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Original paper

Research on evolution of nitrite and nitrate content regarding milk processing in scalded cheese

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Abstract

Nitrates have an insignificant toxicity. They are considered virtual toxic substance because they convert into nitrogen, which have a toxic potential well known. Milk contaminated with nitrates must be severely restricted.

Objectives of our research took into account the wholesomeness of milk to determine nitrates and nitrites. Sixty samples of raw milk (15 samples per season) were taken into consideration, to see the level of nitrites and nitrates from processed milk for consumption. To study the remanence of nitrates and nitrites in the finished product 60 samples of cheese were also analyzed.

Following the conducted research, no evidence has exceeded the maximum permissible limit for nitrates and nitrites which is max. 10 mg/ liter milk. For samples of cheese were not found values above 10 ppm that is the maximum permissible limit for nitrates and nitrites in cheese. Nitrates are concentrated in cheese 1.3 times. The content of nitrites in the cheese falls by 1.9 times, from 0.03367 mg/ l in milk to 0.01728 mg/ kg in cheese.

Keywords Residues of nitrites and nitrates, Griess method, colorimetric method with m-xylenol, cheese.

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Introduction

Nitrates and nitrites added to food can cause formation of cancerous N-nitroso compounds, whereas exposure to perchlorate is especially emphasised as an important risk factor for newborns' health. (SUNGUR [17]) Some epidemiological studies linking intake of nitrate and nitrite with gastric cancer in humans indicated a positive correlation. (SIMION & al [16]).

The main sources of contamination of milk are: water, placing fraudulent ammonium nitrate in milk and nitric acid used to remove "Stone Milk" on pasteurizers with plate. Nitrites from milk are highly toxic, especially for children. Nitrites concentrations grow 5-10 times in severe heat treatment, therefore, to feed infants and children separation techniques through semi-permeable membrane were developed. (ȚIBULCĂ [21]).

Water polluted with nitrates and nitrites can get into milk especially during cleaning operations of equipment and containers. The main source of water contamination is the ammonium nitrate used as basic chemical fertilizer in agricultural practice (SINDILAR [18], JIMBOREAN & al [11]).

Nitrates and nitrites get in animals through feed and water. Nitrogen is absorbed by plants in the form of either ammonium (NH^{4+}) or nitrate (NO^{3-}), and its accumulation is influenced by a series of factors that are depending on the species, cultivar, age and soil conditions (SIMION & al [16]). Although animals can ingests large amounts of nitrates and nitrites, organic tissues contain relatively small amounts, due to rapid metabolism and excretion in the urine. Additionally, in ruminant a large part of nitrates and nitrites is subject to changes by ruminal microflora. (BONDOC [2], COSTIN [7]). The nitrate may naturally present in milk and the level of it depends on the quality of feeding materials (i.e. water, feeds) of cows. (TOPÇU [19]).

Nitrogen content in milk is very low. According to Walker, milk content in nitrogen is between the values of 20 and 40 mg nitrate/ liter. This variation in the analytical data is determined by nature of the feed consumed. (WALKER [23]).

In preparations of milk in 2006, the Brasov area, the average content of nitrite was 0.009 g/ kg and ranged from not detected to 0.64 mg/ kg, and the average nitrate content was 0.95 mg/ kg and ranged from not detected and 12.95 mg/ kg. (CRISTEA [4]).

In some countries may be added nitrate in the milk used to prepare cheese to prevent bloating caused by coliform, but mainly to prevent latter bloating produced by some clostridia. Part of the nitrate is removed in the whey and some are converted intoharmless compounds in the maturation process. (COSTIN [6], JIMBOREAN [10]) In addition, the residual quantity decreases gradually during maturation (GUȘ [8], BONDOC [2]).

In Brazil, the use of these additives is permitted at maximum levels of 50 mg/kg. The basis of the previously validated method is the quantitative reduction of nitrate to nitrite through cadmium column and spectrophotometric determination after nitrite diazotation with sulphanilic acid/alpha-naphtol reagent. From all samples analyzed, 38 (88.37%) showed neither a nitrate nor nitrite detectable content; 5 samples (11.63%) presented nitrate, 4 of them being above the tolerated level and only one (2.33%) showed detectable nitrite, however below the permissible limit. The majority of samples did not show nitrate or nitrite at detectable levels. However, though probably safe from a toxicological point of view, the results can implicate an increased risk of bacteriological contamination. (SERAPHIM [15]). In other researches, the highest level of nitrate and nitrite was found in green cheese (17.52 mg/kg and respectively at 21.16 mg/kg) obtained from sheep milk. (TUDOR & al [20]).

Objectives of our research are to assess the level of nitrites and nitrates from processed milk for their consumption and remains detectable in the final product: cheese.

Materials and Methods

Cheese preparation

Cheeses have the primary feature of the technological process to obtain curd which, after maturation and slicing, is subjected to scalding temperature of 72-74°C (BANU [1] CODOBAN [5], COSTIN [6], ȚIBULCĂ [22], JIMBOREAN [10]).

Curd preparation involves the following: milk reception, cleaning and standardization of milk, milk pasteurization and cooling, preparation for coagulation which consists of adding calcium chloride (clotting ability of milk increases, it adds 10 to 20 g/100 liters of milk) and selected cultures of lactic bacteria (*Lactococcus lactis* ssp. *lactis*, *Streptococcus thermophilus* and *Lactobacillus casei*). *Lactococcus lactis* ssp. *lactis* is a regular agent of milk spontaneous acidification, hydrolyzing lactose into lactic acid. *Streptococcus thermophilus* produce a weak acidification and a specific flavor. *Lactobacillus casei* is a proteolytic bacteria and plays a role in cheese maturation. (BANU [1], CODOBAN [5], COSTIN [6], ȚIBULCĂ [22], JIMBOREAN [10]). Pasteurization is able to destroy essential microflora, enzymes and pathogens in milk. It should be noted that inactivation level of microorganisms depends on the amount of microorganisms, growth phase and other factors. (CIPROVICA [3]).

The coagulation of milk takes place at 32 ... 35°C, for 30-40 minutes. Processing the curd is then made, and the resulting curd maturates at 18-26°C for 6 ... 10 hours after a pH 4.8 to 5.0 is achieved in curd. Mature curd is cut into slices having a thickness of 0.3 to 0.5 cm and a width of 3-6 cm and is scalded at 72 ... 74°C for 50-60 s in brine with 10-12% salt. The slurry is allowed to air in molds, and is maturated. Storage is done at 4 ... 6°C and φ = 85-90%. (GUZUN [9]).

Experimental design

The reference method for the determination of nitrite in milk is Griess method. The reference method for nitrates is colorimetric method with meta-xylenol. This method determines total nitrogen. Nitrate content is calculated by the difference between total nitrogen and the nitrite determined by Griess method and expressed in equivalent nitrate. (JIMBOREAN & al [12], MUREŞAN [14]).

Determination of nitrate and nitrite is done through the following methods: determination of nitrate with diphenylamine (nitrates form with diphenylamine a blue colored complex; color intensity is proportional to the concentration of nitrates in the analyzed sample and is measured colorimetrically); the method for determining the indole nitrite (nitrite formed with indole in sulfuric acid medium, a pink-colored complex; the intensity of the color is proportional to the nitrite concentration in the sample to be analyzed and is measured colorimetrically). In case of dispute the method used is to reduce nitrate to nitrite in cadmium copper environment. The reagents used must be of quality for analysis or of equivalent quality. Water must be distilled or of equivalent purity, free of nitrates and nitrites. (ŢIBULCĂ [21]).

Results and Discussions

NO_3^- and NO_2^- limits for milk and measures to be taken are:

For NO_3^-:

- lack NO_3^-, but correlated with NO_2^- it is freely admitted in consumption;
- up to 10 mg/ liter is allowed in consumption without restrictions;
- from 10 mg/ liter to 100 mg/ liter is excluded from food for children under one year and is admitted for adults;
- more than 100 mg/ liter is excluded from consumption and is processed in fermented cheese products.

For NO_2^-:

- lack NO_2^- but correlated with NO_3^- is freely allowed freely in consumption;
- less than 10 mg/ liter is allowed without restrictions in consumption;
- more than 10 mg/ liter, regardless of the amount of NO_3^- is excluded from consumption and is processed in fermented chesses. (BONDOC [2])

The determined levels of nitrates and nitrites in raw milk are shown in Table 1.

Table 1. Variation of nitrates and nitrites content in raw milk

Crt. No	Season	Samples/ season	Nitrates NO_3^-, mg/1			Nitrites NO_2^-, mg/l		
			0-0.05	0.051-0.1	> 0.1	0-0.05	0.051-0.1	> 0.1
1.	A	15	3	12	-	10	5	-
2.	W	15	-	14	1	8	7	-
3.	Sp	15	4	11	-	13	2	-
4.	Su	15	1	14	-	13	2	-
TOTAL		60	8	51	1	44	16	-
%		100	13.3	85	1,7	73.3	26.7	-

legend: A/ autumn; W/ winter; Sp/ spring; Su/ summer.

Of the 60 milk samples investigated no evidence has exceeded the maximum permissible limit for nitrates and nitrites which is max. 10 mg/l milk. Nitrates had an average (±) of 0.0623 ± 0.004678 mg/l and a coefficient of variation of 58.16%. The determined values ranged between 0.001 and 0.3 mg/l. Statistically analyzing the nitrates variation, per seasons, shows the following:

- In autumn, nitrates, had an average (±) of 0.05287 ± 0.006306 mg/l and a coefficient of variation of 46.19%. The determined values ranged between 0.001 and 0.092 mg/l.
- In winter, nitrates, had an average (±) of 0.08867 ± 0.01547 mg/l and a coefficient of variation of 67.57%. The determined values ranged between 0.057 and 0.3 mg/l.
- In spring, nitrates, had an average (±) of 0.0552 ± 0.004762 mg/l and a coefficient of variation of 33.41%. The determined values ranged between 0.001 and 0.092 mg/l.
- In summer, nitrates, had an average (±) of 0.0007488 ± 0.05247 mg/l and a coefficient of variation of 5.53%. The determined values ranged from 0.05 to 0.061 mg/l.

Nitrites had an average (±) of 0.03367 ± 0.003034 mg/l and a coefficient of variation of 69.81%. The determined values ranged between 0.0 and 0.08 mg/l. Statistically analyzing the nitrites variation, per seasons, shows the following:

- In autumn, nitrites, had an average (±) 0.036 ± 0.007024 mg/l and a coefficient of variation of 75.56%. The determined values ranged between 0.01 and 0.08 mg/l.
- In winter, nitrites, had an average (±) of 0.05 ± 0.004781 mg/l and a coefficient of variation of 37.03%. The determined values ranged between 0.02 and 0.08 mg/l.

- In spring, nitrites, had an average (±) of 0.02667 ± 0.00504 mg/l and a coefficient of variation of 73.19%. The determined values ranged between 0.01 and 0.07 mg/ l.
- In summer, nitrites, had an average (±) 0.022 ± 0.004995 mg/l and a coefficient of variation of 87.94%. The determined values ranged between 0.0 and 0.06 mg/l.

In Romania it is forbidden the use of nitrite in milk and milk products. Nitrites and nitrates are not toxic in their normal concentrations in food. Exceeding certain limits, however, have serious repercussions on the human body (COSTIN [7]).

A large study whose results were presented by Şindilar (ŞINDILAR [18]) included a total of 210 milk samples, of which 95 samples of collected milk, where nitrates had limits of variation between 0 and 7.5 mg NO_3^-/l, with an average of 2.7 NO_3^-/l.

The limits of variation of nitrate and nitrite in studied cheese samples are shown in Tables 2 and 3.

As shown in table 2 values of nitrate were recorded as follows:

- 93.3% of cheese samples had values of nitrate between 0-0.1 ppm;
- 6.7% of cheese samples had values of nitrate between 0.11 to 1 ppm.

Table 2. Variation limits of *nitrates* content in pressed cheese samples investigated

Crt. No	Season	Samples	Nitrates, ppm							
			0-0.1	%	0.11-1	%	1.1-10	%	> 10	%
1.	A	15	14	93.3	1	6.7	-	-	-	-
2.	W	15	9	60	6	40	-	-	-	-
3.	Sp	15	14	93.3	1	6.7	-	-	-	-
4.	Su	15	15	100	-	-	-	-	-	-
Total		60	52	86.7	8	13.3	-	-	-	-

legend: A/ autumn; W/ winter; Sp /spring; Su/ summer

Table 3. Variation limits of *nitrites* content in pressed cheese samples investigated

Crt. No	Season	Samples	Nitrites, ppm							
			0-0.01	%	0.011-0.1	%	0.11-10	%	> 10	%
1.	A	15	8	53.3	7	46.7	-	-	-	-
2.	W	15	4	26.7	11	73.3	-	-	-	-
3.	Sp	15	8	53.3	7	46.7	-	-	-	-
4.	Su	15	8	53.3	7	46.7	-	-	-	-
Total		60	28	46.7	32	53.3	-	-	-	-

legend: A/ autumn; W/ winter; Sp /spring; Su/ summer

As shown in table 3 of nitrite values were recorded as follows:

- 46.7% of cheese samples had values of nitrite ranging from 0 to 0.01 ppm;
- 53.3% of cheese samples had values of nitrite ranging from 0.011 to 0.1 ppm.

No evidence had values > 10 ppm that is LMA for nitrates in cheese, according to Reg. EC 1881/2006.

Statistically, nitrates had an average (±) of 0.08 ± 0.005797 ppm and a coefficient of variation of 56.13%. The determined values ranged between 0.0012 and 0.36 ppm.

Statistically analyzing the nitrates variation, per seasons, shows the following:

- Infall, nitrates, had an average (±) of ± 0.004898 0.06721 ppm and a coefficient of variation of 28.22%. The determined values ranged between 0.0012 and 0.078 ppm.
- In winter, nitrates, had an average (±) of ± 0.008054 0.06873 ppm and a coefficient of variation of 45.39%. The determined values ranged between 0.0014 and 0.11 ppm.
- In spring, nitrates, had an average (±) of 0.1139 ± 0.01823 ppm and a coefficient of variation of 62.01%. The determined values ranged between 0.068 and 0.36 ppm.
- In summer, nitrates, had an average (±) of ± 0.005988 0.07021 ppm and a coefficient of variation of 33.03%. The determined values ranged between 0.0012 and 0.11 ppm.

Statistically, nitrite had an average (±) of ± 0.002279 0.01728 ppm and a coefficient of variation of 102.16%. The determined values ranged between 0.0 and 0,066 ppm.

Statistically analyzing the nitrite variation, per seasons, shows the following:

- In fall, nitrites, had an average (±) of ± 0.009867 0.003567 ppm. The determined values ranged between 0.0 and 0.04 ppm.
- In winter, nitrites, had an average (±) of 0.01867 ± 0.005257. The determined values ranged between 0.0 and 0.066 ppm.
- In spring, nitrites, had an average (±) of ± 0.004916 0.0268 ppm and a coefficient of variation of 71.04%. The determined values ranged between 0.0 and 0.05 ppm.
- In summer, nitrites, had an average (±) of ± 0.003416 0.0138 ppm and a coefficient of variation of 95.87%. The determined values ranged between 0.0 and 0.04 ppm.

No evidence had values > 10 ppm that is LMA for nitrates and nitrites in cheese.

KORÉNEKOVÁ, (2010), in one of his studies on Emmental cheeses, obtained the mean $NaNO_2$ content in untreated and in pasteurized milk of 0.2 and 0.1 $mg \cdot kg^{-1}$, respectively and the mean $NaNO_3$ content of 0.9 and 0.9 $mg \cdot kg^{-1}$ respectively. He also mentioned that the milk with nitrate added had the mean content of 81.2 $mg \cdot kg^{-1}$ $NaNO_3$. The final product had a markedly decreased content of nitrates (3.3 $mg \cdot kg^{-1}$ $NaNO_3$) and nitrites (0.2 $mg \cdot kg^{-1}$ $NaNO_2$) when compared with the values in cheese during maturation (11.3 $mg \cdot kg^{-1}$ $NaNO_3$; 0.4 $mg \cdot kg^{-1}$ $NaNO_2$). The results of his study showed that a considerable quantity of nitrates passed into the whey and that nitrates were added to the milk to prevent the blowing defect of hard cheese by micro organisms. (KORÉNEKOVÁ [13]).

Conclusions

From the 60 investigated milk samples no evidence has exceeded the maximum permissible limit for nitrates and nitrites which is max. 10 mg/l milk. Nitrates had an average (±) of 0.0623 ± 0.004678 mg/l. The determined values ranged between 0.001 and 0.3 mg/l. Nitrites had an average (±) of 0.03367 ± 0.003034 mg/l. The determined values ranged between 0.0 and 0.08 mg/l.

Following statistical analysis of the values obtained regarding nitrate for raw milk samples and cheese final product we found statistically significant differences *** ($p < 0.001$) for fall and winter samples and statistically significant * ($0.01 < p < 0.05$) for samples of spring and summer. Overall, registered statistical difference was significant * ($0.01 < p < 0.05$).

During processing nitrate concentrate into cheese 1.3 times (from 0.0623 mg/l in milk to 0.08 mg/kg in cheese).

Following statistical analysis of the values obtained regarding nitrite for raw milk samples and cheese final product we found statistically significant differences *** ($p < 0.001$) for samples taken in winter distinctively statistically significant ** ($0.001 < p < 0.01$) for the summer samples and statistically significant * ($0.01 < p < 0.05$) for samples of autumn and spring. Overall, registered statistical difference was very significant *** ($p < 0.0001$).

During processing of nitrite, content in cheese decreases by 1.9 times, from 0.03367 mg/l in milk from 0.01728 mg/kg in cheese.

References

1. BANU, C. şi VIZIREANU, CAMELIA. *Procesarea industrială a laptelui*, Editura Tehnică, Bucureşti (1998).
2. BONDOC, I. *Tehnologia şi controlul laptelui şi produselor lactate*, vol. I, Editura „Ion Ionescu de la Brad", Iaşi (2007).
3. INGA CIPROVICA, ALLA MIKELSONE. *The influence of ripening temperature on diversity of non-starter lactic acid bacteria in semi-hard cheeses*, Rom Biotechnol Lett 16 (6) Supplement (2011).
4. CRISTEA COSTACHE. *Study of the Level of Nitrates/Nitrites in Milk Products from Braşov County*, J.M.B. nr. 2, p. 28 (2011).
5. CODOBAN, JEANETA şi CODOBAN, I. *Procesarea laptelui în secţii de capacitate mică*, Editura Cetatea Doamnei, Piatra Neamţ (2006).
6. COSTIN, G.M. şi col. *Ştiinţa şi ingineria fabricării brânzeturilor*, Editura Academica, Galaţi (2003).
7. COSTIN, G. M. Alimente ecologice – Alimentele şi sănătatea, Editura Academica, Galaţi (2008).
8. GUŞ, CAMELIA. *Laptele şi produsele lactate*, Ediţia a II-a revizuită, Ed. Risoprint, Cluj-Napoca (2005).
9. GUZUN, V. şi col. *Industrializarea laptelui*, Editura Tehnică-Info, Chişinău (2001).
10. JIMBOREAN, MIRELA, ŢIBULCĂ, D. *Tehnologia de fabricare a brânzeturilor*, Editura Risoprint, Cluj-Napoca (2006).
11. MIRELA JIMBOREAN, D. ŢIBULCĂ, ADRIANA PĂUCEAN. *Researches of Nitrates Content in Raw Milk*, Buletin USA-MV Cluj-Napoca, vol. 68 (2), p. 540 (2011).
12. MIRELA JIMBOREAN, D. ŢIBULCĂ. *Seasonal Variation of Nitrates And Nitrites In Milk As Raw Material, Analele Universităţii din Craiova*, vol. XVII(LIII) (2012).
13. KORÉNEKOVÁ B, KOTTFEROVÁ J, KORÉNEK M. *The fate of added nitrate used in the manufacture of Emmental cheese*, Food Addit Contam., 17(5):373-7 (2010).
14. CRINA MUREŞAN, MARIA TOFANĂ, SONIA SOCACI, LIANA SALANŢĂ. *Content Evaluation on*

Nitrates and Nitrites in Local and Imported Vegetables, Bulletin UASVM Agriculture, 69(2), p. 510 (2012).
15. SERAPHIM KR[1], de SIQUEIRA ME. *Nitrates and Nitrites in Homemade and Industrial Cheeses commercialized in the Southern region of Minas Gerais*, Brazil, Arch Latinoam Nutr.; 50(1):87-90 (2000).
16. VASILICA SIMION, Gh. CÂMPEANU, GINA VASILE, MIHAELA ARTIMON, LUMINIŢA CATANĂ, MIOARA NEGOIŢĂ. *Nitrate and nitrite accumulation in tomatoes and derived products*, Rom Biotechnol Lett, 13 (4): 3785-3790 (2008).
17. SUNGUR Ş, ATAN MM. *Determination of Nitrate, Nitrite and Perchlorate Anions in Meat, Milk and Their Products Consumed in Hatay Region in Turkey*, Food Addit Contam Part B Surveill, 6(1):6-10 (2013).
18. ŞINDILAR, E. *Controlul igienic al produselor şi subproduselor de origine animală*, vol. 1, 2, Editura I.N.R.C.S., Iaşi (2000).
19. ALI TOPÇU, AYLIN AYAZ TOPÇU, ILBILGE SALDAMLI and MINE YURTTAGÜL. *Determination of nitrate and nitrite content of Turkish cheeses*, African Journal of Biotechnology, vol. 5 (15), pp. 1411-1414 (2006).
20. L. TUDOR, ELENA MITTRĂNESCU, LAURA TUDOR, F. FURNARIS. *Assessment of Nitrate and Nitrite Content of Romanian Traditional Cheese*, Lucrări Ştiinţifice Medicină Veterinară, vol. XL, Timişoara (2007).
21. ŢIBULCĂ AURORA. *Cercetări privind influenţa factorilor tehnologici şi de igienă asupra calităţii caşcavalurilor fabricate în zona de nord a Transilvaniei*, Cluj-Napoca (2011).
22. ŢIBULCĂ, D. şi MIRELA A. JIMBOREAN. *Fabricarea produselor lactate şi a brânzeturilor,* Editura AcademicPres, Cluj-Napoca (2003).
23. WALKER R. Nitrates, *Nitrites and N-nitrosocompounds: a Review of the Occurrence in Food and Diet and the Toxicological Implications*, Food Addit Contam. Nov-Dec; 7(6): 717-68 (1990).

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Research Article

Aloe vera gel microcapsules and essential oils of thyme and oregano incorporated in spreadable goat cheese: impact on its microbiological, physicochemical, and sensory characteristics during storage

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Abstract

The aim of this study was to develop a new assortment of high-quality cream cheese, with a high amount of biologically active compounds, namely a spreadable cheese from goat milk with addition of *Aloe vera* microcapsules and essential oils of thyme and oregano. The research was focused on the testing and optimization of an appropriate manufacturing recipe for this new product. Two assortments were made: one with *Aloe vera* microcapsules and thyme essential oil and the other one with *Aloe vera* microcapsules and oregano essential oil. For each assortment two different concentrations of *Aloe vera* and essential oils were tested. The obtained products were analysed for organoleptic, physico-chemical (fat, protein, total dry matter, sodium chloride and acidity) and microbiological parameters. In terms of consumer preferences, the cream cheese with 3% microcapsules of *Aloe vera* gel and 0.018% thyme essential oil was the most appreciated in terms of commercial aspect, consistency, and taste. We concluded that the addition of *Aloe vera* microcapsules improves the sensory characteristics and leads to an increase nutritional value of the finished product. Also, the addition of essential oils (thyme and oregano) ensured a stable quality during storage.

Keywords: *Aloe vera*; essential oils; goat milk; spreadable cheese

Introduction

Essential oils (EO) are extracted from aromatic plants containing a variety of natural, biologically active components with antimicrobial and antioxidant properties (Amatiste *et al.*, 2014; Hamedi *et al.*, 2014; Yousefi *et al.,* 2017, Khorshidian *et al.*, 2018). Antimicrobial properties of essential oils against various microorganisms (*L. monocytogenes, E. coli* O157:H7, *Aspergillus ochraceus* ZMPBF 318, *Penicillium spp.*) have been reported in various studies (Kotzekidou *et al.*, 2008; Yahyazadeh *et al.*, 2008; Čvek *et al.*, 2010; Jeong *et al.*, 2014).

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The oregano and thyme essential oils have significant antibacterial effects, due to the presence of their phenolic compounds, like carvacrol and thymol (Sakkas and Papadopoulou, 2017). In a study released by Burt and Reinders (2003), including four essential oils (oregano, thyme, bay and clove bud) the oregano and thyme oils showed the greatest antibacterial effect against *Escherichia coli* O157:H7. Various studies related to the antioxidant property of oregano essential oil, showed that it has strong antioxidant activity due to its high thymol and carvacrol content (McKay and Blumberg, 2006).

Thyme essential oil is one of the ten more important essential oils due to its proven antimicrobial, antimycotic, and antioxidant effects. Many studies have shown that thymol and carvacrol, its main constituents, are responsible for these properties. According to regulation (EC) no. 1334/2008 thyme essential oil is on the list of essential oils generally recognized as safe for ingestion.

Aloe vera is known as a perennial herb belonging to the Liliaceous family. It contains a large variety of nutrients and bioactive compounds, such as flavonoids, terpenoids, (Boudreau and Beland, 2006; Harlev *et al.*, 2012) fatty acids, mono-and polysaccharides (pectins and hemicelluloses), tannins and sterols. It also contains a variety of enzymes, minerals (calcium, chromium, copper, iron, magnesium, manganese, potassium, phosphorus, sodium, and zinc) and vitamins (A, C, E, β-carotene, B1, B2, B3, B6, choline, B12 and folic acid) (Sahu *et al.*, 2013; Rodrigues *et al.,* 2018; Marzanna *et al.*, 2019). *Aloe vera* is used in the food industry as a functional ingredient (Kazhal and Samira, 2015).

Microencapsulation may be defined as a packaging technology used for separating and storing materials in microscopic capsules, for later release under controlled conditions. The material that provides protection and controlled release of the bioactive compound is called coating material. In general, these matrices are natural or synthetic polymers (Cock and Castillo, 2013).

Goat milk has high nutritional properties and lower allergenic effect as compared with cow's milk, especially in non-sensitized children, which led to an increased interest in goat's milk as a functional food (Albenzio and Santillo, 2011; Hassan, 2014). Spreadable cream cheeses are fresh cheeses produced by coagulating milk, cream or whey with acid, a mixture of acid and rennet, or a combination of acid and heat (Brčina *et al.*, 2017). Fresh cheeses are characterized as slightly acid, soft, homogeneous products that are white in colour and smooth-textured (Frau *et al.*, 2014).

This study aimed to evaluate the effect of essential oils (i.e. thyme and oregano) and microcapsules with *Aloe vera* incorporation in a spreadable goat cheese product. This would enhance its nutritional value and functional properties by increasing its contents in bioactive compounds and will improve its quality and organoleptic properties.

Materials and Methods

Materials

Three successive cream cheese trials were carried out. Goat's milk was obtained from a local farm. The composition of the goat milk used was determined with an Ekomilk M apparatus (model Milkana KAM98-2A Bulteh 2000 Ltd, Stara Zagora, Bulgaria) and the mean values (±) of three replicates for all batches were as follows: 3.85 ± 0.07% fat, 3.78 ± 0.02% total protein, and 8.75 ± 0.05% non-fat dry matter. Titratable acidity was recorded as Thörner degrees (T°) according to the Thörner method (16.5±1 T°), and pH was measured with a pH meter (HI 99161, Hanna Instruments, Limena, Italia) (6.71±0.02). Dry plant material of two commercial plants (thyme and oregano) was used in this study to obtain essential oils. Starter cultures, including the mesophilic strains *Lactococcus lactis* subsp. *lactis* and *Lactococcus lactis* subsp. *cremoris* (Lyofast MO 142), were used. Powder animal rennet was obtained from Chr-Hansen's Laboratories (Copenhagen, Denmark). Using species-specific primers, a PCR assay was performed to certify the presence of goat milk and to detect possible undeclared cow milk addition into the bulk milk used for cheese production experiments.

Preparation of microcapsules with Aloe vera gel

The matrix encapsulation or microencapsulation was accomplished using a laminar flow through a nozzle and an additional vibration of the nozzles or liquid (Cock and Castillo, 2013). To obtain the gel from *Aloe vera* leaves, they were quickly processed in order to avoid oxidation.

The *Aloe vera* gel was obtained by removing the shell, followed by a thermal treatment (72 °C for 12 sec.). Finally, the gel was chopped and homogenized. For the chemical determinations, extraction of the compounds with methanol was performed.

Microencapsulation was proposed as a successful strategy to improve the *Aloe vera* stabilization, to make easier their handling during cream cheese processing and to ensure its bioavailability when they are used as dairy food bioactive compounds. Thus, another objective of this study was to encapsulate bioactive compounds contained in *Aloe vera* gel in order to obtain microcapsules usable as natural food additives.

Extraction of thyme and oregano essential oils

For the flavouring process of the cream cheese, thyme and oregano essential oils were isolated by hydro distillation, using a Clevenger type apparatus. Fifty grams of dried and minced leaves were weighed and added into a distillation flask together with 750 ml of distilled water. The distillation process was performed during three hours. The yield was calculated as millilitres of essential oils per 100 g dry plant material (thyme or oregano). The obtained essential oils were dried using anhydrous sodium sulphate and stored in sealed vials at 6-8 °C until analysis.

PCR analysis of raw goat's milk

To identify possible undeclared addition of cow milk into goat bulk milk used for cheese production experiments, PCR assays were performed on samples collected from bulk milk under sterile conditions and transported at 4 °C. For the recovery of the somatic cells required for DNA extraction, the milk samples were centrifuged at 3000g for 10 minutes. DNA extraction from the cell pellet was performed with the DNesay Blood & Tissue kit (Qiagen), according the manufacturer instructions. Milk somatic cells recovered from the samples were digested in 90 µl of ATL solution and 10 µl of proteinase K, by incubation at 56 °C. Subsequently, 200 µl of AL solution and 200 µl of absolute alcohol were added to each tube. The lysate was transferred to the purification columns and centrifuged at 8000 rpm for 1 min. The columns were subsequently washed with AW1 and AW2 solutions respectively. After centrifugation the DNA from the column filter was eluted in 50 µl of AE solution. DNA concentration and purity were spectrophotometrically evaluated.

To certify the presence of goat milk and an eventual undeclared cow milk addition into the bulk milk used as raw material we amplified two different size fragments from goat and cow mitochondrial DNA (cytochrome B gene).

PCR amplification was performed with a commercial kit (2X Green Master Mix, Fermentas) in 25 µl reactions, containing: 12.5 µl - 2X reaction mixture (Taq polymerase, buffer, $MgCl_2$ and dNTP), 6.5 µl sterile water, 2 µl of common-sense primer specific for cytochrome B of both species and 1 µl of each antisense primer specific for goat or cow. PCR amplification was performed in a thermocycler, under the following conditions: predenaturation – 95 °C for 3 minutes 1 cycle, followed by 35 cycles at 94 °C - 1 minute, 58 °C - 1 minute, 72 °C - 1 minute.

To differentiate the presumptive amplification products obtained in the amplification reactions, the samples were migrated in 2% concentration agarose gel, in 1X TBE buffer and 1X Gel Red Nucleic Acid Stain Gel, at 75 V for one hour. The gel image was analysed using a UV transilluminator system.

Cream cheese manufacturing

Three fresh cheese making trials were carried out during three successive weeks. In each trial, cheese was made in one vat. Goat's milk, obtained from a local farm, was pasteurized at 63-65 °C for 20-30 min. After pasteurization, the milk was cooled at 30 °C and each milk vat was inoculated with starter culture containing about 1 UC/100 l at the rate of 1% (3.5×10^6 cfu/g). After inoculation 20 g/100L of $CaCl_2$ was added. Milk was fermented in a thermostatically controlled incubator for 1 hour at 31°C. When pH reached ≤ 6.5 (6.46), commercial rennet was added under stirring conditions in all vats. The mixtures were incubated at 25-28 °C for about 12 hours. The curd was set in cheese-cloths and was pressed in a ripen chamber for draining at 16-18 °C. After drainage, the curd was cooled to 10 °C.

Cream cheese with *Aloe vera* gel microcapsules and essential oils was made by blending and cooling at 10 °C. During this procedure the cream cheese (90%) was salted (0.4% w/w) and mixed with sour cream (20% fat), 10% proportion. Two concentration of *Aloe vera* microcapsules (7% and 3%) mixed with thyme/oregano essential oil in ratios of 0.0072% and 0.018% were used. Then, cream cheese samples were packaged in plastic cups (250 g) and stored in the refrigerator at 2-6 °C for 21 days until analysis.

The control cream cheese (M) was produced without essential oils and *Aloe vera* addition. The samples were coded as follows: P1C1-7% *Aloe vera* microcapsules and 0.0072% thyme essential oil, P2C2-3% *Aloe vera* microcapsules and 0.018% thyme essential oil, P1O1-7% *Aloe vera* microcapsules and 0.0072% oregano essential oil, P2O2-3% *Aloe vera* microcapsules and 0.018% oregano essential oil.

Physicochemical and microbiological analysis

Physicochemical analysis was performed using cream cheese (two samples from each cheese making trial). The methods specified in the following standards were used to determine the fat, protein, total dry matter, and sodium chloride content of the spreadable cheeses: SR ISO 3433:2009, SR EN ISO 8968-1:2014, SR EN ISO 5534:2004, SR EN ISO 5943:2007 and SR ISO 1740:2008.

The antibacterial activity of essential oils was tested on the following bacteria: *Staphylococcus aureus* ATCC 25923, *Escherichia coli* ATCC 25922 and *Salmonella enteritidis* ATCC 13076. The objective was to identify the minimum inhibitory concentration (MIC) and the minimum bactericidal concentration (MBC) in accordance with the National Committee for Clinical Laboratory Standards (NCCLS, 1997). The minimum inhibitory concentration (MIC) was determined by using 96-well microtiter plates.

The microbiological determinations of the cream cheeses were performed using the following standard methods: SR ISO 16649-2: 2007 Microbiology of food and feed - Horizontal method for the enumeration of positive *Escherichia coli* beta-glucuronidase. Part 2: Colony enumeration technique at 44 °C using 5-bromo-4-chloro-3-indolyl beta-D-glucuronate; ISO 6881-1: 1999 Microbiology of food and animal feeding stuffs - Horizontal method for the enumeration of coagulase-positive staphylococci (*Staphylococcus aureus* and other species) - Part 1: Technique using Baird-Parker agar medium.

Sensory evaluation

A fifty-member sensory panel (aged 21-50 years, women, and men) evaluated one cream cheese of each type at the end of the manufacturing process. The cream cheeses were evaluated for appearance (creamy white), colour (white colour such as cream - degree of whiteness, low-yellow colour), consistency (Low-hard/High-easy to spread on a cracker, creamy feeling of fullness in the mouth) and taste (fresh sourness-reminding of yoghurt). A five-point hedonic scale was used for appearance, colour, consistency, smell, and taste, ranging from 0 (poor) to 5 (very good).

Data analyses

Data analysis was carried out using Minitab statistical software (version 16.1.0;LEAD Technologies, Inc., Charlotte, NC, USA) by one-way ANOVA with Tukey's post-hoc test, at a 95% confidence level ($p <$

0.05). Statistical significance of the effects was interpreted as follows: $p \geq 0.05^{NS}$, not significant; $p < 0.01^{**}$, very significant; $p < 0.001^{***}$, extremely significant.

Results and Discussion

PCR analysis of raw goat 's milk

A reference cow DNA sample was used to differentiate goat milk from cow milk (Figure 1, sample R1). In this case, a specific 274 bp fragment was obtained, which denotes the presence of mitochondrial cow DNA. In the case of DNA samples extracted and amplified from goat milk used in the preparation of the product, the presence of a single amplification product of 157 bp, specific for goat mitochondrial DNA (Figure 1, samples P1 and P2) was detected.

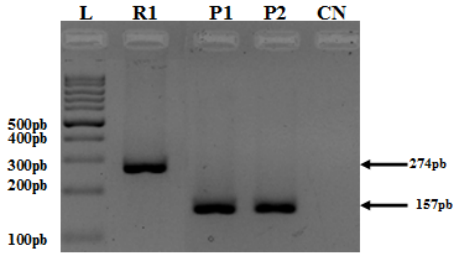

Figure 1. Specific amplification products of the cytochrome B gene from goat's and cow's milk: L-ladder 100bp; R1- cow DNA reference sample, 274 bp PCR product; P1 and P2 - DNA samples amplified from goat milk used in the preparation of the product, 157 bp PCR product; CN-negative control.

Aloe vera gel characterization

In order to characterize the *Aloe vera* samples, the following determinations were achieved: determination of the total phenolic content by the Folin-Ciocâlteu spectrophotometric method (λ = 750 nm), determination of total flavonoid content by spectrophotometric method (λ = 500 nm), determination of antioxidant capacity by DPPH method (Williams *et al.*, 1995), determination of vitamin C by titrimetric method (Deac *et al.*, 2014). Results of the total phenolic, flavonoid, antioxidant capacity and vitamin C content from the *Aloe vera* gel, are presented in Table 1.

Table 1. *Aloe vera* gel characterization

Aloe vera gel	TPC (mg GAE/100g)	Flavonoids (mg QE/100 g)	Antioxidant activity (%)	Vitamin C mg/100 g
	20.68	0.248	3.81	7.04

Determination of antimicrobial activity of essential oils and Aloe vera extract (minimum inhibitory concentration and minimum bactericidal concentration).

The concentration that resulted in complete inhibition of the bacteria (MIC) was the concentration corresponding to the culture wells where the blue colour did not turn pink. Two repetitions for each sample were performed. The minimum bactericidal concentration (MBC) was achieved by inoculating on Mueller Hinton agar from the last three culture wells where bacterial growth was completely inhibited. The results obtained can be found in Table 2.

In our trials, the antimicrobial activity of thyme essential oil against *Staphylococcus aureus* ATCC 25923, *Salmonella enteritidis* ATCC 13076 and *Escherichia coli* ATCC 25922 was found to be lower than the antimicrobial activity of oregano essential oil. Based on these results, it was found that oregano essential oil had a higher antimicrobial activity than thyme essential oil (Olmedo *et al.*, 2013; Carvalho *et al.*, 2015)

Table 2. The susceptibility profile of bacteria (1.5×10^8 CFU/ml) to thyme and oregano essential oils (µl/ml)

Samples		*Staphylococcus aureus* ATCC 25923	*Salmonella enteritidis* ATCC 13076	*Escherichia coli* ATCC 25922
Thyme essential oil	MIC	0.27±0.00	0.13±0.00	0.06±0.00
	MBC	0.27±0.00	0.13±0.00	0.06±0.00
Oregano essential oil	MIC	0.04±0.017	0.01±0.00	0.01±0.00
	MBC	0.04±0.017	0.01±0.00	0.01±0.00
Gentamicin		0.24	0.24±0.00	0.05±0.00

The susceptibility of tested bacteria (1.5×10^6 CFU/ml) to *Aloe vera* extract (mg/ml) is presented in Table 3. The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of the *Aloe vera* extract against microorganisms ranged from 37.79 to 56.58 mg/ml. The study revealed that the *Aloe vera* extract has a greater medicinal potential against *Staphylococcus aureus* and *Escherichia coli*. Similarly, in another study, gram-positive test organisms were found to be more susceptible to the sterile *Aloe vera* extract (Shahzad *et al*., 2009).

Table 3. The susceptibility profile of bacteria (1.5×10^6 CFU/ml) to *Aloe vera* extract (mg/ml)

Aloe vrea extract	*Staphylococcus aureus* ATCC 25923	*Salmonella enteritidis* ATCC 13076	*Escherichia coli* ATCC 25922
MIC	37.79±0.017	56.58±0.00	37.79±0.017
MBC	37.79±0.017	56.58±0.00	37.79±0.017
Gentamicin (µg/ml)	0.05±0.00	0.05±0.00	0.05±0.00

Determination of pathogenic microorganisms (coagulase-positive staphylococci and Escherichia coli) during storage highlighting the influence of essential oils and Aloe vera microcapsules on their evolution.

Table 4 presents the results of the microbiological determinations of the spreadable goat cheeses.

Table 4. Microbiological quality of the spreadable goat cheese assortments supplemented with *Aloe vera* microcapsules and essential oils

Samples	*Staphylococcus aureus*	*Escherichia coli*
P1O1	Absent	Absent
P2O2	Absent	Absent
P1C1	Absent	Absent
P2C2	Absent	Absent
Control sample	Absent	Absent

P1C1-7% *Aloe vera* microcapsules and 0.0072% thyme essential oil, P2C2-3% *Aloe vera* microcapsules and 0.018% thyme essential oil, P1O1-7% *Aloe vera* microcapsules and 0.0072% oregano essential oil, P2O2-3% *Aloe vera* microcapsules and 0.018% oregano essential oil

Physicochemical characteristics

In this work the physicochemical and the sensory properties of all cheese samples were analysed. Cheese composition is shown in Table 5.

The fat and protein content found in the present study is similar to those reported by other authors for this variety of cheese (Frau *et al*., 2014). No significant differences were found in all treatments in regards to moisture, total protein, salt, and fat content of the cheese samples during storage. The addition of thyme and oregano essential oils in cheese did not seem to affect those parameters after spreadable cheese manufacturing and during storage (Table 5).

The moisture and fat in dry matter content of all cheeses (control and *Aloe vera*/ essential oils) have been found to meet the mentioned standards (≤75% moisture and ≥ 40% FDM content) for fresh spreadable cheese (Aktypis *et al.*, 2018). The results were similar to those found in commercial goat cheeses (Gambaro *et al.*, 2017), in French goat milk cheeses (Raynal-Ljutovac *et al.*, 2011) and those found by Vieitez *et al.* (2016). Regarding the salt and total protein content no, significant differences were found among the examined cheeses. The protein content was found to be, almost, constant during their storage and ranged from 10.08 to 10.84% (Table 5).

Table 5. Physicochemical characteristics of spreadable goat cheeses

Sample / Parameter	Fat (%)	Protein (%)	Moisture (%)	Total dry matter (%)	Fat in dry matter (%)	Salt (%)	Acidity (°T)
Control sample	19.68±0.042^{b}	10.84±0.028^{a}	68.20±0.325^{c}	31.80±0.325^{a}	61.90±0.481^{bc}	0.411±0.031^{c}	150±5.657^{a}
P1C1	19.07±0.156^{bc}	10.08±0.057^{b}	70.77±0.580^{a}	29.23±0.580^{c}	65.26±1.824^{ab}	0.741±0.017^{a}	158±5.657^{a}
P2C2	20.92±0.184^{a}	10.40±0.042^{b}	69.52±0.057^{c}	30.48±0.057^{b}	68.63±0.735^{a}	0.684±0.016^{ab}	155±1.414^{a}
P1O1	17.56±0.170^{d}	10.29±0.141^{b}	70.16±0.113^{ab}	29.84±0.113^{bc}	58.85±0.346^{c}	0.694±0.007^{ab}	157±1.414^{a}
P2O2	18.61±0.212^{c}	10.40±0.099^{b}	69.64±0.042^{ab}	30.06±0.042^{bc}	61.30±0.785^{c}	0.635±0.004^{b}	152±2.828^{a}
p	0.000***	0.002**	0.003**	0.003**	0.001**	0.000***	0.332^{NS}

Different letters in the same column indicate statistically significant differences at p < 0.05 (Tukey's test). Significance: $p \geq 0.05^{NS}$, not significant; $p < 0.01^{**}$, very significant; $p < 0.001^{***}$, extremely significant.

Sensory evaluation of cream cheese

Ratings for flavour and textural attributes of spreadable goat cheeses are presented in Figure 2 and Figure 3, respectively.

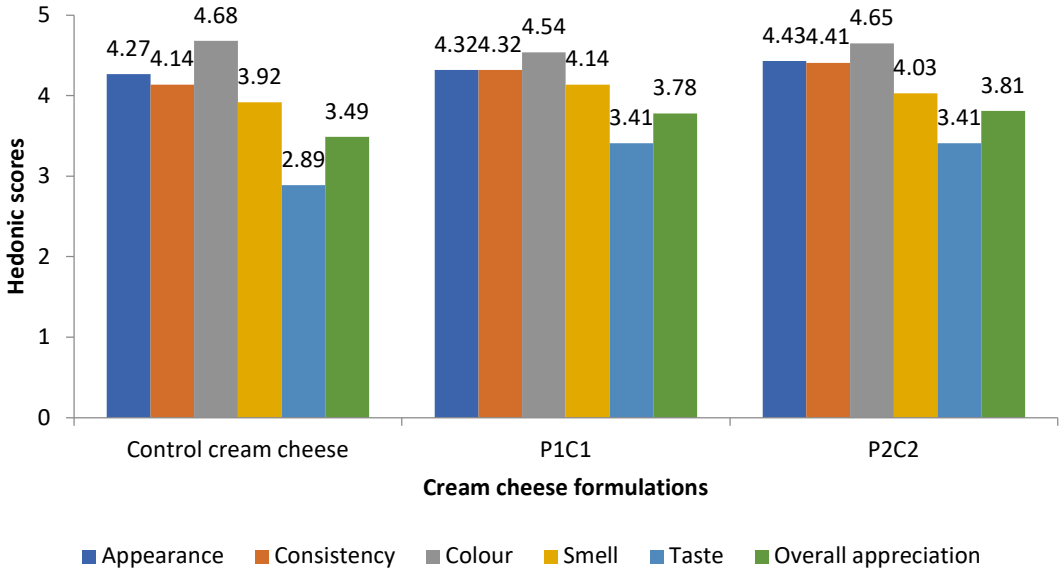

Figure 2. Sensory characteristics of control and spreadable goat cheese with thyme essential oil and *Aloe vera* gel microcapsules

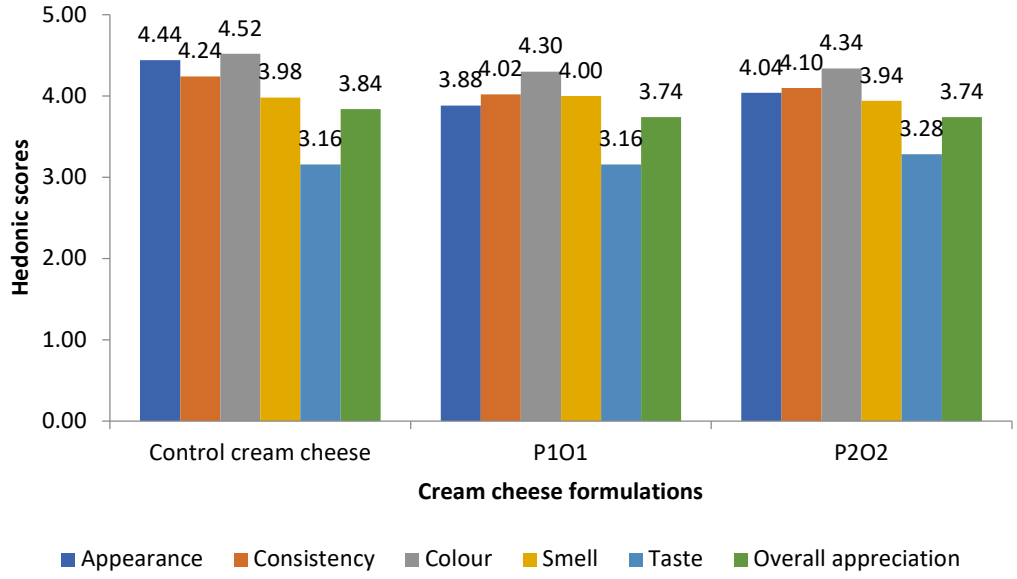

Figure 3. Sensory characteristics of control and spreadable goat cheese with oregano essential oil and *Aloe vera gel* microcapsules

As shown in Figures 2 and 3, the panellists found the differences in appearance, consistency, and colour between control, P1C1 and P2C2 cheese samples.

