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Article

Raw milk Cheeses as Reservoirs of Antimicrobial-Resistant Bacteria: A Comparative Study of Goat and Sheep Milk Products

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Abstract

Raw milk cheeses represent complex microbial ecosystems that may act as reservoirs of antimicrobial-resistant bacteria. This study investigated the microbiological characteristics, bacterial community structure, and antimicrobial resistance (AMR) profiles of artisanal goat and sheep cheeses produced by regional manufacturers in Poland. A total of ten cheeses, including five goat cheeses and five sheep oscypek-type cheeses, were analysed. Culture-dependent enumeration and isolation were combined with molecular identification via 16S rRNA gene sequencing and antimicrobial susceptibility testing by disk diffusion. Total viable bacterial counts ranged from 10⁵ to 10⁸ CFU/mL, revealing considerable variability among individual samples. Microbiological profiling indicated the predominance of lactic acid bacteria, with *Lactococcus*, *Lactobacillus*, and *Streptococcus* representing the dominant genera. Multivariate analysis (PCoA) demonstrated substantial intra-group dispersion and overlapping clustering patterns between goat and sheep cheeses, suggesting that sample-specific ecological factors exerted a stronger influence on microbial composition than milk origin. Among 150 bacterial isolates, multidrug resistance (MDR) was detected in 28.7% of strains. MDR prevalence varied markedly between bacterial groups, reaching 100.0% in Enterobacterales, 73.3% in Enterococcus spp., and 16.2% in lactic acid bacteria. Resistance was most frequently observed for aminoglycosides and β -lactam antibiotics, particularly streptomycin and gentamicin. The results indicate that artisanal cheeses constitute heterogeneous microbial niches and may serve as potential reservoirs of antimicrobial-resistant bacteria. Integrating microbiological and AMR analyses provides valuable insight into the ecological determinants of resistance in traditional dairy products. These findings indicate that artisanal cheeses may represent heterogeneous microbial niches and potential reservoirs of AMR bacteria. The integration of microbiome profiling and phenotypic AMR assessment provides valuable insight into the ecological drivers of resistance in traditional dairy products.

Keywords: antimicrobial resistance; multidrug resistance; raw milk cheese; goat cheese; sheep cheese; microbiome; Enterococcus; Enterobacterales; food safety; LAB

1. Introduction

Dairy products constitute an important component of human nutrition, providing essential proteins, lipids, vitamins, and minerals. Cheeses produced from sheep's and goat's milk are particularly valued for their nutritional quality, distinctive sensory attributes, and strong association with traditional and artisanal food systems. Milk from small ruminants is characterized by specific compositional and bioactive features that contribute to the nutritional and functional properties of derived dairy products [1,2]. In addition, traditional cheeses produced from sheep's and goat's milk are recognized as nutritionally valuable foods in local diets, with their quality being influenced by animal breed and traditional production practices [3].

Cheeses manufactured from raw milk host complex microbial communities that play a crucial role in fermentation, ripening, and product safety. Lactic acid bacteria (LAB), including *Lactobacillus*, *Lactococcus*, *Streptococcus*, and *Enterococcus*, dominate these ecosystems and contribute to acidification, texture development, and inhibition of undesirable microorganisms. However, the heterogeneous microbiota naturally present in raw milk cheeses may also include opportunistic or undesirable microorganisms, reflecting the characteristics of the raw material and artisanal processing conditions [4,5].

Antibiotic resistance (AR) represents a major global public health concern, as it reduces the effectiveness of antimicrobial therapies and complicates the treatment of infectious diseases. The presence of antibiotic-resistant bacteria (ARB) in the food chain further amplifies this challenge, since foodborne exposure may contribute to the dissemination of resistance traits to humans [6]. Within this context, raw milk cheeses have increasingly been identified as potential reservoirs of ARB. Resistant strains of *Enterococcus*, *Staphylococcus*, and *Escherichia coli* have been reported in artisanal cheeses, highlighting the persistence of resistance traits within traditional dairy products [7].

The risk associated with ARB is particularly relevant in unpasteurized cheeses, where diverse microbial populations remain viable throughout production and ripening. Although LAB dominate these ecosystems and contribute positively to cheese quality, some strains may harbor intrinsic or acquired resistance determinants that can be horizontally transferred to other bacteria. Increasing evidence indicates that LAB isolated from fermented foods may exhibit resistance to clinically relevant antimicrobials, raising concerns about their potential role as reservoirs of antimicrobial resistance [8,9].

Previous studies have shown that raw milk harbors a richer resistome than processed milk, which may facilitate the persistence of antimicrobial resistance within cheese microbiota [10]. In addition to antimicrobial resistance, interactions among lactic acid bacteria in raw milk cheeses, including bacteriocin-mediated inhibition of foodborne pathogens, may influence microbial community structure and selection pressures within the cheese matrix [11]. These observations underscore the importance of targeted surveillance of antimicrobial resistance in raw milk dairy products.

Despite growing interest in antimicrobial resistance in dairy foods, comparative data on raw milk cheeses from different animal sources remain limited. Most available studies focus primarily on bovine milk, whereas cheeses derived from sheep and goats are less extensively investigated [4]. Understanding how animal source and microbial composition influence resistance patterns in unpasteurized cheeses is therefore essential for accurate risk assessment and the development of effective food safety strategies. Among antimicrobials, β -lactam and non- β -lactam antibiotics are of particular relevance in the context of antimicrobial resistance, as they are widely used in veterinary medicine and frequently associated with resistant bacteria circulating along the food chain [12].

The aim of this study was to characterize the microbiological profiles, bacterial community composition, and antimicrobial resistance patterns of artisanal cheeses produced from raw goat's and sheep's milk. Particular emphasis was placed on identifying dominant bacterial taxa and evaluating the prevalence of multidrug-resistant (MDR) isolates among culture-dependent bacterial populations. Additionally, the study sought to assess whether milk origin influences microbiome

structure and the distribution of resistance traits in traditional cheeses. By integrating classical microbiology, molecular identification, and phenotypic susceptibility testing, this work provides insight into the ecological and food safety implications of antimicrobial resistance in raw milk dairy products.

