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ORIGINAL ARTICLE

IDENTIFICATION AND ANTIMICROBIAL SUSCEPTIBILITY SCREENING OF LACTIC ACID BACTERIA FROM TRADITIONAL CHEESE *PAŠKI SIR*, CROATIA

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Ethical considerations: When reporting experiments on animals Observation of the ARRIVE guidelines 2.0: Updated guidelines for reporting animal research, published on July 14, 2020 (DOI: 10.1371/journal.pbio.3000410), is applied. The authors ensure that all procedures were performed in compliance with the guidelines for animal care of their institutions or with national/international guidelines.

ABSTRACT

***Paški sir*, a hard sheep's cheese, has been produced for decades in households on the island of Pag, Croatia, and is now manufactured under controlled conditions in local cheesemaking facilities. Extensive and ecological sheep breeding and cheese production result in low chemical input or hazards and final products with high safety properties. The aim of the study was to investigate the diversity of lactic acid bacteria (LAB) in this type of production and their phenotypic profiles of antimicrobial resistance. MALDI-TOF MS identification revealed a high diversity of indigenous LAB populations, including lactobacilli, enterococci, pediococci, and lactococci, in traditional *Paški sir* cheese. Most isolates representing identified species showed cut-off values below the critical limits required for selecting potential probiotic strains suitable for food production.**

Keywords: antimicrobial resistance; diversity; lactic acid bacteria; *Paški sir*

INTRODUCTION

Paški sir hard cheese is produced from the milk of the Croatian autochthonous *Paška ovca* sheep, which graze on pastures rich in various types of medicinal and aromatic herbs on the island of Pag, Croatia. This results in a cheese with a characteristic taste and a pleasant aroma. *Paški sir* cheese has been PDO-protected since 2019 and, according to its specification, can be made from raw or pasteurised sheep's milk. The natural microbiota of the milk consists mainly of heterogeneous species of lactic acid bacteria (LAB), which share a common carbohydrate metabolic pathway with lactic acid as the primary metabolic endpoint. Due to their probiotic properties, LAB have a long history of safe use in traditional and industrial fermented dairy products [1–3]. The dominance of certain LAB species during the production process depends on the processing temperature of the curd and the ripening conditions, which strongly influence the quality of the cheese. LAB initiate complex biochemical reactions that are crucial for

the development of the specific organoleptic properties of this cheese [4, 5].

The indigenous microbiota, i.e. LAB, has not yet been studied in relation to the production chain of traditional *Pa*ški sir cheese. Identification of lactobacilli has only been carried out in one study by Jenserle [6] using the PCR method and reported three identified species in cheese: formerly *Lactobacillus brevis, Lactobacillus plantarum,* and *Lactobacillus paracasei* (now *Levilactobacillus brevis*, *Lactiplantibacillus plantarum*, *Lacticaseibacillus paracasei)*; [7]. Furthermore, to our knowledge, the antimicrobial resistance profile of LAB within this type of production has also not been studied.

Thus, this study aimed to isolate and identify LAB from the traditional *Paški sir* cheese production chain, i.e., from sheep's milk, lamb abomasum (used to obtain natural rennet), cheese curd, and cheese during ripening. An additional aim was to select strains of the identified species and subject them to antibiotic susceptibility testing to gain an initial insight into the extent of antimicrobial resistance in traditional *Paški sir* cheese production.

MATERIALS AND METHODS

Isolation and identification of lactic acid bacteria from the *Paški sir* cheese production chain

Paški sir cheese was traditionally produced in a family-owned cheese dairy located in Kolan (Pag), using milk from sheep raised on the farm and following traditional cheesemaking practices. Cheese was produced from raw sheep milk and aged for 120 days. Samples taken for LAB isolation and identification included raw milk from randomly selected lactating sheep in the herd (n = 18), abomasum contents of slaughtered lambs, curd, and cheese at different stages of ripening (days 13, 50, 80, and 120).

To isolate LAB and determine the count, 10 g/10 mL of the sample was aseptically taken, diluted in 90 mL of saline peptone water, and homogenised for two minutes (Stomacher 400 Circulator, Seward, UK). From the selected decimal dilutions, 0.1 mL was spread on MRS agar (BioMerieux, Marcy l'Etoile, France) and incubated under anaerobic conditions at 30 °C for 48-72 hours. After incubation, the number of bacteria per gram or millilitre of sample was calculated using an automatic colony counter (Scan 1200, Interscience, Paris, France). Morphologically different colonies were then cultured in MRS broth, followed by streaking onto MRS agar for identification. A total of 190 colonies of Gram-positive and catalase-negative bacilli, cocci, and coccobacilli were subjected to matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry (MALDI-TOF MS).

The standard MALDI-TOF MS procedure consisted of plating the colony on the MALDI target plate and adding 1 µL of 70% formic acid (Fisher Chemical, Alcobendas, Spain). After drying at room temperature, each spot was covered with 1 µL of 10 mg/mL alpha-4-cyano-4-hydroxycinnamic acid (CHCA, Bruker Daltonik, Bremen, Germany) in 50% acetonitrile and 2.5% trifluoroacetic acid and allowed to dry. Measurements were performed with a Microflex LT mass spectrometer (Bruker Daltonik, Bremen, Germany), and spectra were recorded in a positive linear mode in the mass range from 2,000 to 20,000 Da. MBT Compass HT version 5.1 software (Bruker Daltonik, Bremen, Germany) was used to compare the spectra with the reference database. External calibration was performed using the Bacterial Test Standard (Bruker Daltonik, Bremen, Germany). The identification criteria were as follows: a logarithmic value of 2.00–3.00 indicated a species identification with high confidence, a value of 1.70–1.99 indicated a species identification with low confidence, while a value of 0–1.69 was considered unreliable [8]. Based on the values of the results representing the highest probability of correct identification, 173 isolates were selected to evaluate the representation of LAB species in the samples.

Testing the antimicrobial susceptibility of LAB species

Sixteen isolates of lactic acid bacteria, representing bacterial species identified by MALDI-TOF MS, were used for antibiotic susceptibility testing: *Lactococcus lactis, Lactiplantibacillus plantarum, Carnobacterium maltaromaticum, Lactococcus raffinolactis, Loigolactobacillus cornyformis, Enterococcus faecalis, Levilactobacillus brevis, Pediococcus pentosaceus, Leuconostoc mesenteroides, Enterococcus durans, Enterococcus faecium, Enterococcus italicus, Lacticaseibacillus paracasei, Companilactobacillus paralimentarius, Latilactobacillus sakei,* and *Latilactobacillus curvatus.*

Susceptibility to nine antibiotics was tested using the E-test (BioMerieux, Marcy l'Etoile, France). Antibiotics were selected according to EFSA [9] recommendations in-

tended for the characterisation of microorganisms used as feed additives or as production organisms, as there are no such criteria for isolates from human food. Pure bacterial cultures (density of 0.5 McFarland) were spread in three layers and three directions on MRS agar using a sterile swab. After 15 minutes, E-test strips containing ampicillin (0.016–256 mg/L), vancomycin (0.016–256 mg/L), tetracycline (0.016–256 mg/L), kanamycin (0.016–256 mg/L), streptomycin (0.064–1024 mg/L), clindamycin (0.016–256 mg/L), chloramphenicol (0.016–256 mg/L), gentamicin (0.016–256 mg/L), and erythromycin (0.016–256 mg/L) were applied. After incubation at 30 °C for 24 hours, the minimum inhibitory concentrations (MICs) were determined by reading the lowest point between the inhibition zone and the MIC values on the scale. The results were interpreted according to the EFSA criteria [9]. Multidrug-resistant (MDR) strains were considered as non-susceptible to at least one agent in three or more different classes of antimicrobial drugs.

RESULTS AND DISCUSSION

The results of the identification of lactic acid bacteria are shown in Table 1. The identified species are listed in order of their proportion within the population of identified colonies. A total of 16 different species were identified from milk, abomasum, curd, and cheese.

In the raw sheep's milk samples, only *Lactococcus lactis* was identified. This species was later displaced in the curd by *Carnobacterium maltaromaticum, Leuconostoc mesenteroides,* and *Lactococcus raffinolactis* and was not detected in the cheese during ripening. An increase in species diversity of the population was observed, with the number of identified species rising as the cheese matured. The mixed population consisted mainly of lactobacilli, followed by enterococci, pediococci, leuconostoc, and carnobacteria. Among the lactobacilli, homofermentative species predominated, which is technologically desirable as they do not produce off-flavours, gases, or other unwanted by-products. However, undesirable enterococci (*E. faecalis*) and, in early stages of maturation, *Leuconostoc* species, which are heterofermentative gas-producing bacteria, were also isolated, although to a lesser extent. The presence of enterococci is also common in various hard cheese types (up to 5 $\log_{10}$ CFU/g), but it can lead to higher levels of biogenic amines in the cheese, especially tyramine [10]. The final product was dominated by the species *P. pentosaceus* and *L. paracasei.*

Studies of the indigenous microbiota in the production of *Paški sir* cheese have only rarely been carried out, which is probably due to the shift to standardised production from pasteurised milk with the addition of starter cultures. Only Jensterle [6] identified three predominant species of lactobacilli in *Paški sir* cheese using the PCR method: *L. brevis, L. plantarum,* and *L. paracasei*. To our knowledge, our research is the first study of the microbiota in the production chain of *Paški sir* cheese in terms of identifying the predominant autochthonous LABs in *Paška ovca* sheep's milk, curd, and cheese during ripening. In addition, *L. plantarum* was isolated from the lamb abomasum, the strain used together with natural rennet in microencapsulated form as an innovation in the production of *Paški sir* cheese [11].

The results of MALDI-TOF MS identification of selected LAB colonies show the predominance of lactobacilli at certain stages of cheese maturation, which is completely different from the microbiota of sheep's milk, in which only the species *Lc. lactis* was present. This species is very important in the dairy industry and has many desirable technological and health properties. In this sense, Pajač et al. [12] confirmed the strong acidification capacity and good proteolytic, lipolytic, and antimicrobial activity of lactococci from the milk of *Paška ovca* sheep. The selected strain *Lc. lactis* was also used experimentally in the production of *Paški sir* cheese in microencapsulated form [11], which did not affect the standard quality of the cheese.

A very high species diversity of the sheep's milk microbiota was found by Quintana et al. [13] using MALDI-TOF MS determination, with lactobacilli predominating. It is also common to find enterococci in sheep's milk, which can persist in the curd and the cheese during ripening [14]. It is known that microbiota composition of raw milk depends on numerous factors, including udder health and production hygiene. Nevertheless, the methodology used for LAB isolation, particularly the method of selecting colonies for identification and the identification method itself, should not be disregarded. Accordingly, the composition of the LAB population determined in our study from different matrices during cheese production should only be compared with studies based on culture-dependent isolation methods. Recently, attention has been drawn to

Table 1. Results of LAB identification in the *Paški sir* cheese production chain

Sample	Number of colonies for identification	Number of identified colonies (MALDI score 2.0–3.0)	Identified LAB species	Number of identified species
Sheep milk (n=18)	18	18	*Lactococcus lactis*	18
Lamb abomasum (n=3)	8	8	*Enterococcus faecalis*	7
			Lactiplantibacillus plantarum	1
Curd (n=3)	60	50	*Carnobacterium maltaromaticum*	24
			Leuconostoc mesenteroides	11
			Lactococcus lactis	9
			Lactococcus raffinolactis	3
			Enterococcus faecalis	3
Cheese during ripening (n=4)				
Day 13	18	17	*Leuconostoc mesenteroides*	13
			Lacticaseibacillus paracasei	2
			Latilactobacillus curvatus	1
			Enterococcus faecalis	1
Day 50	20	14	*Loigolactobacillus coryniformis*	7
			Pediococcus pentosaceus	2
			Leuconostoc mesenteroides	2
			Latilactobacillus sakei	1
			Levilactobacillus brevis	1
			Enterococcus faecalis	1
Day 80	24	24	*Enterococcus faecium*	6
			Lacticaseibacillus paracasei	4
			Enterococcus durans	3
			Latilactobacillus sakei	3
			Pediococcus pentosaceus	2
			Leuconostoc mesenteroides	2
			Enterococcus faecalis	2
			Loigolactobacillus coryniformis	1
			Enterococcus italicus	1
Day 120	42	42	*Lacticaseibacillus paracasei*	12
			Pediococcus pentosaceus	12
			Enterococcus faecalis	7
			Enterococcus faecium	5
			Loigolactobacillus coryniformis	2
			Latilactobacillus sakei	1
			Latilactobacillus curvatus	1
			Companilactobacillus paralimentarius	1
			Leuconostoc mesenteroides	1

methods that bypass the classical isolation of live bacteria on microbiological media and instead isolate bacterial DNA from a food sample and subject it for metagenomic sequencing [15]. It is clear that such methods offer a completely new perspective on the composition of the microbiome of milk or cheese, but in the context of product quality and safety, the focus should still be on the microbiota that can be cultured and isolated from the sample (i.e., live cells) so that it can be accurately associated with the observed desirable or undesirable properties of the final product. Nevertheless, studies on the cultivable microbiota in cheese production can contribute to the selection of viable LAB strains at the specific sampling site and under the process conditions. Thus, the abundance of *Lc. lactis* in raw milk can be explained by the neutral acidity, which is favourable for its growth, compared to the acidic environment, increased salt content, and reduced redox potential in hard cheese, where lactobacilli predominate.

Because of the established diversity of the microbiota, the sensitivity profiles to antibiotics were preliminarily

investigated phenotypically in a selected representative of all 16 isolated LAB species in this study (Table 2). Most LAB species (*Lactococcus lactis, Lactiplantibacillus plantarum, Carnobacterium maltaromaticum, Loigolactobacillus coryniformis, Levilactobacillus brevis, Leuconostoc mesenteroides, Enterococcus durans, Enterococcus faecium,* and *Latilactobacillus sakei*) were sensitive to antimicrobial agents relevant in the horizontal transfer of resistance, such as tetracycline or erythromycin, indicating possible lower antibiotic pressure in extensive sheep farming on the island of Pag. However, seven species, namely *Lactococcus raffinolactis, Enterococcus faecalis, Pediococcus pentosaceus, Enterococcus italicus, Lacticaseibacillus paracasei, Companilactobacillus paralimentarius,* and *Latilactobacillus curvatus,* were resistant to at least one agent according to EFSA criteria [9]. Criteria of multi-drug resistance (MDR; resistance to three or more antibiotic classes) were fulfilled only in two enterococci strains (*E. faecalis, E. italicus*) and *L. paracasei.* Resistance was found most frequently to aminoglycosides kanamycin and streptomycin.

Kanamycin and streptomycin, the antibiotics to which resistance was most frequently detected in this study, were closely followed by gentamicin. They belong to the aminoglycoside group and are classified as *critically important antimicrobials* (CIA) according to the WHO MIA List, meaning they are widely used in human medicine and are used for infections where there is evidence of transmission of resistant bacteria or resistance genes from non-human sources. Given the detected resistance in *L. paracasei*, it should be noted that macrolides, such as erythromycin, are also critically important for humans, as they are among the few treatment options for *Campylobacter* infections in humans [16]. This highlights the importance of monitoring resistance to aminoglycosides and macrolides in non-human sources such as fermented products due to the risk of resistance transfer.

It is known that antimicrobial resistance in commensal food bacteria has recently become an important field of research due to the possible horizontal transmission of resistance genes within the food chain [17]. It has been shown that populations of these bacteria in food can carry resistance genes for clinically relevant antibiotics, which may also be transmissible [18]. The role of fermented foods in the transmission of resistance still needs to be evaluated and consumer exposure and potential public health risks assessed [17]. In our study, *L. paracasei* was found to be the most resistant of the species tested, showing resistance to five of the nine antibiotics tested (gentamicin, kanamycin, streptomycin, erythromycin, and tetracycline). These results

Table 2. MIC values (mg/L) of antibiotics in inhibiting the growth of lactic acid bacteria from *Paški sir* cheese production chain

	Ampicillin	Vancomycin	Gentamicin	Kanamycin	Streptomycin	Erythromycin	Clindamycin	Tetracycline	Chloramphenicol
Lc. lactis	0.19 (2)	1 (4)	2 (32)	6 (64)	2 (32)	0.047 (1)	0.064 (1)	0.094 (4)	1 (8)
L. plantarum	1 (2)	n.r.	0.125 (16)	12 (64)	n.r.	0.032 (1)	0.125 (4)	2 (32)	4 (8)
Cb. maltaromaticum	0.023 (2)	0.5 (2)	12 (16)	**64** (16)	**128** (16)	0.047 (1)	0.125 (4)	1 (4)	1 (4)
Lc. raffinolactis	0.064 (2)	1 (4)	16 (32)	**96** (64)	**128** (32)	0.047 (1)	0.064 (1)	0.064 (4)	1.5 (8)
L. coryniformis	1 (2)	1 (2)	2 (16)	1 (16)	4 (16)	0.032 (1)	0.125 (4)	1 (4)	1 (4)
E. faecalis	0.5 (2)	1.5 (4)	**96** (32)	256 (1024)	**1024** (128)	1.5 (4)	**12** (4)	**16** (4)	1.5 (16)
L. brevis	0.5 (2)	n.r.	1.5 (16)	24 (64)	12 (64)	0.094 (1)	3 (4)	6 (8)	1.5 (4)
P. pentosaceus	0.5 (4)	n.r.	4 (16)	**64** (64)	24 (64)	0.064 (1)	0.125 (1)	2 (8)	1 (4)
Ln. mesenteroides	0.25 (2)	n.r.	1.5 (16)	6 (16)	8 (64)	0.023 (1)	0.125 (1)	1 (8)	1.5 (4)
E. durans	0.094 (2)	0.38 (4)	12 (32)	48 (1024)	96 (128)	0.125 (4)	0.016 (4)	0.124 (4)	1.5 (16)
E. faecium	0.094 (2)	0.38 (4)	8 (32)	64 (1024)	48 (128)	2 (4)	0.032 (4)	0.094 (4)	1.5 (16)
E. italicus	0.5 (2)	1.5 (4)	**64** (32)	256 (1024)	**768** (128)	1.5 (4)	**16** (4)	**8** (4)	1.5 (16)
L. paracasei	0.19 (4)	n.r.	**64** (32)	**256** (64)	**384** (64)	**1** (1)	2 (4)	**4** (4)	0.75 (4)
C. paralimentarius	1.5 (2)	n.r.	12 (16)	**256** (16)	**96** (16)	0.25 (1)	0.064 (4)	1 (4)	1.5 (4)
L. sakei	0.023 (2)	n.r.	2 (16)	2 (16)	4 (16)	0.125 (1)	1 (4)	1 (4)	1.5 (4)
L. curvatus	0.38 (2)	n.r.	**16** (16)	**96** (16)	0.94 (16)	0.94 (1)	0.5 (4)	0.5 (4)	1 (4)

n.r. not recommended; in **bold** values are equal to or above MIC cut-off values (in brackets; mg/L)

are consistent with the study by Honi et al. [19], which emphasises the clinical importance of aminoglycoside resistance in the context of probiotics and their potential impact on resistance in the human gut microbiome. Generally, LAB are sensitive to antimicrobial substances that act as cell wall synthesis inhibitors, such as penicillins and beta-lactamase inhibitors, and are mostly resistant to aminoglycosides such as kanamycin, streptomycin, gentamicin, and neomycin [20], which was also observed in this work. Regarding other lactobacilli species, in our study, representatives of *L. cornyformis, L. brevis, L. sakei,* and *L. plantarum* were sensitive to all antibiotics tested. In other recent studies [21–23], resistance to additional antibiotics was reported in these species, such as oxacillin, fluoroquinolones, or fosfomycin. *Enterococcus faecalis* was the second most resistant species, showing resistance to gentamicin, streptomycin, clindamycin, and tetracycline. Similar results were obtained by Furlaneto-Maia et al. [24], who reported resistance of *E. faecalis* from soft cheese in Brazil to tetracycline, as well as to erythromycin and vancomycin, which was not observed in this study. Chingwaru et al. [25] also recorded a high resistance of *E. faecalis* isolates from milk to ampicillin, vancomycin and a very high resistance to tetracycline. Among the other enterococcal species, *E. italicus* was resistant to streptomycin, clindamycin, and tetracycline, while *E. durans* and *E. faecium* were sensitive to all antibiotics tested. It should be noted that the interpretation of antibiotic resistance in enterococci other than *E. faecium* in this study was performed according to the recommended criteria for *E. faecium* [9]. Teuber et al. [26] reported several decades ago that enterococci are frequently resistant to vancomycin, streptomycin, erythromycin, chloramphenicol, tetracycline, and clindamycin. Similarly, Vesković et al. [27] point out the acquired resistance of enterococci to chloramphenicol, clindamycin, erythromycin, aminoglycosides, beta-lactams, and tetracycline. The concern about enterococcal resistance lies in their ability to horizontally transfer genes for important antibiotics such as vancomycin and methicillin, allowing them to rapidly adapt and spread resistance among microbiota [28, 29]. These findings emphasise the importance of continuous monitoring of enterococcal resistance in the food chain and their ability to transfer resistance to pathogenic bacteria, which poses a serious risk to public health. However, the absence of resistance to vancomycin in our isolates suggests low risk in extensive sheep breeding and traditional cheese production. The use of glycopeptide antibiotics in animals, such as vancomycin, is associated with the development of vancomycin-resistant enterococci that are transmissible from animals to humans. This poses a serious threat, as glycopeptides are among few treatment options for very severe enterococcal infections in humans [16].

Considering lactococci, in this study *Lc. lactis* was sensitive to all antibiotics, while *Lc. raffinolactis* was resistant to kanamycin and streptomycin. Devirgilis et al. [30] reported that lactococci often exhibit resistance to tetracycline, macrolides, glycopeptides, and polymyxins. In contrast to our results, Chen et al. [20] found diverse resistance profiles among *Lc. lactis* isolated from sheep's milk, including resistance to oxacillin, amikacin, neomycin, gentamicin, tetracycline, norfloxacin, furazolidone, sulfamethoxazole, polymyxin, ceftazidime, kanamycin, vancomycin, and doxycycline. Bulajić et al. [31] also concluded that lactococci from cheese (Serbia) are often resistant to streptomycin, ampicillin, vancomycin, and penicillin. The varying degrees of susceptibility and resistance of lactococci reported in the cited studies are likely due to differences in the extent of antibiotic exposure in animal husbandry and environmental factors, assuming that methodological differences and varying criteria for resistance assessment are disregarded.

The *Pediococcus pentosaceus* isolate from Paški sir cheese tested in this study exhibited resistance only to kanamycin, indicating a lower level of resistance compared to other studies on this dairy-derived species. For instance, Bhukya and Bhukya [32] reported resistance to streptomycin, vancomycin, ciprofloxacin, norfloxacin, and trimethoprim, in addition to kanamycin, in *P. pentosaceus* from buttermilk. Monitoring antibiotic resistance in *P. pentosaceus* is important, since it may carry transmissible resistance genes, particularly for erythromycin and tetracycline, as highlighted by Vesković et al. [27]. *Leuconostoc mesenteroides* was also sensitive to all antibiotics tested in our study, which is a favourable result compared to other reports [27].

CONCLUSION

The results obtained indicate a moderate prevalence of antimicrobial resistance among selected representatives of LAB species identified from the production chain of

traditionally produced sheep's cheese. Most isolates were susceptible to the tested antibiotics, suggesting possible lower antibiotic pressure in extensive sheep farming on the island of Pag, Croatia. The isolates that showed complete sensitivity to antibiotics were *L. plantarum*, *Lc. lactis*, *E. faecium,* and *E. durans* and are potential candidates for further characterisation in the selection of cheese cultures. The phenotypically observed resistance of individual isolates requires further investigation regarding the resistance transfer to the exposed, non-resistant microbiota.

Data Availability Statement

The raw data of this article will be made available by the authors, without undue reservation.

Ethical Statement

This research was approved by the ethical committee of the Faculty of veterinary medicine, University of Zagreb, Croatia; approval number: 640-01/22-02/15; 251-61-01/139-22-28

Conflict of Interest

The authors declare no conflict of interest.

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Generative AI Statement

No generative AI and AI-assisted technologies were used in writing the manuscript.

Authors' Contributions

Nevijo Zdolec – conceptualisation, analysis, writing and editing, resources; Marta Kiš – laboratory analysis; Snježana Kazazić – MALDI TOF MS identification; Fabijan Oštarić – cheese production and sampling; Ena Horvat – laboratory analysis, writing; Nikola Čudina – writing and editing; Nataša Mikulec – resources, editing.

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ORIGINAL ARTICLE

THE EFFECT OF HEATHER HONEY ADDITION ON PHYSICOCHEMICAL AND MICROBIOLOGICAL PROPERTIES OF STIRRED-TYPE YOGHURT

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Ethical considerations: When reporting experiments on animals Observation of the ARRIVE guidelines 2.0: Updated guidelines for reporting animal research, published on July 14, 2020 (DOI: 10.1371/journal.pbio.3000410), is applied. The authors ensure that all procedures were performed in compliance with the guidelines for animal care of their institutions or with national/international guidelines.

ABSTRACT

The aim of this study was to investigate the impact of the heather honey concentrations (2.5% and 5%) on the physicochemical and microbial properties of yoghurt samples made from cow milk. Honey, used as a natural fortifier, enhanced antioxidant activity and probiotic viability of experimental samples. Except for water activity (a_w), each physicochemical variable (dry matter, titratable acidity, water-holding capacity and antioxidant activity) differed significantly ($p < 0.001$) between the control and both experimental yoghurt sample groups (2.50% and 5.00%). Throughout the study, the 5.00% heather honey-added yoghurt consistently exhibited the peak antioxidant activity on days 1, 15, and 22. Multiple factor analysis (MFA) revealed correlations among the variables and visualised the similarity of the analysed samples on the 15th and 22nd days. On the other hand, the results of MFA showed differences in control and both experimental yoghurt sample groups with heather honey addition. As the demand for fermented milk grows, the dairy industry is increasingly combining probiotics with functional foods—most notably through the development of probiotic functional yoghurts.

Keywords: antiradical activity; fermented dairy product; quality; storage

INTRODUCTION

Yoghurt, a widely consumed dairy product, has a rich cultural and historical background [1]. It is derived from the fermentation of milk and has become an essential part of many people's diets due to its well-established reputation as a nutritious food [2]. For centuries, fermentation has served as a method to extend the shelf life of perishable foods while simultaneously enhancing the taste and aroma of the final product [3]. Yoghurt is the outcome of fermenting pasteurised or sterilised milk using protosymbiotic cultures of *Streptococcus salivarius* subsp. *thermophilus* and *Lactobacillus* (*L.*) *delbrueckii* subsp. *bulgaricus* [4]. These bacteria produce bioactive compounds with nutritional and therapeutic value, such as antioxidants and antidiabetic agents [5]. Yoghurt naturally contains bioactive compounds produced during fermentation (such as probiotics and health-promoting metabolites). However, it is often not primarily recognized for these compounds compared to other fortified or enriched foods [6].

About 300 types of honey are well known (e.g., acacia, citrus, clover, honeydew, Majra, Manuka, Mountain Sidr, and Tualang honey), which are differentiated by their sources and seasonal and geographical origins [7]. Honey, as a natural sweetener, has gained significant attention in dairy processing, particularly in yoghurt production. It not only enhances the flavor of yoghurt but also offers several additional benefits, including antimicrobial, antioxidant, and preservative properties. It is composed mainly of fructose and glucose and contains bioactive compounds such as polyphenols, which contribute to its antioxidant and antimicrobial effects [2]. Therefore, many studies have focused on yoghurt fortified with honey due to its numerous human health benefits. For example, this combination has been shown to increase antioxidant activity [8].

The integration of honey into yoghurt products offers several potential advantages. Honey serves as a natural preservative, extending the shelf life of yoghurt by inhibiting the growth of harmful bacteria. It also improves the viability of probiotics and enhances the overall quality of fermented dairy products. Additionally, honey's water-binding properties, due to its high fructose content, can improve yoghurt texture, preventing syneresis (the separation of liquid from solid content) and contributing to a creamier consistency [2].

The aim of this study was to evaluate the application of the heather honey in the production of yoghurt and to analyse physicochemical and microbiological changes in experimentally prepared yoghurt samples during storage.

MATERIAL AND METHODS

Starter culture and heather honey

A commercial Bulgarian yoghurt starter culture containing *Streptococcus thermophillus* and *Lactobacillus delbrueckii* subsp. *bulgaricus* (1:1) (Genesis Laboratories Ltd., Bulgaria) was used. Starter cultures were in freeze-dried direct-to-vat set form and stored at -18°C until used. Heather honey (Volansky Honey, Slovakia) was obtained from an online market.

Yoghurt sample preparation

Yoghurt samples were prepared from fresh cow's milk pasteurised and standardised at the dairy plant. The production was performed at the Dairy Technology Laboratory of the Department of Food Hygiene, Technology and Safety, University of Veterinary Medicine and Pharmacy in Košice, Slovakia. Prior to yoghurt production, physicochemical analysis of the raw milk was performed. Three distinct yoghurt sample groups were evaluated. The control group consisted of conventional yoghurt inoculated with a standard starter culture without honey fortification. Two experimental treatments were prepared by incorporating heather honey at concentrations of 2.5% and 5.0% (w/w) post-fermentation.

For yoghurt manufacture, fresh milk was thermally treated at 90 °C for 30 min, subsequently cooled to 40 °C, and inoculated with the yoghurt starter culture. The inoculated milk was transferred into glass containers and incubated at 35 °C until complete coagulation was achieved, corresponding to a target pH of 4.5. Following incubation, the base yoghurt was stored at 4 °C for 24 h.