However, the thyme/oregano spreadable goat cheese analogue with higher content of *Aloe vera* gel microcapsules addition was poorer in consistency and appearance as reflected by their lower score. The lower score in appearance and consistency of P1C1 and P1O1 was likely due to the denser microstructure which made the sample too hard. In addition, the lower score in taste and consistency of P1C1 and P1O1 samples might be due to the too soft feeling of the samples resulting from the high moisture level of the product (Liu *et al.*, 2008).

In terms of consumer preference, the cream cheese sample with 3% *Aloe vera* gel microcapsules and 0.018% thyme essential oil was the most appreciated (P2C2). Regarding cheese samples with the addition of oregano essential oil and *Aloe vera* gel microcapsules, the sample with 3% *Aloe vera* and 0.018% oregano essential oil was the most appreciated (P2O2) as appearance, consistency, and taste, compared to the sample with more microcapsules and less oregano oil.

The cheese sample with 7% microcapsules and 0.0072% oregano essential oil, was more appreciated in terms of smell. The lower score in taste and consistency of the P1O1 sample was likely due to the denser microstructure which made the sample too hard (Jeon *et al.*, 2012).

Conclusions

In this work, the influence of Aloe vera gel microcapsules and essential oils addition on physicochemical, microbiological, and sensory properties of spreadable goat's cheese was investigated. The results highlighted that the best results, from a nutritional point of view, were obtained for the cheese samples with lower Aloe vera and higher thyme essential oil content. In addition, the spreadable goat's cheese with oregano essential oil exhibited a more intensive antimicrobial activity against *Salmonella enteritidis* and *Escherichia coli*. The sensory analysis showed that the lesser Aloe vera gel microcapsules fortification of 3% resulted to an acceptable spreadable cheese, which kept its traditional taste and aroma. Based on the above, Aloe Vera and thyme essential oil could be successfully used as natural flavours in spreadable cheese manufacture providing also antimicrobial

and functional properties. Enrichment of dairy products with polyphenols from Aloe vera plants can positively influence their oxidative stability and it may contribute to a decline in the incidence of degenerative human diseases.

Authors' Contributions

All authors contributed to the review and the editing of the article. MAJ wrote the manuscript and supervised in final reviewer the manuscript.

AMR, CRP and LCS participated in the experiment of this study. VAB performed PCR analysis. CAS supervised data analysis. DT and DM designed and conducted the research. AB in reviewer of the manuscript. All authors read and approved the final manuscript.

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Conflict of Interests

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

References

Aktypis A, Christodoulou ED, Manolopoulou E, Georgala A, Daferera D, Polysiou M (2018). Fresh ovine cheese supplemented with saffron (*Crocus sativus* L.): Impact on microbiological, physicochemical, antioxidant, color and sensory characteristics during storage. Small Ruminant Research 167:32-38. *https://doi.org/10.1016/j.smallrumres.2018.07.016*

Albenzio M, Santillo A (2011). Biochemical characteristics of ewe and goat milk: Effect on the quality of dairy products. Small Ruminant Research 101:33-40. *https://doi.org/10.1016/j.smallrumres.2011.09.023*

Amatiste S, Sagrafoli D, Giacinti G, Rosa G, Carfora V, Marri N, Rosati R (2014). Antimicrobial activity of essential oils against staphylococcus aureus in fresh sheep cheese. Italian Journal of Food Safety 3(3):148-150. *https://doi.org/10.4081/ijfs.2014.1696*

Boudreau MD, Beland FA (2006). An evaluation of the biological and toxicological properties of *Aloe barbadensis* (Miller), *Aloe vera*. Journal of Environmental Science and Health Part C 24:103-154. *https://doi.org/10.1080/10590500600614303*

Brčina T, Vilušić M, Moranjak M (2017). Sensory properties of dairy products based on fresh cheese and fruit. Technologica Acta10(2):1-8.

Burt SA, Reinders RD (2003). Antibacterial activity of selected plant essential oils against *Escherichia coli* O157-H7. Letters Applied Microbiology 36:162-167 *https://doi.org/10.1046/j.1472-765X.2003.01285.x*

Carvalho RJ, de Souza GT, Honorio VG, de Sousa JP, da Conceiçao ML, Maganani M, de Souza EL, (2015). Comparative inhibitory effects of *Thymus vulgaris* L. essential oil against *Staphylococcus aureus*, *Listeria monocytogenes* and mesophilic starter co-culture in cheese-mimicking models. Food Microbiology 52:59-65. *https://doi.org/10.1016/j.fm.2015.07.003*

Cock LS, Castillo VV (2013). Probiotic encapsulation. African Journal of Microbiology Research 7(40):4743-4753. *https://doi:10.5897/AJMR2013.5718*

Čvek D, Markov K, Frece J, Dragičević TL, Majica M, Delaš F (2010). Growth inhibition of *Aspergillus ochraceus* ZMPBF 318 and *Penicillium expansum* ZMPBF 565 by four essential oils. Arhiv za Higijenu Rada i Toksikologiju 61(2):191-196. *https://doi.org/10.2478/10004-1254-61-2010-2009*

Deac LM, Fărcaş A, Vodnar DC, Tofană M, Socaci SA (2014). Antioxidant and Antimicrobial Properties of the Fir Buds Syrup. Bulletin UASVM Food Science and Technology 71(1):77-78. *http://dx.doi.org/10.15835/buasvmcn-fst:10123*

Frau F, Font de Valdez G, Pece N (2014). Effect of pasteurization temperature, starter culture, and incubation temperature on the physicochemical properties, yield, rheology, and sensory characteristics of spreadable goat cheese. Journal of Food Processing Volume 2014:1-8. *https://doi.org/10.1155/2014/705746*

Gambaro A, Gonzalez V, Jimenez S, Arechavaleta A, Irigaray B, Callejas N, ... Vieitez I (2016). Chemical and sensory profiles of commercial goat cheeses. International Dairy Journal 69:1-8. *http://dx.doi.org/10.1016/j.idairyj.2017.01.009*

Hamedi H, Razavi-Rohani SM, Gandomi H (2014). Combination effect of essential oils of some herbs with monolaurin on growth and survival of *Listeria monocytogenes* in culture media and cheese. Journal of Food Processing and Preservation 38(1):304-310. *https://doi.org/10.1111/j.1745-4549.2012.00778.x*

Harlev E, Nevo E, Lansky E, Ofir R, Bishayee A (2012). Anticancer potential of aloes: antioxidant, antiproliferative, and immunostimulatory attributes. Planta Medica 78:843-852. *http://doi.org/10.1055/s-0031-1298453*

Hassan FAM, Hayam MA, Mona AM, Abd El G, Ali KE (2014). Goats dairy products as a potentially functional food. Life Science Journal 11(9s):648-657.

Jeon SS, Lee SJ, Ganesan P, Kwak HS (2011). Comparative study of flavor, texture, and sensory in cream cheese and cholesterol-removed cream cheese. Food Science and Biotechnology 21(1):159-165. *https://doi.org/10.1007/s10068-012-0020-6*

Jeong EJ, Lee NK, Oh J, Jang SE, Lee JS, Bae IH, Jeong YS (2014). Inhibitory effect of cinnamon essential oils on selected cheese-contaminating fungi (*Penicillium* spp.) during the cheese-ripening process. Food Science and Biotechnology 23(4):1193-1198. *https://doi.org/10.1007/s10068-014-0163-8*

Kazhal S, Samira B (2015). Antifungal effect of Aloe Vera gel on *Penicillium Citrinum* in culture media and UF cheese. International Journal of Food Engineering 1(1):61-64. *http://doi.org/10.18178/ijfe.1.1.61-64*

Khorshidian N, Mojtaba Y, Elham K, Amir MM (2018). Potential application of essential oils as antimicrobial preservatives in cheese. Innovative Food Science and Emerging Technologies 45:62-72. *http://dx.doi.org/10.1016/j.ifset.2017.09.020*

Kotzekidou P, Giannakidis P, Boulamatsis A (2008). Antimicrobial activity of some plant extracts and essential oils against foodborne pathogens in vitro and on the fate of inoculated pathogens in chocolate. LWT - Food Science and Technology 41(1):119-127. *https://doi.org/10.1016/j.lwt.2007.01.016*

Liu H, Xu XM, Guo SD (2008). Comparison of full-fat and low-fat cheese analogues with or without pectin gel through microstructure, texture, rheology, thermal and sensory analysis. International Journal of Food Science and Technology 43:1581-1592. *http://doi:10.1111/j.1365-2621.2007.01616.x*

Marzanna H, Krzysztof D, Danuta G, Anna JG, Elżbieta G (2019). *Aloe vera* (L.) Webb.: Natural sources of antioxidants - a review. Plant Foods for Human Nutrition 74:255-265. *http://doi.org/10.1007/s11130-019-00747-5*

McKay D, Blumberg J (2006). A review of the bioactivity and potential health benefits of chamomile tea (*Matricaria recutita* L.). Phytotherapy Research 20:519-530. *https://doi.org/10.1002/ptr.1900*

National Committee for Clinical Laboratory Standards (1997). Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically - fourth edition: approved standard, M7-A4. Villanova, Pennsylvania: National Committee for Clinical Laboratory Standards.

Olmedo RH, Nepote V, Grosso NR (2013). Preservation of sensory and chemical properties in flavoured cheese prepared with cream cheese base using oregano and rosemary essential oils. LWT - Food Science and Technology 53:409-417. *http://dx.doi.org/10.1016/j.lwt.2013.04.007*

Raynal-Ljutovac K, Le Pape M, Gaborit P, Barrucand P (2011). French goat milk cheeses: An overview on their nutritional and sensorial characteristics and their impacts on consumers' acceptance. Small Ruminant Research 101:64-72. *https://doi.org/10.1016/j.smallrumres.2011.09.026*

de Oliveira ACL, Tabrez S, Shakil S, Khan MI, Asghar MN, Matias BD, ... de Carvalho Melo-Cavalcante AA (2018). Mutagenic, antioxidant and wound healing properties of Aloe vera. Journal of Ethnopharmacology 227:191-197. *http://doi.org/10.1016/j.jep.2018.08.034*

Sahu PK, Giri DD, Singh R, Pandey P, Gupta S, Shrivastava AK, ... Pandey KD (2013). Therapeutic and medicinal uses of *Aloe vera*: a review. Pharmacology & Pharmacy 4:599-610. *http://doi.org/10.4236/pp.2013.48086*

Sakkas H, Papadopoulou P (2017). Antimicrobial activity of basil, oregano, and thyme essential oils. Journal of Microbiology and Biotechnology 27(3):429-438. *https://doi.org/10.4014/jmb.1608.08024*

Shahzad K, Ahmad R, Nawaz S, Saeed S, Iqbal Z (2009). Comparative antimicrobial activity of aloe vera gel on microorganisms of public health significance. Pharmacologyonline 1:416-423.

Vieitez I, Callejas N, Saibene M, Cabrera L, Irigaray B, Grompone MA (2016). Fatty acids and triglycerides composition in Uruguayan cow, sheep and goat cheeses. Journal of Food Science and Engineering 3:379-387.

Williams BME, Berset CC (1995). Use of a free radical method to evaluate antioxidant activity. LWT - Food Science and Technology 28(1):25-30. *https://doi.org/10.1016/S0023-6438(95)80008-5*

Yahyazadeh M, Omidbaigi R, Zare R, Taheri H (2008). Effect of some essential oils on mycelial growth of *Penicillium digitatum* Sacc. World Journal of Microbiology and Biotechnology 24(8):1445-1450. *https://doi.org/10.1007/s11274-007-9636-8*

Yousefi AM, Khorshidian N, Mortazavian AM, Hosseini H (2017). A review on the impact of herbal extracts and essential oils on viability of probiotics in fermented milks. Current Nutrition & Food Science 13(1):6-15. https://doi.org/ *10.2174/1573401312666161017143415*

*** Regulation (EC) no 1334/2008 of the european parliament and of the council on flavourings and certain food ingredients with flavouring properties for use in and on foods.

*** SR ISO 3433:2009. Brânză. Determinarea conținutului de grăsime. Metoda Van Gulik

*** SR EN ISO 8968-1:2014. Lapte şi produse din lapte. Determinarea conținutului de azot. Partea 1: Metoda Kjeldahl şi calculul conținutului de proteină brută

*** SR EN ISO 5534:2004. Brânzeturi şi brânzeturi procesate. Determinarea conținutului total de substanță uscată (Metoda de referință)

***SR EN ISO 5943:2007. Brânză şi produse din brânză procesată. Determinarea conținutului de cloruri. Metoda prin titrare potențiometrică

*** SR ISO 1740:2008. Produse din grăsime din lapte şi unt. Determinarea acidității grăsimii (Metodă de referință)

*** SR ISO 16649-2: 2007 Microbiology of food and feed - Horizontal method for the enumeration of positive *Escherichia coli* beta-glucuronidase.

*** SR ISO 6881-1: 1999 Microbiology of food and animal feeding stuffs - Horizontal method for the enumeration of coagulase-positive staphylococci (*Staphylococcus aureus* and other species)

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Drinking Behavior, Taste Preferences and Special Beer Perception among Romanian University Students: A Qualitative Assessment Research

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Abstract: The transition from adolescence to adulthood can be a challenging period for many students. This period is associated with an increase in alcohol consumption (AC) which can develop a drinking behavior or shape the preferences for certain alcoholic beverages. The purpose of this study was to analyze the AC pattern among Romanian university students, by investigating the association between taste and consumption, including preferences for special beer. A 30-item omnibus-type questionnaire was distributed to undergraduate students and used to gather sociodemographic data, alcohol expectancies, drinking motives and consequences, and special beer consumption. Results showed a statistically significant relationship between the age of first alcohol use and the existence of an alcoholic family member. The main reasons for AC are taste, sensation, relaxation, and socialization. Both female and male students tend to drink occasionally, with a preference for public places. Female students prefer a sweet taste, choosing special beers over the regular ones. The students' residence may also influence the choice of special beers. Understanding the students' drinking behavior and taste preferences is essential to create useful strategies to discourage excessive AC. Special beer, a growing segment in the beverage industry, could represent a healthier and safety alternative to AC.

Keywords: alcohol; prevalence; Romanian university students; non-alcoholic beer; low alcohol beer; public health

1. Introduction

The alcohol consumption (AC) among university students is different between countries, due to cultural differences, family socioeconomic status, level of education, social-activity, health, and religious reasons [1–3]. Nowadays, students drink more than earlier

generations, with increasing emphasis on binge drinking and drunkenness [4]. The transition from adolescence to adulthood, living apart from parents, with less parental support can be a stressful time for students, often associated with an increase AC, up to dangerous levels [5,6]. Multiple studies have reported heavy AC among university students as an emerging issue, which is often associated with a significant risk of adverse psychological, social, and physical health consequences, including injuries, unplanned sex, academic failure, and alcohol related problems later in life [7–9]. Motives for alcohol use differ between genders, so the adverse outcomes [10–12].

The sector of special beers: non-alcoholic beers (NAB) and low-alcohol beers (LAB) is one of the fastest growing segments in the beverage industry. Their increased popularity is mostly due to the beers' rich fruity flavor and refreshing properties. Fruits, fruit juices, by-products, and fruit extracts are often used to give special beer assortments various flavors, tastes, and aroma [13]. Recent studies have shown that special beer gained recognition also due to its potential health benefits, associated with the high content of phenolic antioxidants and the low ethanol content. For special beers, the level of ethanol should be below 2.5% alcohol by volume (ABV), and therefore, the associated health risks should be much lower. Despite the fact that special beers represent a healthier option, the brewing industry faces other challenges, such as ensuring the flavor stability of special beers, which stimulates brewery technological innovations [14]. The drinking motives usually serve as endorsers towards alcohol use, and represent, practically, the gateway through which more distal factors such as alcohol expectancies, genetic factors, and personality features are mediated [15,16].

AC is an ongoing problem and a multifaceted topic to research. As a consequence, data interpretation must be carried out carefully and efficient actions must be taken, if needed. Often, the combined intervention of public health organizations and education specialists is required to create proper strategies for reducing AC among students. For example, problematic drinking might be diminished by targeting the drinking motives behind. Irrespective of the chosen strategy, the first most important step is to have an in-depth understanding of the students' perceptions, consumption patterns, and preferences.

In this context, the present study had two main objectives. First, we investigated the drinking behavior among students in order to find gender, age, or residence patterns and evaluate the associations between taste and consumption. Second, we assessed how students relate to special beer (NAB and LAB) as an alternative to regular beer and if taste preferences can shape the desire for special beer consumption.

2. Materials and Methods

2.1. Students Selection and Procedure

The current study was conducted in academic year 2018/2019 at the University of Agricultural Sciences and Veterinary Medicine from Cluj-Napoca (Romania). Undergraduate university students (18–24 years old) were recruited through posters, flyers, and advertisements posted all over the university campus, email messages, personal communications, and social media. Also, the bachelor, master, and PhD students received an email with a short description of the study and a link to the online questionnaire through Google Drive. Eligibility criteria was restricted to participants who had consumed alcohol in the past 12 months, thus abstinent students were excluded from the analysis. A 30-item omnibus-type questionnaire was used to gather sociodemographic data, alcohol expectancies, drinking motives, relevant information about participants' families, questions about special beer, and items related to health lifestyle/risks and AC. The types of questions that were chosen for the self-report questionnaire included closed and open questions, filter questions, multiple choice (single answer), selection list questions, and free text questions. The questionnaire design is presented in Table 1. The average time to complete the web-based questionnaire was about 30 min. No identifiers such as name, identity number, nor internet protocol address were recorded in order to preserve the anonymity of the

participants. The answers of the questionnaire were downloaded in Microsoft Office Excel format from Google Drive.

Table 1. Questionnaire design.

Questions	Explanatory Variables
Q1–Q4	Sociodemographic characteristics: age, gender, the study program, residence;
Q5–Q13	Drinking habits and behaviours: the age of first alcohol use, reasons to drink alcohol, drinking frequency, drinking places, period of highest consumption of alcohol, the existence of an alcoholic family member;
Q14–Q16	Alcoholic taste preferences: the favorite alcoholic beverage, reasons/preferences for alcoholic favorite drink, what tastes appeal most;
Q17–Q20	Risk factors associated with AC: negative consequences, driving under influence of alcohol, drinking problems (altered states of health after drinking episodes, violence-related problems, etc.);
Q21–Q30	Special beer consumption (NAB and LAB): frequency and motives of consumption, sensory preferences, appealing characteristics, expectation attributes.

2.2. Ethics

Ethics approval was obtained from the Faculty of Food Science and Technology, University of Agricultural Sciences and Veterinary Medicine Cluj-Napoca, Romania. Students were informed that participation was voluntary and anonymous, no identifying information was collected. Following the link, participants gave their informed consent to participate. No financial or material incentive was provided for participation in the study.

2.3. Statistical Analysis

The participants of the study were naturally categorized in drinkers and non-drinkers. All variables were analyzed considering drinkers' preferences for different types of alcoholic beverages. In order to analyze the existing relationship between targeted groups of data one have used Chi square test (Phi and Cramer's V). Here the Phi and Cramer's coefficients were used to measure the strength of an eventual association. Comparisons between two ranked groups were assessed using Mann–Whitney–Wilcoxon rank sum test. Ranked data was represented as median (quartile 1, quartile 3). Interactions between variables was assessed using two-way ANOVA. All the analysis were performed with SPSS 19.0 (Statistical Package for the Social Sciences, version 19.0. New York, NY, USA) and R 4.0.1. The charts were created using Prism (v 6.01, GraphPad Software, San Diego, CA, USA).

3. Results and Discussion

3.1. Characteristics of the Study Participants

In the present study, a total number of 1054 of records were received. After applying the eligibility criteria, 1044 valid records were considered for analysis and constituted our study group. The gender structure of the respondents is presented in Table 2. With respect to age, the median age of responders was 21 years (ranging 18–24 years). The analyzed sample was relatively homogenous concerning gender distribution (472 males (45%) and 573 females (55%)). Most responders have their residence in the urban area of Romania (70.30%, of which 44.88% male, and 55.11% female). All responders were of Caucasian race.

3.2. Drinking Behavior and Taste Preferences

The first objective of our study was to characterize the drinking behavior among students and to determine if age, gender, or residence could be associated with certain consumption patterns.

Table 2. Characteristics of the study participants.

	Male		Female	
	N	**%**	**N**	**%**
Eligible respondents	472	45	573	55
Age				
<20	67	14.19	74	12.91
20	76	16.10	102	17.80
21	106	22.67	151	26.35
22	80	16.95	146	25.48
23	66	13.98	57	9.95
24	52	11.02	25	4.36
>24	24	5.08	18	3.14
Residence				
Urban	332	70.33	402	70.15
Rural	140	29.66	171	29.84

General drinking habits and behavior investigations within our study unraveled interesting patterns among male and female responders. As presented in Figure 1a, male students start consuming alcohol in their teenage years, half of the respondents beginning to drink before the age of 16. Our results are in agreement with the 2018 World Health Organization (WHO) global report on alcohol use, which states that in many countries of the Americas and Europe, the AC starts before the age of 15 years. Global data are worrying, as 50–70% of 15-year-old students use alcohol, with nearly no difference between male and female [17]. It is suggested that drinking motives in young age may predict drinking patterns later in life [18]. The study conducted by Hingson et al. emphasizes the need to counsel adolescents about alcohol use and to implement efficient policies that delay AC [19]. Another recent study suggests that it would be of interest to focus on the training of specific skills among adolescents. In the case of males, it is probably more useful to train skills such as assertiveness to help to resist peer pressure. For females, it could be interesting to teach other ways of relating successfully with their colleagues, both favoring the creation of social networks with peers who do not drink, such as training in social and assertive skills, and encouraging reinforcing activities that do not involve the use of alcohol [20]. In our study, we observed that for female students, the age of alcohol use onset is postponed to 16–18 years.

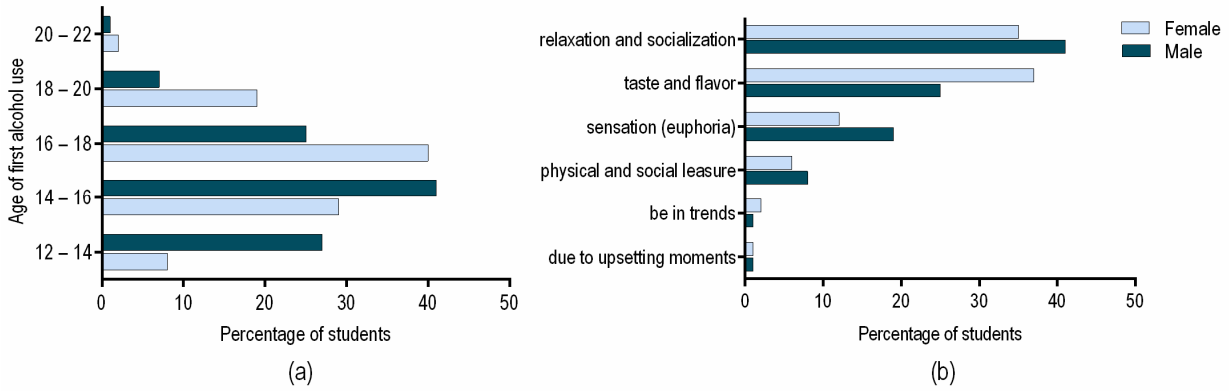

Figure 1. (**a**) The percentages of male and female students related to the age of their first alcohol use; (**b**) The main reasons why male and female students are consuming alcohol.

When we correlated the age of first alcohol use to the existence of an alcoholic family member, we found a statistically significant relationship ($X^2 = 20.64$, $p < 0.001$). Adolescents who started to drink at a very young age (12–16 years) were more likely to have had a family member with alcohol problems (Table 3). When treating the age-intervals as ranked

data we also observed a statistical significance ($p < 0.001$) with subjects having an alcoholic family member having an earlier age of first drink (alcoholic family members median 14–16 (12–14, 16–18); no alcoholic family members median 16–18 (14–16, 16–18)).

Table 3. The interaction between gender, age of first drink and alcoholic member.

Gender				Age of First Alcoholic Drink				Total
				12–14	14–16	16–18	18+	
Female	Alcoholic member	Yes	Count	15	38	35	15	103
			% within Alcoholic member	14.6%	36.9%	34.0%	14.6%	100.0%
		No	Count	33	130	197	110	470
			% within Alcoholic member	7.0%	27.7%	41.9%	23.4%	100.0%
	Total		Count	48	168	232	125	573
			% within Alcoholic member	8.4%	29.3%	40.5%	21.8%	100.0%
Male	Alcoholic member	Yes	Count	45	49	24	7	125
			% within Alcoholic member	36.0%	39.2%	19.2%	5.6%	100.0%
		No	Count	82	144	91	29	346
			% within Alcoholic member	23.7%	41.6%	26.3%	8.4%	100.0%
	Total		Count	127	193	115	36	471
			% within Alcoholic member	27.0%	41.0%	24.4%	7.6%	100.0%
Total	Alcoholic member	Yes	Count	60	87	59	22	228
			% within Alcoholic member	26.3%	38.2%	25.9%	9.6%	100.0%
		No	Count	115	274	288	139	816
			% within Alcoholic member	14.1%	33.6%	35.3%	17.0%	100.0%
	Total		Count	175	361	347	161	1044
			% within Alcoholic member	16.8%	34.6%	33.2%	15.4%	100.0%

Additionally, there was a statistically significant interaction between gender and the presence of alcoholic family members on the age of first alcohol use ($p < 0.01$, Phi and Cramer's V coefficients equals 0.15 for females and $p < 0.05$, Phi and Cramer's V coefficients equals 0.13 for males) indicating a weak association.

The first part of the Table 3 shows the age of the first drink amongst females having or not an alcoholic family member. Most of the girls having an alcoholic family member tend to start drinking between 14–16 years old (36.9%), while for the majority of those not having an alcoholic family member this age is pushed to 16–18 years old (41.9%). Males with alcoholic family member start drinking at 14–16 years old (39.2%), followed closely by even younger age (12–14 years old, 36.0%), while those not having an alcoholic member start drinking alcohol between 14–16 (41.6%).

It is known that individuals with first-degree relatives (mother, father, sister, brother, son, or daughter) with an alcohol problem are two to seven times more likely to develop alcohol problems at some time in their lives than people with nonalcoholic relatives [19,21]. Interestingly, studies including sibling/twin/adopted adolescents aged 12 to 19 years reported that both genetic and environmental factors matter in the development of alcohol use among teenagers, but to a different extent, depending on the time of onset, level of alcohol use, and rates of growth [22,23].

When asked about the reasons for consuming alcohol, more than 70% of the total responders of our study declared that they are consuming alcohol for relaxation, socialization or for its taste and flavor (Figure 1b). Interestingly, most male students drink to relax or socialize, while most female students consume alcohol for the beverages' taste or flavor. Other studies have also shown that drinking motives can differ between male and female students [24]. Among women, associations between drinking motives and the hourly AC rate were observed, and also, interactions between drinking motives and the impact of the number of friends on the hourly consumption rate [25]. Usually, the most common declared drinking motives for both genders include social, enhancement, coping, or conformity motives. A 2015 study confirms that fun, relaxation, and taste are important

motivators for drinking among students. These motives negatively correlate with excessive drinking, while fun was negatively associated with the intention to quit drinking [26]. In turn, if escape, loneliness, social reasons, or recall of alcohol advertising were found to be the main reasons for drinking, a positive association with excessive drinking was identified.

Next, we evaluated the AC frequencies and location among all responders (Table 4).

Table 4. The distribution of alcohol consumption frequency and location among male and female students.

Gender	Alcohol Consumption Frequency				Drinking Location	
	Weekly	Once a Month/Once at 2 Months	Occasionally	Others		
Male	20	11	27	5	Home	63
	13	4	41	1	Clubs&Discos	59
	74	13	250	1	Public places	338
	3	0	8	0	Others	11
Female	13	14	57	8	Home	92
	8	14	80	1	Clubs&Discos	103
	27	29	308	5	Public places	369
	4	0	3	2	Others	9

Concerning the AC frequency, we observed a general tendency for both male and female students to drink occasionally, with a preference for public places, such as restaurants or bars. Our results are in agreement with Zadarko-Domaradzka et al., who found that 70% of the college students in the Carpathian Euroregion (Polish, Slovak, Romanian, and Ukrainian) consume alcohol occasionally [27]. Regarding the consumption location, we obtained a statistically significant difference between genders ($X^2 = 9.13$, $p < 0.03$), with Phi and Cramer's V coefficients equals 0.17, showing a weak association. Also, in the case of home, clubs & discos, and public places, a statistically significant relationship between gender and different alcohol frequency was observed (Home: $X^2 = 8.05$, $p < 0.05$, Phi and Cramer's V = 0.22; Clubs & Discos: $X^2 = 7.78$, $p < 0.05$, Phi and Cramer's V = 0.22; Public places: $X^2 = 36.58$, $p < 0.001$, Phi and Cramer's V = 0.22). Therefore, we could observe that there is a significant relationship regarding the frequency of consumption for the women who choose to consume alcohol inside and the men that are choosing the same type of location.

Gender differences remain one of the most reliable determinants of AC. Males drink and are drunk more frequently than females. Males consume more often because of their higher levels of social and enhancement motives, while women due to their higher levels of coping motives and their lower levels of conformity motives [28].

Our results showed that AC was frequently associated with harmful incidence and altered states of health (Figure 2). Worldwide, studies have reported the heavy AC among college student population is associated with numerous negative consequences, such as health issues (vomiting, headache, dizziness, etc.), physical and violence-related problems (assaults, driving under the influence of alcohol, car accidents, etc.), and other injuries [29–31]. Women drink to socialize, to relax, to improve their mood, or to escape boredom, while males consume alcohol for image and reputation, they drink "to be cool" and to be more popular among their friends [32–34]. The negative consequences of AC are influenced by the body weight, the alcohol tolerance, the speed of consumption, the metabolic rate, the level of hydration, and the food intake [35,36]. Generally, women are more susceptible than men to react to heavy AC due to several reasons. Foremost, women have less enzymes (alcohol dehydrogenase and acetaldehyde dehydrogenase) used to metabolize alcohol, they have a higher body fat to muscle ratio, thus blood and tissue concentrations of alcohol are higher in women, their body size is usually smaller, so women have less water to diffuse the alcohol in their blood stream and, not least, the female hormonal status tends to make women more vulnerable to experience the effects of excessive AC [11]. Men are more likely to take risks while drinking, evidenced by a

high rate of car crashes, drink driving over the legal alcohol limit, increased aggressive behaviors, etc. [37,38].

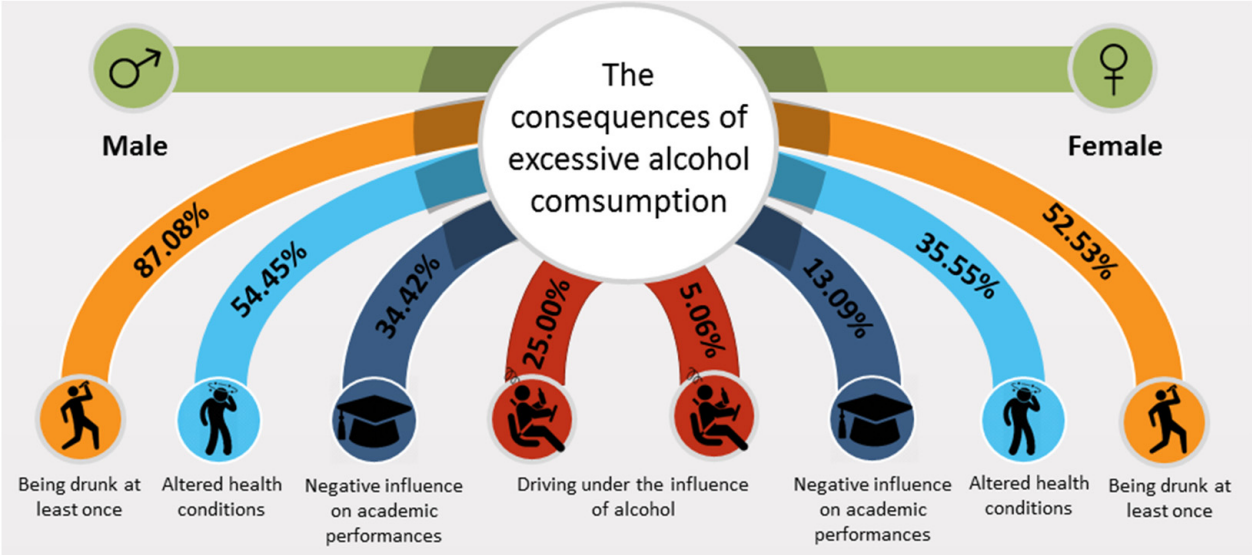

Figure 2. The negative consequences of excessive alcohol consumption among students.

Regarding the favorite alcoholic beverage among college students, our study shows that beer was preferred by male and wine by female (Figure 3a). When we analyzed the consumption behavior, we found that sensory acceptance was the main selection criteria for all the responders. Taste was one of the main features determining the beverage choices (Figure 3b). The flavor of the alcoholic beverage is an important element in explaining drinking behavior patterns, such as overconsumption. The constant consumption of a specific type of beverage can be related to its chemosensory perception [39].

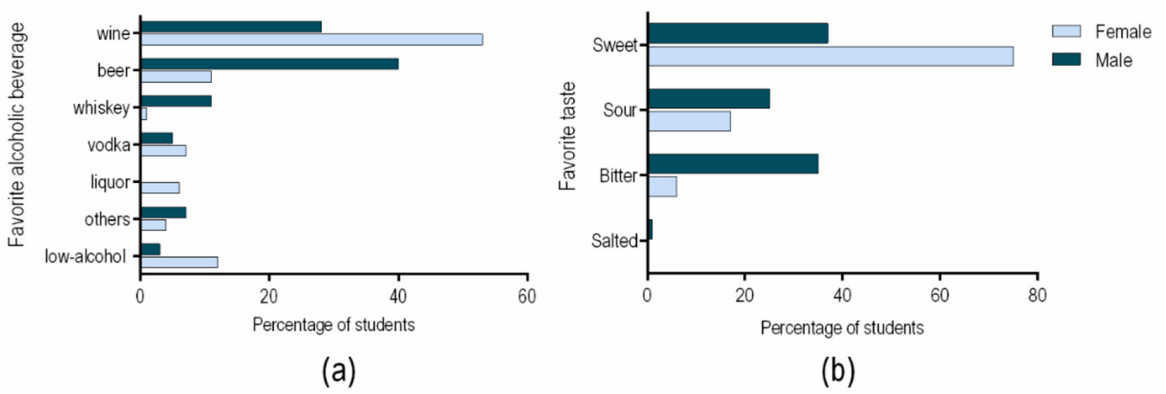

Figure 3. Students' preferences in terms of alcoholic beverages (**a**) and tastes (**b**).

Concerning the taste preferences, our study revealed that male students tend to prefer bitter drinks, while female students prefer the sweet taste, choosing the special beers over the usual ones. Preference for highly concentrated sweet solutions (sweet liking) has been suggested to be a trait and state marker for alcohol dependence [40]. It is well known that women prefer sweeter products, such as wine or flavored beer, instead of classic beer [41]. The complexity of flavor in terms of the scent, the notes and the structure of the wine are factors that may explain women choices. Moreover, women are interested in a healthy lifestyle, and wine is associated with potential health benefits [42]. Beer is an incredibly versatile beverage, served in various locations such as clubs, bars, and

restaurants. Given this context, beer will always be in high demand, particularly for male consumers. Moreover, the men' preference for beer can be partially attributed to advertising, as in many TV commercials men are the ones consuming beer.

3.3. Special Beer: Non-Alcoholic Beer and Low-Alcohol Consumption

Beer is obtained by fermentation of malted barley and it is one of the world's most popular beverage. A moderate beer consume can have positive effects on health [13,43], due to the bioactive compounds from hops (*Humulus Lupulus* L.), such as: xantohumol, isoxantohumol, humulone, 8-prenylnaringenin and lupulone, used for the bitterness and aroma [44,45]. However, lager beer contains 4–5% volumes ethanol and studies have demonstrated its hepatotoxic effects and its potential to promote different types of cancer [46]. As a reaction, the NAB and LAB market has enjoyed significant growth in the past years, these beers becoming the mainstream option for more and more people.

To gain a more accurate insight with respect to special beer consumption among students, the second objective of our study was to understand how Romanian students relate to special beer (NAB and LAB) as an alternative to regular beer or other alcoholic drinks. Results indicated that gender, age, sociodemographic data might influence the preference for special beers, as summarized in Table 5.

Table 5. The preferences for special beers over high alcoholic beverages, classified based on gender, age, and students' residence, along with the statistical interpretation.

Variables		Preference for Special Beers over High Alcoholic Beverages		*p*-Value
		Total-Yes: 59.96%	Total-No: 40.03%	
Gender				
	Male	40.42%	52.15%	$X^2 = 13.476$
	Female	59.58%	47.84%	$p < 0.001$
Age				
	18–20	28.43%	22.52%	$X^2 = 4.129$
	21–24	68.05%	41.37%	$p > 0.05$
	>24	3.51%	2.88%	
Residence				
	Rural	29.23%	25.23%	$X^2 = 6.73$
	Urban	65.50%	65.55%	$p < 0.05$
	No answer	5.27%	9.09%	

In our study, the percentage of respondents who consumed a special beer at least once was over 90% (93.86% in case of female and 91.80% for male). When asked if they would choose special beers at the expense of high-alcoholic beverages, almost 60% of the respondents responded affirmatively. Of this percentage, more than half was represented by female students (59.58%), who are between 21–24 years old (68.05%) and live in an urban area (65.50%).

As presented in Table 5, our study shows that gender can influence the preference for special beers ($X^2 = 13.476$, $p < 0.001$, Phi and Cramer's V coefficients equals 0.12), as female students are more willing to consume this sort of beer. Also, students living in the urban areas of Romania are more likely to choose special beers compared to the students with rural residence ($X^2 = 6.73$, $p < 0.05$, Phi and Cramer's V coefficients equals 0.08). However, the choosing of special beers is not significantly influenced by age.

Because of the differences between males and females considering the reason for consuming alcoholic beverages (especially in the case of the following categories: taste and flavor and sensation (euphoria), relaxation and socialization) we decided to perform subgroup analysis considering these three choices and determining if there was an association in this case between gender and the choice of special beer. There was no association between gender and the choice of special beer in the case of the taste and flavor subgroup

($p = 0.549$), nor in the case of sensation (euphoria) subgroup ($p = 0.202$) or the relaxation and socialization subgroup ($p = 0.156$). This shows that it is possible that the preference in choosing special beers over other alcoholic beverages might be heavily influenced by one person's reason for drinking the alcoholic beverage, which, in turn is associated with their gender.

When we tested the association between gender, reasons for consuming alcoholic beverages and the preferred taste, we found out that the majority of females preferring the sweet taste are also consuming alcohol because of its taste and flavor. On the other hand, males whose preferences are related to the sweet taste tend to consume alcohol for relaxation and socialization. According to the Chi square test's values and to the significance value for both males and females ($p < 0.05$), we may confidently conclude that there is a significant relationship between the favorite taste and the reasons for alcohol consumption.

When asked if they like NAB, 51.86% of responders declared that they do not like it or they have never consumed this type of beer. This high percentage could be explained by the fact that NAB does not deliver a comparable emotional response to the consumers. In order to be accepted by the consumers, a beverage needs to evoke a rich and emotional set of positive associations [47]. While beer and wine are associated with positive emotional responses, the non-alcoholic beer seems to evoke mainly neutral and negative responses such as ration, consciousness, and disappointment [48].

The selection of non-alcoholic versus alcoholic drinks was recently investigated by Blackwell et al., 2020. The results suggested that availability interventions to encourage healthier selection, respectively choosing nonalcoholic rather than alcoholic drinks, may be most effective when changing the relative availability of options, i.e., increasing the proportion of non-alcoholic drinks and consequently decreasing the proportion of available alcoholic drinks [49].

When asked what information they have regarding special beers, the majority of the respondents were familiar with the low alcohol content of special beer (Figure 4a). However, nearly 40% of students have no information about this beer segment. This student category should be targeted by the awareness AC campaigns. By providing accurate information about the benefits of consuming special beers, students might feel encouraged to consume more special beers, reducing AC. Only a small percentage of responders, around 10%, are aware of the nutritional properties of special beer.

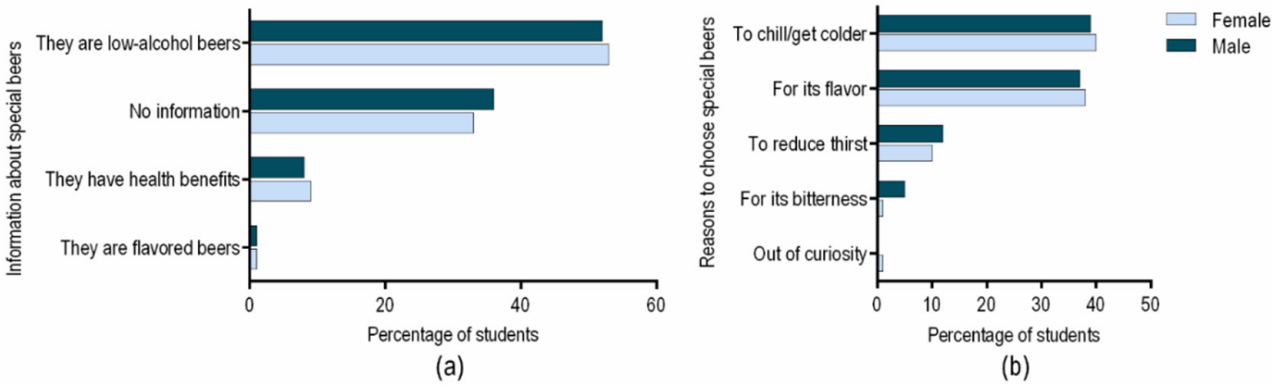

Figure 4. Information about the special beers already knew by students at the time of questionnaire (**a**). The main reasons why male and female students are choosing to drink special beers (**b**).

Concerning the health benefits of special beers, Wright et al. have shown that alcoholic beverages, namely wine and beer, are considered healthier than soda or diet soda [50]. This demonstrates that the alcohol content is not the deciding factor when ranking the healthfulness of a beverage. The same study indicated that the simple positioning of the nutritional information on the label of a beverage, even to an alcohol beverage, significantly influenced the consumers perception of its healthfulness.

The functional and potential health properties of special beer have been extensively reviewed in several studies [13,51,52]. While lacking high contents of alcohol, NAB and LAB can be more nutritious and potentially functional than regular beer [13]. NAB and LAB are a reliable source of vitamins, minerals, soluble fibers, polyphenols, and flavonoids [13]. The non-alcoholic beer fraction might improve bone health in postmenopausal women, and the effects of beer on body hydration [53]. When ingested before physical exercises, beer with lower alcohol content has a better rehydration effect, while alcohol-free beer may even have a positive impact on electrolyte homeostasis [54] or gut microbiota [55]. Different studies have focused on the effects of moderate alcoholic and NAB consumption on health and diseases, including cardiovascular disease, obesity, diabetes, cancer, cognitive decline, osteoporosis, with promising results, nevertheless need further particular in-depth investigations [13,56,57]. The consumption of NAB (0.9% ABV) also seems to have a protective action over learning and memory abilities [58]. Franco et al., have examined the effect of NAB on anxiety levels in a stressed population. Subjects rated their stress levels lower after drinking NAB for 14 nights, compared to a control period when they did not drink. All these studies came to the same conclusion: that drinking 330 mL of NAB during evening meals on two weeks may decrease feelings of anxiety and stress. These results are promising, but it must be stressed related research is still at an early stage [59]. A non-alcoholic beer component, β-pseudouridine, was found to be a potent protector against the damage caused by radiation (radioprotective effect) [60,61]. Potential properties of beers' nonalcoholic fractions are presented in Figure 5.

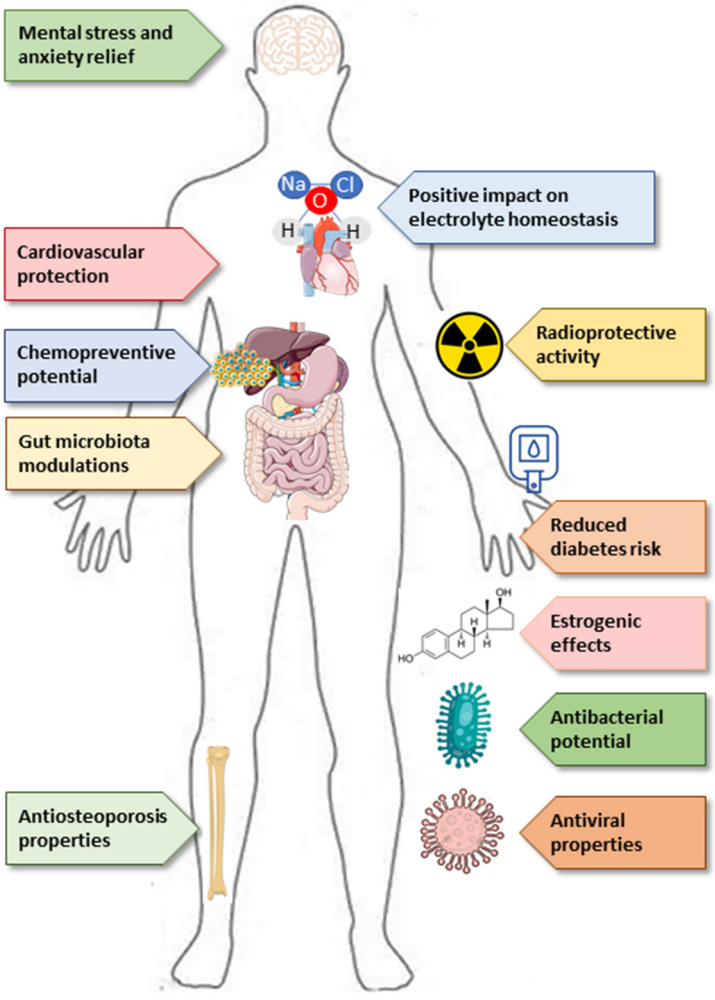

Figure 5. Potential properties of beer's nonalcoholic fractions [13,51–61].

Finally, we wanted to understand how taste preferences can shape the desire for special beer consumption. Thus, participants of our study were asked about the reasons for choosing a special beer. Most students associated special beer consumption with relaxation, sensory properties and refreshing attributes (Figure 4b). If special beer is chosen for these reasons, public health organizations, along with brewers, could exploit this segment of beer as a powerful instrument to combat heavy AC. Special beer consumption could contribute to reduce alcohol related harm, delivering the same refreshing and sensorial benefits [62].

4. Conclusions

The participants in the study were not heavy social drinkers, the majority of students, male and female alike, occasionally reporting AC. Even so, the reported consequences of alcohol intake on health and academic performance were significant. Our results show that AC frequency is related to the contextual influences (places, social contexts, community/friendship, special moments). Most male students start consuming alcohol in their teenage (14–16 years old), while female students are prolonging this period until the first stage of adulthood. We were able to find a statistically significant relationship between the age of first alcohol use and the existence of an alcoholic family member. Concerning the taste preferences, male students tend to prefer bitter drinks, while female students prefer the sweet taste, choosing the special beers over the usual ones. The students' residence may also influence the choice of special beers consumption over the normal ones.

To our knowledge, this is the first study to assess how students relate to special beer as an alternative to the regular beer and how taste can influence the preference for certain beers. The obtained results can serve and guide the Romanian brewers to improve the aroma quality for special beers, to make them more attractive, especially for young people. In Romania, and also in the EU, various strategies can be applied to improve the special beer acceptability. Increasing the availability of NAB and LAB options in public spaces could ease students to identify alternatives to regular beer. Also, in alcohol awareness campaigns and advertisements, the image of drinking special beers (NAB and LAB) should be promoted as a positive, energetic, and less risky experience in order to reach a larger number of consumers.

