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Potential for Biofilm Formation and Antibiotic Resistance of Staphylococci Isolated from Bulk Tank Milk Samples

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Abstract: The prevalence of staphylococci in the environment of humans, including food, may lead to the production of toxins and food poisoning in consumers. Additionally, staphylococci constitute a reservoir of genes determining antibiotic resistance. The study aimed to compare the antibiotic resistance and biofilm-forming ability of staphylococci isolated from bulk tank milk and assess the occurrence of animal-associated methicillin-resistant *Staphylococcus aureus*. The study consisted of isolation of strains from cow milk, confirming their belonging to *Staphylococcus* sp. and *S. aureus*, investigation of the presence of a gene determining the production of coagulase, and genes determining resistance to β-lactams, tetracyclines, vancomycin, natural penicillins, and aminopenicillins, as well as virulence genes determining biofilm production. There were 56 bacterial strains isolated, which belong to *Staphylococcus* spp. All isolates showed resistance to amoxicillin, erythromycin, and tetracycline. The lowest resistance was demonstrated for vancomycin (48.2%). The *mecA* gene was not detected in any of the isolates and there was no positive reaction to the PB2 protein, which indicates that none of the isolated strains was methicillin resistant. Only 26.8% of isolated strains did not show the ability to produce biofilm. Special attention should be paid to the health of dairy cows and the supervision of the use of antibiotics in the dairy herd environment because, even in milk from healthy cows, there is a real risk of the occurrence of multi-drug resistant bacteria.

Keywords: antibiotics; biofilms; bulk tank milk; *Staphylococcus* sp.; *Staphylococcus aureus*; PCR; raw milk

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1. Introduction

The prevalence of staphylococci in the environment of humans and animals has been known for a long time. It is known that they constitute the microbiome of the mucous membranes and skin of both humans and animals [1]; hence, they are frequently present in foods of animal origin [2,3]. Their presence in food in favorable conditions for growth may lead to the production of toxins and food poisoning in consumers [4]. Therefore, they constitute a significant economic problem for farmers and a serious problem for the food industry, especially the dairy industry [5]. The occurrence of staphylococci in milk may be the result of their excretion by infected animals or contamination from the environment where milk is obtained. These bacteria are one of the most important causes of mastitis in farm animals [6]. Broad-spectrum antibiotics are often used in the treatment of mastitis without early identification of the microorganisms causing them and knowledge of their resistance to antimicrobial agents. This is one of the reasons for the phenomenon observed in recent years that not only *Staphylococcus aureus* which can produce

coagulase (coagulase-positive staphylococci—CPS) [7] but also other species belonging to *Staphylococcus* spp. which do not produce coagulase and were previously considered as saprophytic organisms (coagulase-negative staphylococci—CNS) have become the main pathogens causing mastitis in cattle [8].

These bacteria have numerous and diverse virulence factors that can be divided into two groups: Those that are an integral part of the cell structure and those that are secreted into the environment. Their ability to produce biofilms both in tissues and on abiotic surfaces is considered to be the main cause of virulence. Biofilm enables better protection of staphylococci in the host body against immune mechanisms, and in the case of abiotic surfaces, it makes their cleaning and disinfection more difficult, which is important in maintaining the safety of milk processing.

The presence of staphylococci in the production environment [9] and in food of animal origin, including raw milk [10] does not necessarily cause consumer illnesses, but it is a reservoir of bacteria that can be transferred to humans during milk processing and preparation of milk products. Additionally, there is a view that CNS constitute a reservoir of genes determining antibiotic resistance [11,12]. In recent years, there has been a trend among consumers in Europe to reduce the consumption of animal products. According to a report published as part of the "Smart-Protein" project, 51% of Europeans declare that they limit their consumption of meat and other animal products [13]. Despite these declarations, data published by FAO indicate that the production of food of animal origin in the world is still increasing. Therefore, food of animal origin is a significant source of microbiological threats in the environment, including the spread of microbial resistance to antibiotics widely used not only in animal therapy but also in humans. According to the forecast published in the United Nations environmental program report, in the next decade up to 24 million people may be exposed to serious illnesses related to the occurrence of antibiotic resistance of microorganisms, and in 2050 it might be expected up to 10 million deaths resulting from the lack of effective antibiotics [14]. Therefore, in the present study, we investigated whether in milk meeting the requirements for milk intended for food purposes, coming from healthy cows, there is a risk of the presence of bacteria of the *Staphylococcus* genus, which are a reservoir of genes determining antibiotic resistance. The study aimed: (1) To compare antibiotic resistance and the biofilm-forming ability of CNS and CPS isolated from bulk tank milk, and (2) to assess the occurrence of AA-MRSA (animal-associated methicillin-resistant *Staphylococcus aureus*) in samples of bulk tank milk meeting EU legal requirements, intended for food purposes. This is vitally important taking into account that in some situations specified by law, milk may also be marketed as raw milk.

2. Materials and Methods

2.1. Sample Collection

The study used staphylococcal strains isolated from samples of cow milk.

Samples of bulk milk meeting the requirements of Regulation 853/2004 EU [15] with a volume of 50 mL were collected in spring (March–April) 2022 and came from randomly selected 100 dairy farms located in north-eastern Poland, supplying raw milk intended for food purposes. The population of dairy cows in herds was in the range of 25 to 100 cows. According to the agreement with the farm owners and veterinarians supervising the farms, samples were taken automatically in the flow, while pumping milk from the barn tank to the transport tank. The milk came from cows that did not show any signs of mastitis, which was confirmed by milking procedures carried out on the farms (assessment of the udder condition, assessment of the milk on the pre-shedder, and the California test).

Aseptically collected milk samples with a volume of 50 mL were stored at 4 °C for no longer than 24 h until analyses. A total of 100 milk samples were collected.

2.2. Isolation of Staphylococcus spp.

Isolation of staphylococci from milk samples was carried out following the PN-EN ISO 6888-1:2022-03 [16]. Initial identification of staphylococci was made based on the

phenotypic characteristics of the colonies on Baird-Parker agar and Chapman agar, the ability to hemolyze on blood agar. The ability to produce catalase and coagulase and the assessment of the morphology of bacterial cells in a microscopic preparation stained with the Gramm method was carried out following the guidelines contained in PN-EN ISO 6888-3:2004 [17]. Moreover, the affiliation of the strains to the *S. aureus* species was determined by the polymerase chain reaction (PCR) method based on the amplification of species-specific parts of the gene encoding the 23S rRNA with the oligonucleotide primers shown in Table 1.

Table 1. PCR primers used in the study.