The experimental samples were then formulated by blending the matured yoghurt with either 2.5% or 5.0% (w/w) heather honey. Both control and fortified yoghurt samples were maintained under refrigeration at 4 °C. Analytical assessments were conducted on the initial day (Day 0) and after 15 and 22 days of cold storage.

Physicochemical Determination

The milk temperature, fat content, solids non-fat (SNF), density, protein, lactose, added water, freezing point, pH, and mineral salts of the six milk samples (40 ml each) were measured using an ultrasonic milk analyser (Lactoscan MCCW Milk Analyzer, Bulgaria).

The milk used for experimental yoghurt production had an average temperature at 18.30 ± 2.49 °C, fat content of 3.43 ± 0.03%, SNF of 8.65 ± 0.06%, density of 1.03 ± 0.00 g/cm^3, proteins of 3.17 ± 0.03%, lactose of 4.76 ± 0.04%, added water of 0.00%, freezing point of -0.55 ± 0.00 °C, pH of 6.22 ± 0.08 and mineral salts of 0.72 ± 0.01%.

The dry matter (DM) content of yoghurt samples was determined using moisture analyser (Radwag MA50/1, Poland). It was determined in three replicates as the average moisture content, each time by drying a sample of around 2.5 g at a temperature of 102 ± 2 °C.

Titratable acidity (TA) was determined according to the Soxhlet-Henkel method [9]. The titration of a 25 g yoghurt sample was performed in the presence of a phenolphthalein indicator until a light pink colour developed. The value was expressed as an average of three measurements as a percentage of the lactic acid.

The water-holding capacity (WHC) was assessed using the modified centrifugation method described by Saffon et al. [10]. Yoghurt samples were weighed to approximately 30 g into centrifugation tubes (volume 50 mL) and centrifuged using a Jouan BR4 Refrigerated Centrifuge (Jouan Technology for Life, USA) at a temperature of 4 ± 2 °C and 3500 rpm for 5 min. The difference between the weight of the sample in the centrifuge tube before and after centrifugation represented the amount of the separated supernatant, expressed as a percentage. The measurement was performed three times for each sample.

Antiradical activity (AA) was determined by 2,2-diphenyl-1-picryl-hydrazyl (DPPH) inhibition by the modified methods of Brand-Wiliams et al. [11]. Yoghurt samples (10 g) were weighed, and 30 ml of ethanol was added and properly mixed and centrifuged using a Jouan BR4 Refrigerated Centrifuge (Jouan Technology for Life, USA) at a temperature of 4 ± 2 °C and 3500 rpm for 5 min. Ethanol extracts prepared from yoghurts were homogenized with DPPH solution. This mixture was incubated in the dark at room temperature for 30 min. The absorbance was measured at 517 nm using a spectrophotometer, and distilled water was used as a blank. The DPPH free radical scavenging ability was then determined as the percentage inhibition of DPPH free radicals.

The water activity (a_w) was determined with the LabMaster a_w Sprint instrument (Novasina Division, Switzerland) according to ISO 21807 [12].

Microbiological examination

A stock suspension and subsequent 10-fold dilutions were prepared according to ISO 6887-1 [13]. The prepared 10-fold dilutions were used in the subsequent microbiological cultivation sample examinations.

To determine the number of coliform organisms, the stock and 10-fold dilutions were spread on the surface of Endo agar (HiMedia Laboratories, India), and the plates were incubated in a thermostat at 37 °C for 24 hours, respectively.

The number of staphylococci from the examined yoghurt samples was obtained according to ISO 6888-1 [14] using Baird-Parker selective diagnostic medium. The inoculated plates were incubated at 37 °C for 24 h.

To determine the counts of lactic acid bacteria (LAB) on the surface of the selective diagnostic medium of De Man, Rogosa, and Sharpe (MRS, Hi-Media, India), the spread plate method was used. The inoculated plates were then incubated under anaerobic conditions at 37 °C for 48 h using AnaeroGen (Oxoid, UK).

The determination of the number of yeasts and moulds (YM) was done according to ISO 21527-1 [15], using the spread plate method and Dichloran Rose-Bengal Chloramphenicol (DRBC) agar (Hi-Media, India). The inoculated plates were incubated at 25 °C for 5−10 days. After incubation, the counts of yeasts and moulds were obtained and converted to the amount per 1 g of the sample.

Detection of the number of bacteria of the family *Enterobacteriaceae* (EB) was performed according to STN EN ISO 21528-1 [16] using the selective diagnostic medium Violet Red Bile Lactose Agar (VRBL; HiMedia, India).

The counts of enterococci (EC) were determined by cultivation on Slanetz-Bartley agar (HiMedia, India) at 37 ± 1 °C for 48 h.

The results of microbiological variable measurements were converted to decadic logarithms ($\log_{10}$) to approximate the data in order to apply statistical testing methods.

Statistical analysis

The results obtained in this experiment were expressed as mean ± standard deviation (SD). Analysis of variance (ANOVA) and Tukey's test for multiple comparisons of means at a significance level of $p < 0.05$ were carried out via the R-statistics software [17]. The effects of 2.5% and 5.0% heather honey additions, and storage periods were set as the main factors of the statistical evaluation. Multiple factor analysis (MFA) was conducted in R-statistics software with the "FactomineR" [18] and "Factoextra" packages [19] according to Semjon et al. [20].

In the presented experiment, the following hypotheses were tested:

- Hypothesis 1: There exist differences in physicochemical and microbial variables in yoghurt samples with the heather honey addition in two concentrations (2.50% and 5.00%).
- Hypothesis 2: Observed variables change during storage for 15 and 22 days at 4 ± 2 °C.
- Hypothesis 3: Statistically significant correlations exist between individual measured variables.

RESULTS

The results of physicochemical determinations are presented in Table 1. Statistically significant differences were observed in physicochemical variables between control and experimental groups (honey 2.50% and honey 5.00%) in each variable (DM, TA, WHC, AA) ($p < 0.001$), except for the a_W variable, where honey addition did not influence water activity of the samples. On the initial day of the experiment, the lowest dry matter content was observed in the control group. The heather honey addition increased DM of the experimental yoghurt samples. However, the highest lactic acid concentration during the storage period was observed on the initial day of storage in the control group ($p > 0.05$). Based on the measured results, the lowest WHC during this experiment was observed in the control group. Adding heather honey at both concentrations (2.50% and 5.00%) increased the WHC of the experimental yoghurt samples by more than 10%. Antiradical activity increased with the higher concentration of heather honey addition. However, a significant effect of honey concentration was observed exclusively on the initial day of storage ($p < 0.001$). The highest AA was observed in yoghurt samples with a 5.00% heather honey addition during the whole experiment.

Bacterial counts were converted to logarithmic form (log10) and are listed in Table 2. The main factor of heather honey addition to yoghurt samples showed statistical significance at level $p < 0.001$ in microbiological variables: LAB, YM and EC. Storage period influenced these microbiological variables significantly ($p < 0.001$), except for the presence of enterococci ($p > 0.05$). Microbiological analysis revealed that the control and both experimental samples were below the limit of detection, respectively, the coliform organisms (including faecal coliform and *E. coli*) and staphylococci ($< 1.0 \times 10^2$ CFU/g). Samples stored at 4 ± 2 °C for 15 days showed the highest enumeration of viable lactobacilli, reaching a maximum of 8.88 ± 0.18 log CFU/g in the control group. Compared to experimental groups, the control samples exhibited significantly lower retention of viable lactobacilli during the storage period than experimental samples with 2.50% and 5.00% heather honey additions. The results of yeasts and mould determination in yoghurt samples showed that the average presence ranged from 2.88 to 3.89 log CFU/g on the initial day of storage. During the storage, the yeasts and mould count significantly decreased, but in the control group, their presence of 2.40 and 2.82 log CFU/g was observed in samples on 15 and 22 days of storage, respectively. The highest count of enterococci was observed in the control group after 22 days of storage.

Table 1. Physicochemical results of experimental yoghurt samples with heather honey addition

Sample	Day	DM (%)	TA (% LA)	WHC (%)	AA (% DPPH in.)	a_w
Control	0 (1)	14.01 ± 0.18^{cde}	1.33 ± 0.02^{a}	78.45 ± 1.50^{e}	47.76 ± 3.07^{h}	0.87 ± 0.00^{a}
Honey 2.50%		16.92 ± 1.52^{a}	1.19 ± 0.02^{f}	93.31 ± 1.53^{bc}	53.01 ± 0.84^{f}	0.87 ± 0.00^{a}
Honey 5.00%		16.37 ± 0.35^{ab}	1.25 ± 0.04^{cde}	92.09 ± 0.64^{c}	61.48 ± 0.15^{c}	0.87 ± 0.00^{a}
Control	15	13.67 ± 0.12^{de}	1.20 ± 0.01^{ef}	81.35 ± 1.48^{de}	48.03 ± 6.17^{g}	0.87 ± 0.00^{a}
Honey 2.50%		14.36 ± 1.22^{cd}	1.33 ± 0.02^{a}	100.04 ± 6.47^{a}	58.52 ± 1.16^{d}	0.87 ± 0.00^{a}
Honey 5.00%		17.25 ± 0.13^{a}	1.22 ± 0.00^{def}	97.63 ± 0.17^{ab}	62.28 ± 1.04^{a}	0.87 ± 0.00^{a}
Control	22	13.06 ± 0.27^{e}	1.30 ± 0.01^{abc}	85.89 ± 2.17^{d}	57.38 ± 0.76^{e}	0.87 ± 0.00^{a}
Honey 2.50%		15.13 ± 0.01^{bc}	1.30 ± 0.00^{ab}	97.02 ± 0.00^{ab}	60.38 ± 0.38^{d}	0.87 ± 0.00^{a}
Honey 5.00%		17.54 ± 0.07^{a}	1.27 ± 0.05^{bcd}	97.37 ± 0.34^{ab}	61.49 ± 1.91^{b}	0.87 ± 0.00^{a}
p - value	SA	$p < 0.001$	$p < 0.001$	$p < 0.001$	$p < 0.001$	$p > 0.05$
	ST	$p < 0.05$	$p < 0.001$	$p < 0.001$	$p < 0.001$	$p > 0.05$
	SA x ST	$p < 0.001$	$p < 0.001$	$p < 0.05$	$p < 0.001$	$p < 0.001$

DM, dry matter; TA, titratable acidity; LA, lactic acid; WHC, water-holding capacity; AA, antiradical activity; in., inhibition; a_W, water activity; SA, the main factor of heather honey addition; ST, the main factor of the storage period; SA x ST, interaction between main factors. Means in the column with a different superscript letter ($^{a-h}$) are statistically different (Tukey's, $p < 0.05$).

Table 2. The microbiological culture examination of yoghurt samples (log CFU/g mean ± SD).

Sample	Day	COLI	STAP	LAB	YM	EB	EC
Control	1	$< 1.0 \times 10^2$	$< 1.0 \times 10^2$	7.30 ± 0.18^{d}	2.88 ± 0.14^{c}	$< 1.0 \times 10^2$	3.33 ± 0.16^{a}
Honey 2.50%		$< 1.0 \times 10^2$	$< 1.0 \times 10^2$	7.73 ± 0.16^{c}	3.89 ± 0.12^{a}	$< 1.0 \times 10^2$	3.34 ± 0.12^{a}
Honey 5.00%		$< 1.0 \times 10^2$	$< 1.0 \times 10^2$	6.54 ± 0.18^{e}	3.35 ± 0.14^{b}	$< 1.0 \times 10^2$	3.02 ± 0.17^{c}
Control	15	$< 1.0 \times 10^2$	$< 1.0 \times 10^2$	8.88 ± 0.18^{a}	2.40 ± 0.08^{d}	$< 1.0 \times 10^2$	3.30 ± 0.09^{ab}
Honey 2.50%		$< 1.0 \times 10^2$	$< 1.0 \times 10^2$	8.69 ± 0.18^{ab}	$< 1.0 \times 10^{2e}$	$< 1.0 \times 10^2$	3.22 ± 0.18^{abc}
Honey 5.00%		$< 1.0 \times 10^2$	$< 1.0 \times 10^2$	8.52 ± 0.18^{b}	$< 1.0 \times 10^{2e}$	$< 1.0 \times 10^2$	3.03 ± 0.16^{c}
Control	22	$< 1.0 \times 10^2$	$< 1.0 \times 10^2$	8.73 ± 0.18^{ab}	2.82 ± 0.12^{c}	$< 1.0 \times 10^2$	3.40 ± 0.10^{a}
Honey 2.50%		$< 1.0 \times 10^2$	$< 1.0 \times 10^2$	8.63 ± 0.18^{ab}	$< 1.0 \times 10^{2e}$	$< 1.0 \times 10^2$	3.24 ± 0.11^{abc}
Honey 5.00%		$< 1.0 \times 10^2$	$< 1.0 \times 10^2$	8.43 ± 0.18^{b}	$< 1.0 \times 10^{2e}$	$< 1.0 \times 10^2$	3.05 ± 0.03^{bc}
***p* - value**	SA	*n.a.*	*n.a.*	$p < 0.001$	$p < 0.001$	*n.a.*	$p < 0.001$
	ST	*n.a.*	*n.a.*	$p < 0.001$	$p < 0.001$	*n.a.*	$p > 0.05$
	SA x ST	*n.a.*	*n.a.*	$p < 0.001$	$p < 0.001$	*n.a.*	$p > 0.05$

n.a., not applicable; COLI, coliforms; STAP, staphylococci; LAB, lactic acid bacteria; YM, yeasts and moulds; EB, *Enterobacteriaceae*; EC, enterococci; SA, the main factor of heather honey addition; ST, the main factor of the storage period. SA x ST, interaction between main factors. Means in the column with a different superscript letter ($^{a-d}$) are statistically different (Tukey's, $p < 0.05$).

The MFA method was applied to the measurements of the physicochemical and microbiological analyses of the experimental yoghurt samples in order to evaluate the main factors of the presented study (effect of honey addition and storage period). The analysis extracted the most significant variables with a minimum loss of information, where Kaiser's criterion (eigenvalue > 1) was applied to determine the number of final dimensions from the initial ones [21]. The results of the MFA extracted four components of the experimental yoghurt samples that explained 88.11% of the total variation in the data set. The first dimension (Dim1) explains 32.89% of the variation, dimension 2 (Dim2) 26.78%, dimension 3 (Dim3) 14.72%, and dimension 4 (Dim4) explains 13.72% (Figure 1). The contribution of the analysed data in Dim1-related physicochemical variables (35.27%, $r = 0.94$). The following physicochemical variables correlated significantly in Dim1: a_w ($r = 0.78$), DM ($r = -0.72$), WHC ($r = -0.85$) and AA ($r = -0.87$). Microbial variables such as LAB ($r = 0.95$), EC ($r = 0.31$) and YM ($r = -0.62$) contributed significantly to Dim2 (43.40%, $r = 0.96$). The factor of the sample groups and storage period contributed mainly to Dim3 and Dim4 with 62.44% ($r = 0.84$) and 74.50% ($r = 0.88$), respectively.

The combined graph of individuals (Figure 1) shows representations of individuals in which the individuals' sample ellipses are plotted separately (sorted according to the main analysed factor experimental group of samples). The experimental yoghurt samples analysed on days 15 and 22 of storage at 4 ± 2°C were plotted overlapped, which means that they were very similar in their physicochemical and microbial characteristics. On the other hand, the control yoghurt samples on the initial day of the experiment differed in the analysed variables. Based on the MFA results, the main analysed factors of the presented study, the heather honey addition and storage period, significantly affected the properties of the experimental yoghurt samples ($p < 0.001$).

DISCUSSION

The dry matter content was significantly increased in samples with honey addition in both concentrations ($p < 0.001$). Tamime and Robinson [22] report that the presence of the soluble carbohydrates leads to an increase in the total dry matter of yoghurt. DM on the first day of storage was 14.01% in the control sample, 16.92% (experimental group with 2.50% of the heather honey addition), and 16.37% in the experimental group with 5% honey. According to McSweeney and Fox [23], fermentation leads to changes in the casein network that can affect the distribution of free and bound water; therefore, these changes are crucial for the formation of the texture and rheological properties of fermented dairy products.

Anwar, Faiz, and Hou [2] report that introducing honey prior to fermentation enhances the metabolic activity of bacterial cultures due to the additional fermentable sugars,

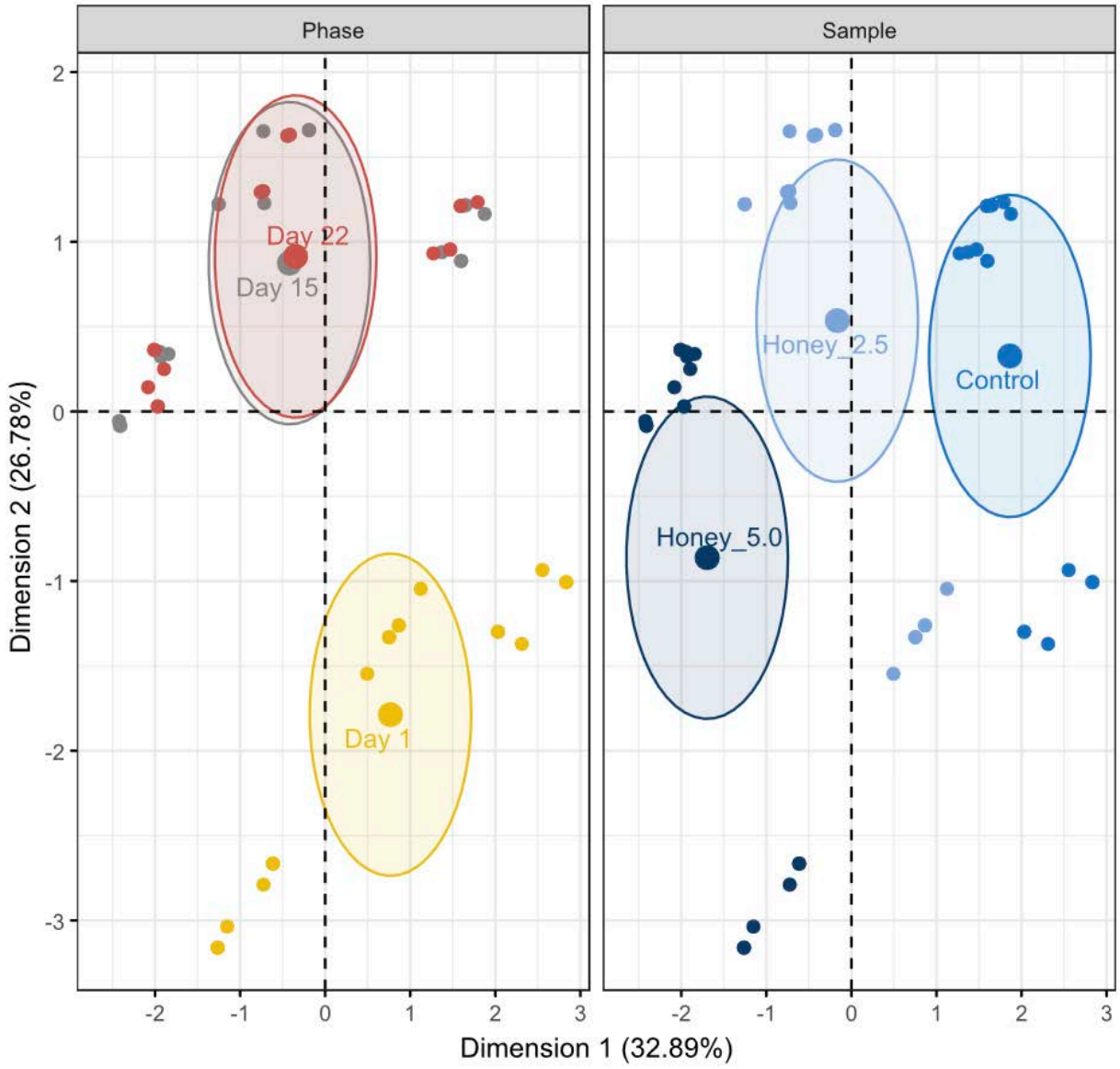

Fig. 1. A combined graph of individual samples with the storage period and experimental group of yoghurt samples as main factors

ultimately leading to higher acidity in the final yoghurt. In contrast, based on our results, incorporating heather honey after fermentation initially led to a decrease in yoghurt acidity on the first day of the storage period, regardless of the concentration used (Table 1).

Experimentally prepared yoghurts were heated during manufacture at temperatures that induce the denaturation of whey proteins (heat-labile), so that they precipitate and coagulate along with casein micelles (heat-stable) on acidification [24]. This step is essential to ensure the yoghurt gel stability, and thus, no major syneresis was observed while the sweetened yoghurts were refrigerated in the containers [25]. Authors Mohan et al. [25] observed statistical difference ($p > 0.05$) in the WHC values among the sweetened yoghurt types when tested at weekly intervals. However, our results of the WHC variable showed a significant effect of honey addition and storage period ($p < 0.001$). Higher WHC values for sunflower honey yoghurts analysed by Sert et al. [26] showed increasing WHC with the concentration of honey (2–6% *w*/*v*).

Honey can have an antioxidant effect in dairy products. Honey contains various antioxidant compounds, including flavonoids, polyphenols, and ascorbic acid. Combining honey with dairy products may result in a synergistic effect on antioxidant activity [27]. Anwar et al. [2] observed that the antioxidant activity of blended yoghurt increased with higher honey concentrations, reaching 42.36 ± 0.01 at 5% honey concentration. This is in consideration of our presented results (Table 1). On the first day of the sample's storage, the experimental yoghurt sample group with

5.00% of the heather honey addition reached 61.48% antiradical activity. During this experiment, the yoghurt samples with 5.00% honey concentration had the highest AA on 1, 15 and 22 days. The antioxidant potential of dairy products is related to the quality of the raw material and, primarily, the presence and activity of natural bioactive compounds in milk [28]. The bacterial cultures also have a high impact on the value of the antioxidant potential [29]. Milk fermentation with lactic acid bacteria contributes to the supply of a huge number of bioactive peptides and free amino acids with diverse biological activities [30]. It was noted that the antioxidant activity increased slightly during storage, even in the control group of yoghurts. This may be due to the slow fermentation process during storage or the increasing number of LAB microorganisms, whose numbers have also risen.

Sert et al. [26] reported that 4–6% honey-added yoghurts had maximum survival of *L. bulgaricus*. However, Roumyan et al. [32] studied the effect of acacia honey and polyfloral honey and found a significant inhibition in the growth of *L. bulgaricus* in honey-added Bulgarian yoghurt. On the other hand, Varga [33] noted that the yoghurt with the addition of honey in the amount of 1.0% to 5.0% did not significantly influence the survival of the starter bacteria in yoghurt during storage at 4 °C. Metry Wedad and Oways [34] determined that the *L. bulgaricus* count in a yoghurt sample with 4% honey decreased from the beginning to the 9th day of storage. Ustunol and Gandhias [35] demonstrated that adding honey at a concentration of 5% in yoghurts is optimal for the growth of probiotics and starter culture bacteria and for their survival during the refrigerated storage period. To provide a precise explanation of the kinetics of microorganism growth and their contribution to physicochemical properties, including antioxidant activity, an in-depth kinetic study of the fermentation process will be mandatory for this kind of study, and moreover, with the identification of microorganisms.

CONCLUSION

The study demonstrates the benefits of adding heather honey to improve the quality of yoghurt made from cow's milk. It provides a comprehensive view of the physicochemical and microbial quality parameters of the experimentally prepared yoghurt. The results showed that yoghurt with heather honey addition is an effective and natural ingredient for enriching probiotic dairy products, such as yoghurt, for at least 22 days of storage. It can be concluded that yoghurt with a 5.00% heather honey addition appears to be the more suitable option than 2.50% for its antiradical activity and better water-holding capacity, which are related to its texture. Honey components and antioxidant properties, which vary by type, enhance the antioxidant properties of the yoghurt. To ensure consumer satisfaction, additional longitudinal sensory testing and testing across several honey types could be conducted to define the exact endpoint of consumer acceptance and honey benefit properties of enriched yoghurts during storage.

Data Availability Statement

The raw data of this article will be made available by the authors, without undue reservation.

Ethical Statement

Ethical approval was not necessary for this work.

Conflict of Interest

The authors have no conflict of interest to declare.

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Authors' Contributions

Conceptualization: B.S., E.K.; Methodology: B.S., Z.M.E., I.R.; Field data and Sampling: B.S., E.K.; Data analysis: B.S.; Resources and Supervision: B.S., Z.M.E., I.R.; Writing-original draft preparation: B.S., E.K.; Writing-review and Editing: Z.M.E., I.R.; All authors read and approved the published version of the manuscript.

Generative AI Statement

No generative artificial intelligence (AI) and AI-assisted technologies were used in writing the manuscript.

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ORIGINAL ARTICLE

EFFECT OF SKIMMED MILK POWDER ADDITION ON SELECTED QUALITY PROPERTIES OF FRESH CHEESES AND NATURAL YOGURTS

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ABSTRACT

Fermented dairy products, due to their nutritional properties, are suitable matrices for further enrichment. This work examined the effect of skimmed milk powder (SMP) addition (0, 3, 6, and 9%) on selected physicochemical, rheological, and organoleptic properties of fresh cheeses and natural yogurts. With increasing SMP content, protein content increased in both products, while fat content decreased. In cheeses, this trend was reflected in increased hardness. In yogurts, higher protein and total solids content led to a significant increase in viscosity (from 1.070 to 3.714 mPa·s). A strong correlation was observed between total solids and viscosity. SMP affected the acidity of both products and the colour of the cheeses, especially yellowness. Sensory evaluation showed that the addition of SMP significantly affected the organoleptic properties (consistency and taste) of both products. Optimal consumer acceptance was achieved at 3–6% in cheeses and 6–9% in yogurts. Further research could focus on the impact of SMP on shelf-life, specific textural characteristics, and consumer acceptance across a broader range of formulations.

Keywords: chemical composition; fresh cheeses; organoleptic properties; protein enrichment; skimmed milk powder; texture; viscosity; yogurts

INTRODUCTION

Milk and dairy products are important components of a balanced diet because they provide essential nutrients. Low consumption of these products during childhood and adolescence can lead to insufficient intake of calcium, magnesium, and iodine, which may negatively affect physical development and bone strength [1–3]. Moreover, fermented dairy products (yogurts, various fermented milks, and cheeses) are an excellent source of probiotics, prebiotics, and bioactive compounds. They offer additional health benefits, such as modulating the gut microbiota, improving intestinal health, and increasing lactose tolerance [4, 5].

This group of foods is also suitable for fortification with other nutritionally desirable components [6, 7]. These components (e.g., vitamin D and antioxidants), which are

often insufficient in the typical consumer diet, play an important role in the proper functioning of the body and enhance the attractiveness of enriched products [4, 8, 9].

The typical way to enrich dairy products is to increase their protein content. Proteins are fundamental nutrients and are important building blocks of cells and muscle tissue [10, 11]. Milk proteins, such as casein and whey proteins, are characterized by high biological value and a complete profile of essential amino acids [10, 12, 13].

In cheeses, increasing the protein content can be achieved by various methods, such as heating milk (above 75 °C), membrane processes (e.g., ultrafiltration), treatment with high hydrostatic pressure (e.g., 400 MPa for 30 min, which can denature up to 90% of whey proteins), ultra-high pressure homogenization (above 100 MPa), or the addition of milk protein concentrates, including skimmed milk powder (SMP), casein concentrates, and whey proteins, as well as buttermilk and its derivatives [14–17].

The protein content of yogurt can be increased prior to fermentation by the addition of dairy powders, evaporation, or membrane filtration. It can also be enhanced after fermentation through straining, mechanical separation, or membrane filtration [7, 10, 12, 18].

Although SMP is commonly used to increase the protein content and improve the texture of fermented dairy products, particularly yogurts [7, 19, 20], limited information is available on the effects of different levels of SMP addition on selected product properties. The optimal level of SMP addition that enhances the nutritional value of both fresh cheeses and natural yogurts while maintaining desirable technological and sensory characteristics has not yet been clearly established. Therefore, the aim of this study was to produce fresh cheeses and natural yogurts with varying SMP levels and to evaluate their physicochemical, technological, and sensory properties.

MATERIALS AND METHODS

Preparation of milk, cheese, and yogurt samples

In two experiments, fresh cheeses and natural yogurts were prepared from cow's milk enriched with different levels of SMP under laboratory conditions. The chemical composition of the SMP was analysed by the supplying dairy company and was as follows: fat, 0.30%; protein, 34.30%; lactose, 50.0%; moisture, 3.53%; and pH, 6.54.

Raw milk (12 l) was pasteurized in the mini pasteurizer FJ 16 (Milky, Farmland, Janschitz GmbH, St. Marienkirchen bei Schärding, Austria) at 72 °C for 20 s, then cooled and enriched with 3, 6, and 9% SMP. The control sample without SMP addition was also prepared. The composition of the raw milk and pasteurized milk samples is given in Table 1.