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References

1. Rose, P.A.; Schuckman, H.E.; Oh, S.S.; Park, E.-C. Associations between Gender, Alcohol Use and Negative Consequences among Korean College Students: A National Study. *Int. J. Environ. Res. Public Health* **2020**, *17*, 5192. [CrossRef]
2. Salanta, C.L.; Tofana, M.; Pop, C.; Coldea, T.; Mudura, E. Beverage Alcohol Choice Among University Students: Perception, Consumption and Preferences. *Bull. Univ. Agric. Sci. Vet. Med. Cluj-Napoca Food Sci. Technol.* **2017**, *74*, 23. [CrossRef]
3. Sudhinaraset, M.; Wigglesworth, C.; Takeuchi, D.T. Social and Cultural Contexts of Alcohol Use. *Alcohol Res. Curr. Rev.* **2016**, *38*, 35–45.

4. Davoren, M.P.; Demant, J.; Shiely, F.; Perry, I.J. Alcohol consumption among university students in Ireland and the United Kingdom from 2002 to 2014: A systematic review. *BMC Public Health* **2016**, *16*, 173. [CrossRef]
5. Chu, J.J.; Jahn, H.J.; Khan, M.H.; Kraemer, A. Alcohol consumption among university students: A Sino-German comparison demonstrates a much lower consumption of alcohol in Chinese students. *J. Health Popul. Nutr.* **2016**, *35*, 25. [CrossRef]
6. Schwartz, S.J.; Petrova, M. Prevention Science in Emerging Adulthood: A Field Coming of Age. *Prev. Sci.* **2019**, *20*, 305–309. [CrossRef]
7. Htet, H.; Saw, Y.M.; Saw, T.N.; Htun, N.M.M.; Mon, K.L.; Cho, S.M.; Thike, T.; Khine, A.T.; Kariya, T.; Yamamoto, E.; et al. Prevalence of alcohol consumption and its risk factors among university students: A cross-sectional study across six universities in Myanmar. *PLoS ONE* **2020**, *15*, e0229329. [CrossRef] [PubMed]
8. Salanţă, L.C.; Tofană, M.; Pop, C.R.; Pop, A.; Coldea, T.; Mihai, M. Risk Factors Associated with Alcohol Consumption Among Romanian University Students- Preliminary Research. *Bull. Univ. Agric. Sci. Vet. Med. Cluj-Napoca Food Sci. Technol.* **2018**, *75*, 86–89. [CrossRef]
9. Larsen, E.L.; Smorawski, G.A.; Kragbak, K.L.; Stock, C. Students' drinking behavior and perceptions towards introducing alcohol policies on university campus in Denmark: A focus group study. *Subst. Abus. Treat. Prev. Policy* **2016**, *11*, 17. [CrossRef] [PubMed]
10. Bratberg, G.H.; Wilsnack, S.C.; Wilsnack, R.; Haugland, S.H.; Krokstad, S.; Sund, E.R.; Bjørngaard, J.H. Gender differences and gender convergence in alcohol use over the past three decades (1984–2008), The HUNT Study, Norway. *BMC Public Health* **2016**, *16*, 723. [CrossRef]
11. Peltier, M.R.; Verplaetse, T.L.; Mineur, Y.S.; Petrakis, I.L.; Cosgrove, K.P.; Picciotto, M.R.; McKee, S.A. Sex differences in stress-related alcohol use. *Neurobiol. Stress* **2019**, *10*, 100149. [CrossRef] [PubMed]
12. Foster, K.T.; Hicks, B.M.; Iacono, W.G.; McGue, M. Gender differences in the structure of risk for alcohol use disorder in adolescence and young adulthood. *Psychol. Med.* **2015**, *45*, 3047–3058. [CrossRef]
13. Salanță, L.C.; Coldea, T.E.; Ignat, M.V.; Pop, C.R.; Tofană, M.; Mudura, E.; Borșa, A.; Pasqualone, A.; Anjos, O.; Zhao, H. Functionality of Special Beer Processes and Potential Health Benefits. *Processes* **2020**, *8*, 1613. [CrossRef]
14. Salanță, L.C.; Coldea, T.E.; Ignat, M.V.; Pop, C.R.; Tofană, M.; Mudura, E.; Borșa, A.; Pasqualone, A.; Zhao, H. Non-Alcoholic and Craft Beer Production and Challenges. *Processes* **2020**, *8*, 1382. [CrossRef]
15. Young-Wolff, K.C.; Wang, P.; Tuvblad, C.; Baker, L.A.; Raine, A.; Prescott, C.A. Drinking experience uncovers genetic influences on alcohol expectancies across adolescence. *Addiction* **2015**, *110*, 610–618. [CrossRef]
16. Baines, L.; Jones, A.; Christiansen, P. Hopelessness and alcohol use: The mediating role of drinking motives and outcome expectancies. *Addict. Behav. Rep.* **2016**, *4*, 65–69. [CrossRef] [PubMed]
17. World Health Organization. Global Status Report on Alcohol and Health. 2018. Available online: https://www.who.int/publications/i/item/9789241565639 (accessed on 20 January 2021).
18. Nehlin, C.; Öster, C. Measuring drinking motives in undergraduates: An exploration of the Drinking Motives Question-naire-Revised in Swedish students. *Subst. Abus. Treat. Prev Policy* **2019**, *14*, 49. [CrossRef]
19. Hingson, R.W.; Heeren, T.; Winter, M.R. Age at drinking onset and alcohol dependence: Age at onset, duration, and severity. *Arch. Pediatr. Adolesc Med.* **2006**, *160*, 739–746. [CrossRef]
20. Prieto-Ursúa, M.; Baena, B.C.; Caperos, J.M.; Falcón, C.M.; Olivares, J.U. Alcohol consumption in adolescents: The predictive role of drinking motives. *Psicothema* **2020**, *32*, 189–196. [PubMed]
21. Grant, B.F. The impact of a family history of alcoholism on the relationship between age at onset of alcohol use and DSM-IV alcohol dependence: Results from the National Longitudinal Alcohol Epidemiologic Survey. *Alcohol Health Res. World* **1998**, *22*, 144–147. [PubMed]
22. Zheng, Y.; Brendgen, M.; Dionne, G.; Boivin, M.; Vitaro, F. Genetic and environmental influences on developmental trajectories of adolescent alcohol use. *Eur. Child Adolesc. Psychiatry* **2019**, *28*, 1203–1212. [CrossRef]
23. Zheng, Y.; Brendgen, M.; Girard, A.; Dionne, G.; Boivin, M.; Vitaro, F. Peer Alcohol Use Differentially Amplifies Genetic and Environmental Effects on Different Developmental Trajectories of Adolescent Alcohol Use. *J. Adolesc. Health* **2019**, *65*, 752–759. [CrossRef] [PubMed]
24. Yoo, H.H.; Cha, S.W.; Lee, S.Y. Patterns of Alcohol Consumption and Drinking Motives Among Korean Medical Students. *Med. Sci. Monit.* **2020**, *26*, e921613–e921621. [CrossRef] [PubMed]
25. Thrul, J.; Kuntsche, E. Interactions Between Drinking Motives and Friends in Predicting Young Adults' Alcohol Use. *Prev. Sci.* **2016**, *17*, 626–635. [CrossRef] [PubMed]
26. Paswan, A.K.; Gai, L.; Jeon, S. Alcohol and college students: Reasons, realization and intention to quit. *J. Bus. Res.* **2015**, *68*, 2075–2083. [CrossRef]
27. Zadarko-Domaradzka, M.; Barabasz, Z.; Sobolewski, M.; Nizioł-Babiarz, E.; Penar-Zadarko, B.; Szybisty, A.; Zadarko, E. Alcohol Consumption and Risky Drinking Patterns among College Students from Selected Countries of the Carpathian Euroregion. *BioMed Res. Int.* **2018**, *2018*, 1–9. [CrossRef] [PubMed]
28. Kuntsche, E.; Wicki, M.; Windlin, B.; Roberts, C.; Nic Gabhainn, S.; Van Der Sluijs, W.; Aasvee, K.; De Matos, M.G.; Dankulincová, Z.; Hublet, A.; et al. Drinking Motives Mediate Cultural Differences but Not Gender Differences in Adolescent Alcohol Use. *J. Adolesc. Health* **2015**, *56*, 323–329. [CrossRef]
29. Castaño-Perez, G.A.; Calderon-Vallejo, G.A. Problems associated with alcohol consumption by university students. *Rev. Latino-Am. Enferm.* **2014**, *22*, 739–746. [CrossRef]

30. White, A.; Hingson, R. The burden of alcohol use: Excessive alcohol consumption and related consequences among college students. *Alcohol Res.* **2013**, *35*, 201–218.
31. Turrisi, R.; Mallett, K.A.; Mastroleo, N.R.; Larimer, M.E. Heavy Drinking in College Students: Who Is at Risk and What Is Being Done About It? *J. Gen. Psychol.* **2006**, *133*, 401–420. [CrossRef]
32. Nasui, B.A.; Popa, M.; Popescu, C.A. Drinking Patterns and Behavioral Consequences: A Cross-Sectional Study Among Romanian University Students. *Slov. J. Public Health* **2016**, *55*, 59–66. [CrossRef] [PubMed]
33. Rada, C.; Ispas, A.T. Alcohol consumption and accentuated personality traits among young adults in Romania: A cross-sectional study. *Subst. Abus. Treat. Prev. Policy* **2016**, *11*, 1–13. [CrossRef]
34. Salanţă, L.C.; Tofană, M.; Mudura, E.; Pop, C.; Coldea, T. The Alcoholic Beverage Consumption Preference of University Students: A Preliminary Romanian Case Study. *Bull. Univ. Agric. Sci. Vet. Med. Cluj-Napoca Food Sci. Technol.* **2016**, *73*, 33–39. [CrossRef]
35. Hoyle, J.; Miller, B.L.; Stogner, J.M.; Posick, C.; Blackwell, B.S. Analyzing Predictors of Drinking and Driving among Gender Cohorts within a College Sample. *Am. J. Crim. Justice* **2018**, *43*, 754–767. [CrossRef]
36. Cederbaum, A.I. Alcohol Metabolism. *Clin. Liver Dis.* **2012**, *16*, 667–685. [CrossRef]
37. Dir, A.L.; Bell, R.L.; Adams, Z.W.; Hulvershorn, L.A. Gender Differences in Risk Factors for Adolescent Binge Drinking and Implications for Intervention and Prevention. *Front. Psychiatry* **2017**, *8*, 289. [CrossRef] [PubMed]
38. Nolen-Hoeksema, S. Gender differences in risk factors and consequences for alcohol use and problems. *Clin. Psychol. Rev.* **2004**, *24*, 981–1010. [CrossRef]
39. Bachmanov, A.A.; Kiefer, S.W.; Molina, J.C.; Tordoff, M.G.; Duffy, V.B.; Bartoshuk, L.M.; Mennella, J.A. Chemosensory Factors Influencing Alcohol Perception, Preferences, and Consumption. *Alcohol. Clin. Exp. Res.* **2003**, *27*, 220–231. [CrossRef]
40. Kranzler, H.R.; Sandstrom, K.A.; Van Kirk, J. Sweet Taste Preference as a Risk Factor for Alcohol Dependence. *Am. J. Psychiatry* **2001**, *158*, 813–815. [CrossRef] [PubMed]
41. Betancur, M.I.; Motoki, K.; Spence, C.; Velasco, C. Factors influencing the choice of beer: A review. *Food Res. Int.* **2020**, *137*, 109367. [CrossRef]
42. Vecchio, R.; Decordi, G.; Grésillon, L.; Gugenberger, C.; Mahéo, M.; Jourjon, F. European consumers' perception of moderate wine consumption on health. *Wine Econ. Policy* **2017**, *6*, 14–22. [CrossRef]
43. Jackowski, M.; Trusek, A. Non-alcoholic beer production—An overview. *Pol. J. Chem. Technol.* **2018**, *20*, 32–38. [CrossRef]
44. Salanță, L.C.; Socaci, S.A.; Tofană, M.; Mudura, E.; Pop, C.R.; Nagy, M.; Odagiu, A. Characterization of volatile components in hop pellets using in-tube extraction GC-MS analysis. *Rom. Biotechnol. Lett.* **2017**, *23*, 13541–13550.
45. Salanţă, L.C.; Tofană, M.; Socaci, S.; Mudura, E.; Fărcaş, A.; Pop, C.; Pop, A.; Odagiu, A. Characterisation of hop varieties grown in romania based on their contents of bitter acids by HPLC in combination with chemometrics approach. *Czech. J. Food Sci.* **2015**, *33*, 148–155. [CrossRef]
46. O'Shea, R.S.; Dasarathy, S.; McCullough, A.J.; Practice Guideline Committee of the American Association for the Study of Liver Diseases; Practice Parameters Committee of the American College of Gastroenterology. Alcoholic liver disease. *Hepatology* **2010**, *51*, 307–328. [CrossRef]
47. Spence, C.; Van Doorn, G. Does the Shape of the Drinking Receptacle Influence Taste/Flavour Perception? A Review. *Beverages* **2017**, *3*, 33. [CrossRef]
48. Silva, A.P.; Jager, G.; Van Bommel, R.; Van Zyl, H.; Voss, H.-P.; Hogg, T.; Pintado, M.; De Graaf, C. Functional or emotional? How Dutch and Portuguese conceptualise beer, wine and non-alcoholic beer consumption. *Food Qual. Prefer.* **2016**, *49*, 54–65. [CrossRef]
49. Blackwell, A.K.M.; De-Loyde, K.; Hollands, G.J.; Morris, R.W.; Brocklebank, L.A.; Maynard, O.M.; Fletcher, P.C.; Marteau, T.M.; Munafò, M.R. The impact on selection of non-alcoholic vs alcoholic drink availability: An online experiment. *BMC Public Health* **2020**, *20*, 526–529. [CrossRef]
50. Wright, C.A.; Bruhn, C.M.; Heymann, H.; Bamforth, C.W. Beer and Wine Consumers' Perceptions of the Nutritional Value of Alcoholic. *J. Food Sci.* **2008**, *73*, 8–11. [CrossRef]
51. Mellor, D.D.; Hanna-Khalil, B.; Carson, R. A Review of the Potential Health Benefits of Low Alcohol and Alcohol-Free Beer: Effects of Ingredients and Craft Brewing Processes on Potentially Bioactive Metabolites. *Beverages* **2020**, *6*, 25. [CrossRef]
52. Osorio-Paz, I.; Brunauer, R.; Alavez, S. Beer and its non-alcoholic compounds in health and disease. *Crit. Rev. Food Sci. Nutr.* **2019**, *60*, 1–14. [CrossRef] [PubMed]
53. Trius-Soler, M.; Vilas-Franquesa, A.; Tresserra-Rimbau, A.; Sasot, G.; Storniolo, C.E.; Estruch, R.; Lamuela-Raventós, R.M. Effects of the Non-Alcoholic Fraction of Beer on Abdominal Fat, Osteoporosis, and Body Hydration in Women. *Molecules* **2020**, *25*, 3910. [CrossRef] [PubMed]
54. Castro-Sepulveda, M.; Johannsen, N.; Astudillo, S.; Jorquera, C.; Álvarez, C.; Zbinden-Foncea, H.; Ramírez-Campillo, R. Effects of Beer, Non-Alcoholic Beer and Water Consumption before Exercise on Fluid and Electrolyte Homeostasis in Athletes. *Nutriments* **2016**, *8*, 345. [CrossRef]
55. Hernández-Quiroz, F.; Nirmalkar, K.; Villalobos-Flores, L.E.; Murugesan, S.; Cruz-Narváez, Y.; Rico-Arzate, E.; Hoyo-Vadillo, C.; Chavez-Carbajal, A.; Pizano-Zárate, M.L.; García-Mena, J. Influence of moderate beer consumption on human gut microbiota and its impact on fasting glucose and β-cell function. *Alcohol* **2020**, *85*, 77–94. [CrossRef]
56. Mahli, A.; Seitz, T.; Freese, K.; Frank, J.; Weiskirchen, R.; Abdel-Tawab, M.; Behnam, D.; Hellerbrand, C. Therapeutic Application of Micellar Solubilized Xanthohumol in a Western-Type Diet-Induced Mouse Model of Obesity, Diabetes and Non-Alcoholic Fatty Liver Disease. *Cells* **2019**, *8*, 359. [CrossRef] [PubMed]

57. Scalbert, A.; Manach, C.; Morand, C.; Rémésy, C.; Jiménez, L. Dietary Polyphenols and the Prevention of Diseases. *Crit. Rev. Food Sci. Nutr.* **2005**, *45*, 287–306. [CrossRef]
58. Merino, P.; Santos-López, J.; Mateos, C.; Meseguer, I.; Garcimartín, A.; Bastida, S.; Sánchez-Muniz, F.; Benedí, J.; González-Muñoz, M. Can nonalcoholic beer, silicon and hops reduce the brain damage and behavioral changes induced by aluminum nitrate in young male Wistar rats? *Food Chem. Toxicol.* **2018**, *118*, 784–794. [CrossRef]
59. Franco, L.M.; Galán, C.; Bravo, R.M.R.; Bejarano, I.; Penaslledo, E.M.; Rodríguez, A.B.; Barriga, C.; Cubero, J. Effect of non-alcohol beer on anxiety: Relationship of 5-HIAA. *Neurochem. J.* **2015**, *9*, 149–152. [CrossRef]
60. Monobe, M.; Arimoto-Kobayashi, S.; Ando, K. β-Pseudouridine, a beer component, reduces radiation-induced chromosome aberrations in human lymphocytes. *Mutat. Res. Toxicol. Environ. Mutagen.* **2003**, *538*, 93–99. [CrossRef]
61. Sohrabvandi, S.; Mortazavian, A.; Rezaei, K. Health-Related Aspects of Beer: A Review. *Int. J. Food Prop.* **2012**, *15*, 350–373. [CrossRef]
62. Burton, R.; Henn, C.; Lavoie, D.; O'Connor, R.; Perkins, C.; Sweeney, K.; Greaves, F.; Ferguson, B.; Beynon, C.; Belloni, A.; et al. A rapid evidence review of the effectiveness and cost-effectiveness of alcohol control policies: An English perspective. *Lancet* **2017**, *389*, 1558–1580. [CrossRef]

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Effect of Goji Berries and Honey on Lactic Acid Bacteria Viability and Shelf Life Stability of Yoghurt

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Abstract

The probiotic properties and the viability of lactic acid bacteria of fermented dairy products can be improved by addition of bioactive compounds originating from natural sources (e.g. goji berries). This study aimed to evaluate how goji berries and honey affect the sensorial quality of yoghurt, the chemical properties, the viability of lactic acid bacteria (LAB) and the concurrent microflora development. Two types of yoghurts (yoghurt with goji berries and yoghurt with honey and goji berries) were developed. The addition of honey affected the entire yoghurt microflora including LAB, manifesting bactericidal effect. The addition of goji berries maintained the viability of LAB at probiotic levels (10^6-10^7 log CFU/ml) during 21 days of storage; compared to classic yoghurt, LAB viability decreased during storage at 10^3 log CFU/ml. Goji berries also improved sensory acceptance of consumers. The results obtained in this study collect information that enables the use of goji berries as enhancer of probiotic levels in yoghurt, while honey can provide bacteriostatic/bactericidal effect for contaminants.

Keywords: consumer acceptance, chemical parameters, plant extracts, probiotic

Introduction

Fermented dairy products are popular because of the health benefits provided by the ingestion of probiotics generated by the consumption of these products (Butel, 2014; Goktepe *et al.*, 2006; Guarner and Schaafsma, 1998; Khalid, 2011; Sanders, 2003; Wang, 2009). Among all of the fermented dairy products yoghourt is the most consumed (Cruz *et al.*, 2010; Cruz *et al.*, 2013; de Oliveira, 2014; Saint-Eve *et al.*, 2006), probably due to the positive perception on the market as being seen by the consumers as a functional dairy product containing living microorganisms like lactic acid bacteria (LAB), streptococci, bifidobacteria or their combinations, coming from the starter cultures, recognised as ingredients that promote human health (Davis, 2014; Goktepe *et al.*, 2006; Kent *et al.*, 2014; Khalid, 2011; Ouwehand *et al.*, 2015; Rastall *et al.*, 2002; Sanders *et al.*, 2010). The market generated a need for fermented milk products that are fermented and processed in new conditions or enriched with bioactive compounds (Sun-Waterhouse *et al.*, 2013; Zamfir *et al.*, 2006).

Studies regarding the addition of different categories of bioactive molecules in yoghurt, including free-cell of probiotics, entrapped in different matrices and symbiotic forms (Brinques *et al.*, 2011; Chavarri *et al.*, 2010; Krasaekoopt *et al.*, 2003; Lourens-Hattingh *et al.*, 2001; Pinto *et al.*, 2012; Stanton *et al.*, 2001) and a wide range of plant extracts with various active properties as red berries (Breme *et al.*, 2014; Cruz *et al.*, 2010; Ścibisz *et al.*, 2012; Sun-Waterhouse *et al.*, 2013), grape and grape seed extracts (Chouchouli *et al.*, 2013; Coda *et al.*, 2012; Karaaslan *et al.*, 2011; Tseng *et al.*, 2013), pomegranate peel extract (El-Said *et al.*, 2014), tea extracts (Jaziri *et al.*, 2009; Ye *et al.*, 2012) could be easily found. Researchers attempted to make the yoghurt a better environment for LAB and a source of bioactive compounds by addition of valuable molecules (Breme *et al.*, 2014; do Espírito Santo *et al.*, 2011).

The nutritional impacts of LAB and health benefits still continue to arise the interest of scientists who discover new potentials as food and valuable ingredients. Systems that can emphasize the great potential of probiotics are of interest. Saccharides are a good source food for these valuable bacteria, being utilized mostly as probiotics (Rastall *et al.*, 2002; Teitelbaum *et al.*, 2002; Wang, 2009). The benefits brought by carotenoid consumption include reduction of cancer risk or cardiovascular diseases (Pintea *et al.*, 2005; Pintea *et al.*, 2011; Socaciu *et al.*, 2000), improving vision (Pintea *et al.*, 2011) and a healthy tan looking effect.

Polyphenols possess strong antioxidant activities being free radical scavengers, electron donors and strong metal chelators (Andjelković *et al.*, 2006), helping in the prevention of lipid peroxidation (Vodnar *et al.*, 2014). Several reports have shown

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Table 1. Experimental design-treatments and responses

Treatments	Responses	Treatments	Responses
Sensory evaluation		Physicochemical and microbiological analysis	
YC (classic yoghurt)	X	YC_i (classic yoghurt-initial)	xx
YG_3 (yoghurt with 3% goji berries)	X	YG_{7i} (yoghurt with 7% goji berries-initial)	xx
YG_5 (yoghurt with 5% goji berries)	X	YHG_{7i} (yoghurt with honey and 7% goji berries-initial)	xx
YG_7 (yoghurt with 7% goji berries)	X	YC_m (classic yoghurt-middle of storage-14th day of storage)	xx
YHG_3 (yoghurt with honey and 3% goji berries)	X	YG_{7m} (yoghurt with 7% goji berries-middle of storage-14th day of storage)	xx
YHG_5 (yoghurt with honey and 5% goji berries)	X	YHG_{7m} (yoghurt with honey and 7% goji berries-middle of storage-14th day of storage)	xx
YHG_7 (yoghurt with honey and 7% goji berries)	X	YC_f (classic yoghurt-final of storage-21st day of storage)	xx
		YG_{7f} (yoghurt with 7% goji berries-final of storage-21st day of storage)	xx
		YG_{7f} (yoghurt with 7% goji berries-final of storage-21st day of storage)	xx

that polyphenols prevent the proliferation of degenerative diseases, clearly improving the condition of oxidative stress biomarkers (Bunea *et al.,* 2013; Chedea *et al.*, 2010).

Lycium barbarum (goji berries or wolfberries, *Solanaceae* family) represent a rich source of chemical, having health promoting properties: ocular neuroprotecti (Srinivasan, 2014), hepato-protective (Liu *et al.,* 2015), antitumoral (How *et al.,* 2014; Martínez *et al.,* 2014), antioxidative and immunomodulatory effects (Xiao *et al.,* 2012). These properties are related to the saccharides, caroteinoids and some phenolics in the soluble fraction (Bondia-Pons *et al.,* 2014; Inbaraj *et al.,* 2010; Wang *et al.,* 2010; Xiao *et al.,* 2012; Yang *et al.,* 2013). Honey contains phenolic acids and their derivates, flavonoids and hydrogen peroxide (Brudzynski, 2006; Brudzynski *et al.,* 2011); it has high osmolarity, low pH and water activity (Voidarou *et al.,* 2011). Thus, honey could provide good bacteriostatic or bactericide effect. Research showed that the redox potential can be reduced by supplementing yoghurt with bioactive compounds from natural sources (Perna *et al.,* 2014; Zalibera *et al.,* 2008). Moreover, the viability of LAB (*L. bulgaricus* and *S. thermophilus*) could be increased by reducing the redox potential with addition of bioactive compounds from natural sources (Zalibera *et al.,* 2008).

In this study it was investigated how goji berries and honey affected the sensorial quality, the chemical properties, the viability of lactic acid bacteria and concurrent microflora in yoghurt. Two types of yoghurt were obtained with different concentrations of goji berries and honey.

Materials and methods

Yoghurt preparation

Whole milk was provided by UASVM farm together with an analysis bulletin (fat content - 3.87%; protein content - 3.40%; crioscopic point: - 0.60 °C; non-fat dry matter - 9.10%; density - 1.0295 g/cm^3). Classic yoghurt was prepared starting from whole milk (3.5% fat), pre-heated (homogenized) at 50-65 °C (150-200 atm), pasteurized at 85-90 °C (maintained for 20-30 min) and cooled at 45-48 °C. Starter mezophylic culture Lyofast Y450B (Sacco, Cadorago, Italy) containing *Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus* (ratio 1:1) was added (5 units at 250 L milk which correspond to 0.5 x 10^{12} CFU/ml) to start fermentation. The yoghurt was stored at 43-45 °C for 3 hours, pre-cooled at 18-20 °C, cooled at 2-8 °C and stored at this temperature for further analysis (Jimborean and Ţibulcă, 2013).

The classic yoghurt was supplemented with 3%, 5% and 7% (w/w) goji berries, after the inoculation with starter culture. The same technology was used for yoghurt with honey and goji berries; polyfloral honey 3% (w/w) was added before fermentation and goji berries in amount of 3%, 5%, and 7% (w/w) after fermentation. Goji berries and honey were purchased from a local market.

Total phenolic content of goji berries

Goji berries (2.5 g) were cut in small pieces, homogenized using a rotary magnetic stirrer with 10 mL distilled water, centrifuged at 3,000×*g* for 10 min. The supernatant was analyzed spectrophotometrically using Folin-Ciocâlteu method. Aliquots of 2.375 mL distilled water were mixed with 0.025 mL extract, 0.150 mL Folin-Ciocâlteu reagent and 0.450 mL Na_2CO_3 (7.5%). Absorbance was read at 750 nm (Biotek multiplate reader) after keeping the samples for 2 hours in the dark. Results were expressed as mg of gallic acid equivalents (GAE) 100 g^{-1} goji berries.

Experimental design

Two types of yoghurt were obtained in this study: yoghurt with goji berries (YG) and yoghurt with honey and goji berries (YHG). Classic yoghurt (YC) was the control sample. Goji berries were added in proportions of 3%, 5% and 7% (w/v) in classic yoghurt (YG_3, YG_5 and YG_7) and in yoghurt with honey (YHG_3, YHG_5 and YG_7). Samples codification and experimental design are shown in Table 1.

Yoghurt with 3%, 5% and 7% goji berries (YG_3, YG_5, YG_7) and yoghurt with honey (3%) and goji berries (YHG_3, YHG_5, YHG_7), were sensory evaluated. Further studies (chemical and microbiological analysis) were conducted on classic yoghurt, yoghurt with 7% goji berries, and yoghurt with honey and 7% goji berries during a shelf life of 21 days at 4 °C. The samples were analyzed initial (i), in the 14th day of storage (m) and in the 21st day of storage (f). Fat, proteins, lactic acid, lactose, glucose, fructose, sucrose, total sugars, total solids and solids non-fat were tested. *Salmonella* spp., *Enterobacter* spp. and *Escherichia coli* were determined as microbial contaminants and *Streptococcus thermophilus* and *Lactobacillus bulgaricus* as lactic acid bacteria.

Sensory evaluation of yoghurt

A 9-point hedonic test was used to determine consumer's preference of yoghurt. Yoghurt with 3%, 5% and 7% goji berries (YG_3, YG_5, YG_7) and yoghurt with honey (3%) and goji berries (YHG_3, YHG_5, YHG_7) were sensory evaluated. A panel of 30 trained assessors (male and female) participated to this study. The response categories ranged from 1-extreme dislike, to 9-extreme like.

Chemical analysis of yoghurt

10 mL of yoghurt were homogenized using a stomacher (Bagmixer®-100MiniMix®, Interscience, Arpents, France) before the chemical analysis, as sample preparation.

The chemical content (fat, proteins, lactic acid, lactose, glucose, fructose, sucrose, total sugars, total solids and solids non-fat) of the yoghurt was determined using the MilkoScan FT2 analyser (Foss, Hillerød, Denmark).

The method was based on a mathematic procedure that allowed splitting the interferogram in sinus functions, each one representing a wavelength. The interferogram was then introduced in a spectrophotometer and converted in a larger spectral image of the sample. The results were expressed as percentage.

Determination of lactic acid bacteria in yoghurt

Lactic acid bacteria in yoghurt with 7% goji berries and in yoghurt with honey and goji berries was initially determined, after the 14th day of storage and after the 21st day of storage. Yoghurt samples were ten-fold diluted, placed on MRS broth/M17 broth, (Oxoid, Basingstoke, UK) and incubated in anaerobic conditions for 72 h at 37 °C (*L. bulgaricus)* and for 48 h at 37 °C (*S. thermophilus)*. Plates containing 30 to 300 CFU were counted. The confirmation was made by specific test (Gram affinity, colony aspect and catalase +).

Determination of microbial pathogens in yoghurt

Three strains of Gram negative bacteria (*Salmonella* spp., *Enterobacter* spp. and *Escherichia coli)* and one Gram positive strain (*Staphylococcus* spp.) were tested for yoghurts and aqueous extract of goji (5 g goji berries in 45 mL physiological serum). Honey was microbiological evaluated according to the same methodology as goji berries.

Identification of Salmonella spp.

The presence of *Salmonella* was determined according to SR ISO 6579/1997 method. For the pre-enrichment stage, the sample was suspended in Buffered Peptone Water (Laboratorios Conda, Madrid, Spain). For the enrichment stage, 1.0 mL of sample was inoculated on RVS broth (Merck, Darmstadt, Germany) and incubated at 42 °C for 24 h. The isolation was made by inoculating the bacterial suspension obtained in the enrichment phase on XLD Agar (Oxoid, Basingstoke, UK) and Brilliant Green Agar (modified CM0329, Oxoid, Basingstoke, UK). The incubation was made at 35-37 °C for 20-24 h (another 24 h if necessary). The confirmation was made on characteristic colonies using selective growth mediums. The results were expressed as colony forming units per gram (CFU 25 ml^{-1}).

Identification of Enterobacter spp.

The presence of *Enterobacter* was made according to SR-ISO 21528-2/2007 method. Briefly, 1.0 mL of the diluted sample was transferred to a sterile Petri dish. Aliquots of 15 mL of Violet Red Bile Glucose Agar (Lab M Ltd., Lancashire, UK) were poured over the sample and maintained at 45±1 °C. In order to ensure semi-anaerobic conditions, another 15 mL of VRBGA agar were poured into the Petri dish. Incubation was made at 35 °C for 24 h. The results were expressed as Log CFU/ml.

Identification of Escherichia coli

The presence of *E. coli* was determined according to SR ISO 7251/1996. 1.0 mL of the diluted sample was uniformly distributed into a sterile Petri dish and then TBX Agar (Oxoid, Basingstoke, UK) was poured and mixed. The incubation was made at 35 °C for 24 h. The results were expressed as Log CFU/ml.

Identification of Staphylococcus aureus

SR EN ISO 6888-2/A-1/2005 standard method was used. Briefly, 1.0 mL of the diluted sample was transferred to a sterile Petri dish covered with Baird-Parker agar (Oxoid, Basingstoke, UK) supplemented with Egg Yolk Tellurite Supplement (SR 00540, Oxoid, Basingstoke, UK) and spread using a Drigalsky spatula. The results were expressed as Log CFU/ml.

Statistical analysis

Statistical analysis of data was performed by Minitab Statistical software version 16.1.0 (LEAD Technologies, Inc.). The analysis of variance was assessed by two-way ANOVA and significant differences among the means of samples were analyzed by Tukey's test with a 95% confidence level.

Results and discussions

Total phenolic content of goji berries

The total phenolic content (TPC) of goji berries water extract obtained in this study was 132.26 mg GAE 100 g^{-1} goji berries. The study of Hunaefi *et al.* (2012) reports that phenolic compounds are secondary metabolites that can interfere with the LAB fermentation process through their antioxidant properties.

Donno *et al.* (2014) evaluated TPC of various cultivars of fresh goji berries and determined values ranging from 255.87 to 281.91 mg GAE g^{-1} fresh weight (FW), while Medina *et al.* (2011) obtained higher values in dry goji berries extracted in ethanol (895 mg GAE g^{-1} DW). Differences can be attributed to genotype, cultivars (Donno *et al.,* 2014), extraction type, mainly solvent polarity (Medina *et al.*, 2011) processing and method sensibility.

Sensory evaluation by Hedonic test

The sensory evaluation showed that the consumers preferred yoghurt with 7% goji berries (8.21 points on hedonic scale). Yoghurt with goji berries and honey was less accepted by consumers. Yoghurt with honey and 7% goji berries scored 7.4 points, while yoghurt with honey and 3% goji berries scored the lowest 6.9 points on hedonic scale (Fig. 1).

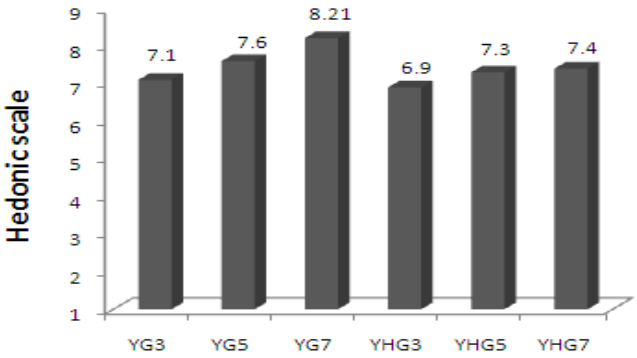

Fig. 1. Graphical representation of the sensory evaluation of yoghurt, according to the hedonic scale

Table 2. Results of the physicochemical evaluation of tested yoghurts

Sample	Fat (%)	Proteins (%)	Lactic acid (%)	Lactose (%)	Sucrose (%)	Glucose (%)	Fructose (%)	Total sugars (%)	Total solids (%)	Solids non-fat (%)
YC_i	4.51±0^{d}	3.66±0^{f}	0.61±0.01^{cd}	4.77±0^{a}	0.04±0.01^{e}	0.00±0^{f}	0.20±0^{f}	4.14±0^{g}	13.84±0^{i}	9.24±0^{h}
YG_{7i}	4.50±0^{d}	3.73±0^{e}	0.61±0^{bcd}	4.72±0.01^{b}	0.02±0^{e}	0.03±0^{e}	0.24±0^{e}	4.17±0^{f}	13.96±0^{g}	9.31±0^{g}
YHG_{7i}	4.52±0.01^{d}	3.67±0.01^{f}	0.60±0.01^{d}	4.74±0^{b}	0.02±0^{e}	0.00±0^{f}	0.21±0^{f}	4.15±0.01^{fg}	13.90±0^{h}	9.25±0^{h}
YC_m	4.32±0^{e}	4.18±0.01^{b}	0.63±0^{bc}	3.82±0^{d}	0.35±0.01^{ab}	1.96±0^{c}	3.45±0.01^{a}	8.76±0.01^{a}	20.91±0.01^{a}	16.40±0.01^{c}
YG_{7m}	4.20±0^{f}	4.22±0^{a}	0.63±0.01^{bc}	3.65±0.01^{f}	0.27±0^{d}	2.06±0^{a}	3.45±0.01^{a}	8.72±0.01^{b}	20.85±0.01^{c}	16.56±0.01^{a}
YHG_{7m}	4.22±0.01^{f}	4.23±0.01^{a}	0.63±0^{b}	3.76±0.01^{e}	0.35±0^{a}	2.01±0^{b}	3.40±0.01^{b}	8.74±0.01^{ab}	20.88±0.01^{b}	16.49±0.01^{b}
YC_f	5.51±0.01^{a}	4.14±0.01^{c}	1.17±0.0^{a}	3.87±0.01^{c}	0.30±0^{c}	1.32±0^{d}	2.35±0^{c}	7.13±0.01^{c}	20.16±0.01^{d}	14.46±0.01^{f}
YG_{7f}	5.42±0.01^{c}	4.08±0.01^{d}	1.17±0.0^{a}	3.82±0.01^{d}	0.27±0^{d}	1.32±0^{d}	2.31±0^{d}	6.99±0^{e}	20.06±0.01^{f}	14.56±0.01^{d}
YHG_{7f}	5.46±0.01^{b}	4.13±0^{c}	1.17±0.0^{a}	3.87±0^{c}	0.33±0^{b}	1.31±0^{d}	2.31±0^{d}	7.04±0.01^{d}	20.13±0.01^{e}	14.52±0.01^{e}

*Values are presented as mean ± standard deviation of three replicates; Different letters within columns indicates statistically significant differences at $p<0.05$ (Tukey's test)

1) YC_i (classic yoghurt-initial); YG_{7i} (yoghurt with 7% goji berries-initial); YHG_{7i} (yoghurt with honey and 7% goji berries-initial); YC_m (classic yoghurt-14th day of storage);

2) YG_{7m} (yoghurt with 7% goji berries-14th day of storage); YHG_{7m} (yoghurt with honey and 7% goji berries-14th day of storage);

3) YC_f (classic yoghurt-21st day of storage); YG_{7f} (yoghurt with 7% goji berries-21st day of storage); YHG_{7f} (yoghurt with honey and 7% goji berries-21st day of storage)

Table 3. Effects of yoghurt type, storage time and their first-degree interaction on fat (%), proteins (%), lactic acid (%), lactose (%), sucrose (%), glucose (%), fructose (%), total sugars (%), total solids (%), solids non-fat (%) and their percentage contribution

Factor	Fat (%)	Proteins (%)	Lactic acid (%)	Lactose (%)	Sucrose (%)	Glucose (%)	Fructose (%)	Total sugars (%)	Total solids (%)	Solids non-fat (%)
Yoghurt type (YT)										
YC	4.8^{a}	4.0^{b}	0.8^{a}	4.2^{a}	0.2^{a}	1.1^{c}	2.0^{a}	6.7^{a}	18.3^{a}	13.4^{c}
YG	4.7^{c}	4.0^{a}	0.8^{a}	4.1^{c}	0.2^{b}	1.1^{a}	2.0^{a}	6.6^{c}	18.3^{b}	13.5^{a}
YHG	4.7^{b}	4.0^{a}	0.8^{a}	4.1^{b}	0.2^{a}	1.1^{b}	2.0^{b}	6.6^{b}	18.3^{a}	13.4^{b}
SD/Contribution (%)	***/0.34	**/0.12	n.s./0	***/0.72	***/2.32	***/0.05	***/0.01	***/0.01	**/0.001	***/0.02
Storage time (ST)										
Initial	4.5^{b}	3.7^{c}	0.6^{c}	4.7^{a}	0.0^{c}	0.0^{c}	0.2^{c}	4.2^{c}	13.9^{c}	9.3^{c}
Middle stage of storage	4.2^{c}	4.2^{a}	0.6^{b}	3.7^{c}	0.3^{a}	2.0^{a}	3.4^{a}	8.7^{a}	20.1^{b}	16.5^{a}
Final stage of storage	5.5^{a}	4.1^{b}	1.2^{a}	3.8^{b}	0.3^{b}	1.3^{b}	2.3^{b}	7.0^{b}	20.9^{a}	14.5^{b}
SD/Contribution (%)	***/99.48	***/98.59	***/99.96	***/98.98	***/96.33	***/99.91	***/99.98	***/99.97	***/99.98	***/99.98
YT x ST										
SD/Contribution (%)	*/0.17	***/1.26	n.s./0.01	***/0.29	***/1.30	***/0.04	***/0.01	***/0.02	***/0.02	***/0.003

1) Different letters indicates statistically significant differences at $p<0.05$ (Tukey's test)

2) Significant differences (SD) are denoted by asterisks: *$p<0.05$; **$p<0.01$; ***$p<0.001$; n.s. $p\geq0.05$, non-significant

3) YC (classic yoghurt); YG (yoghurt with 7% goji berries); YHG (yoghurt with honey and 7% goji berries)

In general, fruity yoghurts are popular among consumers (Kailasapathy *et al.*, 2008). Senaka Ranadheera *et al.* (2012) also reported a higher preference of yoghurts supplemented with fruits. No previous report evaluated the sensory attributes of fruity yoghurt supplemented with honey. In this study the addition of honey influenced negatively the consumer's perception.

Chemical evaluation of yoghurts

Chemical analysis evaluated the effects of fortification and shelf life stability on yoghurt (Table 2). The effects of yoghurt type, storage time and crossed treatment interaction on chemical properties were analyzed by two-way ANOVA and Tukey's test. Significant differences were observed in chemical attributes dependent on yoghurt type, storage time and cross treatment interaction (Table 3).

Sucrose had the highest percentage of contribution of yoghurt type (2.32%, $p<0.001$); sucrose accumulated in yoghurts towards the end of shelf life. The quantity of sucrose increased during storage for all types of yoghurt. Sucrose from honey was solubilized in yoghurt and lead to high values of sugars (Tewari *et al.*, 2004) (Table 2). This increase might occur due to efflux of intracellular carbohydrates associated with the disaccharide metabolism and can be observed in case of LAB. The lowest percentage contribution on yoghurt type was determined for total solids followed by fructose, total sugars and solid non-fat.

An increasing tendency for glucose and fructose was observed until the 14th day of storage because saccharides from honey and goji berries were solubilized. This can be also explained by the fact that lactose was decomposed by lactic acid bacteria. Moreover, LAB have the ability to decrease the carbohydrates content by fermentation process.

Storage influenced the content of lactic acid with a contribution of 99.96%. The content of lactose decreased and lactic acid increased during storage (lactose is decomposed into lactic acid). Lactic acid showed insignificant differences in relation to yoghurt type, but a direct contribution to storage time and an increasing tendency was found towards the end of shelf life (Table 3).

Storage time influenced significantly the content of total solids, solids non-fat, fructose (99.98%), total sugars (99.97%) and glucose (99.91%). Lactic acid bacteria consumed glucose and fructose resulting in low quantities at the end of the storage. In case of yoghurt with 7% goji berries, glucose decreased from 2.06% to 1.32%, while fructose decreased from 3.45% to 2.35%. In yoghurt honey and with 7% goji berries, glucose decreased from 2.01% to 1.31% and fructose from 3.40% to 2.31%. Classic yoghurt showed a similar decreasing tendency for glucose and fructose, from 1.96% to 1.32% and from 3.45% to 2.35%, respectively.

Solid non-fat, total solids and total sugars increased until the 14th day of storage and decreased in the 21st day of storage; the type of yoghurt had little influence on these parameters. The total sugar content for yoghurt with 7% goji berries ranged from 4.17% (initial) to 8.72% in the 14th day of storage and decreased at 6.99% in the 21st day of storage. The same tendency was observed in case of total solids and solid non-fat (Table 2). The higher amount of total solids (including fat and protein content) was found in yoghurts in the 21st day of storage.

Table 4. Microbiological evaluation of yoghurt, goji berries and honey

Contaminant	Samples										
	YC_i	YC_m	YC_f	YG_{7i}	YG_{7m}	YG_{7f}	YHG_{7i}	YHG_{7m}	YHG_{7f}	Goji berries	Honey
Salmonella log CFU/ 25ml	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
E. coli log CFU/ml	ND	ND	ND	ND	$0.35x10^2$±1.77	$0.55x10^2$±1.77	ND	ND	$0.72x10^2$±2.0	$0.35x10^3$±1.77	ND
Enterobacter log CFU/ ml	ND	ND	ND	ND	$0.6x10^2$±0.44	$0.35x10^2$±1.77	ND	ND	$0.4x10^2$±1.11	$1.6x10^3$±0.44	ND
S. aureus log CFU/ ml	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Lactic acid Bacteria											
Str. thermophilus log CFU/ ml	$1.38x10^7$	$1.46x10^6$	$1.50x10^3$	$2.14x10^8$	$1.99x10^7$	$1.76x10^7$	$1.7x10^7$	$1.63x10^6$	$2.06x10^5$	NA	NA
L. bulgaricus log CFU/ ml	$1.59x10^7$	$1.51x10^6$	$1.33x10^3$	$2.17x10^8$	$2.71x10^7$	$1.67x10^7$	$1.30x10^8$	$1.33x10^6$	$1.71x10^5$	NA	NA

*Values are presented as mean ±standard deviation of three replicates

[1] YC_i (classic yoghurt-initial); YG_{7i} (yoghurt with 7% goji berries-initial); YHG_{7i} (yoghurt with honey and 7% goji berries-initial)

[2] YC_m (classic yoghurt-14th day of storage); YG_{7m} (yoghurt with 7% goji berries-14th day of storage); YHG_{7m} (yoghurt with honey and 7% goji berries-14th day of storage)

[3] YC_f (classic yoghurt-21st day of storage); YG_{7f} (yoghurt with 7% goji berries-21st day of storage); YHG_{7f} (yoghurt with honey and 7% goji berries-21st day of storage

Crossed treatment interaction revealed significant differences in the content of sucrose (1.30% contribution, $p<0.001$) and proteins (1.26% contribution, $p<0.001$), while the smallest percentage of contribution was determined in case of solids non-fat (0.003%, $p<0.001$) and fructose (0.02%, $p<0.001$).

Lactic acid bacteria

Lactic acid bacteria concentration remained at probiotic value level (10^6-10^7 Log CFU/ml) (Shiby *et al.,* 2013) in yoghurt with 7% goji berries addition during storage and decreased in yoghurt with honey and 7% goji berries (Table 4).

S. termophillus concentration decreased during storage in all yoghurt types because of its sensitivity to lactic acid. A slight maintenance of *S. termophillus* concentration was observed during the first week of storage. One of the most important properties of lactic acid bacteria is their ability to decrease the carbohydrates content by fermentation. Until the 14th day of storage, lactic acid bacteria had sufficient carbohydrates to synthesize lactic acid (Tables 2 and 4); in the 21st day of storage, lactic acid bacteria viability decreased and once with it the ability to metabolize carbohydrates.

The growth of probiotics/prebiotics and yoghurt starter culture in the presence of fruit juices is strain specific (Vinderola *et al.*, 2002). The data presenting the growth and viability of lactobacilli in this particular medium is scarce (Kailasapathy *et al.,* 2008; Vinderola *et al.,* 2002).

The production of lactic acid by *Lactobacillus* is influenced by the medium pH. Chookietwattana (2014) reported that at an initial pH of 6.5 the lactic acid production was high, whereas at a pH of 5.0/5.5 the production of lactic acid was prohibited.

The same evolution in lactic acid bacteria concentration was observed by Michael *et al.* (2010), while Rotar *et al.* (2007) reported a significant decrease of the viable germs to 10^3 log CFU/ml at the end of the storage period in classic yoghurt.

Microbiological evaluation of yoghurt

Salmonella spp. and *Staphylococcus* spp. were absent in all types of yoghurt (Table 3). Contamination with *E. coli* was determined in goji berries yogurts. In the 14th day of storage the yoghurt with goji berries resulted positive for contamination. The presence of *E. coli* was noted in case of yoghurt with honey and goji berries in the 21st day of storage.