Primer	Sequence (5′-3′)	Product Size	Annealing Temperature	References
23S rRNA Staur4 Staur6	ACG GAG TTA CAA AGG ACG AC AGC TCA GCC TTA ACG AGT AC	1250	55 °C/2 min 72 °C/5 min	[18]
coa coa1-F coa2-R	ATA GAG ATG CTG GTA CAG G GCT TCC GAT TGT TCG ATG C	440, 600, 840 variable	55 °C/2 min 72 °C/5 min	[18]
mecA mecA1-F mecA2-R	GGG ATC ATA GCG TCA TTA TTC AAC GAT TGT GAC ACG ATA GCC	527	56.1 °C/1 min	[19]
tetM tetM1–F tetM2-R	GTT AAA TAG TGT TCT TGG AG CTAAGATATGGCTCTAACAA	656	45 °C/1 min	[19]
tetK tetK1-F tetK2-R	TTA GGT GAA GGG TTA GGT CC GCA AAC TCA TTC CAG AAG CA	718	55 °C/1 min	[19]
tetO tetO1-F tetO1-R	AAC TTA GGC ATT CTG GCTCAC TCC CAC TGT TCC ATA TCG TCA	515	55 °C/1 min	[20]
blaZ blaZ1-F blaZ2-R	CAG TTC ACA TGC CAA AGA G TAC ACT CTT GGC GGT TTC	772	50 °C/1 min	[21]
vanA vanA1-F vanA2-R	GGG AAA ACG ACA ATT GC GTA CAA TGC GGC CGT TA	732	54 °C/1 min	[22]
vanB vanB3-F vanB4-R	ACG GAA TGG GAA GCC GA TGC ACC CGA TTT CGT TC	647	54 °C/1 min	[22]
VanC1/2 vanC5-F vanC8-R	ATG GAT TGG TAY TKG TAT c TAG CGG GAG TGM CYM GTA A c	815/827	54 °C/1 min	[22]
VanD vanD1-F vanD2-R	TGT GGG ATG CGA TAT TCA A TGC AGC CAA GTA TCC GGT AA	500	54 °C/1 min	[22]
Ica Ica-F Ica-R	TATACCTTTCTTCGATGTCG CTTTCGTTATAACAGGCAAG	700	54 °C/1 min	[23]
agr agr-F agr-R	TATGCTCCTGCAGCAACTAA CTTGCGCATTTCGTTGTTGA	1070	50 °C/1 min	[23]
sasC sas-F sas-R	GCAACGAATCAAGCATTGG TGACAGCACTTCGTTAGG	600	54 °C/1 min	[23]

2.3. Molecular Analyses

2.3.1. DNA Isolation

Based on phenotypic characteristics, strains were selected for molecular testing. Isolation of genetic material from a 24 h bacterial culture of strains on TSA medium (Millipore, Sigma-Aldrich, Darmstadt, Germany) with 5% sheep blood (tryptone soya agar with 5% sheep blood) was carried out using a genomic DNA isolation kit from A&A Biotechnology (Gdańsk, Poland), according to the manufacturer's procedure. The obtained genetic material was suspended in 200 µL of H_2O (distilled, free from RNA and DNA).

2.3.2. PCR Assay

The isolated genetic material was subjected to PCR analysis to (1) confirm the identity of isolated strains to the *S. aureus* species based on the amplification of species-specific parts of the gene encoding the 23S rRNA, (2) detect the presence of a gene determining the production of coagulase (parts of the gene encoding the *coa*), (3) detect the presence of genes determining antibiotic resistance: (parts of the gene encoding the *mecA* for β-lactams; *tetM*, *tetK*, *tetO*—tetracyclines; *vanA*, *vanB*, *vanC*, *vanD*—vancomycin; *blaZ*—natural penicillins and aminopenicillins), (4) detect virulence genes determining biofilm production (*agr*; *sasC*; *ica*). Oligonucleotide primers used in the experiment are shown in Table 1.

Amplification in a thermal cycler (Mastercykler vapo.protect, Eppendorf A.G., Hamburg, Germany) was carried out in a reaction mixture with a final volume of 25 µL containing: PCR Mix Plus (standard mixture from A&A Biotechnology) 12.5 µL, Primer F 1 µL, Primer D 1 µL, H_2O 5.5 µL, DNA 5 µL. All PCR reactions were performed under the following conditions: Initial denaturation at 94 °C for 5 min., followed by 30 cycles of denaturation for 1 min at 94 °C, annealing at the temperature appropriate for the primer pair for 1 min (Table 1) and amplification for 1 min at 72 °C. The final amplification step was performed at 72 °C for 5 min.

The PCR product was identified after electrophoresis for 1 h at 70 V on a 1.5% agarose gel (0.75 g of agarose + 50 mL of TAE) with the addition of 25 µL of EtBr.

The control was DNA isolated from strains from the ATCC collection: *S. aureus* MRSA-33591 positive *coa* and *mecA*, *S. aureus*-35923 negative *mecA*, *S. epidermidis* 12228 negative *coa* and *S. saprophiticus* 15305.

2.4. Assessment of Antibiotic Resistance

The antibiotic resistance was performed following EUCAST recommendations using the disk diffusion test on the Mueller-Hinton (MH) medium (Millipore, Sigma-Aldrich, Darmstadt, Germany). Prepared inoculum with a density of 0.5 on the McFarland scale (approx. cell density 1.5×10^8 CFU/mL) by suspending colonies of the tested strain in physiological saline. MH plates were inoculated with the prepared suspension and waited until the substrate surface was dry. Then, the following antibiotics were placed on the surface of the plates: Erythromycin (ERY, 15 µg), amoxicillin (AMK, 10 µg), ampicillin (AMP 10 µg), vancomycin (WAN, 5 µg), and tetracycline (OKS, 30 µg). The selection of antibiotics for the experiment was based on an interview conducted at a dairy farm. These were the antibiotics most often used to treat mastitis occurring in dairy cows on the farms from which the milk samples for testing came.

Strains from the ATCC collection (American Type Culture Collection) were used to control the quality of the assays. As reference materials: *Staphylococcus aureus*-ATCC 29213 and ATCC 25923, for methicillin-resistant *S. aureus*-MRSA ATCC 33591, for *Staphylococcus epidermidis*-ATCC 12228 and *Staphylococcus saprophyticus* ATCC 15305.

2.5. Determination of Methicillin Resistance-Detection of PB2 Protein

PB2 protein was detected using the Oxoid latex test (PENICILIAN-BINDING PROTEIN (PBP-2) according to the manufacturer's instructions. Latex particles coated with monoclonal antibodies against the PBP-2 protein react with methicillin-resistant staphylococci, causing visible agglutination. Strains from the ATCC collection were used as controls:

S. aureus MRSA-33591 *mecA* positive and *S. aureus*-35923 *mecA* negative. The test was assessed based on the presence or absence of agglutination in the test field.

