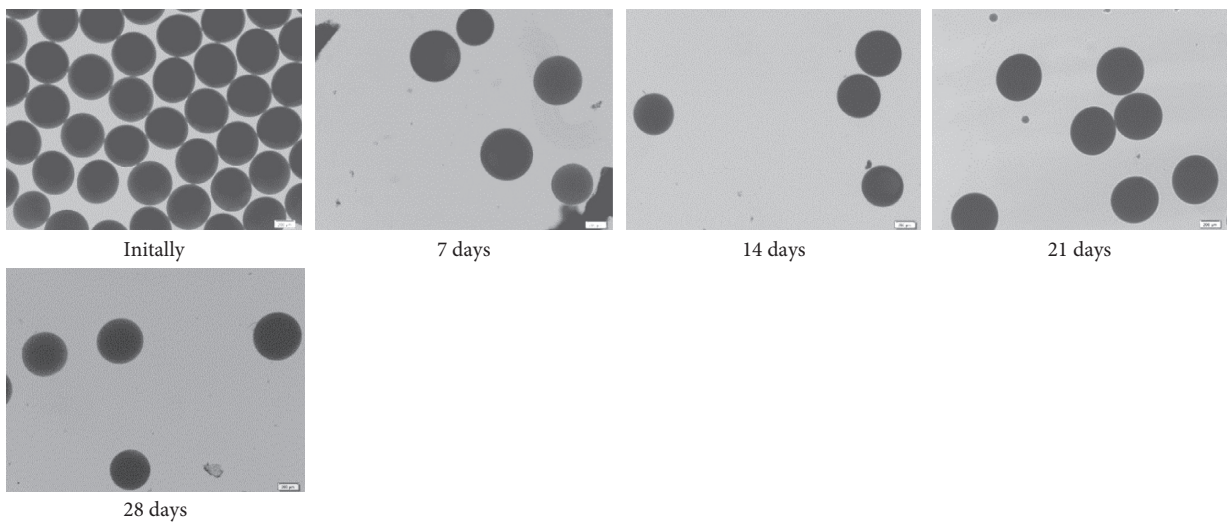

Figure 2: Optical microscopic images (40x): microbeads made with 2% calcium alginate containing immobilized bacterial cells directly after formation using the electrostatic technique and during 4-week storage at a temperature of 4°C in fermented beverage. The irregular fragments visible in the images are the beverage ingredients.

511 μm (D (4,3)) for microbeads and 528 and 568 μm for microcapsules with a liquid core. These diameters did not change significantly after 28 days of storage and amounted to 500 and 520 μm for microbeads and microcapsules, respectively.

The diameters of the alginate microgels obtained using the electrostatic technique were also uniform (Table 3). Moreover, these diameters did not change significantly ($P \leq 0.05$) during storage. Initially, the average diameter of the microbeads was 527 ± 12 μm and after 28 days of storage 517 ± 31 μm (a 2% reduction). The initial outer diameter of the microcapsules was 551 ± 38 μm, and the inner (core) diameter was 424 ± 34 μm. These values decreased slightly during storage at 4°C and after 4 weeks amounted to 520 ±