2. Results

1.1. Microbiological Counts in Goat and Sheep Cheese Samples

The quantitative microbiological analysis included determining the numbers of lactic acid bacteria (LAB), total mesophilic microflora, and Enterobacteriaceae in goat and sheep cheeses. These parameters are basic indicators of the microbiological quality and fermentation stability of the tested products. The results obtained provide context for interpreting the structure of the microbiome and the drug resistance profiles of the isolated strains. Detailed data are presented in Table 1.

Table 1. Microbiological counts (log₁₀ CFU/g) of lactic acid bacteria (LAB), total mesophilic.

Milk Type	Sample	LAB (log ₁₀ CFU/g)	Mesophilic Bacteria (log ₁₀ CFU/g)	Enterobacteriaceae (log ₁₀ CFU/g)
Goat	G1	8.56 ± 0.21	8.19 ± 0.13	2.72 ± 0.26
Goat	G2	8.74 ± 0.30	8.39 ± 0.20	3.41 ± 0.18
Goat	G3	8.34 ± .017	8.05 ± 0.03	1.83 ± 0.08
Goat	G4	7.85 ± 0.11	7.68 ± 0.24	2.49 ± 0.21
Goat	G5	7.65 ± 0.14	7.51 ± 0.31	1.77 ± 0.33
Sheep	S1	4.95 ± 0.19	5.93 ± 0.05	1.94 ± 0.06
Sheep	S2	5.04 ± 0.02	5.26 ± 0.14	2.20 ± 0.15
Sheep	S3	5.48 ± 0.21	5.85 ± 0.09	3.27 ± 0.10
Sheep	S4	6.15 ± 0.06	6.34 ± 0,12	3.11 ± 0.05
Sheep	S5	5.48 ± 0.11	5.66 ± 0,21	3.74 ± 0.27

The microbiological analysis revealed distinct differences in the counts of lactic acid bacteria (LAB), total mesophilic microorganisms, and Enterobacteriaceae between individual goat and sheep cheese samples (Table 1). Goat cheese samples (G1–G5) showed high LAB counts, ranging from 7.65 ± 0.14 to 8.74 ± 0.30 log₁₀ CFU/g. The highest LAB level was observed in sample G2 (8.74 ± 0.30 log₁₀ CFU/g), while the lowest value was recorded in sample G5 (7.65 ± 0.14 log₁₀ CFU/g). Total mesophilic bacterial counts in goat cheeses ranged from 7.51 ± 0.31 to 8.39 ± 0.20 log₁₀ CFU/g, with sample G2 also exhibiting the highest mesophilic count. In sheep cheese samples (S1–S5), LAB counts were lower and ranged from 4.95 ± 0.19 to 6.15 ± 0.06 log₁₀ CFU/g. The lowest LAB level was detected in sample S1, whereas the highest was observed in sample S4. Mesophilic bacterial counts in sheep cheeses varied from 5.26 ± 0.14 to 6.34 ± 0.12 log₁₀ CFU/g, with sample S4 showing the highest value. Enterobacteriaceae counts in goat cheese samples ranged from 1.77 ± 0.33 to 3.41 ± 0.18 log₁₀ CFU/g. The highest Enterobacteriaceae level was recorded in sample G2, while the lowest was observed in sample G5. In sheep cheeses, Enterobacteriaceae counts ranged from 1.94 ± 0.06 to 3.74 ± 0.27 log₁₀ CFU/g, with the highest value detected in sample S5.

1.2. Microbial Community Composition of Individual Goat and Sheep Cheese Samples

The composition of the cheese microbiome at the genus level was further examined by analysing the relative abundance of bacterial taxa in individual goat (G1–G5) and sheep (S1–S5) cheese samples (Figure X). The stacked bar plot representation highlights both inter-group differences and pronounced intra-group variability in microbial community structure. Across all samples, lactic acid bacteria dominated the microbiota, with the genera *Lactobacillus* and *Lactococcus* being the most abundant. However, their relative contributions varied substantially among individual cheeses. Goat cheese samples were generally characterised by a higher relative abundance of *Lactobacillus*, ranging

from 42.3% (G4) to 68.7% (G2), with a mean value of $56.4 \pm 9.8\%$. The relative abundance of *Lactococcus* in goat cheeses ranged from 18.5% (G2) to 37.9% (G5), averaging $27.8 \pm 7.1\%$. In addition, lower proportions of *Staphylococcus* (2.1–8.4%) and *Enterobacter* (1.3–6.7%) were detected. Minor contributions from *Pseudomonas* (<2%) and *Bacillus* (<1.5%) were observed sporadically. In contrast, sheep cheese samples exhibited a more diverse microbial profile. The relative abundance of *Lactobacillus* ranged from 21.4% (S1) to 46.8% (S3), with a mean value of $34.2 \pm 10.7\%$, while *Lactococcus* varied between 14.2% (S3) and 33.5% (S2), averaging $24.6 \pm 8.3\%$. Several sheep cheese samples showed increased *Bacillus* contributions, ranging from 9.8% (S4) to 27.6% (S1), with a mean relative abundance of $18.7 \pm 7.9\%$. Moreover, *Pseudomonas* (3.5–12.9%) and *Bacteroides* (2.4–10.7%) were consistently detected. The relative abundance of *Staphylococcus* remained moderate (1.9–6.3%). Notably, the observed variability among individual samples within each group was comparable to, or greater than, the average differences between goat and sheep cheeses. This pattern was consistent with the ordination analyses based on PCoA, which indicated substantial within-group dispersion and a lack of clear separation between cheese types in multivariate space. Together, these results suggest that individual sample characteristics exert a stronger influence on microbiome composition than cheese type alone. This finding is consistent with the ordination analyses based on PCoA, which indicated substantial within-group dispersion and a lack of clear separation between cheese types in multivariate space. Together, these results suggest that individual sample characteristics exert a stronger influence on microbiome composition than cheese type alone.