The presence of *Enterobacter* was detected in the 21st day of storage for yoghurt with honey and goji berries. The initial levels of *Enterobacter* spp. in goji berries was $1.6x10^3$ log CFU/ml; these values were reduced in the 21st day of storage at $0.4x10^2$ log CFU/ml, proving bacteriostatic effect by adding honey. *Salmonella* spp. and *Staphylococcus* spp. were absent in all types of yoghurt (Table 3). Contamination with *E. coli* was determined in goji berries. In the 14th day of storage the yoghurt with goji berries resulted positive for contamination. The presence of *E. coli* was noted in case of yoghurt with honey and goji berries in the 21st day of storage. Literature reports the bactericide/bacteriostatic effect of honey (Brudzynski *et al.*, 2012; Brudzynski *et al.,* 2011; Voidarou *et al.*, 2011).

Conclusions

The addition of goji berries (7%) improved the sensory quality of classic yoghurt and increased the consumer's acceptance. Quality parameters (chemical parameters) were maintained during storage. Goji berries improved the lactic acid bacteria evolution and maintained the prebiotic value of yoghurt during storage. Concurrent microflora (contaminants) appeared when goji berries were added. The results obtained in this study collect information that enables the use of goji berries as enhancer of probiotic levels in yoghurt.

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References

Andjelković M, Van Camp J, De Meulenaer B, Depaemelaere G, Socaciu C, Verloo M, Verhe R (2006). Iron-chelation properties of phenolic acids bearing catechol and galloyl groups. Food Chemistry 98(1):23-31.

Bondia-Pons I, Savolainen O, Törrönen R, Martinez JA, Poutanen K, Hanhineva K (2014). Metabolic profiling of goji berry extracts for discrimination of geographical origin by non-targeted liquid chromatography coupled to quadrupole time-of-flight mass spectrometry. Food Research International 63 Part B:132-138.

Breme K, Guggenbühl B (2014). Aroma profile of a red-berries yoghurt drink by HS-SPME-GC-MS-O and influence of matrix texture on volatile aroma compound release of flavored dairy products. In: Flavour Science. Lopez VF (Ed) Academic Press, San Diego, pp 101-106.

Brinques GB, Ayub MZ (2011). Effect of microencapsulation on survival of *Lactobacillus plantarum* in simulated gastrointestinal conditions, refrigeration, and yogurt. Journal of Food Engineering 103(2):123-128.

Brudzynski K (2006). Effect of hydrogen peroxide on antibacterial activities of Canadian honeys. Canadian Journal of Microbiology 52(12):1228-1237.

Brudzynski K, Abubaker K, Miotto D (2012). Unraveling a mechanism of honey antibacterial action: Polyphenol/H2O2-induced oxidative effect on bacterial cell growth and on DNA degradation. Food Chemistry 133(2):329-336.

Brudzynski K, Kim L (2011). Storage-induced chemical changes in active components of honey de-regulate its antibacterial activity. Food Chemistry 126(3):1155-1163.

Bunea A, Rugină D, Sconța Z, Pop RM, Pintea A, Socaciu C, Tăbăran F, Grootaert C, Struijs K, Vancamp J (2013). Anthocyanin determination in blueberry extracts from various cultivars and their antiproliferative and apoptotic properties in B16-F10 metastatic murine melanoma cells. Phytochemistry 95:436-444.

Butel MJ (2014). Probiotics, gut microbiota and health. *Médecine et* Maladies Infectieuses 44(1):1-8.

Champagne CP, Green-Johnson J, Raymond Y, Barrette J, Buckley N (2009). Selection of probiotic bacteria for the fermentation of a soy beverage in combination with *Streptococcus thermophilus*. Food Research International 42(5-6):612-621.

Chavarri M, Maranon I, Ares R, Ibanez FC, Marzo F, Villaran MDC (2010). Microencapsulation of a probiotic and prebiotic in alginate-chitosan capsules improves survival in simulated gastro-intestinal conditions. Int J Food Microbiol 142(1-2):185-189.

Chedea VS, Braicu C, Socaciu C (2010). Antioxidant/prooxidant activity of a polyphenolic grape seed extract. Food Chemistry 121(1):132-139.

Chookietwattana K (2014). Lactic acid production from simultaneous saccharification and fermentation of Cassava starch by *Lactobacillus Plantarum* MSUL 903. APCBEE Procedia 8:156-160.

Chouchouli V, Kalogeropoulos N, Konteles SJ, Karvela E, Makris DP, Karathanos VT (2013). Fortification of yoghurts with grape (*Vitis vinifera*) seed extracts. LWT-Food Science and Technology 53(2):522-529.

Coda R, Lanera A, Trani A, Gobbetti M, Di Cagno R (2012). Yogurt-like beverages made of a mixture of cereals, soy and grape must: Microbiology, texture, nutritional and sensory properties. International Journal of Food Microbiology 155(3):120-127.

Cruz AG, Cadena RS, Castro WF, Esmerino EA, Rodrigues JB, Gaze L, Faria JF, Freitas MQ, Deliza R, Bolini HMA (2013). Consumer perception of probiotic yogurt: Performance of check all that apply (CATA), projective mapping, sorting and intensity scale. Food Research International 54(1):601-610.

Cruz AG, Walter EHM, Cadena RS, Faria JF, Bolini HMA, Pinheiro HP, Sant'ana AS (2010). Survival analysis methodology to predict the shelf-life of probiotic flavored yogurt. Food Research International 43(5):1444-1448.

Davis C (2014). Enumeration of probiotic strains: Review of culture-dependent and alternative techniques to quantify viable bacteria. Journal of Microbiological Methods 103:9-17.

de Oliveira MN (2014). Fermented milks-Fermented milks and yogurt. In: Encyclopedia of Food Microbiology (Second Edition). Tortorello CABL (Ed), Academic Press, Oxford pp 908-922.

do Espírito Santo AP, Perego P, Converti A, Oliveira MN (2011). Influence of food matrices on probiotic viability – A review focusing on the fruity bases. Trends in Food Science and Technology 22(7):377-385.

Donno D, Beccaro GL, Mellano MG, Cerutti AK, Bounous G (2014). Goji berry fruit (*Lycium* spp.): antioxidant compound fingerprint and bioactivity evaluation. Journal of Functional Foods doi:10.1016/j.jff.2014.05.020.

El-Said MM, Haggag HF, Fakhr El-Din HM, Gad AS, Farahat AM (2014). Antioxidant activities and physical properties of stirred yoghurt fortified with pomegranate peel extracts. Annals of Agricultural Sciences 59(2):207-212.

Goktepe I, Juneja VK, Ahmedna M (2006). Probiotics in food safety and human health, Taylor and Francis group, Boca Raton, Florida.

Guarner F, Schaafsma GJ (1998). Probiotics. International Journal of Food Microbiology 39(3):237-238.

How CW, Teruel JA, Ortiz A, Montenegro MF, Rodríguez-López JN, Aranda FJ (2014). Effects of a synthetic antitumoral catechin and its tyrosinase-processed product on the structural properties of phosphatidylcholine membranes. Biochimica et Biophysica Acta (BBA) - Biomembranes 1838(5):1215-1224.

Hunaefi D, Akumo DN, Riedel H, Smetanska I (2012). The effect of *Lactobacillus plantarum* ATCC 8014 and *Lactobacillus acidophilus* NCFM fermentation on antioxidant properties of selected *in vitro* sprout culture of *Orthosiphon aristatus* (java tea) as a model study. Antioxidants 1(1):4-32.

Inbaraj BS, Lu H, Kao TH, Chen BH (2010). Simultaneous determination of phenolic acids and flavonoids in *Lycium barbarum Linnaeus* by HPLC–DAD–ESI-MS. Journal of Pharmaceutical and Biomedical Analysis 51(3):549-556.

Jaziri I, Ben Slama M, Mhadhbi H, Urdaci MC, Hamdi M (2009). Effect of green and black teas (*Camellia sinensis* L.) on the characteristic microflora of yogurt during fermentation and refrigerated storage. Food Chemistry 112(3):614-620.

Jimborean MA, Țibulcă D (2013). Dairy Technology (in Romanian). Risoprint, Cluj-Napoca, 246 p.

Kailasapathy K, Harmstorf I, Phillips M (2008). Survival of *Lactobacillus acidophilus* and *Bifidobacterium animalis* ssp. *lactis* in stirred fruit

yogurts. LWT-Food Science and Technology 41(7):1317-1322.

Karaaslan M, Ozden M, Vardin H, Turkoglu H (2011). Phenolic fortification of yogurt using grape and callus extracts. LWT-Food Science and Technology 44(4):1065-1072.

Kent RM, Doherty SB (2014). Probiotic bacteria in infant formula and follow-up formula: Microencapsulation using milk and pea proteins to improve microbiological quality. Food Research International 64:567-576.

Khalid K (2011). An overview of lactic acid bacteria. International Journal of Biosciences 1(3):1-13.

Krasaekoopt W, Bhandari B, Deeth H (2003). Evaluation of encapsulation techniques of probiotics for yoghurt. International Dairy Journal 13(1):3-13.

Liu Y, Cao L, Du J, Jia R, Wang J, Xu P, Yin G (2015). Protective effects of *Lycium barbarum* polysaccharides against carbon tetrachloride-induced hepatotoxicity in precision-cut liver slices *in vitro* and *in vivo* in common carp (*Cyprinus carpio* L.). Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology 169:65-72.

Lourens-Hattingh A, Viljoen BC (2001). Yogurt as probiotic carrier food. International Dairy Journal 11(1-2):1-17.

Martínez V, Mitjans M, Vinardell MP (2014). Cytoprotective effects of polyphenols against oxidative damage. In: Polyphenols in human health and disease, Watson RR, Preedy VR, Zibadi S (Eds), Academic Press, San Diego pp 275-288.

Medina MB (2011). Determination of the total phenolics in juices and superfruits by a novel chemical method. Journal of Functional Foods 3(2):79-87.

Michael M, Phebus RK, Schmidt KA (2010). Impact of a plant extract on the viability of *Lactobacillus delbrueckii* ssp. *bulgaricus* and *Streptococcus thermophilus* in nonfat yogurt. International Dairy Journal 20(10):665-672.

Ouwehand AC, Röytiö H (2015). Probiotic fermented foods and health promotion. In: Advances in fermented foods and beverages, Holzapfel W (Ed), Woodhead Publishing pp 3-22.

Perna A, Intaglietta I, Simonetti A, Gambacorta E (2014). Antioxidant activity of yogurt made from milk characterized by different casein haplotypes and fortified with chestnut and sulla honeys. Journal of Dairy Science 97(11):6662-6670.

Pintea A, Diehl HA, Momeu C, Aberle L, Socaciu C (2005). Incorporation of carotenoid esters into liposomes. Biophysical Chemistry 118(1):7-14.

Pintea A, Rugină DO, Pop R, Bunea A, Socaciu C (2011). Xanthophylls protect against induced oxidation in cultured human retinal pigment epithelial cells. Journal of Food Composition and Analysis 24(6):830-836.

Pinto SS, Fritzen-Freire CB, Munoz IB, Barreto PLM, Prudencio ES, Amboni RDMC (2012). Effects of the addition of microencapsulated *Bifidobacterium* BB-12 on the properties of frozen yogurt. Journal of Food Engineering 111(4):563-569.

Rastall RA, Maitin V (2002). Prebiotics and synbiotics: towards the next generation. Current Opinion in Biotechnology 13(5):490-496.

Rotar MA, Semeniuc C, Apostu S, Suharoschi R, Mureşan C, Modoran C, Laslo C, Guş C, Culea M (2007). Researches concerning microbiological evolution of lactic acid bacteria to yoghurt storage during shelf-life. Journal of Agroalimentary Processes and Technologies 13(1):135-138.

Saint-Eve A, Lévy C, Martin N, Souchon I (2006). Influence of proteins on the perception of flavored stirred yogurts. Journal of Dairy Science 89(3):922-933.

Sanders ME (2003). Probiotics: considerations for human health. Nutrition Reviews 61:91-99.

Sanders ME, Akkermans LMA, Haller D, Hammerman C, Heimbach J, Hörmannsperger G, Huys G, Levy DD, Lutgendorff F, Mack D, Phothirath P, Solano-Aguilar G, Vaughan E (2010). Safety assessment of probiotics for human use. Gut Microbes 1(3):164-185.

Ścibisz I, Ziarno M, Mitek M, Zaręba D (2012). Effect of probiotic cultures on the stability of anthocyanins in blueberry yoghurts. LWT-Food Science and Technology 49(2):208-212.

Senaka Ranadheera C, Evans CA, Adams MC, Baines SK (2012). Probiotic viability and physico-chemical and sensory properties of plain and stirred fruit yogurts made from goat's milk. Food Chemistry 135(3):1411-1418.

Shiby VK, Mishra HN (2013). Fermented milks and milk products as functional foods-A review. Critical Reviews in Food Science and Nutrition 53(5):482-496.

Socaciu C, Jessel R, Diehl HA (2000). Competitive carotenoid and cholesterol incorporation into liposomes: effects on membrane phase transition, fluidity, polarity and anisotropy. Chemistry and Physics of Lipids 106(1):79-88.

Srinivasan K (2014). Polyphenols in vision and eye health. In: Handbook of nutrition, diet and the eye, Preedy VR (Ed), Academic Press, San Diego pp 413-421.

Stanton C, Gardiner G, Meehan H, Collins K, Fitzgerald G, Lynch PB, Ross RP (2001). Market potential for probiotics. Am J Clin Nutr 73(2):476s-483s.

Sun-Waterhouse D, Zhou J, Wadhwa SS (2013). Drinking yoghurts with berry polyphenols added before and after fermentation. Food Control 32(2):450-460.

Teitelbaum JE, Walker WA (2002). Nutritional impact of pre and probiotics as protective gastrointestinal organisms. Annual Review of Nutrition 22:107-138.

Tewari J, Irudayaraj J (2004). Quantification of saccharides in multiple floral honeys using fourier transform infrared microattenuated total reflectance spectroscopy. Journal of Agricultural and Food Chemistry 52(11):3237-3243.

Tseng A, Zhao Y (2013). Wine grape pomace as antioxidant dietary fibre for enhancing nutritional value and improving storability of yogurt and salad dressing. Food Chemistry 138(1):356-365.

Vinderola CG, Costa GA, Regenhardt S, Reinheimer JA (2002). Influence of compounds associated with fermented dairy products on the growth of lactic acid starter and probiotic bacteria. International Dairy Journal 12(7):579-589.

Vodnar DC, Socaciu C (2014). Selenium enriched green tea increase stability of L*actobacillus casei* and *Lactobacillus plantarum* in chitosan coated alginate microcapsules during exposure to simulated

gastrointestinal and refrigerated conditions. LWT-Food Science and Technology 57(1):406-411.

Voidarou C, Alexopoulos A, Plessas S, Karapanou A, Mantzourani I, Stavropoulou E, Fotou K, Tzora A, Skoufos I, Bezirtzoglou E (2011). Antibacterial activity of different honeys against pathogenic bacteria. Anaerobe 17(6):375-379.

Wang CC, Chang SC, Inbaraj BS, Chen BH (2010). Isolation of carotenoids, flavonoids and polysaccharides from *Lycium barbarum* L. and evaluation of antioxidant activity. Food Chemistry 120(1):184-192.

Wang Y (2009). Prebiotics: Present and future in food science and technology. Food Research International 42(1):8-12.

Xiao J, Liong EC, Ching YP, Chang RCC, So KF, Fung ML, Tipoe GL (2012). *Lycium barbarum* polysaccharides protect mice liver from carbon tetrachloride-induced oxidative stress and necroinflammation. Journal of Ethnopharmacology 139(2):462-470.

Yang X, Bai H, Cai W, Li J, Zhou Q, Wang Y, Han J, Zhu X, Dong M, Hu D (2013). *Lycium barbarum* polysaccharides reduce intestinal ischemia/reperfusion injuries in rats. Chemico-Biological Interactions 204(3):166-172.

Ye M, Liu D, Zhang R, Yang L, Wang J (2012). Effect of hawk tea (*Litsea coreana* L.) on the numbers of lactic acid bacteria and flavour compounds of yoghurt. International Dairy Journal 23(1):68-71.

Zalibera M, Staško A, Šlebodová A, Jančovičová V, Čermáková T, Brezová V (2008). Antioxidant and radical-scavenging activities of Slovak honeys – An electron paramagnetic resonance study. Food Chemistry 110(2):512-521.

Zamfir M, Vancanneyt M, Makras L, Vaningelgem F, Lefebvre K, Pot B, Swings J, de Vuyst L (2006). Biodiversity of lactic acid bacteria in Romanian dairy products. Systematic and Applied Microbiology 29(6):487-495.

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Dedicated to Professor Ioan Bâldea on the Occasion of His 80th Anniversary

EVALUATION OF BIOCHEMICAL AND MICROBIOLOGICAL CHANGES OCCURRING IN FRESH CHEESE WITH ESSENTIAL OILS DURING STORAGE TIME

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ABSTRACT. The current study aimed to determine the chemical composition and antibacterial activity of two essential oils extracted from herbs belonging to the family *Lamiaceae* (mint and oregano), and their beneficial impact on the biochemical and microbiological changes occurring in fresh cheese during storage time. Based on the essential oils results three types of fresh cheese were formulated and the consumer prefer sample were sensory evaluated using the 9-point hedonic test. In order to determine the stability during storage, the selected sample and the essential oil free control sample were sampled initially, after 6 and 12 days of storage than subjected to physicochemical (protein, fat, moisture, ash, total carbohydrates, and energy) and microbiological analyses (*S. aureus*, *E. coli*).

Keywords: *Antibacterial activity*, *Biochemical changes, essential oils, microbiologic, sensory evaluation, storage, volatile profile*

INTRODUCTION

In the recent years, cheese manufacturing and processing has transitioned from traditional art to science. Many of the cheese varieties have been developed and tested for different environmental conditions in order to meet the highly pretentious customer standards [1]. Fresh cheeses

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production involves the enzymatic coagulation of milk with rennet and/or other coagulating enzymes, which in some cases are combined with specific lactic bacteria [2]. It is well known that soft texture, low salt content, high acidity and moisture, which are characteristics of fresh cheeses, favor the growth of spoilage microorganisms, leading to consumer rejection and possible economic losses for the industry. Moreover, fresh cheeses are considered potential vehicles for *Escherichia coli*, a pathogen able to survive and grow even at refrigerate temperature. As a result, the manufacturing of cheeses involves the addition of synthetic preservatives, (e.g., potassium and sodium sorbate) in order to ensure the safety of fresh cheeses [3]. However, many studies revealed the negative effects of synthetic food preservatives on human health and the increasing resistance of microorganisms to these compounds. As a result, researchers are focused on the identification and use of natural preservatives like essential oils (EOs), which are volatile liquids distilled from different aromatic plant materials [4-7]. EOs from *Origanum vulgare L.* (oregano – OrEO) and *Mintha piperita* (mint – MiEO) are effective in inhibiting a range of cheese-related bacteria in vitro systems, making them a key alternatives for cheese preservation [8]. Considering the antioxidant activity and food preserving properties of OrEO and MiEO and the fact that they are natural products [7], which can be produced in organic condition, the selected EO could be used in organic food products with short shelf-life. Therefore, this study aimed to evaluate the physicochemical, microbiological and sensory aspects that characterized a fresh cheese made with the incorporation of OrEO and MiEO and the effects of these EOs on the cheese during refrigerated storage.

RESULTS AND DISCUSSION

Chemical Composition of Essential Oils

The volatile compounds detected by ITEX-GC/MS analysis in the two essential oils with their percentage composition are summarized in Table 1.

According to the results, the MiEO contained 39 constituents and the most important ones were α-pinene (3.57%), β-pinene (4.71%), Sabinene (2.04%), D-limonene (13.06%) and Eucalyptol (17.76%), all of them belonging to the monoterpene hydrocarbons class. The obtained results are similar to the aromatic profile presented by de Sousa Barros et al. [8] regarding essential oils extracted from different Mentha species. Similarly, OrEO included 21 major components among which p-cymene (13.76%), gamma terpinene (11.94%), D-limonene (34%) and thymol (19.38%) had the highest concentration which is also confirmed by the data presented by Sahbaz A. et al. [9]. The thymol belongs to the oxygenated monoterpenes class and the rest of them to monoterpene hydrocarbons.

Table 1. Volatile compounds profile of oregano and mint essential oils

Crt. No.	Compound	MiEO, %	OrEO, %
1.	Furan, 2,5-diethyltetrahydro-	0.14	-
2.	2-Hexenal, (E)-	-	0.29
3.	Origanene	-	2.37
4.	alpha.-Thujene	0.53	-
5.	alpha.-Pinene	**3.57**	1.51
6.	Camphene	0.15	0.39
7.	3-Methyl-cyclohexanone	0.20	-
8.	Sabinene	2.04	-
9.	beta.-Pinene	**4.71**	0.24
10.	beta.-Myrcene	0.67	4.78
11.	n.i.	0.04	-
12.	1-Methylene-4-(1-methylethenyl)- cyclohexane	0.07	-
13.	Octanal	-	0.20
14.	alpha.-Phellandrene	0.05	0.69
15.	3-Carene	0.05	0.24
16.	alpha.-Terpinene	0.27	3.49
17.	*p*-Cymene	0.08	**13.76**
18.	n.i.	2.43	-
19.	D-Limonene	**13.06**	**34.08**
20.	Eucalyptol	**17.76**	-
21.	beta-*trans*-Ocimene	0.13	0.06
22.	beta-*cis*-Ocimene	0.03	0.10
23.	gamma.-Terpinene	0.51	**11.94**
24.	n.i.	0.16	-
25.	Terpinolene	0.13	0.32
26.	Benzene, 2-ethenyl-1,3-dimethyl-	-	0.06
27.	beta.-Linalool	-	0.62
28.	Thymol	-	**19.38**
29.	Carvacrol	-	5.28
30.	Cyclohexanone, 5-methyl-2-(1-methylethyl)-	30.54	-
31.	Menthofuran	2.06	-
32.	Cyclohexanone, 5-methyl-2-(1-methylethyl)-, (2R-cis)-	5.99	-

Crt. No.	Compound	MiEO, %	OrEO, %
33.	Cyclohexanol, 5-methyl-2-(1-methylethyl)-, (1.alpha.,2.alpha.,5.alpha.)	0.86	-
34.	Cyclohexanol, 5-methyl-2-(1-methylethyl)-, (1.alpha.,2.beta.,5.alpha.)-(.+/-.)-	9.63	-
35.	Cyclohexanol, 5-methyl-2-(1-methylethyl)-, [1S-(1.alpha.,2.beta.,5.beta.)]-	0.15	-
36.	alpha.-Terpineol	0.09	-
37.	Pulegone	0.38	-
38.	Piperitone	0.13	-
39.	Cyclohexene, 4-methyl-1-(1-methylethyl)-	0.10	-
40.	Menthol, acetate	2.14	-
41.	Cyclohexane, 1-ethenyl-1-methyl-2-(1-methylethenyl)-4-(1-methylethylidene)-	0.07	-
42.	n.i.	0.06	-
43.	Caryophyllene	0.88	0.17
44.	alpha.-Caryophyllene	0.02	-
45.	Germacrene D	0.07	-
46.	n.i.	0.02	-
TOTAL		100	100

MiEO- mint essential oil, OrEO – oregano essential oil

Antibacterial Activity of Essential Oils

In order to evaluate the antibacterial activity, minimum inhibitory concentration tests of the studied essential oils were performed. According to the results shown in Table 2, minimum inhibitory concentrations values differ significantly between the two essential oils.

Table 2. Minimum inhibitory concentrations (MIC) of essential oils

Essential Oil	*E. coli* ATCC 25922	*S. aureus* ATCC 25923	*S. enteritidis* ATCC 13076	*L. monocytogenes* ATCC 19114
	µl/ml			
MiEO	0.56 ± 0.0	2.45 ± 0.0	1.59 ± 0.0	5.14 ± 0.0
OrEO	0.22 ± 0.0	0.13 ± 0.0	0.27 ± 0.0	0.13 ± 0.0

MiEO- mint essential oil, OrEO – oregano essential oil, *E. coli* - *Escherichia coli*, *S. aureus* - *Staphylococcus aureus*, *S. enteritidis* - *Salmonella enteritidis*, *L. monocytogenes* - *Listeria monocytogenes*. Values are results of three replicates. Control negativ, were 0.11 ± 0.0 for *E. coli* (ATCC 25922) and 0.05 ± 0.0 µg GE mL^{-1} for *S. aureus* (ATCC 25923), 0,24± 0.0 for *S. enteritidis,* 0.11 ± 0.0 for *L. monocytogenes* .

The results show that OrEO was the most bacteriostatic against all four of the selected bacteria, considering that it had the lowest MIC values. However, in the case of *E. coli*, the antibacterial activity of the two essential oils is comparable while for the other bacteria the differences are more significant. In the case of bacteria *S. aureus* and *L. monocytogenes* OrEO had the same antibacterial activity (0.13 µl/ml). The antibacterial activity of MiEO was also the most bacteriostatic against *E. coli*, followed by *S. enteritidis, S. aureus* and *L. monocytogenes*.

Sensorial Analysis

Considering that the sensorial quality of food products is a key factor in consumer's decision-making process, the Hedonic testing was used to determine consumer's attitude towards all three fresh cheese formulations samples by measuring the degree of acceptance of the new products. It is very important to note that the organoleptic properties of fresh cheese enhanced with essential oils remained acceptable to consumers and the quality level similar to the current commercially available products. This is also confirmed by the results shown in the Table 3 for sensorial evaluation of fresh cheese samples containing different type and level of essential oils compared to the control sample (without EOs).

Table 3. Results of sensorial evaluation

Sample	Appearance	Color	Texture	Odor	Taste	Overall acceptability
C.S.	7.1	7.0	6.2	7.2	7.1	6.9
CH. 0.03% MiEO	7.5	7.6	8.2	7.6	8	7.8
CH. 0.02% OrEO	7.0	7.0	6.4	6	7	6.6

C.S. – control sample, CH. 0.03% MiEO- cheese with 0.03% mint essential oil, CH. 0.02% OrEO – cheese with 0.02% oregano essential oil

It was found that the acceptability of the fresh cheese with 0.02% oregano essential oil was the lowest which can be attributed to the taste and the intense smell of OrEO. In contrast, the sample with 0.03% MiEO had the highest acceptability score (7.8) as well as for the other organoleptic characteristics. The mint essential oil has given a fresh taste and smell to the product and it reduces the fatty taste of the product. Moreover, the sensorial evaluation revealed that fresh cheese with 0.03% MiEO achieved higher score than the control cheese sample.

The biochemical analysis of the fresh cheese

The results of the biochemical analysis presented in Table 4 revealed that the physicochemical parameters for the cheese samples with and without MiEO were approximately the same during storage. It was observed that the gradual reduction in of cheeses moisture content during the storage led to an increase in total lipids, protein, and ash. According to the literature, this can be explained by the curd shrinkage, as a consequence of the acid production by starter culture, which helps to drain the whey from the cheese mass [3].

Table 4. The compositional parameters percentage values according to the ripening stages

Sample	Control sample			Fresh cheese with 0.03% MiEO		
Storage time, day	1	6	12	1	6	12
Fat, g/100 g	27.5 ± 0.1	28.63 ± 0.3	30.17 ± 0.1	28 ± 0.07	29.04±0.04	30.39 ± 0.09
Protein, g/100 g	17.98 ± 0.08	19.11± 0.1	21.07 ± 0.5	18.14 ± 0.1	19.28± 0.3	20.97 ± 0.2
Moisture, g/100 g	44.87 ± 0.2	43.08±0.06	41.93 ± 0.1	45.48 ± 0.05	44.17± 0.4	42.96 ± 0.03
Ash, g/100 g	2.6 ± 0.04	2.71 ± 0.07	2.85 ± 0.01	2.73 ± 0.06	2.8±0.02	2.97 ± 0.1
Total carbohydrates, g/100 g	7.05 ± 0.01	6.47 ± 0.2	3.98 ± 0.03	5.65 ± 0.02	4.71 ± 0.1	2.71 ± 0.08
Energy, kcal/100 g	347.62±0.09	359.99±0.1	371.73±0.2	347.16±0.07	357.32±0.1	368.23±0.06

MiEO- mint essential oil

The biochemical analysis of the fresh cheese with and without MiEO has revealed that the fat percentage shows a consistent upper trend during the ripening stages, from 28.0 ± 0.76 g/100 g within first day of ripening to 30.39 ±0.09 in the last stage (day 12).

Microbiological evaluation of cheese during storage

Quantitative detection of *Escherichia coli* (*E. coli*) and Staphylococcus *aureus* (*S.aureus*) was performed to establish the contribution of essential oil to the fresh cheese microbial load and to evaluate their safety. The European regulations on microbiological criteria for cheese preparations (European Union, 2005) contain limits for *E. coli* and *S. aureus* only. The results of fresh cheese microbiological examination, Table 5, indicate that MiEO has an important antimicrobial effect on the finished product in comparison to the control sample.

Table 5. Microbiological characteristics of cheese formulations

Sample	Storage time	*E. coli*	*S. aureus*
	day	log CFU/g	
Control sample	1	ABS	2.14
	6	ABS	2.34
	12	ABS	2.57
Fresh cheese with 0.03% MiEO	1	ABS	ABS
	6	ABS	ABS
	12	ABS	ABS

In the case of the fresh cheese with 0.03% MiEO, it can be seen that *S. aureus* is absent during storage which sustains the inhibitory effect of the MiEO. Moreover in the control sample *S. aureus* grow in the product during storage from 2.14-2.57 [log CFU/g. These findings clearly underline the beneficial impact of the addition of 0.03% MiEO on the biochemical and microbiological changes occurring in fresh cheese during storage time.

CONCLUSIONS

The present work proved that the incorporation of OrEO and MiEO can enhance the antimicrobial properties of fresh cheese during storage time, leading to the natural preservation of the product. Based on the results it can be concluded that OrEO was the most bacteriostatic against all four of the selected bacteria, due to the lowest MIC values, being followed by MiEO. On the other hand, sensory analysis revealed that the acceptability of the fresh cheese with 0.02% OrEO was the lowest while the sample with 0.03% MiEO had the highest acceptability score (7.8) due to the fresh taste and smell, which is related to the distinctive volatile profile of MiEO. As an overall conclusion it can be stated, the addition of 0.03% MiEO to the fresh cheese improves significantly its sensorial quality and stability during storage time, but without modifying the physicochemical parameters of the final product.

EXPERIMENTAL SECTION

Plant Materials and Essential Oils Extraction

The dried mint and oregano leaves were purchased from a company that markets food ingredients (Solina Group, Alba Iulia, Romania). Essential oils were obtained by hydrodistillation using 50 g dried leaves for both plants. The

extraction was performed for 3 h with 750 mL distilled water in a Clevenger-type apparatus (S.C. Energo-Metr S.R.L., Odorheiu Secuiesc, Romania). The essential oils were dried over anhydrous sodium sulphate and stored at 4 °C until analysis.

ITEX/GC-MS Analysis of Volatile Components

The extraction of volatile compounds was performed using the in-tube extraction technique (ITEX) as described in our previous work [10] using 1 µL of sample. The analysis of volatile compounds was carried out on a GCMS QP-2010 (Shimadzu Scientific Instruments, Kyoto, Japan) model gas chromatograph-mass spectrometer which can be used for measurement of various components including hydrogen [11], volatile organic compounds [12]. Next, the volatile compounds were separated on a Zebron ZB-5ms capillary column of 30 m × 0.25 mm i.d. × 0.25 µm film thickness. In all determinations, the carrier gas was He, 1 ml/min and the split ratio 1:20. The temperature program used for the column oven was: from 40 oC (kept at this temperature for 2 min) to 160 °C at 4 °C/min, then raised to 240 °C at 15 °C /min (kept at this temperature for 5 min). The injector, ion source and interface temperatures were set at 250 °C and the MS mode was electron impact (EI) at ionization energy of 70 eV. The scanned mass range was 40–650 m/z. Volatile compounds were tentatively identified using the spectra of reference compounds from NIST27 and NIST147 mass spectra libraries and verified by comparison with retention indices drawn from www.pherobase.com or www.flavornet.org (for columns with a similar stationary phase to the ZB-5ms column). Compounds were considered “tentatively identified” only in the case in which their mass spectra similarity value was above 85%. All peaks found in at least two of the three total ion chromatograms (TIC) were considered when calculating the total area of peaks (100%) and the relative areas of the volatile compounds.

Bacterial Strains

The following microorganisms were tested: *Escherichia coli* (ATCC 25922), *Staphylococcus aureus* (ATCC 25923), *Salmonella enteritidis* (ATCC 13076) and *Listeria monocytogenes* (ATCC 19114). All strains were grown into a test tube containing 10 mL sterile nutrient broth (Oxoid Ltd., Basingstoke, Hampshire, England) at 37 °C for 24 h in the case of *E. coli*, *S. aureus* and *S. enteritidis*, while *L. monocytogenes* at 37 °C for 30 h. The purity of the inoculums was confirmed by plating on appropriate selective media and microscopic examination of the Gram-stained smear (Optika microscope, B-252, M.A.D. Apparecchiature Scientifiche, Milan, Italy). A loopful of inoculums was transferred by streaking onto a selective medium: TBX for *E. coli*, BP (baird

parker) for *S. aureus*, XLD agar (Oxoid Ltd., Basingstoke, Hampshire, England) for *S. enteritidis* and Palcam agar base (Oxoid Ltd., Basingstoke, Hampshire, England) with added Palcam selective supplement for *L. monocytogenes*. Plates were incubated at 44 ◦C for 24 h *E. coli*, at 37 ◦C for 24 h in the case of *S. Aureus*, and *S. enteritidis* and at 37 ◦C for 30 h in the case of *L. monocytogenes*. Bacterial morphology was confirmed by optical microscopy. Several colonies were collected with a sterile inoculating loop, transferred into sterile saline solution (8.5 g L^{-1}), and adjusted to match the turbidity of a McFarland 0.5 standard (1.5×108 CFUmL^{-1}) [31]. Then, three serial 10-fold dilutions (107, 106, and 105 CFU mL^{-1}) were prepared using the sterile saline solution as diluent.

Fresh Cheese Manufacture

All cheese samples were elaborated in the Experimental Laboratory of Dairy Products (pilot scale) of the Faculty of Food Science and Technology, Cluj-Napoca (University of Agricultural Sciences and Veterinary Medicine Cluj-Napoca, Romania). The control sample (fresh cheese without EOs) was obtained using 100 l of cow milk with 3.4 % of fat content, which was previously pasteurized at 65˚C/30 min and cooled to 35˚C. Coagulation was performed at 35°C for 60 minutes using calcium chloride and 5 U selected cultures of lactic bacteria *(Lactococcus lactis ssp. lactis, Streptococcus thermophilus and Lactobacillus casei)* and microbial enzyme rennet in form of two cubes [13]. Once the curd was formed, it was smoothly cut into 4-5 cm cubes and shredded with harp up to 6-8 mm. The obtained curd was placed in perforated containers (10 cm diameter) for whey removal for 1 h at 18 °C. The cheese samples were vacuum packaged in sterile polyethylene bags and stored for 12 days in refrigerated storage at 4 °C. The two types of fresh cheese with 0.02% oregano EO and 0.03% mint EO were obtained based on the above steps, like the control sample, with the only difference that the selected EOs were added in the stage of coagulation. The amount of essential oils (mint and oregano) used has been established after the microbiological analyzes results and composition of the EOs.

Sensory evaluation of cheese

Sensory characteristics of cheese samples were evaluated by a panel of 40 untrained assessors, with a mean age of 25, consisted of students and staff members of the department. All samples were coded numerically and supplied in plastic dishes randomly. The 9-point hedonic scale test (1 being “dislike extremely” and 9 being “like extremely”) was used to evaluate all cheese samples. The main sensory attributes used in the assessment of the samples were appearance, color, texture, odor, taste, and overall acceptability.

Physicochemical parameters of cheese

The moisture, ash, total carbohydrates, total sugars, crude fat and crude protein of samples were determined according to AOAC procedures [14]. The cheeses samples moisture content was determined via drying in an oven at 105 °C until constant weight while the ash content was established by incineration at 600±15 °C. The crude protein content of the samples was estimated by the micro-Kjeldahl method, in which the sample was digested with a known quantity of concentrated H_2SO_4 in the Kjeldahl digestion apparatus. The crude fat content was determined in accordance with the Gerber method described by SR ISO 488 [15]. The amount of total carbohydrate resulted as a difference based on the following equation: 100 - (g moisture + g protein + g fat + g ash). The total energy was calculated using the following equation from the literature: energy (kcal) = 4 × (g protein + g carbohydrate) + 9 × (g lipid) [16, 17].

Microbiological evaluation of cheese

Detection of *Escherichia coli* was carried out using the method described in SR EN ISO 16649-2:2007 standard (International Organization for Standardization, 2007, 2007b). *Staphylococcus aureus* was determined using the method described in SR EN ISO 6888-1:2002 standard (International Organization for Standardization, 2002). Total combined yeasts and moulds count (TYMC) was not determined, because the European regulations on microbiological criteria for cheese preparations (European Union, 2005) contain limits only for *E. coli* and *S. aureus*.

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REFERENCES

[1] F.A. Tabaran, S.D. Dan, A. Tabaran, C. Bele, C. Catoi, M. Borzan, G. Valasutean, M. Mihaiu, *Studia UBB Chemia,* **2015**, *60,* 4, 85.
[2] C.M. Asensio, N.R. Grosso, H. Rodolfo Juliani, *LWT - Food Science and Technology,* **2015,** *60,* 2, 664.
[3] H.T. Diniz-Silva, J. Batista de Sousa, J. da Silva Guedes, R.d.C. Ramos do Egypto Queiroga, M.S. Madruga, J.F. Tavares, E. Leite de Souza, M. Magnani, *LWT - Food Science and Technology,* **2019**, 10.1016/j.lwt.2019.01.039

[4] L.-C. Salanta, M. Tofană, S.A. Socaci, E. Mudura, C. Pop, A. Pop, A. Cuceu, M. Nagy, *Hop and Medicinal Plants,* **2014**, *1-2,* 1.
[5] M. Nagy, S.A. Socaci, M. Tofană, C. Pop, C. Mureşan, A.V. Pop Cuceu, L. Salanţă, A.M. Rotar, *Bulletin of University of Agricultural Sciences and Veterinary Medicine Cluj-Napoca. Food Science and Technology,* **2015**, *72,* 1,
[6] C.A. Semeniuc, M.I. Socaciu, S.A. Socaci, V. Muresan, M. Fogarasi, A.M. Rotar, *Molecules,* **2018**, *23,* 9,
[7] N. Khorshidian, M. Yousefi, E. Khanniri, A.M. Mortazavian, Innovative Food Science *& Emerging Technologies,* **2018**, *45,* 62.
[8] A. de Sousa Barros, S.M. de Morais, P.A.T. Ferreira, Í.G.P. Vieira, A.A. Craveiro, R.O. dos Santos Fontenelle, J.E.S.A. de Menezes, F.W.F. da Silva, H.A. de Sousa, *Industrial Crops and Products,* **2015**, *76,* 557.
[9] A. Sahbaz, H. Isik, O. Aynioglu, K. Gungorduk, B.D. Gun, *European journal of obstetrics, gynecology, and reproductive biology,* **2014**, *177,* 44.
[10] S.A. Socaci, C. Socaciu, C. Mureşan, A. Fărcaş, M. Tofană, S. Vicaş, A. Pintea, *Phytochemical Analysis,* **2014**, *25,* 2, 161.
[11] L.-C. Pop, L. Sygellou, V. Dracopoulos, K.S. Andrikopoulos, S. Sfaelou, P. Lianos, *Catalysis Today,* **2015**, *252,* 157-161.
[12] J. Dewulf, H. Van Langenhove, G. Wittmann, **2002**, *21,* 637.
[13] D. Ţibulcă, M. Jimborean, A. Ţibulcă, *Romanian Biotechnological Letters,* **2017**, 10.26327RBL2017.67
[14] Official Methods of Analysis of AOAC INTERNATIONAL, 20th Edition ed., AOAC International; 20 edition.
[15] C.A. Semeniuc, A. Rotar, L. Stan, C.R. Pop, S. Socaci, V. Mireşan, S. Muste, *CyTA - Journal of Food,* **2015**, *14,* 2, 213.
[16] M. Fogarasi, S.A. Socaci, F.V. Dulf, Z.M. Diaconeasa, A.C. Farcas, M. Tofana, C.A. Semeniuc, *Molecules,* **2018**, *23,* 12
[17] M. Nagy, C.A. Semeniuc, S.A. Socaci, C.R. Pop, A.M. Rotar, C.D. Salagean, M. TofanĂ, *Food Science and Technology,* **2017**, *37,* 2, 315.

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Original Article

Inhibitory Effects of Iso-α and β Hop Acids Against *Pediococcus pentosaceus*

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Abstract

The goal of the research was to assess the inhibitory effects of hop extracts, iso-α and β acids, against *Pediococcus pentosaceus* bacteria, during a short incubation period, both in liquid selective media (high pH values) and beer wort fermentation (low pH values) and testing if the identified iso-α acid stress changes the activity of *S. cerevisiae boulardii* yeast and ethanol production. Flow cytometry analysis was used for bacterial and yeast cell viability. In relation to the antibacterial activity of β-acids, a lower viability of *Pediococcus pentosaceus* cells was observed after a short incubation period in selective media, under iso-α acid stress. In beer wort, for a mixed culture with *P. pentosaceus* bacteria and *S. cerevisiae boulardii* yeast, under iso-α acid stress conditions at pH 4.0-5.0, *Pediococcus pentosaceus* exhibited lower cell viability (20.7%) than in selective media (61.4%). Regarding iso-α hop acid on *S. cerevisiae boulardii* yeast, the results showed that iso-α does not change the *S. cerevisiae* activity but prevents the culture from being contaminated by *Pediococcus pentosaceus.* The results highlighted reliable inhibitory effects of iso-α and β-acids against *P. pentosaceus*, both at pH 6.0-7.0 and pH 4.0-5.0, which open the possibility of hops being used as a supplement to prevent beverage contamination with spoilage microorganisms.

Keywords: cell viability; hop acids; inhibition; *Pediococcus pentosaceus*; *Saccharomyces cerevisiae*

Introduction

Hop acids are one of the essential ingredients of beer, imparting its bitterness, flavour, and astringency. They have been known for thousands of years for their anti-inflammatory, antiseptic and sedative properties (Zanoli and Zavatti, 2008; Muthayan *et al*., 2011; Olsovska *et al*., 2016). Depending on the bacterial growth conditions, these bioactive compounds may exhibit either bacteriostatic or bactericidal activity (Muthayan *et al*., 2011; Cermak *et al*., 2017). Behr and Vogel (2009), demonstrated that the main factors affecting the antibacterial activity of hop compounds are the pH value and their ability to bind to the Mn^{2+} cations (Sakamoto and Konings, 2003; Behr and Vogel, 2009; Zhao *et al*., 2017). Low pH enhances antibacterial activity while at high pH values hop acids lose their inhibitory effects (Zhao *et al*., 2017). The antibacterial activity of hop compounds decreases with high pH values, because hop acids are weak acids and only undissociated forms are active (Cleemput *et al*., 2009). Antibacterial hop compounds, mainly iso-α acids, have been described as ionophores, which exchange pH for cellular divalent cations and thus, dissipate ion gradients across the cytoplasmic membrane (Behr *et al*., 2007). An investigation by Buggey *et al*. (2001) showed that more hydrophobic, reduced iso-α acids have greater antimicrobial activity than their naturally occurring analogues. There are various studies pointing out that due to the antimicrobial properties, hop compounds could be utilized in commercial fuel bioethanol industries to control the contaminant bacteria. These bacteria produce lactic and acetic acid and under low pH values adversely affect the viability of yeast (Vaughan *et al*., 2005).

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In terms of beer spoilage microorganisms, lactic acid bacteria are the predominant spoilers, *Lactobacillus* and *Pediococcus* being the most commonly reported strains (Takahashi *et al*., 2014). Regarding *Lactobacillus* and *Pediococcus* bacterial strains, it has been reported that some isolates develop mechanisms that confer resistance to hop compounds, thus apparently facilitating growth in beer (Haakensen *et al*., 2009). Many studies revealed that beer represents an unfavourable growth media for most of the microorganisms because of its low pH, lack of nutrients, presence of hop derived compounds and alcohol (Pinto *et al*., 2004; Iijima *et al*., 2008). The contamination with lactic acid bacteria in the brewery environment is a problem when microorganisms are able to grow in beer and cause off-flavours or turbidity in the final product from metabolites and sediment production (Maifreni *et al*., 2015). It is well known that the contaminant microorganisms compete with the *Saccharomyces cerevisiae* strain for micro and macro-nutrients and produce inhibitory end products such as acetic acid and lactic acid (Obi, 2017). These substances, have been shown to increase "lag" times, decrease growth rates, reduce biomass yields and even lead to *Saccharomyces cerevisiae* death in some media (Bayrock and Ingledew, 2004; Skinner-Nemec *et al*., 2007).

Considering that at high pH values hop bitter acids lose activity and that some strains of *Pediococcus* bacteria develop hop-resistance mechanisms, this study aimed to evaluate the antibacterial activity of iso-α and β-hop acids against *Pediococcus pentosaceus* during a short incubation period in liquid selective media at pH 6.0-7.0.

Furthermore, some factors affecting the growth rate were studied, in order to determine the inhibitory effects of iso-α acids at low pH values and to identify if the iso-α acid stress changes the *S.cerevisiae boulardii* activity and ethanol production. Due to the iso-β-acid degradation during the wort boiling process and their very low values in beer, we chose to analyze the antibacterial activity of iso-α hop acids in beer wort fermentation, contaminated with *Pediococcus pentosaceus*.

Materials and Methods

Bacteria strains and culture conditions

The microbial strains used in the experiment, were *Pediococcus pentosaceus* bacteria and *Saccharomyces cerevisiae* subsp. *boulardii* yeast, belonging to the Collection of Gembloux Agro Bio Tech University, Belgium. *Pediococcus pentosaceus* strain was inoculated in MRS liquid media at 30 °C, pH 6.5, until optical density $O.D._{600nm}$= 0.4 AbsU (*Pediococcus pentosaceus* culture A).

Saccharomyces cerevisiae boulardii strain was grown in selective liquid medium and incubated at 30 °C for 24 h, pH 6.5 (*S. cerevisiae boulardii* culture A). For testing the antibacterial activity during wort fermentation, *Pediococcus pentosaceus* - culture A was inoculated in wort at pH 5.0, until $O.D._{600nm}$ = 0.4 AbsU to obtain the final culture B. The same procedure was applied for the yeast strain, culture A was inoculated in wort and incubated for 2.5 h, until the optical density $O.D._{600nm}$ = 0.7 AbsU.

Hop acid compounds

To evaluate the antibacterial activity of hops, a clear yellow aqueous solution of potassium salts of hop-derived α-acids (30% m/v) and a clear brown aqueous solution of potassium salts of hop-derived β-acids (10% m/v), derived from a pure CO_2 supercritical extracted resin of hop, belonging to the Collection of Gembloux Agro Bio-Tech./Liège University, Belgium, were used.

Antimicrobial activity of iso-α and β-acids from hops against Pediococcus pentosaceus

The antibacterial activity of iso-α and β-acids from hops against *Pediococcus pentosaceus* in selective media, was performed according to the optimized method *(α,β-Inhibition Method)*. Samples were prepared by incubating *Pediococcus pentosaceus* culture in MRS liquid media with iso-α and β hop acids. The inoculation density was $O.D._{600nm}$ = 0.4 AbsU.

Five increasing concentrations of iso-α acids: 50 mg/l, 100 mg/l, 150 mg/l, 200 mg/l, 250 mg/l and β acids: 15 mg/l; 30 mg/l; 60 mg/l; 100 mg/l; 200 mg/l, were used. The control sample was prepared without hop extracts. Samples were incubated at 30 °C for 4.5 h under agitation on a rotary shaker at 100 rpm.