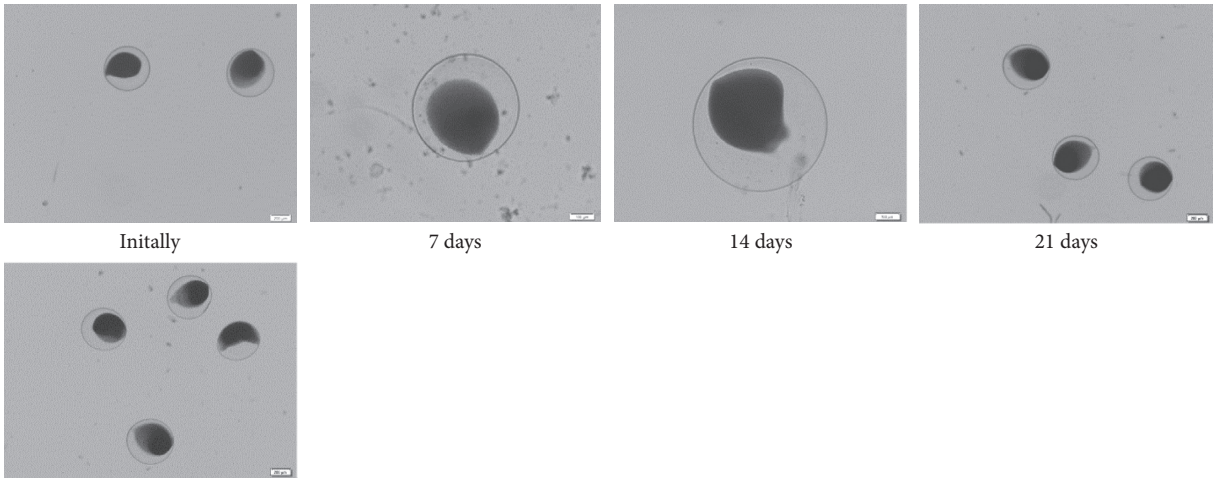

Figure 3: Optical microscopic images (40x: initially and after 21 and 28 days; 100x: after 7 and 14 days) of microcapsules made with 2% calcium alginate with bacteria in an alginate core directly after formation using the electrostatic technique and during 4-week storage at a temperature of 4°C in fermented beverage.

Table 2: Changes in the diameter of the microbeads and microcapsules made with 1.5% calcium alginate using the vibrating technique during refrigerated storage (4°C) of fermented beverage.

Alginate microgels	Diameter (μm)		
	Initially*		28** days
	D (3,2)	D (4,3)	
Microbeads	499 ± 17	511 ± 23	500 ± 42
Microcapsules with liquid core	528 ± 14	568 ± 13	520 ± 44

*The size of the alginate microgels was determined by the laser diffraction method. The values are the mean of three replicates ± standard deviation. **The size of the alginate microgels was measured using the scale from the images. The values are the mean of 20 replicates ± standard deviation.

58 μm and 516 ± 40 μm, respectively (a reduction of 6 and 2%, resp.).

As in the case of alginate microgels, the appearance of pectin and alginate beads obtained using a syringe (Figure 4) as well as their size (Table 4) did not change significantly during the whole storage period. The initial average diameter of pectin beads was 2.47 ± 0.09 mm, and after 28 days, it was 2.56 ± 0.11 mm (an increase of 3.6%); in the case of alginate beads, their initial average diameter was 2.36 ± 0.07 mm, and after 28 days, it was 2.30 ± 0.14 mm (a reduction of 2.5%).

3.2. Acidity and Count of the Population of S. thermophilus T_KM_3 KKP2030p Starter in Beverages. The characteristics of oat and banana-oat fermented beverages, obtained as a result of the lactic acid fermentation of cereal and fruit-cereal matrices using *S. thermophilus* T_KM_3 KKP2030p as a starter culture, are shown in Table 5. The acidity of the beverages was pH 4.22 and 4.26, and the count of the starter culture in the beverage biota was 7.60 and 7.65 log cfu·g^{-1}, respectively. Oat beverage with peach filling achieved a pH of 4.07 and a bacteria count of 7.47 log cfu·g^{-1}.

Acidity, expressed as a pH value, was stable during the whole period of beverage storage (Table 5), which can be attributed to the fact that the streptococci of *S. thermophilus* spp. do not exhibit any postacidification activity [32]. The pH values did not change after supplementation with *B. infantis* ATCC15697 KKP2029p. Similar observations were made by Sohail et al. [33], who supplemented the biota of fermented peach dessert with immobilized and free cells of *L. rhamnosus* GG; these samples retained the same pH during storage (4°C) as the nonsupplemented sample. According to Quero et al. [34], the decrease in the pH of fermented milk beverages after completion of fermentation led to generally unfavourable sensory changes. As shown in Table 5, introducing free and immobilized *B. infantis* ATCC15697 KKP2029p cells into the beverage did not cause a substantial decrease in the count of *S. thermophilus* T_KM_3 KKP2030p starter (only of approximately 0.5 log) during storage, similar to the results obtained in nonsupplemented beverages (a decrease of approximately 0.5 log).