2.6. Determination of the Ability to Produce Biofilm

2.6.1. Congo Red Agar (CRA)

The production of biofilm by the tested strains was examined on CRA medium [24]. The culture was carried out for 24 h at 37 °C and then left overnight at room temperature. The ability to produce biofilm was assessed based on the color of bacterial colonies. Strains forming black colonies on CRA medium were classified as mucus producing, and strains with brown-red, red, or colorless bacterial colonies were classified as lacking the ability to form biofilm.

2.6.2. Spectrophotometric Method

Biofilm production was determined using flat-bottomed polystyrene micropipetting plates using the spectrophotometric technique described by Ebrahimi et al. [25]. Absorbance was measured at a wavelength of 595 nm using an ELISA microplate reader (ASYS UVM340, Biogenet, Józefów, Poland). Columns with TSB medium alone served as a negative control. The *S. aureus* ATCC 25923 strain was used as a positive control. All tests were performed in three columns with 8 replicates for each strain. Biofilm production results were interpreted using the criteria described by Stepanović et al. [26].

3. Results

3.1. Isolation and Identification of Staphylococcus spp.

After analyzing the phenotypic features of bacterial colonies grown on selective microbiological media (Bird-Parker and Chapman), bacterial strains showing phenotypic features characteristic of the *Staphylococcus* genus were isolated from 93 out of 100 cow milk samples. On Bird-Parker medium, the colonies were convex, black, or grey, with a diameter of 1–1.5 mm after incubation for 24 h and a diameter larger than 2.5 mm after 48 h. In 30 cultures, colonies with a typical opalescent ring were observed, in 26, a transparent ring around the colony. On Chapman's medium (7% NaCl content), mannitol-fermenting coagulase-positive staphylococci (CPS) grew as cream to orange colonies, turning the medium yellow. Coagulase-negative staphylococci (CNS) grew as pink colonies, and the medium did not change its original color.

After analyzing Gram-stained microscopic slides and determining the ability to produce catalase and coagulase, 56 bacterial strains isolated from 28 bulk tank milk samples were determined to belong to *Staphylococcus* spp.

The CPS group included 28 isolates showing a positive reaction to coagulase production. All of them were identified as *S. aureus* because they had a fragment of 23S rRNA (*Stuart*) and the *coa* gene in the isolated genetic material. The remaining 28 were considered CNS due to showing a negative reaction to coagulase production. Among the isolated CNS strains, 18 strains were identified as *S. aureus* based on the presence of a 23S rRNA fragment in the genome, while the remaining (10 strains) belonged to other species belonging to *Staphylococcus* spp. (Table 2).

Among CPS, 9 strains showed α hemolysis and the remaining strains on blood agar caused β hemolysis. The vast majority of CNS strains (17) caused α hemolysis (11 strains *S. aureus* and 6 strains *Staphylococcus* sp.), only a few (3) β hemolysis (respectively 2 and 1), and 8 strains (respectively 5 and 3) did not show it at all (Table 2).

Table 2. Results of the molecular species identification and determination of the type of hemolysis and the ability to produce coagulase.

Strain Type	Strains	Species Identification		Hemolysis Type		Coagulase (24 h Test)
		Stuart	Coag	α	β	
Coagulase-negative *Staphylococcus* (CNS) strains	68/2, 30/2, 35/1, 89/1, 13/2, 28/2, 24/1, 37/1, 68/1, 61/1, 75/1	+	−	+	−	−
	73/2, 23/2	+	−	−	+	−
	73/3, 61/2, 72/3, 39/2, 28/1	+	−	−	−	−
	20/3, 18/1, 36/3, 2/3, 72/2, 10/2	−	−	+	−	−
	13/1	−	−	−	+	−
	28/3, 20/2, 20/1	−	−	−	−	−
Coagulase-positive *Staphylococcus* (CPS) strains	13/3, 2/2, 41/1, 72/1, 51/3, 15/1, 11/1, 73/1, 87/2, 97/3, 11/2, 15/3, 41/2, 51/1, 18/2, 74/3, 41/3, 51/2, 97/1	+	+	−	+	+
	75/2, 74/2, 2/1, 87/3, 87/1, 97/2, 15/2, 18/3, 75/3	+	+	+	−	+

+ positive; − negative.

3.2. Antibiotic Resistance

All CPS and CNS isolates showed resistance to amoxicillin, erythromycin, and tetracycline in the disc diffusion test. The majority (92.86%) of the strains were resistant to ampicillin. Three CNS strains and 1 CPS strain sensitive to this antibiotic were identified. The lowest resistance was demonstrated for vancomycin (48.2%) (13 CPS strains and 14 CNS strains). Analyzing the results of the disk diffusion test performed on CNS strains, it was shown that out of all 19 strains identified as *S. aureus*, 3 (15.8%) were sensitive to ampicillin and vancomycin, and 6 (31.6%) only to vancomycin. Of the strains identified as belonging to *Staphylococcus* spp. (not *S. aureus*), 5 (50%) were sensitive to vancomycin, while all were resistant to ampicillin. The *mecA* gene was not detected in any of the isolates and there was no positive reaction to the PB2 protein, indicating methicillin resistance.

Comparing the results obtained in the disk diffusion test to the presence of genes determining antibiotic resistance in the genome of the isolated strains, it was shown that all strains sensitive to ampicillin and vancomycin did not have them. In turn, resistance to antimicrobial substances demonstrated by the disk diffusion test was not strictly correlated with the occurrence of genes determining antibiotic resistance in the genetic material of isolated strains (Table 3).

Table 3. Determination of the antibiotic resistance profile of *Staphylococcus* sp. strains isolated from bulk tank milk.