Fresh cheese samples. Pasteurized milk samples (0, 3, 6, and 9% SMP) were heated to the coagulation temperature (32 °C). A starter culture (10% mesophilic culture; Laktoflora®, MILCOM a.s., Czech Republic) was added to the milk. After 15 min, 0.01% calcium chloride (36%) and 0.01% rennet (NATUREN®, Christian Hansen, NOVONESIS, Lyngby, Denmark) were added. Coagulation was carried out at 32 °C for 45 min. The developed curd was cut into cubes approximately 1 × 1 cm. Then, the curd

Table 1. Composition of raw and pasteurized milk samples enriched by different levels of skimmed milk powder (SMP)*

Parameters[1]	Raw milk	Pasteurized milk				P-value
		0% SMP	3% SMP	6% SMP	9% SMP	
Fat (%)	3.35±0.14	3.34±0.18	3.24±0.19	3.11±0.20	2.99±0.17	ns
Protein (%)	3.26±0.05	3.28±0.06	4.26±0.06	5.27±0.08	6.28±0.12	***
Casein (%)	2.62±0.03	2.64±0.06	3.80±0.06	5.00±0.11	6.18±0.18	***
Lactose (%)	5.00±0.09	5.03±0.06	6.61±0.09	8.24±0.09	9.89±0.10	***
SNF (%)	9.02±0.12	9.05±0.10	11.7±0.10	14.5±0.13	17.2±0.09	***
Total solids (%)	12.4±0.13	12.4±0.22	15.0±0.17	17.6±0.18	20.2±0.11	***
SCC (×10^3 cells/ml)	272±73.2	281±74.6	313±78.1	336±80.7	358±93.3	ns
FPD (°C × -1 000)	532±9.87	530±7.94	695±12.6	859±12.2	1018±12.7	***

* Values are presented as the mean ± standard deviation; * – $P < 0.05$; ** – $P < 0.01$; *** – $P < 0.001$; ns – not significant;
[1] SNF – solids-not-fat; SCC – somatic cell count; FPD – freezing point depression

grains were stirred manually for approximately 25 min and subsequently transferred into conical plastic molds (top ∅ 95 mm, bottom ∅ 75 mm, height 70 mm). From each group (0, 3, 6, and 9% SMP), four cheese samples were prepared. Fermentation proceeded at room temperature (21 °C) until the following day (approximately 18 h), during which the cheese samples were turned twice.

Natural yogurt samples. Pasteurized milk samples (0, 3, 6, and 9% SMP) were heated to the fermentation temperature (42 °C), inoculated with a starter culture (5% yogurt culture; YC-380, Christian Hansen, NOVONESIS, Lyngby, Denmark), and thoroughly mixed. From each group (0, 3, 6, and 9% SMP), four yogurt samples were prepared. Fermentation was carried out at 42 °C for 4 h. The process was terminated at the specified time in accordance with ON 57 0534 [21] to ensure identical conditions for all samples.

Both cheese and yogurt samples were subsequently stored at 4 ± 1 °C until analyses. All analyses were performed within 24 h after the completion of cheese and yogurt preparation.

Analysis of milk, cheese, and yogurt samples

Milk samples (n = 2). The chemical composition (fat, protein, casein, lactose, and solids-not-fat contents (%)), somatic cell counts ($\times 10^3$ cells/ml), and freezing point depression (°C × -1 000) were determined using Fourier transform mid-infrared spectroscopy. The analyses were performed using a Combi Foss™ FT 6000 instrument (Foss Electric, Denmark), comprising a MilkoScan 6000 analyzer and a Fossomatic™ 5000 flow cytometer.

Cheese and yogurt samples (n = 4). The chemical composition (fat, protein, lactose, and total solids contents; %) was determined using near-infrared spectroscopy with Fourier transformation (NIR MasterTM N500; Büchi, Switzerland) and NIRWare version 1.5, according to the manufacturer's instructions. Approximately 30 g of homogenized sample was placed in a Petri dish and analysed.

Active acidity was measured using a pH meter equipped with a combined electrode (Fisher Scientific) and reported in pH units. Titratable acidity was determined according to the Soxhlet-Henkel method and expressed in SH units (ml of 0.25 M NaOH required to titrate 100 g of cheese or yogurt to a standard pink colour) as well as in mmol/l according to the equation: titratable acidity (mmol/l) = SH × 2.5, where SH = SH units and 2.5 = coefficient.

The colour of cheese and whey samples was measured using a ColorEye XTH spectrophotometer (Gretag Macbeth, New Windsor, NY, USA). Measurements were performed on 50 g portions of the sample. The results were expressed as L* (lightness), a* (red–green axis), and b* (yellow–blue axis) values.

The hardness of the cheese samples was analysed using the TA.XT Plus texture analyser (Stable Micro-Systems, Godalming, UK). First, the shape of the cheese samples was standardised (blocks of 75 × 40 × 25 mm). The analyser was equipped with a knife probe that cut the samples in half, and the maximum force (kg) required for cutting was recorded. The following settings of the texture analyser were used: pre-test speed: 3 mm/s; test speed: 2 mm/s; and post-test speed: 10 mm/s. A knife probe used for cutting force analysis was a common approach in food measurement and processing, e.g., as generally described by Brown et al. [22]. Moreover, it was one of the described approaches recommended by the manufacturer of the texture analyser.

The viscosity in mPa·s of yogurt samples was measured with a HAAKE Viscotester 6L/R plus viscometer (Thermo Electron (Karlsruhe) GmbH, Karlsruhe, Germany) with an R5 spindle at 50 rpm and a sample temperature of 20 ± 2 °C (R4 for a sample containing 0 and 3% SMP). Enumeration of lactic acid bacteria in yogurt samples was performed using the standard plate method [23]. A ten-fold series dilution was prepared using 10 g of the sample and 0.9% sterile saline solution. For bacterial enumeration, 1 ml of an aliquot was inoculated on MRS agar for *Lactobacillus delbrueckii* subsp. *bulgaricus* (incubated anaerobically at 37 °C for 48 hours) and on M17 agar for *Streptococcus salivarius* subsp. *thermophilus* (incubated aerobically at 37 °C for 48 hours). The results were expressed as the logarithm of colony-forming units per gram of sample (log CFU/g).

Sensory analysis

The sensory evaluation of both products was conducted by a panel of 10 assessors with a mean age of 42.7±10.8 years (range: 29–57). The panel consisted of 50% men (mean age, 46.4±10.5 years) and 50% women (mean age, 39.0±10.9 years). The evaluation was carried out in accordance with the requirements and principles of sensory analysis [24, 25]. The intensity of individual descriptors (colour, aroma, consistency, acceptability, sour and sweet

taste intensity) for each sample was recorded on a 100-mm unstructured line scale.

Statistical analysis

Statistical analysis was performed using Statistica 12 software (StatSoft CR s.r.o.). The dataset was first tested to verify the assumptions for parametric methods (normality of data and homogeneity of variances). The effect of SMP addition was evaluated using one-way analysis of variance. Relationships among variables were assessed using correlation and regression analyses. Post hoc pairwise comparisons were performed using Tukey's HSD test, and Pearson's correlation coefficient (r) was used in the correlation analysis. Statistical significance was determined at the usual levels of significance (0.05, 0.01, 0.001).

RESULTS AND DISCUSSION

Properties of cheese samples

Table 2 shows the effect of SMP addition on the chemical composition, acidity, hardness, and colour of the cheeses. A clear trend was observed in protein and fat content: with increasing SMP levels, protein content increased (from 15.6 to 16.9%), while fat content decreased significantly (from 19.2 to 9.92%; $P < 0.001$). This trend corresponds to the well-known phenomenon whereby an increase in protein content leads to a relative decrease in fat content and changes in the protein-to-fat ratio in cheese [26].

Rogers et al. [27] reported that such compositional changes affect cheese microstructure. Although cheeses with reduced fat content align with current nutritional trends, fat remains a key component that substantially influences the rheological properties of cheese. According to Sharafi et al. [28], decreasing fat content results in increased hardness and springiness, while adhesiveness and cohesiveness decrease, which may be undesirable from a sensory perspective.

These findings were also confirmed in the present study. With increasing SMP content, the force required to cut the samples increased significantly from 2.52 kg to 5.41 kg ($P < 0.001$), indicating an increase in cheese hardness. This relationship was further supported by a positive correlation between hardness and protein content (Figure 1), with a moderate coefficient ($r = 0.5787$; $P < 0.05$). This correlation suggests that compositional changes are among the primary factors influencing the rheological properties of the products [26].

Table 2. Selected parameters of fresh cheese samples enriched with 0, 3, 6, and 9% skimmed milk powder*

Parameters		Levels of skimmed milk powder				P-value
		0%	3%	6%	9%	
Acidity	active (pH)	$4.61^{a}\pm0.13$	$4.64^{ab}\pm0.15$	$4.77^{bc}\pm0.11$	$4.81^{c}\pm0.11$	**0.0020**
	titratable (SH)	88.6±7.58	95.5±6.49	96.3±7.91	97.0±5.10	0.3262
Hardness (kg)		$2.52^{a}\pm0.51$	$3.41^{a}\pm0.94$	$4.55^{b}\pm1.17$	$5.41^{b}\pm0.98$	**<0.001**
Cheese colour	L*	89.8±7.17	89.2±7.59	89.2±6.09	88.7±5.83	0.9864
	a*	$-0.56^{b}\pm0.31$	$-0.60^{b}\pm0.62$	$-1.06^{ab}\pm0.35$	$-1.30^{a}\pm0.37$	**0.0008**
	b*	$9.52^{a}\pm0.82$	$10.0^{ab}\pm0.38$	$10.3^{ab}\pm0.63$	$10.7^{b}\pm0.88$	**0.0036**
Whey colour	L*	35.2±5.72	35.5±5.99	33.3±2.12	35.5±4.18	0.7390
	a*	-0.46±0.29	-0.69±0.36	-0.67±0.11	-0.74±0.15	0.1115
	b*	$-1.70^{b}\pm0.47$	$-2.21^{ab}\pm0.68$	$-2.73^{a}\pm0.41$	$-2.63^{a}\pm0.56$	**0.0011**
Lactose (%)		$2.17^{a}\pm0.38$	$2.82^{b}\pm0.30$	$3.12^{b}\pm0.42$	$3.31^{b}\pm0.41$	**<0.001**
Fat (%)		$19.2^{c}\pm2.53$	$14.8^{b}\pm0.29$	$12.0^{ab}\pm0.25$	$9.92^{a}\pm0.52$	**0.0001**
Protein (%)		15.6±1.31	16.0±0.71	17.2±0.16	16.9±0.59	0.0584
Total solids (%)		36.5±1.58	33.1±5.07	35.9±1.56	34.5±2.20	0.1817

* Values are presented as the mean ± standard deviation
^{a,b,c} Means with different superscripts in a row differ ($P < 0.05$)

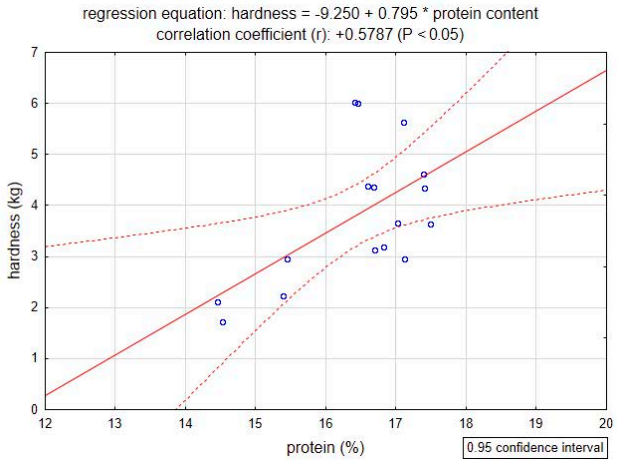

Fig. 1. The relationship between protein content and the hardness of fresh cheese samples

At the same time, a significant increase in lactose content was observed with increasing SMP levels (from 2.17 to 3.31%; $P < 0.001$). In contrast, SMP addition had no significant effect on total solids content. This may indicate that changes in individual components (protein, fat, lactose) partially compensated for each other, resulting in a relatively stable total solids content.

Regarding acid–base properties, increasing SMP levels led to a statistically significant increase in the cheeses' active acidity (from 4.61 to 4.81; $P < 0.01$). This trend is mainly associated with a higher lactose content, which serves as the primary substrate for lactic acid bacteria and thus significantly influences the acidification [29].

Colour measurements of cheese samples showed statistically significant differences in the a* ($P < 0.001$) and b* ($P < 0.01$) coordinates. For the b* coordinate, which describes the transition from yellow (positive values) to blue (negative values), a clear trend was observed: increasing SMP addition led to higher yellow colour intensity in the cheeses. A significant effect was also found for the b* coordinate of whey, where increasing SMP addition resulted in reduced yellow colour ($P < 0.01$). Results reported by Milovanovic et al. [30] further indicate that L* and b* values increase in milk powder.

Properties of yogurt samples

As in fresh cheeses, a similar trend in the fat and protein content was observed in yogurts (Table 3). With increasing SMP content, a significant increase in protein content was detected (from 3.50 to 6.08%; $P < 0.001$), accompanied by a significant decrease in fat content (from 3.58 to 2.96%; $P < 0.01$). In contrast to cheeses, yogurts with higher SMP levels also exhibited a significant increase in total solids content (from 11.6 to 19.7%; $P < 0.001$).

An increase in total solids content is positively associated with improved yogurt texture. Janoušek Honesová et al. [20] observed a positive effect of 10% SMP addition compared to 5%. Damin et al. [7] reported beneficial effects within the range of 12–14% total solids, while Tamime and Robinson [31] identified the most pronounced effects at 12–20%. The improvement in textural properties, together

Table 3. Selected parameters of natural yogurt samples enriched with 0, 3, 6, and 9% skimmed milk powder*

Parameters[1]		Levels of skimmed milk powder				P-value
		0%	3%	6%	9%	
Acidity	active (pH)	4.47±0.36	4.43±0.21	4.39±0.11	4.49±0.14	0.8948
	titratable (SH)	40.7^{a}±4.69	51.2^{b}±4.10	60.1^{c}±2.80	68.4^{d}±3.65	**<0.001**
Lactose (%)		3.13±0.94	2.40±1.01	3.22±1.71	3.13±1.60	0.7627
Fat (%)		3.58^{b}±0.02	3.54^{b}±0.13	3.03^{a}±0.39	2.96^{a}±0.19	**0.0026**
Protein (%)		3.50^{a}±0.05	4.33^{a}±0.46	5.56^{b}±0.11	6.08^{b}±0.92	**0.0001**
Total solids (%)		11.6^{a}±1.55	14.5^{ab}±1.75	16.4^{b}±1.77	19.7^{c}±1.38	**<0.001**
Viscosity (mPa·s)		1070^{a}±554	2174^{ab}±1087	3120^{b}±667	3714^{b}±1060	**0.0027**
ST (log CFU/mL)		8.51±0.36	8.60±0.13	8.64±0.30	8.84±0.07	0.2310
LB (log CFU/mL)		8.38±0.48	8.32±0.22	8.49±0.08	8.41±0.09	0.8058

* Values are presented as the mean ± standard deviation

[a,b,c] Means with different superscripts in a row differ ($P < 0.05$)

[1] ST – *Streptococcus salivarius* subsp. *thermophilus*; LB – *Lactobacillus delbrueckii* subsp. *bulgaricus*; CFU – colony-forming units

with increased protein and total solids content, is attributed to a denser protein network and reduced pore size, resulting in higher viscosity. For example, Damin et al. [7] showed that yogurts enriched with SMP exhibited a higher proportion of smaller pores within the gel structure. A strong influence of SMP addition on the rheological properties of yogurt has also been reported by Yu et al. [32].

A similar trend was confirmed in the present study: the viscosity of yogurt samples without SMP addition was 1.070 mPa·s, whereas the addition of 9% SMP increased the viscosity to 3.714 mPa·s. This increase can be attributed to the higher total solids and protein content provided by SMP, which improved water-binding capacity and promoted the formation of a stronger gel network [7, 12]. The strong relationship between total solids content and yogurt viscosity is further illustrated in Figure 2, which shows a high correlation coefficient ($r = 0.8634$; $P < 0.001$).

Active acidity did not differ significantly among the yogurt samples. Körzendörf and Hinrichs [33] reported that the optimal pH of high-protein yogurts ranges from 4.7 to 4.9, with higher pH values promoting greater viscosity and a more homogeneous structure, whereas lower pH values result in a more granular texture. In contrast, a significant effect of SMP addition on the titratable acidity of the yogurts was observed, with values increasing from 40.7 to 68.4 SH at higher levels of addition ($P < 0.001$).

The different trends in active and titratable acidity were also confirmed by other authors [7, 19, 20]. The discrepancy between pH and titratable acidity can be attributed to the buffering capacity of milk components, particularly proteins and minerals, which stabilizes the concentration of free hydrogen ions. Consequently, the total acid content may increase significantly without a proportional decrease in pH, leading to higher titratable acidity values despite a relatively constant pH.

Sensory evaluation of yogurt and cheeses

The study also included an evaluation of organoleptic properties (Figure 3). As expected, the most pronounced differences were observed in consistency for both cheeses and yogurts (both $P < 0.001$). Among the cheeses, the sample with 9% SMP addition received the highest scores for consistency and colour, but the lowest score for taste. This sample also exhibited the lowest perceived sour taste intensity, whereas the cheese without SMP addition (0%) showed the highest. These findings suggest that sour taste intensity plays a key role in consumer preference, as cheeses with 0% and 9% SMP were rated lowest in taste, while those with 3% and 6% SMP were rated most favourably.

In contrast, yogurt samples containing 6% and 9% SMP achieved the highest ratings for both taste and consistency. Unlike cheeses, the higher sour taste intensity in these samples was positively perceived. Additionally, these samples showed the lowest sweet taste intensity while receiving the highest taste scores, indicating that a combination of higher sour and lower sweet intensities may be preferred by consumers in natural yogurts.

The influence of protein enrichment on organoleptic properties and consumer preferences has also been reported in previous studies for cheese [34, 35] and yogurt [7, 20, 32], which demonstrate that the incorporation of milk proteins improves texture, viscosity, and overall sensory quality, key factors determining consumer acceptance.

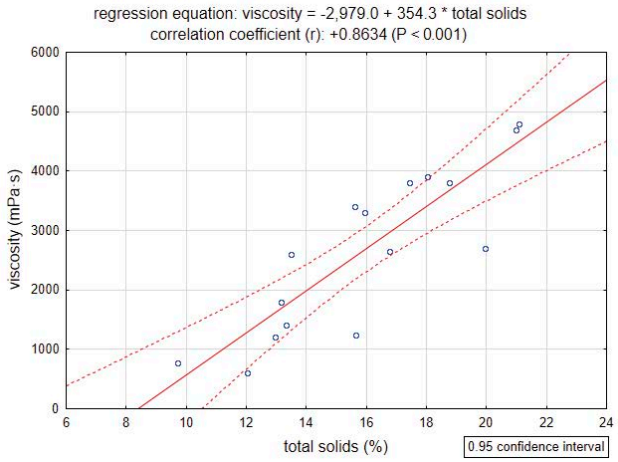

Fig. 2. The relationship between total solids content and the viscosity of natural yogurt samples

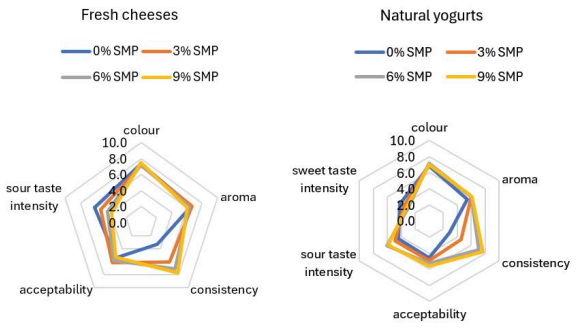

Fig. 3. Sensory profile of fresh cheese samples and natural yogurt samples enriched with 0, 3, 6, and 9% skimmed milk powder (SMP)

CONCLUSION

The study demonstrated a significant effect of SMP addition on the physicochemical and organoleptic properties of the evaluated dairy products. However, the optimal level of SMP addition differed between fresh cheeses and yogurts.

With increasing SMP content, both fresh cheeses and natural yogurts showed increased protein content accompanied by a relative reduction in fat content. In fresh cheeses, this trend was reflected in increased hardness, whereas in yogurts it resulted in increased viscosity.

Sensory evaluation confirmed that SMP addition affects the organoleptic properties of fermented dairy products. In fresh cheeses, samples containing 3% and 6% SMP were rated as the most acceptable, while those with 0% and 9% SMP showed lower consumer preference. In contrast, yogurt samples with higher levels of SMP addition (6% and 9%) were evaluated more favourably.

Based on these findings, it can be concluded that SMP addition is an effective approach to enhance the nutritional value, technological, and sensory properties of fermented dairy products. However, the optimal level of SMP addition should be adjusted based on consumer preferences associated with specific characteristics of each dairy product.

Data Availability Statement

The raw data supporting the findings of this study are available from the authors upon reasonable request.

Ethical Statement

Ethical approval was not required for this study.

Conflict of Interest

The authors declare that there is no conflict of interest.

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Generative AI Statement

No generative AI or AI-assisted technologies were used in the preparation of this manuscript.

Authors' Contributions

Simona Janoušek Honesová: writing – original draft, resources, conceptualization, writing – review & editing. **Eva Samková:** writing – original draft, writing – review & editing, methodology, investigation, conceptualization, funding acquisition, supervision. **Lucie Hasoňová:** methodology, investigation, conceptualization, writing – original draft, data curation. **Kristina Hradilová:** methodology, investigation, writing – original draft. **Tereza Janů:** writing – review & editing, resources. **Lada Švecová:** writing – review & editing, resources. **Eva Baldíková:** writing – review & editing, resources. **Jan Bedrníček:** methodology, investigation, conceptualization, writing – original draft, data curation.

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ORIGINAL ARTICLE

EVALUATION OF CHANGES IN MILK PRODUCTION AND QUALITY IN DAIRY COWS WITH MASTITIS DEPENDING ON ETIOLOGICAL AGENTS

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ABSTRACT

Mastitis negatively impacts both milk production and milk quality. The aim of this study was to evaluate changes in milk production and composition in dairy cows with mastitis (n = 111) compared to controls (n = 111) and to assess the effect of a specific mastitis pathogen on selected milk quality indicators. The most common pathogen was *Streptococcus uberis*. Most mastitis cases were detected in summer (35%), in dairy cows in their fourth and higher lactations (52%), at 101–200 days of lactation (37%). A statistically significant difference (p <0.001) was found between control and mastitis cows in milk yield (32.3 vs 28.4 kg), lactose content (5.01 vs 4.72%), somatic cell count (4.84 vs 5.96 log), and fat-to-lactose ratio (0.83 vs 0.91). The somatic cell count in mastitis caused by *Escherichia coli* (3,270,000/ml) was significantly higher than in mastitis caused by other pathogens (p <0.001). The lactose content was also lowest in *Escherichia coli* mastitis compared to other mastitis cases (4.45%; p <0.001). It was found that the influence of specific pathogens on selected milk parameters varies considerably. The results obtained may provide a basis for optimizing preventive and therapeutic strategies in dairy cattle breeding.

Keywords: dairy cow; mastitis; milk quality; milk yield; somatic cells

INTRODUCTION

Mastitis is a disease with many etiological causes related to the cattle production system and rearing environment. It manifests as various changes in mammary gland tissue [1, 2, 3], resulting from tissue inflammation and other defensive responses to the presence of microorganisms within the mammary gland [4]. Mastitis can be classified as subclinical or clinical. In both cases, there is a significant reduction in milk production [5] and a decline in milk quality, resulting in substantial economic losses. These losses are estimated at approximately €134 to €198 per cow with mastitis per lactation [6, 7, 8, 9]. Common mastitis pathogens include *Staphylococcus aureus, Streptococcus agalactiae, Streptococcus dysgalactiae, Streptococcus uberis,* and *Escherichia coli* [10, 11, 12, 13, 14, 15].

The somatic cell count (SCC) is likely the most important indicator in the diagnosis of mastitis; from a hygiene

perspective, it is also a key parameter for determining milk quality [16]. These are blood cells (specifically white blood cells—leukocytes) and, to a lesser extent, mammary gland epithelial cells, which are released into the mammary alveoli during milk production [17]. Lactose content is another sensitive indicator of mammary gland integrity in mastitis. Lactose content decreases from physiological levels to 4.01–4.51%, a phenomenon the authors attribute to a disruption of the so-called tight junctions between alveolar epithelial cells, which allow lactose to pass back from milk into the bloodstream [18, 19]. Mastitis is also associated with a decrease in casein content and an increase in whey protein concentration, particularly immunoglobulins (especially IgG) and serum albumin [20, 21]. Elevated levels of these proteins are considered part of the mammary gland's defense response, as immunoglobulins neutralize pathogens and promote phagocytosis, while albumin serves as a transport protein [22, 23].

During mastitis, there is a significant increase in the concentration of sodium (up to 40%) and chloride in milk, accompanied by a parallel decrease in potassium [20, 21]. However, some studies point out that sensitivity to individual minerals may differ [24].

Numerous scientific studies have shown that the bacterial pathogen responsible for mastitis significantly affects milk composition. The aim of this study was to compare the quality characteristics of milk from healthy dairy cows and dairy cows with mastitis caused by various bacterial pathogens.

MATERIALS AND METHODS

Selection of experimental groups of dairy cows

The study was conducted on a dairy farm (1,850 head of cattle, including 800 dairy cows) located in the Vysočina Region at an elevation of 566 meters above sea level. Milking takes place twice daily in a parallel milking parlor (2 × 16), and the milk is collected daily.

Data for evaluating the impact of mastitis on milk yield and milk quality parameters were collected from dairy cows with clinical mastitis over the course of one year. The cows met the following criteria: 1) purebred Czech Fleckvieh dairy cows, 2) only one pathogen was detected in the milk sample, specifically *Streptococcus uberis*, *Escherichia coli*, *Streptococcus dysgalactiae*, *Klebsiella pneumoniae*, 3) for each dairy cow meeting the above criteria, a "control" cow in the herd was also selected, matched for parity and stage of lactation, with a SCC of ≤135,000/ml.

A total of 111 cows with mastitis and 111 control cows were included in the study (Table 1).

Mastitis pathogen determination

Milk samples were aseptically collected from infected quarters of cows with clinical mastitis (oedema, redness, pain, changes in milk secretion and appearance, positive NK test results). The samples were analyzed by inoculation into three-part chromogenic culture media (ClearMilk test; LabMedia Servis, s.r.o.; the Czech Republic), which are selective for streptococci, staphylococci, and Gram-negative bacteria. Cultivation conditions were 37 °C for 24–48 hours. A farm-specific atlas was used to identify pathogens; it was compiled based on milk sample cultivation results and subsequent PCR identification of mastitis pathogens performed by the Veterinary Research Institute Brno. For this study, the four most frequently identified pathogens were used.

Milk yield and quality indicators

Data on milk yield and milk quality parameters were evaluated based on the results of performance testing. Milk quality analyses were conducted at the ČMSCH,

Table 1. Characteristics of Czech Fleckvieh dairy cows selected for the study

Group of dairy cows	n	Parity		Days in milk	
		X	S_x	X	S_x
Control	111	3.7	2.0	143	90
Mastitis	111	3.7	2.0	142	87
of which:					
Streptococcus uberis	59	3.3	2.0	139	97
Escherichia coli	18	3.7	1.7	134	73
Streptococcus dysgalactiae	15	3.8	2.2	134	74
Klebsiella pneumoniae	19	4.7	2.2	164	82

a.s. laboratory in Brno-Tuřany. The following parameters were recorded: milk yield (kg), fat, protein, casein, lactose, non-fat solids (NFS), all in g/100 g, urea content (mg/100 ml), citric acid (%), acetone (mmol/l), β-hydroxybutyric acid (BHB) (mmol/l), SCC ($\times 10^3$ cells/ml) using a Combi-Foss™ FT 60000 (Foss Electric, Denmark), comprising a MilkoScan 6000 analyzer and a Fossomatic™ 5000 flow cytometer. In addition, the fat-to-protein (F/P), fat-to-lactose (F/L), and casein-to-protein (C/P) ratios were calculated. SCC were logarithmically adjusted.

Statistical analysis

Statistica 12.0 (StatSoft CR s.r.o.) was used to analyze the collected data. For the relevant datasets, the assumptions required for parametric methods (normal distribution of data and homogeneity of variances) were assessed.

A student's t-test was used to compare milk production and quality between control and mastitis dairy cows, and one-way analysis of variance was used to determine the effect of the bacterial cause of mastitis. Group comparisons were verified using Tukey's HSD test for unequal n at standard significance levels.

RESULTS AND DISCUSSION

The highest proportion of mastitis cases was diagnosed in the summer (35%), while the lowest proportion occurred in the winter (18%). The fact that 52% of mastitis cases occurred in the fourth lactation or later confirms the general trend of an increasing risk of this disease with age – these data are not included in the table. This phenomenon may be related to a decline in the immune function of the mammary gland and to a gradual teat sphincter damage during previous lactations. A higher incidence of mastitis with advancing age has also been reported in other studies [25, 26, 27]. The highest incidence of mastitis (37%) was recorded during the 101–200-day lactation stage, which corresponds to the period when milk production declines slightly after the peak of lactation yet remains at a high level. Moosavi et al. [28] reported that mastitis most commonly occurs in the postpartum period due to metabolic stress associated with the onset of lactation. Other studies also consider the postpartum period to be the time when the incidence of various diseases, including mastitis, is highest [29, 30]. Our study did not confirm this finding.

