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Unprocessed milk as a source of multidrug-resistant *Staphylococcus aureus* strains

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Abstract

Staphylococcus aureus is the most relevant pathogen of animal mastitis and milk-related intoxications. Its presence in directly sold milk is rather not to be expected if strict udder health management and regular microbiological control of raw milk are performed. In this one-year survey, we present the results of monthly microbiological testing of milk from vending machines in Croatia for *S. aureus* and its multi-drug resistance. *Staphylococcus aureus* was detected in 27.58% of the samples. Among 60 tested isolates from 10 farmers, a total of 41 isolates were resistant to at least one antimicrobial agent (68.33%). A Multiple Antibiotic Resistance (MAR) index of 0.2 or higher had 48.8% of the resistant isolates, which is considered a high-risk potential for the spread of antimicrobial resistance. The majority of the isolates were resistant to penicillin and ampicillin followed by ciprofloxacin, ceftazidime, and kanamycin. The results impose the need for improving the control measures in the raw milk distribution chain focused on MAR risk reduction.

Raw milk, Staphylococcus aureus, vending machines, antimicrobial resistance

Antimicrobial resistance is one of the leading public health problems, closely linked to interactions between livestock, farmers, the environment, and food of animal origin (Garipcin and Seker 2015). Of particular concern is the possibility of resistant organisms of animal origin becoming directly pathogenic to humans or transferring their resistance genes to pathogens of medical importance (Teuber 2001). Due to the intensive use of antibiotics in health care and animal husbandry, antibiotic resistance in pathogens has become an increasing medical problem in recent decades (Sharma et al. 2014; Navrátilová et al. 2020).

Besides, there is a possibility that foodborne commensals or opportunistic pathogens are carriers of resistance genes and thus pose a potential risk to consumers. In this context, ready-to-eat foods may pose a particular problem. For example, raw milk sold directly through vending machines is a raw product that is consumed without thermal treatment, despite other recommendations, so we can consider it in the context of ready-to-eat foods (Mikulec et al. 2019). It is well known that raw milk, due to its composition, is a very suitable medium for the growth and development of many microorganisms, including certain pathogens. Numerous pathogenic microorganisms such as *Salmonella* spp., *Campylobacter* spp., Shiga toxin-producing *Escherichia coli*, *Listeria monocytogenes*, *Yersinia enterocolitica*, *Staphylococcus aureus*, *Mycobacterium bovis*, *Brucella* spp., *Coxiella burnettii* and others can be isolated from raw milk and most of them have been identified as major microbiological hazards associated with raw milk consumption using the decision tree approach (EFSA 2015). Some of these foodborne pathogens have their habitat in food-producing animals, e.g., skin and gastrointestinal tract, as well as in the agricultural environment (Oliver et al. 2009). Their occurrence in milk is variable but has been confirmed in many studies (Viltrop and Roasto 2013; Mikulec et al. 2019;

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Zdolec et al. 2019). Coagulase-positive staphylococci, especially *Staphylococcus aureus*, are not only pathogens of mastitis, but also important for dairy (and food) hygiene, as they can cause alimentary intoxication in humans. As a result of the uncritical use of antibiotics, antimicrobial resistance of staphylococci from mastitic milk has increased significantly over the years. This assertion is supported by the fact that successful treatment of mastitis caused by *S. aureus* is achieved in only 10% of cases. *Staphylococcus aureus* strains are usually sensitive to β-lactam antibiotics, cephalosporins, and chloramphenicol, but often become resistant to these and other antimicrobial agents. Lack of hygiene measures in food processing can also increase the number of *S. aureus*, especially in manually prepared foods. As a result, resistant *S. aureus* can also contaminate dairy products made from both raw and heat-treated milk (Medveďová et al. 2014).

Based on the above facts, the presence of coagulase-positive staphylococci in raw milk intended for public consumption is not acceptable, especially if they are resistant to antibiotics. Therefore, this study aimed to investigate the presence and number of *S. aureus* in raw milk directly sold via vending machines and to test the susceptibility of the isolates to antimicrobial agents over one year.

