FOOD SAFETY AND QUALITY ENGINEERING MSC THESIS

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Hungarian University of Agriculture and Life Science

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Food Safety and Quality Engineering MSc

DOMINANT MICROORGANISMS IN MILK AND FERMENTED DAIRY PRODUCTS, AND ANTIBIOTIC RESISTANCE OF LACTIC ACID BACTERIA

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LIST OF ABBREVIATIONS

LAB - Lactic acid bacteria

GRAS - Generally recognized as safe

QPS - Qualified Presumption of Safety

EFSA - European Food Safety Authority

HGT - Horizontal gene transfer

AR - Antibiotic resistance

MALDI-TOF-MS - Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry

AACs - Chloramphenicol acetyltransferases

RAPD -PCR-Random Amplified Polymorphic DNA-Polymerase Chain Reaction

IPTG - Isopropyl-β-D-thiogalactophranoside

MRS - de Man, Rogosa and Sharpe

TSA - Tryptic soy agar

CNS - Coagulase-positive staphylococci

DNA - Deoxyribonucleic Acid

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1. INTRODUCTION

While certain lactic acid bacterial (LAB) strains are utilized as probiotics to prevent and treat intestinal illnesses, others are used as starter cultures to control food fermentations (Saez-Lara et al., 2015; Sirichoat et al., 2020). For a long time of safe application as probiotics and producing natural products to promote health, lactic acid bacteria have gained the label of "Generally regarded as safe" (GRAS) (Nawaz et al., 2011) and the European Food Safety Authority (EFSA) has granted "Qualified Presumption of Safety (QPS)" status to the majority of LAB species. LAB strains only need to lack antibiotic resistance (AR) genes against clinically and veterinary significant antimicrobials to meet the QPS standards (Sirichoat et al., 2020). Antibiotic resistance has emerged, evolved, and spread in humans, animals, and the environment as a result of the widespread use of antibiotics in both pathogenic and non-pathogenic bacteria (Hernando-Amado et al., 2019). LAB can serve as reservoirs for AR genes, which may eventually be transmitted by horizontal gene transfer (HGT) to pathogenic bacteria during the production of food or after consumption, despite the fact that LAB is not harmful. According to recent research, antibiotic resistance may spread throughout the food chain as a result of LAB (Wang et al., 2019). For instance, LAB can spread mobile-resistant genes to other bacteria during food processing after acquiring antibiotic-resistant genes from the resistant bacteria in raw milk. There were also reports of antibiotic-resistant genes on conjugative plasmids or transposons in LAB, which may result in horizontal gene transfer (Wang et al., 2019). Intrinsic resistance is chromosomally encoded and associated with an organism's overall physiology or anatomy; it cannot be transferred horizontally. Horizontally transferable acquired resistance results from genetic alterations caused by mutations or the acquisition of genetic elements (plasmids or transposons), most likely through conjugation (Nawaz et al., 2011). Resistance resulting from chromosomal mutations and intrinsic means is less likely to spread horizontally than resistance resulting from the acquisition of mobile genetic elements (transposons or plasmids) that are more prone to dissemination (Manaia, 2017).Within the past ten years, scientists have also emphasized on characterizing antibiotic resistance in LAB (Belletti et al., 2009). The European Food Safety Authority (EFSA) warns against using bacterial strains carrying genes for transferable antibiotic resistance in animal feed, fermented foods, and probiotic foods intended for human consumption (Ricci et al., 2018). Antimicrobial resistant microorganisms are becoming a bigger concern for public health. According to Ammor et al.

(2007), it has an impact on food production and quality as well as medical care for humans and animals.

This study aims to investigate the dominant microorganisms of Hungarian fermented dairy products and raw milk, and determine the antibiotic resistance of LAB strains typical to these products.

2. GOALS OF THE THESIS

Lactic acid bacteria are a group of aerotolerant anaerobic, Gram-positive, fermentative bacteria that are common in the natural environment and extensively used in the food sector. They are known for their ability to perform fermentation (Wang et al., 2019).

AR has regularly been found in LAB connected to a range of fermented foods. More specifically, LAB strains isolated from cheeses, fermented meats and vegetables are often found to be resistant to erythromycin, penicillin, tetracyclines, and chloramphenicol as well as macrolides (Flórez, Delgado and Mayo, 2005).

Given the potential consequences for human health and food safety, it is justifiable to be concerned about the possibility that lactic acid bacteria used in probiotic applications and food fermentations could be sources of antibiotic-resistance genes that can be transferred to pathogens.

Although probiotics are generally thought to be harmless, instances of infections linked to probiotic lactobacilli have been reported, especially in those with impaired immune systems (Land et al., 2005).

Nevertheless, there isn't much comprehensive research focused on antibiotic resistance in food-borne LAB, such as lactobacilli, *Leuconostoc* or lactococci, among others. The majority of data presented are on opportunistic pathogenic bacteria.

A comprehensive understanding of the AR bacteria in fermented foods, such as dairy products, will shed light on the scope and implications of the problem with these foods.

The goals of this thesis are:

i. isolation of microorganisms from different Hungarian dairy products (e.g., raw milk, cheese, cottage cheese, yogurt, and sour cream), thus mapping their typical and dominant microbiota;
ii. identification of the isolates by MALDI-TOF MS;
iii. characterization of the isolates by biochemical tests and molecular typing of LAB using RAPD-PCR analysis;

iv. characterization of the phenotypic antibiotic resistance of the isolates using different antibacterial substances (assessment of antibiotic resistance in the isolated) for evaluating the risk of the analyzed dairy products.

3. LITERATURE REVIEW

3.1. Importance of LAB in the food industry and microbiota of milk and dairy products

Although the primary metabolic activity of LAB is the production of lactic acid from the fermentation of carbohydrates, i.e. starter cultures, they are also involved in the production of many beneficial compounds such as organic acids, polyols, and exopolysaccharides, and as a result have many applications in the food industry (Bintsis, 2018).

LABs produce a variety of antibacterial/antimicrobial metabolites like bacteriocins, lactic acid, and exopolysaccharides that effectively target bacteria that break down food, safeguarding food and extending its shelf life, as multiple studies have shown (Vuyst and Leroy, 2007).

Producing and the purification of bacteriocins: Although bacteriocins can be produced during food fermentation in the food matrix, with the right physical and chemical circumstances, LAB can produce significantly more bacteriocins during in vitro fermentations (Lahiri et al., 2022).

Milk and dairy products such as cheese, sour cream, yogurt, and others are rich in microbes because of their high nutritional value. It is widely acknowledged that, prior to pasteurization, the lactic acid bacteria, a class of bacteria that ferments lactose to lactate, compose the majority of the population in milk from cows, goats, sheep, and buffalo. *Lactococcus*, *Lactobacillus*, *Leuconostoc*, *Streptococcus*, and *Enterococcus* are the most prevalent LAB genera in milk and milk products. *Pseudomonas* and *Acinetobacter* species are common examples of psychotropic populations, which are very important and specially become dominant during cold storage. Milk also contains several yeasts and molds, which are non-LAB species (Quigley et al., 2013). The microbes in milk and dairy products can enter the products through a variety of channels and then play a variety of roles within the product, including promoting health (like lactobacilli and bifidobacteria*),* disease-causing (like species of *Listeria*, *Salmonella*, *Staphylococcus*, *Campylobacter*, and *Escherichia*, as well as mycotoxin-producing fungi) or facilitating dairy fermentations (like members of *Pseudomonas*, *Clostridium*, *Bacillus* genera, and other spore-forming or thermodurant microorganisms). Additionally, there is concern that the presence of antibiotic residues in milk and dairy products promotes the emergence of resistance in bacteria, especially pathogenic bacteria (Quigley et al., 2013).