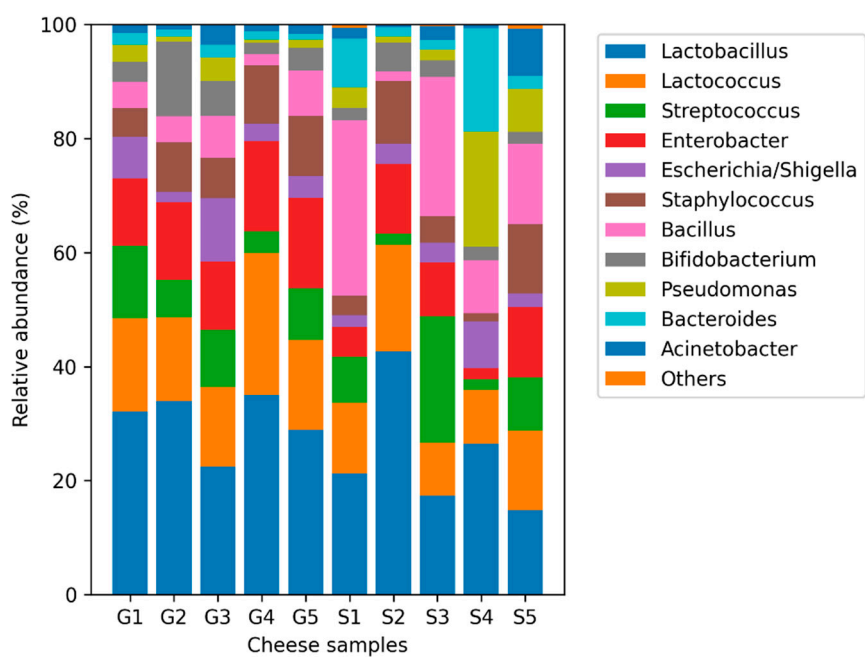


Figure 1. Relative abundance (%) of bacterial genera in individual goat (G1–G5) and sheep (S1–S5) cheese microbiomes.

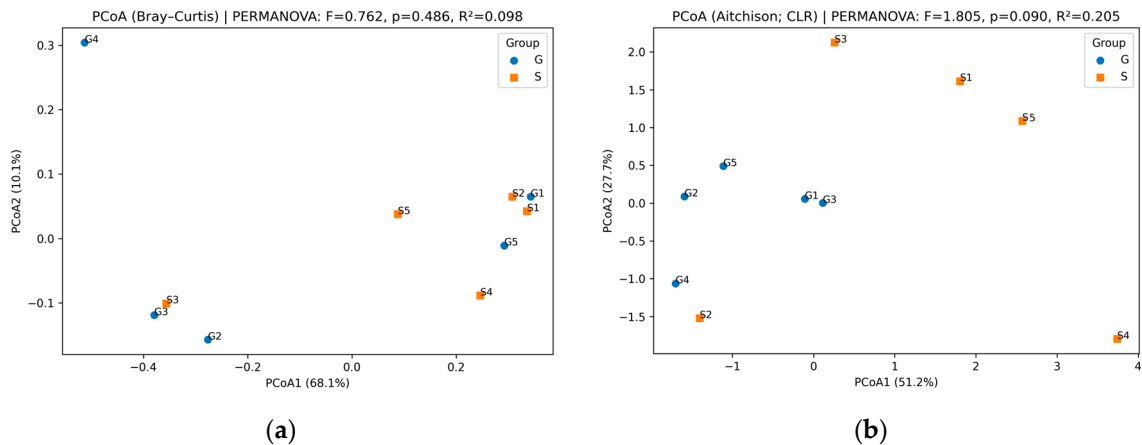


Figure 2. Principal coordinates analysis (PCoA) of goat (G) and sheep (S) cheese microbiomes based on Bray–Curtis and Aitchison dissimilarity.

The similarity structure among samples was evaluated using Principal Coordinates Analysis (PCoA) based on the Bray–Curtis dissimilarity metric (Figure X). The first principal coordinate (PCoA1) explained 68.1% of the total variance, while the second axis (PCoA2) accounted for 10.1%, together capturing over 78% of the variability in the dataset. The ordination plot showed partial clustering of samples from groups G and S. However, no distinct separation between the two groups was observed. Samples within group G exhibited greater dispersion along the primary ordination axis. Permutational multivariate analysis of variance (PERMANOVA) performed on the Bray–Curtis distance matrix did not indicate statistically significant differences between groups G and S (pseudo-F = 0.76, p = 0.486). Group membership explained 9.8% of the total variance (R² = 0.098). SIMPER analysis identified the variables contributing most strongly to between-group dissimilarity. The highest contribution was attributed to variable F1 (25.7%), followed by F7 (15.9%) and F2 (11.8%). The three most influential variables collectively explained more than 53% of the observed dissimilarity. An additional PCoA was conducted using Aitchison distance following CLR transformation (Figure X). This analysis showed a more pronounced tendency toward group separation. PERMANOVA based on Aitchison distance indicated a stronger group effect (pseudo-F = 1.81; R² = 0.205), although the result was not statistically significant (p = 0.090).

1.3. Antibiotic Susceptibility of Bacterial Isolates (Disk Diffusion Method)

The susceptibility of bacterial isolates to antibiotics was evaluated using the disk diffusion method. The analysis included identifying resistance phenotypes and classifying strains as multidrug-resistant (MDR). The obtained results enabled a comparative assessment of antimicrobial resistance profiles among bacterial species isolated from the investigated cheese samples.

Table 2. Antibiotic resistance profiles and prevalence of multidrug-resistant (MDR) isolates among bacterial species recovered from goat and sheep cheeses.