Materials and Methods

Milk samples

Milk samples (n = 319) were collected from vending machines in Zagreb, Croatia and junction counties, over a one-year period. The monthly number of sampled vending machines ranged from 25 to 32. Raw milk samples were placed in a sterile bottle and transported in a mobile refrigerator at + 4 °C. Samples were stored in the laboratory at + 4 °C and analyzed within 12 h after sampling.

Microbiological analyses

For microbiological analysis 25 ml of homogenized raw milk was aseptically taken and decimally diluted in Buffered Peptone Water (Merck, Darmstadt, Germany) to determine the number of staphylococci in 1 ml of sample. Inoculum (0.1 or 0.33 ml) of selected decimal dilutions were plated on Baird-Parker agar (Merck) and incubated at 37 °C for 48 h. After incubation, presumptive colonies were checked for morphology by Gram staining and coagulase test (Merck). The reference strain of *S. aureus* ATCC® 25923 was used as a positive control. Isolates were identified using the API system (bioMérieux, Marcy l'Etoile, France).

Antimicrobial susceptibility testing

Selected strains were tested for their susceptibility to the following 14 antimicrobial agents: penicillin (10 IU), ampicillin (10 µg), ciprofloxacin (5 µg), linezolid (30 µg), ceftazidime (30 µg), kanamycin (30 µg), levofloxacin (5 µg), erythromycin (15 µg), trimethoprim + sulphamethoxazole (25 µg), tetracycline (30 µg), chloramphenicol (30 µg), nitrofurantoin (300 µg), teicoplanin (30 µg) and cefotaxime (30 µg) using Kirby-Bauer disk diffusion test. Bacterial culture (0.5 McFarland) was spread on Mueller-Hinton agar (Bio-Rad, Hercules, USA) and six antibiotic disks were used per plate using a disk dispenser (Bio-Rad). After incubation (18–20 h, 35 °C), zones of inhibition were measured in millimetres (mm) and results were interpreted according to CLSI criteria for staphylococci.

Multiple Antibiotic Resistance index

For multidrug-resistant strains of *S. aureus* the individual Multiple Antibiotic Resistance (MAR) index was calculated according to Krumperman (1983). The MAR index of an individual microorganism, if greater than 0.2, means that the bacteria originated from an environment where multiple antimicrobials were used.

Statistical evaluation

Statistical analysis was performed using Microsoft Excel. Significant differences were evaluated at the 0.05 level and verified by one-way ANOVA for independent samples. Correlation between two antimicrobial agents in a sample was measured using the Pearson correlation method. Principal Component Analysis (PCA) was used to show the dominant presence of each antimicrobial agent by the milk producer.

Results

Concerning the number of milk samples tested (n = 319), a total of 27.58% (88 samples) contained *S. aureus*. Their mean counts ranged from 2.04 to 3.09 log CFU/ml, and there was no significant difference between months of sampling ($P > 0.05$).

In the present study, antimicrobial susceptibility was tested in 60 isolates and the percentage of resistant isolates varied from 33.3 to 100% depending on the month of collection. The majority of the isolates were resistant to penicillin and ampicillin followed by ciprofloxacin, ceftazidime, and kanamycin (Fig. 1). A total of 41 isolates were resistant to at least one antimicrobial agent (68.33%) (Table 1).

