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Changes in the microbiota of Bryndza cheese after frozen storage

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ABSTRACT

Bryndza cheese is a traditional Slovakian product. In this research, we have investigated whether it would be possible to freeze bryndza, store it at a temperature of -18°C, and then thaw and place it on the market during the off-production season. The current legislation in Slovakia does not allow this procedure. The freezing process was chosen based on the request of several small food business operators who would like to replace the process of preserving the primary raw material, matured salted ewe's lump cheese, in barrels due to acrid-sour taste. Bryndza cheese is preferred by consumers due to its unique microbial composition, which is beneficial for their health. Many microorganisms present in bryndza are probiotics. For this reason, we wanted to determine how the microflora in the bryndza cheese changes after freezing. These findings have practical implications for the food industry, particularly for small food business operators, who can potentially adopt freezing to preserve bryndza, thereby extending its shelf life and availability to consumers. Additionally, in many households, people store bryndza in their freezers after purchasing and use it to prepare dishes. Understanding the role of microorganisms in the ripening process and during storage can provide valuable data on Brynzda quality and safety. The present study aimed to analyse the representation of microorganisms in "Bryndza" samples at the beginning of storage and after 6 months of storage at a temperature of -18 °C. A total of 10 samples of "Bryndza" cheese made from pasteurised milk were analysed. Analysis of total viable counts of viable bacteria (TVC), coliform bacteria (CB), lactic acid bacteria (LAB), and microscopic filamentous fungi (MFF) was performed using the plate dilution method. Isolated strains of microorganisms were identified with mass spectrometry MALDI-TOF MS Biotyper. A total of 295 isolates from Bryndza cheese were identified at the start of storage and 220 isolates at the end of storage of samples. The dominant species of microorganisms found in Bryndza cheese were lactic acid bacteria, especially *Lactococcus lactis*, with 68 isolates and *Lactobacillus fermentum*, with 41 isolates at the start of storage. The most frequently isolated species were *Lactococcus lactis*, with 62 isolates, and *Limosilactobacillus fermentum*, with 33 isolates. Our results show that important lactic acid bacteria were present in the bryndza even after 6 months of freezing, but coliform bacteria were absent. Experimental outputs: TVC: showed no significant decrease (p-value = 0.0137); LAB: No significant decrease in lactic acid bacteria counts post-storage; MFF: Significant decrease in microscopic filamentous fungi post-storage; CB: Qualitative analysis indicates a significant reduction to undetectable levels after storage. Long-term storage of bryndza at -18°C is safe from a microbiological point of view.

Keywords: lactic acid bacteria, coliform bacteria, mass spectrometry, identification of microorganisms, Bryndza cheese

INTRODUCTION

Bryndza is a natural, white, mature and spreadable cheese manufactured traditionally in specified mountainous areas of the Slovak Republic and dairy factories. Slovenská bryndza (Slovak bryndza) cheese has been protected by Protected Geographical Indication since 2008 **[1]**. It is manufactured using traditional techniques using either 100 % matured ewe's lump cheese or a mix with a maximum of 50 % cow's lump cheese in dry matter **[2]**. The primary compound used in the production of bryndza is ewes' lump cheese, made by a two-stage ripening process that takes eight to fourteen days **[3]**. Bryndza cheese contains natural microflora in raw ewe's milk and cows's lump cheese, as well as the distinctive production process. The raw raw material for making Slovak Bryndza is either matured ewe's lump cheese or a mix of matured ewe's and cow's **[4]**. Bryndza is usually made from unpasteurised milk with unique microflora in traditional farms. In dairy companies, pasteurised milk is inoculated by starter bacteria. The production process includes renneting, curd cutting, lump cheese forming, fermentation and ripening of the lump cheese. In the first fermentation step, the temperature in the fermentation room is 21 – 25 °C for 2 – 3 days until the pH drops to 5.2. In the second step, the temperature is 8 – 20 °C for 4 – 6 days until the pH is 4.2 – 4.8 **[5]**. The quality of "Bryndza" cheese, including its composition, properties and microbial diversity, depends on the quality of the ewe milk **[6]** and the production process **[5]**, **[7]**.

The manufacturing season of fresh ewe's Bryndza ends in the autumn; animals are stable, and milk production is stopped. To manage the overproduction of ewe's lump cheese during the season, it is preserved by salting. The process involves layering the sliced sheep's lump cheese in foil-lined barrels. Each layer is salted and pressed. The filled barrel is then closed and kept in cold storage. We called it barreled ewe's lump cheese. This barreled cheese is mixed with fresh cow's lump cheese in winter to soften its sharp taste.

Traditional producers have asked us to verify whether this traditional technological phase can be replaced by the freezing process. For this purpose, we examined how this cheese's microflora would change due to freezing and storage.

Numerous research was conducted using Bryndza cheese since it is thought that the composition and activity of microflora give different types of cheese their flavour and aroma. Data from older studies, which identified *Lactobacillus* spp., *Lactococcus* spp., *Streptococcus* spp., *Enterococcus* spp., *Kluyveromyces marxianus* and *Geotrichum candidum* as main components of the microflora of Bryndza cheese **[3]**. Some authors **[8]**, **[9]** revealed the presence *of Lactobacillus delbrueckii*, *Lactobacillus brevis*, *Lactobacillus lactis*, *Lactobacillus raffinolactis*, *Streptococcus macedonicus*, *Streptococcus thermophilus*, *Leuconostoc pseudomesenteroides*, *Debaromyces hansenii*, *Mucor fragilis*, *Yarrowia lipolytica and Galactomyces geotrichum / Geotrichum candidum* (now called *Galactomyces candidus / Geotrichum candidum*) in Bryndza cheese.

