UNIVERSITY OF SINDH JOURNAL OF ANIMAL SCIENCES
DOI: https://doi.org/10.57038/usjas.v8i02.7065
Uni. Sindh. J. Anim. Sci., vol. 8(2); 16-48, 2024
Email: editors.usjas@usindh.edu.pk
ISSN (P): 2521-8328
ISSN (E): 2523-6067
Published by University of Sindh, Jamshoro.

ANALYSIS OF ANTIBIOTIC RESISTANCE IN RAW MILK OF ORGANIC AND CONVENTIONAL COWS IN DISTRICT SIALKOT PAKISTAN

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ARTICLE INFORMATION

Article History:
Received: 5th July 2024
Accepted: 28th November 2024
Published online: 25th December 2024

Author Contributions:
AK, SM, and SKK design the experiment; AK and IA performed the experiment; BK, ZK, SA, and AK analysis data; AK, SM, SKK write the initial draft; SKK, BK, ZK and SM review and edit the final draft.

Key words:
Antimicrobial resistance, raw milk, Conventional farming, Antibiotic susceptibility, Disk diffusion method, livestock.

Similarity Index: *14%*

ABSTRACT

Over the last few decades, antimicrobial resistance (AMR) has been recognized as one of the principal threats to public health. Even though the emerging crisis of AMR was formerly considered a problem to human health, it entails a "One Health" approach, because of the animal, human, and environmental reservoirs. Here of the all-encompassing antimicrobial use (AMU) in the livestock production systems can result in the occurrence of AMR genes in bacteria that can be transmitted in milk, leading them into the food chain. This study aimed to estimate the AMR prevalence in dairy farm, using susceptibility testing of cow's raw milk bacteria as an indicator to evaluate whether AMR patterns differ between conventional and organic dairy herds. For this purpose, an AMR comparison was made between the milk obtained from organic (No AMU) and conventional (AMU) cows for which disk diffusion method was used where AMU data were based on the injections given to cows. The findings revealed that 44% of AMR was found among all the bacterial isolates against five antibiotics i.e., amoxicillin (AMOX), ampicillin + cloxacillin (AMC), cephradine (CED), ciprofloxacin (CFX), and oxytetracycline (OT) at five different concentrations (0.1, 1, 10, 100, and 1000 µg/ml) where organic samples have shown 52% of AMR and in conventional milk samples 39% of AMR was observed, most likely because of the good health of cows and consequently less AMU in both types of dairy farms illustrating the problems in determining associations with AMU. This was a surveillance study of antimicrobial susceptibility in district Sialkot which has provided us with a rundown on AMR in raw milk as well as on the role of AMU in the occurrence of AMR in raw milk. Further research of the complete dairy farm environment is required to unravel the complex web of AMR and its matrices on dairy farms.

1. INTRODUCTION

The growing prevalence of antimicrobial resistance (AMR) in bacteria is posing serious threats to human health (Rubiola, Chiesa, Dalmasso, Di Ciccio, & Civera, 2020).

Globally, approximately 700,000 people die each year due to antibiotic-resistant infections (Oved, 2021). In the O'Neill report composed by the well-known economist Jim O'Neill commissioned by the UK government it is stated that, at present rates, the number of human deaths could rise to approximately 10 million human deaths per year by 2050 (O'Neill, 2021). Like the rest of the globe, an increase in drug-resistant infections has been reported in Pakistan (Kumarasamy et al., 2010; Shah, Wasim, & Abdullah,

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2015). Currently, Pakistan is the third highest antibiotic consumer after India and China among all low to middle-income countries. In Pakistan, the rate of antibiotics consumption has been increased by 65% between 2000 and 2015 (Klein et al., 2018). This upsurge in antibiotic consumption is becoming a foremost healthcare challenge from an AMR perspective. According to a WHO report, Pakistan has been ranked among the top 5 countries in which the highest rate of neonatal deaths due to drug-resistant bacteria have been reported (Laxminarayan et al., 2016). In the past decade, resistance to *Enterobacteriaceae* against quinolones has increased in Pakistan (Yasmin, Akhtar, & Hameed, 2013). One of its examples was the outbreak of XDR *Salmonella* in 2016 that showed 100% resistance against fluoroquinolones (Qamar et al., 2018).

Besides the potentially pathogenic bacteria, antibiotic-resistant microbes are also present in raw milk (Alexa et al., 2020; Burakoff et al., 2018; Caudell et al., 2018; Godziszewska, Pogorzelska-Nowicka, Brodowska, Jagura-Burdzy, & Wierzbicka, 2018; Munsch-Alatossava & Alatossava, 2007), and thus the consumption of raw milk may facilitate the dissemination of ARGs to the human gastrointestinal tract. Currently, there is a lack of comprehensive understanding of the presence of antibiotic resistome in raw milk (Liu, Zhu, Jay-Russell, Lemay, & Mills, 2020). Observational research on cattle production systems has revealed that a higher prevalence of AMR enteric bacteria was found in conventional dairies as compared to organic dairies (Call, Davis, & Sawant, 2008).