Table 2. Comparison of daily milk yield and milk quality parameters in the control group and in the mastitis group

Indicator	Groups				p
	Control (n = 111)		Mastitis (n = 111)		
	Mean	SD	Mean	SD	
Milk yield (kg)	32.3	6.8	28.4	7.6	**<0.001**
SCC (log)	4.84	0.12	5.96	0.46	**<0.001**
Fat (%)	4.13	0.53	4.26	0.62	0.0951
Protein (%)	3.50	0.33	3.66	0.48	**0.0037**
Casein (%)	2.81	0.32	2.89	0.45	0.1278
Lactose (%)	5.01	0.16	4.72	0.40	**<0.001**
NFS (%)	9.19	0.36	9.01	0.60	**0.0052**
Urea (mg/100 ml)	19.83	7.50	19.40	9.27	0.6992
Citric acid (%)	0.17	0.03	0.17	0.04	0.1559
Acetone (mmol/l)	0.09	0.13	0.09	0.19	0.8032
BHB (mmol/l)	0.07	0.18	0.08	0.19	0.5176
F/P	1.19	0.16	1.17	0.17	0.5746
F/L	0.83	0.12	0.91	0.18	**<0.001**
C/P (%)	80.03	2.74	78.66	3.95	**0.0029**

SCC – somatic cell count; BHB – β-hydroxybutyric acid; NFS – non-fat solids; F/P – fat/protein; F/L – fat/lactose; C/P – casein/protein; SD – standard deviation

Milk production and quality in cows with mastitis and in the control group

A comparison of production performance and milk quality parameters between cows with mastitis and control cows revealed statistically significant differences (Table 2). Mastitis-affected dairy cows had significantly lower milk yield ($p < 0.001$) compared to control animals, confirming that mastitis negatively impacts the herd's production capacity. At the same time, a significant change in milk quality was observed in mastitis-affected dairy cows, particularly a decrease in lactose and NFS.

In mastitis, protein content typically increases. The physiological basis for this is an increase in Igs and serum albumin levels, which enter the mammary gland from the bloodstream [20, 21]. Casein content remains unchanged or decreases. Alhussien et al. [31] found higher total protein (3.70%) and lower casein (2.25%) in milk from cows with clinical mastitis than in milk from healthy dairy cows (3.30% and 2.70%, respectively). In our study, milk from cows with clinical mastitis had significantly higher protein content than control milk. In contrast, no significant difference in casein content was found between the two groups. It may be assumed that this is related to differences in the parameters used to define clinical mastitis. The authors included only dairy cows with an SCC above 500,000/ml in the clinical mastitis group, whereas our study also included dairy cows with somewhat lower SCC (the lowest SCC was 201,000/ml, as shown in Figure 1).

The changes observed may affect the technological processability of milk, including its curdling properties and stability during heat treatment [32].

Milk production and quality depending on etiological agents

The most frequently isolated pathogen was *Streptococcus uberis* (53%). To a lesser extent, *Klebsiella pneu-*

Table 3. Effect of the selected mastitis pathogens on daily milk yield and milk quality parameters in dairy cows with clinical mastitis

Indicator	Pathogens								p
	Streptococcus uberis n = 59		Escherichia coli n = 18		Streptococcus dysgalactiae n = 15		Klebsiella pneumoniae n = 19		
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	
Milk yield (kg)	29.4	6.9	29.5	7.5	27.5	7.9	25.2	8.9	0.1739
SCC (log)	5.71^{a}	0.31	6.35^{b}	0.43	6.22^{b}	0.42	6.14^{b}	0.46	**<0.001**
Fat (%)	4.30	0.58	4.50	0.77	4.10	0.40	4.05	0.70	0.1152
Protein (%)	3.59	0.40	3.87	0.64	3.66	0.48	3.68	0.51	0.1929
Casein (%)	2.85	0.39	3.00	0.64	2.86	0.42	2.91	0.42	0.6287
Lactose (%)	4.84^{b}	0.17	4.45^{a}	0.65	4.81^{b}	0.37	4.54^{ab}	0.46	**0.0002**
NFS (%)	9.07	0.42	8.91	0.98	9.13	0.49	8.80	0.65	0.2731
Urea (mg/100 ml)	19.83	10.85	17.72	6.35	19.10	8.21	19.86	7.13	0.8577
Citric acid (%)	0.17^{b}	0.04	0.14^{a}	0.03	0.18^{ab}	0.04	0.16^{ab}	0.03	**0.0058**
Acetone (mmol/l)	0.06	0.05	0.17	0.33	0.18	0.33	0.08	0.05	0.0561
BHB (mmol/l)	0.04	0.03	0.18	0.39	0.13	0.25	0.08	0.08	0.0779
F/P	1.20	0.17	1.17	0.17	1.14	0.17	1.11	0.17	0.1527
F/L	0.89^{a}	0.13	1.04^{b}	0.30	0.86^{a}	0.10	0.90^{ab}	0.20	**0.0077**
C/P (%)	79.09	3.43	77.19	5.65	78.15	3.09	79.12	4.08	0.2974

SCC – somatic cell count; BHB – β-hydroxybutyric acid; NFS – non-fat solids; F/P – fat/protein; F/L – fat/lactose; C/P – casein/protein; $^{a, b}$ – means with different superscripts in rows differ at $p < 0.05$; SD – standard deviation

moniae (17%), *Escherichia coli* (16%), and *Streptococcus dysgalactiae* (14%) – Table 3. Poutrel et al. [33] also identified *Streptococcus uberis* as the most common pathogen, with a prevalence of 18–37%. A similar result was found by Davies et al. [34], where this pathogen was present in 38% of cases of clinical mastitis. In contrast, Cervinkova et al. [35] detected *Streptococcus uberis* in only 7.9% of cases. It can be assumed that this difference was due to their study including only dairy cows without clinical symptoms.

When evaluating the effect of selected mastitis pathogens on daily milk yield and milk quality, statistically significant differences were observed in SCC, lactose, citric acid, and F/L (Table 3).

Differences in milk yield were observed primarily in mastitis caused by *Streptococcus uberis* and *Klebsiella pneumoniae*; however, these differences were not statistically significant. Although more severe changes in milk production are generally observed with coliform mastitis compared to mastitis caused by environmental streptococci [36], the actual cause of the observed differences may not necessarily be related to the pathogen. Another possible explanation could be slightly higher variability, caused, for example, by individual differences among dairy cows or by the stage of lactation [37].

The results suggest that *Streptococcus uberis* constitutes a distinct group that differs significantly from the other three pathogens, among which no significant statistical differences were found.

As previously mentioned, the most significant indicator of mastitis is the SCC. An interesting finding was that *Streptococcus uberis* had a very low average SCC (719,000/ml) compared to other pathogens – Figure 1. Such a low value is typically reported for subclinical mastitis; however, our study included only dairy cows with clinical mastitis. A similar SCC value for mastitis caused by *Streptococcus uberis* was reported by de Haas et al. [38], although they attribute this value specifically to subclinical cases. This finding may provide a new diagnostic approach for the clinical management of mastitis of this etiology.

The highest SCC value (3,270,000/ml) was recorded in milk samples from which *Escherichia coli* was isolated. This result is characteristic of acute mastitis caused by Gram-negative bacteria. The differences between the *Escherichia coli* group and other pathogens were statistically highly significant ($p < 0.001$), confirming unique ability of this pathogen to induce a strong immune response. Hogan and Smith [39] report that SCC above 1,000,000/ml is typical of coliform mastitis. A study by Burvenich et

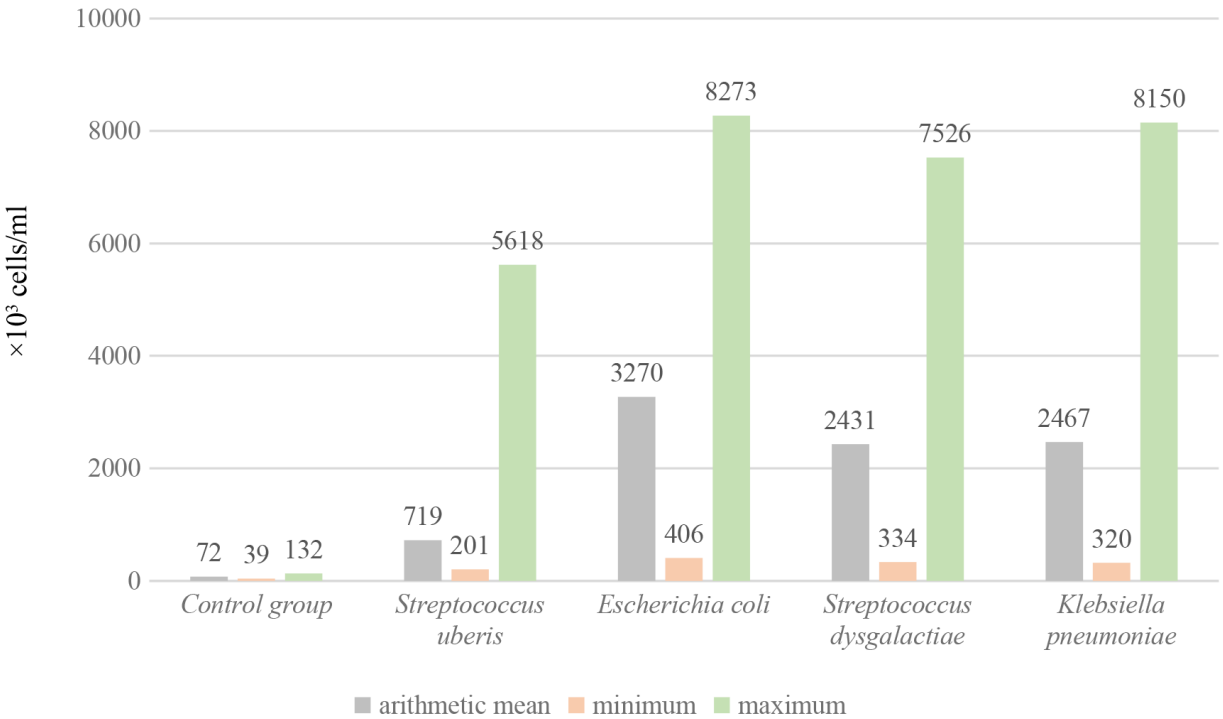

Fig. 1. Somatic cell counts in milk from healthy dairy cows and in milk from dairy cows with mastitis caused by selected pathogens

al. [40] shows that even a low dose of *Escherichia coli* (50 colony-forming units) can cause a dramatic increase in SCC and clinical signs within a few hours. Similarly, Fredebeul-Krein et al. [41] confirm that *Escherichia coli* infections are associated not only with high SCC values but also with severe systemic symptoms, such as elevated body temperature, decreased milk yield, and changes in milk consistency. Compared with Gram-positive pathogens, *Escherichia coli* has a significantly greater ability to trigger an inflammatory response.

The literature indicates that the extent of lactose decline varies depending on the bacterial pathogen. These differences are attributed to differences in the virulence of the pathogens and their effects on the inflammatory response of the mammary gland [42, 43, 44]. In our study, the lowest lactose content was found in milk with mastitis caused by *Escherichia coli* (4.45 g/100 g). Blum et al. [45] reported a decrease in lactose content in *Escherichia coli*-induced mastitis to as low as 3.5–4.0%. The study also notes that this decrease may persist even after mastitis has been cured, due to long-term damage to the mammary gland. Antanaitis et al. [44] state that, given the relatively constant lactose level in healthy dairy cows, a reduction in lactose can be considered a suitable biomarker for mastitis.

The F/L ratios ranged from 0.86 to 1.04. Since this ratio is not a standard indicator in the scientific literature, the value obtained in this study for the control dairy cows (0.83) was used as a physiological reference for discussion. It has been reported that this ratio increases particularly in cases of mastitis caused by *Escherichia coli*, as this bacterium metabolizes lactose as an energy source for its rapid growth in the mammary gland [46]. Paudyal et al. [47] reported values of 1.27–1.37 in dairy cows with mastitis. This higher value compared to our study can be explained by the fact that the authors only included dairy cows up to 60 days of lactation.

The average acetone levels in milk ranged from 0.06 mmol/l (*Streptococcus uberis*) to 0.18 mmol/l (*Streptococcus dysgalactiae*). For BHB, the lowest values were again recorded for *Streptococcus uberis* (0.04 mmol/l) and the highest for *Escherichia coli* (0.18 mmol/l). These findings suggest that specific bacterial pathogens may have distinct effects on milk components, reflecting metabolism in both the mammary gland and the entire body [48]. In milk samples containing Gram-negative bacteria (*Escherichia coli* and *Klebsiella pneumoniae*), higher concentrations of ketone bodies were detected compared to those containing Gram-positive pathogens (*Streptococcus uberis* and *Streptococcus dysgalactiae*). This trend may be associated with the lipopolysaccharides in the cell walls of Gram-negative bacteria, which have an exceptional ability to induce an inflammatory response. Lipopolysaccharides activate the production of pro-inflammatory cytokines, which can affect lipid metabolism in the mammary gland and lead to increased β-oxidation of fatty acids. This fact was described by Du et al. [49], who demonstrated that the inflammatory response induced by the lipopolysaccharides of these pathogens leads to significant metabolic changes in the mammary gland.

Although the study yielded interesting findings, it should be noted that it was conducted on a single commercial dairy farm, which limits the generalizability of the results.

CONCLUSION

An interesting finding from this study is that the traditionally used parameter, somatic cell count, may not always be a sufficiently sensitive indicator of mammary gland health, particularly in mastitis caused by *Streptococcus uberis.* In these infections, monitoring the fat-to-lactose ratio appears to be more appropriate, as it may better reflect changes in milk composition induced by this pathogen. Based on the results obtained, it is recommended that mammary gland health monitoring not be limited to somatic cell count alone but that other indicators, such as lactose and citric acid, be implemented into routine practice. Also, the F/L ratio may serve as a promising complementary indicator warranting further validation. A comprehensive approach to evaluating milk composition can thus contribute to more accurate diagnosis of mastitis and more effective milk quality management in dairy herds.

Ethical Issues

Not applicable.

Data Availability

Data will be made available on request.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Authors' Contributions

Tereza Janů: writing – original draft, resources, conceptualization, writing – review & editing. **David Bílý:** methodology, investigation, writing – original draft. **Eva Samková:** methodology, investigation, conceptualization, writing – original draft, data curation. **Eva Baldíková:** writing – review & editing, resources. **Iliyasu Mohammed Sulaimam:** writing – review & editing, resources. **Lucie Hasoňová:** writing – original draft, writing – review & editing, methodology, investigation, conceptualization, funding acquisition, supervision.

Generative AI Statement

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ORIGINAL ARTICLE

ANALYSIS OF QUALITY AND SAFETY ASPECTS OF TABLE GREEN OLIVES

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ABSTRACT

Table olives are pickled products obtained by partial or complete debittering followed by fermentation. This study evaluated the quality and safety of six commercial green table olive products (TaO1–TaO6) using microbiological, antioxidant, physicochemical, colorimetric, and sensory analyses. Microbiological assessment confirmed generally high hygienic quality. Total bacterial count (TBC) was below the detection limit in TaO1–TaO4 and detected only in TaO5 (2.90 log10 CFU/g) and TaO6 (2.76 log10 CFU/g). Yeasts and moulds were detected only in TaO5, while lactobacilli, *Escherichia coli*, and *Staphylococcus* spp. were not detected in any sample. Enterococci were isolated from TaO5 and TaO6. Antioxidant capacity differed significantly among samples ($p = 0.001$), with TaO4 showing the highest Trolox equivalent antioxidant capacity (TEAC) and TaO3 and TaO6 the lowest. Significant differences ($p < 0.05$) were also found in physicochemical properties, color parameters (L*, a*, b*), and sensory characteristics. TaO1 achieved the highest sensory scores for firmness and palatability, whereas TaO5 showed the poorest quality due to off-odors, soft texture, and poor appearance. Overall, the results demonstrate considerable product-specific variation, highlighting the importance of processing, formulation, and packaging for the quality and safety of commercial table olives.

Keywords: antioxidants activity; food quality; microbial safety; olives; sensory analysis

INTRODUCTION

Table olives represent a globally popular food commodity, with particularly high consumption in Mediterranean regions. They are described as fruits obtained from selected cultivars of the olive tree (*Olea europaea* L.) that possess technological and sensory characteristics suitable for processing. These include appropriate size and shape, a favorable flesh-to-stone ratio, desirable texture and flavor, firmness, and ease of pit removal. To render them edible, the natural bitterness of the fruit is reduced through specific treatments, after which the olives are preserved either by spontaneous or controlled fermentation or by thermal processing, with or without the use of preservatives. The

final product may be marketed in the presence or absence of a covering liquid [1].

Fresh olives are not suitable for direct consumption due to the presence of the phenolic compound oleuropein, which imparts an intense bitterness. Therefore, the primary objective of olive processing is the reduction or elimination of this compound, typically achieved through its hydrolysis, resulting in more palatable products. Several technological approaches have been developed to debitter olives, the most common being the Spanish-style, Californian-style, and natural (Greek-style) methods [2].

In the Spanish-style process, which is the most widely applied for green olives, olives are treated with an alkaline solution (approximately 2–3% sodium hydroxide) for a defined period, facilitating the transformation of oleuropein into non-bitter derivatives. Following this treatment, olives are thoroughly rinsed to remove residual alkali and subsequently immersed in brine solutions (9–10% NaCl), where fermentation is driven by indigenous lactic acid bacteria and yeasts over several months [3, 4].

The Californian-style method involves a more complex, multi-step process. Initially, olives are stored in acidic conditions for an extended period. This is followed by a darkening phase, during which fruits are exposed to dilute sodium hydroxide and then washed. Subsequently, olives are treated with iron salts (such as lactate or gluconate) while aeration is applied to promote color development. The resulting black ripe olives typically exhibit a near-neutral pH (6.0–7.0) and require sterilization to ensure microbiological safety [5].

In contrast, the natural or Greek-style approach avoids chemical treatments altogether, relying solely on direct brining to initiate spontaneous fermentation [6].

Scientific studies addressing the biodiversity of table olive microbiota report a wide range of bacterial, yeast, and mold species identified using both conventional microbiological methods and advanced molecular approaches, including culture-dependent and culture-independent techniques. To ensure microbiological safety and protect consumers from potential hazards, table olive processing facilities are required to implement comprehensive food safety management systems. Among these, the HACCP (Hazard Analysis and Critical Control Points) system, as established by the Codex Alimentarius, represents a fundamental preventive approach focused on identifying, evaluating, and controlling chemical, physical, and biological hazards throughout the production process [7].

In addition, adopting internationally recognized standards, such as ISO certification, is essential for modern food enterprises. These certifications demonstrate that the company has effectively developed, documented, and implemented standardized procedures for production, processing, and packaging, ensuring product safety, quality, and regulatory compliance [1].

Comprehensive physicochemical analysis of table olives is essential for evaluating their quality attributes, detecting defects or spoilage, and ensuring adherence to food safety standards. Additionally, these products are rich in bioactive constituents, particularly polyphenols and triterpenic acids, which significantly influence both sensory characteristics and potential health benefits. Detailed characterization of these compounds offers valuable information that can support process optimization and help maintain consistent product quality [6].

Therefore, the aim of this study was to perform a comprehensive evaluation of the quality and safety of green table olives commercially available on the Slovak market. Six anonymized samples originating from different countries were subjected to microbiological examination, including the determination of total bacteria count, yeasts and moulds, coliform bacteria, *Lactobacillus* spp., *Escherichia coli*, *Staphylococcus* spp. and *Enterococcus* spp. In addition, antioxidant activity, salt content, instrumental colour characteristics, and sensory properties were evaluated. The study aimed to assess the microbiological safety, physicochemical quality, and sensory attributes of table olives and to compare the overall quality of products available to consumers.

MATERIALS AND METHODS

Collection of samples

Analyzed samples of table olives were obtained from the retail market network of the Slovak Republic and originated from various countries. Samples were anonymized and evaluated within a single production batch for each product.

A total of six commercial green table olive products (n = 6), including both plain olives and olives enriched with additional ingredients, were examined. The samples were packaged in three different types of packaging materials: glass jars (TaO1, TaO2, TaO3), polypropylene (PP) plastic containers (TaO5, TaO6), and a metal can (TaO4).

The total product weight ranged from 152 to 295 g, while the drained weight ranged from 75 to 170 g. According to the manufacturer's declaration, the red pepper paste content was 18% in sample TaO2 and 8.2% in sample TaO5. Following purchase, samples were transported to the laboratory and stored in their original unopened packaging under the conditions specified on the product label until analysis. After opening, samples were immediately processed for laboratory analyses.

For sensory evaluation, 35 g of olives (approximately 6–10 fruits, depending on fruit size) were prepared from each sample. Prior to physicochemical and microbiological analyses, additional ingredients (almonds, red pepper, and capers) were removed, and pits were excluded where applicable.

The following samples were analyzed:

TaO1 – table olives stuffed with almonds
TaO2 – green olives stuffed with red pepper 1
TaO3 – plain green table olives in brine
TaO4 – green pitted olives stuffed with capers
TaO5 – green olives stuffed with red pepper 2
TaO6 – green pitted table olives

Product characteristics differed among samples. Sample TaO1 contained 220 g of product, including 120 g of olives, whereas samples TaO2 and TaO3 contained 85 g of olives. Sample TaO4 had a declared volume of 212 mL and contained 85 g of olives, while pouch-packaged samples TaO5 and TaO6 contained 80 g and 75 g of olives, respectively.

Cultivation and microbial analyses

Samples intended for microbiological analysis were randomly selected from collected packages. The packaging material was aseptically opened, and samples were collected under sterile conditions using a scalpel and pipette. The contents were homogenized, and 10 g of each sample was aseptically transferred into sterile containers, followed by the addition of 90 mL of sterile 0.1% peptone water. Homogenization was performed using a Stomacher 400 (Seward Medical, London, UK). Subsequently, tenfold serial dilutions were prepared in accordance with STN EN ISO 6887-1 [8]. Microbiological analyses were then performed following relevant legislative requirements: Commission Regulation (EC) No. 2073/2005 on microbiological criteria for foodstuffs and the Decree of the Ministry of Agriculture of the Slovak Republic No. 06267/2006-SL [9]. The following microbiological parameters were determined: total bacteria count (TBC) (STN ISO 4833-1) [10], yeasts and moulds (STN ISO 21527-1) [11], coliform bacteria (STN ISO 4832) [12], *Lactobacillus* spp. (STN ISO 15214) [13], *Escherichia coli* (ISO 16649-1) [14], *Staphylococcus* spp. (STN ISO 6888-1) [15], and *Enterococcus* spp. by cultivation on Slanetz and Bartley agar according to validated laboratory procedures [16].

Appropriate decimal dilutions of the homogenized olive samples were prepared under aseptic conditions. For the determination of the total bacteria count, coliform bacteria, *Lactobacillus* spp., and *Escherichia coli*, 1.0 mL of the selected dilution was inoculated into sterile Petri dishes using the pour plate technique, followed by the addition of the corresponding molten culture medium according to the respective standards. For the enumeration of yeasts and moulds and *Staphylococcus* spp., 0.1 mL of the selected dilution was inoculated onto the surface of the appropriate solid culture medium using the spread plate technique. *Enterococcus* spp. were determined by inoculating 0.1 mL of the selected dilution onto the surface of Slanetz and Bartley agar using the spread plate technique in accordance with the validated laboratory method. All microbiological analyses were carried out in duplicate. Microbial counts were expressed as log10 CFU/g.

Antioxidant activity

The antioxidant activity of the samples was determined using a slightly modified DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging assay according to Várady et al. [17]. The method was adapted from the original protocol, which was performed in 96-well plates. In the present study the plates were not used. However, the reagent ratios were kept identical, and only the experimental format was modified. Olive samples (1 g) were mechanically crushed using a mortar. Subsequently, 10 ml of methanol was added, and the mixture was thoroughly homogenized for 5 minutes to extract the target compounds. The resulting mixture was filtered through filter paper (Whatman No. 40, Cytiva, Marlborough, USA) to obtain a clear filtrate for further analysis. Serial dilutions (2 mL) of each sample were prepared in absolute methanol. Then, 2 mL of freshly prepared 1 mM DPPH (Sigma-Aldrich, Saint Louis, USA) solution in methanol (MikroCHEM, Pezinok, Slovakia) was added. Trolox (Sigma-Aldrich, Saint Louis, USA) was used as a positive control. The reaction mixture

was incubated in the dark at room temperature for 30 minutes. Absorbance was read at 517 nm on a T65 UV-Visible Spectrophotometer (PG Instruments, Lutterworth, United Kingdom). Results were expressed as Trolox equivalent antioxidant capacity (mg TEAC/g sample). The samples were determined in a wet state without prior drying. All analyses were performed in triplicate.

Determination of salt content

All fortified olive samples and brine samples were analyzed for salt content using the Mohr titration method in six replicates. An aliquot of 1 mL of brine was transferred into an Erlenmeyer flask, followed by the addition of 0.5 mL of 5% potassium chromate as an indicator. The burette was filled with 0.1 N $AgNO_3$ solution. The sample was titrated with standardized $AgNO_3$ until the first appearance of a persistent pale red-brown color, which remained stable for at least 30 seconds. The volume of titrant consumed was recorded. The following formula was used to calculate the amount of salt.

$$NaCl\ content\ (\%) = \frac{V \times N \times F \times 0.0585 \times 100}{m}$$

V: Consumption of 0.1 N $AgNO_3$ in titration (mL)
N: Normality of $AgNO_3$
F: Factor of 0.1 N $AgNO_3$
m: mass of sample (g)

Determination of color

Color parameters were determined using the CIE L*a*b* color space (Commission Internationale de l'Eclairage, 1986) with a Minolta Chroma Meter CR-410 (Minolta, Osaka, Japan), supported by CM-S100w SpectraMagic NX software (Konica Minolta Sensing Inc., Sensing Americas, Inc., Ramsey, USA). For each sample, three parameters were measured: L* (lightness), a* (green–red axis), and b* (blue–yellow axis). Measurements were performed on the surface of the olives. In samples containing almonds, the almonds were mechanically removed to avoid distortion of color measurements. Samples were arranged on Petri dishes to ensure a uniform and representative measurement surface. Each sample was measured six times; the result is the average ± standard deviation [18]. All analyses were performed in triplicate.

Sensory evaluation

Descriptive sensory analysis was performed to determine the sensory profile of olives according to the methodology of Lawless and Heymann [19]. A trained panel of seven evaluators assessed selected sensory attributes, including appearance, aroma, taste, and texture. The intensity of each attribute was rated using a 7-point intensity scale ranging from 0 to 6, where 0 = imperceptible, 1 = very slightly perceptible, 2 = slightly perceptible, 3 = perceptible, 4 = clearly perceptible, 5 = strongly perceptible, and 6 = very strongly perceptible. Table olive samples were presented to the panelists in a randomized order. Results were expressed as mean values obtained from seven assessors.

Statistical evaluation of experiment results

One-way analysis of variance (ANOVA) and Tukey's test for multiple comparisons with a 95% confidence interval ($p < 0.05$) were used for evaluation using GraphPad Prism 10.6.1(892) 2025 statistical software (GraphPad Software, San Diego, CA, USA).

Table 1. Results of quantitative determination of selected microorganisms expressed as log10 CFU/g

Sample	PCA agar	DRBC agar	MRS agar	ENDO agar	TBX agar	BP agar	SB agar
TaO1	-	-	-	-	-	-	-
TaO2	-	-	-	-	-	-	-
TaO3	-	-	-	-	-	-	-
TaO4	-	-	-	-	-	-	-
TaO5	2.90	3.83	-	-	-	-	3.99
TaO6	2.76	-	-	-	-	-	4.14

- = not detected (below detection limit); PCA – Plate Count Agar; DRBC – Dichloran Rose Bengal Chloramphenicol Agar; MRS – de Man, Rogosa and Sharpe Agar; ENDO – Endo Agar; TBX – Tryptone Bile X-Glucuronide Agar; BP – Baird-Parker Agar; SB – Slanetz and Bartley Agar

RESULTS

The microbiological quality of the analyzed samples of table olives, based on the detection of individual microorganisms, was determined to be at a very high level of quality and safety (Table 1). The total number of microorganisms in samples TaO1, TaO2, TaO3, and TaO4 was below the detection limit. Total bacterial count (TBC) was determined in TaO5 and in TaO6. Yeasts and moulds were detected only in sample TaO5. *Lactobacilli* were below the detection limit in all samples. Coliform bacteria were below the detection limit in all samples. *Escherichia coli* was not detected in any of the tested samples. *Staphylococcus* spp. were not detected in any sample. *Enterococci* were successfully cultured from samples TaO5 and TaO6.

The antioxidant activity of the analysed table olive samples is presented in Table 2. Statistically significant differences were observed among all samples ($p = 0.05$). The highest antioxidant activity was recorded in sample TaO4 (3.0705 ± 0.069 mg TEAC/g), which differed significantly from all other samples. In contrast, the lowest values were observed in samples TaO6 (0.570 ± 0.063 mg TEAC/g) and TaO3 (0.7295 ± 0.032 mg TEAC/g), which formed a statistically similar group.

Intermediate antioxidant activity was detected in samples TaO2 with significant differences observed among them as indicated by differing superscripts. The results demonstrate considerable variability in antioxidant capacity among the analysed olive samples, likely reflecting differences in composition, processing methods, or added ingredients.

Table 2. Results of antioxidant activity of table olive samples

Sample	mg TEAC/g of sample (mean± SD)
TaO1	0.902 ± 0.034^{c}
TaO2	1.563 ± 0.092^{b}
TaO3	0.7295 ± 0.032^{d}
TaO4	3.0705 ± 0.069^{a}
TaO5	1.016 ± 0.060^{c}
TaO6	0.57 ± 0.063^{d}
p	0.05

$^{a, b, c, d}$ – values in the column with different superscripts are statistically different ($p < 0.05$).