This work aims to determine whether the process of preserving Bryndza by salting and storing it in barrels can be replaced by freezing and how the freezing process may affect the variability of microorganisms in Bryndza cheese during storage at -18°C.

Figure 1 Ewe's lump cheese, barrelled and salted ewe's lump cheese, Bryndza.

Scientific Hypothesis

Microflora of Bryndza cheese will change after the freezing and storage at -18°C.
Ewes´cheese Bryndza is a valuable source of lactic acid bacteria.

MATERIAL AND METHODOLOGY

Samples

Ten samples of Bryndza cheese from various regions of Slovakia were evaluated for microbiological quality in the present study. Table 1 shows the region of production and ewes´ milk content.

Table 1 Ewe's Bryndza cheese sample

Sample	Producer and region of production	Ewe's and Cow's lump cheese content
1	Veľký Krtíš	100 %
2	Humenné	100 %
3	Liptovský Mikuláš	100 %
4	Turčianske Teplice	50 %
5	Považská Bystrica	50 %
6	Ilava	50 %
7	Dolný Kubín	50 %
8	Zvolen	50 %
9	Detva	50 %
10	Gelnica	50 %

Chemicals

Distilled water (Sigma-Aldrich, St. Louis, MO, USA), absolute ethanol (Bruker Daltonik, Bremen, Germany), 70 % formic acid (v/v) (Sigma-Aldrich, USA), acetonitrile (Sigma-Aldrich, USA), trifluoroacetic acid (Sigma-Aldrich, USA).

Plate count agar (PCA, Oxoid, Basingstoke, UK), Violet Red Bile lactose agar (VRBL, Oxoid, Basingstoke, UK), Rogosa and Sharpe agar (MRS, Oxoid, Basingstoke, UK), Dichloran-rose Bengal chloramphenicol agar (DRBC, Oxoid, Basingstoke, UK), Tryptone Soya Agar (TSA agar, Oxoid, UK).

Instruments

Shaker (GFL 3031, Burgwedel,Germany), centrifuge (ROTOFIX 32A, Ites, Vranov, Slovakia), MALDI-TOF-MS Biotyper (Bruker Daltonics, Bremen,Germany).

Laboratory Methods

The plate dilution method was used to analyze microorganisms from Bryndza cheese. The TVC was determined according to ISO 4833-2:2013 **[10]**, the number of CB was determined according to ISO 4832:2006 **[11]**, the number of LAB was performed according to ISO 15214:1998 **[12]**, and the number of MFF was performed according to ISO 21527-1:2008 **[13]**. A MALDI-TOF MS Biotyper mass spectrometer (Bruker, Daltonics, Bremen, Germany) was used to identify isolated microorganisms from samples.

Description of the Experiment

Sample preparation:

The samples were obtained in May 2023. The samples were placed in sterile sample containers and transported to the microbiological laboratory at 5 °C. Microbiological analyses were performed immediately at the same sampling day and then after 6 months of storage of samples at -18 °C.

Number of samples analyzed: 10
Number of repeated analyses: 3
Number of experiment replication: 3

Design of the experiment:

Microbioloogical analysis:

The primary dilution of the samples was made to prepare them for testing as follows: 5 mL of sample was added to 45 mL of 0.89 % saline solution. The samples were homogenized for 30 minutes using a shaker (GFL 3031, Burgwedel, Germany). Then, the serial dilutions (10^{-2} to 10^{-4}) were done, and 0.1 mL of an aliquot from the appropriate dilution was pipetted and spread on plate count agar media.

Total viable counts (TVC) were determined using plate count agar (PCA, Oxoid, Basingstoke, UK), and inoculated Petri dishes were incubated at 30 °C for 48 – 72 h. Coliform bacteria (CB) were determined using

Violet Red Bile lactose agar (VRBL, Oxoid, Basingstoke, UK) and inoculated Petri dishes were incubated at 37 °C for 24 – 48 h. Lactic acid bacteria (LAB) were determined using Rogosa and Sharpe agar (MRS, Oxoid, Basingstoke, UK), and inoculated Petri dishes were incubated with 5% CO_2 at 30 °C for 48 – 72 h.

Microscopic filamentous fungi (MFF) were determined using Dichloran-rose Bengal chloramphenicol agar (DRBC, Oxoid, Basingstoke, UK). Inoculated Petri dishes were incubated at 25 °C for 5 – 7 days. All measurement analyses were conducted in triplicate.

Preparation of MALDI matrix solution:

The stock solution consisted of acetonitrile (50 %) (Sigma-Aldrich, USA), water (47.5 %) (Sigma-Aldrich, USA), and trifluoroacetic acid (2.5 %) (Sigma-Aldrich, USA). 500 µL of 100 % acetonitrile, 475 µL of distilled water, and 25 µL of 100 % trifluoroacetic acid were pipetted into an Eppendorf tube.