Food animals are treated by AMU that affects human health via 2 mechanisms: 1) growing risk of antimicrobial residues, and 2) influencing the selection or generation of antimicrobial-resistant foodborne pathogens (Yan & Gilbert, 2004). The risk of increasing antimicrobial residues in the environment is widely recognized and has been addressed via the use of appropriate regulatory mechanisms however there is growing concern regarding the AMU impact on the development of AMR in food animals (Ruegg, 2013) (Fig. 1).

An upsurge in antibiotic-resistant infections has been reported in Pakistan which can be a result of over and misuse of antibiotics in livestock production systems therefore this surveillance study of antimicrobial susceptibility was performed in district Sialkot which would provide us with a rundown on antimicrobial resistance in raw milk as well as on the role of antimicrobial use (AMU) in the occurrence of AMR in raw milk. Several international studies had compared AMR patterns in organic and conventional dairy farm (Sjöström et al., 2020). But to the best of our knowledge, there is no published study comparing AMR prevalence in the milk of organic and conventional cows in district Sialkot Pakistan. Because of the association among AMU and AMR, it was assumed that the farm with lesser AMU would also have a lesser AMR prevalence.

2. MATERIALS AND METHODS

This was a comparative and correlational study focusing on the prevalence of antibiotic resistance in the environment. The study involved a quantitative assessment of antibiotic susceptibility by determining the MIC. The sample size was 6 samples.

Sample Collection

Raw milk samples were collected near Power House, Shahabpura, and Abbot Road Sialkot from six lactating cows three of those were organic cows with no antimicrobial treatments and three were conventional cows with antimicrobial treatments in the recent past. For this purpose, the teats of each cow were disinfected with the help of a swab before taking the sample (Wattenburger, Schmidt, Placheta, Middleton, & Adkins, 2020). Samples were taken in a sterilized glass container. Immediately after taking the sample, the glass containers were placed in a bottle containing ice cubes to travel to prevent bacterial growth. Samples were transferred to the lab and refrigerated at -4 degrees Celsius within 2 hours until downstream processing.

Culturing

Serial dilutions of raw milk were prepared in distilled water. Samples were cultured by first applying the milk to the MacConkey agar media (Blood agar was used for reference and nutrient agar for CFU). The samples were plated using the spread plate culture technique so that bacteria spread out evenly on the plate (Kuehn et al., 2013). When the milk samples were plated, the left-over milk was stored in a freezer at -4˚C for further analysis. The plates, after being spread with milk, were placed in the incubator upside down. The incubator was set at 37° C. Incubation was done for 24 hours so that bacteria would be given plenty of time for growth.

Colony-forming Unit (CFU)

For measuring the CFU, samples were grown on nutrient agar. Further, dilutions of these samples were prepared (by keeping the track of dilution factor) and cultured (Pious Thomas, Sekhar, Upreti, Mujawar, &

Pasha, 2015). For serial dilution, 1 ml of sample was aseptically weighed into a sterile glass bottle and 9 ml of sterile diluent was added to provide the initial 1:10 dilution from which serial dilutions were prepared as required (Harrigan & McCance, 2014).

The plates were incubated at 37 °C for 48 hours before counting colonies. The colonies obtained were counted on each per plate of each dilution by hand. The plate consisting of the countable number of colonies was selected along with the dilution factor assigned to that culture plate for measuring CFU. The colony-forming units (CFUs) were counted after 24 hours of incubation and calculated back to CFU/ml in the original sample (Sjöström et al., 2020). Colony counts estimated the number of colony-forming units per 1 ml of the sample (Sarao, 2017). So the number of bacteria (CFU) per milliliter of the sample was obtained by dividing the number of colonies by the dilution factor (P Thomas, Mujawar, Sekhar, & Upreti, 2014). CFU was calculated by applying a specific formula (Terzich, Pope, Cherry, & Hollinger, 2000) which is as follows:

$$Bacteria\ (per\ mL) = \frac{number\ of\ colonies \times dilution\ factor}{volume\ of\ the\ plate}$$

Isolation and Purification of Bacterial Isolates

The bacterial colonies obtained through spreading were isolated by sub-culturing using the same protocol. Each bacterial colony was sub-cultured to identify each bacterial variant. The sub-cultures were labeled to avoid any confusion (Sasidharan, Prema, & Latha, 2011).

Morphological Characterization

Bacterial colony morphology was observed in terms of form (size and surface), elevation, color, texture and margins. Isolates were grown on MacConkey agar and checked for the occurrence of Lactose fermentation that changes the color of MacConkey agar due to pH change (Fig. 3A-D).