Lactate production by microorganisms can cause milk to ferment, and the flavor, texture, and organoleptic qualities of the resulting products can be affected in several ways (Wouters et al., 2002). Additionally, microorganisms can have a detrimental effect on the shelf life and quality of milk. For instance, extracellular lipases and proteases produced by psychrotolerant bacteria can cause spoilage when they multiply during refrigeration (Hantsis-Zacharov and Halpern, 2007). The microbiological composition of milk can also impact a person's health because consuming raw milk contaminated with pathogens can occasionally result in severe sickness (Quigley et al., 2013).

The genera *Carnobacterium, Enterococcus, Lactobacillus, Lactococcus, Leuconostoc, Oenococcus, Pediococcus, Streptococcus, Tetragenococcus, Vagococcus,* and *Weissella* are among the genetically varied group of bacteria known as LAB. *Lactococcus, Enterococcus, Oenococcus, Pediococcus, Streptococcus, Leuconostoc,* and *Lactobacillus* species are important for the food industry (Karaduman et al., 2017).

3.2. Antibiotic resistance in LAB

Antibiotic resistance refers to the characteristic of bacteria that confers the ability to render antibiotics inactive or a system that prevents the antibiotics' potential to inhibit or kill (Davison et al., 2000).

In addition to the potential link between probiotics and infectious processes, there is also a need to consider the risk of these bacteria developing resistance genes. Wide-ranging implications arise from the presence of AR bacteria and genes in fermented foods, including possible impacts on human health, ramifications for the food industry, and environmental ramifications (Vinayamohan et al., 2023).

3.2.1. Lactobacillus genus

Studying the transmission of antibiotic resistance within the *Lactobacillus* genus is challenging because of its taxonomic complexity. The *Lactobacillus* genus is the biggest group among LAB and is found in most foods, primarily dairy products. Twenty-five genera have been identified as a result of a recent proposal to reclassify the genus *Lactobacillus* (Nunziata et al., 2022).

Significant variation in the resistance of this genus to several antibiotic classes has been noted. The majority of *Lactobacillus* species exhibit strong resistance to both teicoplanin and vancomycin; among lactobacilli, penicillins typically cause lactobacilli to become sensitive and prone to other inhibitors of cell wall production, such as β-lactam antibiotics (penicillin G, ampicillin and oxacillin). Nonetheless, unusual resistance to this class of antibiotics has been observed in multiple trials. *Lactobacillus plantarum* isolated from Karst ewe's cheese, one strain of *Lactobacillus rhamnosus* isolated from traditional Italian cheese Valtellina Casera, and one strain of *Lactobacillus helveticus* from Chinese fermented milk were resistant to penicillin G (Nunziata et al., 2022).

3.2.2. Lactococcus species

Since *Lactococcus lactis* is the only species involved in industrial food processing, more concentration was put on it in this context. Additionally, *Lactococcus garvieae*, a zoonotic pathogen that can be a common species in animal-based products such as raw milk cheeses, was also examined.

The genus *Lactococcus* is known to be susceptible to β-lactam antibiotics (penicillin, ampicillin, amoxicillin, piperacillin, ticarcillin, and imipenem), broad-spectrum antibiotics (rifampicin, spectinomycin, and chloramphenicol), and macrolides with a spectrum (lincomycin, bacitracin, novobiocin, teicoplanin, and vancomycin) on Gram-positive bacteria. However, atypical resistance in *Lactococcus* has been observed in ampicillin in five strains from Turkish cow milk and penicillin G in one strain from Turkish cheese. Additionally, *Lactococcus* exhibits variability in terms of resistance regarding erythromycin (Nunziata et al., 2022).

3.2.3. Streptococcus genus

Researchers focused on *Streptococcus thermophilus*, one of the most significant starters for the dairy sector, as the sole species in the genus *Streptococcus* of technological significance.

Ammor et al. (2007) reported variations in resistance of *S. thermophilus* to vancomycin, ampicillin, and penicillin G. Furthermore, a number of strains resistant to these antibiotics have lately been

found in a variety of countries, including in commercial yogurt and fermented milk from China, as well as raw milk cheese from Italy and Spain (Nunziata et al., 2022).

3.2.4. Leuconostoc genus

When it relates to β-lactam antibiotics, various species of *Leuconostoc* are susceptible to ampicillin and penicillin G, however, Italian and Spanish cheeses have been shown to have a number of oxacillin-resistant strains of *Ln. citreum*, *Ln. lactis*, *Ln. mesenteroides*, and *Ln. pseudomesenteroides*. In the past, there have been several reports of rifampicin, erythromycin, clindamycin, and tetracycline susceptibility (Nunziata et al., 2022).

3.2.5. Species of Pediococcus

There are remarkably few studies on the antibiotic resistance profiles of pediococci that are isolated from dairy products. There is not enough information available about clindamycin and rifampicin to create a profile of antibiotic resistance. Moreover, tetracycline sensitivity is also inadequate and variable; in fact, the isolates of *Pediococcus* species from Turkish fermented dairy products accounted for 80% of the tetracycline-resistant *P. acidilactici* examined, while isolates from food supplements did not exhibit any resistance, according to other authors. While isolates of *P. acidilactici* and *P. pentosaceus* from various dairy sources were reactive to amikacin, gentamycin, kanamycin, streptomycin, and neomycin, some investigators found complete resistance to this class of antibiotics. Penicillin G, chloramphenicol, and erythromycin are generally effective against *Pediococcus* species, however, there have been occasional exceptions (Nunziata et al., 2022).

Table 1. The phenotypic resistance profile of some antibiotic-resistant bacteria found in certain fermented dairy products (Vinayamohan et al., 2023).

Food type	**Bacteria present**	**Phenotypic resistance profile**
Cheese	LAB, *Staphylococcus* (CNS)	Erythromycin, chloramphenicol, and tetracycline
Yogurt	*Lactobacillus*	Trimethoprim, colimycin, mycostatin, nalidixic acid, neomycin, polymyxin B, sulfamethoxazole, and sulfonamides
	Streptococcus	Trimethoprim–sulfamethoxazole, streptomycin, gentamicin, kanamycin, Mycostatin, nalidixic acid, neomycin, polymyxin B, and sulfonamides.
Turkish cheese	*Enterococcus*	Vancomycin, oxacillin, erythromycin, and streptomycin
Nono (African traditionally fermented milk product)	*Enterococcus thailandicus*	Tetracycline and streptomycin
	Streptococcus infantarius	Tetracycline

The ability of microorganisms to withstand the effects of antimicrobials, which are intended to either kill or slow their growth, poses a concern to human health today, raising the possibility that common mild illnesses could turn lethal. Global mortality, morbidity, and economics are significantly impacted by the consequences of bacterial antimicrobial resistance (AMR), especially in low- and middle-income nations where unrestricted access to potentially life-saving medications is growing (Ikhimiukor et al., 2022).

3.3. Classification of antibiotics and mechanisms through which antibiotic resistance can develop

Antibiotics can be categorized in several ways, but the most popular ones are based on their molecular structures, modes of action, and range of activities. Based on their chemical or molecular

structures, several common groups of antibiotics include oxazolidinones, beta-lactams, macrolides, tetracyclines, quinolones, aminoglycosides, sulphonamides, and glycopeptides. (Etebu and Ibemologi, 2016).

The mechanisms of action of antimicrobial agents can be classified according to the function they affect. Generally, these included inhibition of the synthesis of cell walls or nucleic acids, inhibition of ribosome function, inhibition of cell membrane function, and inhibition of folate metabolism. However, more and more microorganisms that are resistant to antibiotics are compromising the effectiveness of antimicrobials. There are two ways to characterize resistance:

A). **Intrinsic resistance** is when antimicrobials have no effect on microorganisms and do not have target sites for them(Etebu and Ibemologi, 2016).