3.3. Survival of Free and Immobilized B. infantis ATCC15697 KKP2029p Cells in Fermented Beverages during Refrigerated Storage. For studying the survival of free and immobilized cells of *B. infantis* ATCC15697 KKP2029p during storage, first the oat-banana beverage was used with 0.5 mm microbeads and microcapsules (with liquid and gelled core) obtained with 1.5% and 2% sodium alginate (Table 6). The reduction of living bacterial cells count was significantly lower in all alginate microgels, compared to free cells. Nonencapsulated cells did not survive 14 days of storage. However, bacterial

Table 3: Changes in the diameter of the microbeads and microcapsules made with 2% calcium alginate using the electrostatic technique during refrigerated storage (4°C) of fermented beverage.

Alginate microgels		Diameter (μm)				
		Initially	7 days	14 days	21 days	28 days
Microbeads		527 ± 12	487 ± 53	505 ± 32	490 ± 39	517 ± 31
Microcapsules with alginate core	Outer diameter	551 ± 38	502 ± 39	507 ± 30	510 ± 31	520 ± 58
	Inner (core) diameter	424 ± 34	—	—	407 ± 23	416 ± 40

The values are the mean of 40 replicates ± standard deviation.

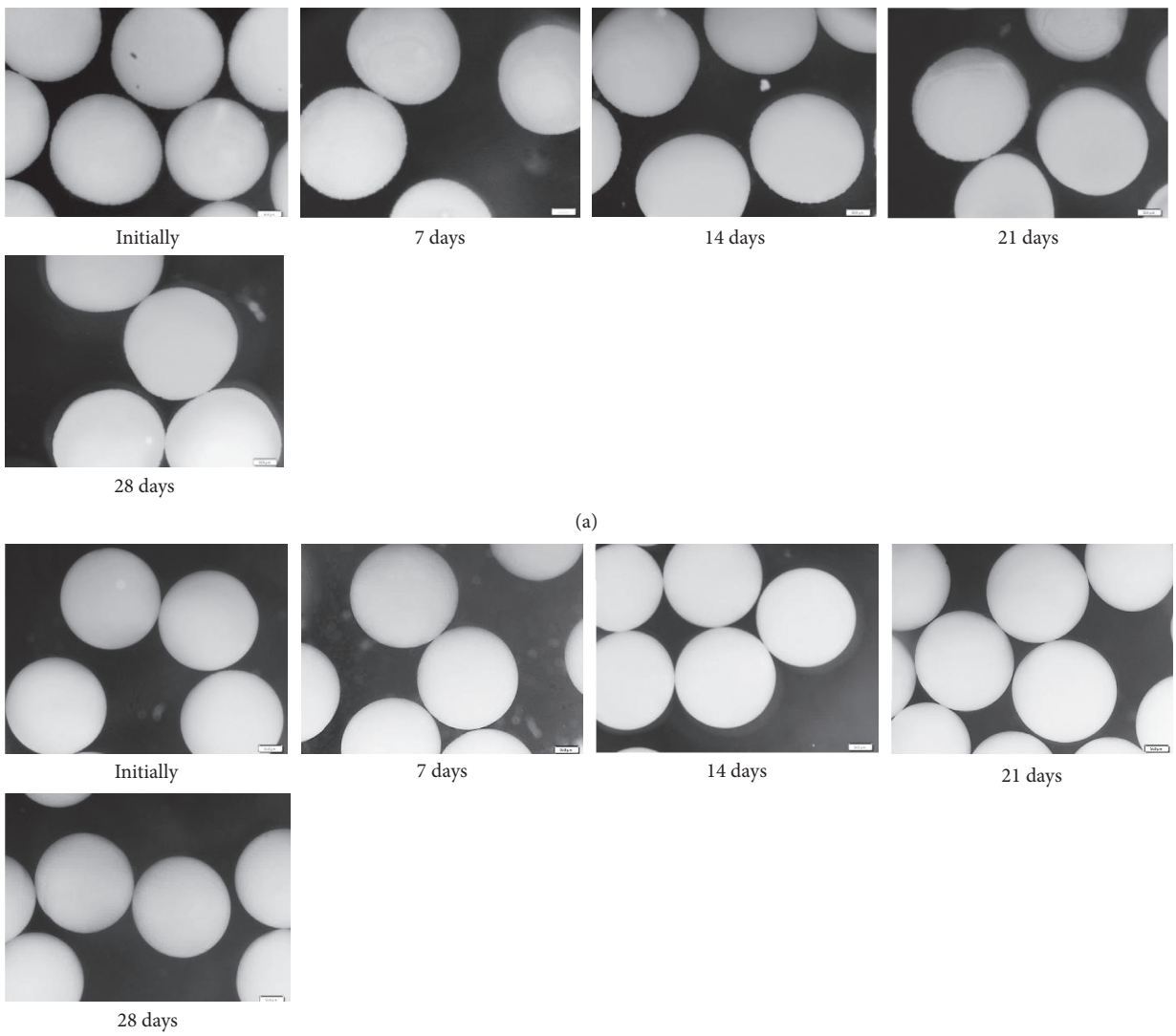

Figure 4: Optical microscopic images (20x reflected light) of beads made with (a) 5% low-methoxylated pectin and (b) 2% alginate-containing immobilized bacterial cells directly after formation with a syringe and after 4-week storage at temperature 4°C in fermented beverage.

