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Research Article

Slovak Sheep Cheese Bryndza as a Reservoir of Antimicrobial-Resistant Bacteria

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Abstract
The aim of this study was to determine antibiotic resistance of bacteria isolated from sheep cheese “bryndza”. The cheese samples were obtained from five Slovak farms and different groups of bacteria isolated from cheese samples. Enterobacteriaceae and Lactobacillaceae were the most distributed bacterial families with other bacterial families as Enterococcaceae, Staphylococcaceae and Moraxellaceae were identified. Enterobacteriaceae were tested to cefepime (cephalosporins) and imipenem (carbapenems), Lactobacillaceae family were tested for resistance to vancomycin (glycosides) and to imipenem. Staphylococcaceae were tested for resistance against tigecycline (tetracycline) and chloramphenicol (amphenicols). Enterococcaceae family was tested against tigecycline and imipenem. Genus *Acinetobacter* of the Moraxellaceae family was tested for resistance to ciprofloxacin (fluoroquinolones) and to imipenem. All tested bacteria showed susceptibility against the chosen antibiotics.

Keywords: antibiotics, antibiotic resistance, bacteria, bryndza sheep cheese

Introduction

Slovak bryndza is Slovak traditional ripened spreadable cheese. It is made by grinding and crushing soured lump sheep's cheese and adding salt or a special salt solution. The production of bryndza is technologically different from the production of other sheep's cheeses [1].
Milk is a very good substrate for the growth and multiplication of various microorganisms. High water activity and neutral pH represent an ideal environment for microorganisms [2].
Traditional Slovak bryndza is made from unpasteurized sheep's milk, so it contains a naturally wide range of microorganisms. Natural microbiota of raw ewe's milk and ewe's lump provide typical sensory characteristics of the products and also production method influences the sensory properties. The basic raw material for the production of Slovak „Bryndza“ is ewe's lump cheese or a mixture of ewe's and cow's lump cheese, or a mixture of cured ewe's and cow's lump cheese aged under specific conditions [3].
Previous study [4] focused on the possible occurrence of human pathogenic microorganisms in raw sheep's milk and the presence of

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Escherichia coli, Staphylococcus aureus, and *Listeria monocytogenes* was identified.
Lactic acid bacteria (LAB), which are naturally found in raw sheep's milk, are largely responsible for the quality and taste of cheeses and other sheep's milk products. During their maturation, *Lactobacillus, Lactococcus* and *Leuconostoc* of LAB group produce important metabolites such as lactic acid and aromatic constituents, which contribute to the organoleptic properties of the products [5].
Lactococcus spp. are group of technologically important bacterial species. They are used as starter cultures in cheese production because they have the ability to produce lactic acid and thereby acidify the substrate. In addition, they convert amino acids into flavour components, thus they contributing to sensory properties of the products. They have anaerobic catabolic metabolism. The most important species are *Lactococcus lactis* subsp. *lactis* and *Lactococcus lactis* subsp. *cremoris* [6].
Other bacteria used in the dairy industry belong to genus *Leuconostoc*. They are used to maintain the nutritional value and prolong the shelf-life of dairy products, but their most important role is to complete the acidity and texture of finished products by proteolysis of dairy proteins [7].
Enterococcus are bacteria that could induce the lactic fermentation. They are Gram-positive bacteria that occur in various environments: soil, water or food. Enterococci can be used as starter dairy cultures as probiotics, but they can also be pathogenic and cause food spoilage [8].
Pathogenic bacteria can cause foodborne infection after ingestion. *Salmonella, Shigella, Staphylococcus* and *Listeria* were most often found in raw milk [8].
Antibiotics are substances with an antimicrobial effect produced by microorganisms or plants [9].
They are natural substances produced mainly by bacteria, microscopic fungi and actinomycetes. *Penicillium, Bacillus, Streptomyces* and *Actinomycetes* are predominant in antimicrobial production. Antimicrobials are considered to be an integral part of the treatment of diseases caused by bacteria in humans and animals. However, the excessive use of antimicrobials can lead to resistance of microorganisms to these substances. Resistant bacteria can spread through direct contact with individuals, contaminated food or feed, and transmission of resistance genes among the bacteria themselves may occur [6].
The aim of this study was to isolate and identify the antimicrobial resistance of microorganisms that were found in bryndza.