B). **Acquired resistance** is when an organism that is naturally susceptible to an antibiotic develops a defense mechanism against it. The existence of an enzyme that renders an antimicrobial agent inactive, post-transcriptional or post-translational change of the antimicrobial agent's target, decreased absorption of the antimicrobial agent, and active efflux of the antimicrobial agents are among the mechanisms of acquired resistance (Etebu and Ibemologi, 2016).

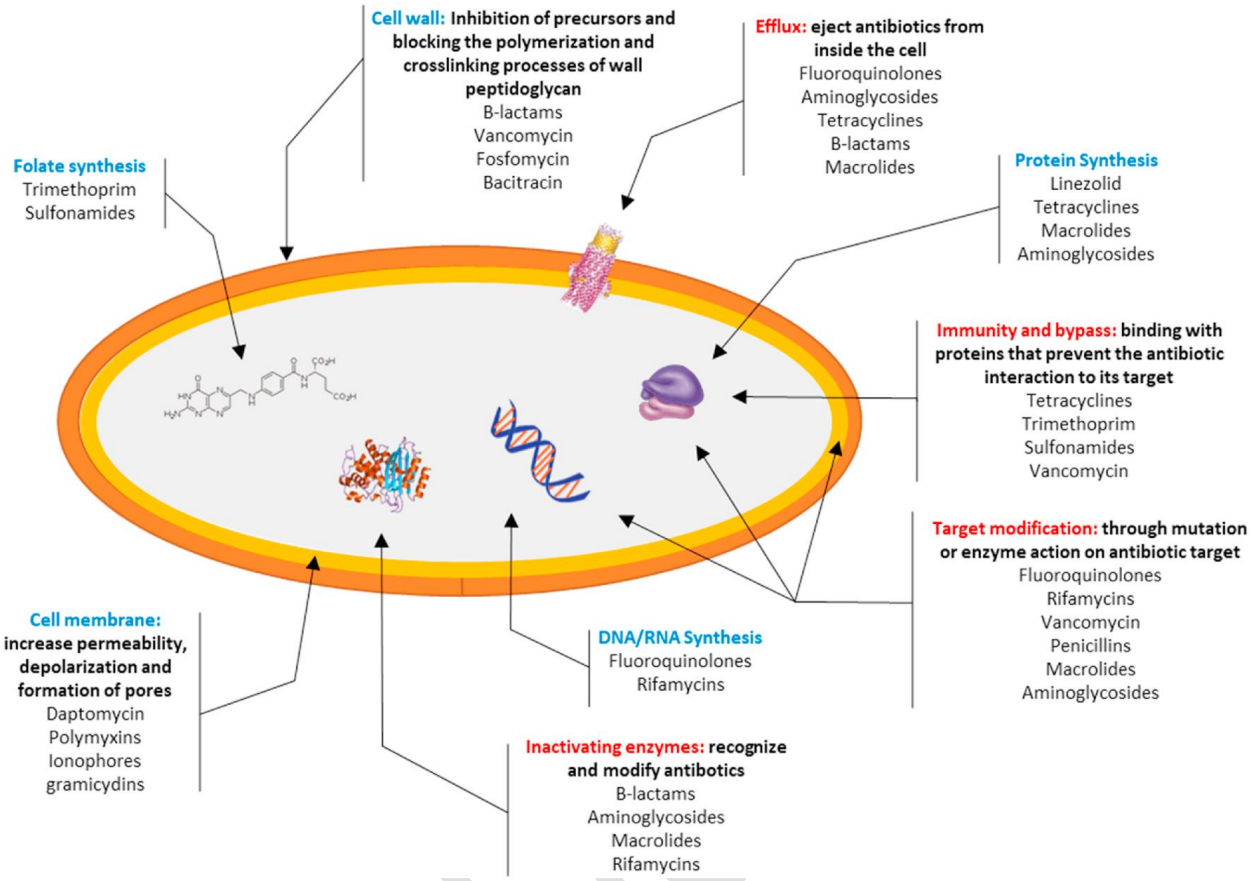

Figure 1. Antibiotic targets (blue) and the cell's defense mechanisms against antibiotics (red) (Nunziata et al., 2022)

Efflux pumps are membrane proteins that export antibiotics from the cell while maintaining their low intracellular concentrations. Efflux mechanisms release these antimicrobials at the same rate they enter the cell, preventing them from reaching their intended target. These pumps are found in the membrane of the cytoplasm. Efflux systems can be activated by any antibiotic, with the exception of polymyxin. Efflux pumps may exhibit antibiotic-specificity. The majority of them are multidrug transporters, which greatly contribute to the emergence of multidrug-resistant organisms by pumping a variety of unrelated antibiotics, such as macrolides, tetracyclines, and fluoroquinolones (Lin et al., 2015).

Antibiotic inactivation: Three primary enzymes, namely beta-lactamases, aminoglycoside-modifying enzymes, and chloramphenicol acetyltransferases (AACs), are responsible for rendering antibiotics inactive. Nearly all beta-lactams with ester and amide bonds, such as penicillins, cephalosporins, monobactams, and carbapenems, are hydrolyzed by beta-lactamases (Kaufman, 2011).

Reduced membrane permeability: Drugs must enter Gram-negative bacteria through their cell pores since they have an outer cell membrane. Changes in the electrical charge or physical structure of pores caused by gene mutations can result in a barrier to the entry of antibiotics into cells. In this way, a microbe can become resistant to several antibiotic classes at once. However, certain Gram-negative bacteria have an inbuilt resistance to large medications like vancomycin because they are too big to fit through the pores, even before a mutation takes place (Lin et al., 2015).

Modification of target site: Many antibiotics work by attaching themselves to a specific molecular target within the microbe. If the target molecule's structure significantly changes and the antibiotic is no longer able to bind to it, the bacterium may lessen the effectiveness of the medication. Tetracyclines, for instance, bind to the transfer RNA access site and inhibit it, which causes microbial resistance to tetracyclines (Kaufman, 2011).

3.4. RAPD-PCR for characterization of LAB in dairy products

The typing technique Random Amplified Polymorphic DNA (RAPD) employs a single, randomly chosen primer consisting of 8-12 bases. In order to identify polymorphisms, this primer randomly hybridizes to many sites on chromosomal DNA sequences that exhibit similarity closest to the bacterial genome. DNA Amplification Fingerprinting (DAF) and Arbitrarily Primed-PCR (AP-PCR) are two examples of RAPD variations that vary according to the length of the primer, annealing temperature, and length of the protocol. The amplification products are separated by agarose gel electrophoresis, which creates a bacterial fingerprint that is used to type and characterise different bacterial strains (Sharma et al., 2020).

3.5. Specific PCR for the identification of bacterial strains

Using specific oligonucleotide primers, PCR is a potent technique for detection or identification of target bacteria in complex environments. The method has been widely utilized in the case of harmful bacteria because it has excellent speed, accuracy, and sensitivity. For the several bacterial species that are known to be present in the fermented dairy products, specific oligonucleotide primers have been designed (Matsuki et al., 2003). Bacteria that are not the target of primers cannot be detected by the specific PCR procedures. As a result, it is essential to create particular primers for each of the major bacterial species and genus, moreover serogroups of some important pathogenic bacteria (Walter et al., 2000).

4. MATERIALS AND METHODS

4.1. Samples

Five different samples, including raw milk and dairy products (sour cream, cottage cheese, yogurt, and cheese) were collected from a local market situated in the 11th district of Budapest, Hungary. The samples were stored at 4 °C until the start of their examination.

4.2. Media used for isolation in this study

M17 agar (Biolab): it is a selective medium commonly used for isolation and cultivation of various LAB, including lactococci and streptococci.