Growth characteristics of Saccharomyces cerevisiae boulardii under iso-α acid stress

The inhibitory effects of iso-α hop compounds in a mixed culture with *P. pentosaceus* and *Saccharomyces cerevisiae boulardii* were also studied. The experiments were carried out under optimal conditions for the *Saccharomyces cerevisiae boulardii* strain, both in wort and yeast selective media, (pH 5.0), at $O.D._{600nm}$ = 0.4 AbsU with five iso-α increasing concentrations: 20 mg/l; 50 mg/l; 100 mg/l; 150 mg/l; 300 mg/l. The contamination was performed with *Pediococcus pentosaceus* strain (culture B) and incubated at 30 °C for 4.5 h.

Cell viability assay

The analyses were carried out on a Genesys 20 Thermo Spectronic, Unicom model 4001/4 spectrofotometer using the optical density method. The cell incubation was monitored for 4.5 h in order to evaluate the antibacterial activity of hop acids. All samples were assessed spectrophotometrically at 600 nm after 30 min, 1.5 h, 2.5 h, 3.5 h, and 4.5 h.

Flow cytometry analysis

The analysis of GFP expression level was performed by Fluorescence Activated Cell Sorting (FACS) on a FACScan (Becton Dickinson) flow cytometer (Delvigne *et al*., 2011). The samples (1 ml) were transferred to microfuge tubes and cells were centrifugated at 14.000 rpm (16.000 xg) for 4 minutes. The supernatant was removed, cells were washed with cold phosphate-buffered saline (1 ml PBS) and after adding 10 µl propidium iodide (PI) the cells were gently vortexed and incubated at 30 °C for 15 min in the dark . Thereafter the cells were centrifugated at 14.000 rpm (16.000 x g) for 4 minutes. After removing the supernatant, cells were resuspended in 1ml PBS.

Analysis by flow cytometry was performed within 1 h. The results were analyzed by the FlowJo 7.6.1 software. For ethanol analysis by UV-method, an Enzymatic Bioanalysis Kit for Ethanol Assays, was used.

Results and Discussion

The inhibitory effects of iso-α and β-hop acids in MRS selective media

The results for accessing the growth of the *Pediococcus pentosaceus* strain under iso-α and β hop acids stress are shown in Fig. 1. It was observed that *Pediococcus pentosaceus* was inhibited by iso-α and β acids in MRS media, during 4 h 30 min incubation at 30 °C/100 rpm and pH 6.0-7.0. The minimal inhibitory concentration of iso-α acids against *Pediococcus pentosaceus*, was 50 mg/l (13.2% inhibition ± 0.5%) and 15 mg/l (2.8% inhibition ± 0.3%) of β acids. The higher inhibitory concentration of iso-α-acids was 200 mg/l (96.3% ± 1.5% inhibition), while for β-acids at the same concentration a lower inhibition (89.6% ± 1.6%) was observed. The results showed that for 250 mg/l iso-α acids the maximal inhibition was achieved (100% after incubation at 30 °C/100 rpm for 4.5h).

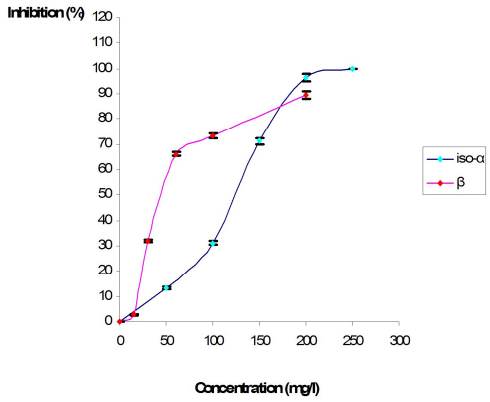

Fig. 1. The inhibitory effect of different concentration of iso-α and β hop acids against *Pediococcus pentosaceus* bacteria

According to the flow cytometry analysis, the profiles were divided in three distinct subpopulations (Fig. 2): "PI first stage" represents the exponential phase of the bacterial cells," PI - thermal stress" represents the cells exposed at 60 °C for 30 min and " PI - second stage" an intermediate subpopulation. The stressed fractions (PI - second stage and PI - thermal stress), were increased under iso-α acids activity after 4.5h incubation. The results of flow cytometry analysis (Fig. 2B and C), highlighted that *Pediococcus pentosaceus* exerted tolerance to the iso-α hop acids, cells (100 and 200 mg/l) showing a higher PI permeability after 4.5 h (Fig. 2A) and a lower PI permeability after 24 h (Fig. 2E and F) than the control sample. At a higher concentration, the bacterial cells were stronger both after 4.5 h and 24 h incubation time. Several studies have showed that this effect can be attributed to the bacteriostatic activity (Hrncic *et al*., 2019). The inhibitory effects of iso-α acids were stronger during the first stage of incubation (4.5 h), while after 24 h the viability of *Pediococcus pentosaceus* strain increased, both at 100 mg/l and 200 mg/l, beginning to develop resistance to the media. The resistance of certain strains of lactic acid bacteria to hop bitter acids is probably caused by a combination of mechanisms influencing the acidity inside the bacterial cell (Cermak *et al*., 2015).

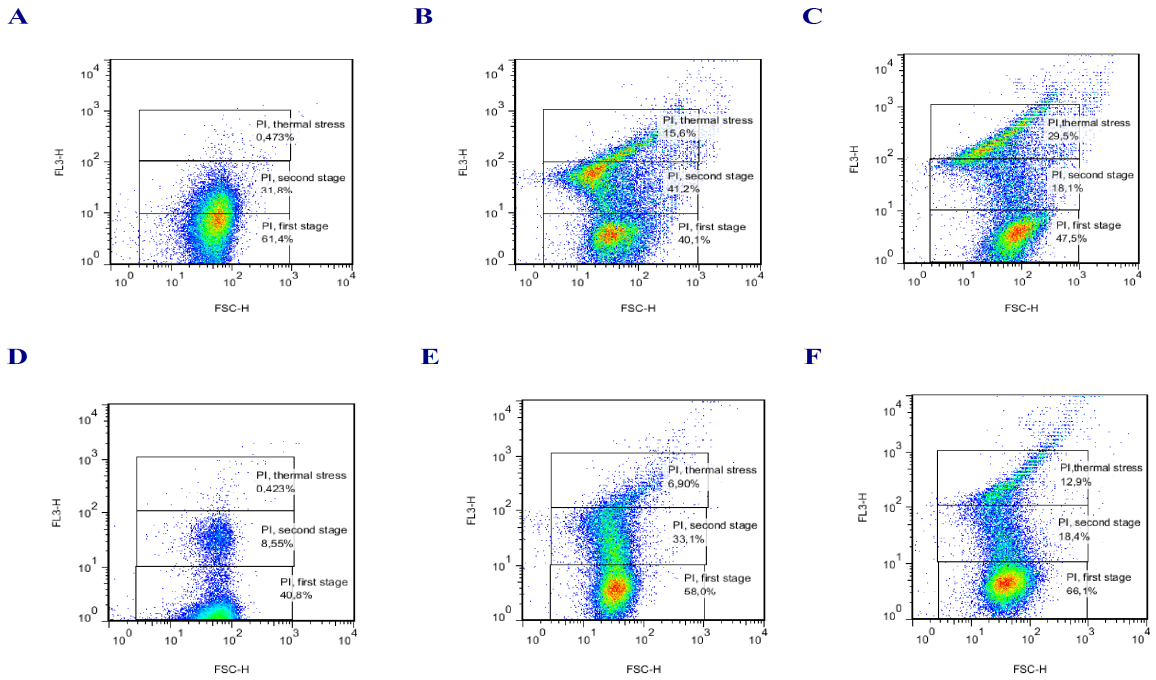

Fig. 2. Flow cytometry dot plots of *Pediococcus pentosaceus* incubated with iso-α hop acids A: control sample, B:100 mg/l iso-α acids, C:200 mg/l iso-α acids (after 4.5 h incubation) /D,E,F (after 24 h incubation)

The flow cytometry analysis provided a comparison between iso-α-acids and β-acids stress conditions on *Pediococcus Pentosaceus* cell viability, after which iso-α-acids were found to be less effective (Cermak *et al*., 2015) according to previous studies. In relation to the iso-α acids, *Pediococcus pentosaceus* cells cultivated under β-acid stress (Fig. 3B and C), exhibited a lower PI permeability after 4.5 h of incubation, compared to the control sample (Fig. 3A), while it also showed a lower PI permeability after 24 h for 100mg/l concentration, (Fig. 3F) (Gerhauser, 2005). It is known that iso-α-acids, as well as the products derived from α-acids act as ionophores, which alter the selective permeability of cytoplasmic membrane and cause intracellular acidification (Behr *et al*., 2007).

The inhibitory effects of β-hop acids were higher, but the viable cells were stronger during the first stage of incubation (4.5 h), indicating that *Pediococcus Pentosaceus* cells were resistant, However, after 24 h of incubation, cell viability decreased, and began to develop tolerance. There are several studies showing that tolerance may be developed when the microbes are exposed to a mild concentration of a weak acid, and rendering them resistant to a stronger dose (Jyoti Das *et al*., 2019).

Effects of simultaneous cultivation of Pediococcus pentosaceus and S. cerevisiae boulardii in beer wort and selective media

In the present study, the bacteriotatic activity of iso-α acids against *Pediococcus pentosaceus* strain in beer wort fermentation with *S. cerevisiae boulardii* strain was also evaluated. The analyses were performed to test whether the iso-α hop compounds change the activity of *S. cerevisiae boulardii* yeast and ethanol production. Many studies showed that *P. pentosaceus* have never been reported to cause any defect in finished beer (Zhao *et al*., 2017). Also in our study, the beer wort was an unfavourable medium for *P. Pentosaceus* cell growth, due to the lower pH (initial pH 5.0 and final pH 3.8-4.2 after 24h of incubation), lower nutrient value, hop-acid stress and alcohol (Pinto *et al*., 2004). The *Pediococcus pentosaceus* strain has slowly grown after 4.5 h, while in relation to the MRS selective medium (61.4%), the cell viability of the control sample (Fig. 4A) was lower (20.7%). In relation to the control samples, at lower pH, the bacteria were more tolerant both to 100mg/l and 300 mg/l iso-α acid concentration. After 24 h at pH 4.0, the viability of the cells was reduced to 4.5% and 5.4% (Fig. 4E and F), while in the MRS medium, the viability increased to 58.0% and 66.1% (Fig. 2E and F). As a result of the iso-α acids addition, a decrease of cell viability was induced at higher concentration, which was more pronounced after a short incubation period. Regarding iso-α acids activity on the *S. cerevisiae boulardii* strain, the results showed that the yeast was not affected by iso-α acids hop extract (30%) and the ethanol concentration was not influenced either (Fig. 5). This may be due to the slow development of *Pediococcus pentosaceus*, which could have competed with the yeast strain for micro and macro-nutrients (Blanco *et al*., 2007).

Because iso-β-acids in beer are in very small amounts due to their degradation during the boiling process, it could be concluded that iso-α acids present higher antibacterial properties in beer wort fermentation, rather than in a selective medium.

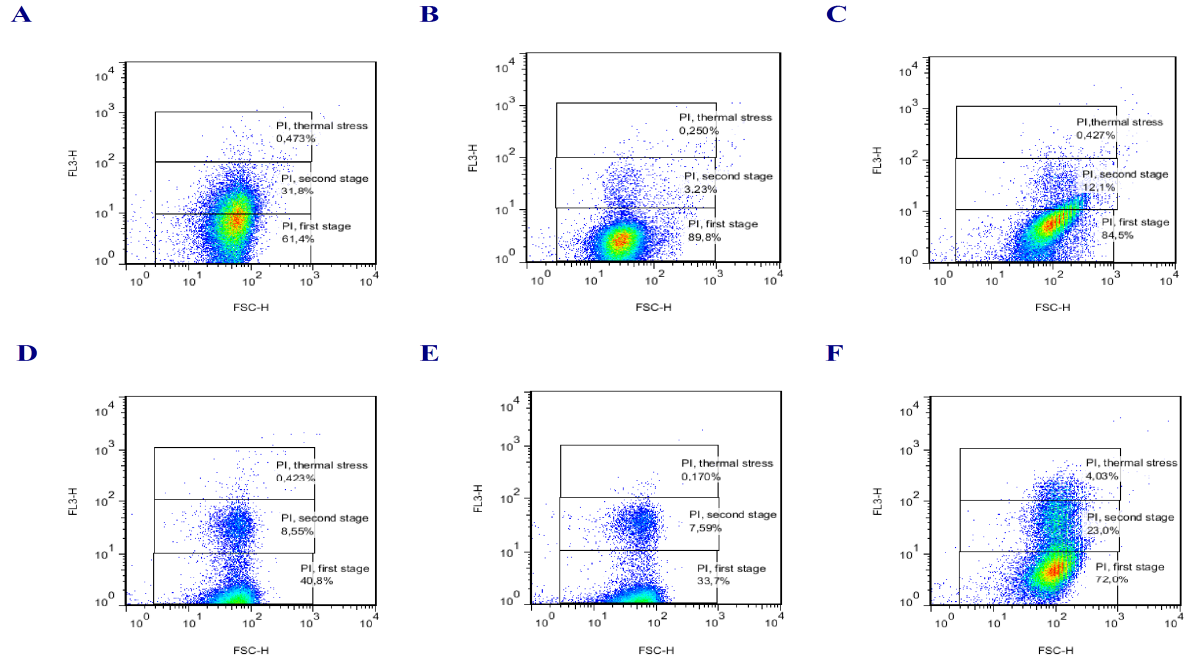

Fig. 3. Flow cytometry dot plots of *Pediococcus pentosaceus* incubated with β hop acids A: control sample, B: 30 mg/l β hop acids, C: 100 mg/l β hop acids (4.5 h); D,E,F (24 h)

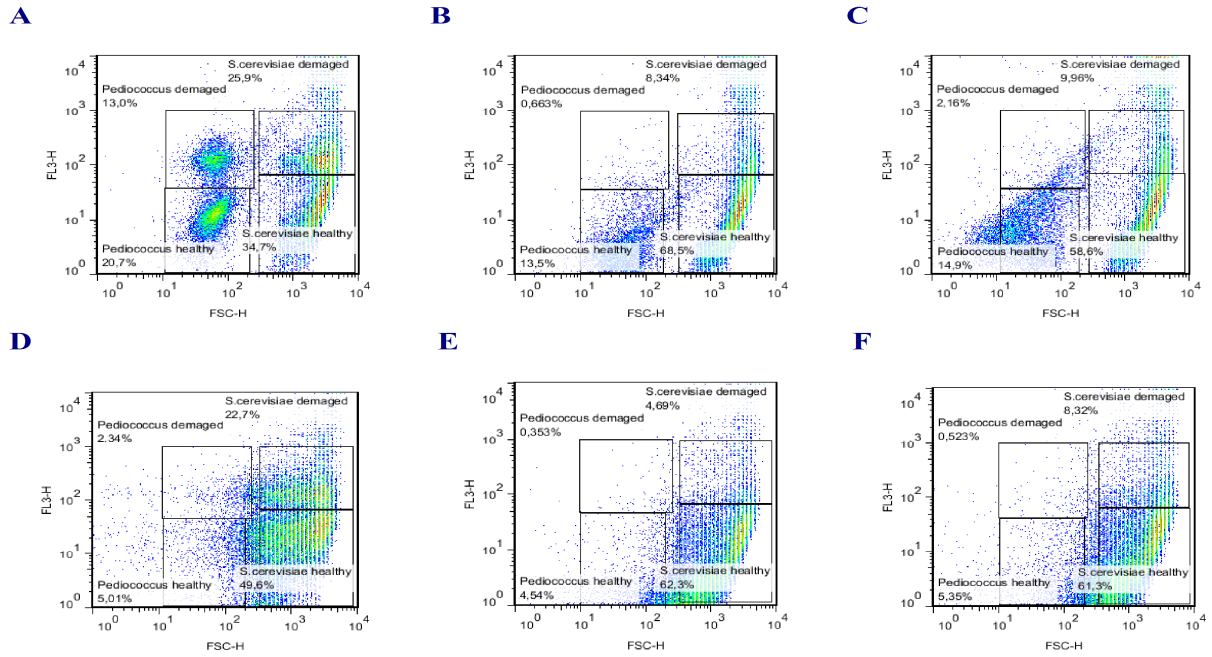

Fig. 4. Effects of iso-α hop acids against *S. cerevisiae* and *P. pentosaceus* in beer wort fermentation by flow cytometry analysis, A: control sample, B: 100 mg/l iso-α acids, C: 300 mg/l iso-α acids (4.5 h); D,E,F- (24 h)

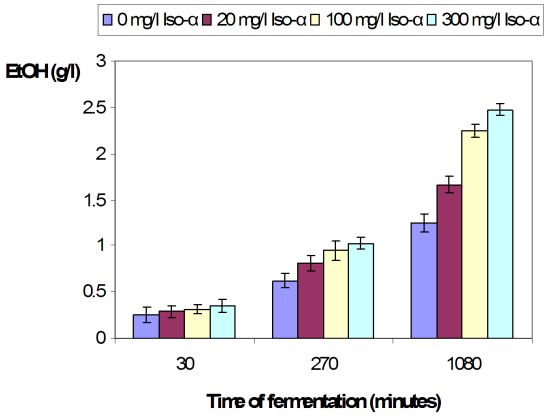

Fig. 5. Ethanol concentrations in beer wort fermentation with *S. cerevisiae boulardii* and iso-α acids hop extract (30%): control sample, 20 mg/l; 100 mg/l; 300 mg/l

Fig. 4C and F ilustrate the viability of *Pediococcus pentosaceus* cells in contact with 300 mg/l iso-α hop acids, which was higher than for 100 mg/l in the inhibition tests in selective medium (Fig. 2C and F). It was concluded that with the increase of hop extract concentration, the *Pediococcus pentosaceus* cells become stronger, revealing their bacteriostatic activity at pH 3.8-4.2.

The antibacterial activities of iso-α acids against the *Pediococcus pentosaceus* strain, during a similar fermentation in a selective medium are illustrated in Fig. 6. It was observed that cells viability was very low due to the unfavourable selective growth media, the antibacterial activity on *P. pentosaceus* being higher after 24h incubation when the yeast cells viability reached almost its maximum (Fig. 6E and F).

After 4.5 h incubation in selective media at pH 6.0-7.0, the iso-α and β-acids exhibits a bacteriostatic activity on *Pediococcus pentosaceus* bacteria. The results indicate that the *Pediococcus pentosaceus* strain was resistant to β-acids hop extract and tolerant to iso-α acids during the short incubation period. After 24 h incubation, under iso-α acid stress, the bacteria developed a resistance, thus reducing their sensitivity and consequently the kinetics of their destruction, while under β acids stress they were tolerant. Similarly, a study released by Garcia *et al.* (2016), showed that the *Pediococcus* bacteria were able to grow and adapt to increasing concentrations of hop compounds in culture media and beer, which shows that it could be considered a potential beer spoiler.

The bacteria were able to tolerate the iso-α acids in a stronger dose, showing to be stronger both after 4.5 h and 24 h incubation.

Regarding iso-α hop acids on the *S. cerevisiae boulardii* strain, the results showed that iso-α compounds do not change yeast activity but prevent the culture to be contaminated by *Pediococcus pentosaceus* bacteria. The yeast cell viability was slightly affected due to the *P. pentosaceus* strain competition, compared to the similar fermentation in a selective medium, where the viability of *P. pentosaceus* cells was very low and yeast cell viability reached almost the maximum (93%). At lower pH, during a short incubation period, the *Pediococcus pentosaceus* strain was more tolerant to the iso-α acids while for β-acids the tolerance started to develop after 24 h.

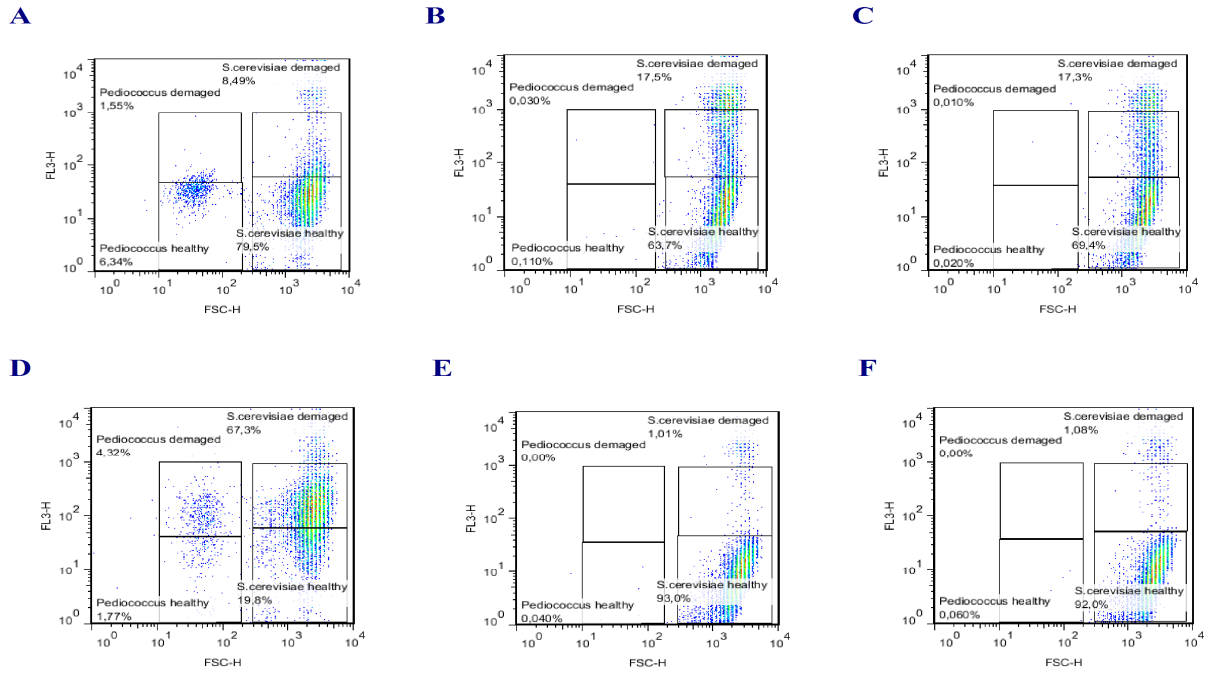

Fig. 6. Effects of iso-α hop acids against *S. cerevisiae* and *P. pentosaceus* in yeast selective media by flow cytometry analysis, A: control sample, B: 100 mg/l iso-α acids, C: 300 mg/l iso-α acids (4.5 h); D,E,F- (24 h)

Conclusions

In conclusion, the results of this study strongly support the likelihood that hop extracts can successfully be used as a bacteriostatic agent against *Pediococcus Pentosaceus* and warrant further research on developing hop bactericidal activity against this strain by testing the inhibitory effects in MRS selective media at low pH (modified pH) under iso-α and β acid stress during a longer incubation period. The results obtained in this study collect information that enables the use of iso-α acids as antimicrobial agent during beer wort fermentation, exhibiting a stronger antimicrobial effect than in a yeast selective medium. The search for new hop compounds with bactericidal properties represents a possible solution to the global problem of resistant spoilage microorganisms. Thus, hops might present themselves as a useful source of potential antimicrobial agents applicable in food industry.

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Conflict of Interest

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

References

Bayrock DP, Ingledew WM (2004). Inhibition of yeast by lactic acid bacteria in continuous culture: nutrient depletion and/or acid toxici. Journal of Industrial Microbiology and Biotechnology 31(8):362-368.

Behr J, Israel L, Gänzle MG, Vogel RF (2007). Proteomic approach for characterization of hop-inducible proteins in *Lactobacillus brevis*. Applied and Environmental Microbiology 73(10):3300-3306.

Behr J, Vogel RF (2009). Mechanisms of hop inhibition: hop ionophores. Journal of Agricultural and Food Chemistry 57(14):6074-6075.

Blanco C, Rojas A, Nimubona D (2007). Effects of acidity and molecular size on bacteriostatic properties of beer hop derivates. Trends in Food Science & Technology 18(3):144-149.

Buggey LA, Price A, Stapely SJ (2001). The antibacterial activity of hop compounds. In: Proceedings of the 48th International Hop Growers Convention, Canterbury, UK.

Cermák P, Palečková V, Houška M, Strohalm J, Novotná P, Mikyška A,...Sikorová M (2015). Inhibitory effects of fresh hops on *Helicobacter pylori* strains. Czech Journal of Food Sciences 33(4):302-307.

Cermak P, Olsovska J, Mikyska A, Dusek M, Kadleckova Z, Vanicek J, ... Bostik P (2017). Strong antimicrobial activity of xanthohumol and other derivatives from hops (*Humulus lupulus* L.) on gut anaerobic bacteria. Apmis 125(11):1033-1038.

Cleemput V, Cattoor MKK, De Bosscher G, Haegeman D, De Keukeleire AH (2009). Hop (*Humulus lupulus*)-Derived bitter acids as multipotent bioactive compounds. Journal of Natural Products 72(6):1220-1230.

Delvigne F, Brognaux A, Gorretb N, Neubauerc P, Delafossed A, Collignond ML, ... Thonart P (2011). Characterization of the response of GFP microbial biosensors sensitive to substrate limitation in scale-down bioreactors. Biochemical Engineering Journal 55(2):131-139.

Garcia-Garcia JH, Damas-Buenrostro LC, Cabada-Amaya JC, Elias-Santos M, Pereyra-Alférez B (2016). Pediococcus damnosus strains isolated from a brewery environment carry the horA gene. Journal of the

Institute of Brewing 123(1):77-80.

Gerhauser C (2005). Broad spectrum antiinfective potential of xanthohumol from hop (*Humulus lupulus* L.) in comparison with activities of other hop constituents and xanthohumol metabolites. Molecular Nutrition and Food Research 49(9):827-831.

Haakensen M, Schubert A, Ziola B (2009). Broth and agar hop-gradient plates used to evaluate the beer-spoilage potential of *Lactobacillus* and *Pediococcus* isolates. International Journal of Food Microbiology 130(1):56-60.

Hrncic MK, Španinger E, Košir IJ, Knez Z, Bren U (2019). Hop compounds: extraction techniques, chemical analyses, antioxidative, antimicrobial, and anticarcinogenic effects. Nutrients 11(2):257-294.

Iijima K, Asano S, Suzuki K, Ogata T, Kitagawa Y (2008). Multiplex PCR to detect beer-spoilage bacteria. Bioscience, Biotechnology, and Biochemistry 72(10):2764-2766.

Jyoti Das A, Jyoti Das M, Miyaji T, Deka SC (2019). Growth and metabolic characterization of four lactic acid bacteria species isolated from rice beer prepared in Assam, India. Access Microbiology 1(4):1-14.

Maifreni M, Frigo F, Bartolomeoli I, Buiatti S, Picon S, Marino M (2015). Bacterial biofilm as a possible source of contamination in the microbrewery environment. Food Control 50:809-814.

Muthaiyan A, Limayem A, Ricke SC (2011). Antimicrobial strategies for limiting bacterial contaminants in fuel bioethanol fermentations. Progress in Energy and Combustion Science 37(3):4-13.

Obi CN (2017). Brewery contaminants, challenges and remedies - a review. Nigerian Journal of Microbiology 31(1):3926-3940.

Olsovska J, Bostikova V, Dusek M, Jandovska V, Bogdanova K, Cermak P, ... Kolar M (2016). *Humulus lupulus* L. (hops) – a valuable source of compounds with bioactive effects for future therapies. Military Medical Science Letters (Voj Zdrav Listy) 85(1):19-30.

Pinto MGV, Pasteris SE, Strasser de Saad AM (2004). Glycerol catabolism by *Pediococcus pentosaceus* isolated from beer. Food Microbiology 21(1):111-118.

Sakamoto K, Konings WN (2003). Beer spoilage bacteria and hop resistance. International Journal of Food Microbiology 89(2-3):112-114.

Skinner-Nemec KA, Nichols NN, Leathers TD (2007). Biofilm formation by bacterial contaminants of fuel ethanol production. Biotechnology Letters 29(3):379-383.

Takahashi M, Kita Y, Kusaka K, Mizuno A, Goto-Yamamoto N (2014). Evaluation of microbial diversity in the pilot-scale beer brewing process by culture-dependent and culture-independent method. Journal of Applied Microbiology 118(2):454-469.

Vaughan A, O'Sullivan T, Van Sinderen D (2005). Enhancing the Microbiological stability of malt and beer - a review. Journal of the Institute of Brewing 111(4):355-31.

Zanoli P, Zavatti M (2008). Pharmacognostic and pharmacological profile of *Humulus lupulus* L. Journal of Ethnopharmacology 116(3):383-396.

Zhao Y, Knøchel S, Siegumfeldt H (2017). Heterogeneity between and within strains of *Lactobacillus brevis* exposed to beer compounds. Frontiers in Microbiology 8(239):1-13.

sustainability

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Article

Food Safety System (HACCP) as Quality Checkpoints in a Spin-Off Small-Scale Yogurt Processing Plant

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Abstract: The present study describes the implementation of a food safety system in the dairy pilot plant "Gourmeticus Academicum," a spin-off within the University of Agricultural Sciences and Veterinary Medicine of Cluj Napoca, Romania. In order to improve Hazard Analysis of Critical Control Points (HACCP) the preliminary programs were integrated into the quality management system (QMS) by monitoring the biological hazards. The process provides future specialists with good practice hands-on and educational tools. This study focused on hazard analysis, the determination and establishment of prerequisite programs, and the role of critical control points (CCPs) based on HACCP and the challenges found during the process as a critical thinking model on education programs. The determination of the CCPs in the processing of yogurt was made by applying the decision tree method. Besides, biological hazards are included as a by-control of the system's implementation performance. For the successful implementation of HACCP principles, prerequisite programs (PRPs) and operational prerequisite programs (OPRPs) were initially implemented. This process could be challenging but feasible to be reached in small-scale food industries with remarkable results as educational tools.

Keywords: yogurt; PRP; OPRP; HACCP; critical thinking model; education

1. Introduction

Yogurt is one of the most popular fermented dairy products, with a wide acceptance worldwide and whose nutritional and health benefits have been known for centuries [1]. As a general definition, yogurt is a fermented dairy product obtained from lactic acid fermentation by lactic acid bacteria (*Streptococcus thermophilus* and *Lactobacillus delbrueckii* ssp. *bulgaricus*). After fermentation, the milk acidifies and coagulates and increases the shelf life due to the low pH [2].

According to the available literature, yogurt is considered a functional food. The complexity of nutrients and digestibility gives this classification. It is a food that can be recommended for people with gastrointestinal disorders (irritable bowel disease, inflammatory bowel disease) and people with lactose intolerance. It helps increase the immune system and lose weight [3]. Yogurt and dairy products foster

a significant concern to the dairy industry and public health authorities [4]. Yogurt is a good source of probiotics, but it could also be an essential source of foodborne pathogens [5]. Several authors have reported the outbreaks or incidents of foodborne diseases associated with dairy products: *Brucellosis, Salmonella, Listeria, Clostridium botulinum* [6–9]. In industrialized countries, milk and dairy are involved in 2–6% of outbreaks of foodborne diseases [10].

The classical methods regarding the hygienic quality of the finished products are inadequate to control hazards occurring at early stages of the process [11]. Food safety requires compliance with good manufacturing practices (GMP), sanitation standard operating procedure (SSOP), good hygiene practices (GHP), also called operation prerequisite programs (OPRPs), and the principles of Hazard Analysis of Critical Control Points (HACCP) [12].

The concept of critical control points originated in 1959, when the National Aeronautics and Space Administration (NASA), Pillsbury, and US Army laboratories collaborated to provide safe food for future space expeditions. This scientific concept is based on the assessment of food safety hazards through a control system. This system is a preventive one that analyzes the biological, chemical, and physical hazards that affect the entire food chain [11,13]. Several reports indicated the effects of implementing HACCP on the microbiological quality of food products [11,14–16].

Note that the implementation of HACCP is mandated for all small- and medium-sized food companies in the European Union (EU), and HACCP is recognized in the international food safety community as a worldwide guideline for controlling foodborne safety hazards [17]. Its principles, detailed in the Codex Alimentarius guidelines, are integrated with International Standard ISO 22000:2018 [18]. The application of HACCP systems does not imply the existence of a traceability system as a direct consequence of the documentation procedures. However, the implementation of such a system is of particular importance. Even if Principle 7 of the HACCP system requires established documentation and record-keeping procedures, traceability systems are not mandatory under this system [19].

ISO 22000:2018, which was introduced worldwide on 19 June, 2018, states that organizations must conduct a risk analysis to identify significant hazards [18]. ISO 22000 was not recognized by the Global Food Safety Initiative as a standardized reference for food manufacturers in the past, as it imparts no detailed PRP (prerequisite program)-related information. Hence, ISO 22000:2018 comes with improvements essentially looking to determine a PRP for and the CCP (critical control point) of the significant hazards, having as fundamental principle risk-based thinking and risk reduction [18]. In food industries, identifying the hazards was the one of the 12 application steps for the HACCP approach that were considered critical. It also agrees with the first principle of Codex HACCP and ISO 22000:2018, which calls for the execution of hazard analysis. HACCP systems aim to identify, evaluate, and control hazards [16].

This work aims to implement a food safety system (HACCP) under the ISO 22000:2018 [18] standard by conducting a hazard analysis in a small-scale dairy pilot plant and yogurt production to develop a critical thinking model as an educational tool for food engineering students (FES) as well to identify CCPs, thus setting up an effective preventive system that will lead to a safer and more efficient production of yogurt and providing an example of good practice and educational tools for FES education programs.

2. Materials and Methods

2.1. Small-Scale Dairy Pilot Plant Description

This study was conducted at the small-scale dairy pilot plant (DPP) of the Faculty of Food Science and Technology, University of Agricultural Sciences and Veterinary Medicine of Cluj Napoca, Romania. This DPP is part of the food pilot chain consisting of six pilot plants, founded in 2012. The main goal is to implement the EN ISO 22000:2018 food safety management systems [18] within the pilot plant where the practical works are carried out with the FES (as internship in traineeship programs—integrated

education programs), thus setting up an effective preventive system that will lead to a safer and more efficient production of yogurt. Management commitment was realized by communicating to the organization the importance of meeting the International Standard statutory and regulatory requirements as well as customer requirements relating to food safety, and by ensuring the availability of financial, material, and human resources for the establishment of the necessary work environment, complying with the EU food standards and regulation. The products are directed exclusively to the internal market. DPP has implemented ISO 22000:2018 to improve the quality and safety of its products, customer expectations, the product image on the market, and to develop good practice as an educational tool. The identification, analysis, monitoring, and corrective actions established for CCPs and the verification of the effectiveness of the entire HACCP plan were performed according to the procedures underlying ISO 22000:2018. This standard has been implemented in production lines. However, the present study aims to integrate microbiological parameters (the total colony forming unit (CFU), somatic cell count (SCC), and *Enterobacteriaceae*) in the food safety system (HACCP) as quality parameters in a spin-off small-scale yogurt processing plant.

2.2. Materials

This manuscript analyzes the implementation of ISO 22000:2018 for natural yogurt with 3.6% fat made in a DPP.

Qualitative and quantitative reception of milk. Milk is transported from Cojocna farm in secured aluminum cans.

From the reception valve the milk is passed to an acid dairy products plant (IPI tank with 100 L capacity) using a pump (202 MHI type) a milk flow of 2000 L/h.

The acid dairy products plant is used for milk pasteurization and inoculation with the starter culture.

Milk pasteurization. Pasteurization is performed at high temperatures (85–90 °C) for 20–30 min. Pasteurization aims at the following:

- improvement of hygienic quality of milk;
- environment improvement for the development of lactic bacteria;
- yogurt consistency improvement: high temperatures of pasteurization favors a softer curd that retains more whey.

From the pasteurization device the milk is continuously passed through the meanings of a pump (MHI 202 type) in a heat exchanger placed above the tank until complete pasteurization of the milk. The pasteurization is done under continuous stirring (the valve is provided with an agitator).

Milk cooling. The cooling of the milk is done in the same valve as pasteurization by recycling the milk through the heat exchanger until the yogurt reaches a temperature of 45–46 °C. The heat exchanger uses water from the regular city supplies network to cool the milk.

Milk inoculation. This is done with starter cultures of lyophilized lactic bacteria. The culture is diluted in milk and then the milk is strongly stirred until uniform distribution of the culture is reached for 10–15 min. With the help of the second pump (MHI 202 type) the inoculated milk is sent to a packaging device (ADL-ATS 200 type, 200–250 cups/h capacity for 200 mL cups).

Packaging. The dosage in sales packaging is performed in a manual device (ADL-ATS 200 type). The yogurt has to be continuously stirred in the valve during packaging. The cups are thermosealed with aluminum foil after filling.

Tempering. The packaged products are placed in a thermostatic aluminum cabinet (with a capacity of 400 cups for each 200 mL). The yogurt tempering takes place at 43–45 °C for 2.5–3 h.

Precooling at 18–20 °C for 2 h.

Cooling and storing at 2–6 °C for 12 h.

2.3. Methods

2.3.1. Elaboration of Critical Thinking Model

The critical thinking learning model developed and applied is described in Figure 1. Through the learning process three stages were identified: (1) Evaluation of information; (2) description/identification of problems as main concept; and (3) analysis (interpretation and inferences). The evaluation of information is based on gathering and reporting data, facts, observations, and experiences that should be clear, relevant, accurate, adequate, and consistent. The description is focused on identifying the most important concepts, theories, ideas, regulations, procedures, principles, models, and definitions that should be clear, relevant, and accurately presented. The analysis is centered on interpretation and inferences and elaborate conclusions and solutions that should be clear, logical, justifiable, and consistent. The application of the model during the learning process of FSMS (food safety management systems) to food engineering students leads to achieving the ability of critical and design thinking.

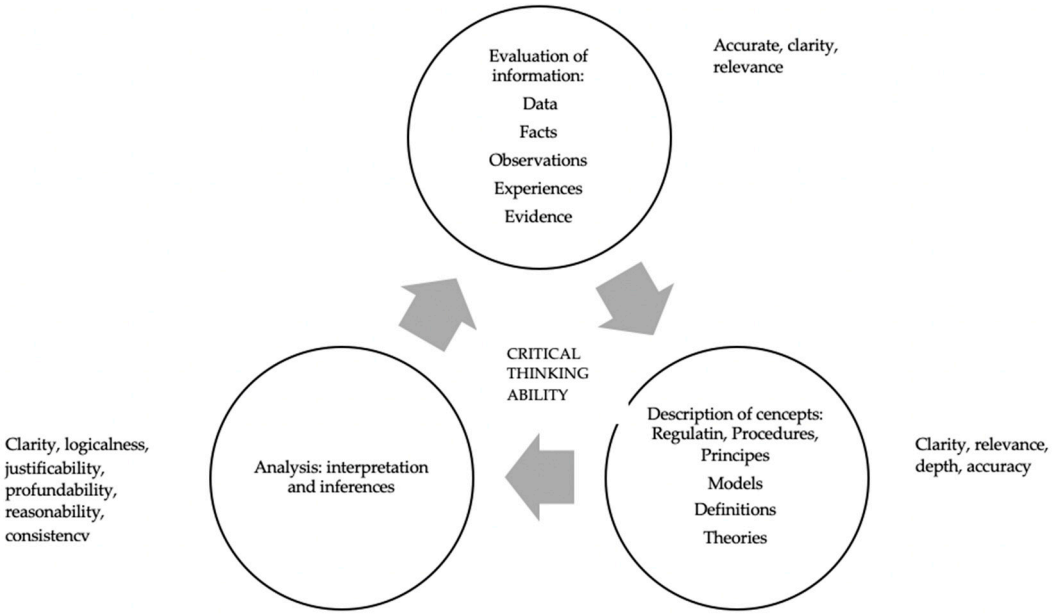

Figure 1. Critical thinking model developed and applied as an educational tool to learning FSMS (food safety management systems).

2.3.2. Elaboration of PRPs

The HACCP team was responsible for coordinating and implementing the corrective measures to improve the adaptation to the PRPs (GMPs, GHPs, and SSOPs). The definition of the production chain—from the farm to final consumer; the definition of food safety and contamination; types of contamination; the importance of microbiological contamination; optimal conditions for the growth of microorganisms; contamination by microorganisms—elimination, inhibition, and prevention; the application of GMP principles (personal hygiene, environment, and equipment); habits for the correct handling of foods; benefits of GMPs (food safety, longer shelf life, reduced losses, better working environment, and consumer satisfaction); the need to change the behavior and commitment of all employees; work instructions; the importance of hygiene (how to avoid contamination); conditions for effective cleaning; phases of the hygiene process; and the presentation of work instructions as described by Cusato [20] were followed.

2.3.3. Elaboration of the HACCP Plan

Based on ISO 22000:2018 [18] and HACCP principles, according to Codex Alimentarius, the overall technical process of yogurt production was drawn and a hazard analysis was performed following the 12 steps for developing an HACCP plan (Table 1). The identification of hazards is made according to their nature (biological, chemical, and physical). The analysis is done according to the likelihood occurrence level and its severity (Table 2) [12]. Hazard rating is calculated by multiplying likelihood by severity. The determination of CCPs is done with the help of the decision tree (DT) (Figure 2), in which only the stages with a hazard rating ≥3 are introduced [16].

Table 1. Steps of Hazard Analysis and Critical Control Points.

Step 1	Assemble HACCP [1] team
Step 2	Describe product
Step 3	Identify intended use
Step 4	Construct flow diagram
Step 5	On-site confirmation of flow diagram
Step 6. Principle 1	List all potentioal hazards, conduct a hazard analysis, and consider control measures
Step 7. Principle 2	Determine CCPs [2]
Step 8. Principle 3	Establish critical limits for each CCPs
Step 9. Principle 4	Establish a monitoring system for each CCPs
Step 10. Principle 5	Establish corrective actions
Step 11. Principle 6	Establish verification procedures
Step 12. Principle 7	Establish documentation and record-keeping

[1] HACCP, Hazard Analysis of Critical Control Points; [2] CCP, Critical Control Point. Adapted from Kamboj et al., 2020 [12].

Table 2. Level of likelihood of occurrence and hazard severity.

	Likelihood of Occurrence	**Hazard Severity**
High (3)	Highly probable; known history in the sector	Life-threatening or long-term chronic illness (e.g., infection, intoxication, or anaphylaxis), chronic effects or death
Medium (2)	Could occur; minimal history within the sector but has happened	Injury or intolerance; not usually life-threatening
Low (1)	Unlikely to occur; no known examples	Minor or no effect; short duration

Adapted from Kamboj et al., 2020 [12].

2.3.4. Microbiological Analyses

The mandatory analyses according to Romanian legislation were performed according to Regulation No. 853/2004 as amended and supplemented by Regulation No. 1020/2008 [21] for raw material milk and pasteurized milk, and according to Regulation No. 2073/2005 as amended and supplemented by Regulation No. 365/2015 [22] for yogurt, which is in conformity with the EU Council Directive 2002/99/EC [23], Regulation (EC) No 178/2002 [24], Regulation (EC) No 852/2004 [25], Regulation (EC) No 853/2004 [26], Regulation (EC) No 854/2004 [27], and Regulation (EC) 882/2004 [28] for the public health rules and safety food trade.

The total colony forming unit (CFU) was analyzed according to the SR EN ISO 4833-1: 2014 method [29], the somatic cell count (SCC) was analyzed according to the SR EN ISO

13366-1:2008/AC:2010 method [30], and the *Enterobacteriaceae* were analyzed according to the ISO 21528-1: 2017 method [31].

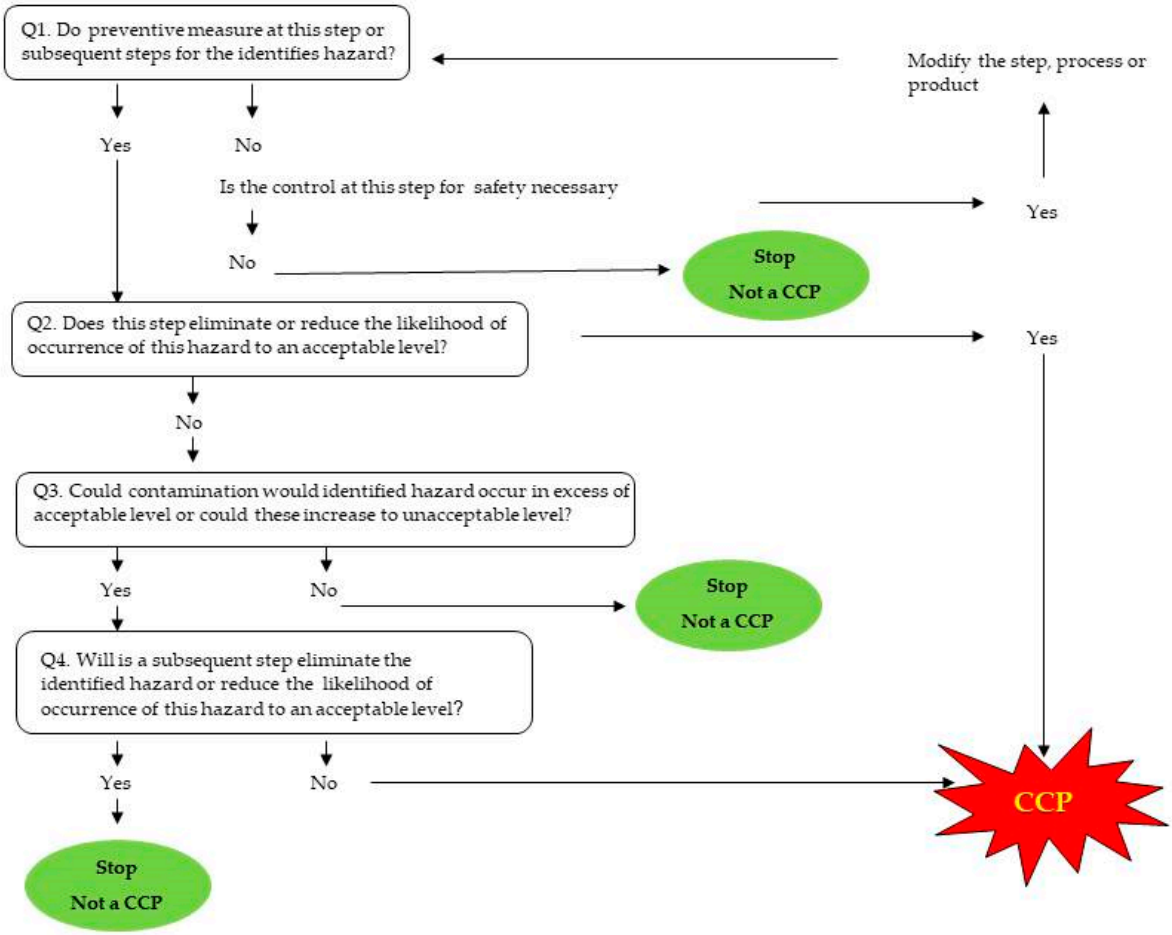

Figure 2. Decision tree (DT) protocol. The DT protocol was used to established CCPs.

3. Results and Discussion

3.1. Assessment and Implementation of the PRPs

The PRPs implemented in the DPP are hygiene of personnel and food hygiene, disinfection and cleaning, prevention of cross-contamination, the importance of maintaining a cold chain during food storage, hygiene premises and buildings, control pests, equipment maintenance, quality control of raw material at reception, food with water, waste and wastewater disposal, storage and transportation, product management, and supply management. A well-defined plan includes these programs. PRPs are fundamental conceptual programs for establishing security bases. There are more basic programs and assistance programs that provide foundations for HACCP [32]. The programs' basis is GMP and GHP for products and the handling and delivery of finished products, to be provided by technology [18,33].