cells immobilized in microbeads and microcapsules made with 1.5% calcium alginate completely disappeared after 21 days while the cells protected by microbeads and microcapsules made with 2% calcium alginate died after 28 days of storage. There was also a significant ($P \leq 0.05$) difference in the survival of bacterial cells immobilized in 1.5% and 2% calcium alginate between microcapsules and microbeads. The count reduction of living cells in microcapsules was approximately 1 log lower compared to the reduction in microbeads up to 21 days of storage. This finding was confirmed by other researchers: Bakuła et al. [35] reported that capsules better sustain a bacterial population count, more efficiently preventing their migration into the environment. Mokarram et al. [36] also found that bifidobacteria were better

Table 4: Changes in the size of beads made with 5% low-methoxylated pectin and 2% alginate during refrigerated storage (4°C) in fermented beverage.

Beads	Diameter (mm)				
	Initially	7 days	14 days	21 days	28 days
Beads with 5% pectin	2.47 ± 0.09	2.52 ± 0.08	2.51 ± 0.11	—	2.56 ± 0.11
Beads with 2% alginate	2.36 ± 0.07	2.34 ± 0.10	2.34 ± 0.09	2.36 ± 0.07	2.30 ± 0.14

The values are the mean of 20 replicates ± standard deviation.

Table 5: The acidity and *S. thermophilus* T_KM_3 KKP2030p count of fermented beverages without supplementation and of beverages supplemented with free and immobilized *B. infantis* ATCC15697 KKP2029p during storage at 4°C.

Beverage	Storage time (days)	Acidity (pH)			*S. thermophilus* T_KM_3 KKP2030p (cfu·g^{-1})		
		A	B	C	A	B	C*
Oat	0	4.22	4.22	4.22	7.65 ± 0.08	7.65 ± 0.08	7.65 ± 0.08
	28	4.19	4.20	4.18	7.19 ± 0.17	7.16 ± 0.15	7.14 ± 0.16
Oat-banana	0	4.26	4.26	4.26	7.60 ± 0.06	7.60 ± 0.06	7.60 ± 0.06
	28	4.20	4.24	4.25	7.18 ± 0.24	7.06 ± 0.22	7.12 ± 0.12
Oat with peach filling	0	4.07	4.08	4.07	7.47 ± 0.10	7.47 ± 0.10	7.47 ± 0.10
	28	4.04	4.09	4.05	6.88 ± 0.19	6.86 ± 0.28	6.94 ± 0.06