Gram Staining

On a grease-free slide, a thin spread was prepared by blending a small quantity of organisms selected from the stocked colony of 18–24 hours old pure culture into a drop of sterile distilled water in the first step. By moving it slowly over the flame, the spread was air-dried and heat-fixed. For 30–60 seconds, the slide was cautiously located on the staining rack and was swamped with primary stain (crystal violet). The addition of a gram's iodine was done (mordant) for 30 seconds. Later, the smear was moderately rinsed with tap water in the second step. The 70% ethanol that was used as a decolorizer for 10–30 seconds was essential for the third step. The smear was stained with the secondary stain (safranin) for 30 seconds before getting rinsed with tap water and later it was given the due time to get dry in the final step. Through the help of an oil immersion objective (x100) the spread was observed under the microscope. Gram-positive organisms exhibited the purple color and gram-negative exhibited the red color (Dawodu & Akanbi, 2021). Microscopic appearance of bacteria was also observed.

Biochemical Testing for Identification

Microbial identification was performed by applying several biochemical tests. The biochemical test applied for each subculture of colonies obtained were oxidase test (OX) (Cheesbrough, 2005; Hussain et al., 2013), catalase test (CT) (Bennion et al., 1990; Dawodu & Akanbi, 2021), indole test (IND) (Hussain et al., 2013; Kuffner et al., 2010), motility test (Mot) (Bello, 2002; Islam et al., 2017), citrate utilization test (CIT) (Bello, 2002; Dawodu & Akanbi, 2021), gas, and urease test (URE) (Cheesbrough, 2005; Hussain et al., 2013).

By culturing the microorganisms in peptone water medium having tryptophan in a screw-capped tube, incubated for 24 h at 37°C, IND test was carried out and later on the addition of Kovac's reagent (0.5 ml) was done through which the positive results were identified not later than seconds of adding Kovac's reagent through the development of a pink-red layer on the broth (Hussain et al., 2013). Commonly, the fact that the bacteria has moved away from the stab mark (were motile) was identified if the entire tube was turbid. However, the organism was tending to be non-motile in the case, if the stab mark was evidently visible and the rest of the tube was not disorganized (Islam et al., 2017).

Simmon's citrate agar medium was formulated in a bijou bottle and was given the time to set in a slanting position to perform citrate test. To inject the test organism onto the slant medium and incubated at 37°C for 48 hours after which it was observed for color change, a sterile wire loop was utilized. A positive citrate test was determined by a bright blue color in the medium (Dawodu & Akanbi, 2021).Urea broth was immunized and incubated at 37°C for 24 to 48 h, for the URE test. The transformation of color of the broth from yellow-orange to bright pink was measured to be positive (Hussain et al., 2013).

To evaluate the bacteria which produce the enzyme cytochrome Oxidase, OXI test was carried out. Just a few drops of 1% tetramethyl-p-phenylenediamine

dihydrochloride were used to moisten the filter paper. The growth from the plate was spread over the paper by a wooden applicator. The development of the purple color proposed an encouraging result. No change in color specified a negative result (Hussain et al., 2013). To classify the bacterial strains that produce the enzyme catalase, catalase test was utilized. The addition of the drop of 3 percent hydrogen peroxide solution was done to the sterile slide containing a loopful of the organism. Foaming or bubble indicated a positive result (Dawodu & Akanbi, 2021).

To make a distinction among organisms on the basis of peculiarities in carbohydrate fermentation that was stipulated by the production of gas, the triple sugar-iron agar test utilizing Triple Sugar Iron Agar was structured. The production of gas (formation of $CO_2$and H_2) was shown by bubbles or cracks in the agar (formation of $CO_2$and H_2).

Minimal Inhibitory Concentration (MIC)

Serial dilutions of each antibiotic were prepared in distilled water. To determine the MIC value of antibiotics, bacterial isolates were grown on nutrient agar. A total of 5 antibiotics were used on each bacterial variant obtained from organic and conventional cows and each antibiotic is used in 5 different concentrations as shown in Table 3. The antibiotics of different classes were selected on the basis of their frequent use in cows. The antibiotics used were of commercial grade. First of all stock solution of each antibiotic was prepared from which further dilutions were made.

Following the disk diffusion method (Kowalska-Krochmal & Dudek-Wicher, 2021), filter paper disks were prepared for each concentration of each antibiotic by dipping in the particular dilution (Table 1). The filter paper disk was then placed onto the media on which particular bacterial isolate has been inoculated. The plates were then incubated for 24 hours at 36°C. After incubation, the plates were examined and the zone of inhibition (if formed) was measured. The particular concentration of a given antibiotic at which the bacterial growth was completely inhibited was considered the MIC value of that antibiotic. Following the CLSI guidelines, the bacterial isolates obtained from raw milk samples were reported as sensitive, intermediate, or resistant to a particular antibiotic (Weinstein, 2021).