For the implementation of PRPs, buildings, facilities, equipment, utensils, food handlers, production, transportation of food, and documentation were evaluated. Following the evaluation and the observed non-conformities, operational procedures were performed. As an educational tool, the essential stage of the implementation of FSMS is the training. Although most people involved, especially interns, know about food contamination, theoretical training is not sufficient to implement FSMS in practice. The theoretical and practical training applied was observed by changing habits and behavior regarding GMPs and GHPs (by applying the principles of GMP (personal hygiene, environment, and equipment), habits for the correct handling of foods, how to

avoid contamination, types of surfaces to be cleaned and cleaning agents, conditions for effective cleaning (solution concentration, water temperature, exposure time, and mechanical action), phases of the hygiene process (pre-rinsing, detergent solution, rinsing, and sanitizing), and the presentation of work instructions). Another aspect that encounters difficulties in DPP is the large rotation of the interns, delaying a team's consolidation with the desired standard work and resulting in improvements taking longer than expected. To improve this aspect, a technological engineer (a university assistant responsible for student practice) was delegated to do theoretical and practical training and verify the activity on PRPs and the necessary monitoring. A similar approach was noted by Cusato [20] in a small dairy factory and by Karaman [34] in a dairy factory in Turkey.

3.2. Implementation of HACCP Plan

Preliminary steps to enable hazard analysis (Step 1–6).

3.2.1. Food Safety Team

A multidisciplinary team composed of nine people was created to implement the requirements of the system. The team members were trained thoroughly on the HACCP system and ISO 22000:2018 standard [18]. The food safety team members are an HACCP team leader, dairy technological engineer, technological engineer (university assistant responsible for student practice), testing laboratory manager, hygiene manager (responsible), maintenance manager, supply manager, sales manager, and HACCP team secretary.

3.2.2. Product Characteristics and Intended Use

The food safety team preceded a complete description of the yogurt, identifying its composition; chemical, biological, and physical characteristics; treatments; durability; storage conditions; and distribution methods. Table 3 summarizes the yogurt's characteristics, and its use is recommended for all segments of the population, except sensitive people (people with a milk allergy or intolerance).

Table 3. Gourmeticus yogurt product description.

1	**Product name**	Gourmeticus yogurt
2	**Composition and ingredients**	Pasteurized milk and cultures of selected dairy bacteria (Lyofast Y 450 B, Lyofast Y 452 B)
3	**Organoleptic characteristics**	Compact, homogeneous curd, without gas bubbles or zircon; the ruptured clot has a porous granular appearance. Milk-specific white, uniform or yellowish in color. The specific smell and taste of yogurt, pleasantly sour, without foreign taste or smell.
4	**Physio-chemical characteristics**	It must not have any physical impurities. Fat minimum 3.0 ± 0.1%, total solids content minimum 11%, acidity minimum 0.6% lactic acid, protein substances minimum 2.8%
5	**Microbiological characteristics**	*Salmonella, E coli., Enterobacter, Shigella, Klebsiella*—absent
6	**Treatments**	Pasteurization
6	**Nutritional values**	Energy value 55.8 kcal 3% fat, of which 2 g saturated fatty acids, 4 g carbohydrates, 3.2 g protein, and 0.2 g salt.
7	**Packing method**	In 200 g plastic cups and the closure is made with heat-sealable metallic foil.
8	**Terms of validity**	21 days
9	**Storage instructions**	Refrigerated rooms, clean, disinfected, ventilated, no foreign smell at temperatures between 2–8 °C.

Table 3. *Cont.*

10 **Labelling instructions**	Labelling must be carried out following the regulations and include the following aspects: the name of the product, list of ingredients, any ingredient or technological adjuvant that causes allergies or intolerances used in the respective factory, the number of certain ingredients or ingredient categories, net quantity of food, date of minimum durability or expiration date, special storage conditions, name or trade name and address of the food business operator, country of origin or place of origin, instructions for use, nutrition statement, date of manufacture (day, month, batch).
11 **Instructions for use**	It is consumed as such.
12 **Delivery/sales conditions**	Authorized means of transport, isothermal, refrigerated, clean, ventilated, in the absence of toxic substances or a pungent smell at temperatures between 2–8 °C. The product is sold at the university store. The temperature in the storage refrigerator is between 2–8 °C.

3.2.3. Flow Diagram

The flow diagram includes all the technological process stages for making Gourmeticus yogurt (Figure 3). In addition to the technological process stages, the diagram shows the stages until delivery to consumers (storage on the market). This detail is essential for a better presentation of the environmental conditions that could affect the product's quality and safety. These aspects must be taken into account due to their importance for consumer health [35]. The flow diagrams were checked on-site by the food safety team.

3.2.4. HACCP Plan Principles (Steps 7–12): Hazard Identification and Determination of Acceptable Levels

The identification and assessment of hazards is a crucial principle for all HACCP systems [36] and a prerequisite to protecting public health. To achieve this step, the food safety team established a procedure specifying the methodology for hazard analysis, described in Table 4. Hazard analysis is applied from the receipt of raw materials to the delivery of the finished product. The dangers can have a direct or indirect impact on yogurt. They are based on the implementation of PRPs and aim to identify CCPs.

The identified hazards are classified according to pathogens (biological hazards), toxic substances (chemical hazards), and external particles (physical hazards) and are due to contamination, multiplication, and persistence. The HACCP team's identification and analysis of the dangers of yogurt were performed for all stages of the production process.

Assessment of hazards based on the severity (S) of known effects on consumer health and the likelihood of these hazards occurring in DPP. The probability (P) is established according to the history and expertise of the DPP. Each hazard is evaluated and receives a score between 1 and 3. A hazard is considered significant if the resulting hazard rating (HR) score from the multiplication of the probability by the severity is above 3 [12,16,37,38]. A significant hazard is one of such a nature that its elimination or reduction to an acceptable level is essential to the production of safe food.

Following the hazard analysis, an HR is established. For hazards of HR ≤ 2, which are considered low or almost non-existent hazards, control measures are made using PRPs, with no CP (control point) or CCP required [39].

The PRPs control the potential chemical hazards associated with milk, such as veterinary drug residues, food additives, residue of migration of substances from packaging materials, heavy metals, and oil-free air compressors or potential biological hazards in order to reduce the probability of occurrence [40].

Even if greater importance is given to chemical and biological hazards, physical hazards in dairy products are just as significant [40]. Physical hazards can easily occur through non-compliance with PRPs or accidental contamination [41], and are related to contact with various objects, packaging, or incorrect labelling [40].

Milk cleaning is not considered in our unit, with HR ≥ 3 (CCP or CP), but is periodically checked for the presence of external particles (glass, plastic, wood, metal, etc.) [39].

When significant hazards are identified as having HR ≥ 3, a 4Q (Questions) decision tree is used to decide whether a particular hazard is a CCP or control point (CP), analyzed in Table 5. Although it is not mandatory to use the CCP decision tree method of ISO 22000:2018, the decision tree, a clear, well-organized, and understandable visual analysis tool, should be used to determine [41] and to prioritize [42] the CCPs.

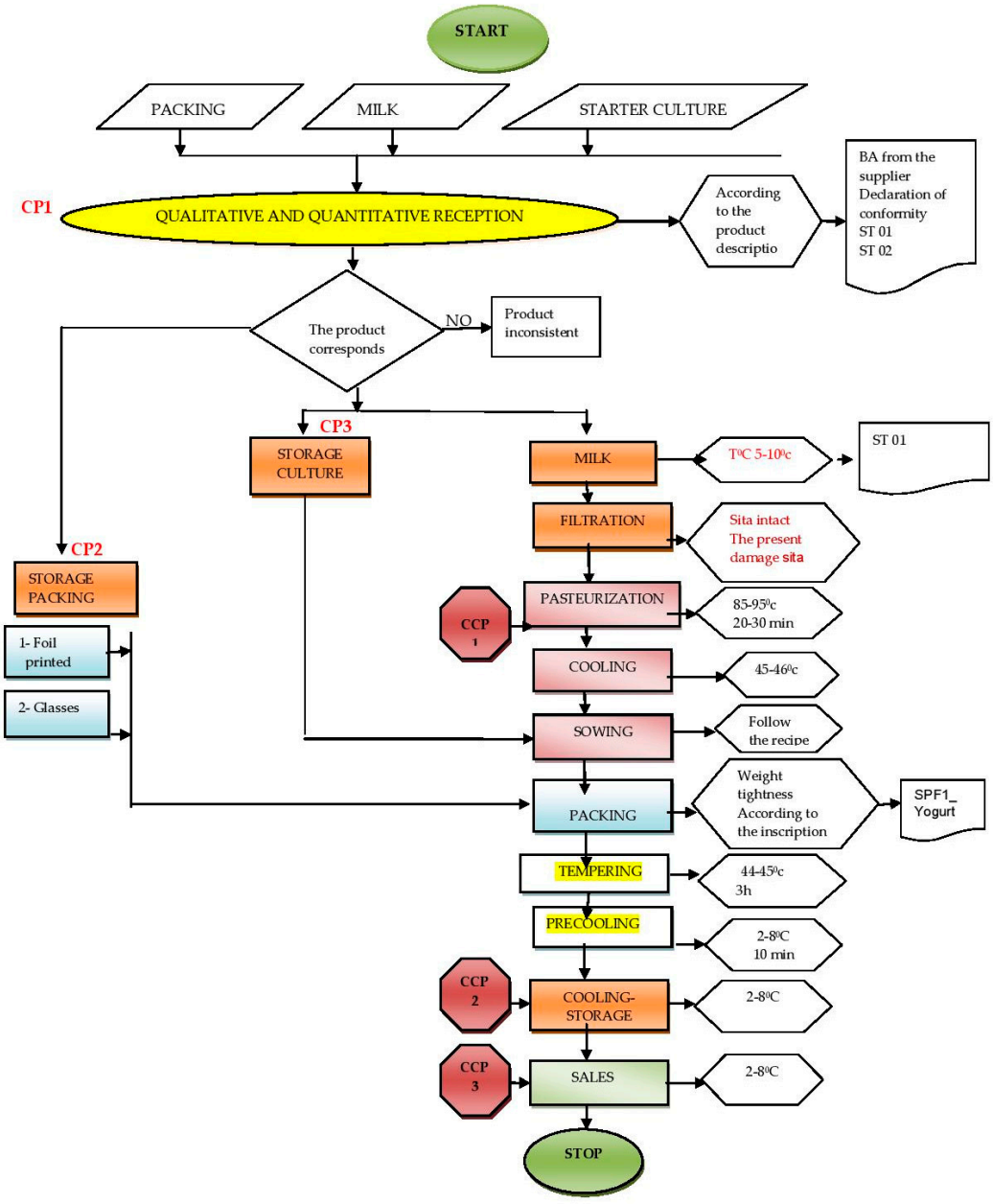

Figure 3. Flow diagram describing the technological steps of the Gourmeticus yogurt process.

Table 4. Hazard analysis and assessment. The table presents the hazard analysis of each steps of the technological flow diagram.

The Stage of the Technological Process	.2 Potential Hazards		.4 Is the Danger Potentially Significant?	Hazard Assessment			.2 Preventive Measures/Control Measures
				S [1]	P [2]	HR [3]	
1. Reception of milk	B [4]	*Mycobacterium tuberculosis*, *Salmonella*, *E coli*, *Staphylococcus aureus*, *Brucella campylobacter*, *Listeria monocytogenes*, *Bacillus cereus*, *Mycobacterium bovis* **CFU [7] max 100,000/mL, SCC [8] max 400,000 mL**	Yes—non-compliant milk can lead to obtaining an inappropriate product or even to the production of diseases.	3	1	3	-Compliance with GMP measures and training of staff on compliance with GMP measures -Performing a second party audit at the supplier to verify compliance with GHP measures -Checking the analysis reports, the declarations of conformity, and the sanitary approvals that accompanies the raw milk -Rejection of inadequate raw milk from a microbiological and physio-chemical point of view -Cooling the milk immediately after milking on the farm and transporting to a refrigerator -Checking the temperature and transport conditions
	C [5]	Antibiotics, pesticides, neutralizers, nitrates, mycotoxins, drugs, growth hormones, the presence of detergents and disinfection substances	Yes—it can lead to obtaining an inappropriate product or even to a health impact causing different diseases.	3	1	3	
	P [6]	Hair, straw, feces	No—the presence of foreign bodies cannot cause injury to the consumer.	2	1	1	
2. Reception and packaging storage	B	*E coli.*, *Staphylococcus*, *B. cereus*, molds	Yes—infected and infested packaging can lead to an unsuitable product or even disease.	3	1	3	-Compliance with GMP measures and training of staff on compliance with GMP measures -Evaluation and selection of suppliers -Verification of declarations of conformity accompanying packaging -Rejection of improper packaging from a microbiological and physio-chemical point of view -Proper storage of packaging
	C	-	-	-	-	-	
	P	-	-	-	-	-	

Table 4. *Cont.*

.5 3. Reception of lactic acid bacteria starter cultures	B	*Salmonella, E coli., Staphylococcus*	Yes—contamination can lead to an unsuitable product or even disease.	3	1	3	-Compliance with GMP measures and training of staff on compliance with GMP measures -Verification of the declarations of conformity that accompany the starter cultures -Monitoring the storage temperature and following the validity period written on the label -Observing the FIFO [9] principle and disinfecting the refrigerator after each defrost -Keeping in its own closed packaging
	C	-	-	-	-	-	
	P	-	-	-	-	-	
4. Milk filtration	B	-	-	-	-	-	-Compliance with GMP measures and staff training -Maintenance filters -Checking the hygiene and operation of the filter
	C	Contamination with detergent residues	No—the presence of residues of washing substances cannot cause serious illness.	2	1	2	
	P	Filtering surface	Yes—the presence of metallic impurities can cause illness and injury to the consumer.	2	1	2	
5. Pasteurization	B	*M. tuberculosis, Brucella, E coli.*	Yes—contamination can lead to an unsuitable product or even disease.	3	1	3	-Compliance with GMP measures and training of staff on compliance with GMP measures -Checking the equipment's hygiene and utensils by performing quarterly sanitation tests and by visual inspection before each pasteurization. -Respecting and monitoring the pasteurization conditions (time and temperature)—thermograms -Maintenance of washing and disinfection substances in specially arranged places, kept under lock and key -Control of washing solutions
	C	Contamination with detergent residues	No—the presence of residues of washing substances cannot cause serious illness.	2	1	2	
	P	-	-	-	-	-	

Table 4. *Cont.*

6. Cooling	B	-	-	-	-	-	-
	C	-	-	-	-	-	
	P	-	-	-	-	-	
7. Inoculation with the starter culture of lactic acid bacteria and fermentation	B	-	-	-	-	-	-Compliance with GMP measures and training of staff on compliance with GMP measures -Performing quarterly sanitation tests to check the hygiene of equipment, utensils, staff -Employee staff must have regular medical check-ups performed according to the legislation in force -Performing disinfection operations according to the planning
	C	Contamination with detergent residues	No—the presence of residues of washing substances cannot cause serious illness.	2	1	2	
	P	The presence of foreign bodies in the production space, from the staff, from the utensils	Yes—the presence of foreign bodies can cause injury to the consumer.	2	1	2	
.5 8. Packaging	B	-	-	-	-	-	-Compliance with GMP measures and training of staff on compliance with GMP -Performing quarterly sanitation tests to check the hygiene of equipment, staff, packaging -Employee staff must have regular medical check-ups performed following the legislation in force -Checking the heat seal of the lids
	C	Contamination with detergent residues	No—the presence of residues of washing substances cannot cause serious illness.	2	1	2	
	P	-	-	-	-	-	
9. Tempering	B	-	-	-	-	-	-
	C	-	-	-	-	-	
	P	-	-	-	-	-	

Table 4. *Cont.*

10. Pre-cooling	B	-	-	-	-	-	-
	C	-	-	-	-	-	
	P	-	-	-	-	-	
11. Storage Cooling	B	*Salmonella, E coli., Enterobacter, Shigella, Klebsiella*	Yes—contamination can lead to an unsuitable product or even disease.	3	1	3	-Compliance with GMP, GHP measures and training of staff in compliance with GMP, GHP -Performing disinfection operations according to the planning made by the HACCP coordinator -Monitoring the temperature in the cold storage and following the shelf life written on the label -Respect the FIFO principle -Carrying out the disinfection operations according to the planning
	C	-	-	-	-	-	
	P	Pests, mice	Yes—contamination can lead to an unsuitable product or even disease.	2	1	2	
12. Sales	B	*Salmonella, E coli., Enterobacter, Shigella, Klebsiella*	Yes—contamination can lead to an unsuitable product or even disease.	3	1	3	-Compliance with GMP, GHP measures and training of staff on compliance with GMP, GHP -Respecting the sales parameters and checking the validity term written on the label -Respecting the FIFO principle and sanitizing the refrigerator after each defrost -Performing disinfection operations according to the planning made by the HACCP coordinator
	C	-	-	-	-	-	
	P	-	-	-	-	-	

[1] **S**, severity; [2] **P**, probability; [3] **HR**, hazard rating; [4] **B**, biological; [5] **C**, chemical; [6] **P**, physical; [7] **CFU**, colony forming units; [8] **SCC**, somatic cell count; [9] **FIFO**, first-in first-out.

Table 5. CCP [1]/CP [2] identification.

Stage	Q[3]1	Q2	Q3	Q4	CCP/CP
Qualitative and quantitative reception of milk	Yes	No	No	-	CP
Qualitative and quantitative reception and packaging storage	Yes	No	No	-	CP
Qualitative and quantitative reception of lactic acid bacteria starter cultures	Yes	No	No	-	CP
Pasteurization	Yes	Yes	-	-	CCP1
Cooling, storage	Yes	No	Yes	No	CCP2
Sales	Yes	No	Yes	No	CCP3

[1] CCP, Critical Control Point; [2] CP, Control Point; [3] Q, Question.

The first CCP identified was pasteurization, because non-compliance with the parameters of this stage could lead to the survival of pathogenic bacteria, which has the consequence of causing health problems to consumers. Several publications have been identified that describe the effect of term treatment on the inactivation of toxins and bacteria [43–45].

The second CCP is considered cooling, followed by storage. At this stage of the technological process, the temperature is reduced from 85 °C to 2–8 °C in 1 h. This CCP is considered essential because keeping it under control prevents the growth of potentially present thermotolerant bacteria. After pasteurization, product cross-contamination can be controlled by applying strict cleaning and disinfection rules [11].

The growth of bacteria can be controlled by strict time–temperature control. Consequently, time and temperature must be carefully monitored during the storage process [46]. The same strict conditions must be observed for the delivery and sales stages—CCP 3.

After the correct performance of the CCPs, the critical limits are established for each, monitoring procedures and actions to be taken if critical limits or action limits or action criteria are exceeded, as illustrated in Table 6.

Table 6. Identifying critical limits, monitoring procedures, and corrective actions.

		Pasteurization	Storage, Cooling	Sales
Target value		85–95 °C 20–30 min	2–8 °C	2–8 °C
Critical value		≤85 °C; ≤20 min	≥8 °C	≥8 °C
Monitoring	Responsible	Technological engineer	Technological engineer	Refrigerator driver
	Method	Physical method, visual	Physical method, visual	Physical method, visual
	Frequency	Continue	Continue	Continue
	Document	Monitoring sheet	Monitoring sheet	Monitoring sheet
Correction/ Corrective action	Correction	For parameters (temperature, time)	For parameters (temperature)	For parameters (temperature)
	Corrective action	Bringing the parameters to the critical value (increasing the temperature and time)	Bringing the parameters to the critical value (temperature drop)	Bringing the parameters to the critical value (temperature drop)
	Responsible	Technological engineer	Technological engineer	Technological engineer

To check whether the HACCP plan is functioning as envisaged, the food safety team established a verification plan in Table 7, which specifies the application domain, frequencies, and responsibilities for the verification activities.

Table 7. Establishing verification procedures.

Crt. No	Field of Verification	Check Frequency	Responsible for Verification
1.	Verification of compliance with the procedure for selecting suppliers and procurement of raw milk and materials	Monthly	HACCP team leader/FES
2.	Checking the quality and safety of food	Monthly	Responsible for hygiene and quality/FES
3.	Checking the mode of transport of raw milk and materials	Monthly	Technological engineer/FES
4.	Checking the storage and output mode for processing raw milk and materials	Monthly	Technological engineer/FES
5.	Drinking water supply check	Annually	Responsible for hygiene and quality/FES
6.	Verification of compliance with the stages of preparation of raw milk and materials	Monthly	Technological engineer/FES
7.	Verification of compliance with equipment maintenance	Biannually	Maintenance manager/FES
8.	Verification of calibration of measuring and control devices	Biannually	HACCP team leader/FES
9.	Checking the hygiene of production spaces, annexes, and social groups	Monthly	HACCP team leader/FES
10.	Checking the control of the health status of the staff	Biannually	HACCP team leader/FES
11.	Checking the hygiene of the work equipment	Monthly	HACCP team leader/FES
12.	Checking the way to ensure the disposal of waste	Biannually	HACCP team leader/FES
13.	Verification of compliance with the pest control procedure	Monthly	HACCP team leader/FES
14.	Verification of CCP records; deviations from critical limits; execution of corrective measures	Daily	HACCP team leader/FES
15.	Checking CP records	Daily	HACCP team leader/FES
16.	Checking the way to ensure staff training	Biannually	HACCP team leader/FES
17.	Checking the quality control and safety of the finished products	Monthly	Responsible for hygiene and quality/FES
18.	Checking the registration activity	Monthly	HACCP team secretary/FES
19.	Checking the registration and settlement mode of complaints	Monthly	HACCP team secretary/FES

FES—food engineering students.

In this study, to achieve the last principle of the HACCP plan, the documents and records prepared during the implementation of the plan are used. These documents represent evidence regarding the realization of the HACCP principles, the monitoring of the parameters of the CCPs, and the proposed corrective actions. These documents are divided into instructions and procedures and consist of the documents elaborated for the educational tool [11]. Their structural elements are title, purpose,

application/scope, definitions, abbreviations, authorities, responsibilities, description of activities, records, related documents, references, and annexes.

3.3. Microbiological Analysis Results of Yogurt

The microbiological characteristics of raw milk, pasteurized milk, and yogurt samples are shown in Table 8. The samples were analyzed before and after the implementation of ISO 22000:2018 to verify the advantages of FSMS.

Table 8. Microbiological characteristics of raw milk, pasteurized milk, and yogurt samples quantified before and after the HACCP implementation.

	Analyze/Sample	Before/After HACCP Implementation	Raw Milk	Pasteurized Milk	Yogurt
.5	CFU SR EN ISO 4833-1:2014	Before HACCP implementation	250,000 cfu/mL	754 cfu/mL	-
		After HACCP implementation	80,182 cfu/mL	97 cfu/mL	-
.5	SCC SR EN ISO 13366-1:2008/AC:2010	Before HACCP implementation	345,000 NCS/mL	-	-
		After HACCP implementation	14,000 NCS/mL	-	-
.5	*Enterobacteriaceae* ISO 21528-1:2017	Before HACCP implementation	-	6 cfu/mL	3 cfu/mL
		After HACCP implementation	-	0 cfu/mL	0 cfu/mL

Following the HACCP plan's implementation, a decrease in the specific microbiological load is observed, as shown in Table 8. In the case of raw milk, CFU decreases from 250,000 cfu/mL to 80,182 cfu/mL. In the case of pasteurized milk it decreases from 754 cfu/mL to 97 cfu/mL. These values are within the maximum allowed [21] of 300,000 cfu/mL for raw milk and 100,000 cfu/mL for pasteurized milk. In the case of NCS there is a decrease in raw milk from 345,000 NCS/mL to 14,000 NCS/mL, with the maximum allowed [21] being 400,000 NCS/mL. Spectacular decreases are also observed in the case of *Enterobacteriaceae*: In the case of pasteurized milk it decreases from 6 cfu/mL to 0 cfu/mL, and in the case of yogurt it decreases from 3 cfu/mL to 0 cfu/mL, within the maximum allowed values [22] of 10 cfu/mL. In the literature, the HACCP system application in dairy establishments has improved the microbial quality of the dairy product [14,20]. A study by Cusato [20] show similar results and showed the reduction of total coliform, mold, and yeast count in yogurt after the application of the HACCP plan in a dairy factory.

4. Limitation of the Study

The study integrates the microbiological parameters as a quality control (QC) tool of FSMS (Food Safety Management Systems) (HACCP) concerning good hands-on practice for FES implemented on-site in a small-scale yogurt pilot plant as educational programs. The model is adapted to a small-scale yogurt pilot plant, implementing only a simple FSMS (Food Safety Management System) involving HACCP principles and PRPs. These limitations help define new good practice and thinking models for teaching and learning FSMS in food-scale yogurt plant production.

5. Conclusions

The implementation of PRPs has a significant impact on the implementation of the HACCP system. The decision tree application shows that pasteurization, cooling/storage, and distribution processes are the selected hazard control measures, classified as CCP. The results of microbiological analysis

of packed yogurt showed that the implementation of HACCP could improve the microbial quality of yogurt. The implementation of the HACCP plan in a small-scale yogurt pilot plant has brought benefits to food security. This system allows immediate action to be taken when safety issues are reported from the receipt of the raw milk to the delivery of the yogurt and the basis of educational tools for practice and learning the implementation of FSMS.

The results obtained following the implementation of ISO 22000:2018 regarding the processing of yogurt in a small-scale yogurt pilot plant have implications for the yogurt industry and education programs. The HACCP approach in DPP and the results obtained can be easily applied in pilot stations or food industry factories at a food scale-up, assessing the advantages and drawbacks of implementing FSMS in the food industry. This study's conclusions underlie future research regarding the development of FSMS by applying predictive microbiology models and risk-assessment schemes, being an integrated model of good practice and education tools.

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Abbreviations

DPP	Dairy pilot plant
CCP	Critical control point
CP	Control point
PRPs	Prerequisite programs
OPRPs	Operational prerequisite programs
GMP	Good manufacturing practices
SSOP	Sanitation standard operating procedure
GMP	Good manufacturing practices
GHP	Good hygiene practices
HACCP	Hazard Analysis Critical Control Points
FSMS	Food safety management system
QMS	Quality management system
QS	Quality system
CFU	Colony forming units
SCC	Somatic cell count
FES	Food engineering students

References

1. Zhang, S.S.; Xu, Z.S.; Qin, L.H.; Kong, J. Low-sugar yogurt making by the co-cultivation of *Lactobacillus plantarum* WCFS1 with yogurt starter cultures. *J. Dairy Sci.* **2020**. [CrossRef]
2. Corrieu, G.; Béal, C. Yogurt: The Product and its Manufacture. *Encycl. Food Health* **2016**, 617–624. [CrossRef]
3. Aryana, K.J.; Olson, D.W. A 100-Year Review: Yogurt and other cultured dairy products. *J. Dairy Sci.* **2017**, *100*, 9987–10013. [CrossRef]
4. Melo, J.; Andrew, P.W.; Faleiro, M.L. *Listeria monocytogenes* in cheese and the dairy environment remains a food safety challenge: The role of stress re-sponses. *Food Res. Int.* **2015**, *67*, 75–90. [CrossRef]
5. Soni, R.; Jain, N.K.; Shah, V.; Soni, J.; Suthar, D.; Gohel, P. Development of probiotic yogurt: Effect of strain combination on nutritional, rheological, organoleptic and probiotic properties. *J. Food Sci. Technol.* **2020**, 1–13. [CrossRef]

6. Garcell, H.G.; Garcia, E.G.; Pueyo, P.V.; Martín, I.R.; Arias, A.V.; Serrano, R.N.A. Outbreaks of brucellosis related to the consumption of unpasteurized camel milk. *J. Infect. Public Health* **2016**, *9*, 523–527. [CrossRef]
7. Gould, L.H.; Mungai, E.; Barton Behravesh, C. Outbreaks attributed to Cheese: Differences between outbreaks caused by unpasteurized and pasteurized dairy products, United States, 1998-2011. *Foodborne Pathog. Dis.* **2014**, *11*, 545–551. [CrossRef]
8. Lindstrom, M.; Myllykoski, J.; Sivela, S.; Korkeala, H. Clostridium botulinum in cattle and dairy products. *Crit. Rev. Food Sci. Nutr.* **2010**, *50*, 281–304. [CrossRef]
9. Motarjemi, Y.; Moy, G.G.; Jooste, P.J.; Anelich, L.E. Milk and dairy products. In *Food Safety Management—A Practical* Guide for the Food Industry; Motarjemi, Y., Lelieveld, H., Eds.; Academic Press: New York, NY, USA, 2014; pp. 83–117.
10. Claeys, W.L.; Cardoen, S.; Daube, G.; De Block, J.; Dewettinck, K.; Dierick, K.; De Zutter, L.; Huyghebaert, A.; Imberechts, H.; Thiange, P.; et al. Raw or heated cow milk consumption: Review of risks and benefits. *Food Control* **2013**, *31*, 251–262. [CrossRef]
11. Allata, S.; Valero, A.; Benhadja, L. Implementation of traceability and food safety systems (HACCP) under the ISO 22000:2005 standard in North Africa: The case study of an ice cream company in Algeria. *Food Control* **2017**, *79*, 239–253. [CrossRef]
12. Kamboj, S.; Gupta, N.; Bandral, J.D.; Gandotra, G.; Anjum, N. Food safety and hygiene: A review. *Int. J. Chem. Stud.* **2020**, *8*, 358–368. [CrossRef]
13. Manley, D. Quality management systems and hazard analysis critical control point (HACCP) in biscuit manufacture. *Manley's Technol. Biscuitscrackers Cookies* **2011**, 23–28. [CrossRef]
14. El-Hofi, M.; El-Tanboly, E.S.; Ismail, A. Implementation of the hazard analysis critical control point (HACCP) system to UF white cheese production line. *Acta Sci. Pol. Technol. Aliment.* **2010**, *9*, 331–342.
15. Nada, S.; Ilija, D.; Igor, T.; Jelena, M.; Ruzica, G. Implication of food safety measures on microbiological quality of raw and pasteurized milk. *Food Control* **2012**, *25*, 728–731. [CrossRef]
16. Chen, H.; Chen, Y.; Liu, S.; Yang, H.; Chen, C.; Chen, Y. Establishment the critical control point methodologies of seven major food processes in the catering industry to meet the core concepts of ISO 22000:2018 based on the Taiwanese experience. *J. Food Saf.* **2019**, 1–10. [CrossRef]
17. Panghal, A.; Chhikara, N.; Sindhu, N.; Jaglan, S. Role of Food Safety Management Systems in safe food production: A review. *J. Food Saf.* **2018**, *38*. [CrossRef]
18. ISO 22000:2018. *ISO 22000-Food Safety Management Systems Requirements for Any Organization in the Food Chain*; ISO: Geneva, Switzerland, 2018.
19. Chhikara, N.; Jaglan, S.; Sindhu, N.; Anshid, V.; Veera, M.; Charan, S.; Panghal, A. Importance of traceability in food supply chain for brand protection and food safety systems implementation. *Ann. Biol.* **2018**, *34*, 111–118.
20. Cusato, S.; Gameiro, A.H.; Corassin, C.H.; Sant'Ana, A.S.; Cruz, A.G.; Faria, J.d.A.F.; de Oliveira, C. AF Food Safety Systems in a Small Dairy Factory: Implementation, Major Challenges, and Assessment of Systems' Performances. *Foodborne Pathog. Dis.* **2013**, *10*, 6–12. [CrossRef]
21. Regulation (EC) No 853/2004 of the European Parliament and of the Council of 29 April 2004 Laying Down Specific Hygiene Rules for Food of Animal Origin as Amended and Supplemented by Reg. Nr. 1020/2008. Available online: https://eur-lex.europa.eu/legal-content/EN/TXT/PDF/?uri=CELEX:32004R0853&from=en (accessed on 11 May 2020).
22. Regulation (EU) No. 365/2010 of the Commission of 28 April 2010 Amending Regulation (EC) No Regulation (EC) No 2073/2005 on Microbiological Criteria for Food as Regards Enterobacteria in Pasteurized Milk and Other Liquid Pasteurized Milk Products and Listeria Monocytogenes in Food Salt. Available online: https://eur-lex.europa.eu/legal-content/EN/TXT/PDF/?uri=CELEX:32010R0365&from=EN (accessed on 11 May 2020).
23. EU Council Directive 2002/99/EC. Council Directive 2002/99/EC of 16 December 2002 Laying Down the Animal Health Rules Governing the Production, Processing, Distribution and Introduction of Products of Animal Origin for Human Consumption. Available online: https://eur-lex.europa.eu/legal-content/EN/TXT/PDF/?uri=CELEX:32002L0099&from=DE (accessed on 11 May 2020).

24. Regulation (EC) No 178/2002. Regulation (EC) No 178/2002 of the European Parliament and of the Council of 28 January 2002 Laying Down the General Principles and Requirements of Food Law, Establishing the European Food Safety Authority and Laying Down Procedures in Matters of Food Safety. Available online: https://eur-lex.europa.eu/legal-content/EN/TXT/PDF/?uri=CELEX:32002R0178&from=EN (accessed on 11 May 2020).
25. Regulation (EC) No 852/2004. Regulation (EC) No 852/2004 of the European Parliament and of the Council of 29 April 2004 on the Hygiene of Foodstuffs. Available online: https://eur-lex.europa.eu/legal-content/EN/TXT/PDF/?uri=CELEX:32004R0852&from=EN (accessed on 11 May 2020).
26. Regulation (EC) No 854/2004. Regulation (EC) No 854/2004 of the European Parliament and of the Council of 29 April 2004 Laying Down Specific Rules for the Organisation of Official Controls on Products of Animal Origin Intended for Human Consumption. Available online: https://eur-lex.europa.eu/legal-content/EN/TXT/PDF/?uri=CELEX:32004R0854&from=EN (accessed on 11 May 2020).
27. Regulation (EC) 882/2004. Regulation (EC) No 882/2004 of the European Parliament and of the Council of 29 April 2004 on Official Controls Performed to Ensure the Verification of Compliance with Feed and Food Law, Animal Health and Animal Welfare Rules. Available online: https://eur-lex.europa.eu/legal-content/EN/TXT/PDF/?uri=CELEX:32004R0882&from=EN (accessed on 11 May 2020).
28. SR EN ISO 4833-1:2014 Microbiology of the Food Chain—Horizontal Method for the Enumeration of Microorganisms—Part 1: Colony Count at 30 Degrees c by the Pour Plate Technique. Available online: https://www.iso.org/standard/53728.html (accessed on 11 May 2020).
29. SR EN ISO 13366-1:2008/AC:2010 Milk—Enumeration of Somatic Cells—Part 1: Microscopic Method (Reference Method). Available online: https://www.iso.org/standard/40259.html (accessed on 11 May 2020).
30. *ISO 21528-1:2017 Microbiology of the Food Chain—Horizontal Method for the Detection and Enumeration of Enterobacteriaceae—Part 1: Detection of Enterobacteriaceae*; ISO: Geneva, Switzerland, 2017.
31. Da Cruz, A.G.; Cenci, S.A.; Maia, M.C. Quality assurance requirements in produce processing. *Trends Food Sci. Technol.* **2006**, *8*, 406–411. [CrossRef]
32. Gaaloul, I.; Riabi, S.; Ghorbel, R.E. Implementation of ISO 22000 in cereal food industry "SMID" in Tunisia. *Food Control* **2011**, *22*, 59–66. [CrossRef]
33. Karaman, A.D.; Cobanoglu, F.; Tunalioglu, R.; Ova, G. Barriers and benefits of the implementation of food safety management systems among the Turkish dairy industry: A case study. *Food Control* **2012**, *25*, 732–739. [CrossRef]
34. Martínez-Rodríguez, A.J.; Carrascosa, A.V. HACCP to control microbial safety hazards during winemaking: Ochratoxin A. *Food Control* **2009**, *20*, 469–475. [CrossRef]
35. Mortimore, S. How to make HACCP really work in practice. *Food Control* **2001**, *12*, 209–215. [CrossRef]
36. Fernandez-Segovia, I.; Perez-LIacer, A.; Peidro, B.; Fuentes, A. Implementation of a food safety management system according to ISO 22000 in the food supplement industry: A case study. *Food Control* **2014**, *43*, 28–34. [CrossRef]
37. McSwane, D.; Rue, N.; Linton, R. *Essentials of Food Safety and Sanitation*, 3rd ed.; Pearson Education: Upper Saddle River, NJ, USA, 2003.
38. Arvanitoyannis, I.S.; Varzakas, T.H.; Koukaliaroglou-van Houwelingen, M. Implementing HACCP and ISO 22000 for Foods of Animal Origin—Dairy Products. In *HACCP and ISO 22000-Application to Foods of Animal Origin*; Arvanitoyiannis, I.S., Ed.; Wiley-Blackwell: Oxford, UK, 2009; pp. 91–180.
39. Papademas, P.; Bintsis, T. Food safety management systems (FSMS) in the dairy industry: A review. *Int. J. Dairy Technol.* **2010**, *63*, 489–503. [CrossRef]
40. MacSwane, D.; Rue, N.; Linton, R. Food safety. In *Essentials of Food Safety and Sanitation*, 2nd ed.; McSwane, D., Rue, N., Linton, R., Eds.; Prentice Hall: Upper Saddle River, NJ, USA, 2000; pp. 1–75.
41. Van Asselt, E.D.; Noordam, M.Y.; Pikkemaat, M.G.; Dorgelo, F.O. Risk-based monitoring of chemical substances in food: Prioritization by decision trees. *Food Control* **2018**, *93*, 112–120. [CrossRef]
42. Trevisani, M.; Mancusi, R.; Valero, A. Thermal inactivation kinetics of Shiga toxin-producing *Escherichia coli* in buffalo mozzarella curd. *J. Dairy Sci.* **2014**, *97*, 642–650. [CrossRef]
43. Valero, A.; Cejudo, M.; García-Gimeno, R.M. Inactivation kinetics for *Salmonella* Enteritidis in potato omelet using microwave heating treatments. *Food Control* **2014**, *43*, 175–182. [CrossRef]

44. Van Lieverloo, J.H.M.; de Roode, M.; Fox, M.B.; Zwietering, M.H.; Wells- Bennik, M.H. Multiple regression model for thermal inactivation of *Listeria monocytogenes* in liquid food products. *Food Control* **2013**, *29*, 394–400. [CrossRef]
45. Lu, J.; Pua, X.H.; Liu, C.T.; Chang, C.L.; Cheng, K.C. The implementation of HACCP management system in a chocolate ice cream plant. *J. Food Drug Anal.* **2014**, *22*, 391–398. [CrossRef]
46. Kassem, M.; Salem, E.; Ahwal, A.M.; Saddik, M.; Gomaa, N.F. Application of hazard analysis and critical control point in dairy industry. *Rev. Sante Mediterr. Orient.* **2002**, *8*, 114–128.

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Review

Food Security during the Pandemic and the Importance of the Bioeconomy in the New Era

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Abstract: One of the biggest challenges in managing the food sector during a pandemic crisis is sustaining a robust food security system and adopting the right strategies in correlating the consumers' needs and requirements with those of food safety, the producers, the distribution chain, the economic environment, and waste management. The restrictions on people's global movement, commodities, and services and the measures taken to reduce the spread of COVID-19 have disrupted food environments around the world and forced us to collectively redesign and optimize our systems using existing resources from a more sustainable perspective. This paper offers an overview of the implications of COVID-19 for the food supply chain and discusses several potential strategies for tackling short- and long-term adverse effects resulting from the pandemic.

Keywords: COVID-19; SARS-CoV-2; pandemic crises; food security; health implications; bioeconomy

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1. Introduction

The most recent outbreak of coronavirus disease (identified from a wholesale seafood and wildlife market from the Wuhan region of China) resulted in a highly transmittable viral infection with pandemic dimensions. Although the zoonotic source of SARS-CoV-2 is not yet fully elucidated, genomic analysis suggests bats as the vital reservoir from which the worldwide spread started [1].

The new coronavirus pandemic has exposed many vulnerabilities across various domains, far beyond the medical system and its related clinical aspects. Besides the urgency of designing a medical treatment and developing an effective vaccine, issues related to patient testing, the safety of and facilities for professional healthcare providers, social and economic safety, food safety, and even mental/psychological health and domestic safety require increased attention and the implementation of a range of crisis and postcrisis management tools [2,3].

The strict lockdowns, social distancing, the halting of all nonessential economic activities, and all of the protectionist restrictions imposed by authorities to control the coronavirus outbreaks have caused disruptions in most production and supply chains around the world [4]. These disruptions are, specifically, the restrictions on people's global movement, delays in the supply of raw materials and import–export transactions, price fluctuations, the lack of workers in agriculture, increased farming costs, and distribution

deficiencies. Other similar consequences have forced many companies to relocate, restructure, find alternative sources, and rapidly redesign their production processes, or in some cases, temporarily or permanently suspend their activities. Hence, in many developing countries and beyond, COVID-19 has had a severe impact on employment and income, generating a significant food crisis and insecurity for a growing number of people unable to afford their basic daily food [5,6]. What the pandemic has highlighted is the necessity of using resources by adopting the principles of sustainability to mitigate the devastating effects of the crises [7].

This paper offers an overview of the implications of COVID-19 for the food supply chain, highlighting global food security issues and the behavior of consumers under the pressures of a lockdown. It also emphasizes the importance of managing resources by applying sustainable principles and explains several potential strategies that could be useful for minimizing short- and long-term pandemic drawbacks (e.g., the bioeconomy and food bank concepts).

2. A Brief Overview of SARS-CoV-2 Health Implications

In December 2019, the Wuhan region of China faced an alarming number of cases of pneumonia of unknown etiology. Shortly afterward, it was assumed that a small local fish and wild animal market was the starting point of the unknown viral infection [8]. Following multiple investigations, a genome sequencing of respiratory tract samples from the pneumonia patients led to the isolation of a new, novel coronavirus and placed it in the *Betacoronavirus* genus (betaCoV), which also includes SARS-CoV and MERS-CoV [9].

Further epidemiological and genomic analysis revealed that 2019-nCoV shares 89% nucleotide identity with bat SARS-like CoVZXC21 and 82% identity with human SARS-CoV. Therefore, the International Committee on Taxonomy of Viruses and the World Health Organization (WHO) decided to name it SARS-CoV-2 [10,11], while the disease caused is called COVID-19.

Despite rigorous global containment and quarantine restrictions, the incidence of COVID-19 continues to rise, with 66,422,058 laboratory-confirmed cases and 1,532,418 deaths reported worldwide to date (7 December 2020). Moreover, 9 months after the WHO declared the new coronavirus a pandemic, the contamination rate is still alarming, with almost 550,000 new positive tests reported daily globally [12].

Usually, the most common symptoms of COVID-19 are a fever, a dry cough and sore throat, fatigue, diarrhea, breath shortness, and headaches. However, a large proportion of COVID-19 patients are exposed to a severe form of the disease with various complications, which can cause acute respiratory distress syndrome; hypoxia, dyspnea, and neurological deficiencies; acute heart injury, acute kidney, liver, and gastrointestinal problems; secondary infection with bacteria; and even death [13–16]. The maximum incubation period is assumed to be up to 14 days, while the average period between the onset of symptoms and death can range from 2 to 8 weeks [17]. However, the incidence and incubation period for mild or severe cases may differ, being closely related to the age of the patient; their immune response; or other pre-existing comorbidities including diabetes, cardiovascular disease, cerebrovascular disease, endocrine disease, chronic lung or kidney disease, and respiratory deficiencies [9,18].

Indeed, the pandemic severity of COVID-19 demands rigorous surveillance and ongoing monitoring to accurately track its evolution, transmissibility, and pathogenicity [19]. Clinical care for a confirmed COVID-19 case prioritizes early recognition, immediate isolation, and the implementation of appropriate measures to prevent the spread of the infection, followed by the provision of symptomatic care for those with a mild illness and optimized supportive care for those with severe symptoms. WHO is also monitoring the accelerated global vaccine development effort; of the 52 vaccine candidates in clinical evaluation, 13 were in phase 3 of clinical trials by the end of November 2020 [20].

To date, the most widely used identification technique is real-time reverse transcription polymerase chain reaction (RT-PCR) on a nasopharyngeal swab or sputum samples.

Serological tests can only be applied to identify antibodies generated by the body's defense system, not the virus's genetic material. It can take anywhere between several days to several weeks to develop enough antibodies to be detected in a blood test; therefore, the molecular method remains the safest method to diagnose an active coronavirus infection [21].

3. Food Security and Insecurity

As a result of the emergence of the coronavirus outbreak, the crisis has exacerbated all existing discrepancies in the food sector, leading to a significant destabilization of the global food security system. The food systems comprise a complex of closely correlated stages meant both to ensure the "farm to fork traceability" and security of the final products in all processing, distribution, consumption, and waste management activities and to maintain a connection between all involved parts [22]. Thus, one of the biggest challenges in managing the food sector during a pandemic crisis is to adopt an effective strategy in correlating the needs of consumers, food safety, producers, the distribution chain, the economic environment, and waste management. Even if the amount of food produced today would theoretically be enough to sustain all of humanity, the socioeconomic differences and the unequal distribution of resources (in different regions around the world) would generate discrepancies in living standards. Consequently, in developed countries, food abundance inevitably leads to a large amount of food waste, while in underdeveloped countries, more than one billion people have difficulty in purchasing their essential daily commodities [23].

The COVID-19 pandemic has had a significant impact on the food industry not only in developing countries, where the repercussions are already being felt, but also in developed countries with relatively stable food sectors, where the repercussions will be felt in the long term. From an economic point of view, pandemic effects have been intensified by the loss of more than 200 million jobs. Consequently, according to the prediction of the United Nations World Food Programme (WFP), the number of people across the planet facing acute food insecurity stands to rise to 265 million in 2020, almost doubling from the 135 million reported in 2019 [24].

According to the official Food and Agriculture Organization (FAO) definition, "Food security is a situation that exists when all people, at all times, have physical, social and economic access to sufficient, safe and nutritious food that meets their dietary needs and food preferences for an active and healthy life." When people do not have adequate physical, social, or economic access to food as defined above, the phenomenon of food insecurity will inevitably occur [25]. Before the coronavirus outbreak, food insecurity was already a severe problem, mainly due to the increased number of people with limited access to food. Under the current pandemic, food security has declined significantly [26].

At the community level, food access and availability have been significantly affected, primarily due to disruption in the transportation, distribution, and delivery chain. At the individual level, nutritional status, also considered an indicator of food security, has been perturbed by variations in consumption behavior [27]. Moreover, due to the restrictions on people's movement imposed by the pandemic, reductions in regulatory control measures and oversight of inspection agencies in the food sector have occurred. As a consequence, food authenticity vulnerabilities have been exposed by creating suitable premises for food fraudsters to operate, thus increasing the risk of food fraud (e.g., substitution, misrepresentation, document fraud, waste diversion, illegal processing) [28].

As shown schematically in Figure 1, the consequences of pandemic disruptions have been severe and have exposed the principal vulnerabilities of the security system.

Looking to the future, increasing investment in agriculture and biotechnology research would undoubtedly have a significant impact on enhancing and maintaining more stable food security in the event of future pandemics. Advanced technologies and innovative approaches need to be adopted globally in each region to optimize regional food production capability in order to provide not only a safer but also a more sustainable source of food [5]. For example, management systems such as Vulnerability Assessment and Critical Control

Points (VACCP) plans have already been implemented in several large food chains. Still, in the actual pandemic context, their inclusion in every supplier's food safety assurance program is likely to be accelerated [28].

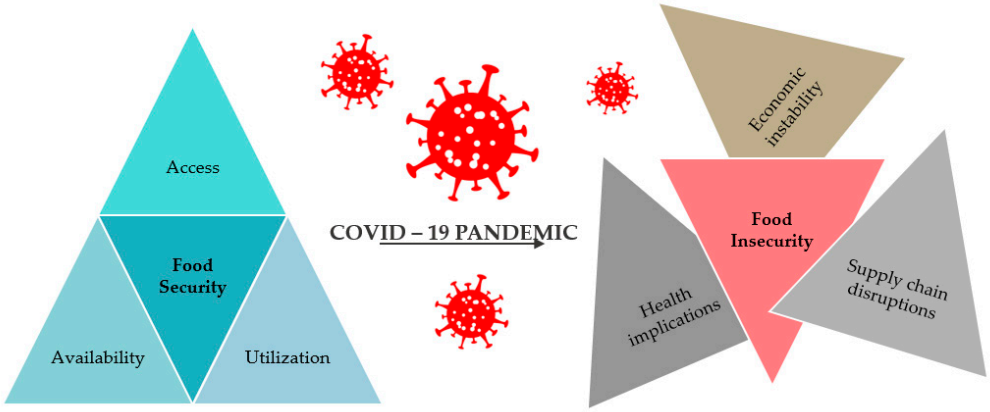

Figure 1. The repercussions of a pandemic on the stability of food security.