Species	n isolates	MDR (%)	MDR (n)	Resistance pattern
<i>Lactococcus lactis</i>	28	0.0	0	STR-TMP
<i>Streptococcus thermophilus</i>	20	0.0	0	AMP-OXA-GEN
<i>Lactiplantibacillus plantarum</i>	18	0.0	0	AMP
<i>Lacticaseibacillus paracasei</i>	14	0.0	0	AMP-STR-CIP
<i>Enterococcus faecalis</i>	12	100.0	12	ERY-GEN-VAN
<i>Lactobacillus helveticus</i>	10	0.0	0	AMP-STR
<i>Enterococcus faecium</i>	10	100.0	10	AMX-ERY-TET-GEN
<i>Lactobacillus delbrueckii</i>	8	0.0	0	AMP-ERY
<i>Leuconostoc mesenteroides</i>	8	100.0	8	PEN-TET-STR-VAN
<i>Enterococcus durans</i>	8	0.0	0	OXA-GEN

<i>Pediococcus pentosaceus</i>	6	100.0	6	AMP-ERY-CIP
<i>Staphylococcus equorum</i>	6	100.0	6	AMX-OXA-GEN
<i>Staphylococcus saprophyticus</i>	4	0.0	0	OXA
<i>Bacillus subtilis</i>	4	0.0	0	CLI-CIP
<i>Lacticaseibacillus rhamnosus</i>	3	100.0	3	PEN-AMP-CLI-STR
<i>Micrococcus luteus</i>	3	100.0	3	AMX-OXA-GEN-STR-TMP
<i>Lactococcus garvieae</i>	2	100.0	2	AMP-ERY-STR
<i>Enterobacter cloacae</i>	2	100.0	2	AMP-AMX-GEN-CIP-VAN
<i>Escherichia coli</i>	2	100.0	2	ERY-CLI-GEN
<i>Citrobacter freundii</i>	1	100.0	1	VAN
<i>Klebsiella oxytoca</i>	1	100.0	1	PEN-AMP-VAN

Table 2 summarizes the antimicrobial resistance profiles of bacterial isolates recovered from the analyzed cheese samples, including the total number of isolates per species, the proportion classified as multidrug-resistant (MDR), and the absolute number of MDR strains.

The most frequently identified species was *Lactococcus lactis* (n = 28), for which no MDR isolates were detected (0%). Similarly, no multidrug resistance was observed in *Streptococcus thermophilus* (n = 20), *Lactiplantibacillus plantarum* (n = 18), *Lacticaseibacillus paracasei* (n = 14), *Lactobacillus helveticus* (n = 10), and *Lactobacillus delbrueckii* (n = 8). In addition, all analyzed isolates of *Enterococcus durans* (n = 8), *Staphylococcus saprophyticus* (n = 4), and *Bacillus subtilis* (n = 4) were susceptible to multiple antibiotic classes and did not exhibit an MDR phenotype. In contrast, a high prevalence of multidrug resistance was recorded among enterococci. All isolates of *Enterococcus faecalis* (12/12; 100%) and *Enterococcus faecium* (10/10; 100%) were classified as MDR. An analogous resistance pattern was observed in *Leuconostoc mesenteroides* (8/8; 100%), *Pediococcus pentosaceus* (6/6; 100%), and *Staphylococcus equorum* (6/6; 100%), with each isolate demonstrating resistance to multiple antimicrobial categories. Complete MDR prevalence was also noted in less frequently detected species, including *Lacticaseibacillus rhamnosus* (3/3), *Micrococcus luteus* (3/3), and *Lactococcus garvieae* (2/2). Among Gram-negative bacteria, all isolates of *Enterobacter cloacae* (2/2), *Escherichia coli* (2/2), *Citrobacter freundii* (1/1), and *Klebsiella oxytoca* (1/1) were likewise classified as MDR. The highest absolute number of MDR isolates was associated with the genus *Enterococcus*, particularly *E. faecalis* (n = 12) and *E. faecium* (n = 10), indicating that enterococci constituted the principal reservoir of multidrug resistance within the studied cheese microbiota.

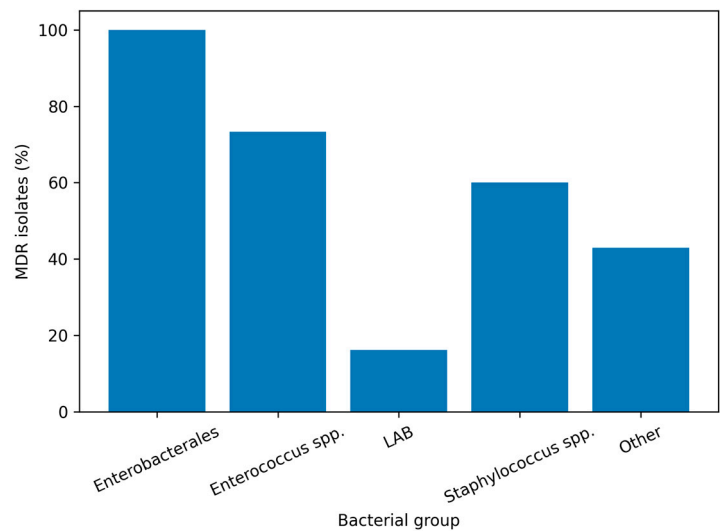


Figure 3. Prevalence of multidrug-resistant (MDR) isolates among major bacterial groups recovered from goat and sheep cheeses.

Figure 3 illustrates the distribution of multidrug-resistant (MDR) isolates among the major bacterial groups recovered from cheese samples. Enterobacterales exhibited the highest MDR prevalence (100%), followed by Enterococcus spp. (73%) and Staphylococcus spp. (60%). The “Other” bacterial group showed an MDR proportion of 43%. In contrast, lactic acid bacteria (LAB) demonstrated the lowest MDR prevalence, with only 16% of isolates classified as multidrug-resistant.