Material and Methods

Bryndza cheese samples
In this study, 20 samples of sheep cheese bryndza were evaluated. Samples were collected from primary production, placed in sterile sample containers and brought to laboratory with icebox for microbiological investigation. Samples were kept in a refrigerator (4±1°C) until the testing began. The primary dilution of the milk products was made by adding of 5 g of sample material to 45 ml of 0.89 % sterile saline. Then, the serial dilutions (10^{-2} to 10^{-4}) were done and 100 µl of each dilution was plated out on to agars.
Isolation of total microbial counts
Plate count agar (PCA, Sigma-Aldrich, St. Louis, USA) for total microbial counts was used. Inoculated plates were incubated for 24-48 h at 37 °C and then examined for the presence of microbial colonies [10].
Isolation of coliforms bacteria
Violet red bile lactose agar (VRBGA, Sigma-Aldrich, St. Louis, USA) for enumeration of coliforms bacteria was used. Inoculated agars were incubated for 24-48 h at 37 °C and then examined for the presence of typical colonies [10].
Isolation of enterococci
Enterococcus selective agar (ESA, Sigma-Aldrich, St. Louis, USA) for enumeration of enterococci was used. Inoculated agars were incubated for 24-48 h at 37 °C and then examined for the presence of typical colonies [10].
Isolation of Lactic Acid Bacteria (LAB)
MRS (Main Rogose Sharp agar, Sigma-Aldrich, St. Louis, USA) agar was used for LAB identification. Inoculated agars were incubated for 72 h at 30 °C anaerobically and then the bacterial growth was evaluated [11].
Isolation of microscopic fungi and yeasts
Malt extract agar (Sigma-Aldrich, St. Louis, USA) was used for microscopic fungi and yeasts identification. Inoculated agars were incubated for 5 days at 25 °C aerobically and then evaluated [12].
The different colonies from total microbial count, coliforms bacteria, enterococci, LAB, fungi and yeasts were selected for further confirmation with MALDI-TOF. Selected colonies were cultured overnight on TSA agar aerobically or anaerobically and used for identification.
Identification of bacteria with MALDI-TOF MS Biotyper
Samples for analysis with MALDI-TOF MS analysis were prepared in accordance with extraction procedure provided by the manufacturer (Bruker Daltonik, Bremen, Germany) [11].
Detection of antimicrobial resistance

The disk diffusion method was performed by inoculating a bacterial suspension with a standard density of 0.5 McF ° onto Müller-Hinton agar.
Sensitivity and resistance in Lactobacillaceae were tested using the E-test for detection of MIC (minimum inhibitory concentration) of antimicrobials. Discs of antimicrobials or E-tests strips were placed onto inoculated agars and incubated for 24-48 h at 37 °C aerobically or anaerobically. The antibiotic sensitivity or resistance were evaluated by EUCAST [13].

Results and Discussion

In cheeses made from raw sheep's milk after 30 days of maturation, Pintado et al. [14] described the total number of microorganisms of 9.0 log cfu/g. In our study, the highest number of microorganisms of 4.78 log cfu/g was identified in sample 7 (Table 1). Pangallo et al. [15] detected counts of LAB up to 10.95 log cfu/g in sheep bryndza in comparison to 3.18 log cfu/g identified in the present study. The coliform bacteria counts were in line with Pangallo et al. results [15] who reported coliform bacteria counts in bryndza was 3.87 log cfu/g vs. 3.95 log cfu/g were found in the present study, which is a comparable result to that of Pangallo et al. [15]. However, the total highest number of yeast colonies was 5.97 log cfu/g in contrast to 2.65 log cfu/g in the present study. Vrabec et al. [16] reported 7-8 log cfu/g of enterococci in bryndza that was higher than in our report - 3.97 log cfu/g.
A total of 1061 isolates was identified in our study, and the most abundant were *Lactococcus lactis, Lactobacillus paraplantarum, Lactobacillus plantarum, Lactobacillus paracasei, Lactobacillus brevis, Lactobacillus harbinensis* and *Hafnia alvei* from Enterobacteriaceae family (Table 2). The similar results were found in Kačániová et al. [10] study.
Acinetobacter baumannii and *Acinetobacter tandoii* were isolated in the present study. Both species showed sensitivity to ciprofloxacin and imipenem (Table 4).
Mahmoudi et al. [17] reported resistance of Acinetobacter baumannii isolated from hospitals. Up to 94.7% of the isolates were resistant to imipenem and 97.4% were resistant to ciprofloxacin.
Antimicrobial resistance of *Acinetobacter* spp. was reported also in isolates from goat sample, including resistance to carbapenems [18].
In our study, 9 yeasts species were isolated from bryndza (Table 3).