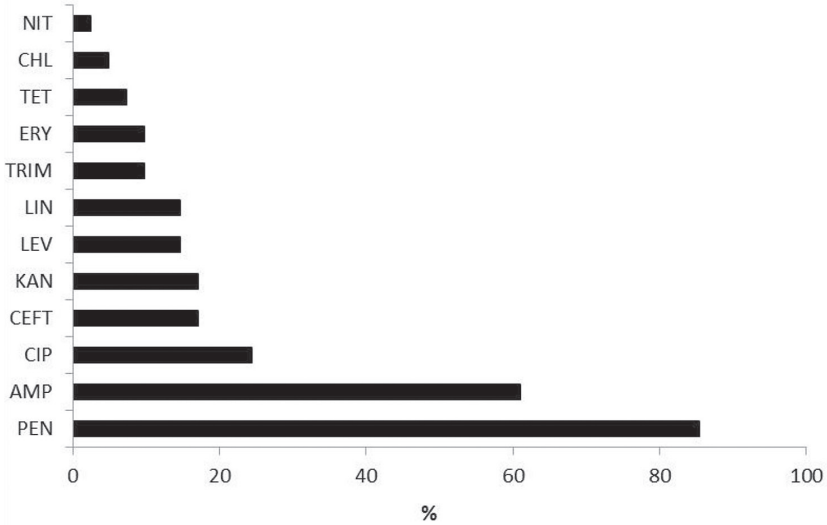

Fig. 1. The frequency of each antimicrobial agent resistance in the total number of measurements (%)
PEN = penicillin, AMP = ampicillin, CIP = ciprofloxacin, LIN = linezolid, CEF = ceftazidime, KAN = kanamycin, LEV = levofloxacin, ERY = erythromycin, TRIM = trimethoprim + sulphamethoxazole, TET = tetracycline, CHL = chloramphenicol, NIT = nitrofurantoin

Table 1. *Staphylococcus aureus* findings in vending machine milk and the count of resistant strains.

Month of sampling	Number of vending machines (samples)	Number (%) of vending machines with *S. aureus*	The average count of *S. aureus* (log CFU/ml; x ± SD)	Number of tested *S. aureus* on ATB*	Number of resistant *S. aureus***
December	26	4 (15.4%)	3.09 ± 0.43	4	4 (100.0%)
January	25	10 (40.0%)	2.47 ± 0.52	10	7 (70.0%)
February	25	5 (20.0%)	2.65 ± 0.53	5	4 (80.0%)
March/April	32	13 (40.6%)	2.51 ± 0.80	9	4 (44.4%)
May	27	6 (22.2%)	2.31 ± 1.08	4	3 (75.0%)
June	26	5 (19.2%)	2.76 ± 0.56	5	4 (80.0%)
July	25	13 (52.0%)	2.04 ± 0.46	-	-
September	25	8 (32.0%)	2.65 ± 0.54	8	6 (75.0%)
October	27	7 (25.9%)	2.73 ± 0.54	-	-
November	28	6 (21.4%)	2.81 ± 0.43	6	2 (33.3%)
December	26	4 (15.4%)	3.09 ± 0.59	3	2 (66.6%)
January	27	7 (25.9%)	3.27 ± 0.32	6	5 (83.3%)

*Penicillin, ampicillin, ciprofloxacin, linezolid, ceftazidime, kanamycin, levofloxacin, erythromycin, trimethoprim + sulphamethoxazole, tetracycline, chloramphenicol, nitrofurantoin, teicoplanin, cefotaxime
** Number of resistant *S. aureus* on at least one antimicrobial agent

Resistance to penicillin and ampicillin was significantly more common than to other antimicrobial agents ($P < 0.05$), which was confirmed by correlation analysis where the correlation factor obtained (r = 0.73) showed a strong positive relationship between these two antimicrobial agents. The lower proportion of *S. aureus* strains were resistant to trimethoprim/sulphamethoxazole, erythromycin, tetracycline, chloramphenicol, and nitrofurantoin, while all isolates were sensitive to teicoplanin and cefotaxime. However, among the 41 resistant isolates, 20 (48.8%) isolates had a MAR index value of 0.2 or higher, which is considered a high-risk potential for the spread of antibiotic resistance.

For the vendors included in the study, it was found that there was no significant difference ($P > 0.05$) between the producers (n = 10) in the number of antimicrobial agents on which resistance occurred. The frequency of occurrence of antimicrobial agents resistance according to the manufacturer is shown in Fig. 2.