Modified MRS (de Man, Rogosa and Sharpe)-BPB agar (55g; Becton, sparks, MD, USA): it is a medium for isolation and differentiation of LAB in a mixed culture, particularly lactobacilli and pediococci.

CATC agar (Biolab): it is a selective medium for the isolation and cultivation of enterococci.

Chromobio (Biolab): it is a selective and differential medium for detection of coliforms and *Escherichia coli*.

Palcam agar (Biolab): Selective medium for *Listeria* spp.

Harlequin (Biolab): Selective medium for isolation of *Salmonella* spp.

Baird-Parker (Biolab): Selective medium for isolation of *Staphylococcus* spp. and *S. aureus*

Muller Hilton agar (Biolab): Test medium for antimicrobial susceptibility.

TSA agar (Biolab): It is an extremely nourishing all-purpose medium that can be used to cultivate a broad range of microorganisms.

4.3. Isolation, characterization, and maintenance of the LAB and other dominant bacteria

The purchased samples were weighed in 10 g or 10 ml aliquots, diluted by 1% sterile saline (preparing a ten-times dilution), and homogenized using stomacher bag mixer. A decimal serial dilution was also prepared in the case of each sample.

Dominant microorganism were isolated from the samples using selective media mentioned in subchapter 4.2. without any selective pre- and enrichment. 0.1 ml of the appropriate dilutions of the samples were spread onto plates containing different culture media and incubated at 30 °C for 48 hours for LAB under anaerobic conditions, and 37 °C for 24 hours for other bacteria under aerobic conditions. After incubation, 44 colonies were selected. Pure cultures were prepared by taking single colonies from each plate after the incubation time and inoculating them onto M17, mMRS-BPB agar, and TSA plates.

Microscopic examination of the single colonies obtained from the pure cultures was also done to determine the morphology of the cells as well. The purified colonies were subsequently preserved in 20% glycerol containing Nutrient broth at -80 °C for further application.

KOH test (for determining the Gram-property based on cell wall composition) was performed to examine whether the bacteria were Gram-negative or Gram-positive by the use of 3% KOH. 10 µl of the solution was dropped on a microscopic slide, and using a sterile loop, a small amount of the cells was taken and mixed with the KOH. After that the loop was elevated to check if there is stickiness or not which can refer to the cell wall composition of bacteria (Karami et al., 2017).

4.4. Identification of the isolates using MALDI-TOF MS

Using MALDI-TOF MS (Becton-Dickinson, USA), the identification of dominant microbes (40) isolated from milk and dairy product samples was made based on the examination of their protein and peptide composition. Overnight cultures were cultivated on M17, mMRS-BPB and TSA agar plates as it is recommended by Karaduman et al. (2017). A colony from each culture was taken off and smeared on the ground steel MALDI target. After drying at room temperature, 1µL of 70% formic solution was added on the target and dried again at room temperature, which step was followed by the addition of 1µL matrix solution. The prepared samples were analyzed automatically using a MALDI-TOF mass spectrometer (Becton-Dickinson USA) running Flex Control 3.4 software. Calibration of mass spectrometer was achieved with the Bruker's bacterial test standard.

4.5. RAPD-PCR and specific PCR analyses for LAB and dominant bacteria

Genomic DNA was extracted from overnight cultures, according to Pavlidou et al., (2011). The purity and concentration of the DNA were measured by NANO drop instrument. The DNA samples were diluted to obtain 50 ng/µL before the start of the analysis. The reaction mixture for RAPD-PCR was prepared from 1-time *Taq* buffer, 1.25 mM $MgCl_2$, 0.3 mM deoxynucleotide triphosphate, 0.5 µM primer, 0.75 units of *Taq* DNA polymerase, 14.8 µl dd H_2O and 50 ng of DNA. 1 µL of DNA (from 50 ng/µL) was added to 6µL of the reaction mixture, and amplified by using ESCO thermocycler.

RAPD-PCR analysis was performed using primer D8635 (Pavlidou et al., 2011). Gel electrophoresis was used to separate the amplification products on 1.5% (w/v) agarose gels in 0.5 × TBE buffer (27 g-Tris-base, 13.75 g-boric acid, 2.02 g-EDTA, pH 8.5). GelCompar II software was used to analyze the RAPD-PCR profiles of the photos of the gels exposed to UV light that were taken.

Specific PCR analysis was performed using simple PCR detection system. The primers were designed to detect *Escherichia* species, as *E. alberti*, *E. fergusonii* and *E. coli*. In this study, primers EC-F:5'-CCAGGCAAAGAGTTTATGTTGA-3' and EC-R:5'-GCTATTTCCTGCCGATAAGAGA-3' were used for the amplification of *E. coli* with amplicon size of 212 bp, EF-F:5'-AGATTCACGTAAGCTGTTACCTT-3' and EF-R:5'-CGTCTGATGAAAGATTTGGGAAG-3' for *E. fergusonii* with amplicon size of 595 bp, and EA-F:5'-GTAAATAATGCTGGTCAGACGTTA-3' and EA-R: 5'-AGTGTAGAGTATATTGGCAACTTC-3' for *E. albertii* with amplicon size of 393 bp. The reaction mixture was prepared from 1 times Taq buffer, 1.5 mM MgCl2, 100 µM deoxyribonucleoside triphosphate, 0.375 µM of each primer, 1.25 units of Taq DNA polymerase, dd H2O, and 50 ng of target DNA. Amplification was done using C1000 Thermal cycler, Amplification products were separated by gel electrophoresis on 2% (w/v) agarose gels in 0.5 × TBE buffer. The thermal profile was the following: initial denaturation at 95 °C for 10 minutes, followed by 30 cycles of denaturation at 92 °C for 1 min, 57 °C for 1 min, and annealing at 72°C for 30 s, and one final cycle at 72 °C for 5 minutes.

4.6. Antibiotic susceptibility testing

According to Wang et al., (2019), The Kirby-Bauer disk diffusion method was used to evaluate the LAB and some other bacterial isolates' susceptibility to 12 different antibiotics, including Ampicillin, Tetracycline, Kanamycin, Erythromycin, Azithromycin, Streptomycin, Ciprofloxacin, Vancomycin, Aztreonam, Chloramphenicol, Clindamycin, and Gentamicin.

Overnight cultures were grown on M17 and mMRS (de Man, Rogosa, and Sharpe)-BPB agar in the case of lactic acid bacteria. At the same time, TSA was used for the other tested bacterial strains. 2 ml of saline solution was prepared in test tubes and cells of the overnight cultures were added to them and vortexed. The OD was then adjusted to a density corresponding to 0.5 McFarland standard. A sterile cotton swab was dipped into the prepared suspension, vigorously pressed and rotated against the tube's inside to remove any excess liquid. The swab was then streaked over the whole surface of M17 and mMRS (de Man, Rogosa, and Sharpe)-BPB agar plates for LAB and Muller Hilton agar (Biolab) plates for other bacteria to ensure even distribution of the suspension on the media. To assure that the antibiotic disks made contact with the M17, mMRS (de Man, Rogosa and Sharpe)-BPB agar and Muller Hilton agar surfaces, they were applied gently on the surfaces of the plates and incubated at 37 °C under anaerobic condition for 48 hours for LAB, and 30 °C for 24 h in aerobic environment for the other bacteria. After the incubation time, the inhibition zones were measured using a ruler. The breakpoints suggested in a prior study were used to interpret the results (Wang et al., 2019; Charteris et al., 1998).

Table 2. The observed diameters for the antibiotic resistance test and their interpretation (Wang et al., 2019; Charteris et al., 1998).