Strains	Molecular Method												Disc Diffusion Test			
	mecA	*tetM*	*tetK*	*tetO*	*ermA*	*ermC*	*aph*	*blaZ*	*vanA*	*vanB*	*vanC1/2*	*vanD*	Erytromycin	Amocsycylin	Ampicylin	Vancomycin
72/3, 39/2, 2/2, 74/2, 15/1, 11/1, 18/2, 97/2, 18/3,	−	−	−	−	−	−	−	−	−	−	−	−	R	R	R	S
11/2	−	−	−	−	−	−	−	−	−	−	−	−	R	R	S	S
68/2, 61/2, 72/2, 15/2, 41/3	−	−	+	−	−	−	−	−	−	−	−	−	R	R	R	S
35/1, 24/1, 37/1, 23/2,	−	−	+	−	−	−	−	−	−	−	−	−	R	R	S	S
30/2, 61/1,	−	−	−	−	−	−	−	−	+	−	−	−	R	R	R	R
89/1	−	+	−	−	−	−	−	−	+	−	−	+	R	R	R	R
13/2	−	−	+	−	−	−	−	−	+	−	−	+	R	R	R	R

Table 3. *Cont.*

Strains	Molecular Method												Disc Diffusion Test			
	mecA	*tetM*	*tetK*	*tetO*	*ermA*	*ermC*	*aph*	*blaZ*	*vanA*	*vanB*	*vanC1/2*	*vanD*	Erytromycin	Amocsycylin	Ampicylin	Vancomycin
28/2	−	+	+	+	−	−	+	−	+	−	−	+	R	R	R	R
68/1	−	−	+	−	−	−	−	−	+	−	−	−	R	R	R	R
73/2	−	−	−	−	−	−	−	+	−	−	−	−	R	R	R	S
73/3	−	+	−	−	−	−	+	−	+	−	−	−	R	R	R	R
75/1	−	+	−	−	−	−	−	−	+	−	−	−	R	R	R	R
28/1	−	+	+	−	−	−	+	−	+	−	−	−	R	R	R	R
20/3	−	−	+	−	−	+	+	+	−	−	−	−	R	R	R	S
13/1	−	−	+	−	−	−	−	−	+	−	−	+	R	R	R	R
28/3	−	−	−	−	−	−	+	−	+	−	−	+	R	R	R	R
20/2, 36/3	−	+	−	−	−	−	−	−	−	−	−	−	R	R	R	S
20/1	−	−	+	−	−	+	+	−	−	−	−	−	R	R	R	S
18/1	−	−	−	−	−	+	−	−	+	−	−	+	R	R	R	R
2/3	−	−	−	−	−	−	−	−	+	−	−	+	R	R	R	R
10/2	−	+	+	−	−	+	−	−	+	−	−	−	R	R	R	R
13/3, 51/3, 87/2, 2/1, 41/2, 51/1	−	−	−	−	−	−	−	−	−	+	−	−	R	R	R	R
75/2	−	−	+	−	−	−	−	−	−	+	−	−	R	R	R	R
41/1	−	−	+	−	−	+	−	−	−	−	−	−	R	R	R	S
72/1	−	−	−	−	−	−	−	+	−	+	−	−	R	R	R	R
73/1	−	−	+	−	−	−	−	+	+	−	−	−	R	R	R	R
97/3, 87/1, 51/2, 97/1, 75/3	−	−	+	−	−	−	−	−	−	+	−	−	R	R	R	R
87/3, 15/3	−	−	+	−	−	−	−	−	−	−	−	−	R	R	R	S
74/3	−	−	+	−	−	−	−	+	−	+	−	−	R	R	R	R

+ Positive: the gene is present in the genetic material isolated from the tested strain; − negative: the gene is absent in the genetic material isolated from the tested strain; R: resistant; S: sensitive.

By analyzing the occurrence of individual genes in the genetic material isolated from the tested strains, a total of 18 antimicrobial resistance profiles were determined. Twelve were observed among CNS strains and 7 among CPS strains. One antimicrobial resistance profile (*tetK*) was present among both CNS and CPS (Table 4).

Table 4. Distribution of antimicrobial resistance profile in CNS and CPS from bulk tank milk.

Strain Type	Gene Pattern	Strains
CNS	*tetK*	68/2, 35/1, 24/1, 37/1, 20/1, 61/2, 23/2, 72/2, 13/2, 28/2, 68/1, 28/1, 20/3, 13/1, 10/2
	tet M	20/2, 36/3, 89/1, 28/2, 73/3, 75/1, 28/1, 10/2
	blaZ	73/2, 20/3
	vanA	30/2, 61/1, 89/1, 13/2, 28/2, 68/1, 73/3, 75/1, 28/1, 13/1, 28/3, 18/1, 2/3, 10/2
	tetM, van A	89/1, 28/2, 73/3, 75/1, 28/1, 10/2
	vanA, vanD	89/1, 28/2
	tetM, vanA, vanD	89/1, 28/2
	tetK, vanA, vanD	13/1, 13/2, 28/2
	tetK, vanA	13/1, 13/2, 28/2, 68/1, 28/1, 10/2
	tetM, tetK, vanA	28/1, 28/2, 10/2
	tetK, blaZ	20/3
	tetM, tetK, tetO, vanA, vanD	28/2

Table 4. *Cont.*

Strain Type	Gene Pattern	Strains
CPS	*tetK*	75/2, 41/1, 73/1, 97/3, 87/3, 15/3, 87/1, 74/3, 15/2, 41/3, 51/2, 97/1, 75/3
	vanB	13/3, 75/2, 72/1, 51/3, 87/2, 2/1, 97/3, 41/2, 87/1, 51/1, 74/3, 51/2, 97/1, 75/3
	tetK, vanB	75/2, 97/3, 87/1, 74/3, 51/2, 97/1, 75/3
	blaZ, vanB	72/1, 74/3
	tetK, vanA	73/1
	tet K, blaZ, vanA	73/1
	tetK, blaZ, vanB	74/3

CNS: coagulase-negative *Staphylococcus* strains; CPS: coagulase-positive *Staphylococcus* strains.

3.3. Biofilm Production

Of the 56 strains tested on CRA medium, a total of 12 tested strains (6 CNS strains and 6 CPS strains) showed changes in the color of the medium indicating the ability to produce biofilm. The number of isolates with mucus production properties was in the CNS group (6 strains) and in the CPS group (6 strains).

By assessing biofilm production using the spectrophotometric method by measuring absorbance according to Ebrahimi et al. [25], one strain (1.77%) showed the ability to intensively produce biofilm (OD > 0.8); 30 strains (53.57%) were moderate (0.4 < OD < 0.8), while 10 (17.87%) were weak (0.2 < OD < 0.4). The remaining 15 strains (26.80%) did not show the ability to produce biofilm (OD $\leq$ 0.2). Among the CNS strains tested using the spectrophotometric method, 19/28 strains showed the ability to produce biofilm—one strain showed an intense ability (3.57%), 14 (50%) a moderate ability, and 4 (14.28%) a weak ability. The inability to produce biofilm was found in 9 cases (32.15%). Analyzing the species affiliation of CNS strains, in the group of strains belonging to *S. aureus*, 13 strains with the ability to produce biofilm were found (1 strain intense, 10 strains moderate, and 2 strains weak). In the group of CNS strains belonging to the *Staphylococcus* genus, the ability to produce biofilm was confirmed in 6 cases (4 moderate and 2 weak). Among the CPS strains tested using the spectrophotometric method, the ability to produce biofilm was demonstrated by 22/28 strains, including 16 strains with a moderate ability to produce biofilm and 6 with a weak ability. In the case of 6 strains, the ability to produce biofilm was not demonstrated in the test performed using the spectrophotometric method.