The results of the physicochemical analysis of brine and olive samples are summarized in Table 3. In the case of brine samples, statistically significant differences ($p < 0.05$) were observed among all analyzed groups. The highest mean value was recorded in sample TaO6 (5.446 ± 0.063 NaCl %), which differed significantly from all other samples. Similarly, sample TaO5 (4.693 ± 0.048 NaCl %) showed significantly higher values compared to the remaining groups. In contrast, sample TaO3 (2.976 ± 0.066 NaCl %) exhibited the lowest mean value and formed a distinct statistical group. Samples TaO1 (3.460 ± 0.025 NaCl %), TaO2 (3.390 ± 0.064 NaCl %), and TaO4 (3.570 ± 0.063 NaCl %) did not differ significantly from each other and were classified within the same homogeneous group. Overall, the brine samples showed considerable variability between individual products.

For olive samples, statistically significant differences ($p < 0.05$) were also identified; however, the variability among samples was less pronounced compared to brine. The highest mean values were observed in samples TaO5 (1.359 ± 0.042 NaCl %) and TaO6 (1.361 ± 0.043 NaCl %), which formed a homogeneous group. The lowest values were recorded in samples TaO2 (1.252 ± 0.043 NaCl %) and TaO3 (1.254 ± 0.043 NaCl %), without significant differences between them. Samples TaO1 (1.340 ± 0.045 NaCl %) and TaO4 (1.299 ± 0.045 NaCl %) showed intermediate values and overlapped statistically with both lower and higher groups. These findings indicate that while significant differences exist among samples, the extent of variability was notably higher in brine than in olive flesh. Although samples with the highest NaCl concentrations in brine (TaO5 and TaO6) also contained the highest NaCl levels in olive flesh, the differences observed in olive flesh were much less pronounced, suggesting that the salt concentration of the brine is not directly reflected in the final salt content of the olives.

Table 3. Salt content determination in brine and table olives (mean ± SD)

Sample	Brine (NaCl %)	Table olives (NaCl %)
TaO1	3.460 ± 0.025^{c}	1.340 ± 0.045^{ab}
TaO2	3.390 ± 0.064^{c}	1.252 ± 0.043^{c}
TaO3	2.976 ± 0.066^{d}	1.254 ± 0.043^{c}
TaO4	3.570 ± 0.063^{c}	1.299 ± 0.045^{bc}
TaO5	4.693 ± 0.048^{b}	1.359 ± 0.042^{a}
TaO6	5.446 ± 0.063^{a}	1.361 ± 0.043^{a}

Values are presented as mean ± SD (n = 3). SD – standard deviation. $^{a, b, c, d}$ – values within a column with different superscript letters differ significantly ($p < 0.05$).

The color parameters (L*, a*, b*) of the analyzed table olive samples are presented in Table 4. Statistically significant differences ($p < 0.05$) were observed among samples for all measured color coordinates.

For the lightness parameter (L*), the highest value was recorded in sample TaO2 (47.55 ± 1.2), indicating the brightest appearance, while the lowest values were observed in samples TaO3 (44.17 ± 0.63) and TaO6 (44.16 ± 0.30), which formed a statistically similar group.

Regarding the a* parameter (green–red axis), sample TaO4 (2.81 ± 0.21) showed significantly higher values, indicating a shift toward red coloration, whereas sample TaO3 (−2.48 ± 0.5) exhibited negative values, reflecting a greenish hue. The remaining samples showed positive but lower a* values and formed a statistically similar group.

For the b* parameter (yellow–blue axis), the highest yellowness was observed in sample TaO5 (23.07 ± 0.52), which differed significantly from most other samples. In contrast, the lowest b* values were found in samples TaO1 (19.44 ± 0.17) and TaO6 (19.42 ± 1.28), indicating lower intensity of yellow coloration. The remaining samples (TaO2, TaO3, and TaO4) exhibited intermediate values with partial statistical overlap.

Overall, the results demonstrate notable variability in color characteristics among the olive samples, which may be influenced by differences in raw material, processing methods, or the presence of added ingredients.

The sensory profile of the analyzed green olive samples is presented in Figure 1. Based on sensory evaluation, differences were observed among the olive samples in terms of appearance, aroma, taste, and texture. Sample TaO1 was assessed as the largest and firmest, whereas sample TaO5 exhibited the poorest visual quality, being soft, broken, and disintegrated. Samples TaO2 and TaO6 showed more uniform size and good firmness, with TaO6 also displaying color variability within the fruits. Regarding aroma, sample TaO5 was characterized by the presence of off-odors, whereas the remaining samples exhibited a typical olive aroma without noticeable defects.

In terms of taste, sample TaO1 received the highest rating, being perceived as palatable and free of bitterness, while samples TaO2 and TaO4 were characterized by a bitter taste. Sample TaO3 exhibited defects, including the presence of stems and difficulties in pit removal, which reduced its overall quality. Sample TaO5 was negatively evaluated due to its atypical taste and overall poor sensory quality, whereas sample TaO6 showed a less intense but also less appealing flavor profile.

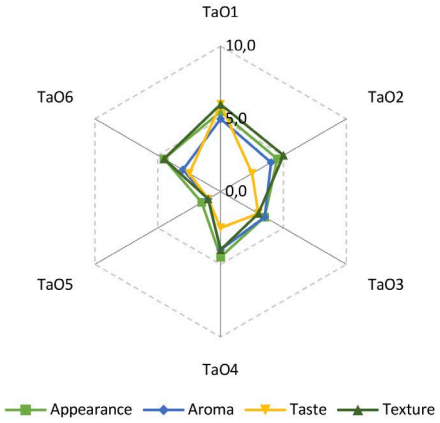

Fig. 1. Sensory profile of green olive samples

Regarding packaging, differences were identified in both type and labeling quality. Samples TaO1–TaO3 were packaged in glass containers, TaO4 in a metal can, and TaO5–TaO6 in flexible pouches. In samples TaO4 and TaO6, issues with label readability were noted, specifically an illegible product name (gold lettering on a dark background), which may negatively affect consumer information.

Table 4. Results of color measurement (L*, a*, b*) of table olive samples

Sample	L* (mean ± SD)	a* (mean ± SD)	b* (mean ± SD)
TaO1	46.88 ± 0.37^{ab}	0.69 ± 0.06^{b}	19.44 ± 0.17^{c}
TaO2	47.55 ± 1.2^{a}	0.96 ± 0.06^{b}	20.07 ± 1.10^{bc}
TaO3	44.17 ± 0.63^{c}	-2.48 ± 0.5^{c}	21.76 ± 3.17^{ab}
TaO4	46.25 ± 0.86^{b}	2.81 ± 0.21^{a}	20.46 ± 1.08^{bc}
TaO5	45.58 ± 0.10^{bc}	0.70 ± 0.03^{b}	23.07 ± 0.52^{a}
TaO6	44.16 ± 0.30^{c}	0.19 ± 0.01^{b}	19.42 ± 1.28^{c}

Values are presented as mean ± SD (n = 3). Different upper indices ($^{a–c}$) within the bars indicate statistically significant differences between samples ($p < 0.05$). SD – standard deviation.

DISCUSSION

The present study evaluated the quality and safety aspects of commercially available green table olives, focusing on microbiological status, antioxidant activity, physicochemical properties, color parameters, and sensory attributes. The results revealed considerable variability among samples, which is likely attributable to differences in processing technologies, formulation, and added ingredients. Moreover, the observed differences were not limited to a single quality parameter but were consistently reflected across microbiological, physicochemical, and sensory analyses, indicating substantial product-specific variation among commercially available olives.

From a microbiological perspective, the analyzed samples demonstrated a generally high level of safety and quality. The absence of *Escherichia coli*, *Staphylococcus* spp., and coliform bacteria in all samples indicates good hygienic practices during processing and handling. Similar findings were reported by Benítez-Cabello et al. [20] and Mougiou et al. [21], who observed low levels of pathogenic microorganisms in properly fermented table olives. The detection of total bacterial count together with yeasts and moulds in sample TaO5 may suggest ongoing microbial activity or incomplete fermentation processes. Although lactic acid bacteria were not detected in the present study, previous studies have highlighted that yeasts and lactic acid bacteria are the dominant microorganisms during olive fermentation [4]. The presence of enterococci in samples TaO5 and TaO6 may be associated with environmental contamination or insufficient process control, as also discussed by De Castro et al. [22]. Among all analysed products, TaO5 showed the least favourable microbiological profile, as it was the only sample in which total bacterial count, yeasts, moulds and enterococci were detected simultaneously. In contrast, TaO6 contained only total bacterial count and enterococci, while the remaining products showed no detectable microbial contamination. These findings demonstrate considerable variability in microbiological quality among commercial products despite their belonging to the same product category. The antioxidant activity results showed significant variability among samples, with TaO4 exhibiting the highest values. This variability is likely related to differences in phenolic compound content, particularly oleuropein derivatives and hydroxytyrosol, which are known to contribute to antioxidant activity. According to Brenes et al. [5], processing methods such as alkaline treatment and fermentation significantly influence the retention or degradation of phenolic compounds. The lower antioxidant activity observed in samples TaO3 and TaO6 may be explained by processing conditions or dilution effects caused by brining and storage. Similar trends have been reported by Panagou et al. [23], who emphasized that both cultivar and technological processing strongly affect antioxidant potential. Interestingly, the product with the highest antioxidant activity (TaO4) also showed no detectable microbiological contamination, whereas TaO6 combined one of the lowest antioxidant activities with detectable microbial counts, illustrating that commercial processing may influence multiple quality parameters simultaneously.

Physicochemical analysis revealed greater variability in brine composition compared to olive flesh. This finding suggests that brine formulation and processing conditions play a crucial role in determining the final product characteristics. High variability in brine parameters has also been documented by Sánchez-Gómez et al. [2], who highlighted the influence of salt concentration, pH, and fermentation dynamics on product quality. The relatively lower variability observed in olive flesh indicates a more stable internal composition, less affected by external processing conditions. In the present study, TaO5 and TaO6 contained the highest salt concentrations in brine, confirming that manufacturers apply different brining strategies. However, although higher NaCl concentrations were observed in samples TaO5 and TaO6, no consistent relationship between salt concentration and microbiological, colour, or sensory characteristics could be identified based on the present results. This suggests that product quality is influenced by multiple technological factors rather than salt concentration alone. Color analysis demonstrated statistically significant differences among samples in all measured parameters (L*, a*, b*). The highest lightness observed in TaO2 suggests better preservation of visual quality, whereas the negative a* values in TaO3 indicate a shift toward green coloration, which is typical for less oxidized or differently processed olives. According to McLaren [24], color parameters in food products are influenced by pigment composition, processing, and storage conditions. The high b* value in TaO5 indicates increased yellowness, which may be associated with ingredient addition or pigment degradation. These findings are consistent with studies showing that processing methods, such as

oxidation and fermentation, significantly affect olive color [1]. Although colour differences do not necessarily indicate reduced product safety, they further demonstrate the considerable technological variability among the analysed commercial products.

Sensory evaluation further confirmed the variability among samples, particularly in texture, taste, and overall acceptability. Sample TaO1 was rated as the most favorable, likely due to its firm texture and balanced flavor profile. In contrast, sample TaO5 exhibited defects such as soft texture, off-odor, and atypical taste, which may be linked to microbial activity or improper processing. Similar sensory defects have been described by Panagou et al. [23], who reported that spoilage microorganisms and fermentation imbalances can negatively affect organoleptic properties. The bitterness observed in samples TaO2 and TaO4 may be associated with incomplete hydrolysis of oleuropein, as described by Perpetuini et al. [1]. Importantly, the poor sensory quality of TaO5 corresponded well with its microbiological findings, making it the only product that consistently exhibited inferior quality across both microbiological and sensory analyses. Although causality cannot be confirmed within the present study, this agreement suggests that inadequate fermentation or storage conditions may have contributed to the observed deterioration in product quality. Conversely, TaO1 combined the best sensory acceptability with the absence of detectable microbial contamination, supporting the importance of appropriate processing and preservation practices.

Additionally, differences in packaging type and labeling quality were identified, which may influence consumer perception and product quality. Glass packaging (TaO1–TaO3) is generally associated with better product stability and consumer preference, whereas flexible packaging may increase the risk of mechanical damage and quality deterioration. Variability in olive content relative to packaging size further highlights inconsistencies in product standardization across manufacturers [20, 21]. However, the relatively small number of analysed products does not allow definitive conclusions regarding the influence of packaging material alone, and further studies including a larger number of commercial samples would be required.

The results of this study are in agreement with previously published research, confirming that the quality of table olives is strongly influenced by processing technology, microbial activity, and formulation factors. The detected variability among commercial products underscores the importance of standardized production practices and strict quality control to ensure consistent product safety and quality. In particular, TaO5 consistently represented the least favourable product, combining the highest microbial counts with the poorest sensory evaluation. The agreement between independent microbiological and sensory analyses strengthens the conclusion that processing conditions substantially influence the final quality of commercial table olives.

CONCLUSIONS

The present study demonstrated considerable variability among commercially available green table olives in terms of microbiological safety, antioxidant activity, physicochemical properties, color characteristics, and sensory quality. Most products exhibited high microbiological quality, whereas TaO5 and TaO6 showed detectable microbial counts, with TaO5 also exhibiting yeasts, moulds, and the poorest sensory quality. In contrast, TaO1 achieved the highest sensory scores, while TaO4 showed the highest antioxidant capacity. These findings indicate that processing technology, formulation, and packaging substantially influence the final quality of commercial table olives. Particular attention should therefore be paid to fermentation control, microbial stability during storage, and optimization of brine composition to ensure consistent product quality. Further studies correlating processing variables with microbiological, chemical, and sensory characteristics are warranted to support the development of best-practice guidelines for commercial table olive production.

Data Availability Statement

All data generated or analyzed during this study are included in this published article.

Conflict of Interest

The authors declare no conflict of interest.

Generative AI Statement

The authors used ChatGPT (OpenAI) solely for language editing and improving grammar.

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Authors' Contributions

M.K., J.V.: writing – original draft, resources, conceptualization, writing – review & editing. JV: methodology, investigation, writing – original draft. **M.V., M.K., J.V., J.Z.:** methodology, investigation, conceptualization, writing – original draft, data curation. **E.D., J.V.:** writing – review & editing, resources.

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ORIGINAL ARTICLE

SYNERESIS, TEXTURE AND MICROBIOLOGICAL QUALITY OF YOGHURT AFTER THE ADDITION OF BLACKBERRY (*RUBUS FRUTICOSUS*)

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Ethical considerations: When reporting experiments on animals Observation of the ARRIVE guidelines 2.0: Updated guidelines for reporting animal research, published on July 14, 2020 (DOI: 10.1371/journal.pbio.3000410), is applied. The authors ensure that all procedures were performed in compliance with the guidelines for animal care of their institutions or with national/international guidelines.

ABSTRACT

This study evaluated the effect of blackberry pomace extract (*Rubus fruticosus*) on the microbiological quality, water holding capacity (WHC), and texture of set-type yoghurt. Four formulations were prepared: a control and three experimental groups supplemented with the extract at concentrations of 0.5%, 0.75%, and 1% prior to fermentation. Streptococci and lactobacilli viability, WHC, and firmness were assessed one day after production and after 7 days of storage at 6 °C. All samples maintained viable counts above 7 log CFU.g^{-1} throughout storage, with values remaining stable at approximately 8 log CFU.g^{-1}. No statistically significant differences ($p > 0.05$) were observed in WHC or firmness between control and enriched yoghurts. The results indicate that blackberry pomace extract can be incorporated into set-type yoghurt without adversely affecting microbiological stability or key texture-related properties.

Keywords: blackberry pomace extract; lactic acid bacteria; set-type yoghurt; syneresis; texture

INTRODUCTION

Yoghurt is a fermented dairy product produced through the activity of lactic acid bacteria, primarily *Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus* [1–3]. Compared with other dairy products, yoghurt consumption continues to increase due to its favourable sensory and nutritional properties, as well as its perceived health benefits [4, 5]. The fermentation process and the metabolites of lactic acid bacteria contribute to product stability and may support the gut microbiota, representing an important factor driving consumer interest in fermented dairy products [6, 7].

At the same time, consumers increasingly prefer foods with a “clean label” and natural additives, which has promoted the use of plant extracts owing to their antioxidant and antimicrobial potential [8, 9]. Plant extracts are therefore being investigated as functional ingredients capable of improving the physicochemical, microbiological, and nutritional properties of fermented dairy products.

Blackberry (*Rubus fruticosus*) is a rich source of bioactive compounds, including polyphenols, flavonoids and anthocyanins, which are associated with antioxidant activity. Blackberry pomace, generated as a by-product of fruit processing, retains a substantial proportion of these bioactive compounds and therefore represents a potentially valuable source of

functional ingredients [10–12]. The valorisation of berry pomace as a source of bioactive compounds and functional food ingredients has received increasing attention [13]. The extraction procedure used in the present study was adapted from Shirahigue et al. (2010) [14], with minor modifications. Moreover, blackberry pomace extracts have demonstrated antioxidant and antimicrobial potential in food systems [12].

Nevertheless, there is limited information available on the specific effect of blackberry pomace extract on the stability of the yoghurt gel and the viability of starter cultures in set-type yoghurt. Therefore, the aim of this study was to evaluate the effect of blackberry pomace extract at different concentrations on the microbiological quality, water-holding capacity, and texture of yoghurt.

MATERIAL AND METHODS

Preparation of Blackberry (*Rubus fruticosus*) Pomace Extract

The extract was prepared according to Shirahigue et al. (2010) [14], with minor modifications. Fresh fruits were purchased from a local supplier, juiced, and the obtained pomace was subsequently freeze-dried. The freeze-dried pomace (20 g) was extracted in 100 mL of 80% ethanol for 24 hours in the dark at laboratory temperature using a shaker. After filtration, the volume of the extract was adjusted to 100 mL with ethanol and subsequently evaporated to dryness at 65 °C under reduced pressure. The dry residue was dissolved in 50 mL of distilled water and stored in the dark at 4 °C. The obtained aqueous extract represented the stock solution used for yoghurt manufacture. Before application, the stock solution was mixed thoroughly and directly added to the milk mixture at the required concentrations (0.5%, 0.75%, and 1%, w/v). The extract was stored in sterile amber bottles at 4 °C and used within seven days of preparation.

Materials Used for Yoghurt Manufacture

The following materials were used for yoghurt production:

- full-fat homogenised high-temperature pasteurised milk (≥ 3.6% fat; Tatranská mliekareň a.s., Slovakia),
- skimmed milk powder (Levické mliekarne a.s., Slovakia),
- yoghurt starter culture Laktoflora (Milcom a.s., Czech Republic) containing species *Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus*.

Commercially available pasteurised milk was selected to ensure standardised composition and high reproducibility of yoghurt manufacture under laboratory conditions. The use of raw milk was avoided because variations in its microbiological quality and composition could introduce additional variability unrelated to the effect of blackberry pomace extract.

Experimental Yoghurt Manufacture

Four groups of experimental yoghurts were prepared: one control and three experimental groups with the addition of blackberry extract at concentrations of 0.5%, 0.75%, and 1% (w/v), added prior to fermentation. The milk was heated to 40 °C and fortified with 60 $g.L^{-1}$ of skimmed milk powder. The mixture was heat-treated at 85 °C for 10 minutes, cooled to 40–43 °C, and inoculated with the starter culture according to the manufacturer's instructions.

The inoculated milk was divided into four groups (K, A, B, C). Extract was added to samples A (0.5%), B (0.75%), and C (1%). The samples (125 mL) were fermented at 40–43 °C until a pH of 4.7 was reached, then cooled and stored at 6 °C. A storage period of 7 days was selected in order to evaluate post-fermentation stability and short-term technological effects.

pH Measurement

The pH value was measured using a calibrated digital pH meter (Hanna Instruments, USA) following two-point calibration (pH 4.00 and 7.00).

Texture Analysis

Texture was evaluated using a TA.XT.plus texture analyser (Stable Micro Systems, UK) 1 day after production and after 7 days of storage at 6 °C. Firmness was measured directly in the original containers without stirring.

Water-Holding Capacity (WHC)

Water-holding capacity was determined by centrifuging 15 g of yoghurt at 4000 rpm for 10 minutes. WHC (%) was calculated according to Remeuf et al. (2003) [15]:

$$WHC = \frac{weight\ after\ whey\ separation}{initial\ weight} \times 100$$

Microbiological Analysis

Yoghurt samples (5 g) were aseptically transferred into sterile flasks containing 45 mL of sterile physiological saline (0.85% NaCl supplemented with 0.1% peptone; HiMedia, India) to obtain the initial 10^{-1} dilution. Decimal dilutions were subsequently prepared using sterile physiological saline. Appropriate dilutions were inoculated in duplicate (1 mL) into sterile Petri dishes using the pour plate method.

Streptococcus thermophilus was enumerated on M17 agar (HiMedia, India) supplemented with β-glycerophosphate according to ISO 7889/IDF 117 (2003) [16]. Plates were incubated aerobically at 37 ± 2 °C for 72 ± 2 h.

Lactobacillus delbrueckii subsp. *bulgaricus* was enumerated on MRS agar (HiMedia, India) according to ISO 7889/IDF 117 (2003) [16]. After inoculation, the plates were overlaid with a thin layer of MRS agar to provide anaerobic conditions and incubated at 37 ± 2 °C for 72 ± 2 h.

Colonies were counted according to ISO recommendations and expressed as log CFU g^{-1}.

Statistical Analysis

All experiments were performed in triplicate (n = 3). Microbiological counts were expressed as log CFU·g^{-1} and presented as geometric means together with geometric standard deviations (GSD). Statistical analysis was performed using one-way analysis of variance (ANOVA), and differences were considered statistically significant at $p < 0.05$.

RESULTS AND DISCUSSION

The effect of plant extracts on yoghurt quality is complex and influenced by multiple interacting factors. During the production of yoghurts enriched with extracts, it is essential to evaluate their impact, which is influenced by the extract concentration, the type of yoghurt, and the production technology used.

Microbiological Analysis – Streptococci

According to the regulation of the Slovak Republic [17], fermented dairy products must contain at least 7 log CFU.g^{-1} of viable microorganisms. The counts of streptococci determined one day after yoghurt production are presented in Table 1. All yoghurt samples met the legislative requirement, with streptococcal counts exceeding 8 log CFU.g^{-1} (8.20–8.38 log CFU.g^{-1}). No significant differences were observed between the control and experimental samples.

Table 1. Streptococci counts in yoghurts with the addition of blackberry pomace extract (*Rubus fruticosus*) – one day after production

Samples	Control (log CFU.g^{-1})	A – 0.5% (log CFU.g^{-1})	B – 0.75% (log CFU.g^{-1})	C – 1% (log CFU.g^{-1})
Trial 1	8.29	8.31	8.29	8.25
Trial 2	8.34	8.20	8.24	8.37
Trial 3	8.35	8.38	8.34	8.32
Geometric mean	8.33	8.30	8.29	8.31
GSD	1.004	1.011	1.006	1.007

Note: Trials 1–3 represent three independent yoghurt production experiments (n = 3). Microbiological analyses were performed in duplicate. Values are expressed as geometric mean and geometric standard deviation (GSD). No statistically significant differences were found between the control and experimental groups ($p > 0.05$).

Streptococcal counts after 7 days of storage at 6 °C are presented in Table 2. The values remained stable at approximately 8 log CFU.g^{-1}, with no statistically significant differences observed between the individual samples ($p > 0.05$). These results suggest that the addition of blackberry (*Rubus fruticosus*) pomace extract did not affect the growth of bacteria of the genus *Streptococcus*.

Table 2. Streptococci counts in yoghurts with the addition of blackberry pomace extract (*Rubus fruticosus*) – after 7 days of storage at 6 °C

Samples	Control (log CFU.g^{-1})	A – 0.5% (log CFU.g^{-1})	B – 0.75% (log CFU.g^{-1})	C – 1% (log CFU.g^{-1})
Trial 1	8.15	8.26	8.25	8.32
Trial 2	8.28	8.26	8.33	8.26
Trial 3	8.16	8.29	8.17	8.28
Geometric mean	8.20	8.27	8.25	8.29
GSD	1.009	1.002	1.010	1.004

Note: Trials 1–3 represent three independent yoghurt production experiments (n = 3). Microbiological analyses were performed in duplicate. Values are expressed as geometric mean and geometric standard deviation (GSD). No statistically significant differences were found between the control and experimental groups ($p > 0.05$).

In general, streptococci maintain high viability during storage in yoghurts containing fruit juices as well as in plain yoghurts, which is related to their ability to survive at low temperatures.

For comparison, the counts of *Streptococcus thermophilus* in yoghurts enriched with modified starch and a polyphenolic preparation from honeysuckle (*Lonicera*

caerulea) at concentrations of 0.1% and 0.2% have been reported in the range of 8.94–9.04 log CFU.g^{-1} [18].

Szoltysik et al. (2020) reported higher streptococcal counts compared with our findings, reaching 9.23 log CFU.g^{-1} after 24 hours in yoghurts supplemented with extracts from *Rosa spinosissima* fruits at concentrations of 0.1% and 0.2%. They also observed significantly higher streptococcal counts in yoghurts with added extracts compared with the control group. A similar trend was noted after 7 and 14 days of storage at 4 °C, although a slight decrease in streptococcal counts occurred during storage [19].

A starter culture composed of *Streptococcus thermophilus* strains producing exopolysaccharides and with a lower proportion of *Lactobacillus delbrueckii* subsp. *bulgaricus* has also been reported by several authors [20, 21]. Dimitrellou et al. (2020) determined initial streptococcal counts in yoghurt samples at approximately 7.5 log CFU.g^{-1}, whereas lactobacilli counts were around 2.5 log CFU.g^{-1}. A decline in lactobacilli counts was observed particularly in samples enriched with fruit juices [21].

Microbiological Analysis – Lactobacilli

Lactobacilli counts determined one day after production are presented in Table 3, while the values after 7 days of storage at 6 °C are shown in Table 4. One day after production, lactobacilli counts ranged from 7.92 to 8.20 log CFU.g^{-1}.

Table 3. Lactobacilli counts in yoghurts with the addition of blackberry pomace extract (*Rubus fruticosus*) – one day after production

Samples	Control (log CFU.g^{-1})	A – 0.5% (log CFU.g^{-1})	B – 0.75% (log CFU.g^{-1})	C – 1% (log CFU.g^{-1})
Trial 1	8.08	8.10	8.03	7.94
Trial 2	8.05	8.13	8.20	8.05
Trial 3	7.92	8.00	8.05	8.00
Geometric mean	8.02	8.08	8.09	8.00
GSD	1.011	1.008	1.012	1.007

Note: Trials 1–3 represent three independent yoghurt production experiments (n = 3). Microbiological analyses were performed in duplicate. Values are expressed as geometric mean and geometric standard deviation (GSD). No statistically significant differences were found between the control and experimental groups ($p > 0.05$).

All samples met the legislative requirement of at least 7 log CFU.g^{-1}, and no statistically significant differences were observed between the control and experimental yoghurts with added extract ($p > 0.05$). These results demonstrate that blackberry (*Rubus fruticosus*) pomace extract at concentrations of 0.5%, 0.75%, and 1% did not affect the growth of lactobacilli.

Table 4. Lactobacilli counts in yoghurts with the addition of blackberry pomace extract (*Rubus fruticosus*) – after 7 days of storage at 6 °C

Samples	Control (log CFU.g^{-1})	A – 0.5% (log CFU.g^{-1})	B – 0.75% (log CFU.g^{-1})	C – 1% (log CFU.g^{-1})
Trial 1	7.93	7.95	7.85	7.98
Trial 2	7.82	8.07	8.12	8.05
Trial 3	7.71	7.92	8.09	7.91
Geometric mean	7.82	7.98	8.02	7.98
GSD	1.014	1.010	1.019	1.009

Note: Trials 1–3 represent three independent yoghurt production experiments (n = 3). Microbiological analyses were performed in duplicate. Values are expressed as geometric mean and geometric standard deviation (GSD). No statistically significant differences were found between the control and experimental groups ($p > 0.05$).

Szoltysik et al. (2021) investigated the counts of *Lactobacillus bulgaricus* in yoghurts enriched with modified starch in combination with a polyphenolic preparation from honeysuckle (*Lonicera caerulea*) at concentrations of 0.1% and 0.2% [19]. Lactobacilli counts in the experimental samples ranged from 8.08 to 8.22 log CFU.g^{-1} for the respective concentrations (0.1% and 0.2%), compared with 7.75 log CFU.g^{-1} in the control sample. After 14 days of storage, lactobacilli counts decreased by a maximum of 0.5 log CFU.g^{-1}.

In the case of yoghurts supplemented with extracts from *Rosa spinosissima* fruits at concentrations of 0.1% and 0.2%, Szoltysik et al. (2020) reported lactobacilli counts of 8.65 log CFU.g^{-1} in samples with 0.2% extract compared with 8.13 log CFU.g^{-1} in control samples [19]. After 14 days of storage at 4 °C, a slight decrease in lactobacilli counts was observed, reaching 8.23 log CFU.g^{-1} in yoghurts with 0.2% extract and 7.65 log CFU.g^{-1} in control samples. The authors concluded that both extract addition and storage time had a statistically significant effect ($p < 0.05$) on the counts of yoghurt starter culture microorganisms.