Antibiotics	**Concentration (µg)**	**Zone of inhibition(mm)**			**Reference**
		R	**I**	**S**	
Ampicillin (AMP)	10	≤12	13-15	≥16	(Wang et al., 2019; Charteris et al., 1998).
Azithromycin (AZM)	15	≤13	14-17	≥18	
Aztreonam (ATM)	30	≤15	16-21	≥22	
Chloramphenicol (CHL)	30	≤13	14-17	≥18	
Ciprofloxacin (CIP)	5	≤13	14-18	≥19	
Clindamycin (CMN)	2	≤8	9-11	≥12	
Erythromycin (ERY)	15	≤13	14-17	≥18	
Gentamicin (HLG)	120	≤12	0	≥13	
Kanamycin (KMN)	30	≤13	14-17	≥18	
Streptomycin (HLS)	300	≤11	12-14	≥15	
Tetracycline (TET)	30	≤14	15-18	≥19	
Vancomycin (VAN)	30	≤14	15-16	≥17	

Intermediate (I, zone diameter ± SD), sensitive (S) and resistant (R)

4.7. Data Analysis and visualization

The phenotypic AMR panel among identified genera was illustrated as a complex heatmap with hierarchical clustering using R studio and "Complex Heatmap" package in version 4.3.1 of R software (https://www.r-project.org/).

4.8. Clustering of the isolated bacterial strains

From the molecular fingerprints generated by RAPD-PCR, a dendrogram was constructed using Gelcompar II software (BioNumerics, Belgium). The dendrogram was used to group the LAB strains into different clusters, and based on this result, the clonal relationship can be determined among the isolates originated from different samples.

5. RESULTS AND DISCUSSION

5.1. Isolation and characterization of the dominant microorganisms

A total of 40 isolates were obtained from the dairy products: from cheese (n=19), raw milk (n=12), cottage cheese (n=3), sour cream (n=5) and yoghurt (n=1) (Table 3.).

Table 3. Summary of the isolates obtained from the investigated samples

Agar used for isolation	**Number of isolated colonies**					**Total number**
	raw milk	**cottage cheese**	**sour cream**	**yoghurt**	**cheese**	
M17	3	3	2	1	3	**12**
MRS	2	n.i.	3	n.i.	4	**9**
CATC	2	n.i.	n.i.	n.i.	5	**7**
Harlequin	2	n.i	n.i.	n.i.	2	**4**
Baird-parker	2	n.i.	n.i.	n.i.	2	**4**
Chromobio	1	n.i.	n.i.	n.i.	3	**4**
Palcam agar	0					
Total	**12**	**3**	**5**	**1**	**19**	**40**

n.i.: not isolated from the sample by the application of the indicated agar

As it can be seen from Table 3. majority of the isolates were derived from cheese and raw milk (31 in total), which exhibited the highest microbial content. The most efficient agars for isolation were M17 and MRS, as 21 out of 40 isolates (52.5%) were derived from these media.

Gram-staining and KOH test results indicated that 25 isolates are Gram-positive and 15 isolates are Gram-negative. Results of microscopic examination revealed the presence of bacteria (40 isolates) with bacillus (n=10), coccus (n=28), coccoid (n=2), morphologies as it is summarized in a table found in Annex 1 and Annex 2.

5.2. Results of identification using MALDI-TOF MS

On M17 medium, both LAB and non-LAB isolates were able to grow. However, only LAB could grow on modified MRS supplemented by bromophenol blue (mMRS-BPB). There was no growth in the case of Palcam agar, while *Enterococcus* and other bacteria, like *Staphylococcus*, were isolated from CATC medium.

E. coli, Staphylococcus and *Enterobacter* were able to grow on Chromobio medium, and *E. coli* could be easily distinguished as it had different color on it. *Staphylococcus, Enterobacter,* and *Enterococcus* were isolated from Baird Parker medium. Harlequin enabled the growth of *Hafnia alvei* and *Enterobacter* as well. *Salmonella* was not isolated on it.

From M17 medium, the isolates were identified as LAB: out of the 12 isolates 4 were *Lactococcus lactis* and 8 were identified as non-LAB. From MRS medium, 9 isolates were identified as LAB belonging to *Lactococcus lactis* (n=2), *Lactobacillus curvatus* (n=2), *Lacticaseibacillus paracasei* (n=4) and *Leuconostoc lactis* (n=1) species. Moreover, as it was expected, isolates originated from CATC, Harlequin, Baird Parker and Chromobio agar plates were non-LAB (n=23). Results of MALDI-TOF MS analyses are summarized in a table found in Annex 1.

In our study, most of the LAB isolates were identified as *Lactococcus lactis* (isolated from sour cream, raw milk and cheese), followed by *Lacticaseibacillus paracasei* (from sour cream and cheese), *Lactobacillus curvatus* (from cheese) and *Leuconostoc lactis* (from raw milk) by MALDI-TOF MS.

In many previous studies, MALDI-TOF MS has been properly used for identification of microorganisms isolated from food products, thus this technique was also used for species identification in our work.

In the study of Nacef et al., (2017) French cheeses from raw or pasteurized milk were investigated, and based on their results mesophilic lactic acid bacteria were identified by MALDI-TOF MS and classified into three genera, namely *Lactobacillus, Enterococcus,* and *Leuconostoc*. Lactic acid bacteria found in cheese were also determined with MALDI-TOF MS and the results indicated that they were members of the following species: *Lactobacillus curvatus, Lactobacillus diolivorans, Lactobacillus paracasei, Lactobacillus plantarum, Lactobacillus rhamnosus, Lactococcus lactis,* and *Leuconostoc mesenteroides* (Sánchez et al. 2020; Kanak and Yilmaz., 2019). In another study, non-starter lactic acid bacteria (NSLAB) were identified to species level using MALDI-TOF analysis in Naxos, Greece (Gantzias et al., 2020). Isolates from non-commercial yogurt samples in southern Turkey contained two genera, *Enterococcus* and *Lactobacillus,* and four species including *Enterococcus hirae, Enterococcus faecium, Enterococcus durans,* and *Lactobacillus paracasei* after MALDI-TOF identification (Karaduman et al., 2017). In Slovakia, milk and milk products were collected, and isolates from the products were identifiable by MALDI-TOF MS (Kačániová et al., 2017). Based on evidences from all these research work, MALDI-TOF MS is a good tool for the identification of LAB at the species and genus level.

5.3. Molecular typing of the isolates by RAPD-PCR analysis

RAPD-PCR yielded evaluable bands that were used to cluster the LAB strains by constructing a similarity tree using GelCompar II software (Version 5.1). Based on the dendrogram the strains were grouped into four bigger clusters each containing isolates of different species. Significant heterogeneity was observed in the case of the LAB strains using primer D8635, and only two strains (MRS_11 and MRS_5) of *Lacticaseibacillus paracasei* showed 100% similarity, both were isolated from cheese and sour cream samples. For more accurate characterization of the LAB strains it would be necessary to use additional RAPD primers.