The tests carried out for the presence of three genes responsible for biofilm production (*ica, agr, sasC*) did not reveal the *agr* gene in any of the 56 CNS and CPS isolates tested. The *ica* gene was detected in 39 tested isolates (69.64%) and the *sasC* gene in 38 (67.86%). It was determined that 15 isolates did not contain any of the tested genes, which constituted 26.79% of all tested strains.

Of the 28 CPS isolates tested, all of them contained the *ica* and *sasC* genes. The *agr* gene was not found in the tested group of strains. Of the 28 CNS isolates tested, 11 strains contained the *ica* gene (39.29%) and 10 strains contained the *sasC* gene (35.71%). CNS isolates had significantly lower numbers of both the *ica* 11 gene (39.29%) and the *sasC* 10 gene (35.71%). Detailed results are summarized in Table 5.

Table 5. Comparison of biofilm production results by CNS and CPS isolated from bulk tank milk.

CNS				CPS			
Strain	CRA	Spectrophotometric Method		Strain	CRA	Spectrophotometric Method	
		OD	Result			OD	Result
68/2	N	0.634	++	75/2	P	0.676	++
30/2	N	0.352	+	2/2	N	0.579	++
89/1	N	0.602	++	41/1	P	0.551	++
13/2	N	0.275	+	74/2	N	0.552	++
68/1	N	0.620	++	51/3	N	0.586	++
73/3	N	0.470	+	15/1	N	0.294	+
61/2	P	1.184	+++	11/1	N	0.246	+
72/3	P	0.536	++	73/1	N	0.238	+
23/2	N	0.685	++	87/2	N	0.220	+
75/1	P	0.688	++	2/1	P	0.752	++
28/1	N	0.527	++	87/3	P	0.679	++
20/3	P	0.611	++	11/2	N	0.615	++
28/3	N	0.395	+	15/3	N	0.559	++
20/2	P	0.519	++	41/2	N	0.392	+
20/1	P	0.662	++	87/1	P	0.524	++
18/1	N	0.608	++	57/1	N	0.651	++
2/3	N	0.329	+	18/2	N	0.595	++
72/2	N	0.003	−	97/2	P	0.539	++
35/1	N	0.024	−	74/3	N	0.456	++
28/2	P	0.468	++	15/2	N	0.242	+
24/1	N	0.164	−	13/3	N	0.003	−
37/1	P	0.689	++	72/1	N	0.003	−
73/2	N	0.004	−	97/3	N	0.090	−
61/1	N	0.032	−	41/3	N	0.015	−
39/2	N	0.075	−	51/2	N	0.021	−
13/1	N	0.175	−	97/1	N	0.090	−
10/2	N	0.113	−	18/3	P	0.595	++
36/3	N	0.157	−	75/3	P	0.676	++

CPS: coagulase-positive strain; CNS: coagulase-negative strain; CRA: Congo Red Agar test; N (negative): lack of ability of biofilm production demonstrated on CRA medium; P (positive): confirmed ability of biofilm production demonstrated on CRA medium; OD: optical density; + positive: confirmed ability of biofilm production demonstrated in the spectrophotometric test (+ weak; ++ moderate; +++ intensive); − negative: lack of ability of biofilm production demonstrated in the spectrophotometric test.

4. Discussion

Currently, 54 species of bacteria of the *Staphylococcus* genus are known, and their number is increasing, as indicated by the addition of six more in the last 5 years [27]. Unlike many other bacterial pathogens, which often rely on one or a few toxins to develop disease, *S. aureus* produces multiple virulence factors. These include both toxins and immune evasion factors, as well as proteinaceous and non-proteinaceous factors that enable host colonization during infection [28]. It is believed that persistent colonization of the intestines by *S. aureus* may lead to gastrointestinal diseases and even systemic diseases because the intestine may constitute a reservoir for the distribution of *S. aureus* to other sites of epithelial colonization [29,30]. *S. aureus* opportunistically exploits primary damage caused by other pathogens or predisposing factors [31,32].

An important means of maintaining *S. aureus* infection is the formation of biofilms [33]. They can form on abiotic material of permanently located medical devices, but also on tissue surfaces. The main role of biofilm formation during infection is to protect bacteria from phagocyte attacks [34]. Furthermore, *S. aureus* biofilms have been shown to skew the host immune response towards an anti-inflammatory state [28,34,35]. The ability to form biofilm is an important virulence attribute due to its potential to induce persistent antibiotic resistance, delay phagocytosis, and attenuate or promote inflammation [36].

Antimicrobial resistance is an increasingly global problem around the world. The growth of drug-resistant virulent strains of *S. aureus*, especially methicillin-resistant *S. aureus* (MRSA), is a serious problem in the treatment and control of staphylococcal infections [37,38]. It should be remembered that bacteria of the genus *Staphylococcus*, and especially *S. aureus* may be resistant, often in combination, to almost all available antibiotics [28].

S. aureus is a microorganism ubiquitous in the milking parlor environment [39]. It was classified by Cobirka et al. [40] as an infectious and environmental pathogen. This is because it spreads through various channels, such as litter, urine, feces, and other contaminants, as well as contaminated milk from infected cows or poor hygienic conditions during milking. Milk from *S. aureus*-infected dairy cows may be a continuing source of *S. aureus* contamination of milking equipment and other surfaces. Subsequent surface colonization may lead to the eventual formation of a biofilm. The results published in 2023 by Vargová et al. [36] confirmed a statistically significant relationship between the occurrence of *S. aureus* on monitored surfaces—the floor, teat cups, and milking equipment for cows-and the occurrence of mastitis caused by *S. aureus*. In our work, we showed that 73.3% of *Staphylococcus* sp. strains isolated from milk can produce biofilm. These results are consistent with those of Qi Chen et al. [41], who showed that 72% of *S. aureus* strains isolated from various food samples could produce biofilm. Like other research groups, we observed that both CPS and CNS strains have a similar ability to produce biofilm. The findings of the present study indicate that the smallest number of strains isolated from milk showed the ability to produce strong biofilm. Generally, this is consistent with results obtained on strains isolated from food. However, we observed an inverse correlation in the case of the ability to produce moderate and weak biofilm production. Our observations show that 53.57% of the tested strains showed moderate biofilm production and 17.8% showed poor biofilm production, while Qi Chen et al. [41] showed moderate biofilm production in only 14.43% of isolated strains and weak biofilm production in 54.64% of *S. aureus* strains. Interestingly, studies conducted on *Staphylococcus* sp. strains isolated from clinical cases showed that all (100%) have the ability to form biofilm [42].