Sample Preparation and MALDI-TOF MS Measurement:

The identification of microorganisms isolated from „Bryndza“ ewes' cheese samples was performed using MALDI-TOF (matrix-assisted laser desorption/ionization time of flight) MS Biotyper (Daltonics, Bremen, Germany). Before identification, bacterial and yeast colonies were subcultured on Tryptone Soya Agar (TSA agar, Oxoid, UK) for 18 – 24 hours. Out of the eight bacterial isolates, one colony was chosen. Colonies of bacteria and yeast were suspended in a solution containing 900 µL of absolute ethanol (Bruker Daltonik, Bremen, Germany) and 300 µL of distilled water (Sigma-Aldrich, St. Louis, MO, USA). The mixture was centrifuged at 13,000 rpm for 2 min. After draining the supernatant, the pellet was combined with 50 µL of 70 % formic acid (v/v) (Sigma-Aldrich, USA) and 50 µL of acetonitrile (Sigma-Aldrich, USA). Following another centrifugation, 1 µL of the supernatant was applied to a steel plate and air-dried at 20 °C. Subsequently, 1 µL of MALDI matrix was applied to the samples. The MALDI Biotyper 3.0 program evaluated the mass spectra data (Bruker Daltonik, Germany). A score between 2.000 and 2.299 indicated a secure genus identification with probable species identification, a score between 1.700 and 1.999 suggested probable identification at the genus level, and a score below 1700 was considered unreliable for identification. These scores were used to determine the identification criteria.

Statistical Analysis

All experiments were carried out in triplicate. The means and standard deviations were calculated for microbial counts, lactic acid bacteria counts, coliform bacteria counts, and microscopic filamentous fungi counts. Krona charts were used to visualise the relatedness of the identified microbial isolates. We have used XLSTAT 2024.1 (Lumivero).

Firstly, we performed the Wilcoxon signed-rank test to analyse if there was a statistically significant decrease in Total Viable Counts (TVC), Coliform Bacteria (CB), Lactic Acid Bacteria (LAB), and Microscopic Filamentous Fungi (MFF) in Brynzda cheese stored at -18 °C during 6 months. Null Hypothesis (H0): There is no decrease in the TVC, CB, LAB and MFF after freezing and storage at -18 °C. Alternative Hypothesis (H1): Freezing and storage at -18 °C for 6 months decreases the TVC, CB, LAB and MFF.

Thereafter, we performed the chi-squared test to assess whether the proportions of microorganism species significantly changed from the beginning of storage to after 6 months.

RESULTS AND DISCUSSION

Freezing is a popular food preservation method that applies low temperatures to the product, which converts liquid water into ice crystals. The low water activity (a_w) and decreased molecular mobility resulting from freezing significantly slow down the kinetics of chemical and enzymatic reactions like proteolysis and oxidation, as well as physical changes such as mass transfers, including recrystallisation or phase separations **[14]**, **[15]**. Generally, lower freezing temperatures slow the kinetics of these deteriorative reactions **[16]**. Moreover, during frozen storage, the growth of microorganisms is stopped or delayed. The freezing process and the storage at subzero temperatures may increase the mortality of microorganisms because of the mechanical damage caused by the intracellular and extracellular ice crystal formation to the microbial membranes, dehydration of the cells caused by water pressure differences and the presence of osmotic gradients **[17]** and **[18]**. A significant challenge in the ewes' milk and goats' milk cheese manufacturing industry is the seasonal variation in milk production, leading to considerable differences in cheese output between summer and winter. To address this issue and ensure a consistent cheese supply, curds and fresh, brined, unripened cheeses produced during peak milk production periods are frozen and stored. These can then be thawed and ripened during times of lower milk production. This approach maintains a steady market supply throughout the year **[19]**.

In our research, we have found that freezing impacted the viability of the microbiota in the cheese. Although there was a slight recovery, reaching normal growth levels during the subsequent ripening period was insufficient.

The average number of total viable count (TVC) was in the range from 4.26 ±0.01 log CFU.g^{-1} in sample no. 8 to 5.03 ±0.02 log CFU.g^{-1} in sample no. 4. In the study of Kačániová et al. **[20]**, the total viable counts in the samples of Bryndza cheese ranged from 3.87 to 4.32 log CFU.g^{-1}. Coliforms exhibit a high mortality rate during freezing **[21]**, **[22]** and **[23]**. In our study, coliform bacteria (CB) were not present in sample no. 1. The highest number of CB was 2.87 ±0.02 log CFU.g^{-1} in sample no. 3. Other authors **[6]** reported the numbers of coliform bacteria in spring Bryndza at the level of 3.87 log CFU.g^{-1}, which represents higher numbers compared to our results. The average value of lactic acid bacteria (LAB) ranged from 4.00 ±0.03 log CFU.g^{-1} in sample no. 7 to 4.90 ±0.02 log CFU.g^{-1} in sample no. 3. Other authors **[6]** and **[24]** detected higher numbers of lactic acid bacteria (from 10^8 to 10^9 CFU.g^{-1}, respectively) in Bryndza cheese made from unpasteurised ewe milk. Yeasts belong to the natural microbiota of Bryndza cheese and contribute to the ripening of the cheese **[25]**. The average number of microscopic filamentous fungi (MFF) was in the range from 1.00 ±0.01 log CFU.g^{-1} in sample no. 8 to 2.30 ±0.02 log CFU.g^{-1} in sample no. 4. Yeasts and moulds significantly contribute to the deterioration of dairy products, which can lead to changes in the taste, texture and colour of the products. In addition, the spread of moulds in dairy products poses health risks to consumers **[26]**. Table 2 shows the results of the microbial analysis of "Bryndza" cheese at the beginning of storage.

Table 2 Microbiota of Bryndza cheese at the beginning of storage (average ± SD log CFU.g^{-1}).