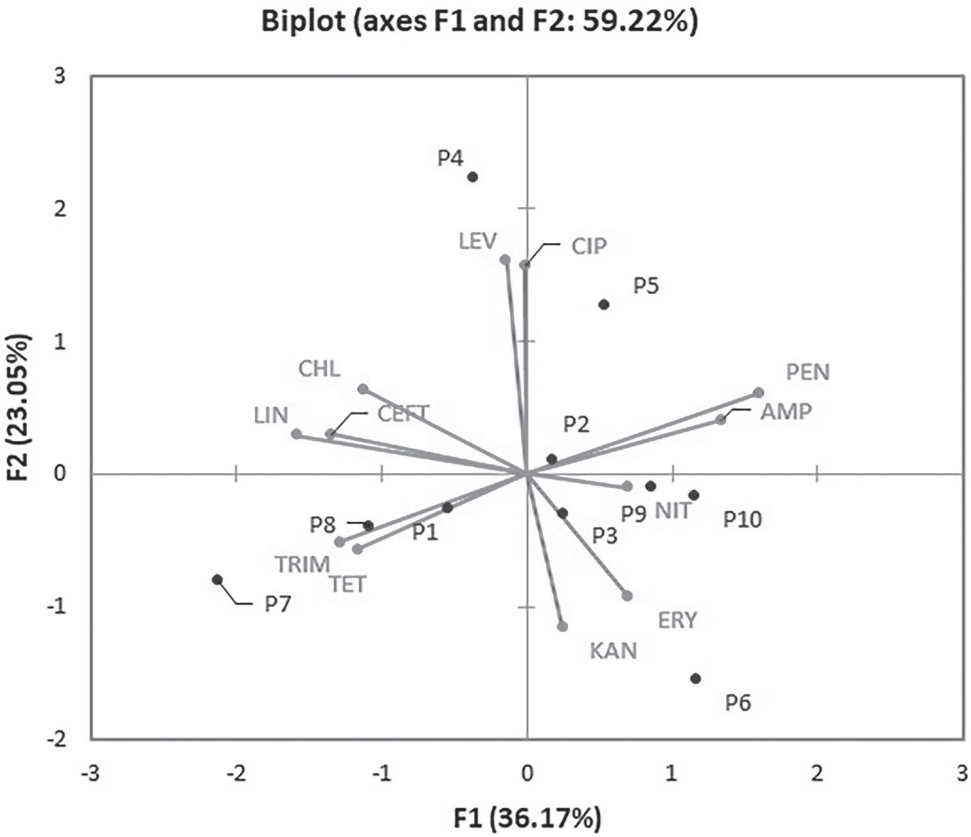

Fig. 2. Principal component analysis (PCA) biplot (axes F1 and F2: 59.22%) for the frequency of resistance to antimicrobial agents by the manufacturer (P1–P10)
PEN = penicillin, AMP = ampicillin, CIP = ciprofloxacin, LIN = linezolid, CEF = ceftazidime, KAN = kanamycin, LEV = levofloxacin, ERY = erythromycin, TRIM = trimethoprim + sulphamethoxazole, TET = tetracycline, CHL = chloramphenicol, NIT = nitrofurantoin

Discussion

The microbiological criteria for directly sold raw milk (at the farm, vending machines) are set by national law, and differ among the European countries that allow the selling of raw milk to consumers (EFSA 2015). In Croatia, the recommended criteria for *S. aureus* are as follows: m = 10 CFU/ml, c = 1, M = 10^2 CFU/ml). Recently, Jaki Tkalec et al. (2020) did not find *S. aureus* strains in raw milk from local producers in Croatia, but 24% of their traditional products of fresh cheese and cream were contaminated with the pathogen. The *S. aureus* counts observed in our survey indicate the non-compliance of raw milk in vending machines with the set criteria. In terms of risk to the consumer, these counts are of no concern because only a population above 10^5 CFU/ml can synthesize enterotoxins and cause food poisoning (Heidinger et al. 2009). The composition of the raw milk microbiota depends on several factors, such as farm management, milking procedures, udder health and medical treatment, environmental hygiene, as well as lameness (Hisira et al. 2020). Animal health, welfare (e.g. lameness, mastitis), and poor farming hygiene significantly affect the microbiological status of cow's milk. In general, higher bacterial counts are found in raw milk from previously medicated cows with currently healed mastitis (Dobranić et al. 2016). The same case may be seen for the appearance of bacterial strains resistant to clinical antibiotics (Zdolec et al. 2016).