A: beverage without supplementation with *B. infantis* ATCC15697 KKP2029p; B: beverage supplemented with free cells of *B. infantis* ATCC15697 KKP2029p; C*: -beverage supplemented with immobilized cells of *B. infantis* ATCC15697 KKP2029p; oat and oat with peach filling: supplementation with beads made with 5% pectin; oat-banana: supplementation with microbeads made with 1.5% alginate.

Table 6: Survival of free *B. infantis* ATCC15697 KKP2029p cells immobilized in calcium alginate and pectinate in fermented beverages during storage at 4°C.

B. infantis ATCC15697	Initial cell count (log cfu·g^{-1})	Cell count reduction (log)				
		4 days	7 days	14 days	21 days	28 days
Oat-banana beverage						
Free	9.28 ± 0.27	3.75 ± 0.24^{d}	5.12 ± 0.13^{d}	9.28 ± 0.27^{h}	—	—
Microcapsules[(1)] with 1.5% alginate	9.21 ± 0.26	2.24 ± 0.25^{c}	3.67 ± 0.23^{b}	6.20 ± 0.24^{a}	9.21 ± 0.26	—
Microbeads with 1.5% alginate	9.71 ± 0.20	3.24 ± 0.29^{a}	4.82 ± 0.06^{g}	7.02 ± 0.11^{g}	9.71 ± 0.20	—
Microcapsules[(2)] with 2% alginate	8.75 ± 0.18	1.47 ± 0.20^{e}	2.53 ± 0.19^{e}	5.10 ± 0.23^{f}	>7	8.75 ± 0.18
Microbeads with 2% alginate	9.14 ± 0.35	2.05 ± 0.38^{c}	3.16 ± 0.24^{f}	5.91 ± 0.46^{a}	>8	9.14 ± 0.35
Oat beverage						
Free	9.41 ± 0.10	4.06 ± 0.17^{d}	5.40 ± 0.37^{d}	9.41 ± 0.10^{h}	—	—
Beads with 5% pectin	9.77 ± 0.15	0.93 ± 0.08^{b}	2.11 ± 0.07^{a}	3.32 ± 0.29^{b}	5.08 ± 0.24^{a}	>7
Beads with 2% alginate	9.62 ± 0.04	1.09 ± 0.07^{b}	2.19 ± 0.12^{a}	3.76 ± 0.10^{c}	5.60 ± 0.16^{b}	>8
Oat-peach beverage						
Free	9.41 ± 0.10	5.15 ± 0.32^{f}	6.88 ± 0.29^{h}	9.41 ± 0.10^{h}	—	-
Beads with 5% pectin	9.77 ± 0.15	2.88 ± 0.15^{a}	3.93 ± 0.24^{bc}	4.28 ± 0.26^{d}	5.96 ± 0.10^{c}	>8
Beads with 2% alginate	9.62 ± 0.04	2.92 ± 0.10^{a}	4.08 ± 0.19^{c}	4.70 ± 0.11^{e}	6.51 ± 0.16^{d}	9.62 ± 0.04

The values are the mean of two replicates. The values with the same superscript letter in columns do not differ significantly ($P \leq 0.05$). [(1)]Microcapsules with liquid core. [(2)]Microcapsules with alginate core.

protected in microcapsules with a double alginate shell, which provides anaerobic conditions more favourable for microorganisms than microbeads. Taking into account the results presented in this study, it can be concluded that *B. infantis* ATCC15697 cells immobilized in 2% calcium alginate microcapsules with an alginate core remain alive in fermented oat-banana beverages at a significantly higher level compared to cells encapsulated/immobilized in other microgels (a reduction of 2.52 log).