	TVC	CB	LAB	MFF
1	4.71 ±0.04	< 1	4.79 ±0.02	2.18 ±0.02
2	5.01 ±0.02	2.44 ±0.02	4.58 ±0.02	1.68 ±0.02
3	4.89 ±0.01	2.87 ±0.02	4.90 ±0.02	2.02 ±0.02
4	5.03 ±0.02	2.73 ±0.02	4.71 ±0.01	2.30 ±0.02
5	4.67 ±0.01	2.65 ±0.02	4.76 ±0.02	1.99 ±0.01
6	5.02 ±0.03	2.00 ±0.01	4.76 ±0.01	1.68 ±0.02
7	4.64 ±0.02	1.46 ±0.01	4.00 ±0.03	1.93 ±0.01
8	4.26 ±0.01	1.11 ±0.01	4.77 ±0.02	1.00 ±0.01
9	4.41 ±0.01	1.86 ±0.04	4.83 ±0.02	1.47 ±0.02
10	4.80 ±0.01	2.42 ±0.02	4.78 ±0.02	1.02 ±0.01

Note: TVC – total viable counts, CB – coliforms bacteria, LAB – lactic acid bacteria, MFF – microscopic filamentous fungi, SD – standard deviation.

The number of individual groups of microorganisms was determined after 6 months of storage at a temperature of -18 °C. The average number of TVCs ranged from 4.86 ±0.01 (sample no. 1) to 5.31 ±0.01 log CFU.g^{-1} (sample no. 9). Coliform bacteria were not present in any of the analyzed samples after 6 months of storage. The average number of LAB were in the range from 4.03 ±0.02 (sample no. 7) to 5.44 ±0.01 (sample no. 3). The average number of MFF ranged from 1.51 ±0.02 (sample no. 10) to 2.69 ±0.01 log CFU.g^{-1} (sample no. 1).

Food cultures produced by microorganisms include yeasts, fungi, and bacteria. These microorganisms determine the fermented food's flavour, texture, and acidity and provide health advantages beyond basic nourishment **[27]**. Table 3 shows the results of the microbial analysis of "Bryndza" cheese at the end of storage.

Table 3 Microbiota of Bryndza cheese at the end of storage (average ± SD log CFU.g^{-1}).

	TVC	CB	LAB	MFF
1	4.86 ±0.01	< 1	4.45 ±0.01	2.69 ±0.01
2	4.90 ±0.01	< 1	4.67 ±0.01	1.89 ±0.02
3	5.09 ±0.02	< 1	5.44 ±0.01	2.02 ±0.02
4	5.09 ±0.01	< 1	5.03 ±0.01	2.17 ±0.02
5	4.97 ±0.01	< 1	4.66 ±0.01	2.13 ±0.02
6	4.86 ±0.01	< 1	4.10 ±0.01	1.76 ±0.03
7	4.96 ±0.01	< 1	4.03 ±0.02	2.03 ±0.02
8	5.00 ±0.01	< 1	4.82 ±0.01	1.72 ±0.03
9	5.31 ±0.01	< 1	4.93 ±0.02	1.66 ±0.02
10	5.10 ±0.01	< 1	4.90 ±0.02	1.51 ±0.02

Note: TVC – total viable counts, CB – coliforms bacteria, LAB – lactic acid bacteria, MFF – microscopic filamentous fungi, SD – standard deviation.

The Wilcoxon signed-rank test was conducted to evaluate whether there was a decrease in the TVC, CB, LAB and MFF after 6 months of storage at -18 °C. The test results yielded p-values of 0.0137 (TVC), 0.3125 (LAB), and 0.0104 (MFF), not calculated for (CB). This result indicates a statistically significant reduction in TVC, not a statistically significant decrease in LAB, but a statistically significant reduction in MFF. Since the CB counts are reported as "<1" for all samples after storage, this indicates a reduction to below the detection limit in all cases. This qualitative result strongly suggests a significant decrease in CB due to storage, although a specific p-value cannot be provided without exact counts.

A total of 295 isolates from cheese "Bryndza" were identified using MALDI-TOF Biotyper at the beginning of storage. The dominant species of microorganisms found in Bryndza cheese were lactic acid bacteria, especially, *Lactococcus lactis* (68 isolates), *Lactobacillus fermentum* (41 isolates), *Lactobacillus delbrueckii* (33 isolates), *Lactiplantibacillus plantarum* (32 isolates), *Lacticaseibacillus rhamnosus* (30 isolates) and *Lactiplantibacillus paraplantarum* (28 isolates). Other authors **[24]** determined that during one production season, the genus *Lactobacillus* was present in every sample of bryndza cheese manufactured in seven specialised factories in various Slovakia locations. The bacteria *Lactococcus lactis* was isolated and detected in every sample of bryndza cheese. The absence of pathogenic and spoilage bacteria in ewes cheese could depend on the quality of raw milk utilised **[28]**; moreover, the thermal treatment applied during stretching **[29]** and the microbial biofilms of the wooden vats **[30]** also contribute to the safety of the resulting cheeses. Isolated species of microorganisms from Bryndza cheese at the beginning of storage are shown in Table 4.

Table 4 Number of isolates of microorganisms from Bryndza cheese at the beginning of storage.