A decrease in starter culture bacterial counts during storage has been reported by several authors and has been associated with decreasing pH and increasing acidity during refrigerated storage [20, 22, 23].

Flavoured yoghurts with added fruit extracts meet legislative microbiological requirements despite variability in starter culture counts. Even markedly lower lactobacilli counts do not necessarily indicate product deficiencies or non-compliance with legislation, as fermentation intensity may be intentionally adjusted to produce less acidic

yoghurts while still meeting the required microbiological criteria [22].

Determination of Water-Holding Capacity

For consumers, the absence of visible syneresis represents an important quality attribute of set-type yoghurts. Syneresis is considered a key quality parameter influencing yoghurt stability and consumer acceptability [24]. A higher degree of syneresis is associated with reduced yoghurt quality [25]. Findings suggest that syneresis is significantly affected by the addition of fruit pulp, its concentration, and storage duration.

The mean values of water-holding capacity (WHC) after production and after 7 days of storage are shown in Fig. 1. WHC values ranged from 70.77–72.23% after production and from 66.65–69.32% after storage. No statistically significant differences were observed between the control and experimental samples, nor between the different storage times ($p > 0.05$). These results indicate that the addition of blackberry (*Rubus fruticosus*) pomace extract did not negatively affect the stability of the yoghurt gel.

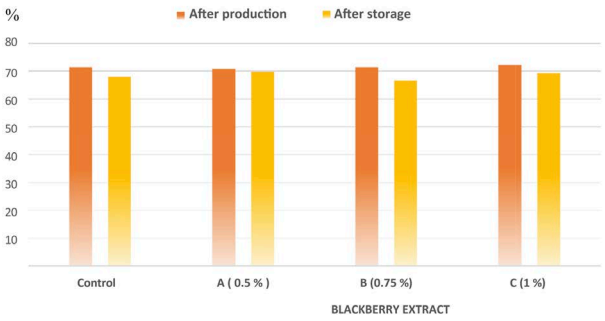

Fig. 1. Water-holding capacity in yoghurts with the addition of blackberry pomace extract (*Rubus fruticosus*) – after production and after 7 days of storage

Several methodologies for determining water-holding capacity (WHC) in yoghurts have been reported in the literature, and the results may vary depending on the method used [26].

A significant increase in WHC values during storage ($p < 0.01$) has been observed in yoghurts supplemented with maca (*Lepidium meyenii*) powder or propolis extract. The highest WHC values during storage were found in samples containing propolis (10%), whereas the lowest values were recorded in samples with maca (3%) [27].

The addition of fruit pulp to yoghurt may lead to a reduction in syneresis, probably due to the increased water-binding capacity of fruit components [28]. The absence of significant changes in WHC in the present case may be related to limited interactions between the phenolic compounds present in the extract and casein micelles within the yoghurt matrix. Polyphenols are known to interact with milk proteins through hydrogen bonding and hydrophobic interactions, which may influence gel structure and water retention [29, 30]. However, the concentrations used were likely insufficient to induce measurable structural changes.

Although the phenolic composition of the extract was not determined in this study, the literature indicates that blackberry (*Rubus fruticosus*) pomace contains significant amounts of phenolic compounds and anthocyanins, which contribute to its biological activity [10–12].

Texture Determination – Yoghurt Firmness

Firmness values of the yoghurts determined one day after production and after 7 days of storage at 6 °C are presented in Fig. 2. Firmness ranged from 577 to 626 g after production, while a slight increase to 662–746 g was observed after storage. This increase was not statistically significant ($p > 0.05$), and no significant effect of the addition of blackberry (*Rubus fruticosus*) pomace extract on yoghurt firmness was demonstrated.

The textural properties of yoghurt are strongly influenced by technological processing conditions [31]. The final structure is primarily determined by interactions among milk proteins, dry matter content, and processing parameters, which together define the strength and stability of the gel network [32].

In practice, texture improvement is often achieved through the use of stabilisers such as pectin, starch, or gelatine. However, the use of modified starch remains controversial from a consumer perspective [32].

An increase in firmness during storage has also been reported in yoghurts enriched with plant extracts. Key textural parameters in yoghurt evaluation include hardness, adhesiveness, cohesiveness, gumminess, and springiness [33, 34].

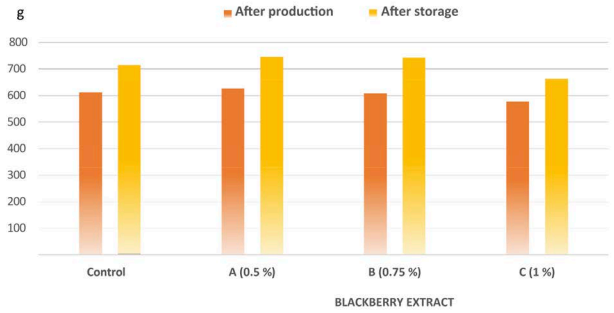

Fig. 2. Firmness (hardness) in yoghurts with the addition of blackberry pomace extract (*Rubus fruticosus*) – one day after production and after 7 days of storage at 6 °C

In contrast, the addition of fruit juices generally results in only minimal changes in the physicochemical properties of yoghurt, with the exception of colour parameters [22].

CONCLUSION

The aim of this study was to evaluate the suitability of blackberry (*Rubus fruticosus*) pomace extract as an additive in set-type yoghurt at concentrations of 0.5%, 0.75%, and 1%. The obtained results demonstrated that the addition of the extract did not have a negative effect on the viability of starter culture microorganisms. Both streptococci and lactobacilli counts remained stable at approximately 8 log CFU.g^{-1} during 7 days of refrigerated storage and met legislative requirements. At the same time, no statistically significant differences ($p > 0.05$) were observed in water-holding capacity between the control and experimental samples, confirming that the extract did not contribute to syneresis or disrupt the stability of the yoghurt gel. Yoghurt firmness slightly increased during storage; however, no statistically significant effect of extract addition on textural properties was observed compared with the control. Based on these findings, it can be concluded that blackberry pomace extract at the tested concentrations represents a technologically suitable ingredient for set-type yoghurt without negative effects on microbiological quality, gel stability, or texture. Furthermore, it shows potential as a natural functional ingredient with possible applications in dairy products.

Data Availability Statement

The raw data of this article will be made available by the authors, without undue reservation.

Ethical Statement

No ethical approval was necessary for this study.

Conflict of Interest

The authors declare no conflicts of interest.

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Generative AI Statement

No generative AI and AI-assisted technologies were used in writing the manuscript.

Authors' Contributions

Conceptualization: M.K., A.H.; methodology: V.D., M.K.; sample collection: V.D, investigation: M.K., V.D.; data collection: V.D.; data curation: M.K.; supervision: M.K.; writing – original draft preparation: M.K., A.H.; writing – review and editing: M.K., A.H. All authors have read and agreed to the published version of the manuscript.

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DOI: 10.2478/fv-2026-0027

FOLIA VETERINARIA, 70, 3: 55—61, 2026

ORIGINAL ARTICLE

EFFECT OF TWO *LIMOSILACTOBACILLUS* STRAINS AND *SALMONELLA* CHALLENGE ON IMMUNE MARKERS IN A POULTRY CAECAL MODEL

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Ethical considerations: When reporting experiments on animals Observation of the ARRIVE guidelines 2.0: Updated guidelines for reporting animal research, published on July 14, 2020 (DOI: 10.1371/journal.pbio.3000410), is applied. The authors ensure that all procedures were performed in compliance with the guidelines for animal care of their institutions or with national/international guidelines.

ABSTRACT

Probiotic microorganisms are increasingly investigated for their capacity to influence host physiology, particularly through interactions with the intestinal immune system. The present study aimed to assess the effects of selected probiotic strains, *Limosilactobacillus reuteri* CCM 9425 and *Limosilactobacillus reuteri* B2/6, applied simultaneously in an *ex vivo* model of poultry caecal explants, with a focus on their impact on immune-related gene expression. In addition to probiotic treatment, explants were challenged with *Salmonella* Enteritidis PT4 to evaluate immune responses under infectious conditions and following probiotic pre-treatment. The transcriptional profiles of selected cytokines (IL-15, IL-1β, IL-8, and IL-18) indicated that probiotic exposure did not trigger an exaggerated inflammatory response. Instead, a consistent and significant increase in IL-15 expression was observed in both the probiotic-treated group and the group receiving probiotic pre-treatment ($p < 0.01$). The upregulation of IL-15, a cytokine involved in early immune defense and activation of innate immune cells, may reflect enhanced immune readiness without accompanying tissue damage. Taken together, these findings demonstrate that the investigated probiotic strains can modulate intestinal immune responses in a controlled manner, supporting gut homeostasis while avoiding harmful inflammation.

Keywords: caecal explants; cytokines, gut immunity; probiotics; *Salmonella* Enteritidis

INTRODUCTION

Probiotic bacterial strains are widely recognized for their beneficial effects on both human and animal health. Numerous studies highlight the importance of lactic acid bacteria (LAB) not only for the intestinal environment and digestion but also for the overall condition of the organism, including immunity, behavior, and the nervous system [1]. Their relevance is particularly pronounced in the context of the emerging post-antibiotic era, in which

the use of antimicrobial treatments should be strictly limited to necessary cases. However, in practice, antibiotics are often used excessively and unjustifiably, not only in livestock production but also in human medicine, thereby contributing to the spread of antimicrobial resistance [2]. Probiotics and other natural substances therefore represent a promising strategy for supporting gut health and the immune system. At the same time, there is increasing emphasis on the protection of experimental animals due to stricter legislation and growing societal awareness [3]. Despite this, the study of zoonotic pathogens in poultry still frequently relies on murine *in vivo* models or heterologous *in vitro* systems, which exhibit significant limitations due to the species-specific nature of host–pathogen interactions [4]. The replication capacity and infectivity of many microorganisms are closely linked to a specific host, which reduces the relevance of the obtained results. Moreover, research on the poultry gastrointestinal tract is limited by the lack of suitable cellular models, as available epithelial cell lines fail to reproduce the complex cellular heterogeneity and full functionality of intestinal tissue [5]. For this reason, alternative experimental approaches are currently being intensively developed, particularly *ex vivo* and advanced *in vitro* models, which allow for a better simulation of *in vivo* physiological conditions and provide more relevant results in the study of gut health, immunity, and host–microorganism interactions [4].

In the present study, we focused on the isolation and experimental use of the poultry caecum to investigate the immunomodulatory potential of the probiotic strain *Limosilactobacillus reuteri* CCM 9425 in combination with *Limosilactobacillus reuteri* B2/6 alone and in combination with experimental infection induced by the pathogenic strain *Salmonella* Enteritidis PT4. Our previous studies demonstrated good reproducibility and applicability of an ileal explant model derived from the poultry intestine [6, 7]. The cecum represents a highly specialized part of the digestive tract with an important role in microbial fermentation, immune processes, and water absorption.

MATERIAL AND METHODS

Isolation of caecal explant cultures

Sample isolation was performed according to the procedure described in our previous study [7]. Briefly, Ross 308 poultry (one 21-day-old female) was humanely euthanized by decapitation following prior anesthesia with carbon dioxide (CO_2). After opening the coelomic cavity, the caecal portion of the intestine was collected. Intestinal explants from the cecum were obtained using reusable biopsy punches with plungers. Bacterial cultivation: The probiotic strains *Limosilactobacillus reuteri* CCM 9425 and *Limosilactobacillus reuteri* B2/6 (PP) were isolated from the intestinal contents of healthy pheasants at the Department of Microbiology and Immunology (University of Veterinary Medicine and Pharmacy in Košice, Slovakia). The strains were cultivated in de Man, Rogosa, and Sharpe (MRS) broth (Merck) at 37 °C overnight under static conditions. Salmonella enterica serovar Enteritidis (S. Enteritidis; SE), phage type PT4 (Veterinary Research Institute, Brno, Czech Republic), was cultured in Luria–Bertani broth (LB; Sigma-Aldrich, St. Louis, MO, USA) overnight at 37 °C with continuous shaking (160 rpm). Overnight cultures were subsequently inoculated into fresh LB broth and incubated for 4 hours at 37 °C with constant agitation. Prior to the experiments, bacterial concentrations were quantified by measuring optical density at 600 nm, according to the procedure described by Kiššová et al. (2025) [6].

Experimental design

The experimental setup and the distribution of individual experimental groups are presented in Table 1. Following the indicated treatments, explants from all experimental groups were incubated for an additional 1 h in medium supplemented with 100 µg/mL gentamicin (Sigma-Aldrich, St. Louis, MO, USA) to eliminate any remaining extracellular bacteria and ensure identical treatment conditions across all groups.

Analysis of relative gene expression

The total RNA from each sample was isolated from the explants using the RNeasy Mini Kit (Qiagen, Hilden, Germany) exactly according to the manufacturer's instructions. The quantity and purity of RNA (free of DNA and proteins) were determined at 260/280 nm, using NanoDrop 8000 (Thermo Scientific, Waltham, MA, USA). The cDNA was reverse-transcribed from RNA, using the iScript cDNA Synthesis kit (Bio-Rad, Hercules, CA, USA) as outlined by the manufacturer. The qPCR analysis was performed using the CFX96 Touch Real-Time PCR Detection System (Bio-Rad, USA) in a 10 µL reaction volume

Table 1. Experimental scheme of the treated ceacal explant cultures

Experimental group	Type of treatment	Application of Gentamicin 100 ug/mL/1h	Abbreviation
Control	Without treatment	✓	-
Probiotic group	Simultaneous application of *L. reuteri* CCM 9425 and *L. reuteri* B2/6, each at a concentration of 5 × 10^7 CFU/mL (2 h; 37 °C).	✓	PP
Infection group	Infection with SE at a concentration of 10^7 CFU/mL (2 h; 37°C)	✓	S
Pre-treatment group	Pre-treatment with simultaneous application of *L. reuteri* CCM 9425 and *L. reuteri* B2/6, each at a concentration of 5 × 10^7 CFU/mL (2 h; 37 °C) and subsequent infection with SE at a concentration of 10^7 CFU/mL (2 h; 37°C	✓	PP+S

The table describes each type of treatment applied in the individual experimental groups.

containing 1 × iQ™ SYBR® Green Supermix (Bio-Rad), 0.5 µM forward and reverse primers, and 40 ng/µL of cDNA. Ubiquitin was used as a reference gene for internal control. All reactions were performed in triplicate. The experimental protocol consisted of the initial denaturation at 95 °C for 5 min, followed by amplification – 40 cycles of 3 steps (denaturation at 94 °C for 30 s, annealing at 60 °C for 30 s, extension at 72 °C for 30 s), and final extension at 72 °C for 15 min, followed by melting curve analysis to confirm the amplification of a specific product. Relative normalized expression was calculated using the 2−ΔΔCT method. Results of the gene expression experiment conducted in triplicate were expressed as mean ± standard deviation (SD). Primers for the mRNA expression analysis are listed in Table 2.

Statistics

Significant differences in gene expression analyses were determined in GraphPad Prism 9.0.0 software (GraphPad Software Inc., San Diego, USA) by one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparisons. The level of significance was set as P-value ≤0.05 considered significant (*), P-value ≤0.01 considered highly significant (**), P-value ≤0.001 (***) considered very highly significant.

RESULTS

The aim of the present study was to evaluate the immunomodulatory potential of simultaneously applied LAB strains and their prospective protective effect in a model infection with *Salmonella* Enteritidis using an *ex vivo* model of poultry caecal explants.

Caecal explants were isolated using 4 mm biopsy needles, and the obtained tissue punch samples were subsequently placed into a 96-well plate containing DMEM/F12 medium supplemented with growth factors (Figure 1). The use of this standardized isolation approach enabled the preparation of uniform explants and ensured consistent experimental conditions across all samples.

Table 2. Table of used primers

Gene name	Abbreviation	Primer sequence 5´→ 3´	Reference
Interleukin-18	IL-18	F: ACGTGGCAGCTTTTGAAGAT R: GCGGTGGTTTTGTAACAGTG	[8]
Interleukin-8	IL-8	F: ATGAACGGCAAGCTTGGAGCT R: GCAGCTCATTCCCCATCTT	[8]
Interleukin-15	IL-15	F: TGGAGCTGATCAAGACATCTG R: CATTACAGGTTCCTGGCATTC	[9]
Interleukin-1 beta	IL-1β	F: GAAGTGCTTCGTGCTGGAGT R: ACTGGCATCTGCCCAGTTC	[8]
Ubiquitin	UB	F: GGATGCAGATCTTCGTGAAA R: CTTGCCAGCAAAGATCAACCTT	[10]
Glyceraldehyde 3-phosphate dehydrogenase	GAPDH	F: GGCACGCCATCACTATC R:CCTGCATCTGCCCATTT	[10]

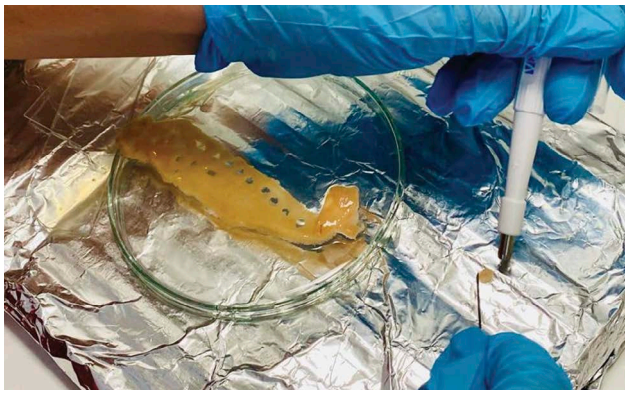

Fig. 1. Isolation of explants
Representative image of explant isolation from the poultry intestine.

The main finding was that the application of probiotics, either alone or in the experimental group referred to as the pre-treatment group, where caecal explants were pre-treated with both lactobacilli strains during a 2-hour incubation and subsequently exposed to infection with the pathogen *S*. Enteritidis did not lead to an increased expression of the monitored pro-inflammatory cytokines such as IL-1β, IL-8, and IL-18 (Fig. 2).

However, a significant upregulation was observed in mRNA encoding the cytokine IL-15, both in the probiotic group ($p < 0.01$) and in the pre-treatment group ($p < 0.01$) compared to the control group (Fig. 2). Conversely, in the infection group, where intestinal inflammation was induced by the pathogenic strain S. Enteritidis, an increased expression of genes encoding IL-8 ($p < 0.001$) and IL-1β ($p < 0.05$) was detected.

DISCUSSION

The absence of increased expression of conventional pro-inflammatory cytokines suggests that the applied combination of LAB does not induce an excessive inflammatory response in intestinal tissue. This aspect is particularly important, as uncontrolled inflammation may lead to epithelial damage and disruption of the intestinal barrier [11, 12]. In addition to preventing tissue injury, the maintenance of a balanced inflammatory tone is essential for pre-

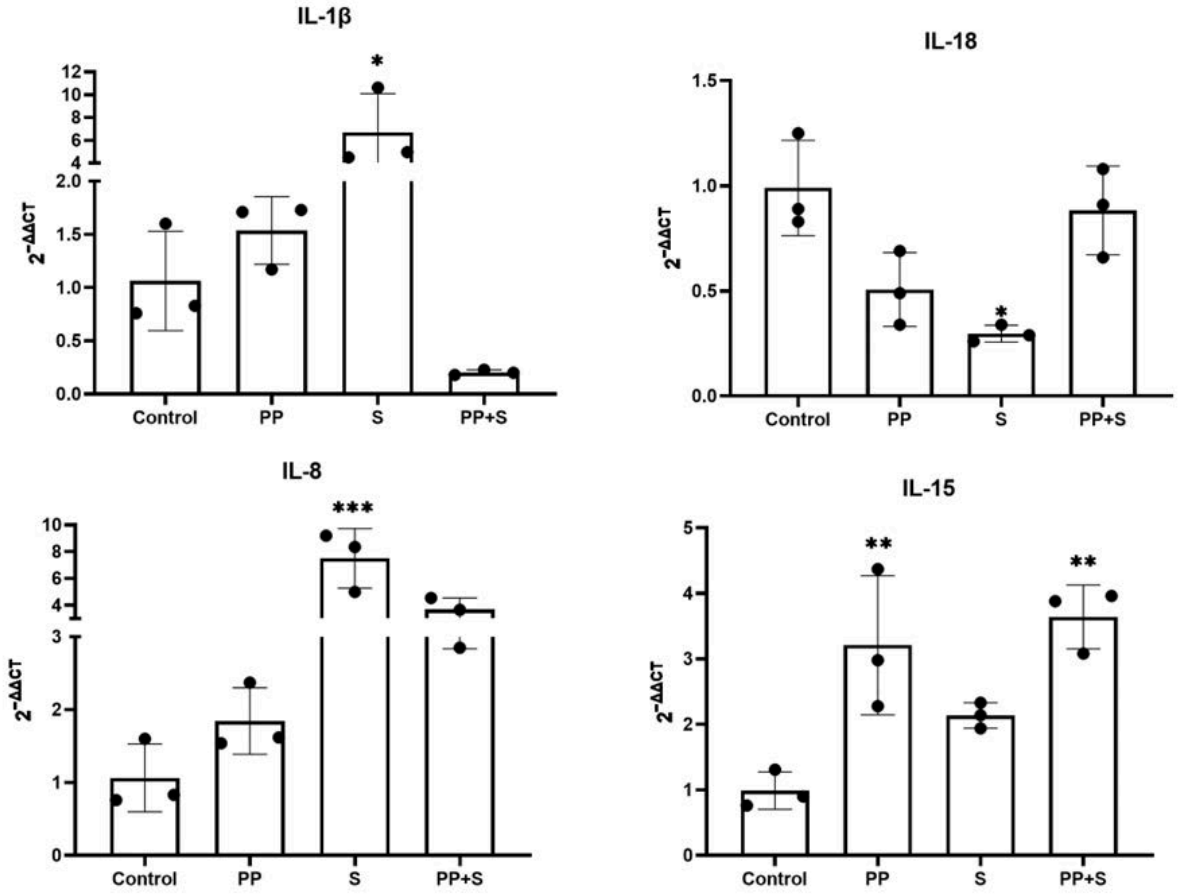

Fig. 2. Gene expression analysis
Results are expressed as mean ± SD (n = 6). Statistical significance of differences was assessed using Dunnett's multiple comparison test. Results were significantly different compared to the control experimental group at statistical significance levels (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$).

serving epithelial integrity and proper nutrient absorption. Similar findings have been reported in previous studies, where probiotic bacteria, particularly strains of *Limosilactobacillus reuteri*, demonstrated the ability to modulate immune responses without inducing strong pro-inflammatory signaling [13, 14]. These observations support the concept that probiotics can fine-tune host immunity rather than simply activating or suppressing it.

Notably, a significant upregulation of IL-15 was observed in probiotic-treated groups. IL-15 plays a key role in the activation and proliferation of NK cells and memory $CD8^+$ T lymphocytes and is crucial for early phases of mucosal immunity [15, 16]. Moreover, IL-15 is involved in the maintenance of intraepithelial lymphocytes, which are essential for epithelial surveillance and rapid immune responses at mucosal surfaces. The increased expression of IL-15 may therefore indicate that probiotic application does not trigger inflammation but rather "primes" the immune system for a more effective response against pathogens. This effect aligns with the concept of immunological priming, where host readiness is enhanced without tissue damage [16]. Such priming may be particularly advantageous in production animals, where rapid responses to enteric pathogens are critical for maintaining health and performance.

In the context of *Salmonella* infection, it is particularly noteworthy that even in the pre-treated experimental group, no increase in the expression of genes encoding IL-1β, IL-18, and IL-8 was observed. These cytokines are typically associated with inflammasome activation and neutrophil recruitment during bacterial infections [17, 18]. Although infection with the pathogenic strain *S.* Enteritidis was confirmed by increased expression of mRNA encoding genes such as IL-1β, IL-8, and IL-15, similar findings were observed in our previous study using a porcine epithelial cell line, where infection with *Salmonella Typhimurium* resulted in elevated expression of pro-inflammatory cytokines such as TNF-α, IL-8, and IL-6 [19]. The unchanged expression in pre-treated probiotic group suggests that probiotic pre-treatment may prevent excessive activation of innate immune pathways that are often linked to tissue damage. Therefore, our results indicate that LAB may attenuate pathogen-induced inflammatory responses while still allowing the activation of protective immune mechanisms. This selective modulation of the immune response could represent a key advantage of probiotic application over conventional antimicrobial strategies.

This effect may be mediated by several mechanisms, including competitive exclusion of the pathogen, inhibition of its adhesion to epithelial cells, or modulation of host signaling pathways such as NF-κB and MAPK [20]. In addition, probiotic-derived metabolites, including short-chain fatty acids and bacteriocins, may contribute to the suppression of pathogen growth and virulence [21].

The intestinal explant model represents a suitable *ex vivo* system that preserves the complex architecture of intestinal tissue, including epithelial and resident immune cells, thereby providing more physiologically relevant results compared to conventional *in vitro* models. This model allows for the investigation of host–microbe interactions in a controlled environment while retaining key structural and functional features of the intestine [22]. However, due to its complex microbiota and dominant immune tolerance mechanisms, it remains questionable whether it is an optimal model for studying early host–pathogen interactions. Future studies may benefit from combining explant models with simplified systems or microbiota-controlled conditions to better dissect the initial stages of infection and immune activation.

CONCLUSION

Our findings suggest that the probiotics used in this study are capable of subtly modulating the intestinal immune response without triggering an undesirable pro-inflammatory reaction. At the same time, this type of immune modulation contributes to the maintenance of intestinal homeostasis and supports the integrity of the gut environment. Moreover, the results demonstrate the suitability of the chicken intestinal explant model as a valuable *ex vivo* platform for the initial screening of probiotic immunomodulatory properties before *in vivo* validation.

Data Availability Statement

The raw data of this article will be made available by the authors, without undue reservation.

Ethical Statement

This study was performed in accordance with the guidelines for animal welfare and was approved by the

Ethics Committee for the approval of research involving animals by the legislative requirements applicable at the UVMP in Košice with permit No. EKVP/2025–05.

Conflict of Interest

The authors declare no competing interests.

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Generative AI Statement

There were no generative AI and AI-assisted technologies used in writing the manuscript. All scientific content, interpretation, and conclusions remain the responsibility of the author. AI-based language model were used only to assist with English language editing and grammar correction. The authors reviewed and approved all modifications and take full responsibility for the final content.

Authors' Contributions

Conceptualization, ZK, VK, and VV. Methodology, ZK, VV, VK and CT. Data curation, ZK, VK, and VV. Formal analysis, writing – original draft preparation, ZK, VV and CT. Supervision, VK. The cell cultures were prepared by VV, and VK. Writing – review and editing, ZK. The management for research activity planning and the financial support for the project leading to this publication were performed by VK and ZK. All authors have read and agreed to the published version of the manuscript.

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ORIGINAL ARTICLE

MICROSCOPIC EXAMINATION OF FOODS CONTAINING INSECTS

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Ethical considerations: When reporting experiments on animals Observation of the ARRIVE guidelines 2.0: Updated guidelines for reporting animal research, published on July 14, 2020 (DOI: 10.1371/journal.pbio.3000410), is applied. The authors ensure that all procedures were performed in compliance with the guidelines for animal care of their institutions or with national/international guidelines.

ABSTRACT

Edible insects have traditionally been consumed in many regions of the world and are currently reconsidered as a sustainable alternative to conventional animal protein sources. In food products, insects are commonly used in powdered form, which complicates their identification in final products. This study aimed to evaluate the applicability of microscopic methods for the detection of insect structures in foods and to compare the effectiveness of the histochemical staining methods PAS-Calleja and Pasini. A total of 14 commercially available insect-based food products were analysed using standard histological techniques. In all samples, the presence of insect structures, particularly fragments of cuticle and muscle tissue, was confirmed. In addition to their detection, the size of individual fragments was evaluated, providing supplementary information on the degree of technological processing and the preservation of insect structures in the final products. The PAS-Calleja staining method provided a stronger contrast of chitinized structures compared to the Pasini method. The results indicate that microscopy, combined with appropriate histochemical staining and evaluation of fragment size, represents a suitable and practical method for the detection and assessment of insect components in foods.

Keywords: cuticle fragments; food control; light microscopy

INTRODUCTION

The consumption of insects has a long tradition and is still common today in many parts of the world, where insects represent an important source of protein and other nutrients. In recent years, interest in entomophagy has been increasing again, primarily in connection with the search for more sustainable alternatives to conventional sources of animal protein [1]. There are currently four species of insect authorised for human consumption in the European Union: *Tenebrio molitor* [2], *Locusta migratoria* [3], *Acheta domesticus* [4], and *Alphitobius diaperinus* [5]. However, the authorisation does not apply solely to specific insect species but also to specific forms and processing methods in which these products may be placed on the market. Insects can be consumed whole; however, espe-

cially in European countries, they are more often technologically processed, for example, into powders or protein extracts that are subsequently added to foods. The significance of insects is in their nutritional value and production efficiency. Insects are also considered an alternative source of animal protein with potential use in the future food system [6, 7]. In addition to their nutritional value, insects also contain bioactive compounds with antioxidant, antimicrobial, and anti-inflammatory effects, which support their use in functional foods [8].