Antimicrobial resistance patterns found in our study are in agreement with several authors, where resistance to penicillin and ampicillin was most common in *S. aureus* (Kalmus et al. 2011; Leskovec et al. 2015; Sudhanthiramani et al. 2015). In a Hungarian study (Petróczki et al. 2018), *S. aureus* occurred in raw milk from all vending machines (n = 13) and most strains were resistant to penicillin (34.6%) and tetracycline (23.1%). Sudhanthiramani et al. (2015) found *S. aureus* in 39% of the samples from the milking machines (n = 110). Most of the isolates showed high resistance to penicillin and ampicillin which is also in agreement with our results. In the study of Akindolire et al. (2015), 30 out of 40 raw milk samples (75%), 25 out of 85 whole milk samples (29%), and 10 out of 75 pasteurized milk samples (13%) were positive for *S. aureus*. Besides, a large proportion (60–100%) of isolates were resistant to penicillin G, ampicillin, oxacillin, vancomycin, teicoplanin, and erythromycin. The high proportion of multidrug-resistant *S. aureus* strain found in our study was also reported before in raw milk. Recently, Sharma et al. (2020) found all *S. aureus* isolates from milk as multidrug-resistant, but their group index MAR was less than 0.2.

In Fig. 2, the observed producers whose milk samples (P1–P10) were analyzed for the presence of antimicrobial agents resistance (n = 12) were presented by Bi-plot. In the observed data, 59.22% of all variations were included and the first principal component (F1 = 36.11%) was dominated by producers while the second principal component (F2 = 23.05%) was dominated by antimicrobial agents, indicating a significant difference between these two sets of observations. The positioning of a producer in a given square is an indicator of the occurrence frequency of antimicrobial agents. For example, resistance to ciprofloxacin and levofloxacin was most frequently detected in milk from producers 4 (P4) and 5 (P5). Since P4 is positioned in the second quadrant of Bi-plot, where linezolid, chloramphenicol, and ceftazidime are also positioned, this means that resistance to these antimicrobials is more frequently detected in P4 than in P5. Likewise, P2 is positioned in the first quadrant, as is P5, indicating that resistance was detected to the same antimicrobials as in P4 and P5, but in a lower percentage. It is interesting to note that resistance to penicillin was detected in all samples, but the Bi-plot shows that P1, P7, and P8 are located in the opposite quadrant (quadrant III), which is indicative of the lowest values. More precisely, at P8 every second sample showed resistance to penicillin, while at P7 every fourth. On the other hand, in the third quadrant, resistance to trimethoprim/

sulphamethoxazole and tetracycline was found in P1 and P7, while in P8 there was only trimethoprim/sulphamethoxazole resistance, which can be seen from the positioning of P8 on the line itself. In P3, resistance to tetracycline was also found but in a lower percentage. From these data, it appeared that most of the farmers developed resistance to their group of antimicrobials, which generally did not change during the year as well as within the same month in different vending machines of the same farmer. For some of them, resistance increased during the year of sampling in terms of the number of antimicrobials. The use of antimicrobials is one of the drivers of antimicrobial resistance and is generally related to the type of farming and milking systems (Deng et al. 2020).

The presence of bacteria resistant to antimicrobial agents in primary milk production is a significant public health problem due to the dissemination of resistance genes to consumers. In the present study, *S. aureus* was successfully isolated from raw milk samples and a large proportion of the isolates exhibited multiple antibiotic resistance characteristics. Moreover, the average count of *S. aureus* in raw milk was found not to meet the recommended microbiological criteria. In conclusion, there is an urgent need to implement appropriate control measures to reduce the microbiological risk and associated antibiotic resistance in the milk distribution chain through vending machines.

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