Species	**Sample**										
	1	2	3	4	5	6	7	8	9	10	Total
Geotrichum silvicola	2										2
Lactococcus lactis	5	9	8	7	8	7	6	4	6	8	68
Yarrowia lipolytica		1	3	2			2	1			9
Escherichia coli				1		8		2			11
Raoultella ornithinolytica							1				1
Pichia cactophila							1				1
Enterococcus faecium									4		4
Enterobacter cloacae										2	2
Enterobacter xiangfangensis										1	1
Lactiplantibacillus plantarum	4	3	2	6	2	8	3	1	1	2	32
Limosilactobacillus fermentum	2	5	4	3	4	3	6	3	5	6	41
Lactobacillus delbrueckii	2	3	3	3	4	5	4	5	2	2	33
Lactiplantibacillus paraplantarum	2	1	3	2	3	4	2	3	5	3	28
Lacticaseibacillus rhamnosus	2	2	3	3	4	3	2	3	5	3	30
Hafnia alvei									1		1
Latilactobacillus curvatus	2	1	1	3	2	4	2	3		1	19
Leuconostoc lactis	3		1		1		3				8
Leuconostoc pseudomesenteroides				1			1	2			4
Total											**295**

Diverse microbiota comprises the *Lactococcus, Streptococcus, Lactobacillus*, and *Enterococcus genera. Lactococcus lactis* subsp. *lactis and Lactococcus lactis* subsp. *cremoris* were present in all tested Bryndza cheese samples **[31]**. In another study, *Lactococcus, Pediococcus, Enterococcus*, and *Streptococcus* were abundant in Bryndza from different Slovak regions **[32]**.

Our research identified 220 isolates from Bryndza cheese after 6 months of storage at -18 °C (Table 5). The most frequently isolated species included *Lactococcus lactis* (62 isolates), *Limosilactobacillus fermentum* (33 isolates), *Lactiplantibacillus plantarum* (30 isolates), and *Levilactobacillus brevis* (18 isolates).

Table 5 Number of isolates of microorganisms from Bryndza cheese after 6 months of storage at -18 °C.

Species	Sample										
	1	2	3	4	5	6	7	8	9	10	Total
Limosilactobacillus fermentum	1	4	4	1	3	6	5	2	4	3	33
Pediococcus pentosaceus	1					1			2	1	5
Levilactobacillus brevis	2		3	1		2		4	2	4	18
Lactiplantibacillus plantarum	3	3	5	4	6	3	2	2	1	1	30
Leuconostoc lactis	2	1	1	3	1			2	1	1	12
Candida krusei		3									3
Candida rugosa		1									1
Enterococcus faecalis	5	1							1		7
Lactococcus lactis		9	10	8	4	1	2	7	10	11	62
Yarrowia lipolytica		1	2	4	2			2	1		12
Geotrichum silvicola					2						2
Enterococcus faecium	6					7		3	1		17
Streptococcus salivarius ssp. *thermophilus*				1							1
Enterococcus durans					2						2
Geotrichum candidum	4	2			3	2					11
Kluyveromyces lactis						1					1
Candida intermedia									2	1	3
Total											**220**

Table 6 The classification of microorganisms into families.

Bacteria	Family
Lactobacillus plantarum	*Lactobacillaceae*
Limosilactobacillus fermentum	
Lactobacillus delbrueckii	
Lactiplantibacillus paraplantarum	
Lacticaseibacillus rhamnosus	
Latilactobacillus curvatus	
Leuconostoc lactis	
Leuconostoc pseudomesenteroides	
Pediococcus pentosaceus	
Levilactobacillus brevis	
Lactococcus lactis	*Streptococcaceae*
Streptococcus salivarius ssp. thermophilus	
Escherichia coli	*Enterobacteriaceae*
Raoultella ornithinolytica	
Enterobacter cloacae	
Enterobacter xiangfangensis	
Hafnia alvei	
Enterococcus faecium	*Enterococcaceae*
Enterococcus faecalis	
Enterococcus durans	
Yeasts	
Yarrowia lipolytica	*Dipodascaceae*
Geotrichum candidum	
Geotrichum silvicola	
Pichia cactophila	*Saccharomycetaceae*
Candida krusei	
Candida rugosa	
Candida intermedia	
Kluyveromyces lactis	

The key findings of the chi-squared test were:

- *Lactococcus lactis*: Minor decrease, not statistically significant (P-value = 0.205061).
- *Enterococcus faecium* showed a significant change in its count, increasing from 4 to 17 (p-value = 0.000654). This indicates a significant increase in proportion relative to other microorganisms over the storage period.
- Other species, like *Limosilactobacillus fermentum and Yarrowia lipolytica*, did not show significant changes, although their p-values were relatively low (P-value = 0.205061 and 0.247978). This suggests some change, but not statistically significant.

These results suggest that while some species exhibit notable changes in their population dynamics, only *Enterococcus faecium* showed a statistically significant increase. This could be due to adaptive advantages or changes in the cheese environment favouring its growth over time.