While coagulase-positive *S. aureus* (CPS) is often the subject of research because it is the predominant isolate in bovine mastitis milk [43], CNS strains both belonging to the *S. aureus* species and other species of the *Staphylococcus* genus (generally considered non-pathogenic) are of less interest to researchers. These bacteria, generally considered non-pathogenic, constitute a reservoir and a vector for transferring antibiotic resistance genes to subsequent populations of microorganisms. Bulk tank milk samples that meet veterinary requirements for milk intended for food are of even less interest to researchers. It is considered that this milk comes from healthy animals, so it is free from pathogens. However, it should be remembered that the term "healthy animals" is not equivalent to the term "uninfected animals". Therefore, it can and often happens that in a herd of dairy cows, there are individuals that shed bacteria of the *Staphylococcus* genus into the environment. Therefore, due to their ability to colonize abiotic surfaces, these microorganisms are common in the environment, and being a reservoir of antibiotic-resistance genes, they may contribute to its growth in the microbial population [28,36]. Resistant bacteria can therefore spread rapidly through milk to many hosts, including humans. It should also be remembered that such milk can be consumed by humans without thermal treatment to eliminate microbiological contamination (for personal use on the farm). In situations specified by law, milk may also be marketed as raw milk.

Comparing the results obtained in the disk diffusion test to the presence of genes determining antibiotic resistance in the genome of the isolated strains, it was shown that all strains sensitive to ampicillin and vancomycin did not have them. In turn, resistance to antimicrobial substances demonstrated in the disk diffusion test was not correlated with the presence of genes determining antibiotic resistance in the genetic material of the isolated strains. This is probably because, in addition to specific, genetically determined resistance

to antibiotics, bacteria of the *Staphylococcus* genus have the ability to form biofilms, which generate non-specific resistance to antibiotics [33].

Most published works describe the antibiotic resistance profile of *Staphylococcus* spp. strains isolated from mastitis. Researchers have shown that *Staphylococcus* spp. isolated from the milk of cows with mastitis are resistant to most antibiotics commonly used in the treatment of cattle [44,45]. Performed by Brahma et al. [46], cross-antibiotic resistance profiling revealed that most *S. aureus* isolates were resistant to multiple antibiotics. Considering that the *S. aureus* isolates were resistant to more than three antibiotics, they were MDR (multi-drug resistant) bacteria. We observed a similar relationship in the strains we tested, isolated from bulk tank milk. A total of 29 out of 56 *Staphylococcus* spp. strains isolated from bulk tank milk showed resistance to all antibiotics selected for the experiment (erythromycin, amoxicillin, tetracycline, ampicillin, vancomycin), 52 strains showed resistance to four antibiotics (erythromycin, amoxicillin, tetracycline, vancomycin). All strains we tested (56) were resistant to at least three antibiotics (erythromycin, amoxicillin, and tetracycline). Similar results were obtained by Capri et al. [47] in a study of the antibiotic resistance of *Staphylococcus* sp. strains isolated from sheep milk samples. Researchers showed that 77.7% of *Staphylococcus* sp. strains could be classified as MDR. In the case of *Staphylococcus* sp. isolated from the milk of cows with mastitis, methicillin resistance is also frequently observed [11,48,49]. In the present study conducted on strains isolated from milk meeting the requirements for milk intended for food purposes, we did not demonstrate the presence of methicillin-resistant strains. This is important because the consumer is directly at risk when resistant pathogenic microorganisms are present in the food chain. According to WHO data, MDR bacteria cause over 700,000 deaths annually worldwide, especially among immunocompromised consumer groups [50]. Antibiotic resistance is a life-threatening global health problem affecting humans and animals. An important vector for the transmission of microbiological threats to humans is food of animal origin (including milk) because antibiotic residues and resistant bacteria quickly spread through food to humans. Due to the widespread use of antibiotics in the treatment of animals, their residues may occur in food of animal origin, including milk. So far, there are no technological possibilities to eliminate antibiotics from the raw material if it is already present, so even pasteurization of milk does not eliminate the risk. This is dangerous because the uncontrolled introduction of even trace amounts of antibacterial substances into the environment increases the risk of increasing antibiotic resistance among environmental bacteria.

5. Conclusions

As we have shown in the present study, special attention should be paid to the health of dairy cows and supervision of the use of antibiotics in the dairy herd environment, because even in milk from healthy cows there is a real risk of the occurrence of multi-drug resistant bacteria. The presence of CNS strains in the milk of both cows with mastitis and healthy cows should be monitored. These strains may contribute to the spread of genes determining biofilm formation and antibiotic resistance in the environment.