4. The Importance of Nutritional Behavior during a Pandemic

Nowadays, different forms of malnutrition affect one in three people globally. This is concerning because nutritional imbalances and a deficient immune system are, in many cases, starting points for the generation or aggravation of other diseases.

Both underdeveloped and high-income countries face problems caused by inadequate nutrition. These issues are reflected by undernutrition, anorexia, and micronutrient deficiencies, as well as an overweight population, obesity, and diet-related noncommunicable diseases [29]. Consequently, nutritional deficiencies of high-quality macro- and micronutrients are strongly associated with a depressed immune function and increased susceptibility to infection [27,30]. Under the pressure of the pandemic, people have had to rethink how they procure and select food resources and should have been more aware of the importance of maintaining a healthy organism. Despite that, panic buying affected diet quality, as people may have encountered various impediments in purchasing healthy fresh food [7]. There is clear evidence that the increase in prices for fresh vegetables, fruits, and rich protein foods (such as meat and fish) has led to a shift towards ready-to-eat snacks, frozen or processed meat, and canned and other nonperishable foods. From a nutritional point of view, this can be translated into a higher caloric intake but a lower nutritional quality [27,31]. Associated with prolonged sedentary time, all of these imbalances can increase the risk of developing obesity and nutrient imbalance in the long term, as well as other related complications like diabetes, dyslipidemia, and cardiovascular diseases [32,33]. Recent evidence suggests that patients with different malnourishment deficiencies or who have specific comorbidities have a high risk of requiring hospitalization in intensive care units or conditions of mechanical ventilation, presenting a higher rate of mortality from SARS-CoV-2 syndrome [34].

Figure 2 explains the potential interdependence between the economic and food crisis generated by the measures applied against the spread of the virus and the fact that nutritional deficiencies caused by limited access to a balanced diet make the human organism more exposed to the risk of infection. People suffering from malnutrition are at a greater risk of developing severe COVID-19 symptoms as a result of associated health conditions and noncommunicable diseases, which compromise the immune system. As a consequence, a weakened immune system reduces the chances of recovery and significantly increases the period of convalescence; therefore, food safety can be considered a vital pawn in the fight against the new coronavirus from this point of view.

Figure 2. The potential connection between the availability of resources, health status, nutrition, and susceptibility to infection.

The response of human cellular immunity mechanisms to the SARS-CoV-2 viral infection is strongly influenced by host nutrition, mainly by the presence or absence of specific micronutrients and bioactive molecules [14,35–38]. An indirect approach is to use different mechanisms that sustain the absorption and bioavailability of bioactive molecules. In this sense, the most efficient and widely used means are fermentation (fermented foods, pro- and prebiotics) [39], enzymatic treatments (use of enzyme supplements and foods rich in enzymes) [40], or micro- and nanoencapsulation (targeted and controlled release and protection) [41].

In the COVID-19 fight, the viral host resistance of the human body consists of support from both macro- and micronutrients (but especially from micronutrients), namely vitamins A, C, D, E, B6, B9, and B12; copper (Cu); magnesium (Mg); selenium (Se); iron (Fe); and zinc (Zn) [42–46]. The wall that forms the first defensive barrier against the virus is the respiratory tract, with its physical and biochemical functions sustained by the presence of vitamin A and Fe [47,48]. The mechanisms of the micronutrient action are complicated. Concerning the vitamins and minerals that directly influence the respiratory functions, vitamins A, C, and D and the mineral Zn can modulate membrane functionality (integrity, fluidity, communication, and capacity to repair) [48–50]. Dietary selenium supplementation can increase the antiviral immune responses by, for example, cutting down the pathogenicity of virus infection in the avian case [51]. Similarly, selenium supplement intake seems to yield good results in COVID-19-susceptible hosts [15]. Thus, suitable and equilibrate nutrition, with properly regulated health monitoring, can significantly improve the immune response to SARS-CoV-2 viral infection.

In addition to the medical health and general safety of the population, psychological wellbeing and mental safety are subjects that cannot be overlooked [2]. Various psychological problems and related consequences, including panic, stress, anxiety, depression, and uncertainty have emerged progressively during the COVID-19 outbreak [52]. Besides the fear of contamination and socioeconomic imbalances, anxiety and uncertainty have also tended to be exacerbated by an inadequate supply of necessary resources (e.g., food, water, clothing, etc.) and restricted access to some commodities during the quarantine period [53].

5. Bioeconomy Concept Importance in Pandemic

The instability of our global supply chains began to be felt from the early stages of the pandemic, starting with the acute crisis of medical equipment and immediately extending to other indispensable areas such as the food industry. Under the global experience of these pandemic emergencies, there has never been a more proper moment to prioritize and accelerate the implementation of the principles of a circular economy vision.

Food loss and agroindustrial byproducts represent a continuously growing global issue, posing a challenge to food safety and security and negatively impacting the economy and environmental stability. According to FAO, approximately one-third of the world's food is inefficiently managed and wasted every year [4]. In the current crisis, the dominant system of the linear economy model, based on the "take–make–consume–dispose" approach, has proved to be inefficient in supporting the sustainable management of resources. Contrary to the principles of the linear economy, the bioeconomy model considers waste as

a new resource with high potential to be converted into various biomaterials, biofuels, and other added-value products [54].

While in low-income countries waste occurs mainly during the processing steps of raw materials, in middle- and high-income countries, waste is generated in the highest proportion in the distribution and consumption stages. Nevertheless, the impact of the pandemic has significantly changed consumer behavior and attitudes towards purchasing and consumption activities, and consumers are also showing increased attention to reducing food waste [4]. Although the population tended to invest in substantial reserves of nonperishable food at the beginning of the quarantine, their purchasing power has since decreased significantly. Therefore, food producers and retailers may face stock imbalances and losses caused by the degradation of perishable products.

If the residues and byproducts of the food industry were considered to be worthless until recently, now the attitude has begun to change, with new research directions being oriented towards their exploitation. Implementing the reintegration concept facilitates the conversion of agroindustrial waste into high-value products with relevant potential applications for human consumption and other necessities of daily living [55]. In this context, the biggest challenge of the scientific world is to develop efficient valorization strategies with a high degree of industrial feasibility [56]. In prioritizing resource efficiency, the closed-loop value chains that convert waste and byproducts into resources can lead to the achievement of a sustainable food system in all its three dimensions: economic, social, and environmental protection [23,57]. In this context, the management of food byproducts is encouraged to move from a linear model to a much more complex circular chain. As illustrated in Figure 3, reintegrated into a circular economy system, residues and agrifood byproducts can generate a series of products with applicability in the food, cosmetics, pharmaceutical, agriculture, or fuel industries.

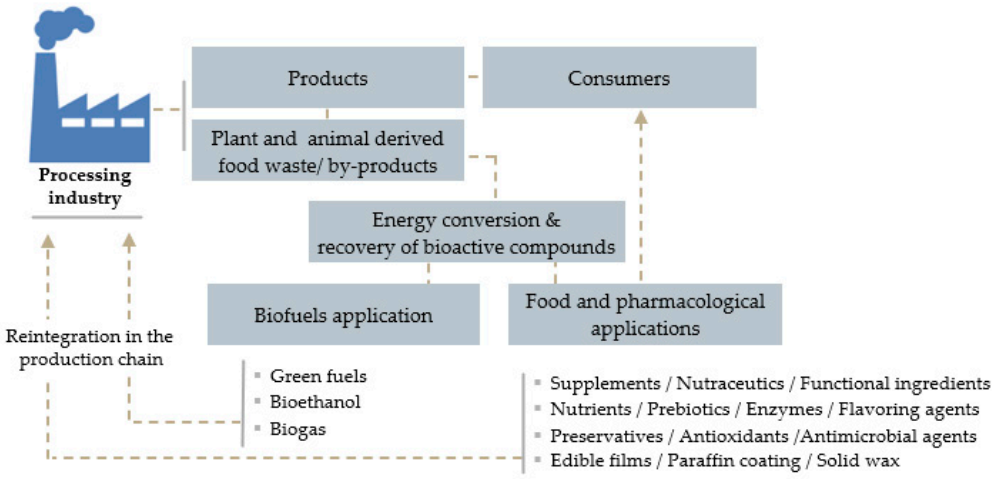

Figure 3. Bioeconomy and agroindustrial waste conceptual diagram.

The most complicated decision in reintegrating waste into a biorefinery model is to establish the usefulness and new destination of the novel products. In this sense, the greatest challenge for researchers is to develop and optimize extraction and conversion protocols with a degree of feasibility to ensure their sustainability when implemented at the industrial level [58,59].

The bioeconomy approach could have a significant impact not only on sustaining the resource demands of a post-COVID world but also on the long-term challenges of climate change, population growth, environmental degradation, and food security [5]. The implementation of the concept can also be seen as a perspective to promote a business model that could generate new employment opportunities [60].

6. Food Banks Concept

Considering the concept of the bioeconomy, the principles of which focus on reuse, redesign, waste minimization, and recycling, the opportunity to leverage the benefits of the immediate community instead of global supply chains seems to be a sustainable approach that deserves to be investigated [61]. A well-developed and stable local distribution chain, once integrated with the principles of the bioeconomy, could temporarily support the problems of the community in different crises through charitable actions. As a result, various volunteer-based associations have begun to emerge, collecting surplus resources and redistributing them where needed [62].

Food banks are a typical community-based response to household food insecurity [63]. The concept of the food bank began to materialize in 1960 in the United States. Its purpose, namely to redirect surplus products to social categories with vulnerabilities in procuring the basic food for their needs, was established from the beginning [64]. Over time, this concept was extended, addressing all segments of the food-insecure population. In Europe, the idea was first initiated in 1984 in France, and in 1986 the European Federation of Food Banks (FEBA) began the process of implementing the concept and developing a network of food banks across the continent. However, in many countries, the concept was expanded significantly only in 2010, when the repercussions of the global economic crisis affected a large part of the population. For example, the Food Waste Romania Organization reported that approximately one-third of all food products are wasted annually. This amount corresponds to about 2.55 million tons of food, while 5 million Romanians live at the poverty line and 66% of rural families cannot afford basic daily food. Thus, in 2016, Romania became a member of FEBA and began the development of a network of food banks that currently operates in five of its main cities [65].

Through food banks, edible and nonfood food products are collected from economic agents that for various reasons have lost their economic market value and are redirected to various nongovernmental organizations that provide support to disadvantaged people. This process is supported through the involvement and support of volunteers, economic agents and retailers, supermarkets, food manufacturers and processors, and any other companies and individuals who are willing to support the disadvantaged social class and reduce food waste [66,67].

Figure 4 shows a simplified diagram of the food bank concept and the main entities involved in the process.

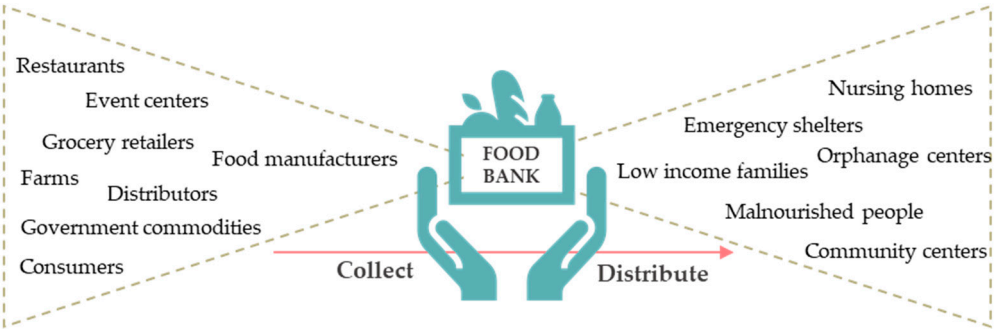

Figure 4. Food bank conceptual diagram.

In the context of a pandemic crisis, the segment of the population that needs the facilities of a food bank is gradually expanding, with the emergence of financial problems generated mainly by the temporary suspension or loss of many jobs. Without the bank collection point, the redirection of surplus food to its beneficiaries is not safe in terms of traceability and food safety. Therefore, the distribution of products is not conducted directly from supermarkets to individuals, but only through eligible partner organizations [68]. Thus, the food bank is not just a simple collection point, as its responsibilities are much more complex and its rules of operation are based on the principles of transparency and food

safety. Throughout this process, principles such as social equity, product nutritional quality, safe and hygienic storage and transport, and environmental protection are mandatory to consider.

In Europe, over the last 9 months, the COVID-19 crisis has caused not only chaos in the medical system but also a new food emergency. According to a report of a survey conducted amongst European Food Banks, the demand for food has increased by up to 50% compared to the precoronavirus period, and lockdown consequences continue to increase the number of people experiencing difficulties. Although collection and distribution activities are hampered by the limitations caused by the pandemic such as a reduction in the number of volunteers and donors involved, transportation difficulties, or economic difficulties, food banks have managed to adapt to the new conditions and continue to support the affected social categories [69].

7. Conclusions and Future Trends

The COVID-19 pandemic has exposed the weaknesses and amplified the instability in medical, social, and economic systems. Confronting the effects and chaos generated by the pandemic crisis has forced us to not only urgently identify an effective treatment scheme and a vaccine to immunize the population, but also streamline and implement a series of sustainable strategies to rebalance all affected sectors. In this sense, in addition to the medical and research fields, the food sector must be considered an essential pillar of survival that must be prioritized for the implementation of effective support strategies. As the COVID-19 pandemic persists, considerable attention has been focused on the stability of food supply chains and their impact on global food security. Understanding of the various safety implications of the current pandemic has raised awareness among consumers, scientists, and authorities that similar (or even more severe) crises may occur and that proper management is crucial for minimizing their negative effects.

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References

1. Letko, M.; Seifert, S.N.; Olival, K.J.; Plowright, R.K.; Munster, V.J. Bat-borne virus diversity, spillover and emergence. *Nat. Rev. Microbiol.* **2020**, *18*, 461–471. [CrossRef]
2. Haghani, M.; Bliemer, M.C.; Goerlandt, F.; Li, J. The scientific literature on Coronaviruses, COVID-19 and its associated safety-related research dimensions: A scientometric analysis and scoping review. *Saf. Sci.* **2020**, *129*, 104806. [CrossRef]
3. Rizou, M.; Galanakis, I.M.; Aldawoud, T.M.; Galanakis, C.M. Safety of foods, food supply chain and environment within the COVID-19 pandemic. *Trends Food Sci. Technol.* **2020**, *102*, 223–229. [CrossRef] [PubMed]
4. Jribi, S.; Ismail, H.B.; Doggui, D.; Debbabi, H. COVID-19 virus outbreak lockdown: What impacts on household food wastage? *Environ. Dev. Sustain.* **2020**, *22*, 3939–3955. [CrossRef] [PubMed]
5. Henry, R. Innovations in agriculture and food supply in response to the COVID-19 pandemic. *Mol. Plant* **2020**, *13*, 1095–1097. [CrossRef] [PubMed]
6. Hobbs, J.E. Food supply chains during the COVID-19 pandemic. *Can. J. Agric. Econ.* **2020**, *68*, 171–176. [CrossRef]
7. Torero, M. Without food, there can be no exit from the pandemic. *Nature* **2020**, *580*, 588–589. [CrossRef]
8. Chen, W.H.; Strych, U.; Hotez, P.J.; Bottazzi, M.E. The SARS-CoV-2 vaccine pipeline: An overview. *Curr. Trop. Med. Rep.* **2020**, *7*, 61–64. [CrossRef]
9. Kaul, D. An overview of coronaviruses including the SARS-2 coronavirus—Molecular biology, epidemiology and clinical implications. *Curr. Med. Res. Pract.* **2020**, *10*, 54–64. [CrossRef]

10. Chan, J.F.W.; Kok, K.H.; Zhu, Z.; Chu, H.; To, K.K.W.; Yuan, S.; Yuen, K.Y. Genomic characterization of the 2019 novel human-pathogenic coronavirus isolated from a patient with atypical pneumonia after visiting Wuhan. *Emerg. Microbes Infect.* **2020**, *9*, 221–236. [CrossRef]
11. Coronaviridae Study Group of the International Committee on Taxonomy of Viruses. The species severe acute respiratory syndrome related coronavirus: Classifying 2019-nCoV and naming it SARS-CoV-2. *Nat. Microbiol.* **2020**, *5*, 536–544. [CrossRef] [PubMed]
12. WHO Coronavirus Disease (COVID-19) Dashboard. Available online: https://covid19.who.int/ (accessed on 8 December 2020).
13. Acter, T.; Uddin, N.; Das, J.; Akhter, A.; Choudhury, T.R.; Kim, S. Evolution of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) as coronavirus disease 2019 (COVID-19) pandemic: A global health emergency. *Sci. Total Environ.* **2020**, *730*, 138996. [CrossRef] [PubMed]
14. Ali, I.; Alharbi, O.M. COVID-19: Disease, management, treatment, and social impact. *Sci. Total Environ.* **2020**, *728*, 138861. [CrossRef] [PubMed]
15. Zhang, J.; Taylor, E.W.; Bennett, K.; Saad, R.; Rayman, M.P. Association between regional selenium status and reported outcome of COVID-19 cases in China. *Am. J. Clin. Nutr.* **2020**, *111*, 1297–1299. [CrossRef]
16. Shereen, M.A.; Khan, S.; Kazmi, A.; Bashir, N.; Siddique, R. COVID-19 infection: Origin, transmission, and characteristics of human coronaviruses. *J. Adv. Res.* **2020**, *24*, 91–98. [CrossRef]
17. Baud, D.; Qi, X.; Nielsen-Saines, K.; Musso, D.; Pomar, L.; Favre, G. Real estimates of mortality following COVID-19 infection. *Lancet Infect. Dis.* **2020**, *20*, 773. [CrossRef]
18. Abd El-Aziz, T.M.; Stockand, J.D. Recent progress and challenges in drug development against COVID-19 coronavirus (SARS-CoV-2)—An update on the status. *Infect. Genet. Evol.* **2020**, *83*, 104327. [CrossRef]
19. Sohrabi, C.; Alsafi, Z.; O'Neill, N.; Khan, M.; Kerwan, A.; Al-Jabir, A.; Iosifidis, C.; Agha, R. World Health Organization declares global emergency: A review of the 2019 novel coronavirus (COVID-19). *Int. J. Surg.* **2020**, *76*, 71–76. [CrossRef]
20. World Health Organisation (WHO). Draft Landscape of COVID-19 Candidate Vaccines. Available online: https://www.who.int/publications/m/item/draft-landscape-of-covid-19-candidate-vaccines (accessed on 9 December 2020).
21. Huang, C.; Wang, Y.; Li, X.; Ren, L.; Zhao, J.; Hu, Y.; Zhang, L.; Fan, G.; Xu, J.; Gu, X.; et al. Clinical features of patients infected with 2019 novel coronavirus in Wuhan, China. *Lancet* **2020**, *395*, 497–506. [CrossRef]
22. Galanakis, C.M. The food systems in the era of the coronavirus (COVID-19) pandemic crisis. *Foods* **2020**, *9*, 523. [CrossRef]
23. Segneanu, A.; Grozescu, I.; Cepan, C.; Cziple, F.; Lazar, V. Food security into a circular economy. *J. Food Sci. Nut.* **2018**, *4*, 1–3. [CrossRef]
24. United Nations World Food Programme. COVID-19 Will Double Number of People Facing Food Crises Unless Swift Action Is Taken. Available online: https://www.wfp.org/news/covid-19-will-double-number-people-facing-food-crises-unless-swift-action-taken (accessed on 20 August 2020).
25. Food and Agriculture Organization of the United Nations (FAO Rome). Trade Reforms and Food Security: Conceptualizing the Linkages. 2003. Available online: http://www.fao.org/3/a-y4671e.pdf (accessed on 14 August 2020).
26. Udmale, P.; Pal, I.; Szabo, S.; Pramanik, M.; Large, A. Global food security in the context of COVID-19: A scenario-based exploratory analysis. *Prog. Disaster Sci.* **2020**, *7*, 100120. [CrossRef]
27. Naja, F.; Hamadeh, R. Nutrition amid the COVID-19 pandemic: A multi-level framework for action. *J. Clin. Nutr.* **2020**, *74*, 1117–1121. [CrossRef]
28. Crew, S. Food Safety Risk during the Pandemic. *JFST* **2020**, *34*, 14–17. Available online: https://ifst.onlinelibrary.wiley.com/doi/10.1002/fsat.3402_4.x (accessed on 9 December 2020).
29. Turner, C.; Aggarwal, A.; Walls, H.; Herforth, A.; Drewnowski, A.; Coates, J.; Kalamatianou, S.; Kadiyala, S. Concepts and critical perspectives for food environment research: A global framework with implications for action in low-and middle-income countries. *Glob. Food Sec.* **2018**, *18*, 93–101. [CrossRef]
30. Rusu, I.G.; Suharoschi, R.; Vodnar, D.C.; Pop, C.R.; Socaci, S.A.; Vulturar, R.; Istrati, M.; Moroșan, I.; Farcas, A.C.; Kerezsi, A.D.; et al. Iron supplementation influence on the gut microbiota and probiotic intake effect in iron deficiency—A literature-based review. *Nutrients* **2020**, *12*, 1993. [CrossRef]
31. Batlle-Bayer, L.; Aldaco, R.; Bala, A.; Puig, R.; Laso, J.; Margallo, M.; Vázquez-Rowe, I.; Maria Antó, J.; Fullana-i-Palmera, P. Environmental and nutritional impacts of dietary changes in Spain during the COVID-19 lockdown. *Sci. Total Environ.* **2020**, *748*, 141410. [CrossRef]
32. Bracale, R.; Vaccaro, C.M. Changes in food choice following restrictive measures due to Covid-19. *Nutr. Metab. Cardiovasc. Dis.* **2020**, *30*, 1423–1426. [CrossRef]
33. Mattioli, A.V.; Sciomer, S.; Cocchi, C.; Maffei, S.; Gallina, S. Quarantine during COVID-19 outbreak: Changes in diet and physical activity increase the risk of cardiovascular disease. *Nutr. Metab. Cardiovasc. Dis.* **2020**, *30*, 1409–1417. [CrossRef]
34. Handu, D.; Moloney, L.; Rozga, M.; Cheng, F.W. Malnutrition care during the COVID-19 pandemic: Considerations for registered dietitian nutritionists. *J. Acad. Nutr. Diet.* **2020**. [CrossRef]
35. Im, J.H.; Je, Y.S.; Baek, J.; Chung, M.-H.; Kwon, H.Y.; Lee, J.-S. Nutritional status of patients with coronavirus disease 2019 (COVID-19). *Int. J. Infect. Dis.* **2020**, *100*, 390–393. [CrossRef] [PubMed]
36. Khan, I.; Haleem, A.; Javaid, M. Analysing COVID-19 pandemic through cases, deaths, and recoveries. *J. Oral Biol. Craniofac. Res.* **2020**, *10*, 450–469. [CrossRef]

37. Quiles, J.L.; Rivas-García, L.; Varela-López, A.; Llopis, J.; Battino, M.; Sánchez-González, C. Do nutrients and other bioactive molecules from foods have anything to say in the treatment against COVID-19? *Environ. Res.* **2020**, *191*, 110053. [CrossRef] [PubMed]
38. Taghizadeh-Hesary, F.; Akbari, H. The powerful immune system against powerful COVID-19: A hypothesis. *Med. Hypotheses* **2020**, *140*, 109762. [CrossRef] [PubMed]
39. Pop, O.L.; Salanță, L.C.; Pop, C.R.; Coldea, T.; Socaci, S.A.; Suharoschi, R.; Vodnar, D.C. Prebiotics and Dairy Applications. In *Dietary Fiber: Properties, Recovery, and Applications*; Galanakis, C.M., Ed.; Academic Press: London, UK, 2019; pp. 247–277. [CrossRef]
40. Sandberg, A.S. Bioavailability of minerals in legumes. *Br. J. Nutr.* **2002**, *88*, 281–285. [CrossRef] [PubMed]
41. Diaconeasa, Z.; Barbu-Tudoran, L.; Coman, C.; Leopold, L.; Mesaros, A.; Pop, O.; Rugină, D.; Ştefan, R.; Tăbăran, F.; Tripon, S.; et al. Cerium oxide nanoparticles and its cytotoxicity human lung cancer cells. *Rom. Bio. Lett.* **2015**, *20*, 10679–10687.
42. Ali, N. Role of vitamin D in preventing of COVID-19 infection, progression and severity. *J. Infect. Public Health* **2020**, *13*, 1373–1380. [CrossRef]
43. De Almeida Brasiel, P.G. The key role of zinc in elderly immunity: A possible approach in the COVID-19 crisis. *Clin. Nutr. ESPEN* **2020**, *38*, 65–66. [CrossRef]
44. Gasmi, A.; Noor, S.; Tippairote, T.; Dadar, M.; Menzel, A.; Bjørklund, G. Individual risk management strategy and potential therapeutic options for the COVID-19 pandemic. *J. Allergy Clin. Immunol.* **2020**, *215*, 108409. [CrossRef]
45. Kieliszek, M.; Lipinski, B. Selenium supplementation in the prevention of coronavirus infections (COVID-19). *Med. Hypotheses* **2020**, *143*, 109878. [CrossRef]
46. Shakoor, H.; Feehan, J.; Al Dhaheri, A.S.; Ali, H.I.; Platat, C.; Ismail, L.C.; Apostolopoulos, V.; Stojanovska, L. Immune-boosting role of vitamins D, C, E, zinc, selenium and omega-3 fatty acids: Could they help against COVID-19? *Maturitas* **2021**, *143*, 1–9. [CrossRef] [PubMed]
47. Haryanto, B.; Suksmasari, T.; Wintergerst, E.; Maggini, S. Multivitamin supplementation supports immune function and ameliorates conditions triggered by reduced air quality. *Vitam. Miner.* **2015**, *3*, 1000128. [CrossRef]
48. Maggini, S.; Pierre, A.; Calder, P.C. Immune function and micronutrient requirements change over the life course. *Nutrients* **2018**, *10*, 1531. [CrossRef] [PubMed]
49. Chew, B.P.; Park, J.S. Carotenoid action on the immune response. *J. Nutr.* **2004**, *134*, 257–261. [CrossRef]
50. Clark, A.; Mach, N. Role of vitamin D in the hygiene hypothesis: The interplay between vitamin D, vitamin D receptors, gut microbiota, and immune response. *Front. Immunol.* **2016**, *7*, 627. [CrossRef]
51. Shojadoost, B.; Kulkarni, R.R.; Yitbarek, A.; Laursen, A.; Taha-Abdelaziz, K.; Alkie, T.N.; Barjesteh, N.; Quinteiro-Filho, W.M.; Smith, T.K.; Sharif, S. Dietary selenium supplementation enhances antiviral immunity in chickens challenged with low pathogenic avian influenza virus subtype H9N2. *Vet. Immunol. Immunopathol.* **2019**, *207*, 62–68. [CrossRef]
52. Duan, L.; Zhu, G. Psychological interventions for people affected by the COVID-19 epidemic. *Lancet Psychiatry* **2020**, *7*, 300–302. [CrossRef]
53. Serafini, G.; Parmigiani, B.; Amerio, A.; Aguglia, A.; Sher, L.; Amore, M. The psychological impact of COVID-19 on the mental health in the general population. *QJM Int. J. Med.* **2020**, *113*, 531–537. [CrossRef]
54. Teigiserova, D.A.; Hamelin, L.; Thomsen, M. Towards transparent valorization of food surplus, waste and loss: Clarifying definitions, food waste hierarchy, and role in the circular economy. *Sci. Total Environ.* **2020**, *706*, 136033. [CrossRef]
55. Campos, D.A.; Gómez-García, R.; Vilas-Boas, A.A.; Madureira, A.R.; Pintado, M.M. Management of fruit industrial by-products—A case study on circular economy approach. *Molecules* **2020**, *25*, 320. [CrossRef]
56. Farcas, A.C.; Socaci, S.A.; Diaconeasa, Z.M. Introductory Chapter: From Waste to New Resources. In *Food Preservation and Waste Exploitation*; Socaci, S.A., Farcas, A.C., Aussenac, T., Eds.; IntechOpen: London, UK, 2019; pp. 1–11. [CrossRef]
57. Sherwood, J. The significance of biomass in a circular economy. *Bioresour. Technol.* **2020**, *300*, 122755. [CrossRef] [PubMed]
58. Mak, T.M.; Xiong, X.; Tsang, D.C.; Iris, K.; Poon, C.S. Sustainable food waste management towards circular bioeconomy: Policy review, limitations and opportunities. *Bioresour. Technol.* **2020**, *297*, 122497. [CrossRef] [PubMed]
59. Socaci, S.A.; Farcas, A.C.; Vodnar, D.C.; Tofana, M. Food Wastes as Valuable Sources of Bioactive Molecules. In *Superfood and Functional Food—The Development of Superfoods and Their Roles as Medicine*; Shiomi, N., Ed.; Intech Open: London, UK, 2017; pp. 75–93. [CrossRef]
60. Stavropoulos, S.; Burger, M.J.; Dufourmont, J. Urban circular policies and employment through greenfield FDI. *Sustainability* **2020**, *12*, 1458. [CrossRef]
61. Korhonen, J.; Honkasalo, A.; Seppala, J. Circular economy: The concept and its limitations. *Ecol. Econ.* **2018**, *143*, 37–46. [CrossRef]
62. Wetherill, M.S.; White, K.C.; Seligman, H. Charitable food as prevention: Food bank leadership perspectives on food banks as agents in population health. *Community Dev.* **2019**, *50*, 92–107. [CrossRef]
63. Loopstra, R.; Lambie-Mumford, H.; Fledderjohann, J. Food bank operational characteristics and rates of food bank use across Britain. *BMC Public Health* **2019**, *19*, 561. [CrossRef]
64. Mook, L.; Murdock, A.; Gundersen, C. Food banking and food insecurity in high-income countries. *Voluntas* **2020**, *31*, 833–840. [CrossRef]
65. Banca Pentru Alimente Bucuresti. Available online: http://bancapentrualimente.ro (accessed on 15 August 2020).

66. Barker, M.; Russell, J. Feeding the food insecure in Britain: Learning from the 2020 COVID-19 crisis. *Food Secur.* **2020**, *12*, 865–870. [CrossRef]
67. Thompson, C.; Smith, D.; Cummins, S. Understanding the health and wellbeing challenges of the food banking system: A qualitative study of food bank users, providers and referrers in London. *Soc. Sci. Med.* **2018**, *211*, 95–101. [CrossRef]
68. Pulker, C.E.; Trapp, G.S.; Scott, J.A.; Pollard, C.M. Global supermarkets' corporate social responsibility commitments to public health: A content analysis. *Glob. Health* **2018**, *14*, 121. [CrossRef]
69. European Food Banks Federatioan (FEBA). Available online: https://www.eurofoodbank.org (accessed on 15 August 2020).

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GAS CHROMATOGRAPHY

Determination of Volatile Markers from Magnum Hops in Beer by In-Tube Extraction—Gas Chromatography—Mass Spectrometry

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ABSTRACT

A chromatographic method was developed for the identification of volatile markers from Magnum hops in two types of beer. The study was initially performed with Magnum hop pellets and hop essential oil and subsequently with traditional and flavored beer during the primary fermentation. The volatile compounds were isolated employing the in-tube extraction (ITEX) technique followed by identification and quantification through gas-chromatography—mass spectrometry (GC-MS) operating in scan mode. The main authentication markers identified in traditional beer were from aromatic compounds, aldehydes and alcohol esters. The most predominant authentication marker compounds in beer flavored with Magnum hop essential oil were obtained from terpenoids, followed by acid esters, alcohol esters and alcohol classes. A unique feature of this study was represented by the discriminant markers for the authentication of Magnum hop variety, identified in hop pellets, hop essential oil and flavored beer. The application of this methodology can be used for optimization of brewing technology and process parameters in view of prolonging fruity hop flavor stability of Romanian beers.

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Introduction

Hops (Humulus lupulus L.), are one of the basic raw materials used for beer production to impart bitterness and aroma (Sakamoto and Konings 2003; Salanţă et al. 2016). The female hop cones contain glandular structures, such as as lupulin glands, that are rich in secondary metabolites (Faragó, Psenáková, and Faragová 2009; Muthaiyan, Limayem, and Ricke 2011). The metabolites of hop compounds, classified as bitter acids (resinous), volatile oil, and polyphenols are responsible for the existence of three elements in beer: bitterness, hop flavor and hop aroma (Štěrba et al. 2015). Depending on the variety, hops contribute to beer flavor (Heras and Gonzalez-Sanjose 2003). Hop essential oils also play an important role in brewing, while the volatile oil of hops comprises terpenoids, such as myrcene (30–50%), humulene (15–25%), caryophyllene and farnesene, which together account for more than 90% of the total hop oil (King and Dickinson 2003; Tofană 2006, Hofmann et al. 2013). The oxygen fraction of hop essential oils represents a mixture of alcohols,

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esters, aldehydes, epoxides, ketones and acids. These compounds are very important in relation with the flavor of beer due to their higher solubility in aqueous solutions. (De Schutter et al. 2008; Štěrba et al. 2015).

The compounds identified in hop oil are not all the same as those in hopped beer, as the boiling and fermentation processes affect hop characteristics. During the brewing process, hop compound losses can occur due to the adsorption of iso-α acids on the solid material or yeast cells and also by oxidative transformations (Cleemput et al. 2009).

The majority of hop flavor compounds are largely evaporated during the boiling process. Brewery production involves the use of various traditional practices of aromatisation as kettle hopping, late hopping associated to lager-style products or dry hopping, associated to some ales (Bamforth 2003; Vanderhaegen et al. 2003; De Cooman et al. 2004). Off-flavors in beer can be produced by lipid oxidation reactions which generate linear aldehydes, such as pentanal and hexanal (Vanderhaegen et al. 2004; Hong et al. 2015).

The predominant classes of volatile compounds in beer are higher alcohols, esters, aldehydes, ketones and organic acids (Branyik et al. 2008). Esters represent the flavor compounds which contribute to the fruity-flowery aroma of beers. Esters can be classified into three groups: acetic esters (acetate esters—fruity aroma), ethyl esters (medium-chain fatty acid esters—apple aroma) and furanic esters (harsh solvent aroma). (Vanderhaegen et al. 2003; Rossi et al. 2014).

The hops aroma in beer can be described as follows: citrus flavors which are attributed to esters and linalool; green flavors attributed to aldehydes; floral and fruity flavors attributed to geraniol, citronellol, linalool, ketones, epoxides and esters (Schönberger and Kostelecky 2011) Linalool and sesquiterpenoids hop compounds represent analytical markers for hoppy aroma in beer. Their levels depend on the hopping technology and in terms of advanced hopping, on the nature of the hop oil fraction (Steinhaus, Fritsch and Schieberle 2003).

A relatively new dynamic headspace sampling technique is in-tube extraction (Laaks et al. 2010). This technique requires minimal sample preparation, allowing simple and rapid enrichment of volatile or semi-volatile compounds during headspace extraction (Socaci et al. 2013; Sandra, David, and Vanhoenacker 2008) and may be coupled with gas chromatography—mass spectrometry for the separation and identification of compounds.

To characterize the Magnum hop variety volatile profile in beer, a method employing in-tube extraction and gas chromatography–mass spectrometry (ITEX-GC-MS) was optimized in previous work (Michiu et al. 2012). The optimized ITEX-GC-MS method was suitable for the characterization of hop volatile markers in two types of beer, especially for the discriminant markers of the Magnum variety.

The main objective of this study was to employ in-tube extraction with gas chromatography—mass spectrometry to fingerprint the volatile profile of beer, hopped with Magnum hop variety, from Romania, by identifying and comparing volatile markers in traditional and flavored beer fermentations. Using beer wort flavoring and the optimized ITEX/GC-MS method, the authentication markers from Magnum hop variety were identified in both types of beers, with a higher concentration in flavored beer, until the end of the primary fermentation process. These findings represents a characteristic flavor profile of Romanian Magnum hop variety which contributes to the fruity-flowery aroma of beers.

Materials and methods

Hop pellets, hop essential oil and beer wort

This study was performed on Magnum hop pellets, hop essential oil and beer hopped with Magnum hop variety, cultivated in the pedo-climatic areas of Sighisoara, Transylvania, Romania. For the flavoring process of beer wort, hop essential oil was isolated by hydrodistillation, using a Clevenger type apparatus, 50 g of T90 pellets (ground in a coffee mill) were weighed into a distillation flask and 700 ml distilled water were added. The distillation time was 3 h. The yield was calculated as ml of essential oil per 100 g dry plant material (MG-P_{eo}).

Laboratory-scale production of traditional and flavored beer

Wort was produced from a 100% pilsner malt prepared on laboratory scale (2 L) as described in a previous study (Michiu et al. 2012). Mashing was performed as follows: 45°C (10 min), 50°C (20 min), 63°C (60 min), 72°C (20 min) and finally an increase to 78°C (1 min). After filtration, wort was boiled for 60 minutes with Magnum hop addition (α-acids: 12.9% (w/w). Hops were added in 1.45 g pellet proportion after 5 minutes of boiling and in 0.36 g pellets proportion after 55 minutes of boiling, aiming at a final beer bitterness of 10 g α-acids/hL. The wort (12 P) was divided in two fermentation flasks and cooled to 16°C. Both worts were fermented for 5 days with Saccharomyces cerevisiae W34/70 brewer's yeast, 10^6 viable cells/mL wort concentration, resulting in beer 1: traditional (unflavored) and beer 2, respectively. They were flavored through Magnum hop essential oil addition at the beginning of the primary fermentation process using a method described by Opstaele et al. (2010) with some modifications. Specifically, a higher concentration of hop essential oil was used at 0.9 mg/L.

Instrumentation

The separation and identification of volatile compounds was carried on a Shimadzu GC-MS QP-2010 (Shimadzu Scientific Instruments, Kyoto, Japan) model gas chromatograph–mass spectrometer equipped with a CombiPAL AOC-5000 autosampler (CTC Analytics, Zwingen, Switzerland). A Zebrone ZB-5 ms capillary column of 50 m × 0.32 mm i.d. and 0.25 µm film thickness, Phenomenex, USA, was used for the separation.

Samples and sample preparation

Three samples of boiled wort were collected, W1-after 5 min of boiling immediately before hops were added, W2-after 20 minutes of boiling with 80% hop addition and W3-after 60 min of boiling immediately before cooling. Wort samples were cooled down with ice water and subsequently frozen at −25°C. The trub was removed by filtration after defrosting, to remove all possible precipitates before preparing the wort samples for injections.

For the identification of hop authentication markers during the primary fermentation process, five samples of both traditional and flavored beer were collected, one sample daily, after 24, 48, 72, 96, and 120 h.

ITEX/GC-MS analysis was performed in triplicate depending on the sample type. The following weight or volumes were placed into 20 mL glass vials.: for Magnum hop pellets,

0.15 g, Magnum hop essential oil, 500 µL, and boiled wort and beer (unflavored and flavored), 5 mL.

GC-MS analysis

The extraction of volatiles from hop pellets, hop essential oil, wort and beer was performed using the ITEX technique. GC-MS was used to separate and identify the volatile compounds.

Magnum hop pellets and essential oil were analyzed according to the following parameters: injector temperature 250°C; pressure 93,1 kPa; column flow 2,42 ml/min; linear velocity 44,0 cm/s; split ratio 1:200. Carrier gas helium; detector: MS, ion source temperature 250°C; interface temperature 250°C; MS mode: electron ionization; mass range: 50–400 m/z, scan speed: 2000 µ/s (Tofană et al. 2009; Salanță et al. 2012). The program for column oven temperature was: 60°C (3 min) with 3°C/min to 150°C (10 min). Sample conditioning was performed at 60°C for 20 min. The number of extraction strokes was 30 × 500 µL; This method was used to highlight the major volatile compounds of Magnum hop pellets and essential oil that are representative for beer production and that could represent some authentication markers during the main fermentation of beer.

The analysis of boiled wort and beer during primary fermentation was based on the mMB1 method described in a previous study (Michiu et al. 2012) with some modifications (start time 5.5/ split ratio 100).

The parameters for mMB1 method were: injector temperature 250°C; pressure 51.7 kPa; column flow 1.7 ml/min; linear velocity 36.4 cm/s; split ratio 1:25 (traditional beer); 1:100 (flavored beer); carrier gas helium; detector: MS, ion source temperature 250°C; interface temperature 250°C; MS mode: electron ionization; mass range: 40–400 m/z; scan speed: 2000 µ/s. The program for column oven temperature was: 35°C (2 min), 10°C/min to 70°C, 4°C/min to 200°C and 20°C/min to 270°C (2 min). Sample conditioning was performed at 80°C for 15 min. The number of extraction strokes was 30 × 500 µL.

The identification of volatile compounds was performed by comparing the obtained mass spectra with NIST27 and NIST147 library information and verified by comparison with retention indices obtained at www.pherobase.com or www.flavornet.org for columns with a similar stationary phase to the ZB-5 ms column (Tofană et al. 2009).

Results and discussion

Volatile profile of hop pellets and hop essential oil

Forty-one volatile compounds were identified from Magnum hop pellets and forty nine compounds from hop essential oil. The most quantitatively predominant compounds in Magnum hop pellets and essential oil were: β–Myrcene; Propanoic acid,2-methyl,2-methylbutyl ester; β-Caryophyllene; 2-Pentanol,propanoate; Propanoic acid,2-methyl,2-methylpropyl ester; Propanoic acid,2-methyl,3-methylbuthyl ester; β-pinene; D-Limonene and α-Caryophyllene.

The compounds separated and identified from hop pellets and essential oil are listed in Table 1 and expressed as percentages of total peak area.

Analytical marker compounds in traditional (unflavored) beer

According to Malfliet et al. (2008) and Hanke et al. (2010) the the hoppy aroma in beer can be mainly ascribed to the chemical and biological conversions of hop oil compounds that

Table 1. Concentrations (peak area percent) of the volatile compounds from Magnum hop pellets and essential oil. (* discriminant markers for the authentication of the Magnum hop variety).

Analyte	Retention time (min)	Peak area percent	
		Magnum hop pellets	Magnum essential oil
2-Hexenal, (E)-	4.971	–	0.29
Propanoic acid, 2-methyl-, butyl ester	4.998	0.13	–
Propanoic acid, 2-methylpropyl ester	5.287	0.93	0.48
1-Butanol, 3-methyl-, formate	5.571	0.57	0.30
Hexanal, 4-methyl- 4-Methylhexanal	6.213	–	0.08
Propanoic acid, 2-methyl-, 2-methylpropyl ester	6.612	5.71	3.06
Hexanoic acid, methyl ester	6.920	0.79	0.42
1,3,6-Octatriene, 3,7-dimethyl-, (E)-	7.045	0.63	–
alpha- Pinene	7.317	2.48	0.86
Thiopivalic acid-Propanethioic acid, 2,2-dimethyl	7.532	0.48	0.25
Propanoic acid, 2-methyl-, butyl ester	7.909	0.41	0.25
2-Pentanol, propanoate	8.614	5.81	5.36
beta-Pinene	8.906	5.25	4.49
beta-Myrcene	9.043	42.58	18.24
Butanoic acid, 2-methyl-, 2-methylpropyl ester	9.854	0.17	0.05
Butanoic acid, 3-methyl-, 2-methylpropyl ester	10.031	0.55	–
Butanoic acid, 2-methylbutyl ester-2-Methylbutyl butyrate	10.157	0.08	–
Propanoic acid, 2-methyl-, 3-methylbutyl ester	10.266	5.67	4.97
***Propanoic acid, 2-methyl-, 2-methylbutyl ester**	10.400	8.36	6.60
Heptanoic acid, methyl ester	10.731	1.63	1.64
D-Limonene	10.994	2.09	1.10
1,4-Cyclohexadiene, 1-methyl-4-(1-methylethyl)	11.010	–	0.71
beta-Phellandrene	11.057	1.42	–
1,3,6-Octatriene, 3,7-dimethyl-, (E)-	11.280	0.03	0.09
1,3,6-Octatriene, 3,7-dimethyl-, (Z)-	11.738	0.97	0.56
Propanoic acid, 2-methyl-, pentyl ester	11.825	–	0.04
Butanoic acid, pentyl ester-Butyric acid, pentyl ester	11.890	0.05	–
Methyl 6-methyl heptanoate	13.564	0.30	0.52
3-Carene	13.979	–	1.64
1,6-Octadien-3-ol, 3,7-dimethyl-, 2-aminobenzoate	14.030	0.10	0.53
2,6-Octadien-1-ol, 3,7-dimethyl-, (Z)-	14.170	0.28	–
Butanoic acid, 2-methyl-, 2-methylbutyl ester	14.341	0.17	–
Pentanoic acid, 2-methylbutyl ester	14.411	–	0.91
n-Amyl isovalerate	14.596	0.41	–
Octanoic acid, methyl ester	15.310	0.24	0.83
***Propanoic acid, 2-methyl-, hexyl ester**	16.457	0.04	0.20
Propanoic acid, heptyl ester	19.039	–	0.21
3-Nonenoic acid, methyl ester	19.355	–	0.15
Nonanoic acid, methyl ester	19.983	–	0.68
Propanoic acid, 2-methyl-, heptyl ester	21.059	–	0.42
beta-Pinene	21.253	–	0.28
2-Undecanone	21.557	–	1.15
Decanoic acid, methyl ester	23.022	–	0.13
Propanoic acid, octyl ester	23.755	–	0.28
4-Decenoic acid, methyl ester	24.034	0.41	3.47
2,6-Octadienoic acid, 3,7-dimethyl-, methyl ester	24.637	0.04	0.77
Decanoic acid, methyl ester-Capric acid methyl ester	24.728	–	0.51
Propanoic acid, 2-methyl-, octyl ester	25.692	–	0.58
Ylangene	26.760	–	0.27
Copaene	27.106	0.14	0.84
2-Dodecanone	27.927	–	0.14
Caryophyllene	29.067	5.82	17.56
1,6-Cyclodecadiene, 1-methyl-5-methylene-8	29.529	0.13	–
alpha-Caryophyllene	30.645	0.73	2.71
Naphthalene, 1,2,3,4,4a,5,6,8a-octahydro-7-methyl-4-methylene-1-(1-methylethyl)-, (1.alpha.,4a.alpha.,8a.alpha.)-	31.437	–	1.31
Naphthalene, 1,2,3,4,4a,5,6,8a-octahydro-7-methyl-4-methylene-1-(1-methylethyl)-, (1.alpha.,4a.alpha.,8a.alpha.)-	31.509	0.57	0.55

(*Continued*)

Table 1. Continued.

Analyte	Retention time (min)	Peak area percent	
		Magnum hop pellets	Magnum essential oil
Eudesma-4(14),11-diene-7-Isopropenyl-4a-methyl-1-methylenedecahydronaphthalene	32.026	–	0.52
Naphthalene, 1,2,3,4,4a,5,6,8a-octahydro-4a,8-dimethyl-2-(1-methylethenyl)-, [2R-(2.alpha.,4a.alpha.,8a.beta.)]	32.094	0.11	–
Naphthalene, 1,2,4a,5,6,8a-hexahydro-4,7-dimethyl-1-(1-methylethyl)-, (1.alpha.,4a.alpha.,8a.alpha.)	32.524	0.10	0.43
3,6-Dodecadienoic acid, methyl ester	32.805	–	0.14

can undergo along the brewing process. Previous studies have shown that the most abundant terpene hydrocarbons in hop essential oil that include monoterpene: β-Myrcene, sesquiterpenes: α-Humulene and β-Caryophyllene, are almost completely removed during the beer wort fermentation by adsorption due the hydrophobic yeast cells and migration to the foam layer (Praet et al. 2012).