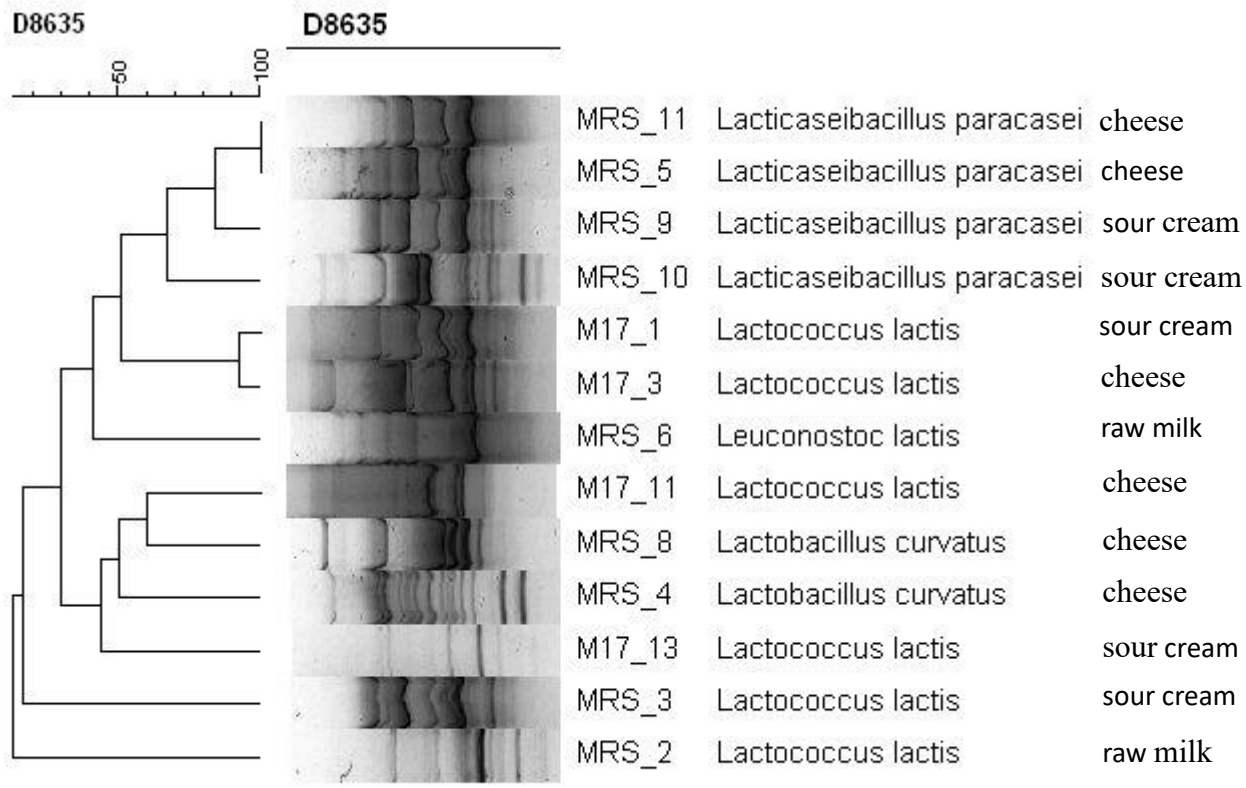

Figure 2. Dendrogram illustrating cluster analysis of RAPD-PCR results of 13 LAB strains isolated in this study. The dendrogram was created using Gel Compar II software (Version 5.1).

In the current study, LAB strains were grouped into four bigger clusters, and the isolates were classified in 12 separated branches. The result indicated that few isolates have strong relationships among them, i.e. *Lactococcus lactis* and *Lacticaseibacillus paracasei*, while some isolates belonging to *Lactococcus lactis* were located on a completely separate branch of the dendrogram. Moreover, it can be seen that isolates from sour cream belonging the *Lactococcus lactis* were clonally distinct strains despite being isolated from the same sample.

In a previous study, LAB originated from yogurt samples in Bangladesh were grouped; isolates from geographically distinct places displayed greater genetic diversity, while isolates from the same site were closely linked, and displayed less genetic variation using RAPD-PCR method (Hossain et al., 2021). Cluster analysis results indicated the existence of a plant and breed-specific LAB ecosystem from fermented sausages (Muzzin et al., 2020). Using the RAPD-PCR, LAB from a common ancestor were found from Sumbawa horse milk and wild honey bee, indicating the vast

diversity of LAB in these samples (Prastyowati et al. 2021). Strains of lactobacilli from milk and cheese samples were compared, and it was discovered that the majority of the strains were of cheese origin (Oneca et al., 2003).

5.4. Results of species-specific PCR reactions

Species-specific PCR analysis yielded bands in the case of two DNA samples which indicated that presence of *Escherichia coli* in the investigated cheese samples. The lines with negative results correspond to strains of *Hafnia alvei* originated from raw milk samples.

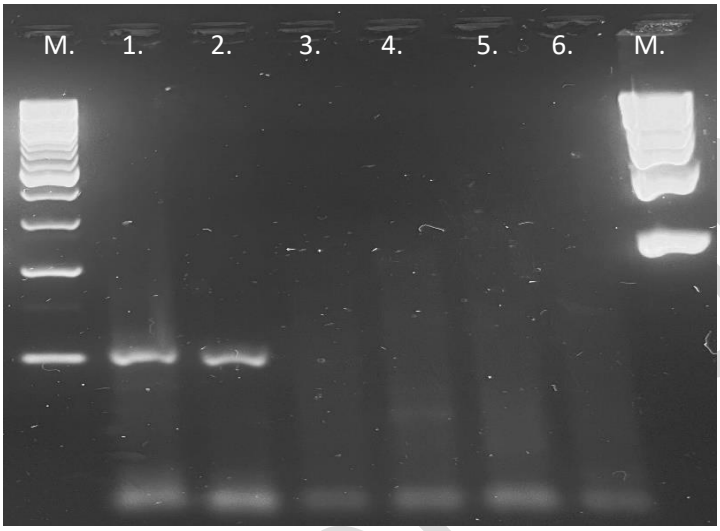

Figure 3. Results of species-specific PCR for *E. coli* isolated from cheese during this study.

Order of samples: M: marker, 1: DNA of *E. coli* isolated from cheese, 2: DNA of *E. coli* isolated from cheese, 3: DNA of *Hafnia alvei* isolated from raw milk, 4: DNA of *Hafnia alvei* isolated from raw milk, 5: DNA of *Hafnia alvei* isolated from raw milk, 6: DNA of *Hafnia alvei* isolated from raw milk, M: marker

In several studies, species-specific PCR reactions have been used for the identification of *E. coli* in different food products. According to Lindsey et al., (2017) a species-specific PCR test provided a quick and precise method for identifying *E. coli, E. alberti,* and *E. fergusonii* in a single reaction, and was 100% sensitive and specific for detecting the predicted species. The original classification

for *E. albertii* was eae-positive *Hafnia alvei.* Owing to the lack of defined isolation and identification techniques, *E. albertii* is frequently misidentified (as *Hafnia alvei*) and may be more important in instances of infectious diarrhea worldwide than previously thought.

The results of species-specific PCR confirmed the presence of *E. coli* in the investigated samples, and supported the earlier findings that *E. coli* can easily be identified using species-specific PCR, and that the isolated *Hafnia alvei* strains were correctly identified by MALDI-TOF MS. Moreover, the presence of *E. coli* and coliform bacteria (like species of *Hafnia, Enterobacter,* and *Serratia*), and some other enteric bacteria (e.g. *Enterococcus*) indicate sever hygienic deficiencies, thus based on our results it can be said, that raw milk and cheese samples were contaminated during the production, or more likely during handling of the products.

5.5. Antibiotic susceptibility of the investigated bacterial strains

Sensitivity and resistance of the isolated LAB were determined in the cases of 12 antibiotics. The results of the disc diffusion tests can be seen in Figure 3.