Statistical Analysis

Statistical software SPSS 16.0 (Statistical Package for the Social Sciences) was used for the analysis of results. For graphical analysis, a scatter plot was used. Correlation analysis between antibiotics was also performed. A comparison between the MIC value of different antibiotics tested against bacteria obtained from organic and conventional samples was made through a t-test. Correlation between MIC values of antibiotics against each sample was analyzed using Pearson correlation that investigated the degree of association between the antibiotics (Table 8). All calculations were performed at a level of significance of 0.05 or 0.01 (Table 9).

3. RESULTS AND DISCUSSION

Colony-forming Unit (CFU) Measurement

As the optimal count while evaluating CFU was 30-300 (i.e. a best single plate) we selected the 3rd dilution that falls in this range (Fig. 2).

For each sample, the original volume (initial volume) of the culture plate was 1ml and the final volume was 10ml through which the dilution factor was calculated. For each of the samples, the dilution factor was 10^3. The number of colonies, the volume of the culture plate, and the dilution factor were required to measure the CFU (CFU/ml) which was the number of bacteria (CFU) per milliliter of the sample (Table 2).

$$F = \frac{V_f}{V_i} = \frac{10}{1} = 10$$

$$Total\ dilutions = Current\ dilution \times Previous\ dilution$$

$$2^{nd} dilution = 10^1 \times 10^1 = 10^2$$

$$3^{rd} dilution = 10^1 \times 10^2 = 10^3$$

In all raw cow milk samples, the SPC (Standard plate count) was below 250,000 CFU/ml. In conventional raw milk samples (210,000-230,000 CFU/ml) bacterial consortium was higher than organic raw milk samples (195,000-206,000 CFU/ml). Similarly in Serbia, Nada Smigic et al. reported that the bacterial consortium for organic raw milk samples was lower by approx. 1 log CFU/ml compared to conventional raw milk samples (Smigic et al., 2017). In contrast, Juraj Čuboň et al. reported in a similar study performed in the south part of Slovakia that the bacterial consortium was higher in organic milk (86.103 CFU/ml) than conventional (51.103 CFU/ml) (Čuboň et al., 2008). In a similar study in Wisconsin, M. Pol and P.L. Ruegg reported no association between the bacterial consortium and herd type as the SPC of raw cow's milk above 5,000 CFU/ml for 7 conventional and 10 organic herds and below 5,000 for 13 conventional and 10 organic herds (Pol & Ruegg, 2007).

As the minimum SPC of raw milk observed in this study was 195,000 CFU/ml which emphasizes the need to pasteurize the raw milk before consumption in

Pakistan. The regulatory requirements have been contributory in ensuring the raw milk's quality; however, most sectors of the dairy industry perceived that more strictly standards should be established. It was reported in some US states by Burke et al. that for direct consumption of unpasteurized milk there is a requirement of SPC value below 10,000 CFU/ml, which is regulated by state law. In England and Wales, for direct consumption of unpasteurized milk, the SPC must be below 20,000 CFU/ml, whereas 50,000 CFU/ml is the limit for certified raw milk in Germany (Brodziak et al., 2021).

Morphological Characterization

The colony morphological characteristics exhibited by the bacterial colonies were determined after 48 hours of incubation, which showed several variations regarding color, elevation, form, margin, texture, and lactose fermentation (Table 3).

Margins which are the edges of the bacterial colonies were soft i.e. entire except for two isolates that have irregular edges i.e. undulate. Bacterial colonies have also shown elevation as most of them were convex and remaining isolates were simply raised. All the colonies of bacterial isolates were circular while there was an exception for two that have shown slight variation in their shape and were irregular. Bacterial colonies had a smooth texture except for two isolates that were mucoid and one moist. Except for lactose fermentation, all the isolates have shown distinguishable morphological characteristics as all the isolates were lactose positive. As grown on MacConkey agar, most of the bacterial colonies have shown red color but some were dark pink in color (Fig. 3A-D).

Gram Staining

The microscopic appearance of bacterial isolates was studied with the help of the gram staining technique. The prepared slides of bacterial isolates were observed under a binocular light microscope (Fig. 3F-G).

All bacterial isolates were gram-positive. False-negative gram stain also occurred may be because of inadequate specimen or smear preparation Fig. 3F-G (Samuel, Balada-Llasat, Harrington, & Cavagnolo, 2016). The microscopic shape of bacteria was also observed as rod-shaped bacteria were detected in O1M, O2M, A2M-K, and A3M-K while in A1M, O3M, A2M, and A3M cocci in pairs and short chains were observed.

Identification of Bacterial Isolates

All the bacterial isolates were identified using several biochemical tests (Table 4) (Fig. 3H). Our present study reported eight isolates out of which four microorganisms were detected after screening. The bacteria identified were *Streptococcus, Enterococcus, Lactobacillus,* and *Bifidobacteria*. All these bacteria were gram-positive, catalase, indole, and oxidase negative with no gas production. Among all isolates, *Lactobacillus* was the only motile bacteria present. Except for *Bifidobacterium*, all isolates were urease negative. On the other hand, *Streptococcus and Bifidobacterium* were the only citrate-positive bacteria among all isolates.