The range of foods containing insect ingredients has expanded significantly in recent years and includes both whole edible insects and products enriched with insect powder, such as crisps, protein bars, pasta, breakfast cereals, and confectionery [9]. Edible insects are most commonly used in bakery and cereal products, particularly in bread, biscuits, crackers, muffins, and pasta. Consumers generally accept the addition of insect meal at levels of up to 10 per cent in bread and around 5 per cent in biscuits and muffins [10], while concentrations of 2–10 per cent have been successfully tested in pasta [11]. Higher levels have also been investigated in pasta (up to 30 per cent) and biscuits (up to 20 per cent), with some of these products retaining acceptable technological and sensory properties [12, 13].

The growing use of insects in food is increasing the need for reliable analytical methods to verify authenticity and determine the content of insect-derived ingredients in products. The microscopic methods we use are important tools for quality control and the identification of ingredients of insect origin [14]. Microscopic analysis is one of the methods used for the detection of insects or their fragments in food. The principle of this method is based on the direct observation of insect morphological structures in food samples using microscopic techniques [15]. Among the most commonly observed structures are fragments of the cuticle, setae (hairs), and parts of the respiratory system, such as tracheoles. Cuticular fragments are relatively well recognizable even after technological processing of food, as chitin is chemically and mechanically stable [16].

The aim of this study was to analyse the applicability of microscopic methods for insect structures detection in selected insect-containing foods and to compare the histochemical staining methods PAS–Calleja and Pasini.

MATERIALS AND METHODS

Food samples (n = 14, with cricket powder) containing insects as an ingredient were obtained from retail markets in the Czech Republic. These were commercially available products declaring the presence of insects (e.g., protein bars or snacks with the addition of insect powder).

The food samples were processed by histological techniques before microscopic examination. An AFA fixative (alcohol–formalin–acetic acid) solution was used for fixation [17]. From each sample, four paraffin blocks were prepared and subsequently cut on a rotary microtome RM2255 (Leica, GER) into 4 µm thick sections. From all samples, two sections from each block were prepared for each staining method. For each sample, eight sections were randomly selected and stained using the Pasini histochemical staining method [18]. The remaining eight sections were stained using the PAS–Calleja (PC) method modified according to Lukášková et al. [19]. The samples were then mounted using Solacryl (Penta, CZE). For each sample, eight sections per staining method were examined at a magnification of 100× and higher using a Nikon Eclipse Ci-L microscope (Nikon, JAP) equipped with a Proscan III motorized table (Prior, USA).

During sample examination, attention was primarily focused on the presence of characteristic morphological characters of insects, especially fragments of the cuticle (chitinous exoskeleton) and muscle tissue. These structures were identified in terms of their characteristic appearance after staining and were used as indicators of the presence of an insect component in the analysed food samples. The size of the fragments was evaluated using NIS Elements software (Laboratory Imaging, CZE) based on the image analysis.

RESULTS AND DISCUSSION

Of the samples analysed, the packaging of two products (pasta and granola) did not specify the type of insect used or its proportion in the product. For the other products, powder or flour made from the house cricket (*Acheta domesticus*) was declared as the insect ingredient. Commission Implementing Regulation (EU) 2022/188 [4] lists the approved forms of *Acheta domesticus* as frozen, dried, and powdered forms. In three cases, the protein bars contained

10 per cent cricket powder and in two cases 17 per cent, which complies with the legal requirements. In all cases, the crisps and triangular snacks analysed were labelled as containing 7 per cent cricket powder, which contravenes the aforementioned regulation, which permits a maximum amount of 5.0 g per 100 g. The bars contained 5 per cent, which is in accordance with the legislation. Two samples of protein bars incorrectly listed "cricket flour"; according to current European Union legislation, the correct designation for this ingredient is "house cricket (*Acheta domesticus*) powder" or "powdered form," not "cricket flour" [4]. The declared concentrations of the insect component in the analysed products correspond to the ranges reported in the scientific literature for baked goods, cereal products, and snack products containing edible insects [10, 11]. The inclusion of 10–17% cricket powder in protein bars confirms the trend toward using insects as a protein source in functional foods, while concentrations of up to 10% are more commonly used in pasta and other cereal products [11, 12]. These results highlight the importance of proper labeling and control of products containing insect ingredients in accordance with novel food requirements.

Microscopic examination of samples stained using the histochemical methods PAS–Calleja and Pasini confirmed the presence of insect-derived structures in all analysed samples. In most samples, these structures were present as larger fragments, which could be readily distinguished from other components of the food matrix under microscopic observation. These findings are generally consistent with results reported in the literature, which describe microscopy as a reliable method for detecting insect particles in food and feed [1, 15]. Our results are consistent with published studies reporting that the most commonly identified insect structures in food include the cuticle, parts of the tracheal system, muscle fibres, setae, and other morphological features characteristic of insect bodies [20, 15, 21]. Microscopic analysis is often used to detect contamination by insects and foreign particles in grains, flours, animal feed, and food [22]. However, its usefulness decreases when applied to finely ground insect powders and technologically processed products, where diagnostic morphological features are lost and the risk of raw material substitution or adulteration increases [23, 14]. Histochemical staining can enhance the contrast of insect structures and, according to Pečová et al. [21], enable detection of insect fragments in food even at concentrations as low as 1% of the insect component.

With respect to the size of insect muscle fragments, the largest were observed in protein bars, whereas the smallest were found in insect-based triangles. Based on the size measurements of insect cuticle fragments, the largest fragments were detected in chips, while the smallest fragments were measured in the sample of protein bars (Table 1). Particle size measurement is important for foods containing edible insects, because this matrix is highly heterogeneous due to the presence of the chitinous exoskeleton. Schiel et al. [24] demonstrated that particle size after milling significantly affects sample homogeneity and the reproducibility of subsequent analytical results. Similarly, Lisboa et al. [25] emphasize that technological processes, particularly drying and grinding, significantly affect the structure of insect powders and their functional properties in food matrices.

Table 1. Size of cuticle and muscle fragments identified by microscopic examination

	Muscle tissue				Cuticle fragments			
	Minimum size		Maximum size		Minimum size		Maximum size	
Staining Group (number of samples)	PC	Pasini	PC	Pasini	PC	Pasini	PC	Pasini
Bars (2)	279.83	336.20	955.94	951.25	313.68	290.78	626.49	631.27
Chips (4)	387.43	389.55	1207.72	1080.49	383.27	311.57	1440.59	963.04
Granola (1)	350.32	264.90	698.02	640.74	371.20	462.61	942.38	620.92
Triangles (1)	340.50	230.68	549.26	571.23	383.27	359.04	617.27	680.27
Protein bars (5)	279.86	418.82	1029.58	1335.11	356.07	228.24	1016.35	856.02
Pasta (1)	273.95	432.41	724.85	694.34	471.83	237.31	713.81	549.71

PC - PAS–Calleja staining method

Differences in fragment size are likely related to the intensity of grinding the insect raw material and the processing methods used for individual products, as particle size and morphology significantly influence the structure and properties of the final food product [26].

The presence of larger cuticle fragments can lead to greater heterogeneity in the food, and it has been shown that the particle size of insect powders affects not only the homogeneity of the mixture but also the texture, sensory properties, and stability of the final products [27].

Using the PAS–Calleja method, both chitinized parts of the exoskeleton (cuticle) and fragments of muscle tissue were determined. The PAS–Calleja staining showed high intensity and provided strong contrast between the cuticle and other sample structures. The cuticle fragments were elongated, without distinct segmentation, and were mostly still attached to the muscle tissue. They exhibited an intense blue coloration, occasionally with a slight greenish tint (Figure 1). The muscle tissue appeared light to dark brown in color (Figure 2).

Comparable results were also obtained using the Pasini staining method, in which structures corresponding to insect tissues were likewise observed. Microscopic slides most frequently contained fragments of muscle tissue and parts of chitinized structures. The insect cuticle exhibited an intense blue color (Figure 3), while the muscle tissue appeared pink (Figure 4).

PAS–Calleja staining provided higher contrast between cuticle, muscle tissue, and surrounding matrix, particularly for the cuticle. In contrast, the Pasini staining method showed weaker contrast. Similar results were reported by Pečová et al. [21], who described the PAS–Calleja staining method

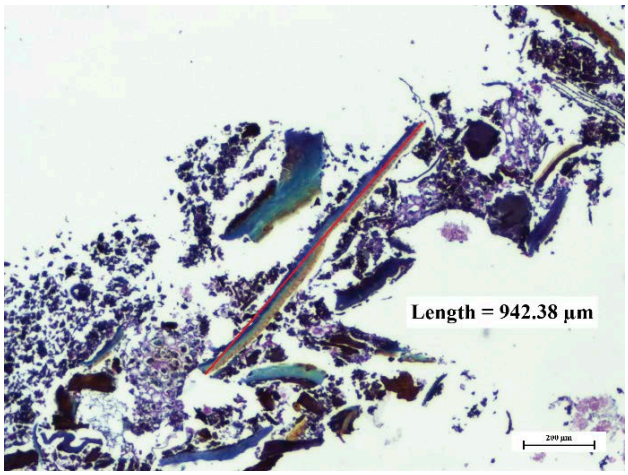

Fig. 1. Chocolate granola with cricket powder, magnification 40×, PC (cuticle)
PC - PAS–Calleja staining method

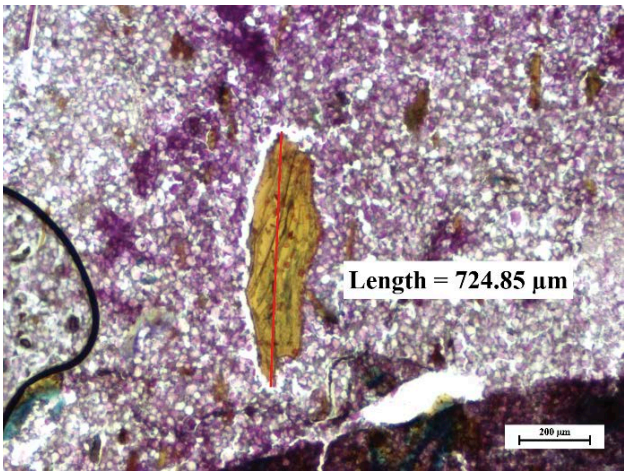

Fig. 2. Wheat egg pasta with cricket powder, magnification 40×, PC (muscle tissue)
PC - PAS–Calleja staining method

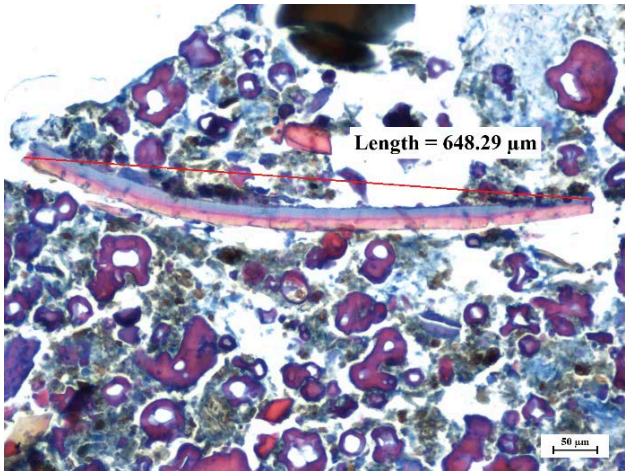

Fig. 3. Protein bar containing insects, magnification 100×, Pasini (cuticle)

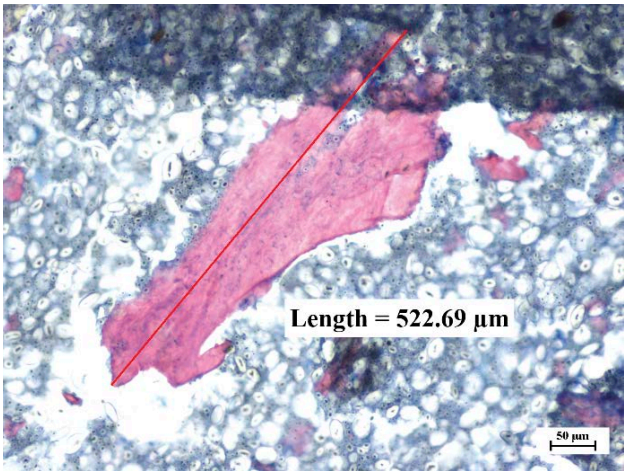

Fig. 4. Wheat egg pasta with cricket powder, magnification 100×, Pasini (muscle tissue)

as more sensitive. Ottoboni et al. [28] used light microscopy in combination with differential staining in their study. They also demonstrated that this combination allows for more reliable differentiation of insect fragments from marine arthropod material by highlighting chitinized structures.

In addition to microscopy, other methods suitable for the detection of insects have been described, including molecular and spectroscopic approaches. Although these methods can be highly sensitive, they do not allow direct visualization of structures. In contrast, an advantage of microscopy is that it enables the observation of specific insect fragments, which may be more practical in certain cases [15, 29, 30].

Some authors also report that microscopy represents a relatively simple and cost-effective method of analysis that can be applied in routine laboratory practice [15]. This is particularly advantageous compared to molecular methods, which, although more precise, are more demanding in terms of equipment and sample processing.

CONCLUSION

The presence of insect-derived structures was confirmed in all tested samples, most commonly in the form of cuticle fragments and muscle tissue, which could be clearly distinguished from other components of the examined samples. Both staining methods proved suitable for the detection of insect-derived structures. However, the PAS–Calleja method produced clearer, more distinct staining, particularly of chitinized structures.

It was also observed that the size of the fragments depends on the degree of technological processing of the food, although the structures remain preserved despite processing. The measurement of fragment size represents an additional important parameter that contributes not only to a more objective assessment of the presence of insects in food but also to a better understanding of the effect of technological processing on other food properties.

The results indicate that microscopy, in combination with appropriate histochemical staining and fragment size measurement, represents a suitable tool for the control of food products containing insects.

Ethical Statement

No ethical approval was necessary for this study.

Conflict of Interest

None of the authors has any conflicts of interest.

Data Availability Statement

The raw data of this article will be made available by the authors, without undue reservation.

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Generative AI Statement

This manuscript was not written using generative AI.

Authors' Contributions

Z.J. contributed to the study design, provided methodological supervision, assisted with data interpretation, and critically revised the manuscript; R.H. performed the laboratory analyses; M.P. contributed to the methodology, sample processing, and critically revised the manuscript; M.B. performed the laboratory analyses; L.H. conceived and designed the study; J.D. carried out sampling; B.T. critically revised the manuscript.

All authors have read and approved the final published version of the manuscript.

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ORIGINAL ARTICLE

SELECTIVE INHIBITORY EFFECT OF CANNABIDIOL FROM *CANNABIS* SP. PLANTS ON TUMOUR CELL PROLIFERATION

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Ethical considerations: When reporting experiments on animals Observation of the ARRIVE guidelines 2.0: Updated guidelines for reporting animal research, published on July 14, 2020 (DOI: 10.1371/journal.pbio.3000410), is applied. The authors ensure that all procedures were performed in compliance with the guidelines for animal care of their institutions or with national/international guidelines.

ABSTRACT

Cannabidiol is a non-psychoactive phytocannabinoid with growing interest as a potential antitumour agent in veterinary medicine due to its modulatory effects on cell proliferation and survival. The aim of this preliminary *in vitro* study was to evaluate the impact of cannabidiol on non-tumourigenic MCF-10A and tumourigenic MCF-7 breast cell lines using colorimetric measurement of metabolic activity (MTS assays) and the real-time cell analyser xCELLigence system (RTCA). Cannabidiol stimulated the metabolic activity of MCF-10A cells at all tested concentrations and time points, with maximal effects at 24 and 48 hours, while enhanced adhesion was observed after 72 hours. In contrast, MCF-7 cells showed a decrease in metabolic activity at 20 µM after 24 and 48 hours, alongside a consistent decrease in adhesion across all conditions, most pronounced at 48 hours. The Pearson correlation test revealed a strong positive correlation (r = 0.7768) between the MTS assay and xCELLigence system results. These findings suggest the selective effects of cannabidiol on tumour cells and support its potential as a targeted bioactive agent. To fully understand its efficacy in the context of various tumour types, more robust research into its mechanisms and safety is needed.

Keywords: adherence; breast cancer; MTS; proliferation; xCELLigence

INTRODUCTION

Cannabinoids are substances of the *Cannabis* sp. plant with a variety of pharmacological effects, including cannabidiol (CBD), a phytocannabinoid isolated from the *Cannabis sativa* plant, which exhibits a wide spectrum of biological effects. Unlike Δ9-tetrahydrocannabinol (THC), it does not have psychotropic properties. Its effect is mediated by interaction with the endocannabinoid system and other regulatory mechanisms of cells. CBD is used in both human and veterinary medicine and is characterized by anti-inflammatory, antioxidant, analgesic, and neuro-

protective effects [1]. Recently, attention has also been focused on the potential anti-tumour effects of CBD. Cannabinoids have been found to inhibit tumour proliferation, neovascularization, invasion, and chemoresistance [2, 3]. Many studies have shown that CBD exhibits high antitumour activity in breast cancers, reducing the proliferation of tumour cells [4, 5] and reducing breast cancer metastasis [6]. Some studies also report a positive effect of CBD in combination with conventional chemotherapy and radiotherapy, thereby improving antitumour efficacy [7, 8]. The mechanism of CBD's antiproliferative effect lies in its ability to induce apoptosis and influence cell migration and adhesion [9].

However, the specific molecular mechanisms underlying these multi-target effects remain unclear in the current literature. Furthermore, there is a lack of veterinary *in vitro* models for researching anti-cancer drugs for mammary gland tumours in animals; therefore, comparative veterinary oncology utilises well-characterized human cell lines. In this study, the MCF-7 cell line (a model of human epithelial breast adenocarcinoma expressing estrogen receptors) and the MCF-10A cell line (a non-tumourigenic human epithelial breast cell line serving as a standard for healthy tissue cells) were selected. Although these are human cell models, mammary gland tumours in companion animals (particularly canine mammary carcinomas) exhibit clinical, histological, and molecular-biological similarities to human breast cancer. Direct extrapolation of data from *in vitro* studies to animal systems has its limitations, primarily due to potential interspecies differences; nevertheless, this comparative approach serves to suggest future possibilities for in vivo studies.

Given the increasing incidence of mammary gland tumours in companion animals, the high toxicity of commercial chemotherapeutics, and the growing chemoresistance of tumours to them, investigating the mechanisms of the antiproliferative effect of CBD is necessary. In addition to the previously described effects in adjuvant therapy for pain relief and its direct antiproliferative effect against tumours, CBD may also potentially enhance the efficacy of conventional chemotherapeutic agents when used in combination, thereby helping to reduce the development of chemoresistance [10]. The aim of our preliminary experiment was to investigate the potential selective effect of CBD on tumour cells by monitoring the cellular response in real time. At the same time, the effect of CBD on the metabolic activity of tumour and non-tumour epithelial cell lines was evaluated.

MATERIALS AND METHODS

Preparation of the tested substance

A standard solution of cannabidiol – 1 mg/ml in methanol, certified reference material, 1 ml ampoule, Cerilliant®; CAS number: 13956-29-1 (Sigma-Aldrich, Germany) was used. The tested concentrations (2.5, 5, 10, and 20 µM) were selected based on relevant published literature mapping the in vitro therapeutic range of phytocannabinoids and our preliminary screening, ensuring clinical relevance without causing immediate non-specific solvent toxicity [2, 4]. The maximum concentration of methanol in the final culture medium did not exceed 0.5% (v/v), and an equivalent vehicle control (solvent control containing 0.5% methanol) was included in all assays to rule out vehicle-induced cytotoxicity.

Cell cultivation

Experiments were performed using the human breast cancer cell line MCF-7 and the non-cancerous epithelial breast cell line MCF-10A (American Type Culture Collection (ATCC, Manassas, VA, USA)). MCF-7 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM; Biosera, Kansas City, MO, USA) supplemented with 2% antibiotic–antimycotic solution (ATB/ATM; penicillin (100 UI/mL), streptomycin (100 µg/mL), amphotericin B (2.5 µg/mL), Lonza, Switzerland), 10% foetal bovine serum (FBS), 1% L-glutamine, and 0.1% gentamicin (Sigma, United States). A high-glucose DMEM/F12 growth medium (Biosera, Kansas City, MO, USA) supplemented with 10% FBS, 2% ATB/ATM solution, insulin (10 µg/mL), epidermal growth factor (EGF; 20 ng/mL), and hydrocortisone (0.5 µg/mL) was used for the cultivation of the MCF-10A cell line. Cells were maintained in an incubator under standard culture conditions in a humidified atmosphere containing 5% CO_2 at 37 °C. The culture medium was replaced every 48 hours with freshly prepared medium, and cells were monitored using an inverted microscope (Zeiss Axiovert 200) equipped with a digital camera. When cell confluence reached approximately 80 – 90%, cells were passaged by enzymatic dissociation using trypsin (Sigma, United States). Cell viability exceeded 95% prior to each experiment.

Evaluation of metabolic activity changes using the MTS assay

The MTS assay (CellTiter 96 non-radioactive cell proliferation assay, Promega, Madison, WI, USA) quantifies the metabolic activity (MA) of cells *in vitro* based on their viability in a cytotoxic assay. It relies on the reduction of a tetrazolium component (MTS) by viable cells into a coloured, soluble formazan product that absorbs light. The intensity of this colour is directly proportional to the number of living cells and measured spectrophotometrically. In this study, both cell lines were plated in three 96-well plates (3,000 cells per well in 100 µl growth medium). Cells were treated with the test substance at the selected concentrations in a 1:10 ratio to the medium. The MTS assay was performed after 24, 48, and 72 hours of incubation according to the manufacturer's instructions. The plates were then incubated for an additional 4 hours. The amount of formazan formed was evaluated spectrophotometrically using an LB 913 Apollo 11 ELISA reader (Berthold Technologies, Bad Wildbad, Germany) at a wavelength of 450 nm. The absorbance obtained was compared with the control (100%) and expressed as a percentage according to the formula: *% metabolic activity = (Sample absorbance) x 100 / (Control absorbance)*.

Real-time cell monitoring and EC50 determination using the xCELLigence system

The xCELLigence system, a real-time cell analyser (RTCA) dual-plate instrument (ACEA Bioscience Inc., San Diego, CA, USA), allows continuous monitoring of cell adhesion properties *in vitro* in a non-invasive and label-free manner. Two 16-well E-plates (ACEA Bioscience Inc., San Diego, CA, USA) were used in the experiment – one for each cell line. First, 100 µl of growth medium was added to the wells for background measurement. Then, a suspension containing 3,000 cells (in 80 µl of medium) was added. The plates were placed back into the incubator and incubated for 24 h to allow the proliferating cells to form a 90% monolayer. After 24 h, selected concentrations of CBD (2.5 – 20 µM) were added to the cells at a ratio of 1:10 with the medium. Any changes in adhesion generate a value known as electrical impedance, which is measured throughout the experiment (72 h). These values are converted by the software into dimensionless values known as Cell Index (CI) and recorded by the xCELLigence software as a graph and displayed as "Normalised Cell Index" (CI = 1) versus time. The CI values were used to calculate the proliferative activity (PA), expressed as a percentage according to the formula: *Proliferative activity % = CI sample x 100 / CI control*. All calculations were performed based on a minimum of three independent biological replicates, with each condition tested in triplicates.

Statistical evaluation

The acquired data were statistically analysed using GraphPad Prism version 10.3.1 (GraphPad Software, San Diego, CA, USA). The values were expressed as percentages of metabolic activity (MTS) and cell adherence (RTCA), with control cells (unaffected) set at 100%. The values were compared to the control using the ANOVA multiple comparisons test with Dunnett's corrections; we also compared the results at 24, 48, and 72-hour intervals at different concentrations between the MCF-10A (non-tumour) and MCF-7 (tumour) cell lines using multiple t-tests. The significance level was set at $p < 0.05$. We determined the relationship between MTS and RTCA values using Pearson's correlation test, where coefficients less than 0.3 represent a weak linear correlation, 0.3 – 0.5 a moderate one, and greater than 0.5 a strong linear correlation.

RESULTS

The results of both our analyses (MTS, RTCA) of the cells demonstrated that cannabinoids have a stimulating effect on both the adhesion and metabolic activity of non-tumour MCF-10A cells compared to control cells not exposed to CBD. Regarding metabolic activity, this was observed at all tested cannabinoid concentrations across all time intervals (Figure 1), with the highest values observed after 24 and 48 hours. In the case of adhesion, we observed stimulation only after 72 hours of CBD exposure (Figure 2). In contrast, however, we did not observe any metabolic activity or adhesion-promoting effects in the MCF-7 tumour cell model. At a concentration of 20 µM, we observed significantly reduced metabolic activity after 24 and 48 hours compared to the control (without CBD). Regarding adhesion, we observed a significant decrease in adhesion compared to the control group at all concentrations tested and at all time points, with the most pronounced decrease occurring after 48 hours of CBD exposure. The Pearson correlation coefficient $r = 0.7768$ confirmed a highly significant, strong positive linear correla-

tion ($P < 0.001$) between MTS and RTCA results (Table 1).

We confirmed the selectivity of CBD toward tumour cells using paired t-tests, in which metabolic activity (MTS) and adhesion (RTCA) were compared between MCF-10A and MCF-7 cells at the same time points and concentrations (Table 1). The results confirmed significant differences in metabolic activity and adhesion between non-tumor and tumor cells at all observed CBD concentrations for metabolic activity after 48 and 72 hours of cultivation. Similarly, we observed significant differences in adhesion at all concentrations tested after 48 and 72 hours of cultivation, with the greatest difference observed after 72 hours at 5 µM CBD.

DISCUSSION

Interest in the study and use of phytocannabinoids has been growing, and their potential use in various diseases is currently being explored. Regarding cancer, cannabinoids are primarily used to alleviate the side effects of chemotherapy, but a growing number of studies now attribute antiproliferative, proapoptotic, anti-invasive, and antiangiogenic effects to these compounds [11, 12, 13].

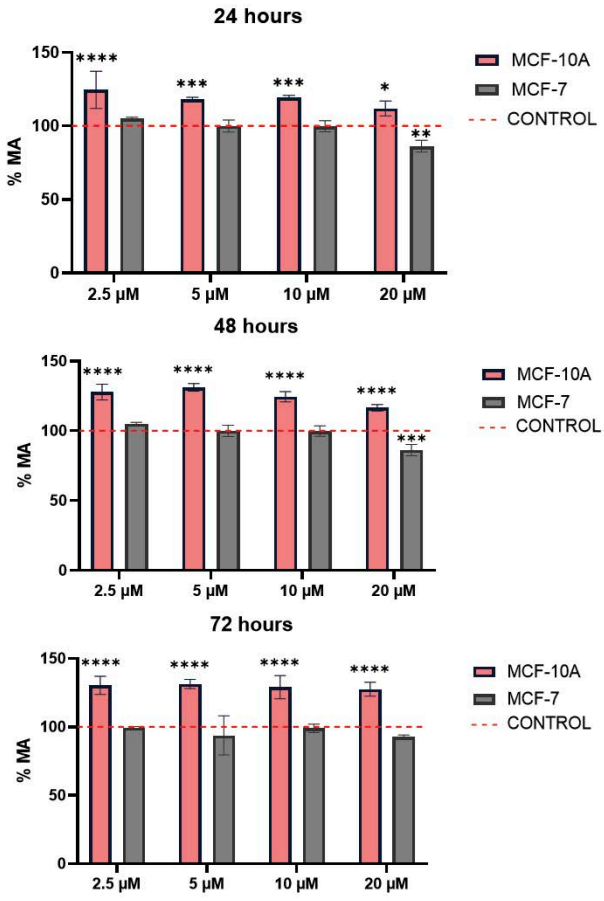

Fig. 1. The effect of CBD on the metabolic activity of MCF-10A and MCF-7 cell lines after 24, 48 and 72 hours of incubation. The graph represents the percentage of metabolic activity (MA) as measured by the MTS test in comparison with control cells (no agent added). ANOVA * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$ statistically different to control cells.

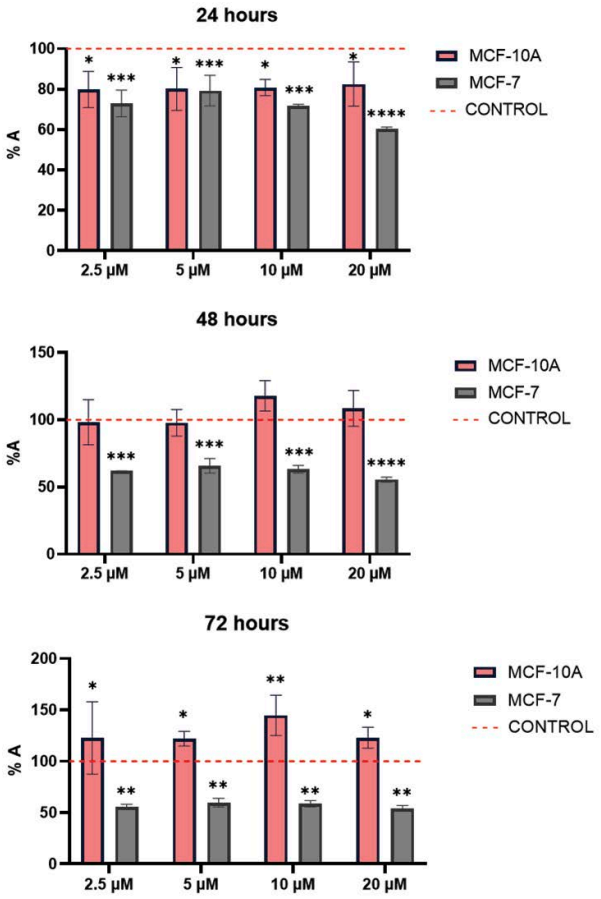

Fig. 2. The effect of CBD on cell adherence of MCF-10A and MCF-7 cell lines after 24, 48 and 72 hours of incubation. The graph represents the average percentage of cell adherence (A) as measured by the RTCA test in comparison with control cells (no agent added). % A – percentage of adherence, ANOVA * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$ statistically different to control cells.