Table 1.
Microbial counts in sheep cheese bryndza (log cfu/g).

Sample	CB	E	TCM	LAB	Y
1.	3.80±0.10	2.75±0.03	4.11±0.05	3.05±0.01	2.35±0.03
2.	3.87±0.06	2.97±0.02	4.07±0.07	3.07±0.05	2.32±0.04
3.	3.67±0.04	2.78±0.07	4.08±0.07	3.06±0.07	2.22±0.22
4.	3.64±0.08	2.81±0.04	4.15±0.07	3.12±0.02	2.2.±0.02
5.	3.52±0.15	2.76±0.08	4.22±0.09	3.16±0.05	2.11±0.05
6.	3.47±0.16	2.82±0.04	4.72±0.12	3.11±0.08	2.65±0.08
7.	3.51±0.13	2.86±0.04	4.78±0.05	3.18±0.05	2.20±0.02
8.	3.42±0.04	2.83±0.03	4.67±0.13	3.09±0.13	2.15±0.05
9.	3.45±0.07	2.85±0.06	4.62±0.11	3.11±0.11	2.22±0.06
10.	3.52±0.12	2.79±0.14	4.72±0.12	3.13±0.13	2.25±0.07
11.	3.57±0.05	2.77±0.11	4.15±0.08	3.08±0.11	2.31±0.03
12.	3.61±0.05	2.83±0.02	4.22±0.05	3.09±0.02	2.35±0.04
13.	3.65±0.04	2.81±0.02	4.23±0.15	3.16±0.05	2.33±0.08
14.	3.71±0.02	2.82±0.04	4.18±0.05	3.10±0.02	2.24±0.02
15.	3.76±0.08	2.76±0.06	4.19±0.06	3.12±0.12	2.15±0.06
16.	3.46±0.04	2.81±0.10	4.06±0.02	3.12±0.04	2.33±0.05
17.	3.49±0.02	2.88±0.06	4.07±0.07	3.14±0.07	2.25±0.02
18.	3.52±0.04	2.92±0.01	4.04±0.04	3.09±0.09	2.09±0.08
19.	3.54±0.06	2.85±0.07	4.15±0.11	3.11±0.01	2.10±0.09
20.	3.58±0.03	2.88±0.06	4.21±0.05	3.14±0.02	2.32±0.05

CB-coliform bacteria, E-enterococci, TCM-total count of microorganisms, LAB-lactic acid bacteria, Y-yeasts

Table 2.

The microbial isolates from sheep cheese bryndza

Bacterial species	No. of isolates	No. of isolates, %
Acinetobacter baumannii	11	1.04
Acinetobacter tandoii	16	1.51
Citrobacter braakii	18	1.70
Citrobacter koseri	17	1.60
Enterobacter cloacae	22	2.07
Enterobacter ludwigii	10	0.94
Escherichia coli	11	1.04
Hafnia alvei	40	3.77
Klebsiella oxytoca	35	3.30
Klebsiella pneumoniae ssp. *ozaenae*	19	1.79
Klebsiella pneumoniae ssp. *pneumoniae*	21	1.98
Raoultella ornithinolytica	28	2.64
Serratia liquefaciens	25	2.36
Stenotrophomonas maltophilia	18	1.70
Bacillus pumilus	26	2.45
Enterococcus faecalis	15	1.41
Enterococcus faecium	25	2.36
Enterococcus hirae	25	2.36
Lactobacillus brevis	44	4.15
Lactobacillus harbinensis	45	4.24
Lactobacillus johnsonii	41	3.86
Lactobacillus plantarum	45	4.24
Lactobacillus paracasei ssp. *paracasei*	45	4.24
Lactobacillus paraplantarum	45	4.24
Lactobacillus suebicus	5	0.47
Lactococcus lactis ssp. *lactis*	58	5.47
Lactococcus lactis	59	5.56
Microbacterium liquefaciens	19	1.79
Pediococcus acidilactici	25	2.36
Staphylococcus aureus ssp. *aureus*	18	1.70
Staphylococcus pasteuri	15	1.41

Table 3.