The important objective in this research was to ensure the good sensory acceptance of milkless beverages enriched with immobilized cells as well as high bacterial count during storage. Literature data show that the bigger the gel particles, the better the cell protection they provide [8, 23]; but, at the same time, they are more sensed during consumption, what is not accepted by the consumers. The sensory scores of oat-banana beverage with 0.5 mm microcapsules were high (Figure 5), but preliminary supplementation of this beverage with microbeads of high diameter (ca. 2.5 mm) was not positively perceived by the panel. Therefore, at the next stage of experiments, the oat beverage with big particles of peach filling (of pH similar to oat-banana beverage, Table 5) was used to make microbeads not detectable in the mouth. For comparison, the survival of bacteria was also checked in oat beverage without fruit filling.

On this stage of research, we decided to check the protective action of pectin on cells of *B. infantis* ATCC15697 KKP2029p strain compared to 2.5 mm alginate beads.

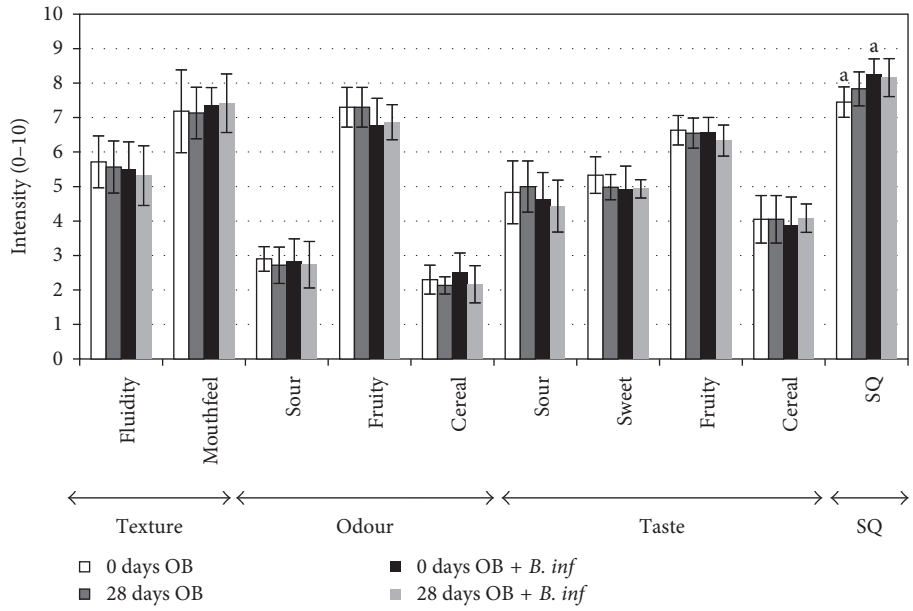

Figure 5: Sensory profile of oat-banana beverage with biotum supplemented with *B. infantis* ATCC15697 KKP28p cells immobilized in calcium alginate (OBan. + *B. inf.*) compared with beverage without supplementation (OBan.) after storage at 4°C (SQ is the overall sensory quality). (a) Differences of mean (of two independent experiments) values of descriptors significant at $P \leq 0.05$.

Studying the survival of *B. infantis* ATCC15697 KKP2029p in oat-peach beverage and oat beverage during refrigerated storage (Table 6), a significantly lower reduction of immobilized cells compared with free cells was observed in beverages. Nonencapsulated cells did not survive 14 days of storage, while the lowest reduction of immobilized bacteria in this period was observed in the pectin beads introduced into the oat beverage (a decrease of 3.32 log) and slightly higher in the pectin beads introduced into the oat-peach beverage (a decrease of 4.28 log). A similar trend was observed in alginate beads, although the reduction of cell count was significantly higher compared to pectin beads, both in oat (a decrease of 3.76 log) and in oat with peach-filling beverage (a decrease of 4.70 log). The pH of all the beverages was similar (4.22–4.07) (Table 5), so it can be stated that factors other than acidity influenced the viability of bacteria, such as the phenolic compounds present in fruit [2, 37].