Our results were in complete agreement with a similar study performed in the Bahir Dar district by Yeshambel Taye et al. where they isolated and identified *Lactobacillus, Bifidobacterium,* and *Streptococcus* from raw milk, yogurt, and cheese by morphological characteristics and biochemical testing (Taye, Degu, Fesseha, & Mathewos, 2021). In Qena city of Egypt, Margret Y. Shafeek et al. isolated *Enterococcus* from raw milk of cow and some dairy products including Kareish cheese, ice cream, and yogurt that were manufactured locally and then identified through biochemical testing and multiplex PCR and concluded that milk and dairy products can play a major role in the spread of *Enterococci* virulent strains to the humans through the food chain (Shafeek, El-Malt, Abdel Hameed, & El-Zamkan, 2018).

Except for *Enterococcus*, all isolates were detected in both conventional and organic raw milk samples. Similar to our results, Kouřimská et al. also observed no differences in terms of the number of coliforms in milk from conventional and organic farming (KouřimsKá, Legarová, Panovská, & Pánek, 2014). On the other hand, *Enterococcus* was the bacteria that existed in only conventional raw milk samples not in organic samples. In Italy, Tiziana Silvetti et al. also isolated *E. faecalis* strains in period (1997–2009) when higher rates of antibiotics were used (Silvetti, Morandi, & Brasca, 2019). The reason may belong to the fact that *E. faecalis* is one of the most important active players of AMR because of its acquired or intrinsic resistance along with dissemination of resistance determinants beyond and within the genus (Silvetti et al., 2019). In Gram-positive microorganisms, *E. faecalis* is an ideal indicator of AMR (Authority, Beloeil, Guerra, & Stoicescu, 2018). AMR exhibited by foodborne *E. faecalis* displayed a public health hazard since *E. faecalis* exposure to humans through food could lead to acquisition of AMR bacteria or bacteria-borne AMR genes (Andreoletti et al., 2008). Jamet et al. (2012) also reported that AMR is prevalent in enterococci isolated from French cheese, with chloramphenicol, erythromycin, and tetracycline as the most common

AMRs detected, emphasizing the origin of erythromycin- and tetracycline -resistant genes from the farms and environment (Jamet et al., 2012).

Minimal Inhibitory Concentration (MIC)

For the quantitative study of antibiotic susceptibility, the minimal inhibitory concentration of bacterial isolates was measured (Table 4). In vitro susceptibility testing was performed by the disk diffusion method. Like the MIC value, the category of susceptibility or resistance of the microbe to a given antibiotic was assessed by the bacterial growth inhibition zone (Kowalska-Krochmal & Dudek-Wicher, 2021). Inhibition zones were created by the antibiotics on an agar medium against susceptible bacteria. The diameter of the inhibition zones was measured (in millimeters) around each antibiotic disc and the diameter of the disc was also calculated (Benkova, Soukup, & Marek, 2020). The size of inhibition zones predicted the MIC value of antibiotics. Cultivation of resistant bacteria showed visible growth against antibiotics (Fig. 3E).

The MIC value was calculated using different parameters for each sample against a particular antibiotic such as the size of the disc which was 5mm for all samples. The X-value of each sample at a particular concentration of a given antibiotic was calculated by subtracting the size of the disc from the inhibition zone created on that sample. X^2 was then calculated from the X-value. Log of concentrations of antibiotic was taken and used to determine MIC. Calculation of MIC of each sample against a particular antibiotic was performed on SPSS software 16.0. Inhibition zones created at each concentration of a particular antibiotic were used to obtain the MIC value through a scatter plot. Inhibition zones were the dependent variable while concentrations of antibiotics were independent. X^2 was plotted on the y-axis and Log of concentrations on the x-axis. A fit line was added to the scatterplot which originates the X-intercept.

In each scatterplot, the R^2 value was given which was the coefficient of determination and its value ranged from 0 and 1. R^2 value closer to 1 indicated a stronger linear association (Kiernan, 2014) (Fig. 9). Antilog of X-intercept gave the value of MIC (Nahar, Khatun, & Kabir, 2020). The determined MIC value was compared with MIC clinical breakpoints to evaluate whether the bacterial strain was resistant or susceptible to the antibiotic (Kowalska-Krochmal & Dudek-Wicher, 2021) (Table 5 and 6). Inhibition zones of all samples created by different Antibiotics at each concentration (µg/ml) were measured in mm. All inhibition zones created on a particular sample at each concentration were used to calculate MIC for that sample. Higher R^2 values indicated stronger linear associations (Kiernan, 2014).