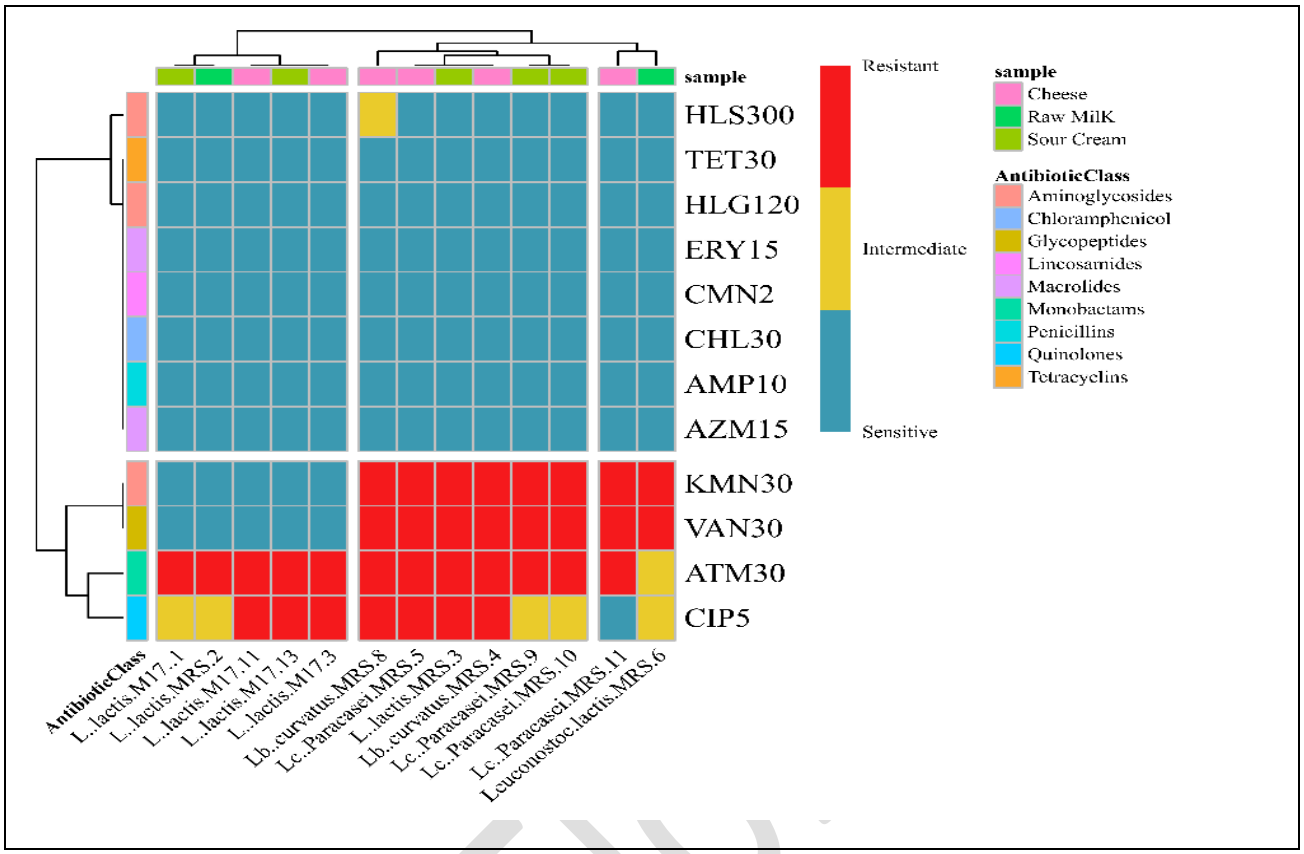

Figure 3. Complex heatmap with hierarchical clustering illustrates the phenotypic antimicrobial resistance patterns of lactic acid bacterial isolates (n=13). The X axis shows genera of lactic acid bacteria and their ID numbers, and the Y axis represents the tested antibiotic discs. Red cells indicate complete resistance; yellow cells indicate intermediate resistance; and blue cells indicate complete susceptibility.

Diameters of the corresponding zones used for the evaluation of sensitivity results are summarized in Table 2. The results of the antibiotic susceptibility analysis are compiled in Figure 3., where the isolates were classified as sensitive (S), intermediate (I) or resistant (R) based on breakpoints suggested by other earlier researches (Wang et al., 2019; Charteris et al., 1998).

Antibiotic susceptibility test results indicated that *Lactococcus lactis, Lactobacillus curvatus, Lacticaseibacillus paracasei* and *Leuconostoc lactis* from raw milk, cheese and sour cream were found to be resistant to Kanamycin and Vancomycin. *Lactococcus lactis, Lactobacillus curvatus,* and *Lacticaseibacillus paracasei* were resistant to Ciprofloxacin. *Lactococcus lactis, Lactobacillus curvatus,* and *Lacticaseibacillus paracasei* were resistant to Aztreonam.

Lactococcus lactis were susceptible to Kanamycin and Vancomycin. All strains of LAB from raw milk, cheese and sour cream were susceptible to Ampicillin, Azithromycin, Chloramphenicol, Clindamycin, Erythromycin, Streptomycin, Gentamicin, Tetracycline. *Lactobacillus curvatus* showed intermediate resistance to Streptomycin. *Lactococcus lactis*, *Lacticaseibacillus paracasei* and *Leuconostoc lactis* exhibited intermediate resistance to Ciprofloxacin. *Leuconostoc lactis* showed intermediate resistance to Aztreonam.

As it can be seen on Figure 3. seven antibiotics could inhibit the growth of all tested strains, while four out of the 12 antibiotics proved to be inefficient to majority of the LAB strains. Furthermore, from the 13 LAB strains 7 were multidrug resistant: four strains had resistance against 4, while three strains showed resistance against 3 antibiotics. Aztreonam proved to be the most ineffective as out of 13 LAB 12 were resistant against it, while 8, 8 and 7 strains were resistant to Kanamycin, Vancomycin and Ciprofloxacin, respectively.

LAB can serve as reservoirs for AR genes, which may eventually be transmitted by HGT to pathogenic bacteria during the production of food or after consumption, despite the fact that LAB is not harmful (Wang et al., 2019). The ability of microorganisms to withstand the effects of antimicrobials, which are intended to either kill or slow their growth, poses a concern to human health today, raising the possibility that common mild illnesses could turn lethal (Ikhimiukor et al. 2022). A widespread resistance of many strains of *Lactobacillus* isolates obtained from the human vagina towards Kanamycin was reported (Sirichoat et al., 2020), and increased resistance of LAB isolated from fermented dairy products was observed in the case of Kanamycin, Streptomycin, and Gentamycin, respectively (Vorlová and Karpíšková, 2020). LAB strains of 16 different species of *Lactobacillus,* and *Streptococcus thermophilus* from fermented products were found in Xi'an, China. All organisms exhibited resistance to Kanamycin and Vancomycin (Nawaz et al., 2011). *Streptococcus thermophilus* and *Lactobacillus bulgaricus* identified from commercial yogurt and cheese showed significant resistance to Vancomycin, Neomycin, Gentamycin, and Streptomycin (Wang et al. 2019). Commercial strains of *Lactobacillus, Streptococcus* and *Bifidobacterium* that are utilized to produce industrial dairy products are often resistant to Tetracycline, Gentamycin, Kanamycin, and Chloramphenicol (Nunziata et al., 2022). *Lactobacillus* species frequently exhibit intrinsic resistance to Tetracycline, Vancomycin, and Erythromycin (Sciences and Campus, 2011). Isolates of dairy origin, including *Leuconostoc lactis* and *Leuconostoc carnosum*, were found to

be resistant to Kanamycin, Tetracycline, and Chloramphenicol, as well as Erythromycin, Clindamycin, Virginiamycin, Ciprofloxacin, and Rifampicin (Flórez et al., 2016). In another study, lactobacilli, lactococci, and streptococci were all resistant to Vancomycin (Gad, Abdel-hamid and Farag, 2014). On the other hand, *Lb. rhamnosus, Lb. acidophilus, Lb. casei, Lb. reuteri, Lb. plantarum,* and *Lb. fermentum* showed high levels of resistance to Amikacin, Streptomycin, Vancomycin, and Kanamycin (Sharma et al., 2016). LAB isolates of dairy origin showed resistance to Kanamycin, Tetracycline, Chloramphenicol, Erythromycin, Clindamycin, Virginiamycin, Ciprofloxacin, and Rifampicin (Flórez et al., 2016). Resistance to Vancomycin and Kanamycin by lactobacilli is related to the cell structures of the bacteria, efflux mechanism, or target mutation (Nunziata et al. 2022). In our study 16.7% of *Lactococcus lactis,* 100% of *Lactobacillus curvatus,* 100% of *Lacticaseibacillus paracasei* and 100% of *Leuconostoc lactis* were found to be resistant to Kanamycin and Vancomycin.