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References

1. Velasco, V.; Buyukcangaz, E.; Sherwood, J.S.; Stepan, R.M.; Koslofsky, R.J.; Logue, C.M. Characterization of *Staphylococcus aureus* from humans and a comparison with isolates of animal origin in North Dakota, United States. *PLoS ONE* **2015**, *20*, e0140497. [CrossRef]
2. Vitale, M.; Scatassa, M.L.; Cardamone, C.; Oliveri, G.; Piraino, C.; Alduina, R.; Napoli, C. Staphylococcal food poisoning case and molecular analysis of toxin genes in *Staphylococcus aureus* strains isolated from food in Sicily, Italy. *Foodborne Pathog. Dis.* **2015**, *12*, 21–23. [CrossRef] [PubMed]
3. Castro, A.; Santos, C.; Meireles, H.; Silva, J.; Teixeira, P. Food handlers as potential sources of dissemination of virulent strains of *Staphylococcus aureus* in the community. *J. Infect. Public Heal.* **2016**, *9*, 153–160. [CrossRef] [PubMed]
4. Hennekinne, J.A.; De Buyser, M.L.; Dragacci, S. *Staphylococcus aureus* and its food poisoning toxins: Characterization and outbreak investigation. *FEMS Microbiol. Rev.* **2012**, *36*, 815–836. [CrossRef] [PubMed]
5. Heikkilä, A.M.; Liski, E.; Pyörälä, S.; Taponen, S. Pathogen-specific production losses in bovine mastitis. *J. Dairy Sci.* **2018**, *101*, 9493–9504. [CrossRef] [PubMed]
6. Holko, I.; Tančin, V.; Vršková, M.; Tvarožková, K. Prevalence and antimicrobial susceptibility of udder pathogens isolated from dairy cows in Slovaki. *J. Dairy Res.* **2019**, *86*, 436–439. [CrossRef] [PubMed]
7. Li, T.; Lu, H.; Wang, X.; Gao, Q.; Dai, Y.; Shang, J.; Li, M. Molecular characteristics of *Staphylococcus aureus* causing bovine mastitis between 2014 and 2015. *Front. Cell Infect Microbiol.* **2017**, *7*, 127. [CrossRef] [PubMed]
8. Gravelanda, H.; Duim, B.; van Duijkeren, E.; Heederik, D.; Wagenaar, J.A. Livestock-associated methicillin-resistant *Staphylococcus aureus* in animals and humans. *Int. J. Med. Microbiol.* **2011**, *301*, 630–634. [CrossRef]
9. Moretro, T.; Langsrud, S. Residential bacteria on surfaces in the food industry and their implications for food safety and quality. *Compr. Rev. Food Sci. Food Safety* **2017**, *16*, 1022–1041. [CrossRef]
10. Mørk, T.; Jørgensen, H.J.; Sunde, M.; Kvitle, B.; Sviland, S.; Waage, S.; Tollersrud, T. Persistence of staphylococcal species and genotypes in the bovine udder. *Vet. Microbiol.* **2012**, *159*, 171–180. [CrossRef]
11. Garza-González, E.; Morfín-Otero, R.; Llaca-Díaz, J.M.; Rodriguez-Noriega, E. Staphylococcal cassette chromosome mec (SCCmec) in methicillin-resistant coagulase-negative staphylococci. A review and the experience in a tertiary-care setting. *Epidemiol. Infect.* **2010**, *138*, 645–654. [CrossRef] [PubMed]
12. Hanssen, A.M.; Ericson Sollid, J.U. SCCmec in staphylococci: Genes on the move. *FEMS Immunol. Med. Microbiol.* **2006**, *46*, 8–20. [CrossRef] [PubMed]
13. Evolving Appetites: An In-Depth Look at European Attitudes towards Plant-Based Eating. Available online: www.smartproteinproject.eu (accessed on 10 November 2023).
14. Antimicrobial Resistance: A Global Threat, United Nations Environment Programme. Available online: https://www.unep.org/topics/chemicals-and-pollution-action/pollution-and-health/antimicrobial-resistance-global-threat (accessed on 25 March 2024).
15. Regulation (EC) No 853/2004 of the European Parliament and of the Council of 29 April 2004 Laying down Specific Hygiene Rules for Food of Animal Origin. *OJ L* **2004**, *139*, 1–55. Available online: https://eur-lex.europa.eu/legal-content/EN/TXT/?uri=CELEX:02004R0853-20230215 (accessed on 12 January 2023).
16. *PN-EN ISO 6888-1:2022-03*; Microbiology of the Food Chain—Horizontal Method for the Enumeration of Coagulase-Positive Staphylococci (Staphylococcus aureus and Other Species)—Part 1: Method Using Baird-Parker Agar Medium. The Polish Committee for Standardization: Warsaw, Poland, 2022.
17. *PN-EN ISO 6888-3:2004*; Microbiology of Food and Animal Feeding Stuff—Horizontal Method for the Enumeration of Coagulase-Positive Staphylococci (Staphylococcus Aureus and Other Species)—Part 3: Detection and Enumeration of Small Numbers by the Most Probable Number (MPN) Technique. The Polish Committee for Standardization: Warsaw, Poland, 2004.
18. Akineden, O.; Annemuller, C.; Hassan, A.A.; Lammler, C.; Wolter, W.; Zschock, M. Toxin genes and other characteristics of *Staphylococcus aureus* isolated from milk of cows with mastitis. *Clin. Diagn. Lab. Immun.* **2001**, *8*, 959–964. [CrossRef] [PubMed]
19. Klimiene, I.; Virgailis, M.; Pavilonis, A.; Siugzdiniene, R.; Mockeliunas, R.; Ruzauskas, M. Phenotypical and genotypical antimicrobial resistance of coagulase-negative staphylococci isolated from cow mastitis. *Pol. J. Vet. Sci.* **2016**, *19*, 639–646. [CrossRef]
20. Ng, L.-K.; Martin, I.; Alfa, M.; Mulvey, M. Multiplex PCR for the detection of tetracycline resistant genes. *Mol. Cell. Probes* **2001**, *15*, 209–215. [CrossRef] [PubMed]
21. Schnellmann, C.; Gerber, V.; Rossanno, A.; Jaquer, V.; Panchaud, Y.; Doherr, M.; Thomann, A.; Straub, R.; Perreten, V. Presence of new mecA and mph(C) variants conferring antibiotic resistance in *Staphylococcus* spp. isolated from the skin of horses before and after clinic admission. *J. Clin. Microbiol.* **2006**, *44*, 4444–4454. [CrossRef]