Inhibition zones obtained were used for the susceptibility interpretation of each antibiotic against each bacterial isolate (Table 7). The bacterium was considered susceptible (growth inhibited), intermediate (growth partially inhibited), or resistant (growth not inhibited) against a particular antibiotic depending upon the size of the inhibition zone created by that antibiotic (Kowalska-Krochmal & Dudek-Wicher, 2021; Reller, Weinstein, Jorgensen, & Ferraro, 2009). The interpretive standards used were established by the Clinical and Laboratory Standards Institute (CLSI) 2020 ((CLSI), January 2020). The findings revealed that all isolates had shown resistance towards oxytetracycline and cephradine. Bacterial strains resistant to oxytetracyline such as *Streptococcus* (Jeljaszewicz & Hawiger, 1966), *Enterococcus* (Yu et al., 2009), *Lactobacillus*, (Klare et al., 2007) and *Bifidobacterium* (Gueimonde et al., 2010) and bacterial strains resistant to cephradine such as *Streptococcus* (Barie, Eachempati, & Shapiro, 2008), *Enterococcus* (Yameen, Iram, Mannan, Khan, & Akhtar, 2013), *Lactobacillus* (Saleem et al., 2018), and *Bifidobacterium* (Charteris, Kelly, Morelli, & Collins, 1998) have previously been reported by several studies. Besides, *Lactobacillus* has shown resistance towards each antibiotic. Resistant *Lactobacillus* strains were also detected in several studies against oxytetracycline (Klare et al., 2007), cephradine (Saleem et al., 2018), Amoxicillin (Wang et al., 2018), Ciprofloxacin (Saleem et al., 2018), Ampicillin + Cloxacillin (Reuben, Roy, Sarkar, Alam, & Jahid, 2020). *Streptococcus* was resistant to both ciprofloxacin and amoxicillin. Samir N. Patel et al. and Viveka Schaar et al. also reported *Streptococcus* resistant strains against ciprofloxacin (Patel et al., 2011) and amoxicillin (Schaar, Uddbäck, Nordström, & Riesbeck, 2014) respectively (Fig. 4).

Resistance towards amoxicillin and ampicillin + cloxacillin by *Enterococcus* was also detected. Resistant *Enterococcus* strains to amoxicillin (Sanlibaba & Senturk, 2018) and ampicillin + cloxacillin (Bulajić & Mijačević, 2004; Różańska, Lewtak-Piłat, Kubajka, & Weiner, 2019) have been observed in different studies. *Bifidobacteria* was resistant against ciprofloxacin ampicillin + cloxacillin (Fig. 5). W.P. Charteris et al. in a similar study isolated *Bifidobacterium* from the human gastrointestinal tract and investigated its antibiotic susceptibility and reported ciprofloxacin resistant *Bifidobacteria* strains (Charteris et al., 1998). *Enterococcus* resistant strains to ampicillin + cloxacillin were also observed. Hanna

Różańska et al. and Snežana BULAJIĆ et al. while investigating AMR in *Enterococcus*, reported it resistant to ampicillin + cloxacillin (Bulajić & Mijačević, 2004; Różańska et al., 2019). However, variability in resistance among samples was observed by *Streptococcus*, *Enterococcus*, and *Bifidobacterium* towards ampicillin + cloxacillin, ciprofloxacin, and amoxicillin respectively. There are several studies that also reported variability in resistance by bacteria towards antibiotics such as variability in resistance was detected by Hsieh J. C. et al. in *Streptococcus* towards ampicillin + cloxacillin (Hsieh, Yen, & Chuang, 2019). Daojin Yu et al. in *Enterococcus* towards ciprofloxacin (Yu et al., 2009), and W.P. Charteris et al. in *Bifidobacterium* towards amoxicillin (Charteris et al., 1998).

At lower concentrations (0.1 and 1 µg/ml), all isolates were resistant to amoxicillin, cephradine, and oxytetracycline while for ciprofloxacin and ampicillin + cloxacillin all isolates were resistant at only 0.1 (µg/ml). At 1 (µg/ml), resistance was observed for ciprofloxacin and ampicillin + cloxacillin by *Lactobacillus* and *Bifidobacteria* while *Streptococcus* has shown resistance towards only ciprofloxacin. At higher concentrations (1000, 100, 10 µg/ml), *Enterococcus* and *Streptococcus* were resistant towards cephradine, *Bifidobacteria*, and *Enterococcus* towards ampicillin + cloxacillin, while for amoxicillin only *Streptococcus* and Bifidobacteria were found resistant to oxytetracycline and ciprofloxacin. At 10 and 100 (µg/ml) of amoxicillin, resistant *Enterococcus* strains were detected. For ciprofloxacin, *Streptococcus* and *Lactobacillus* were resistant at 10 (µg/ml) only (Fig. 6).