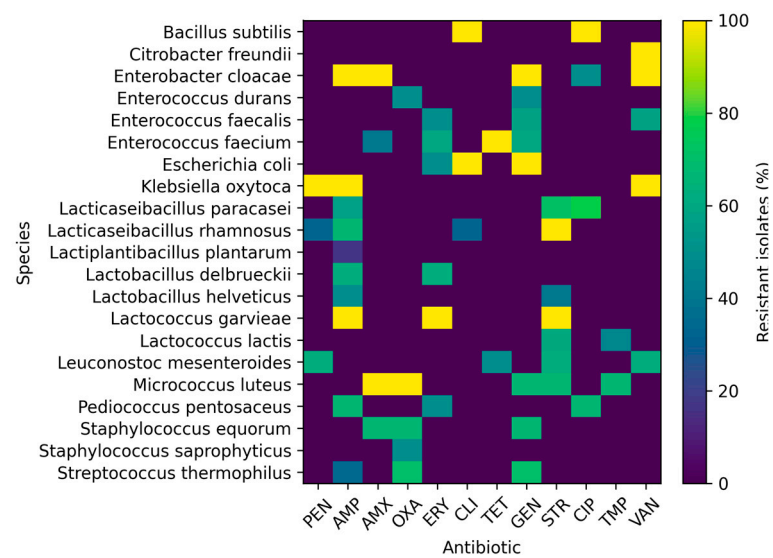


Figure 4. Heatmap of antimicrobial resistance profiles (% resistant isolates) among bacterial species isolated from goat and sheep cheeses.

Figure 4 presents a heatmap showing the proportions of bacterial isolates resistant to individual antibiotics in the dataset of strains recovered from the analyzed cheese samples. The visualization highlights pronounced interspecies variability in resistance prevalence and differential resistance intensities across the investigated antibiotic classes. The highest proportions of resistant isolates were observed for streptomycin (STR; 25.29%), gentamicin (GEN; 24.12%), and ampicillin (AMP; 22.94%). Moderate resistance frequencies were recorded for oxacillin (OXA; 15.88%), erythromycin (ERY; 13.53%), and ciprofloxacin (CIP; 11.76%). Lower resistance rates were noted for vancomycin (VAN; 9.41%), trimethoprim (TMP; 8.82%), tetracycline (TET; 8.24%), and amoxicillin (AMX; 7.65%). The lowest proportions of resistant isolates were associated with penicillin (PEN; 4.12%) and clindamycin (CLI; 4.12%). The heatmap further indicates that antimicrobial resistance was predominantly concentrated within specific taxonomic groups. Isolates of *Enterococcus faecalis* and *Enterococcus faecium* exhibited consistently high resistance frequencies spanning multiple antibiotic classes, including macrolides, aminoglycosides, and glycopeptides. Elevated resistance levels were likewise observed for *Leuconostoc mesenteroides*, *Pediococcus pentosaceus*, and *Staphylococcus equorum*. Notably, all analyzed Gram-negative isolates exhibited extensive resistance patterns, consistent with multidrug resistance. In contrast, the dominant lactic acid bacteria typically associated with dairy fermentation — including *Lactococcus lactis*, *Streptococcus thermophilus*, *Lactiplantibacillus plantarum*, *Lactocaseibacillus paracasei*, *Lactobacillus helveticus*, and *Lactobacillus delbrueckii* — displayed low resistance prevalence, generally limited to single antibiotics rather than multiple drug classes. Moreover, the heatmap reveals that resistance to β -lactam antibiotics and aminoglycosides was the most frequently encountered resistance pattern among the analyzed isolates, suggesting selective pressure associated with these antimicrobial categories.

3. Discussion

The findings of the present study confirm that artisanal cheeses produced from raw goat's and sheep's milk represent complex, heterogeneous, and ecologically dynamic microbial ecosystems. The absence of statistically significant separation between goat and sheep cheeses in Bray-Curtis-based multivariate analyses indicates that milk origin alone does not constitute the dominant determinant of bacterial community structure. Similar observations have been reported in studies of traditional cheeses, where variability associated with artisanal production practices, environmental microbiota, farm-specific conditions, and ripening parameters frequently exceeds that attributable solely to animal species [13–15]. Traditional cheeses are widely recognised as evolving microbial habitats in which physicochemical gradients, including acidification, salt concentration, oxygen availability, and moisture reduction, drive microbial succession and ecosystem stabilisation [13,14]. Across all analysed samples, lactic acid bacteria dominated the microbiomes, with *Lactobacillus*, *Lactococcus*, and *Streptococcus* representing the most abundant genera. This predominance aligns with the established technological and ecological functions of LAB in cheese fermentation, including lactose metabolism, organic acid production, proteolysis, and inhibition of undesirable microorganisms [13,16]. LAB-driven acidification contributes fundamentally to microbial stability; however, the relative abundance profiles revealed substantial intra-group dispersion, particularly among goat cheeses. Such heterogeneity is characteristic of artisanal dairy products and has been attributed to variability in raw milk microbiota, differences in starter and non-starter LAB populations, and inconsistencies in processing conditions [14,16,17]. Comparable variability has been documented in metagenomic studies of fermented foods, highlighting the sensitivity of microbial communities to subtle environmental and technological factors [17]. Sheep cheeses exhibited comparatively higher microbial diversity, including elevated contributions from *Bacillus*, *Pseudomonas*, and occasionally *Bacteroides*-related taxa. The presence of *Bacillus* spp. is microbiologically plausible in *osypek*-type cheeses, where environmental exposure, traditional smoking, and spore persistence favour the survival of stress-resistant taxa [14,15]. Spore-forming bacteria are known to withstand salting, dehydration, and temperature fluctuations encountered during artisanal ripening [14]. Detection of *Pseudomonas* spp., although not dominant, is noteworthy given their psychrotrophic nature and frequent association with raw milk environments. These organisms have been described both as contributors to spoilage processes and as potential reservoirs of antimicrobial resistance determinants [18]. The occasional identification of *Bacteroides*-related sequences, although atypical for cheese matrices, has been reported in metagenomic surveys and may reflect environmental contamination or limitations inherent to sequencing-based taxonomic assignment [17].