Number of isolated yeasts from sheep cheese bryndza

Yeast	No. of isolates	No. of isolates, %
Candida catenulate	25	2.36
Candida krusei	25	2.36
Candida lusitaniae	25	2.36
Candida rugose	25	2.36
Candida utilis	20	1.89
Dipodascus candidum	20	1.89
Dipodascus silvicola	15	1.41
Pichia cactophila	15	1.41
Yarrowia lipolytica	45	4.24

Table 4.

Antimicrobial resistance of *Acinetobacter* isolated from sheep cheese bryndza

Species of bacteria	Antimicrobial	Zone of inhibition (mm, average±SD)	Sensitivity/Resistance
Acinetobacter baumannii	CIP	53±0.05	S
	IMP	28±0.06	S
Acinetobacter tandoii	CIP	55±0.07	S
	IMP	30±0.02	S

CIP-ciprofloxacin, IMP-imipenem

From 20 samples of bryndza we isolated 14 species of bacteria from the family Enterobacteriaceae. We applied two types of antibiotics to this family of bacteria, namely cefepime and imipenem (Table 5).

Cotton et al. [19] isolated and identified a total of 173 Gram-negative bacteria from milk and dairy products, of which almost half were represented by bacteria of the Enterobacteriaceae family, including species such as *Acinetobacter baumannii, Serratia liquefaciens, Klebsiella oxytoca, Hafnia alvei, Escherichia coli* and *Citrobacter braakii*. The detection of antimicrobial resistance indicated that susceptibility to aminosides, imipenems and quinolones was relatively high, but more than half of all isolates tested were resistant to monobactams, cefepime (67%), fosfomycins, colistin, fennel, sulfonamides, and carbopenems (23% to imipenem).

In our study, 63.6% of tested bacteria of the Enterobacteriaceae family were sensitive to cefepime. 100% of the tested Enterobacteriaceae were sensitive to imipenem (Table 5).

In our study, *Enterococcus faecalis, Enterococcus faecium* and *Enterococcus hirae* of the genus *Enterococcus* were isolated. Resistance to tigecycline (tetracyclines), and imipenem (carbapenems) were tested (Table 6)..

Table 5.

Antimicrobial resistance of Enterobacteriaceae isolated from sheep cheese bryndza

Bacterial species	Antimicrobial	Zone of inhibition (mm average±SD)	Sensitivity/Resistance
Citrobacter braakii	FEP	27±0.04	S
	IMP	23±0.03	S
Citrobacter koseri	FEP	25±0.05	I
	IMP	25±0.07	S
Enterobacter cloacae	FEP	30±0.06	S
	IMP	22±0.04	S
Enterobacter ludwigii	FEP	15±0.02	R
	IMP	24±0.01	S
Escherichia coli	FEP	22±0.05	R
	IMP	25±0.06	S
Hafnia alvei	FEP	25±0.05	R
	IMP	22±0.08	S
Klebsiella oxytoca	FEP	28±0.05	S
	IMP	25±0.02	S
Klebsiella pneumoniae	FEP	25±0.04	S
ssp. *ozaenae*	IMP	26±0.03	S
Klebsiella pneumoniae	FEP	28±0.06	S
ssp. *pneumoniae*	IMP	28±0.02	S
Raoultella	FEP	30±0.08	S
ornithinolytica	IMP	51±0.06	S
Serratia liquefaciens	FEP	33±0.07	S
	IMP	53±0.05	S

FEP-cefepime, IMP-imipenem; I – intermediate resistance

Table 6.