The results revealed that 44% of AMR was found among all the bacterial isolates against five antibiotics at five different concentrations. Slight variation was reported in AMR among both conventional and organic raw milk bacteria. The AMR present in conventional samples was found to be 39% while organic samples have shown 52% of AMR. Conceivably, this unpredictability of AMR prevalence was because the association among AMR and AMU may be apparent on a larger scale, even though not easily revealed on an individual farm level (Ceccarelli et al., 2020). In a similar study in 2020, Karin Sjöström et al. investigated the difference in AMR patterns between Swedish conventional and organic dairy farms however observed no apparent difference in AMR prevalence among conventional and organic dairy farms (Sjöström et al., 2020). A possible AMR route for livestock may be from humans excreting antibiotic particles (and AMR strains) into the effluent (Hirsch, Ternes, Haberer, & Kratz, 1999) and then further into surface water that is eventually used by grazing livestock (Atterby et al., 2017). This fact is supported by a study performed by Ivan Literak et al. in which AMR has been reported in bacteria isolated from rats, flies, and other animals that exist in farm environments (Literak et al., 2009). Considering the antibiotics, 47.5% of resistance was shown towards amoxicillin, 45% towards cephradine, 42.5% towards ampicillin + cloxacillin, ciprofloxacin, and oxytetracycline (Fig. 7).

4. CONCLUSION

The issues regarding emerging antibiotic resistance in raw milk bacteria are complex and of high relevance to humans, animals, and the environment. The findings have shown 13% variation in AMR prevalence in both farm types i.e., 39% in conventional raw milk samples while 52% in organic raw milk samples. In this study, no obvious difference in AMR prevalence was detected among organic and conventional cows, most likely because of the good health of cows and consequently less AMU in both types of dairy farms. Although the number of farms was limited and minor variations may have been problematic to determine, the strict AMU regulation in conventional and organic production systems in district Sialkot highlights the question of whether there is a greater influence exerted by other farm level factors on AMR prevalence in this perspective.

5. CONFLICT OF INTEREST

All authors have declared that there is no conflict of interests regarding the publication of this article.

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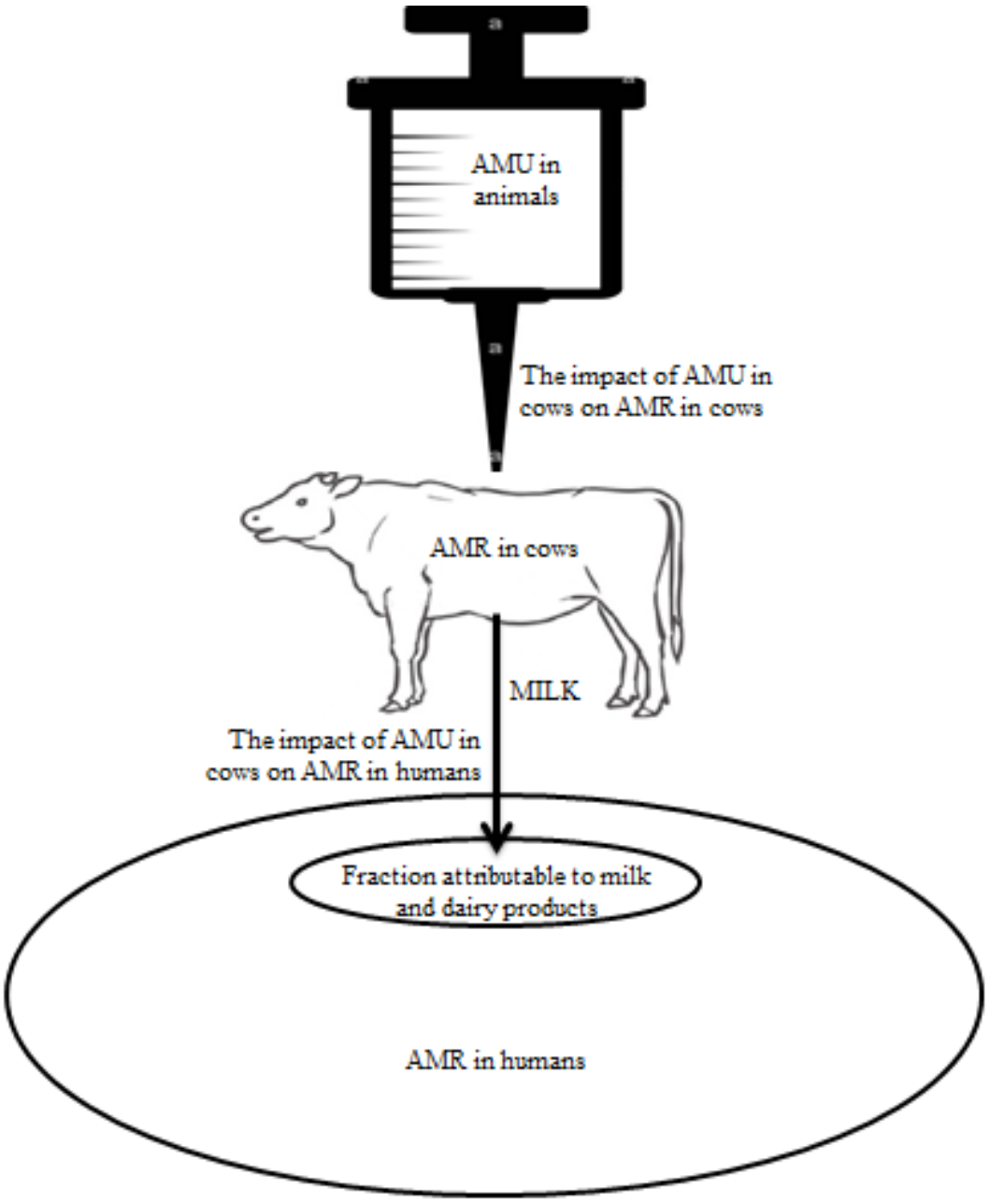