Eighty-seven volatile compounds were identified from the traditional beer samples. The most important are listed in Table 2. Fifteen volatile compounds were selected as process marker compounds, while the monitoring of these compounds was based on their initial presence in boiled wort and their subsequent losses during the primary fermentation process. Kleinová, Geršl, and Mareček (2015) have shown that the content of hop volatiles in the beer wort is not proportional to their production during wort boiling and fermentation and that hop compounds can be removed by resulting carbon dioxide.

The identified process marker compounds from Magnum hops decreased greatly and disappeared during the primary fermentation process (e.g., β-Myrcene; α-Caryophillene; Propanoic acic 2-methyl,3-methylbuthyl ester; Propanoic acic 2-methyl,2-methylpropyl ester).

As authentication markers of the wort boiling and beer fermentation processes, 5 volatile compounds were identified from boiled wort to beer samples after five days of fermentation, as follows: Benzaldehyde; Propanoic acid 2-methyl,2 methylbuthyl ester (hop marker); Nonanal; Cyclohexanol,5-methyl; and 2,5 Cyclohexadiene 1,4 dione,2,5 diphenyl (Figure 1).

Volatile profile of flavored beer

According to the literature, hop oil is rich in sesquiterpene hydrocarbons which give the earthy, herbal, woody, or spicy aromas while the monoterpenoids are more fruity or citrusy (Marcelina and Setzer 2011). It has also been reported that hops are also rich in esters, especially the group of fatty acid methyl esters with linear chains that seem to provide additional floral and fruity characteristics (Salanță et al. 2012).

Seventy nine volatile compounds were identified from the flavored beer samples. Eighteen volatile compounds were identificed as authentication markers: 1-Butanol 3-methyl; 1-Hexanol; Propanoic acid 2-methyl 2-methylpropyl ester; 2-Pentanol propanoate; β-Mircene; Butanoic acid,3-methyl,2-methylpropyl ester; Propanoic acid 2-methyl,3-methylbuthyl ester; Propanoic acid 2-methyl,2-methylbuthyl ester; Hexanoic acid 4-methylene; D-limonene; 2-Nonanone heptyl methyl ketone; Linalyl isobutyrate;

Table 2. Concentrations (peak area percent) of the volatile compounds from wort and traditional (unflavored) beer during primary fermentation (*discriminant marker for the authentication of the Magnum hop variety).

		Peak area percent							
		W1	W2	W3	TB1	TB2	TB3	TB4	TB5
Analyte	Retention time (min)	5 minutes of boiling	20 minutes of boiling	60 minutes of boiling	24 h fermentation	48 h fermentation	72 h fermentation	96 h fermentation	120 h fermentation
Hexane, 2,2-dimethyl-	4.177	5.08	–	4.11	–	–	–	–	–
Pentanal	4.357	6.33	0.53	–	–	–	–	–	–
1-Butanol, 3-methyl-	5.039	5.65	–	–	–	–	–	–	–
Methyl Isobutyl Ketone	5.060	–	–	1.32	–	–	–	–	–
Disulfide, dimethyl	5.130	–	–	0.17	–	–	–	–	–
Toluene	5.568	–	–	0.22	–	–	–	–	–
Butanoic acid, ethyl ester	6.180	–	–	–	–	–	–	2.70	–
Hexanal	6.262	38.36	8.75	5.60	2.18				
Acetyl valeryl	7.073	–	–	–	0.89	–	–	–	–
Propanoic acid, 2-methylpropyl ester	7.840	–	0.24	–	–	0.41	–	–	–
1-Hexanol	7.910	2.30	0.46	0.30	4.26	1.13	0.21	0.10	–
1-Butanol, 3-methyl- acetate	8.040	–	–	–	15.93	51.32	61.45	61.86	–
1-Butanol, 2-methyl- acetate	8.113	–	0.39	0.27	–	–	–	–	–
2-Heptanone	8.393	–	–	–	2.15	–	–	–	–
Heptanal	8.749	1.63	0.35	0.31	–	–	–	–	–
Propanoic acid, 2-methyl-, 2-methylpropyl ester	9.053	–	2.66	1.86	1.20	0.41	0.21	–	–
Hexanoic acid, methyl ester	9.332	–	0.45	0.33	–	–	–	–	–
Propanoic acid, 2-methyl-, butyl ester	10.157	–	0.28	–	–	–	–	–	–
Benzaldehyde	10.524	5.39	1.75	0.92	3.03	1.06	0.42	1.09	2.46
2-Pentanol, propanoate	10.719	–	5.15	3.54	3.16	1.17	0.28	–	–
β-Myrcene	11.307	–	22.10	20.71	2.60	0.76	0.26	–	–
Octanal	11.769	1.16	0.25	0.21	–	–	–	–	0.59
Propanoic acid, 2-methyl-, 3-methylbutyl ester	12.013	–	9.06	6.42	5.95	–	–	–	–
***Propanoic acid, 2-methyl-, 2-methylbutyl ester**	12.121	–	13.62	9.55	7.46	2.09	0.75	0.45	0.56
D-Limonene	12.657	–	0.19	0.09	0.88	1.43	0.68	0.22	–
Benzeneacetaldehyde-Acetaldehyde, phenyl	13.140	3.56	0.80	0.98	1.62	0.54	0.21	–	–
Acetophenone	13.877	–	1.31	0.30	3.06	0.72	0.50	0.79	1.55
2,7-Dimethyl-2,7-octanediol	14.041	1.83	0.79	2.04	2.67	0.67	–	–	–
2-Nonanone-Heptyl methyl ketone	14.680	–	0.28	0.41	1.74	0.51	0.23	–	–
Linalyl isobutyrate	15.000	–	4.09	9.08	10.74	2.94	0.91	0.98	–
Nonanal	15.193	3.67	1.86	1.88	1.42	0.31	0.20	0.33	3.47
Propanoic acid, 2-methy, hexyl ester	16.640	–	–	0.36	–	–	–	–	–

(*Continued*)

Table 2. Continued.

Analyte	Retention time (min)	Peak area percent							
		W1	W2	W3	TB1	TB2	TB3	TB4	TB5
		5 minutes of boiling	20 minutes of boiling	60 minutes of boiling	24 h fermentation	48 h fermentation	72 h fermentation	96 h fermentation	120 h fermentation
Cyclohexanol, 5-methyl	17.829	–	–	0.45	2.45	1.76	0.52	0.68	0.47
2,6-Octadienoic acid, 3,7-dimethyl-, methyl ester,	22.867	–	0.67	0.48	–	–	–	–	–
alpha-Caryophyllene	27.639	–	0.61	1.51	–	–	–	–	–
Pentadecane	32.060	–	–	–	–	–	–	–	0.71
Hexanoic acid, 2-phenylethyl ester	33.307	–	–	–	–	–	1.07	0.78	–
Heptadecane	35.050	–	–	–	–	–	–	–	0.55
Isopropyl Myristate	38.480	–	–	–	0.80	–	–	–	–
Octanoic acid, 2-phenylethyl ester	38.999	–	–	–	0.55	2.49	2.82	1.41	–
2,5-Cyclohexadiene-1,4-dione, 2,5-diphenyl	40.579	3.53	0.54	0.88	1.94	0.54	0.21	1.19	2.57
Dibutyl phthalate	40.791	–	–	0.24	0.69	–	–	–	–
Hexadecanoic acid, ethyl	41.293	–	–	–	–	–	–	0.36	0.91

Table 3. Concentrations (peak area percent) of the volatile compounds from wort and flavored beer during primary fermentation.*represents discriminant markers for the authentication of the Magnum hop variety.

		Peak area percent							
		W1	W2	W3	FB1	FB2	FB3	FB4	FB5
Analyte	Retention Time (min)	5 minutes of boiling	20 minutes of boiling	60 minutes of boiling	24 h fermentation	48 h fermentation	72 h fermentation	96 h fermentation	120 h fermentation
Hexane, 2,2-dimethyl-	4.177	5.08	–	4.11	–	–	–	–	–
2,3-Pentanedione	4.303	–	–	–	–	–	0.32	0.28	0.23
Pentanal	4.357	6.33	0.53	–	–	–	–	–	–
1-Butanol, 3-methyl-	5.039	5.65	–	–	9.83	26.84	26.91	24.15	25.57
Methyl Isobutyl Ketone	5.060	–	–	1.32	–	–	–	–	–
Disulfide, dimethyl	5.130	–	–	0.17	–	–	–	–	–
Hexanal	6.262	38.36	8.75	5.60	–	–	–	–	–
Propanoic acid, hexyl ester	7.830	–	–	–	–	0.19	–	–	–
Propanoic acid, 2- methylpropyl ester	7.840	–	0.24	–	–	–	–	–	–
1-Hexanol	7.910	2.30	0.46	0.30	0.10	0.10	0.06	0.11	0.91
1-Butanol, 2-methyl-, acetate	8.109		0.39	0.27	0.99	0.17	0.15	–	–
1-Butanol, 3-methyl- acetate	8.113	–	–	–	–	0.71	0.75	1.43	1.96
Heptanal	8.749	1.63	0.35	0.31	–	–	–	–	–
Propanoic acid, 2-methyl-, 2-methylpropyl ester	9.053	–	2.66	1.86	3.02	1.91	1.07	1.62	1.53
Hexanoic acid, methyl ester	9.332	–	0.45	0.33	–	0.07	0.04	–	–
Propanoic acid, 2-methyl-, butyl ester	10.157	–	0.28	–	–	–	–	–	–
Benzaldehyde	10.524	5.39	1.75	0.92	–	0.01	0.04	–	–
2-Pentanol, propanoate	10.719	–	5.15	3.54	9.24	5.83	4.82	4.84	4.57
Hexanoic acid, 5-methyl-, methyl ester	11.226	–	2.22	1.66	3.24	1.65	0.90	–	–
β-Myrcene	11.307		22.10	20.71	8.85	8.60	9.60	12.74	11.69
Hexanoic acid, ethyl ester	11.546	–	–	–	–	0.46	0.38	0.71	0.96
Butanoic acid, 3-methyl-, 2-methylpropyl ester	11.834	–	0.52	0.24	0.59	0.42	0.38	0.49	0.49
Propanoic acid, 2-methyl-, 3-methylbutyl ester	12.013	–	9.06	6.42	9.40	7.02	7.04	8.21	8.24
***Propanoic acid, 2-methyl-, 2-methylbutyl ester**	12.121	–	13.62	9.55	13.73	10.36	10.44	12.35	12.32
Heptanoic acid, methyl ester	12.371	–	4.02	2.81	1.62	0.28	–	–	–
Hexanoic acid, 4-methylene	12.477	–	2.27	1.59	4.83	3.28	2.73	2.25	2.12
D-Limonene	12.657		0.19	0.09	–	0.05	0.05	0.12	0.09
Benzeneacetaldehyde-Acetaldehyde, phenyl	13.140	3.56	0.80	0.98	–	–	–	–	–
Propanoic acid, 2-methyl-, pentyl ester	13.239	–	–	–	–	–	–	0.18	–
Acetophenone	13.877	–	1.31	0.30	0.05	–	–	–	–
Methyl 6-methyl heptanoate	14.508	–	1.28	0.84	0.94	0.32	0.18	0.10	–
2-Nonanone-Heptyl methyl ketone	14.680	–	0.28	0.41	0.97	0.60	0.47	0.36	0.37

(*Continued*)

Table 3. Continued.

Analyte	Retention Time (min)	Peak area percent							
		W1	W2	W3	FB1	FB2	FB3	FB4	FB5
		5 minutes of boiling	20 minutes of boiling	60 minutes of boiling	24 h fermentation	48 h fermentation	72 h fermentation	96 h fermentation	120 h fermentation
Linalyl isobutyrate	15.000	–	4.09	9.08	8.70	7.91	5.82	4.79	5.32
Nonanal	15.193	3.67	1.86	1.88	–	–	–	–	–
n-Amyl isovalerate	15.289	–	1.27	–	1.61	–	1.57	1.88	2.02
***Propanoic acid, 2-methyl-, hexyl ester**	16.640	–	–	0.36	0.57	0.49	0.57	0.73	0.82
Cyclohexanol, 5-methyl	17.829	–	–	0.45	–	–	–	–	–
2-Decanone	18.243	–	–	0.66	0.52	0.41	0.43	0.38	0.41
4-Decenoic acid, methyl ester	22.390	–	1.79	1.95	1.81	1.26	1.18	1.06	1.07
Caryophyllene	26.443	–	–	–	1.77	2.30	1.90	2.32	0.97
alpha-Caryophyllene	27.639	–	0.61	1.51	4.67	7.80	6.80	8.11	4.08
2,5-Cyclohexadiene-1,4-dione, 2,5-diphenyl	40.579	3.53	0.54	0.88	–	0.25	0.25	0.25	1.93
Dibutyl phthalate	40.791	–	–	0.24	–	–	–	–	–

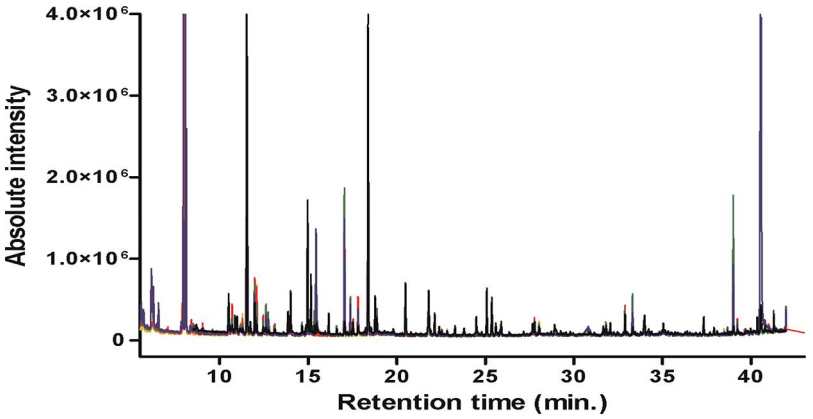

Figure 1. ITEX/GC-MS chromatograms of traditional beer during the primary fermentation: TB1 (24 h), TB2 (48 h), TB3 (72 h), TB4 (96 h), and TB5 (120 h).

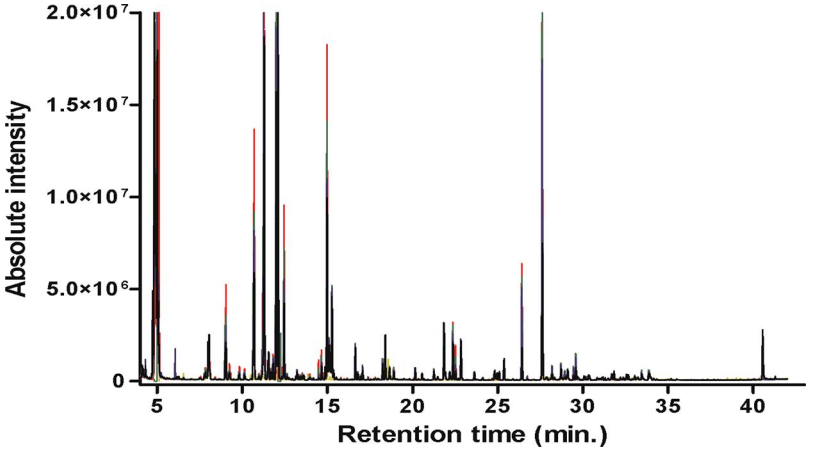

Figure 2. ITEX/GC-MS chromatograms of flavored beer during the primary fermentation: FB1 (24 h), FB2 (48 h), FB3 (72 h), FB4 (96 h), and FB5 (120 h).

n-Amylisovalerate; 2-Decanone; 4-Decenoic acid,methyl ester; Caryophyllene; α-Caryophyllene; and 2,5-Cyclohexadiene-1,4-dione,2,5-diphenyl. These compounds were identified based both on their initial presence in Magnum hop variety and their resulting presence from boiled wort to beer samples (Table 3). Some of these compounds were identified as process marker compounds in traditional (unflavored) beer, for example: β-Myrcene; D-Limonene; Propanoic acid-2methyl-2methylpropyl ester; Propanoic acid-2 methyl-2 methylbuthyl ester; Propanoic acid-2 methyl-3 methylbuthyl ester and Propanoic acid-2 methyl hexyl ester. These compounds and their evolution in flavored beer are shown in Figure 2.

Conclusion

The content of Magnum hop volatile markers was determined in relation to beer production, traditional and flavored beer. In traditional beer, the content of hop volatile

markers gradually decreased during the main fermentation process below the threshold values of sensory perception. The reason may be the removal of the hop compounds by resulting carbon dioxide. Wort aromatization increases the percentage of hop markers: β-Myrcene; D-Limonene; Propanoic acid-2methyl-2methylpropyl ester; Propanoic acid-2 methyl-2 methylbuthyl ester; Propanoic acid-2 methyl-3 methylbuthyl ester and Propanoic acid-2 methyl hexyl ester.

A significant finding of this study was represented among discriminant markers for authentication of Magnum hop variety: Propanoic acid 2 methyl hexyl ester (Hexyl isobutyrate) identified in hop pellets, essential oil and subsequently in flavored beer and Propanoic acid 2 methyl-2methylbuthyl ester, the only authentication marker in both traditional and flavored beer. These compounds provide a characteristic floral and fruity odor of Magnum hop variety in beer. The results show that in traditional beer, the hop flavor profile can be improved using the hop essential oil flavoring at the beginning of the primary fermentation process and that the ITEX-GC/MS method is suitable for the determination of volatile marker compounds from hop in flavored beer. A total of 79 volatile and 18 authentication marker compounds were separated and identified during the main fermentation process.

This methodology will be applied for the evaluation of the quality of Romanian hop varieties and for optimisation of brewing technology and process parameters in view of prolonging fruity hop flavor stability of Romanian beers.

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References

Bamforth, C. W. 2003. Beers – Chemistry of brewing. In *Encyclopedia of food sciences & nutrition*, ed. B. Caballero, L. Trugo, and P. Finglas, 440–7. London: Academic Press.

Branyik T, A. Vicente, P. Dost´alek, and J.-A. Teixeira. 2008. A review of flavour formation in continuous beer fermentations. *Journal of the Institute of Brewing* 114:3–13. doi:10.1002/j.2050-0416.2008.tb00299.x.

Cleemput, V., M. K. K. Cattoor, G. De Bosscher, D. Haegeman, and A. H. De Keukeleire. 2009. Hop (*Humulus lupulus*) - Derived bitter acids as multipotent bioactive compounds. *Journal of Natural Products* 72:1220–30. doi:10.1021/np800740m.

De Cooman, L., G. Aerts, F. Van Opstaele, K. Goiris, E. Syryn, G. De Rouck, M. De Ridder, and D. De Keukeleire. 2004. New trends in advanced hopping-Part 2: Application of varietal hop aromas. *Cerevisia* 29 (1):81–87.

De Schutter, P., D. Saison, F. Delvaux, G. Derdelinckx, J. M. Rock, H. Neven, and F. R. Delvaux. 2008. Optimisation of wort volatile analysis by headspace solid-phase microextraction in combination with gas chromatography and mass spectrometry. *Journal of Chromatography A* 1179:75–80. doi:10.1016/j.chroma.2007.11.103.

Faragó, J., I. Psenáková, and N. Faragová. 2009. The use of biotechnology in hop (*Humulus lupulus L.*) improvement. *Nova Biotechnologica* 9:279–93.

Hanke, S., V. Ditz, M. Herrmann, W. Back, T. Becker, and M. Krottenthaler. 2010. Influence of ethyl acetate, isoamyl acetate and linalool on off-flavor perception in beer. *Brewing Science* 63:94–99.

Heras, M. O., and M. L. Gonzalez-Sanjose. 2003. Beers: Wort production. In *Encyclopedia of food sciences and nutrition*, ed. B. Caballero, L. Trugo, and P. M. Finglas. San Diego, CA: Academic Press.

Hofmann, R., S. Weber, N. Rettberg, S. Thörner, L. A. Garbe, and R. Folz. 2013. Optimization of the hop kilning process to improve energy efficiency and recover hop oils. *Brewing Science* 66 (3): 23–30.

Hong, L., F. Liu, X. He, Y. Cui, and J. Hao. 2015. A study on kinetics of beer ageing and development of methods for predicting the time to detection of flavour changes in beer. *Institute of Brewing & Distilling*. 121:38–43. doi:10.1002/jib.194.

King, A. J., and R. Dickinson. 2003. Biotransformation of hop aroma terpenoids by ale and lager yeasts. *FEMS Yeast Research* 3:53–62. doi:10.1111/j.1567-1364.2003.tb00138.x.

Kleinová, J., M. Geršl, and J. Mareček. 2015. Monitoring volatile substances in beer in relation to beer production technology. *Journal of Advanced Agricultural Technologies* 2 (2):134–7. doi:10.12720/joaat.2.2.134-137.

Laaks, J., M. A. Jochmann, B. Schilling, and T. C. Schmidt. 2010. In-tube extraction of volatile organic compounds from aqueous samples: An economical alternative to purge and trap enrichment. *Analytical Chemistry* 82 (18):7641–8. doi:10.1021/ac101414t.

Malfliet, S., F. V. Opstaele, J. De Clippeleer, E. Syryn, K. Goiris, L. De Cooman, and G. Aerts. 2008. Flavour instability of pale lager beers: determination of analytical markers in relation to sensory ageing. *Journal of the Institute of Brewing* 114 (2):180–92. doi:10.1002/j.2050-0416.2008.tb00324.x.

Marcelina, R. N., and W. N. Setzer. 2011. Volatile components of aroma hops (Humulus lupulus L.) commonly used in beer brewing. *Journal of Brewing and Distilling* 2 (2):16–22.

Michiu, D., M. Tofană, S. A. Socaci, E. Mudura, L. C. Salanță, and A. C. Fărcaș. 2012. Optimization of ITEX/GC-MS method for beer wort volatile compounds characterization. *Journal of Agroalimentary Processes and Technologies* 18 (3):229–35.

Muthaiyan, A., A. Limayem, and S. C. Ricke. 2011. Antimicrobial strategies for limiting bacterial contaminants in fuel bioethanol fermentations. *Progress in Energy and Combustion Science* 37:351–70. doi:10.1016/j.pecs.2010.06.005.

Opstaele, F. V., G. De Rouck, J. De Clippeleer, G. Aerts, and L. De Cooman. 2010. Analytical and sensory assessment of hoppy aroma and bitterness of conventionally hopped and advanced hopped pilsner beers. *Journal of the Institute of Brewing* 116 (4):445–58. doi:10.1002/j.2050-0416.2010.tb00796.x.

Praet, T., F. Van Opstaele, B. Jaskula-Goiris, A. Guido, and L. De Cooman. 2012. Biotransformations of hop-derived aroma compounds by Saccharomyces cerevisiae upon fermentation. *Cerevisia* 36:125–32. doi:10.1016/j.cervis.2011.12.005.

Rossi, S., V. Sileoni, G. Perretti, and O. Marconia. 2014. Characterization of the volatile profiles of beer using headspace solid-phase microextraction and gas chromatography–mass spectrometry. *Journal of the Science of Food and Agriculture* 94:919–28. doi:10.1002/jsfa.6336.

Sakamoto, K., and W. N. Konings. 2003. Beer spoilage bacteria and hop resistance. *International Journal of Food Microbiology* 89:112–14. doi:10.1016/S0168-1605(03)00153-3.

Salanță, L. C., M. Tofană, S. A. Socaci, C. Lazar (Pop), D. Michiu, and A. Fărcaș. 2012. Determination of the volatile compounds from hop and hop products using ITEX/GC-MS technique. *Journal of Agroalimentary Processes and Technologies* 18 (2):110–15.

Salanță, L. C., M. Tofană, S. Socaci, E. Mudura, C. Pop, A. Pop, and A. Fărcaș. 2016. Determination of volatiles in hops from Romania by solid phase fiber microextraction and gas chromatography – Mass spectrometry. *Analytical Letters* 49:477–87. doi:10.1080/00032719.2015.1075129.

Sandra, P., F. David, and G. Vanhoenacker. 2008. Advanced sample preparation techniques for the analysis of food contaminants and residues. *Comprehensive Analytical Chemistry* 51:131–72. doi:10.1016/s0166-526x(08)00005-6.

Schönberger, C., and T. Kostelecky. 2011. 125th anniversary review: The role of hops in brewing. *Journal of the Institute of Brewing* 117 (3):259–67. doi:10.1002/j.2050-0416.2011.tb00471.x.

Steinhaus, M., H. T. Fritsch, and P. Schieberle. 2003. Linalool—A key contributor to hop aroma. *Journal of Agricultural and Food Chemistry* 51:7100–05.

Štěrba, K., P. Čejka, J. Čulík, and M. Jurková. 2015. Determination of linalool in different hop varieties using a new method based on fluidized-bed extraction with gas chromatographic–mass spectrometric detection. *Journal of the American Society of Brewing Chemists*. 73 (2):151–8. doi:10.1094/ASBCJ-2015-0406-01.

Socaci, S. A., C. Socaciu, M. Tofană, I. V. Raţi, and A. Pintea. 2013. In-tube extraction and GC–MS analysis of volatile components from wild and cultivated sea buckthorn (Hippophae rhamnoides L. ssp. Carpatica) berry varieties and juice. *Phytochemical Analysis* 24 (4):319–28. doi:10.1002/pca.2413.

Tofană, M. 2006. Biosinteza constituenţilor uleiului volatil. In *Substanţe amare şi de aromă din hamei*, I. Ed., Cluj-Napoca, Alma Mater, 86–96.

Tofană, M., S. A. Socaci, C. Socaciu, D. E. Mihăiescu, C. Semeniuc, and D. Truţa. 2009. Optimization of HS/GC-MS method for the determination of volatile compounds from some indigenous hop varieties. *Bulletin UASVM Agriculture* 66 (2):500–05.

Vanderhaegen, B., H. Neven, S. Coghe, K. J. Verstrepen, H. Verachtert, and G. Derdelinckx. 2003. Bioflavoring and beer refermentation. *Applied Microbiology and Biotechnology* 62:140–50. doi:10.1007/s00253-003-1340-5.

Vanderhaegen, B., H. Neven, K. J. Verstrepen, F. R. Delvaux, H. Verachtert, and G. Derdelinckx. 2004. Influence of the brewing process on furfuryl ethyl ether formation during beer aging. *Journal of Agricultural and Food Chemistry* 52:6755–64. doi:10.1021/jf0490854.

Microbiological Profile of Kashkaval Cheese During Production Season

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Abstract

Although the scalded cheeses are not an ideal substrate for microorganisms, these are a suitable habitat for starter cultures and indigenous microbiota, the latter being able to survive during processing. The aim of this study was to highlight the qualitative and hygienic aspects of kashkavals as a finished products. The microbiological assays aimed the evaluation of sanitary-hygienic quality in kashkaval cheeses obtained during one year, by the following indicators: determination of coliform bacteria*, E. coli*, monitoring of pathogenic microorganisms: *Stafylococcus aureus coagulase-positive* and total number of micromycetes. Sixty samples from five units which process the milk into scalded cheeses, were collected. 10% from the analyzed cheese samples, had values of coliform counts between 10^2-10^3 cfu/g, while the remaining samples had values less than 10^2 cfu/g. 8.3% of cheese samples had values of *E. coli* ranging from 11 to 100 cfu/g and the remaining samples had lower values. No sample registered over 1,000 cfu/g, which is the upper marginal limit for coagulase-positive staphylococci in cheeses, according to the Reg. EC 1881/2006. 18.3% of cheese samples had values of total number of micromycetes over 1000 cfu/g, while the remaining samples were below 1000 cfu/ g.

Keywords: coliform bacteria, micromycetes, scaled cheese

Introduction

The main feature of the technological process of Kashkaval cheese is to obtain the curd, which, after ripening and cutting (into slices of 3-5 mm thickness and 3-6 cm width), is scalded at 74-80° C (Costin et al., 2003).

Cheese making is a major industry worldwide, and a large part of it is still practiced on a relatively small scale, which accounts for the rich diversity of available cheeses (Fox et al., 2004).

Bărzoi and Apostu (2002), showed that cheeses have a very microbiologically complex system. Considering this, the assessment of their general microbiological quality is very difficult, sometimes impossible to perform, due to the intervention of microflora used in their production.

Coliform bacteria is a sanitary microbiological indicator with a special meaning. Coliform bacteria constitutes a dangerous group among microorganisms, that infect cheeses through various sources (Giammanco et al., 2011).

The occurrence of psychrotrophic bacteria in cheeses causes defects like lipolysis, which may lead to excessive accumulation of free fatty acids and rancid aromas in brined cheese. This is the frequent result of milk contamination

with psychotrophic bacteria that produces heat-resistant lipolytic enzymes. (Bintsis and Papademas, 2002).

Yeast and molds can appear on the cheeses during the manufacturing process, from the air, machinery, installations, containers, packings, or during storage and packaging. According to the Ministry's Order (M.S.) from 1998, the maximum limit for cheeses is 1000 yeasts and molds/g of product. The new Regulation EC 1881/2006, does not provide any informations for this indicator. In this study, we analised the microbiological quality and sanitary status of Kashkaval cheese during a year of production, the main purpose being to determine the number of coliform bacteria and *E. coli*, coagulase-positive staphylococci and the total number of micromycetes. (Prates et al., 2017).

Materials and Methods

Sampling

In this study, the manufacturing process of curd involved the following steps: cow's milk reception, cleaning and standardization of milk, milk pasteurization (62-65°C for 20-30 minutes) and cooling (35-38°C), preparation for coagulation which consisted in adding calcium chloride (10 - 20 g/100 liters of milk, increasing the coagulation ability of the milk) and selected cultures of lactic bacteria (*Lactococcus lactis* subsp. *lactis*, *Streptococcus thermophilus* and *Lactobacillus casei*). The coagulation of milk was achieved at 32-35°C, for 30–40 minutes. Processing of curd was then made and the resulting curd was ripened at 18-26°C for 6-10 hours until a pH between 4.8-5.0. The ripened curd was cut into slices having a thickness between 0.3 - 0.5 cm and width of 3-6 cm and was scalded at 72-74°C for 50–60 s in brine with 10–12% salt. The scalded pasta was then ripened. Storage was done at 4-6°C and φ = 85–90%. (Costin et al., 2003).

Sixty samples of kashkaval (semi-hard texture) from five units were analysed, fifteen samples per season. For the microbiological analysis, all samples were transported under refrigerated conditions and were stored in a refrigerator at 2-4ºC and analyzed within 24 hours.

Microbiological analysis

Total coliform counts was performed according to the SR ISO 4831/2009 standard.

Starting with the number of positive test tubes, the most probable number of coliform bacteria (NCP) per ml (g) sample was calculated.

In order to establish the number of *E. coli*, according to SR ISO 16649-2 / 2007, the most probable number technique was used.

SR EN ISO 6888-1-2/A1/2005 standard establishes the horizontal method for counting colonies of coagulase-positive staphylococci on Baird-Parker medium after aerobic incubation (35-37°C), from products intended for human or animal consumption. (Rotar et al., 2015).

The coagulase-positive staphylococci counts per ml or g of sample was made from the number of typical and/or atypical colonies obtained on the selective medium and subsequently confirmed by coagulase-positive assay. Yeasts and molds are microorganisms which at 25°C form colonies on selective medium. SR ISO 7954/2001 standard provides the counting of yeasts and molds according to the counting technique at 25° C. The result was expressed as a number between 1.0 and 9.9 multiplied by 10^X, where x is the power of 10.

Statistical analysis

The data were analyzed by Two-way ANOVA test, to compare the microbial levels between different time points.

Results and discussions

The statistical analysis of microorganisms in kashkaval samples is presented in table 1. Statistically, in relation to the total number of samples, the average ($\overline{X} \pm s_{\overline{X}}$) of coliform bacteria was log. 1.153 ± 0.09592 with values between 0.0 and 460.0 cfu/g. The average of *E.coli* was log 0.2172 ± 0.06007 for values ranging from 0 to 30.0 cfu/g while for coagulase-positive staphylococci was recorded 0.3723 ± 0.06958. The values were between 0.0 and 130.0 cfu/g. Regarding micromycetes, the average was log. 2.349 ± 0.1042 for 0.0 and 2.1 x 10^4 cfu/g.

Coliform bacteria

In this study, a seasonal analysis of coliform bacteria /g in cheese was performed. In the autumn, 73.3% of samples had coliform counts between 0-10 cfu/g and 26.7% between 11-10^2 cfu/g while in the winter, 80% with a mean value of 0-10 cfu/g and 20% ranging from 11 to 10^2 cfu/g.

Table 1. The statistical analysis of microorganisms in pressed kashkaval Dalia during production season

	Season	Samples	Minimum value, ppm	Maximum value, ppm	Log. of Mean, $\overline{X}$	Log. of Standard error of the mean, $s_{\overline{X}}$
Coliform bacteria	Winter	15	0.00	50.00	0.586	0.148
	Spring	15	0.00	100.00	1.140	0.144
	Summer	15	8.00	460.00	1.989	0.126
	Autumn	15	0.00	70.00	0.897	0.137
E.coli	Winter	15	0.00	0.00	0.0	-
	Spring	15	0.00	0.00	0.0	-
	Summer	15	0.00	30.00	0.802	0.154
	Autumn	15	0.00	10.00	0.067	-
Coagulase-positive staphylococci	Winter	15	0.00	20.00	0.087	-
	Spring	15	0.00	130.0	0.563	-
	Summer	15	0.00	20.00	0.649	0.131
	Autumn	15	0.00	9.00	0.191	0.091
Micromycetes	Winter	15	0.00	520.00	1.671	0.194
	Spring	15	10.00	820.00	2.209	0.149
	Summer	15	20.00	21000	3.151	0.174
	Autumn	15	40.00	1100	2.365	0.111

In the spring, 40% of samples registered 0-10 cfu/g and 60%, 11-10^2 cfu/g coliforms counts, in relation to the summer, where, 6.7% of samples were in the range of 0-10 cfu/g, 53.3% between 11-10^2 cfu/g and 40% of samples with coliform counts ranging from 10^2 to 10^3 cfu/g.

Detection of coliform bacteria is not a compulsory test, but their presence in cheeses involves inadequate pasteurization of raw milk, poor hygiene conditions and a lower activity of lactic microflora in manufacturing. The total coliform count is considered a good predictor of hygienic and sanitary practices during food production (Costello et al. 2014).

Wolfe et al. (2014) reported that cheese product characteristics, including moisture content, pH, salt content, ripening conditions, age of product, and culture, all influence potential levels and roles for coliforms and other microorganisms in the final product.

Even though coliforms are considered thermolabile and do not survive pasteurization, Trmčić et al. (2016), found that 21% of pasteurized milk cheeses tested positive for coliforms (>10 cfu/g).

Sources of coliforms in cheese products can vary depending on the product. Due to the nature of raw milk cheeses, the presence of coliforms is not unexpected as coliforms are common in raw milk. However, in pasteurized cheese products, coliforms present in raw milk should have been eliminated by pasteurization, implying that any coliforms present in the finished product resulted from post-processing contamination (Martin et al. 2016).

Escherichia coli

Seasonal analysis of *E. coli* counts/g in kashkaval showed the following results: in the autumn, 93.3% of samples had values in the range of 0-5 cfu/g, and 6.7% had values between 6-10 cfu/g, while in the winter, 100% of samples had values in the range of 0-5 cfu/g. In spring, 100% of samples registered *E. coli* counts between 0-5 cfu/g in relation with the summer, where 46.7% ranged between 0-5 cfu/g, 20% ranged between 6-10 cfu/g and 33.3% between 11-10^2 cfu/g.

Pasteurization of milk destroys pathogenic bacteria, but *L. monocytogenes* and *E. coli* O157: H17 can survive and contaminate the finished product. Survival depends on many factors: initial level of

Table 2. The statistical differences regarding the microorganisms in kashkaval samples during production season (Two-way ANOVA)

Parameter	Comparison	Difference between means	% of total variation	P value	Significance of differences
Coliform bacteria. log. cfu/g	A - W	0.3113 ± 0.2021	7.82	0.0996	ns
	A - Sp	-0.2427 ± 0.1987	5.06	0.1375	ns
	A - Su	-1.092 ± 0.1864	55.06	P<0.0001	***
	W - Sp	-0.5540 ± 0.2067	20.42	0.0108	*
	W - Su	-1.403 ± 0.1949	64.94	P<0.0001	***
	Sp - Su	-0.8493 ± 0.1914	41.28	0.0002	***
E. coli. log. cfu/g	A - W	-	-	-	-
	A - Sp	-	-	-	-
	A - Su	-0.7353 ± 0.1675	40.78	0.0009	***
	W - Sp	-	-	-	-
	W - Su	-0.7353 ± 0.1675	40.78	0.0009	***
	Sp - Su	-0.7353 ± 0.1675	40.78	0.0009	***
Coagulase-positive staphyloccoc. log. cfu/g	A - W	0.1040 ± 0.1253	2.40	0.4519	ns
	A - Sp	-0.3727 ± 0.2016	10.88	0.0904	ns
	A - Su	-0.4580 ± 0.1594	22.77	0.0195	*
	W - Sp	-0.4767 ± 0.1999	16.89	0.0059	**
	W - Su	-0.5620 ± 0.1573	31.32	0.0007	***
	Sp - Su	-0.08533 ± 0.2228	0.52	0.6774	ns
Micromycetes. log. cfu/g	A - W	0.6947 ± 0.2238	25.61	0.0066	**
	A - Sp	0.1560 ± 0.1862	2.45	0.2403	ns
	A - Su	-0.7853 ± 0.2068	34.00	0.0004	***
	W - Sp	-0.5387 ± 0.2448	14.75	0.0548	ns
	W - Su	-1.480 ± 0.2608	53.50	P<0.0001	***
	Sp - Su	-0.9413 ± 0.2293	37.56	0.0001	***

Legend: A/ autumn; W/ winter; Sp/ spring; Su/ summer.
ns – not significant ($p > 0.05$); * – significant ($0.01 < p < 0.05$); ** – very significant ($0.001 < p < 0.01$); *** – extremely significant ($p < 0.001$).

contamination, pasteurization temperature, acid and salt tolerance of microorganisms, competitive microflora, cheese composition and manufacturing conditions.

Pasteurization is able to destroy essential microflora, enzymes and pathogens in milk. It should be noted that the inactivation level of microorganisms depends on the amount of microorganisms, growth phase and other factors (Ciprovica and Mikelson. 2011).

Giammanco et al. (2011), analyzing the microbiological quality of Pecorino Siciliano cheese, found 27 of the 50 samples (54%) that appeared not to be satisfactory according to European Regulation 2005/2073/EC. This was mainly due to high levels of glucuronidase-positive *E.coli*. In 44% of the samples. *E. coli* exceeded 10^3 CFU g^{-1} and in 8% of the samples its presence was detected in quantities between 10^2 and 10^3 CFU g^{-1}.

The results of a study released by Kwenda, 2015, showed that poor hygiene practices had played a major role to the contamination of the cheddar cheese with *E. coli* and coliform bacteria as well as other factors like pasteurization efficiencies.

Baranceli et al. (2014), studied the percentages of *E. coli* counts in packaged Minas cheeses and showed that up to 93% of samples commercially available at sales points were detected with fecal coliforms above the tolerance limit established by legislation. The higher percentages were indicative of lower hygienic conditions in the final

steps of cheese manufacture, including molding, packaging.

Istrati et al. (2006), in one of their studies, have analyzed 11 Ruc and Dalia cheese samples, sold in the county of Bacău and have found a single sample with *E. coli* values below 10 cfu/g.

Some of the microorganisms that grow in dairy products are able to produce undesirable reactions that deteriorate the organoleptic characteristics of cheese, while other can potentially cause food-borne diseases (Lu et al.. 2013).

Coagulase-positive staphylococci

No sample had values > 10^3 cfu/g which is the upper marginal limit (M) for coagulase-positive staphylococci in cheeses, according to Reg. EC 1881/2006.

The presence of these microorganisms in dairy products depends by factors such as the quality of raw material, the respect of good practices of production and storage conditions (Ledenbach and Marshall. 2009).

Micromycetes

The determinations of micromycetes counts/g during the four seasons showed the following dates: in the autumn, 26.7% of the samples had values ranging from 0 to 100 cfu/g, 53.3% between 101-500 cfu/g, 13.3% between 501-1000 cfu/g and 6.7% were over 1000 cfu/g. In the winter, 66.7% of samples had values between 0-100 cfu/g, 26.7% between 101-500 cfu/g and one sample between 501-1000 cfu/g. In the spring, 33.3% of samples had values ranging from 0 to 100 cfu/g, 40% between 101-500 cfu/g and 26.7% between 501-1000 cfu/g while in the summer, 6.7% of samples had a range of 0-100 cfu/g, 26.7% were between 501-1000 cfu/g and 66.7% with micromycetes counts over 1000 cfu/g, 18.3% of all collected and analyzed samples had values > 1000 cfu/g.

Most problems raised by the contamination of kashkavals with yeasts and molds have related to their shelf life. These microorganisms have a strong proteolytic and lipolytic activity. In order to present no risks for human health and to extend their shelf-life, the kashkaval must not contain such microorganisms, or their number should be less than 1000/g of product (Bărzoi and Apostu. 2002).

Ostry et al., 2004, reported that micromycetes are important factors with a potentially negative effect on human health, especially if are toxigenic, with toxic effects like carcinogenic, developmental toxicity. Many studies showed that the foodstuffs contaminated with the toxigenic micromycetes have presented a serious hazard of the „hidden mycotoxins" (Ostry et al., 2004).

Regarding the statistical analysis of the logarithmic mean values of coliforms, *E. coli* and micromycetes, from the kashkaval samples during the production season, have been observed extremely significant differences for (A-Su), (W-Su) and (Sp-Su), while coagulase-positive staphyloccoc have been reported very significant differences for (W-Sp).

Insignificant levels of (A-W), (A-Sp) production were induced by coliform bacteria, (A-W), (A-Sp) and (Sp-Su) by coagulase-positive Staphyloccoc and (A-Sp), (W-Sp) by micromycetes.

Conclusions

According to the results of this study the highest coliform bacteria values in kashkaval cheeses were detected in the summer, ranging from 8.0 to 460.0 cfu/g, and the lowest in the winter, between 0.0 and 50.0 cfu/g.

Analyzing the seasonal variations of *E.coli*. in the winter and spring was absent, while the highest values were in the summer, ranging between 0.0 and 30.0 cfu/g. According to the Ministry of Health Regulations from 1998, up to 10 coliforms/ g product are allowed, maximum 1000 yeasts and molds/g product. The new regulations do not provide any information for these indicators.

The highest level of coagulase-positive staphylococci group of bacteria were detected in the spring, between 0.0 and 130.0 cfu/g and the lowest in the autumn, between 0.0 and 9.0 cfu/g while the highest Micromycetes counts were in the summer, 20.0 - 21000.0 cfu/g and the lowest were in the winter between 0.0 and 520.0 cfu/g.

The microrganisms that are indicators of the sanitary-hygienic quality, such as coliform bacteria, *E.coli* and micromycetes were present in kashkaval samples at relatively high levels during the summer.

The results of this study highlight that the high temperatures during the summer influenced the microbial load. Due to the risks that these microorganisms represent to public health, it is necessary to improve hygienic practices during the warm season, both at the reception and during the manufacturing process.

References

1. Baranceli G V, Oliveira CAF, Corassin CH, Camargo T M. Santos MG, Novotny LCM, Porto E. (2014). Occurrence of Escherichia coli and Coliforms in Minas Cheese Plants from São Paulo, Brazil. J Adv Dairy Res. 2:2. Doi:10.4172/2329-888X.1000120.
2. Bintsis T, Papademas P. (2002). Microbiological quality of white-brined cheese: A review. Int. Journal of Dairy Technology. 55 (3): 113 – 119.
3. Bărzoi D, Apostu S. (2002). The Microbiology of food products. (3th ed.) Cluj-Napoca: Risoprint Publishing House. (Chapter A1).
4. Ciprovica I , Mikelsone A. (2011). The influence of ripening temperature on diversity of nonstarter lactic acid bacteria in semi-hard cheeses. Romanian Biotechnological Letters. 16 (6):155-162.
5. Costello M, Dougherty RH, Kang D. (2001). The relationship between standard plate counts and coliform counts in raw milk. Dairy Food Environ Sanit. 21: 749-751.
6. Costin GM, Florea T, Popa C, Rotaru G, Segal R, Bahrim G, Botez E, Turtoi M, Stanciu S, Turtoi G.(2003). Ştiinţa şi ingineria fabricării brânzeturilor. Galaţi: Editura Academica. (Chapter 8).
7. Fox PF, McSweeney PLH, Cogan TM, Guinee TP. (2004). Cheese: An Overview. In Cheese Chemistry. Physics and Microbiology. (3rd ed.). General Aspects (pp. 52-66). London. UK: Elsevier Academic Press.
8. Giammanco GM, Pepe A, Aleo A, D'Agostino V, Milone S and Mammina C. (2011). Microbiological quality of Pecorino Siciliano "primosale" cheese on retail sale in the street markets of Palermo, Italy. New Microbioliology. 34:179-185.
9. Istrati L, Ciobanu G, Harja M, Gavrilă L, Ceaunaş R. (2006). Study Concerning Microbiological Contamination of Some Cheese. Journal of Agroalimentary Processes and Technologies. 12 (1): 75-82.
10. Kwenda A. (2015). An Investigation on the Causes of Escherichia Coli and Coliform Contamination of Cheddar Cheese and How to Reduce the Problem. Global Journal of Science Frontier Research: E Interdiciplinary. 15 (2): 2249 - 4626.
11. Lu M, Shiau Y, Wong J, Lin R, Kravis H, Blackmon T, Pakzad T, Jen T, Cheng A, Chang J, Ong E, Sarfaraz N, Wang NS. (2013). Milk spoilage: methods and practices of detecting milk quality. Food Nutr Sci. 4: 113-123.
12. Ledenbach LH, Marshall RT. (2009). Microbiological spoilage of dairy products. Sperber WH. Doyle MP (Eds.) Compendium of the Microbiological Spoilage of Foods and Beverages. Food Microbiology and Food Safety (pp. 41-67). Dordrecht. Netherlands: Springer Science Business Media B.V.
13. Ostry V, Skarkova J, Ruprich J. (2004). The experimental contamination of foodstuffs with the spores of toxigenic micromycetes and the production of mycotoxins. Mycotoxin Research. 20:31-35.
14. Prates DF, Würfel SR, Goldbeck JC, Lima AS, Lopes GV, Silva WP. (2017). Microbiological quality and safety assessment in the production of moderate and high humidity cheeses. Ciência Rural. Santa Maria. Doi: 10.1590/0103-8478cr20170363.
15. Rotar A, Vodnar D, Bunghez F, Cătunescu G, Pop C, Jimborean M, Semeniuc C. (2015). Effect of Goji Berries and Honey on Lactic Acid Bacteria Viability and Shelf Life Stability of Yoghurt. Not Bot Horti Agrobo. 43 (1):196-203.
16. Trmčić A, Chauhan K, Kent DJ, Ralyea RD, Martin NH, Boor KJ, Wiedmann M. (2016). Coliform detection in cheese is associated with specific cheese characteristics, but no association was found with pathogen detection. J. Dairy Sci. Doi: 10.3168/jds.2016-11112.
17. Martin NH, Trmčić A, Hsieh TH, Boor KJ, Wiedmann M. (2016). The Evolving Role of Coliforms As Indicators of Unhygienic Processing Conditions in Dairy Foods. Front Microbiol. Doi: 10.3389/fmicb.2016.01549.
18. Wolfe BE, Button JE, Santarelli M, Dutton RJ. (2014). Cheese rind communities provide tractable systems for in situ and in vitro studies of microbial diversity. Cell. 158 (2): 422–433.
19. *** EC Regulation no.2073/15.11.2005 regarding the microbiological criteria for foods.
20. *** 2006 – Regulament European (CE) nr.1881/19.12.2006 al Comisiei de stabilire a nivelurilor maxime pentru anumiţi contaminanţi din produsele alimentare.
21. *** 1998 – Ordinul M. S. numărul 976. privind aprobarea Normelor de igienă privind producţia. prelucrarea. depozitarea. păstrarea. transportul şi desfacerea alimentelor.