Quantitative microbiological analyses further demonstrated higher LAB and mesophilic bacterial counts in goat cheeses compared with sheep cheeses. LAB levels exceeding 7 log CFU/g are generally indicative of stable fermentation ecosystems and efficient acidification [15]. Lower LAB counts observed in certain sheep cheeses may be associated with technological variables, such as salt diffusion dynamics, moisture reduction, and smoking intensity, which influence microbial growth and succession [14,15]. Although Enterobacteriaceae counts remained within ranges commonly reported for raw milk cheeses, their detection remains relevant from both food safety and antimicrobial resistance perspectives, given the ecological role of Gram-negative bacteria as carriers of transferable resistance genes [18,19]. A central outcome of this study concerns the uneven distribution of antimicrobial resistance phenotypes across taxa. Multidrug-resistant isolates were predominantly associated with *Enterococcus* spp. and Gram-negative Enterobacterales, whereas dominant LAB taxa exhibited low MDR prevalence. Enterococci are well recognised for their dual significance in dairy systems, functioning both as technologically relevant adjunct microbiota and as opportunistic organisms characterised by remarkable genomic plasticity [20–22]. The universal detection of MDR phenotypes among *Enterococcus faecalis* and *Enterococcus faecium* isolates observed in the present study corroborates extensive literature identifying enterococci as major reservoirs of intrinsic and acquired resistance mechanisms [20–23]. Resistance to aminoglycosides, macrolides, tetracyclines, and β -lactams is frequently documented among dairy-associated

enterococci, reflecting their ability to acquire resistance determinants carried by plasmids, transposons, and other mobile genetic elements [23]. The persistence of enterococci in cheese matrices can be explained by their high tolerance to environmental stressors characteristic of cheese ripening, including low pH, elevated salt concentrations, and nutrient limitations [21]. These conditions may favour stress-adapted taxa while exerting selective pressures that maintain resistance traits. Comparable resistance patterns have been described in artisanal cheeses from diverse geographical regions [18,22]. The detection of MDR Enterobacterales isolates, including *Escherichia coli* and *Enterobacter cloacae*, although based on a limited number of strains, remains epidemiologically relevant. Studies investigating antimicrobial resistance in raw milk and raw milk cheeses have repeatedly documented the presence of resistant Gram-negative bacteria [19,24]. Even low-abundance Gram-negative populations may contribute disproportionately to the resistome due to their association with highly mobile resistance determinants and their capacity for horizontal gene transfer [23]. In contrast, dominant LAB species displayed low levels of multidrug resistance, supporting earlier conclusions that resistance traits in LAB are predominantly intrinsic, species-specific, and generally non-transferable [25,26]. Nevertheless, the detection of MDR phenotypes among *Leuconostoc mesenteroides* and *Pediococcus pentosaceus* isolates warrants careful interpretation. Although these genera are commonly regarded as technologically beneficial, acquired resistance determinants have occasionally been described, particularly involving tetracycline and glycopeptide resistance [26,27]. Distinguishing intrinsic resistance from acquired and potentially transferable mechanisms remains essential for accurate safety assessment of food-associated LAB [27]. Recent shotgun metagenomic investigations provide further insight into the dynamics of the resistome in cheeses. Comparative analyses of pasteurised and raw milk cheeses demonstrated that while pasteurisation reduces microbial diversity and ARG abundance, resistance determinants may still persist, particularly those associated with spore-forming bacteria [28]. Resistome variability during ripening has also been linked to microbial succession, highlighting that antimicrobial resistance patterns in cheeses represent temporally dynamic ecological traits rather than static characteristics [28]. Environmental contamination in cheesemaking facilities is another critical factor influencing microbiome composition and the dissemination of resistance. Studies tracking microbial quality and contamination sources in artisanal cheesemaking environments have identified equipment surfaces, brining systems, and air as reservoirs of opportunistic pathogens, including *Staphylococcus aureus* [29]. Similarly, investigations conducted in dairy processing plants revealed the persistence and circulation of resistant opportunistic bacteria across multiple environmental niches [30]. These findings reinforce the interpretation that microbiome variability and resistance signatures may partly reflect environmental contributions rather than exclusively milk-derived microbiota. The detection of antimicrobial-resistant *Staphylococcus aureus* strains in artisanal cheeses further contextualises the AMR risk. Studies analysing toxigenic potential and resistance profiles of coagulase-positive staphylococci isolated from cheeses reported high CPS counts, widespread multidrug resistance, and the presence of MRSA [31]. Experimental studies have also demonstrated that MRSA may survive and proliferate during the production of raw milk cheese, although starter cultures may exert inhibitory effects [32]. Another ecological dimension relevant to resistance maintenance involves antibiotic residues. Investigations tracking enrofloxacin during cheesemaking demonstrated that curd formation may concentrate antibiotic residues, whereas high-temperature treatments reduce their levels [33]. Even sub-inhibitory concentrations persisting within cheese matrices may exert selective pressures affecting microbial populations and resistance dynamics. Collectively, these findings emphasise that artisanal raw milk cheeses function as multifaceted microbial ecosystems in which beneficial fermentation-associated bacteria coexist with opportunistic and multidrug-resistant microorganisms. The clustering of MDR phenotypes primarily among enterococci and Enterobacterales suggests that microbial ecology, technological practices, environmental exposure, and antibiotic-related selective pressures exert stronger influences on resistance distribution than milk species alone. From a One Health perspective, the detection of MDR

bacteria in traditional dairy products underscores the importance of continuous antimicrobial resistance surveillance integrating culture-dependent and metagenomic approaches [18,23,24,28].