22. Depardieu, F.; Perichon, B.; Courvalin, P. Detection of the van Alphabet and Identification of Enterococci and Staphylococci at the Species Level by Multiplex PCR. *J. Clin. Microbiol.* **2004**, *24*, 5857–5860. [CrossRef]
23. Vitale, M.; Galluzzo, P.; Buffa, P.G.; Carlino, E.; Spezia, O.; Alduina, R. Comparison of antibiotic resistance profile and biofilm production of *Staphylococcus aureus* isolates derived from human specimens and animal-derived samples. *Antibiotics* **2019**, *8*, 97. [CrossRef]
24. Arciola, C.R.; Campoccia, D.; Gamberini, S.; Cervellati, M.; Donati, E.; Montanaro, L. Detection of slim production by means of an optimised Congo red agar plate test based on a colorimetric scale in *Staphylococcus epidermidis* clinical isolates genotyped for *ica* locus. *Biomaterials* **2002**, *23*, 4233–4239. [CrossRef]
25. Ebrahimi, A.; Mohamed, A.; Lotfalian, S.; Mishokrei, P. Biofilm formation, hemolysin production and antimicrobial susceptibilities of *Streptococcus agalactiae* isolated from the mastitis milk of dairy cows in Shahrekord district. *Iran. Vet. Res. Forum* **2013**, *4*, 269–272.
26. Stepanović, S.; Vukovi, D.; Hola, V.; di Bonaventura, G.; Djukić, S.; Cirković, I.; Ruzicka, F. Quantification of biofilm in microtiter plates: Overview of testing conditions and practical recommendations for assessment of biofilm production by staphylococci. *APMIS* **2007**, *115*, 891–899. [CrossRef]
27. Genus Staphylococcus. Available online: http://www.bacterio.net/staphylococcus.html (accessed on 12 January 2023).
28. Gordon, Y.C.C.; Bae, J.S.; Otto, M. Pathogenicity and virulence of *Staphylococcus aureus*. *Virulence* **2021**, *12*, 547–569. [CrossRef]
29. Senn, L.; Clerc, O.; Zanetti, G.; Basset, P.; Prod'hom, G.; Gordon, N.C.; Sheppard, A.E.; Crook, D.W.; James, R.; Thorpe, H.A.; et al. The Stealthy Superbug: The role of asymptomatic enteric carriage in maintaining a long-term hospital outbreak of ST228 methicillin-resistant *Staphylococcus aureus*. *mBio* **2016**, *7*, e02039-15. [CrossRef] [PubMed]
30. Fisher, E.L.; Otto, M.; Cheung, G.Y.C. Basis of virulence in enterotoxin-mediated staphylococcal food poisoning. *Front. Microbiol.* **2018**, *9*, 436. [CrossRef] [PubMed]
31. McCullers, J.A. The co-pathogenesis of influenza viruses with bacteria in the lung. *Nat. Rev. Microbiol.* **2014**, *12*, 252–262. [CrossRef] [PubMed]
32. Morens, D.M.; Taubenberger, J.K.; Fauci, A.S. Predominant role of bacterial pneumonia as a cause of death in pandemic influenza: Implications for pandemic influenza preparedness. *J. Infect. Dis.* **2008**, *198*, 962–970. [CrossRef]
33. Otto, M. Staphylococcal Biofilms. *Microbiol. Spectr.* **2018**, *6*, 10. [CrossRef]
34. Thurlow, L.R.; Hanke, M.L.; Fritz, T.; Angle, A.; Aldrich, A.; Williams, S.H.; Engebretsen, I.L.; Bayles, K.W.; Horswill, A.R.; Kielian, T. *Staphylococcus aureus* biofilms prevent macrophage phagocytosis and attenuate inflammation in vivo. *J. Immunol.* **2011**, *186*, 6585–6596. [CrossRef]
35. Hanke, M.L.; Kielian, T. Deciphering mechanisms of staphylococcal biofilm evasion of host immunity. *Front. Cell Infect. Microbiol.* **2012**, *2*, 62. [CrossRef]
36. Vargová, M.; Zigo, F.; Výrostková, J.; Farkašová, Z.; Rehan, I.F. Biofilm-producing ability of *Staphylococcus aureus* obtained from surfaces and milk of mastitic cows. *Vet. Sci.* **2023**, *10*, 386. [CrossRef] [PubMed]
37. Livermore, D.M. Antibiotic resistance in staphylococci. *Int. J. Antimicrob. Agents* **2000**, *16*, 3–10. [CrossRef]
38. Duran, N.; Ozer, B.; Duran, G.G.; Onlen, Y.; Demir, C. Antibiotic resistance genes and susceptibility patterns in staphylococci. *Indian J. Med. Res.* **2012**, *135*, 389–396. [PubMed]
39. Patel, K.; Godden, S.M.; Royster, E.; Crooker, B.A.; Timmerman, J.; Fox, L. Relationships among bedding materials, bedding bacteria counts, udder hygiene, milk quality, and udder health in US dairy herds. *J. Dairy Sci.* **2019**, *102*, 10213–10234. [CrossRef] [PubMed]
40. Cobirka, M.; Tancin, V.; Slama, P. Epidemiology and classification of mastitis. *Animals* **2020**, *10*, 2212. [CrossRef] [PubMed]
41. Qi Chen, Q.; Xie, S.; Lou, X.; Cheng, S.; Liu, X.; Zheng, W.; Zheng, Z.; Wang, H. Biofilm formation and prevalence of adhesion genes among Staphylococcus aureus isolates from different food sources. *Microbiol. Open.* **2020**, *9*, e946.
42. Pishtiwan, A.H. Phenotypic and molecular detection of biofilm formation in methicillin-resistant Staphylococcus *aureus* isolated from different clinical sources in Erbil city. *Mediterr. J. Hematol. Infect. Dis.* **2023**, *15*, e2023016. [CrossRef]
43. Sahoo, S.; Behera, M.R.; Mishra, B.; Sahoo, P.; Kar, S. Antibiotic-resistant bacteria in bovine milk in India. *J. Adv. Vet. Anim. Res.* **2023**, *10*, 21–29. [CrossRef] [PubMed]
44. Mubarack, H.M.; Doss, A.; Vijayasanthi, M.; Venkataswamy, R. Antimicrobial drug susceptibility of Staphylococcus aureus from subclinical bovine mastitis in Coimbatore, Tamil Nadu, South India. *Vet. World* **2012**, *5*, 352–355. [CrossRef]
45. Sharma, I.; Brinty, A. Isolation and identification of *Staphylococcus aureus* from bovine mastitis milk and their drug resistance patterns in Silchar town dairy farms, NE India. *Online Int. Interdiscipl. Res. J.* **2014**, *4*, 256–260.
46. Brahma, U.; Suresh, A.; Murthy, S.; Bhandari, V.; Sharma, P. Antibiotic resistance and molecular profiling of the clinical isolates of *Staphylococcus aureus* causing bovine mastitis from India. *Microorganisms* **2022**, *10*, 833. [CrossRef] [PubMed]
47. Capri, F.C.; Di Leto, Y.; Presentato, A.; Mancuso, I.; Scatassa, M.L.; Alduina, R. Characterization of *Staphylococcus* species isolates from sheep milk with subclinical mastitis: Antibiotic resistance, enterotoxins, and biofilm production. *Foodborne Pathog. Dis.* **2024**, *21*, 1. [CrossRef] [PubMed]
48. Hamid, S.; Bhat, M.A.; Mir, I.A.; Taku, A.; Badroo, G.A.; Nazki, S.; Malik, A. Phenotypic and genotypic characterization of methicillin-resistant *Staphylococcus aureus* from bovine mastitis. *Vet. World* **2017**, *10*, 363–367. [CrossRef] [PubMed]

49. Shah, M.S.; Qureshi, S.; Kashoo, Z.; Farooq, S.; Wani, S.A.; Hussain, M.I.; Banday, M.S.; Khan, A.A.; Gull, B.; Habib, A.; et al. Methicillin resistance genes and in vitro biofilm formation among *Staphylococcus aureus* isolates from bovine mastitis in India. *Comp. Immunol. Microbiol. Infect. Dis.* **2019**, *64*, 117–124. [CrossRef]
50. Buowari, Y.D. Antibiotic resistance in the elderly. *J. Aging Res. Healthc.* **2017**, *1*, 1. [CrossRef]