Antimicrobial resistance of *Enterococcus* spp. species isolated from sheep cheese bryndza

Bacterial species	Antimicrobial	Zone of inhibition (mm, average±SD)	Sensitivity/Resistance
Enterococcus faecalis	TGC	24±0.04	S
	IMP	28±0.03	I
Enterococcus faecium	TGC	23±0.02	S
	IMP	30±0.05	I
Enterococcus hire	TGC	25±0.05	S
	IMP	52±0.04	S

TGC-tigecycline, IMP-imipenem; I-intermediate resistance

Pfaller et al. [20] reported that tigecycline was more effective against *Enterococcus faecium* than against *Enterococcus faecalis* with 0.06 mg/L MIC values for *E. faecium* and 0.12 mg/L for *E. faecalis.* Sensitivity to tigecycline was identified also in the present study.
Two isolated species - *Staphylococcus aureus* ssp. *aureus* and *Staphylococcus pasteuri* - of the genus *Staphylococcus* were tested for antimicrobial resistance to chloramphenicol and tigecycline (Table 7).
Staphylococcus spp. are among the Gram-positive bacteria that are sensitive to tetracyclines. Tigecycline belongs to the group of tetracyclines. Moffa and Brook [21] found that up to 94.2% of methicillin-resistant staphylococci tested in the United States were sensitive to tetracyclines that was in agreement with our results.
Danesh et al. [22] showed *Staphylococcus aureus* sensitivity to chloramphenicol, but only in combination haloduracin.
Of seven *Lactobacillus* spp. we were able to isolate, all showed sensitivity to antimicrobials (Table 8). Sharma et al. [23] showed that their isolated species of *Lactobacillus* spp., which they isolated from commercially available probiotics, were highly resistant to vancomycin. Differences between the studies could be linked to examination of different species of *Lactobacillus* spp. Danielsen and Wind [24] examined antimicrobial susceptibility of *Lactobacillus* spp. *Lactobacillus plantarum* in the authors' work showed the highest sensitivity to imipenem at 0.12 µg/mL, while *Lactobacillus paracasei* was sensitive only at 4 µg/mL. Our *L. plantarum* isolates were sensitive to imipenem at 0.5 mg/mL and *L. paracasei* at a concentration of 1 µg/mL. According to research by Danielsen and Wind [24], *Lactobacillus* spp. were sensitive to penicillins and more resistant to oxacillin, cefoxitin, cephalotein and imipenem. Our experiment shows susceptibility of *Lactobacillus* spp. to imipenem.
The authors of Ma et al. [25] investigated the antibiotic resistance of *Lactobacillus* spp. isolated from fermented foods and the human digestive tract. The tests were performed with 17 antibiotics, including imipenem and vancomycin. We can compare the results of the species *Lactobacillus plantarum*. In the authors' research, most isolates of this species were sensitive to imipenem at 2 µg/mL. For vancomycin, the largest number of isolates was already sensitive at 1 µg/mL. Our *L. plantarum* isolates showed sensitivity to imipenem at 0.5 mg/mL and to vancomycin 1 µg/mL. Comparing our results with the authors, we can state that the differences in MIC values for imipenem are different because bacteria isolated from the human digestive tract could develop higher resistance to given antibiotics due to their more frequent administration by humans.

Table 7.

Antimicrobial resistance of *Staphylococcus* species isolated from sheep cheese bryndza

Bacterial species	Antimicrobial	Zone of inhibition (mm, average±SD)	Sensitivity/Resistance
Staphylococcus aureus	C	22±0.03	S
ssp. *aureus*	TGC	24±0.02	S
Staphylococcus pasteuri	C	24±0.04	S
	TGC	25±0.03	S

TGC-tigecycline, C-chloramphenicol

Table 8.

Antimicrobial resistance of *Lactobacillus* species isolated from sheep cheese bryndza

Bacterial species	Antimicrobial	MIC (µg/mL)	Sensitivity/Resistance
Lactobacillus brevis	VA	1	S
	IMP	1	S
Lactobacillus harbinensis	VA	1	S
	IMP	0.5	S
Lactobacillus johnsonii	VA	1	S
	IMP	1.5	S
Lactobacillus paracasei	VA	0.5	S
ssp. *paracasei*	IMP	1	S
Lactobacillus	VA	0.5	S
paraplantarum	IMP	1	S
Lactobacillus plantarum	VA	1	S
	IMP	0.5	S
Lactobacillus suebicus	VA	1	S
	IMP	1	S

VA-vancomycin, IMP-imipenem

Conclusions

The aim of this study was to detect the antimicrobial resistance of microorganisms isolated from sheep cheese bryndza. All bacteria of Enterobacteriaceae family were sensitive to carbapenem class antimicrobial imipenem. All tested *Lactobacillus* spp. were susceptible against vancomycin and imipenem. Enterococci were tested for antimicrobial resistance against tigecycline and imipenem and intermediate suspeceptible of isolates were identified.

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