Although after 4 and 7 days of beverage storage there was no significant difference in the decrease of bacterial count in pectin and alginate beads, the protective effect of alginate weakened in the following terms.

Nualkaekul et al. [13] studied the viability of *Bifidobacterium longum* in cranberry juice (pH 2.77) and in pomegranate juice (pH 3.16) at a temperature of 4°C. They observed the complete inactivation of free cells and cells immobilized in 4% low-methoxylated pectin and in 4% sodium alginate with a low viscosity already after 7 days of storage in cranberry juice, while in the pomegranate juice the count of free cells decreased by more than 4 log after 7 days, and after 14 days, they were completely inactivated, with only a slight decrease in the immobilized cells. After 14 days of storage in pomegranate juice, the count of cells immobilized in 4% pectin decreased by approximately 1 log, while the cells immobilized in 4% sodium alginate by approximately 1.5 log. Phoem et al. [38] noted a decrease in the *B. longum* free cell count of more than 6 log after 15 days of storage in pineapple juice (pH 3.8), and 1 log in the case of cells encapsulated in 2% sodium alginate with *Eleutherine americana* extract.

Maintaining the population of probiotic bacteria in food at a desirably high level is not an easy task. Even in fermented milk products, which provide the best protection for bacteria because of their buffer properties, the survival of bacteria still remains a challenge for the industry, requiring new technological solutions [2, 39]. Hansen et al. [40] studied the viability of two bifidobacteria strains during storage in milk with a 2% fat content. They observed that the count of free *B. longum* Bb-46 cells decreased by 3 log after 14 days, and cells immobilized in calcium alginate (microbeads of 20 μm diameter) by 1.5 log, unlike in the case of the *B. lactis* Bb-12 strain, whose cell count, both free and immobilized, increased slightly during that period.

Taking into account all results presented in Table 6, it can be said that the survival of immobilized *B. infantis* ATCC15697 KKP2029p cells was significantly higher in all milkless fermented beverages compared to free cells. The best protective effect was observed in beads made with 5% low-methoxylated pectin; hence, it can be stated that pectin is a promising biopolymer for immobilization of bacterial strains in this kind of beverages. Further research is needed to optimize the biopolymer concentration and the size of microgel particles.

3.4. Assessment of the Sensory Profile of Fermented Beverages with Biota Supplemented with Immobilized B. infantis ATCC15697 KKP2029p Cells. A sensory assessment was carried out on two fermented beverages: oat-banana with microcapsules made with 1.5% calcium alginate of approximately 0.5 mm diameter containing *B. infantis* ATCC15697 KKP2029p cells