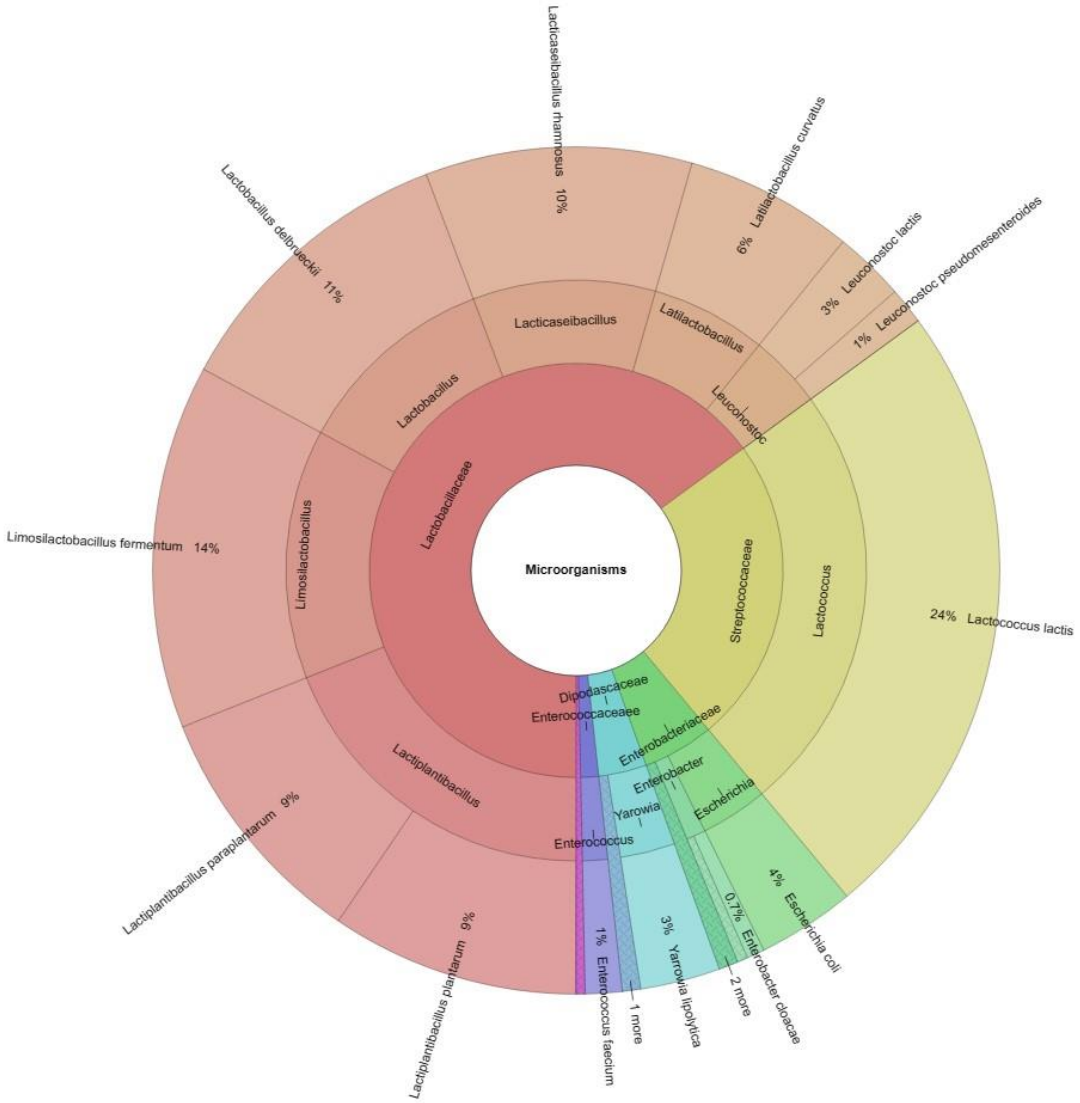

Figure 2 Krona chart for microorganisms isolated from "Bryndza" cheese at the begining of storage.
Note: outermost ring: species, middle ring: genus, innermost ring: family.

Lactic acid bacteria produce organic acids (lactic and acetic acid), hydrogen peroxide, ethanol, diacetyl, acetaldehyde, acetoin, carbon dioxide, and bacteriocins—metabolites categorised as antimicrobial agents. Additionally, the low pH caused by organic acid synthesis limits pathogenic microorganisms' action **[33]**.

In the study of **[34]**, the genus *Enterococcus*, belonging to the family *Enterococcacae*, was detected in 3.18 % of tested cheeses. While enterococci are sometimes thought of as pathogens in cheeses, they can also operate as probiotic microbiota, producing antimicrobial substances known as bacteriocins **[35]**, **[36]**.

The classification of microorganisms into families is shown in Table 6.
Lactic acid bacteria, mainly *Lactobacillus* species, *Lactococcus, Pediococcus, Enterococcus*, and *Streptococcus*, were the dominant group of bacteria found in Bryndza from several locations in Slovakia **[37]**.

Our results show that 9 species of bacteria belonging to the *Lactobacillaceae* family (63 %) were isolated from Bryndza cheese samples immediately after opening. Bacteria belonging to the *Streptococcaceae* family represented 24 % of the isolated microorganisms. Microorganisms belonging to *Enterobacteriaceae* (6 %) were also represented, where 4 bacterial species were isolated. The family *Dipodascaceae* (4 %) with 2 species, *Enterococcaceae* (1 %) with 1 species, and the family *Saccharomycetaceae* (0.34 %) represented by 1 species (Figure 2).

Lactic acid bacteria belong to the helpful microbiota in milk. Representatives of the genera *Streptococcus, Leuconostoc*, or *Lactobacillus* can utilize lactose, broken down in the lactic acid **[38]**.

Lactic acid bacteria (i. e. *Lactococcus* spp., *Streptococcus* spp., *Lactobacillus* spp. and *Leuconostoc* spp.) dominated, forming a share of 57.8–99.6 % of all identified bacteria. After 6 months of sample storage, 5 species belonging to the family Lactobacillaceae (45 %) were isolated, followed by microorganisms belonging to the family Streptococcaceae (28 %) with 1 species, the family *Enterococcaceae* (12 %) with 3 species, the family *Dipodascaceae* (11 %) with 3 species and the family *Saccharomycetaceae* (4 %) with 4 species (Figure 3).