The results of our study demonstrated a selective antiproliferative effect of CBD in a concentration-dependent manner on the MCF-7 mammary tumour cell line compared to the control. In the case of the non-tumourous breast epithelial cell line MCF-10A, CBD showed a concentration-dependent effect on cell proliferation compared to the control. These results were confirmed by MTS and RTCA methods. The most significant changes in cell proliferation inhibition were observed in the MCF-7 tumour cell line and in proliferation promotion in the MCF-10A cell line after 72 hours. A similar antiproliferative effect was also noted in a study [2], which demonstrated inhi-

Table 1. Pairwise comparations (t-test)

Variables (MCF-10A vs. MCF-7)	MTS			RTCA		
	24 h	48 h	72 h	24 h	48 h	72 h
CBD 2.5 µM	0.055387	0.002264	0.001312	0.343158	0.01991	0.029784
CBD 5 µM	0.001904	0.000398	0.011385	0.919528	0.00794	0.000201
CBD 10 µM	0.001057	0.00127	0.004551	0.018149	0.001254	0.00165
CBD 20 µM	0.002326	0.000359	0.000326	0.024255	0.002406	0.00036

* The table shows the corresponding p-values from the multiple t-tests comparing the percentage of metabolic activity (for MTS) and the mean percentage CI (for RTCA) between MCF-10A (non-tumour) and MCF-7 (tumour) cells at 24, 48, and 72 h. Multiple T-tests, statistical significance $P < 0.05$.

bition of proliferation of both MDA-MB-231 and MCF-7 breast tumour cell lines, with an IC50 value of 11.37 ± 1.82 µM and 20.04 ± 2.97 µM after 48 hours of CBD incubation. The cytotoxic effects of CBD in a range of concentrations (0–15 µM) were recorded after 24, 48 and 72 hours in the Head and neck squamous cell carcinoma (HNSCC) tumour cell line, which includes four tumour cell lines (FaDu human hypopharyngeal squamous cell carcinoma line, SNU899 human larynx squamous cell carcinoma line, SCC15 human tongue squamous cell carcinoma line, and Hep2 human epidermoid laryngeal carcinoma line). The greatest decrease in viability was recorded after 48 – 72 hours after treatment of cells with CBD (< 6 µM) compared to the control normal human oral keratinocyte cell line (HOK), indicating an antitumour effect of CBD. At the same time, the antimigratory and anti-invasion effect of CBD on human HNSCC cell lines was recorded [14].

Cannabinoids confer anti-tumour activity to them by inducing apoptosis through cannabinoid receptor 1 and 2-dependent stimulation. Although this mechanism is less clear for CBD, studies suggest that CBD may promote apoptotic death of cancer cells independently of CB1 and CB2 receptors. Regardless of the involvement of cannabinoid receptors, the promotion of apoptotic cell populations in cancer cells appears to be due to the ability of cannabinoids to stimulate ROS production. Furthermore, studies have shown that CBD can trigger glutathione (GSH) depletion, which exacerbates oxidative stress and cell death in human glioma cells. Since these specific downstream molecular pathways (ROS accumulation and GSH depletion) were not experimentally verified in our current study, it is crucial to emphasize that while these mechanisms are widely described in other cancer models [15], this premise remains a theoretical assumption that must be validated for our specific lines in future research.

An essential factor is the selective effect of CBD on tumour and healthy tissues. Comparable to our findings, several preclinical studies have shown that CBD concentrations cytotoxic to cancer cell lines did not significantly reduce the viability of healthy cells, such as primary glial cultures (up to 50 µM CBD), a human oral keratinocyte cell line (up to 15 µM for 24 hours), rat preadipocytes, and mouse monocyte and macrophage cell lines (10 µM for 72 hours) [15]. CBD should therefore be considered a multi-targeted bioactive compound capable of eliciting various responses depending on the cellular microenvironment, which includes the specific receptor profile, CBD concentration, and bioavailability [15].

There are several critical limitations in this preliminary study. First, we evaluated the response of only one non-tumourous and one tumourigenic cell line, and second, a direct veterinary cell line model was absent. This limits immediate species-specific conclusions. Although research on cannabinoids, particularly products containing CBD, has a long history in human medicine, this field is still in its early stages in veterinary medicine. Future studies should therefore employ animal models to explore potential applications of cannabinoids in adjuvant therapy for companion animals.

CONCLUSION

Cannabinoids have recently gained scientific interest due to their wide range of pharmacological effects. In our experiment, CBD demonstrated increased metabolic activity and adhesion in non-tumour MCF-10A cells, while inhibiting these parameters in tumour MCF-7 cells. These findings suggest that CBD can selectively influence cell behaviour, promoting the activity of non-tumour cells while suppressing the activity of tumour cells

and support the hypothesis that CBD is a promising agent in anti-cancer therapy with a selective effect. While these preliminary in vitro results provide an important baseline for comparative oncology, they do not support immediate or direct therapeutic application in clinical veterinary practice. In the context of veterinary pharmacology, future systematic in vitro studies utilizing specific veterinary cell lines, followed by robust in vivo animal trials, are strictly required to thoroughly elucidate the precise molecular pathways and establish clinical safety and pharmacokinetic efficacy.

Data Availability Statement

The raw data of this article will be made available by the authors, without undue reservation.

Ethical Statement

Ethical approval was not necessary for this work.

Conflict of Interest

No conflict of interest.

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Generative AI Statement

No generative artificial intelligence (AI) and AI-assisted technologies were used in writing the manuscript.

Authors' Contributions

DM designed the study and analysed the data; NN carried out the study; NH collected the data; MK and NH provided literature search and resources; DČ and JL proofread and approved the manuscript.

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ORIGINAL ARTICLE

Phthalates in breast milk

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Ethical considerations: When reporting experiments on animals Observation of the ARRIVE guidelines 2.0: Updated guidelines for reporting animal research, published on July 14, 2020 (DOI: 10.1371/journal.pbio.3000410), is applied. The authors ensure that all procedures were performed in compliance with the guidelines for animal care of their institutions or with national/international guidelines.

ABSTRACT

Breast milk collected from the 10th day through the 5th month after delivery was analyzed for DEHP (di-2-ethylhexyl phthalate) and DBP (dibutyl phthalate) content. Dibutyl phthalate concentrations ranged from 5.17 µg/L to 2.81 µg/L, and di-2-ethylhexyl phthalate was not detected in breast milk. Statistical analysis showed that the time factor influenced phthalate levels in breast milk, with a gradual decrease observed. The expected trend of increasing phthalate levels with the duration of breastfeeding was not statistically confirmed. In none of the monthly milk samples did phthalate intake exceed the tolerable daily intake (TDI) for infants. The estimated daily intake of dibutyl phthalate for infants ranged from 0.848 µg/kg to 0.355 µg/kg of body weight. The tolerable daily intake for DBP is set at 10 µg/kg of body weight per day and for DEHP at 50 µg/kg of body weight per day.

Keywords: breast milk; liquid chromatography; phthalic acid esters; plasticizers

INTRODUCTION

Phthalates are a group of widely used industrial chemicals that are primarily employed as plasticizers and are therefore found in a wide range of everyday products–from food and cosmetic packaging to flooring and medical devices [1–6].

Due to their widespread use, they are released into the air, dust, food, and water, leading to continuous exposure of the human population [7, 1]. Phthalates can enter the human body via inhalation, skin contact, and ingestion and are considered endocrine disruptors that can interfere with hormonal balance and immune system function [8, 9]. Scientific studies report their potential impact on reproductive health, fetal development, and metabolic processes, as well as links to certain chronic diseases [10–13].

One of the most vulnerable groups is breastfeeding mothers and their infants, as phthalates, being lipophilic substances, can accumulate in adipose tissue and subsequently pass into breast milk [14, 15].

Breast milk is a complex food for infants containing a significant amount of biologically active substances. The composition of breast milk undergoes changes during lactation to meet the biological needs of the growing child. The mother's diet and lifestyle also significantly influence the composition of her milk [16, 17].

Breast milk contains approximately 3–5% fat, which increases the likelihood of phthalate transfer from the mother to the newborn through breastfeeding. Through this exposure, phthalates can exert toxic effects and negatively impact the health and development of newborns. It is therefore

important to educate mothers to minimize the risk of children's exposure to these harmful substances [18, 19, 7].

A team of researchers Gajdůšková et al. [20] investigated the concentrations of polychlorinated biphenyls (PCBs), chlorinated pesticides, and phthalate-like contaminants in the breast milk of women from the Brno area. The authors found that the measured concentrations varied significantly between individual samples and in the days following childbirth. Contamination of breast milk was detected in all samples, with some showing higher levels of PCBs, while others were more heavily contaminated with pesticides. Significant differences in the levels of toxic substances were also demonstrated among individual mothers. This can be attributed to the variability of factors such as individual exposure to environmental contaminants, age, lifestyle, and overall health status.

The aim of the study was to verify the presence and levels of phthalates in real breast milk samples and to monitor their concentrations over a 5-month breastfeeding period following the mother's delivery.

MATERIALS AND METHODS

Materials and Methodology

To collect breast milk samples, three participants were contacted during their pregnancies and briefed on the sampling procedure. Breast milk from one participant served as a control sample. Another participant who had planned to provide experimental samples had to be excluded from the experiment because she was unable to provide samples continuously throughout the sampling period.

The third participant, who provided samples for the experiment, was not occupationally exposed to phthalates. She was 31 years old, weighed 64 kg before giving birth, and had a body mass index (BMI) of 23. The participant reported that she had always consumed a varied diet and did not use cosmetic products in excessive amounts.

The participants for milk collection (both test and experimental) used exclusively manual expression to minimize the potential risk of secondary contamination of the milk with phthalates from the use of manual breast pumps. During the milk collection process itself, emphasis was placed on the participant's comfort and individual preferences (it was not necessary to express milk, let the baby nurse, or specifically collect foremilk or hindmilk).

The experiment took place in 2023. The first sample collection occurred on the 10th day postpartum and lasted for 5 consecutive days. Subsequent collections were performed once a month until the fifth month postpartum, with each collection lasting 5 days within a given week. Due to the small volume of milk collected (10 to 50 mL per day), the five-day collection period was divided into two samples. Sample I was collected in the first half of the collection week; Sample II was collected in the second half of the week.

The five-month period, beginning on the 10th day postpartum, was chosen based on the assumption that if phthalates are present in milk, they may exhibit variability during lactation. For this reason, it was more appropriate to collect samples at different time intervals after delivery, which allowed for a more accurate estimate of exposure in breastfed infants and better reflected potential changes in phthalate levels during lactation.

The breast milk samples were stored by the participants in the refrigerator. After the sampling period ended, they were transferred to a freezer and stored at -18 °C.

Methods Used

Sample analysis was conducted at the Institute of Food Technology at Mendel University in Brno. Milk samples were analyzed for DEHP and DBP because the legislative limits for food were established specifically for these two, most commonly occurring phthalates. Milk fat content was determined according to the ČSN ISO 1211 (570534) [21] standard for the gravimetric determination of milk fat.

After thawing, the milk samples were tempered to 38 °C for 10 minutes. Then, 30 g of homogenized milk was weighed and quantitatively transferred to a separating funnel; the fat was extracted three times using ethanol, diethyl ether, and hexane.

The combined (three) extracts were evaporated on a vacuum rotary evaporator at 47 °C and 60 revolutions per minute and subsequently dried under a stream of nitrogen. The fat content in the milk was determined gravimetrically by weighing the evaporating flask before and after extraction. The fat was transferred to a vial and subsequently stored in a freezer (-18 °C) for further analysis using gel permeation chromatography (GPC). A blank test was prepared with each series of samples, which did not contain milk, only mixtures of organic solvents.

The separation of phthalates from the fat was performed by GPC using a mobile phase consisting of a mix-

ture of organic solvents (dichloromethane : cyclohexane, 1:1), with a mobile phase flow rate of 1 mL/min. The mobile phase was evaporated on a vacuum rotary evaporator, and the phthalate residue was quantitatively transferred to a vial for determination by high-performance liquid chromatography with UV detection. The mobile phase was acetonitrile with isocratic elution at a flow rate of 0.8 $mL \cdot min^{-1}$, and the sample injection volume was 10 µL. A Zorbax Eclipse chromatographic column with a length of 150 mm, a width of 4.6 mm, and a particle size of 5 µm was used. The column temperature was 25 °C. A diode array detector (DAD) was used for the detection of DEHP and DBP, and measurements were taken at a wavelength of 224 nm. The analysis software was Agilent OpenLAB CDS (Agilent Technologies, USA).

The detection limit providing a statistically significant signal was set at 0.02 $mg \cdot kg^{-1}$ for breast milk samples.

Phthalate quantification was performed using a calibration curve. Microsoft Excel and Statistica 14 software tools were used to quantify the results. A significance level of $p = 0.01$ (1%) was selected for the analysis, representing a strict criterion for the statistical evaluation of the results. All $p < 0.01$ values were considered statistically significant. This level was chosen to minimize the risk of error. The rationale for this approach was that breast milk is a biologically valuable matrix for which a high level of statistical reliability must be ensured ($p = 0.01$ is standard in pharmacological and medical studies). To analyze relationships between variables in data exhibiting a normal distribution, Pearson's correlation coefficient was used, which quantifies the linear dependence between two continuous variables.

Furthermore, one-way analysis of variance (ANOVA) was used to analyze the results. Tukey's test was applied for multiple comparisons (post hoc analysis).

Equipment Used

Analytical balances (OHAUS®, USA).

Ultrasonic bath (Ultrasonic Compact Cleaner 10L PS 10000).

Vacuum rotary evaporator (LabTech EV400H), water bath (HandyLab® System).

Gel permeation chromatography, precolumn 50 x 8 mm, LCP 400 pump, LCD 2083 spectrophotometric detector, WARTEX column (ECOM, spol. s.r.o., Czech Republic).

Agilent Technologies 1260 Infinity II liquid chromatograph (Agilent Technologies, USA).

RESULTS AND DISCUSSION

Results of the Analysis of Fat in Breast Milk

Twelve samples were analyzed to determine the fat content in breast milk. Table 1 shows the fat content in milk from the 10th day postpartum through the 5th month postpartum. The fat content in milk ranged from 2.33% to 4.26%. The highest fat percentage (4.73%) was recorded as early as the 1st month postpartum and then in the 5th month postpartum (4.26%). Conversely, the lowest fat content (2.12%) was found in a sample collected 3 months postpartum.

Table 1. Fat content in breast milk from the 10th day postpartum to the 5th month postpartum

Postpartum milk sample	Fat content in breast milk [g/100g]	
	Sample I	Sample II
10th day	2.33	2.34
1st month	2.52	4.73
2nd month	3.04	3.29
3rd month	2.12	3.51
4th month	2.63	2.38
5th month	4.26	3.79

Given the small amount of breast milk collected on individual days throughout the week, composite samples were created. Sample I – first half of the week (Monday–Wednesday); Sample II – second half of the week (Thursday and Friday)

A statistical analysis was performed to determine whether there is a significant relationship between the duration of lactation and the fat content of breast milk. Pearson's correlation coefficient was used to determine the strength of this correlation, and a linear regression model was subsequently constructed.

The results of the analysis did not show a statistically significant relationship between the duration of lactation and the fat content in milk ($p > 0.01$). The calculated correlation coefficient was close to zero, indicating a very weak linear relationship. The statistical analysis did not show a significant increase in fat content in breast milk with the duration of lactation. The results of the regression analysis are presented in Table 2.

Table 2. Results of the regression model showing the relationship between fat content and the duration of lactation

Dependent variable	Independent variable	Correlation coefficient R	Coefficient of determination R^2	Regression model	p-value
Fat in milk	The Days Following Childbirth	0	0	y = 2,67+0,0046·x	p > 0,01

p > 0.01 – the independent variable (duration of lactation in days) was found to be statistically insignificant

Although the expected trend of increasing fat content with advancing lactation was not clearly confirmed, the average fat values in the analyzed breast milk ranged from 3% to 4.5%. This amount corresponds to physiological norms and is consistent with values reported by a number of experts [16, 19].

Results of Phthalate Analysis in Breast Milk

To determine the content of DBP and DEHP in breast milk, 24 samples were analyzed. Samples from each measurement were analyzed in duplicate. Including the analysis of blank samples, a total of 30 analyses were performed.

DEHP was not detected in any of the analyzed samples collected from the 10th day to the 5th month postpartum. The presence of DBP in breast milk was detected from the 10th day to the 2nd month postpartum. During the lactation phase, the concentration of DBP in milk gradually decreased until it fell below the limit of detection. The average results of the phthalate analysis in breast milk are presented in Table 3.

A regression model was constructed to analyze the statistical relationship between DBP concentration and the number of days postpartum. The degree of linear correlation was assessed using Pearson's correlation coefficient. The independent variable (number of days postpartum) was found to be statistically significant ($p < 0.01$). The coefficient of determination R^2 was 0.81. This indicates that the time since delivery accounted for 81% of the variation in phthalate levels in breast milk.

Based on the above results, it can be concluded that the time factor had a statistically significant effect on the reduction of DBP levels in breast milk. The regression results are presented in Table 4.

No statistical analysis was performed for DEHP. DEHP was not detected in the breast milk samples.

DBP and DEHP levels in the breast milk of Canadian mothers were analyzed over a six-month period. Samples were collected manually to avoid potential contamination from manual breast pumps [22]. The authors recorded the highest DBP level in the 6th month postpartum (1.86 µg/L), with the most significant increase in concentrations occurring after the 3rd month. In contrast, in our subject, the highest DBP concentrations were detected as early as the first month postpartum (5.17 µg/L), followed by a gradual decline to below the limit of detection.

The authors observed the opposite trend, with DBP concentrations in breast milk increasing over time, whereas in our subject, the highest concentrations were recorded at the beginning of lactation and subsequently decreased. In the case of DEHP, the highest measured concentration was 483 µg/L, also in the 6th month postpartum. However, the authors' statistical analysis did not confirm that time

Table 3. Concentrations of DEHP and DBP in breast milk from the 10th to the 15th day postpartum through 5th month postpartum

Postpartum milk sample	Sample I		Sample II	
	DEHP	DBP	DEHP	DBP
	[µg/L milk]		[µg/L milk]	
10th day	ND	4.83	ND	4.18
1st month	ND	5.17	ND	4.52
2nd month	ND	3.52	ND	2.81
3rd month	ND	ND	ND	ND
4th month	ND	ND	ND	ND
5th month	ND	ND	ND	ND

ND – not detected; Given the small amount of breast milk collected on individual days throughout the week, composite samples were created. Sample I – first half of the week (Monday–Wednesday); Sample II – second half of the week (Thursday and Friday)

Table 4. Results of the regression model for DBP concentration as lactation progresses

Dependent variable	Independent variable	Correlation coefficient R	Coefficient of determination R^2	Regression model	p-value
DBP	The Days Following Childbirth	0,827	0,810	y = 5,41−0,0376·x	p < 0,01

had a significant effect on its increase. In comparison with our subject's results, DEHP was not detected at all in the analyzed breast milk samples.

Main et al. [23] analyzed the levels of phthalate metabolites, specifically mono(2-ethylhexyl) phthalate (MEHP) (a metabolite of DEHP) and mono-butyl phthalate (MBP) (a metabolite of DBP), in breast milk from mothers in Finland (n = 65) and Denmark (n = 57). The aim was, similarly to the case of the subject we studied, to assess the temporal evolution of phthalate exposure over a period of several months. Due to the difficulty of regular sampling, a single composite sample was ultimately created, consisting of milk collected once a month over a three-month period.

The average concentration of MEHP was 9.5 µg/L and MBP 4.3 µg/L in the Danish milk samples. Higher average concentrations were measured in the Finnish samples, namely 13 µg/L MEHP and 12 µg/L MBP.

The authors noted significant variations in phthalate concentrations. Although 45.6% of Danish participants used a manual breast pump, their average phthalate concentration was lower compared to the Finnish samples. The results indicate that the use of a manual breast pump did not affect the variability of the measured values.

Analysis of the measured DBP concentrations in our subject during the period from day 10 to 2 months postpartum averaged 4.17 µg/L. Metabolites typically reach higher concentrations than their parent compounds (they are more hydrophilic). This is a possible explanation for why DBP concentrations (4.17 µg/L) in our subject were approximately two to five times lower than those in Danish and Finnish mothers. At the same time, repeated handling of a single composite sample could have significantly contributed to higher phthalate levels due to secondary contamination.

Breast milk samples for DBP and DEHP analysis were provided by 35 mothers from China. Breast milk samples were collected manually one week after delivery, and the mothers were not professionally exposed to phthalates, just as with our subject [24]. DBP was detected in 80% of all milk samples one week after delivery, with concentrations ranging widely from 5.3 to 137.3 µg/L. The range of DEHP concentrations was not as wide but still significant, ranging from 0.5 to 66.5 µg/L, and it was detected in 74% of the samples. The mean contamination levels were 32.9 µg/L for DBP and 13.6 µg/L for DEHP.

In our subject, DBP concentrations of 4.83 µg/L and 4.18 µg/L were detected starting on the 10th day postpartum. This comparison shows that the measured concentrations in our subject were significantly lower than those in the study by Fan et al. [24].

The research team of Cao et al. [14] analyzed DBP and DEHP levels in breast milk from 305 women in Canada. The mothers provided a single milk sample, which was collected during the second month postpartum. DBP was detected in 21 of the 305 samples, with an average concentration of 13.29 µg/L. DEHP was detected in 23 samples, with an average concentration of 244.11 µg/L.

Compared to the subject we studied, the DBP concentration in the second month postpartum was 3.52 µg/L and 2.81 µg/L, which is approximately 4 times lower than the concentration (13.29 µg/L) reported in the Canadian study. At the same time, the DEHP concentration in the Canadian study was up to 200 times higher, as the DEHP concentration in our subject was below the limit of detection.

This discrepancy in results is not attributed to the sampling method, as the sampling procedure was identical to the one described in our study methodology. Environmental factors and the subjects' dietary habits, which can significantly influence phthalate exposure, are considered the more likely cause of these differences. Regional differences in the use of plastic packaging, the presence of phthalates in food and personal hygiene products, as well as various lifestyle habits, can significantly influence phthalate concentrations in breast milk.

Cao et al. [14] attribute the difference in DEHP and DBP concentrations to the primary uses of these phthalates. DEHP is primarily used as a plasticizer in food packaging, which leads to significantly higher exposure through the food chain. In contrast, DBP is most commonly used in

cosmetic products, where exposure occurs primarily via the dermal route, resulting in significantly lower accumulation compared to DEHP.

Results of Estimated Daily Intake and Risk Assessment of Phthalates

The results of the analysis of phthalates in breast milk demonstrated the presence of DBP, while DEHP was not detected in the milk. The presence of phthalates in breast milk has also been confirmed by a number of other authors [25, 26]. This fact underscores the importance of assessing the estimated daily intake of phthalates in infants and its potential impact on their health. Based on these results, the estimated daily intake of phthalates was calculated.

The estimated daily intake of phthalates (EDI) was calculated using the following equation:

$$EDI = \frac{c \times DI}{BW} [\mu g \, . \, kg^{-1}]$$

where:

c……. amount of detected phthalate in [µg/L of milk]

DI…...estimated daily milk intake [l]

BW….infant weight [kg]

The equation for calculating EDI and the average daily breast milk intake, which was set at 0.7 L, are based on studies by Liu et al. [25] and Kim et al. [26]. The infant's weight was recorded monthly by our study participant.

The EDI results for DBP are presented in Table 5. To assess the health risk of DBP and DEHP exposure to infants, the EDI results were compared with tolerable daily intake (TDI) values. For DEHP, the TDI is set at 50 µg/kg body weight per day, while for DBP, the TDI is 10 µg/kg body weight per day [27, 28].

The results of the phthalate analysis in breast milk showed that DEHP was not detected in any sample. For this reason, DEHP did not exceed the TDI (50 µg/kg) in any month following delivery.

The estimated daily intake for DBP was 0.848 µg/kg starting on the 10th day postpartum, 0.748 µg/kg in the first month postpartum, and 0.355 µg/kg in the second month. Starting from the third month postpartum, DBP was no longer detected in breast milk. A comparison of the EDI and TDI values confirms that the established TDI for DBP (10 µg/kg) was not exceeded in any month postpartum.

Liu et al. [25] also conducted an analysis of phthalates in breast milk, but only in the first month postpartum. They then compared the EDI with the TDI. The EDI was 0.228 µg/kg for DEHP and 0.048 µg/kg for DBP. The authors ruled out any exceedance of the TDI, indicating that the use of phthalates during breastfeeding does not pose any apparent health risk to infants. However, they note that risk assessment based solely on dietary intake may underestimate the actual health risk of phthalate exposure. The EDI values for DBP in the first month postpartum (0.748 µg/kg) in the subject we monitored were up to 16 times higher than those reported by Liu et al. [25].

The estimated daily intake of DBP and DEHP in breast milk samples from Korea, collected between the 1st and 2nd month postpartum, showed a very wide range of measured concentrations. For DEHP, the EDI ranged from 0.61 µg/kg to 6.72 µg/kg, and for DBP, it ranged from 0.08 µg/kg to 0.58 µg/kg. The authors ruled out EDI exceedances and attributed the wide range of measured concentrations to various factors, such as lifestyle, diet, or daily exposure to toxic substances [26]. For comparison, the EDI values for DBP in our subject in the second month postpartum were 0.355 µg/kg, which is close to the average EDI value reported by Kim et al. [25].

The estimated daily intake of phthalates during the six-month analysis conducted by Zhu et al. [22] showed that

Table 5. Estimated daily intake of DBP depending on the period of breast milk collection and infant weight

Postpartum milk sample	Average value DBP [µg/L milk]	Weight of an infant [g]	Estimated daily intake (EDI) DBP [µg/kg]
10th day	4.509	3 720	0.848
1st month	4.845	4 540	0.748
2nd month	3.165	5 550	0.355
3rd month	ND	6 180	-
4th month	ND	6 580	-
5th month	ND	6 815	-

ND – not detected

values for DBP ranged from 0.38 to 0.65 µg/kg and DEHP reached values of 82–167 µg/kg. In this case, the TDI for DEHP was exceeded. The study authors describe these findings as alarming and emphasize that infants' actual exposure to phthalates could have been significantly higher. They highlight the ubiquitous presence of these contaminants. In addition to breast milk, infants may be exposed to phthalates through the air, cosmetics, packaging materials, and toys, which further increases their overall exposure. In our study participant, the average EDI value for DBP from day 10 to the 5th month postpartum was 0.18 µg/kg, which is lower than the value reported by Zhu et al. [22].

CONCLUSION

Based on the results of DBP concentrations in breast milk, DBP was present in the samples from the 10th day to the 2nd month postpartum. During this period following delivery, a gradually decreasing concentration of DBP in milk was observed. The average DBP concentration was 4.5 µg/L from the 10th to the 15th day postpartum, 4.84 µg/L in the first month postpartum, and 3.16 µg/L in the second month postpartum. From the 3rd to the 5th month postpartum, DBP was no longer detected in breast milk samples. Statistical analysis confirmed that the duration of lactation had a significant effect on phthalate levels in breast milk, with a gradual decrease observed in 81% of cases. In all analyzed samples collected from the 10th day to the 5th month postpartum, the presence of DEHP was not detected.

The results showed that even higher fat content in the samples did not cause increased accumulation of phthalates in breast milk.

To assess the health risk of DBP and DEHP exposure in infants, the estimated daily intake results were compared with the tolerable daily intake (TDI) values. The results of this study indicate that the tolerable daily intake for DBP was not exceeded in any month following delivery. Since DEHP was not detected in the breast milk samples, the tolerable daily intake was not exceeded in this case either. None of the analyzed breast milk samples exceeded the tolerable daily intake.

The fact that only DBP was detected in breast milk, while DEHP was not, can be explained by the different physical-chemical properties of these two phthalates. DEHP is much more lipophilic, has a higher molecular weight, and breaks down in the body into its hydrolyzed and oxidized metabolites, which get into milk differently than the original compound. On the other hand, DBP is less lipophilic, more stable in its original form, and its transfer into biological samples, including breast milk, is more common. These differences may explain why only DBP was found in the analyzed samples.

The main limitation of this study is that breast milk analyses were ultimately done on just one donor. Such a small number of samples doesn't allow the results to be generalized to the wider population and doesn't let us assess individual differences in DEHP and DBP concentrations.

The obtained data should be interpreted as pilot results, serving primarily to verify the analytical methodology and to highlight the potential health risk associated with the presence of phthalates in breast milk. To confirm these findings, it is necessary to analyze a larger set of samples.

At the same time, it is important to note that in the Czech Republic, very little scientific attention has been devoted to the issue of phthalates in breast milk. The limited amount of data and research studies in this area points to the need for more systematic monitoring and more thorough investigation of phthalate exposure via breast milk.

Data Availability Statement

The raw data of this article will be made available by the authors, without undue reservation.

Ethical Statement

Ethical approval was not necessary for this work.

Conflict of Interest

No conflict of interest.

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Authors' Contributions

A.J. – experiment design, methodological supervision, manuscript drafting

V.K. – procurement of samples, conducting analyses, data processing

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