4. Materials and Methods

4.1. Cheese Samples

The cheese samples analyzed in this study were obtained from regional artisanal producers operating in Poland. Traditional cheeses manufactured using craft-based technologies were selected to represent two types of milk raw material. A total of 10 cheeses were included in the investigation, comprising 5 sheep's milk oscypek cheeses and 5 goat's milk cheeses.

The sheep's milk cheeses represented traditional products characteristic of mountainous regions, produced in accordance with established local cheesemaking practices. The goat's milk cheeses consisted of artisanal ripened or semi-ripened products sourced from small-scale regional manufacturers. Each cheese was treated as an independent analytical sample.

All samples were transported to the laboratory under refrigerated conditions (4 ± 1 °C) and analyzed immediately upon arrival. From each cheese, a representative portion was aseptically collected, grated, and subsequently homogenized in sterile physiological saline to obtain working suspensions used for further microbiological analyses.

4.2. Microbiological Analyses

Cheese samples were subjected to microbiological examination after homogenization. From each sample, 10 g of cheese was aseptically collected and transferred into 90 mL of sterile physiological saline (0.9% NaCl), yielding an initial 10^{-1} dilution. The suspensions were thoroughly mixed, and subsequent tenfold serial dilutions were prepared as appropriate for quantitative enumeration and microbial isolation.

Aliquots (1 mL) from selected dilutions were plated in triplicate onto selective and differential microbiological media. Lactic acid bacteria (LAB) were enumerated using MRS agar. The total counts of mesophilic and psychrotrophic bacteria were determined on GBA agar. Detection and quantitative assessment of bacteria belonging to the family Enterobacteriaceae, including *Escherichia coli*, were performed using MacConkey agar and VRBG agar. Additionally, M17 agar was employed to support the growth of *Lactococcus* spp. and selected spore-forming bacteria.

The inoculated plates were incubated at 30–37 °C for 24–72 h, depending on the growth requirements of the target microorganisms and the characteristics of the applied media. Following incubation, colonies were counted on plates containing 20–300 colonies. The results were expressed as colony-forming units (CFU/mL) and recalculated to the original sample, in accordance with ISO 6611.

Representative colonies with distinct morphologies were selected from the culture plates and subcultured to obtain pure isolates. These cultures were subsequently used for further identification and antibiotic susceptibility testing.

4.3. Antibiotic Panel and Materials

Antibiotic susceptibility testing was performed by disk diffusion method. Commercial antibiotic disks (Oxoid, UK/Germany) were employed, comprising the following panel: penicillin (10 U), trimethoprim (5 µg), streptomycin (25 µg), oxacillin (1 µg), ampicillin (10 µg), ciprofloxacin (5 µg), oxytetracycline (30 µg), clindamycin (2 µg), gentamicin (10 µg), vancomycin (30 µg), and amoxicillin (10 µg). This selection enabled the evaluation of isolate susceptibility to representatives of multiple therapeutic classes, including β -lactams, aminoglycosides, fluoroquinolones, tetracyclines, lincosamides, glycopeptides, and folate pathway inhibitors.

In parallel, stock solutions of selected antibiotics were prepared at 1 g/L in sterile distilled water. The solutions were sterilized by membrane filtration (0.22 µm), aliquoted under aseptic conditions, and stored at -20 °C until further use.

The disk diffusion assay was conducted according to standard laboratory procedures. Freshly grown cultures of the isolates were uniformly inoculated onto the surface of an appropriate agar test medium to obtain confluent growth. Antibiotic disks were then placed onto the agar surface. Following incubation, the inhibition zone diameters were measured, and isolates were categorized as susceptible (S), intermediate (I), or resistant (R) according to established interpretative criteria for the disk diffusion method.

4.4. Genomic DNA Extraction

Genomic DNA was extracted from pure bacterial cultures using the Genomic Mini AX Bacteria+ Spin Kit (A&A Biotechnology, Poland) according to the manufacturer's protocol, with minor modifications to improve DNA yield and quality. Specifically, the enzymatic and chemical lysis steps were extended to ensure efficient disruption of Gram-positive cell walls, and centrifugation times were increased to enhance column performance. DNA concentration was determined fluorometrically using a Qubit 3.0 fluorometer with the dsDNA HS Assay Kit (Thermo Fisher Scientific, USA), providing accurate quantification of double-stranded DNA. Working DNA solutions were prepared by dilution in nuclease-free water to a final concentration of approximately 1 ng/µL for downstream PCR applications.

4.5. PCR Amplification of the 16S rRNA Gene

Molecular identification of bacterial isolates was performed by amplification of the nearly full-length 16S rRNA gene using universal primers 27F (5'-AGAGTTTGATCMTGGCTCAG-3') and 1492R (5'-TACGGYTACCTTGTACGACTT-3'). PCR reactions were performed using a commercial PCR Master Mix PLUS (2×) (EURx, Poland) according to the manufacturer's recommendations. The reactions were performed in a final volume of 25 µL containing template DNA, primers, and the PCR Master Mix PLUS, which includes Taq DNA polymerase, reaction buffer, MgCl₂, dNTPs, and a tracking dye enabling direct electrophoretic analysis. Thermal cycling conditions consisted of an initial denaturation step at 95 °C for 2 min, followed by 30 cycles of denaturation at 95 °C for 30 s, primer annealing at 54 °C for 30 s, and extension at 72 °C for 1 min. A final extension step at 72 °C for 5 min was included to ensure complete synthesis of PCR products. The presence and expected size of the amplicons (~1,500 bp) were verified by agarose gel electrophoresis. PCR products were purified and subjected to Sanger sequencing (IGS, Bydgoszcz, Poland). The obtained nucleotide sequences were quality-checked, assembled, and compared with reference sequences available in the NCBI GenBank database using the BLAST algorithm. Taxonomic identification at the species level was considered reliable when sequence similarity reached ≥99%, in accordance with commonly accepted thresholds for 16S rRNA gene-based identification reported in the literature.