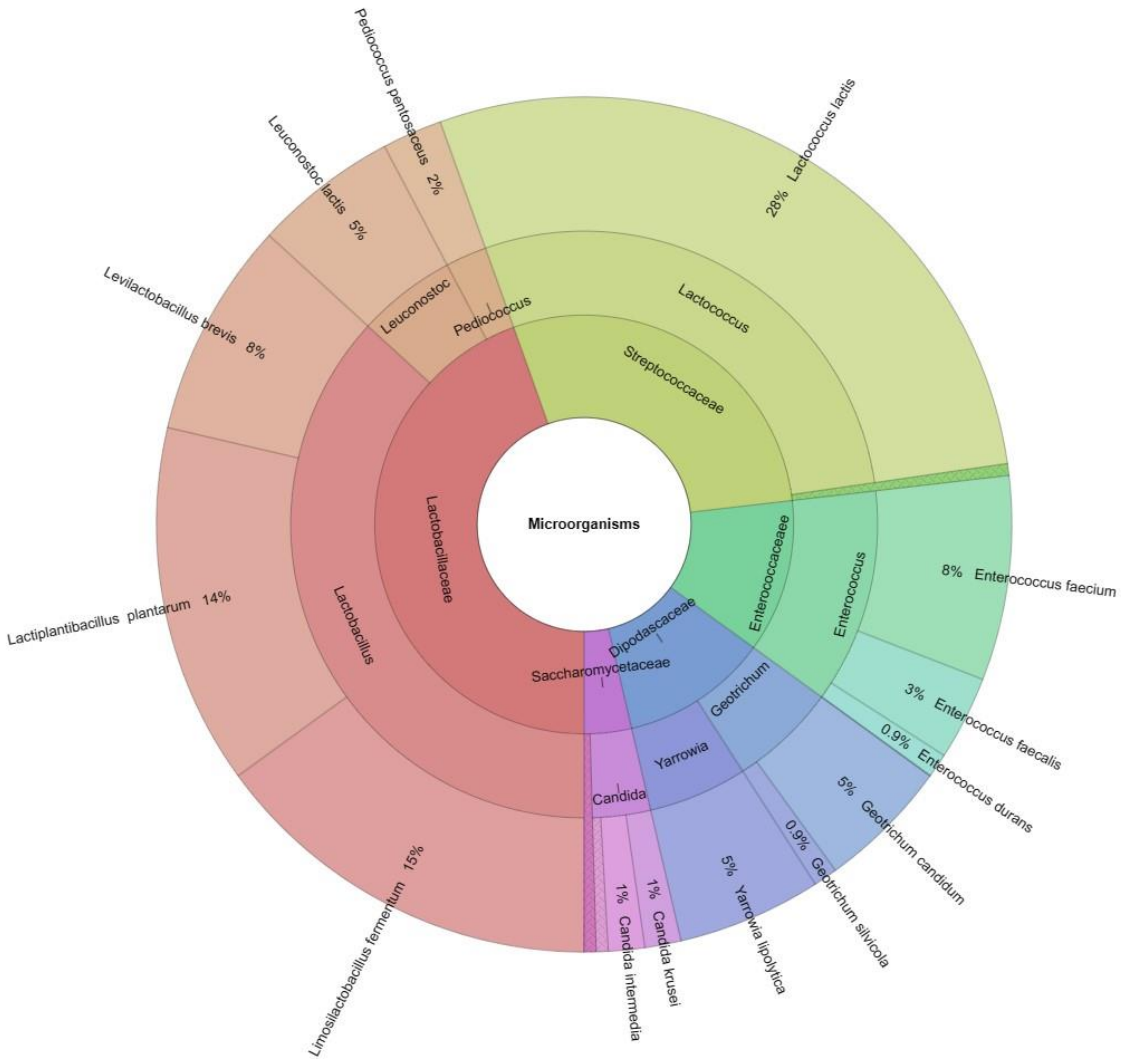

Figure 3 Krona chart for microorganisms isolated from "Bryndza" cheese at the end of storage.
Note: outermost ring: species, middle ring: genus, innermost ring: family.

Compared with other cheeses, Gouda cheese-associated microbial population analyzed by the sequencing approach represents the genera *Lactococcus*, *Streptococcus*, *Staphylococcus*, and *Lactobacillus.* These microorganisms were predominant in cheese **[39]**. In comparison with Bryndza cheese. We and other authors identified lactococci and staphylococci as dominant microflora in Bryndza cheese **[37]**. *Lactobacillus casei, Enterococcus* spp., and *Lactobacillus delbrueckii* ssp. *bulgaricus* were the dominant lactic acid bacteria in

Brazilian mozzarella cheese obtained from whole raw milk and natural whey culture **[40]**. In the study of **[41]**, *Streptococcus* spp. and *Lactobacillus* spp. were the dominant bacteria of sheep's cheeses, while *Phyllobacterium* spp. and *Staphylococcus* spp. were the most common genera in milk. Genus *Enterococcus* **[42]**, *Staphylococcus* **[43]** and fungal species **[44]** were identified in Bryndza cheese in several studies.