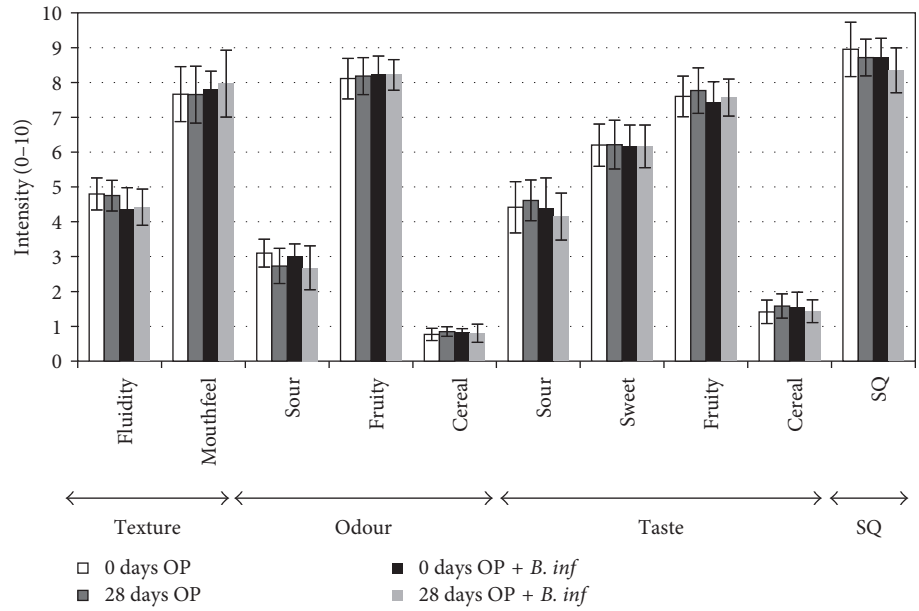

Figure 6: Sensory profile of fermented oat-peach beverage with biotum supplemented with *B. infantis* ATCC15697 cells immobilized in pectin beads (OP + *B. inf.*) compared with beverages without supplementation (OP) stored at 4°C. The values are the mean of two independent experiments.

and oat-peach beverage with the same strain immobilized in pectin beads of approximately 2.5 mm diameter. The aim of the evaluation was to check whether the introduction of gel microbeads or beads into the fermented cereal or fruit-cereal beverage would influence their sensory quality after 4 weeks of storage at 4°C.

The sensory profile assessment of the oat-banana beverage without biotum supplementation and with biotum supplemented with *B. infantis* ATCC15697 cells immobilized in microcapsules did not show any significant ($P \leq 0.05$) differences with regard to texture, odour, and taste during the entire storage period (Figure 5). However, the beverage with microcapsules obtained higher scores for overall sensory quality SQ (8.3 points) compared to the reference without supplementation (7.4 points), but the difference was significant ($P \leq 0.05$) only after the first storage period. Some authors suggest that the capsule size affects the product's sensory quality; that is, microcapsules (made with alginate and corn starch) of 0.3 mm diameter were considered the cause of better yoghurt smoothness compared to samples with free cells [41]. Generally, it can be stated that the addition of microcapsules of a size of approximately 0.5 mm to fermented cereal-fruit beverages did not affect the sensory assessment of the product, and microcapsules were not perceptible by the panelists.

The sensory profile of the oat-peach beverage without supplementation and with biotum supplemented with *B. infantis* ATCC15697 immobilized in 2.5 mm low-methoxylated pectin beads (Figure 6) did not show any significant ($P \leq 0.05$) differences between the samples for any storage period. Supplementing the biotum of the fermented beverage with bacterial cells immobilized in pectin beads of a diameter of approximately 2.5 mm did not affect the product's overall sensory quality (SQ). Fermented beverages received high scores in terms of SQ, both directly after preparation, 8.95–8.73 points, and after 4 weeks of storage, 8.72–8.35 points, for beverages without supplementation and beverages supplemented with *B. infantis* ATCC15697, respectively.

It should be emphasized that the results presented indicate that there were no perceptible differences in the texture attributes (fluidity and mouthfeel) between a beverage with biotum supplemented with bifidobacteria encapsulated in relatively large pectin beads and a beverage without beads. This finding has not been confirmed by other researchers [6, 42], who observed that capsules of a diameter of over 1 mm gave a sandy texture mouthfeel. This divergence and lack of differences noted herein between the sensory assessment of a beverage with 2.5 mm pectin beads and a beverage without beads probably arise from the fact that the panelists associated the pectin beads with fruit particles present in the beverage, and they were not negatively perceived.

4. Conclusions

The immobilization of *B. infantis* ATCC15697 KKP2029p cells in alginate or low-methoxylated pectin hydrogel particles significantly increased the survival rate of these strains in fermented nonmilk beverages during storage compared with free cells. The highest survival of the strain was noted in cells immobilized inside low-methoxylated pectin beads of 2.5 mm diameter. It was proved that microcapsules provided better protection to bacterial cells compared to microbeads. It was also stated that the higher the alginate concentration, the better the protection effect was observed. Supplementation of fermented beverages with immobilized bacterial

cells did not affect the sensory quality of beverages during the whole storage period.

The results of this work create a basis for further research on the technologies for the production of probiotic supplements using pectin, stable in the environment of fermented nonmilk beverages.

Conflicts of Interest

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

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