4.6. DNA Amplification

Amplification of the bacterial 16S rRNA gene was performed using PCR with universal primers targeting the V3–V4 hypervariable region. The reactions were carried out using primers Pro341F and Pro805R, enabling amplification of a fragment suitable for taxonomic profiling. PCR amplification was conducted using SsoFast EvaGreen Supermix (Bio-Rad, USA) under optimized conditions. Each reaction mixture (25 µL total volume) contained genomic DNA template, forward and reverse primers, nuclease-free water (Thermo Fisher Scientific, USA), and the PCR master mix comprising DNA polymerase, reaction buffer, MgCl₂, dNTPs, and EvaGreen dye. Amplification was performed in a thermal cycler (ABI 2720/2724, Applied Biosystems, USA) using the following cycling program: initial denaturation at 95 °C for 3 min, followed by 35 cycles of denaturation at 95 °C for 1 min, primer

annealing at 52 °C for 30 s, and extension at 72 °C for 1 min. A final extension step was carried out at 72 °C for 10 min. Successful amplification was verified by agarose gel electrophoresis.

4.7. PCR Product Purification and Quantification

Following amplification, PCR products were purified using AMPure XP magnetic beads (Beckman Coulter, USA), according to the manufacturer's protocol. Purified amplicons were eluted in 20 µL of nuclease-free water. DNA concentration was determined fluorometrically using a Qubit® 3.0 fluorometer (Thermo Fisher Scientific, USA).

4.8. Library Preparation and MinION Sequencing

Sequencing of 16S rRNA gene amplicons was performed using the MinION platform (Oxford Nanopore Technologies, UK). Libraries were prepared using the Native Barcoding Kit 24 V14 and Flongle Sequencing Expansion (Oxford Nanopore Technologies), following the manufacturer's instructions. The required DNA input was calculated based on fluorometric quantification. Purified 16S amplicons were normalized to the target concentration prior to end-preparation. End-repair and dA-tailing reactions were performed using Ultra II End-Prep Reaction Buffer and Ultra II End-Prep Enzyme Mix (New England Biolabs, USA). The reactions were incubated at 20 °C for 5 min followed by enzyme inactivation at 65 °C for 5 min. The products were subsequently purified using AMPure XP beads. Native barcode ligation was performed using Blunt/TA Ligase Master Mix (New England Biolabs, USA). Barcoded samples were pooled in equimolar ratios and purified using AMPure XP beads. Adapter ligation was carried out using Quick T4 DNA Ligase (New England Biolabs, USA), followed by final bead-based purification.

4.9. Flow Cell Loading and Sequencing

The sequencing library was prepared by combining the sequencing buffer with the purified DNA library. The mixture was loaded onto a primed Flongle flow cell installed in the MinION device. Sequencing was performed according to Oxford Nanopore Technologies standard protocols.

4.10. Bioinformatic Analysis

The sequencing output was imported into CLC Genomics Workbench 8.5 with the CLC Microbial Genomics Module 1.2 (Qiagen, Germany). Readings were trimmed, demultiplexed and paired ends were joined. Then, the chimeric readings were identified and removed. The data were clustered independently against two reference databases: SILVA v119 and GreenGenes 13.5, at 97% similarity for operational taxonomic units (OTUs).

5. Conclusions

This study showed that artisanal goat and sheep cheeses produced by regional manufacturers exhibit highly heterogeneous microbial ecosystems, with substantial differences in bacterial counts and community composition. Lactic acid bacteria dominated most samples, confirming their key technological and ecological role in raw milk cheeses. The pronounced variability among cheeses suggests that production practices and local environmental factors influence microbial structure more strongly than milk origin alone. The analyses also revealed the presence of antimicrobial-resistant bacteria. Multidrug-resistant (MDR) phenotypes were primarily associated with *Enterococcus* spp. and Gram-negative Enterobacterales, whereas lactic acid bacteria exhibited relatively low MDR prevalence. Resistance patterns, particularly to aminoglycosides and β -lactams, suggest that traditional cheeses may serve as reservoirs of antimicrobial resistance. Although multivariate analyses did not reveal a clear separation between goat and sheep cheeses, integrating microbiological and AMR data provided important insights into the ecological distribution of resistant bacteria. These findings highlight the importance of ongoing surveillance of antimicrobial

resistance in artisanal dairy products and support the use of combined microbiological and molecular approaches in microbial risk assessment.

Supplementary Materials: The following supporting information can be downloaded at: Table S1. The results of read-filtering in sequencing data analysis (goat and sheep cheese samples); Table S2. Disk diffusion antimicrobial susceptibility results (R/I/S) for bacterial isolates from goat (G) and sheep (S) cheeses.

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Abbreviations

The following abbreviations are used in this manuscript:

AMR	Antimicrobial Resistance
AR	Antibiotic Resistance
ARB	Antibiotic-Resistant Bacteria
LAB	Lactic Acid Bacteria
MDR	Multidrug-Resistant
MIC	Minimum Inhibitory Concentration
PCoA	Principal Coordinates Analysis
PERMANOVA	Permutational Multivariate Analysis of Variance
SIMPER	Similarity Percentage Analysis
CLR	Centered Log-Ratio
CFU	Colony Forming Units
PCR	Polymerase Chain Reaction
DNA	Deoxyribonucleic Acid
rRNA	Ribosomal Ribonucleic Acid
OTU	Operational Taxonomic Unit
BLAST	Basic Local Alignment Search Tool
NCBI	National Center for Biotechnology Information
AMP	Ampicillin
AMX	Amoxicillin
PEN	Penicillin
OXA	Oxacillin
GEN	Gentamicin
STR	Streptomycin
CIP	Ciprofloxacin
TET	Tetracycline
ERY	Erythromycin
VAN	Vancomycin
TMP	Trimethoprim
CLI	Clindamycin

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