When we compare the representation of microorganisms at the beginning of storage and after 6 months of storage, we can conclude that lactic acid bacteria were represented by 10 species at the beginning of storage and 6 species at the end of storage. Four species of bacteria belonging to the family *Enterobacteriaceae* were initially represented in bryndza cheese samples, but no bacteria belonging to this family were identified at the end of storage.

From the point of view of food safety, the increase in the number of bacteria from the *Enterocaccaceae* family appears to be a problem; specifically, the *Enterococcus faecalis* species was detected only in frozen samples in the number of 4 isolates. The number of identified *Enterococcus faecium* isolates increased from 4 to 17. Dairy products must fulfil the legislation requirements **[45]**, **[46]**.

Our findings align with Chebeňová-Turcovská et al. **[9]**, where *Lactococcus lactis* was prevalent in Bryndza, contributing to the cheese's typical sensory properties by producing lactic acid and other aromatic compounds. Similarly, *Enterococcus* spp. Our study has noted their role in probiotic formulations, owing to their resilience and beneficial interactions within the human gut, despite concerns about their safety **[9]**. Even after frozen storage, these probiotics' survival and functional activity in Bryndza cheese indicate robust adaptability crucial for maintaining viable probiotic populations in dairy products. Our results corroborate findings from Pangallo et al. **[6]**, who noted that the microbiota dynamics in Bryndza adapt well to storage conditions without significant loss of viable probiotic bacteria.

Our study extends the understanding of Bryndza's microbiota by highlighting these microorganisms' survival and functional stability under frozen storage conditions. This aspect is crucial for extending the shelf life of Bryndza while maintaining its health benefits, which aligns with the findings from **[6]**, where they emphasise the role of microbial communities in influencing the sensory and textural profiles of Bryndza during and after storage. Furthermore, the microbial profile changes noted in our study during the storage period reflect adaptive responses that could potentially enhance the probiotic efficacy of Bryndza cheese. This is supported by Pangallo et al. **[6]**, who discuss how microbial dynamics are not merely about survival but also the interaction between microbial species that can lead to enhanced probiotic functions.

The resilience of probiotic strains in Bryndza cheese, such as *Lactococcus lactis* and *Limosilactobacillus fermentum*, during frozen storage, underscores their potential to maintain gut health and modulate the immune system. These strains have been widely recognised for their abilities to survive gastrointestinal conditions and adhere to intestinal cells, thus making them effective at maintaining intestinal barrier integrity and modulating the host's immune response. *Lactococcus lactis*, known for its immunomodulatory effects, can enhance mucosal immunity and has been used in vaccine delivery. Its presence in Bryndza, even post-freezing, suggests that consuming frozen Bryndza might confer these immunological benefits to consumers. *Limosilactobacillus fermentum*, another significant strain found in Bryndza, contributes to gut health by improving the balance of gut microbiota and enhancing the intestinal barrier. It also plays a role in fermentation, contributing to the cheese's flavour and texture over time.

The stability of these microorganisms during frozen storage, as indicated by the minimal changes in viable counts, suggests that Bryndza cheese can serve as an effective carrier of live probiotics. This could be particularly beneficial in dietary applications where probiotics are recommended for maintaining or restoring gut health.

Preserving Bryndza cheese through freezing maintains its microbial integrity and ensures the continuance of its probiotic benefits, making it a valuable functional food product. The study's findings can be applied to improve the storage and distribution of Bryndza cheese, helping it reach a broader market without losing its health-promoting properties.

This enhanced discussion integrates the beneficial properties of probiotic microorganisms found in Bryndza cheese and highlights the potential health benefits these microorganisms can confer, even after extended frozen storage periods. By focusing on these specific probiotic properties and linking them to the health benefits they can confer, the discussion provides valuable insights for readers, highlighting the significance of these findings in both food science and nutritional health contexts.

CONCLUSION

Freezing impact on the microbiological composition of Bryndza cheese highlights several crucial findings that could reshape storage practices for seasonal cheese products. Detailed analysis over six months determined that key microbial communities, particularly lactic acid bacteria, remain viable and largely unaffected by extended periods of freezing at -18 °C during 6 months. This preservation of microbial integrity ensures that the cheese retains its beneficial probiotic qualities even beyond typical production seasons. Interestingly, while total viable counts and lactic acid bacteria showed no significant decline, specific groups, such as microscopic filamentous fungi, experienced a marked reduction. Additionally, the complete suppression of coliform bacteria to undetectable levels underscores the effectiveness of freezing as a storage method, maintaining quality and enhancing product safety. These outcomes support the potential for freezing as a viable alternative to traditional salting and barrel storage, managing seasonal overproduction while maintaining the cheese's safety and nutritional value. This study extends our understanding of Bryndza cheese microbiological dynamics under freezing conditions. It supports similar applications in other dairy products, potentially leading to more flexible and economically viable practices for cheese producers. This research underscores the need for updated regulatory perspectives and legislation to accommodate new scientific insights into food preservation, ensuring that traditional foods can meet modern standards of safety, quality, and accessibility.

The average number of microorganisms in Bryndza stored at -18 °C during 6 months were: TVCs ranged from 4.86 ±0.01 to 5.31 ±0.01 log CFU.g^{-1}. Coliform bacteria were absent in any of the analysed samples after 6 months of storage. The average number of LAB ranged from 4.03 ±0.02 to 5.44 ±0.01. The average number of MFF ranged from 1.51 ±0.02 to 2.69 ±0.01 log CFU.g^{-1}.

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