Additionally, another study found resistances to Ciprofloxacin and Aminoglycosides (Gentamicin and Streptomycin) to be higher than 70%, suggesting that these may be inherent resistances (Hummel et al. 2007). *Lb. rhamnosus, Lb. acidophilus, Lb. casei, Lb. reuteri, Lb. plantarum,* and *Lb. fermentum* obtained from commercial dairy products was found resistant to Ciprofloxacin (Sharma et al. 2016). All LAB strains isolated from fermented foods in Nigeria showed significant phenotypic resistance to beta-lactam antibiotics including Ciprofloxacin (Duche et al., 2023). In this current study *Lactococcus lactis, Lactobacillus curvatus,* and *Lacticaseibacillus paracasei* were resistant to Ciprofloxacin. However, all *Lactococcus lactis, Lactobacillus curvatus,* and *Lacticaseibacillus paracasei* were resistant to Aztreonam. There is no available data in the scientific literature concerning the antibiotic resistance of LAB to Aztreonam.

However, it was observed that LAB isolates obtained from kefir were susceptible to Tetracycline (Budiati, Suryaningsih, and Yudiastuti, 2022). LAB belonging to *Lactobacillus* and *Streptococcus thermophilus* from fermented foods in Xi'an, China, showed susceptibility to Ampicillin and Vancomycin (Nawaz et al., 2011). In a study conducted with sausages and pickles, as well as fermented Chinese foods, LABs in the pickle samples were susceptible to Ampicillin, Clindamycin, and Tetracycline, although they were resistant to other antibiotics (Heo, Lee and Jeong, 2020). Lactobacilli, lactococci, and streptococci were found to be susceptible to Tetracycline, Erythromycin, and Clindamycin (Gad et al., 2014). LAB strains isolated from

fermented foods in Nigeria showed significantly high sensitivity to the subgroups of beta-lactam antibiotics belonging to carbapenems, sulphonamides, and macrolides (Duche et al., 2023). *Lactobacillus* strains isolated from dairy and human sources were found susceptible to Chloramphenicol and Tetracycline (Charteris et al., 1998), and in another study, none of the LAB strains showed resistance to Clindamycin and Erythromycin (Sharma et al., 2016).

On the other hand, some studies showed results that are in contrast with our current findings. LAB isolates from Indonesian traditional fermented foods (dadih, tape ketan, bekasam, and tempoyak) were found to be resistant to Erythromycin and Chloramphenicol in studies that examined the bacteria (Sukmarini et al., 2014). Research has revealed that lactobacilli isolated from fermented foods have acquired resistance to Tetracycline, Erythromycin, Clindamycin, and Chloramphenicol (Vinayamohan et al., 2023). Resistance to Penicillin, Erythromycin, Clindamycin, and Tetracycline was acquired by certain LAB strains, although species-specific resistance to Gentamycin, Ciprofloxacin, Streptomycin, and Chloramphenicol has been observed (Nawaz et al., 2011). In the current study, all strains of LAB were susceptible to Ampicillin, Azithromycin, Chloramphenicol, Clindamycin, Erythromycin, Streptomycin, Gentamicin, Tetracycline except 50% of *Lactobacillus curvatus* (n=1) which showed intermediate resistance to Streptomycin.

Isolates belonging to *Lactobacillus* genus originated from commercial dairy products, showed intermediate resistance to Ciprofloxacin, Cloxacillin, Ampicillin, Methicillin, Penicillin, Tetracycline, Azithromycin, Chloramphenicol, and Novobiocin (Sharma et al., 2016), while no previous data exist on the intermediate resistance of LABs to Aztreonam and Streptomycin. In the current study, all *Lactococcus lactis, Lacticaseibacillus paracasei* and *Leuconostoc lactis* exhibited intermediate resistance to Ciprofloxacin, and 100% of *Leuconostoc lactis* showed intermediate resistance to Aztreonam.

5.6. Conclusions and proposals

In conclusion, the study's findings demonstrate that LAB from raw milk, sour cream, and cheese showed multidrug resistance to 4 different tested antibiotics. The safety of these bacteria is crucial since they are consumed through food, while the presence of *E. coli* in the products indicate that there was fecal contamination in the case of cheese samples, and this could pose possible threat to public health.

Recommendation

When a bacterial strain exhibits antibiotic resistance, as shown by the phenotypic approach, it is preferable to investigate the genetic basis of this resistance to ascertain if it is acquired or intrinsic. This can be done by performing whole genome sequencing or whole meta genome sequencing to easily identify the resistant genes in the strains. Testing for horizontal transfer of resistance genes in strains used as starter cultures is necessary. LAB species harboring antibiotic resistance encoding genes are commonly found in food, as it was previously shown, especially in fermented food, despite not being classified as harmful bacteria. This fact could be detrimental to the transmission of antibiotic resistance genes in the food chain.

6. SUMMARY

The aims of this investigation were to determine the dominant microorganisms of different Hungarian fermented dairy products and raw milk, and their antibiotic resistance, and clonal relationship that exist among the strains of LABs. Thirteen lactic acid bacteria and twenty-seven non-lactic acid bacteria isolated from raw milk, sour cream, cottage cheese, yogurt, and cheese were investigated. They were isolated using M17 and modified MRS (mMRS-BPB), CATC, Harlequin, Palcam, Baird-Parker and Chromobio agar plates incubated at 37 ^{0}C under anaerobic condition for 24 hours for LAB, and 30 ^{0}C in aerobic atmosphere for other dominant microbes.

Isolates were maintained at -80 ^{0}C in glycerol for further investigation. The cell morphology and the composition of cell wall were determined for classification purposes, while MALDI-TOF MS was used for the identification of the bacterial isolates. The Kirby-Bauer disk diffusion method was applied for determining the phenotypic antibiotic resistance pattern of the LAB strains using 12 antibiotic discs. Moreover, a dendrogram was constructed from molecular fingerprints of LAB generated by primer D8635 in RAPD-PCR to establish their clonal relationships.

During the study the following observations were made:

- Modified MRS-BPB agar is a suitable medium for isolation of lactic acid bacteria. On the other hand, selectivity of M17 proved to be low, as non-LAB were also able to grow on it.
- MALDI-TOF MS identified the LAB isolates as *Lactococcus lactis* (n=6)*, Lactobacillus curvatus* (n=2)*, Lacticaseibacillus paracasei* (n=4) and *Leuconostoc lactis* (n=1).
- Based on the results of molecular characterization the isolates proved to be clonally distinct except two *Lacticaseibacillus paracasei*, which had 100% similarity using the D8635 RAPD primer.
- Presence of *E. coil* was confirmed by species-specific PCR in cheese sample, which is a threat to the consumers' health.
- Seven strains (*Lactococcus lactis* (1), *Lactobacillus curvatus* (2), *Lacticaseibacillus paracasei* (4)) showed multi-drug resistance, as their growth was not inhibited by four antibiotics, namely Aztreonam, Ciprofloxacin, Kanamycin and Vancomycin.

Screening for bacterial strains with antibiotic resistance in milk and fermented dairy products should be done continually to reduce the risk associated with them and for the safety of the food products since our current results indicated that there is antibiotic resistant of LAB in these products, however, further studies, mainly on a genetic basis, are needed to accurately determine their impact on consumer health.

Annexes

Annex 1

Table summarizing gram property based

Annex 2

characteristeristic typical to the isolates.

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