Figure 1. Framework for externality cost of AMR from AMU in cows. Cows treated with antimicrobials may develop AMR that can be transmitted to humans via milk and dairy products [17].

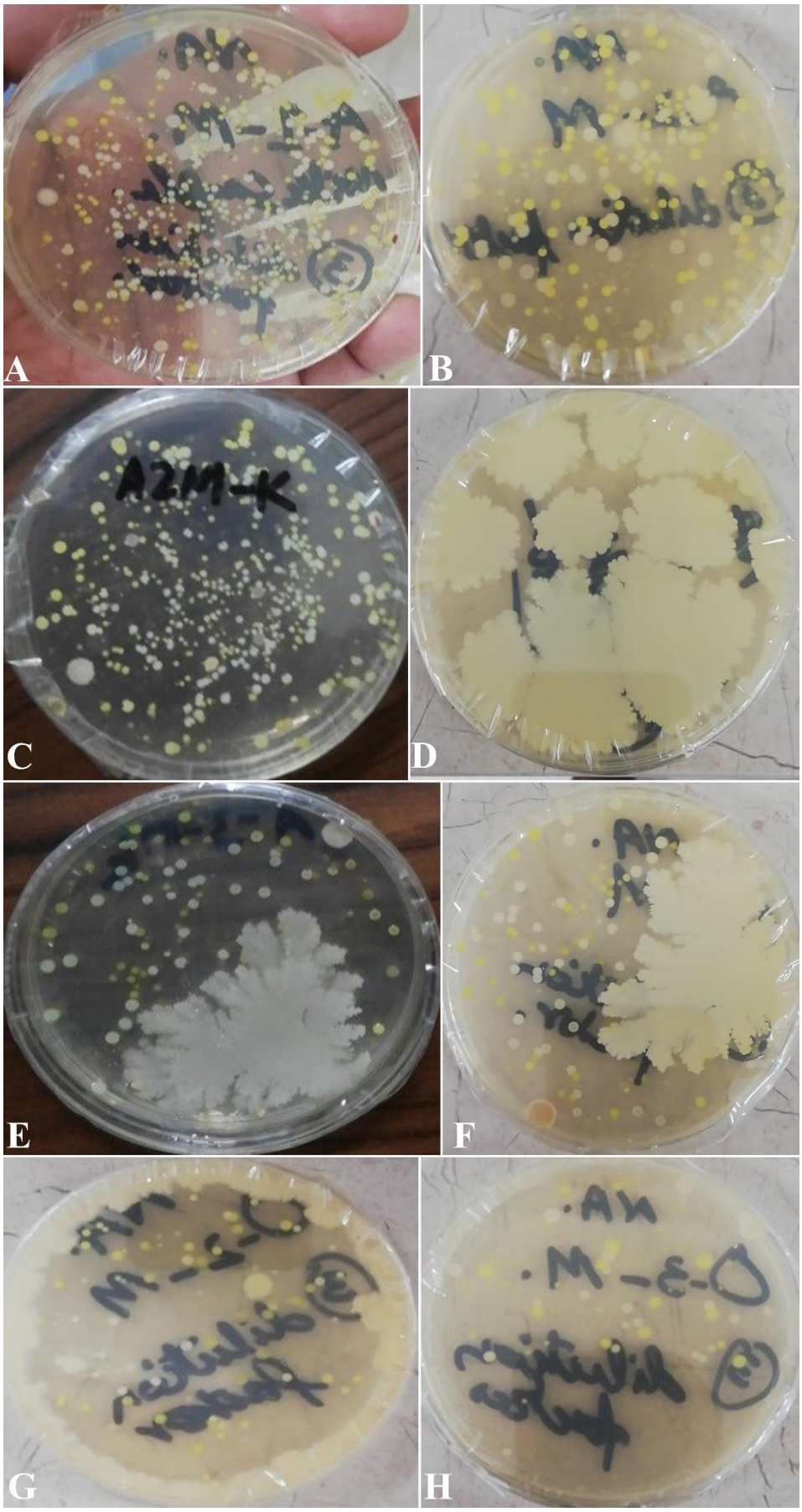
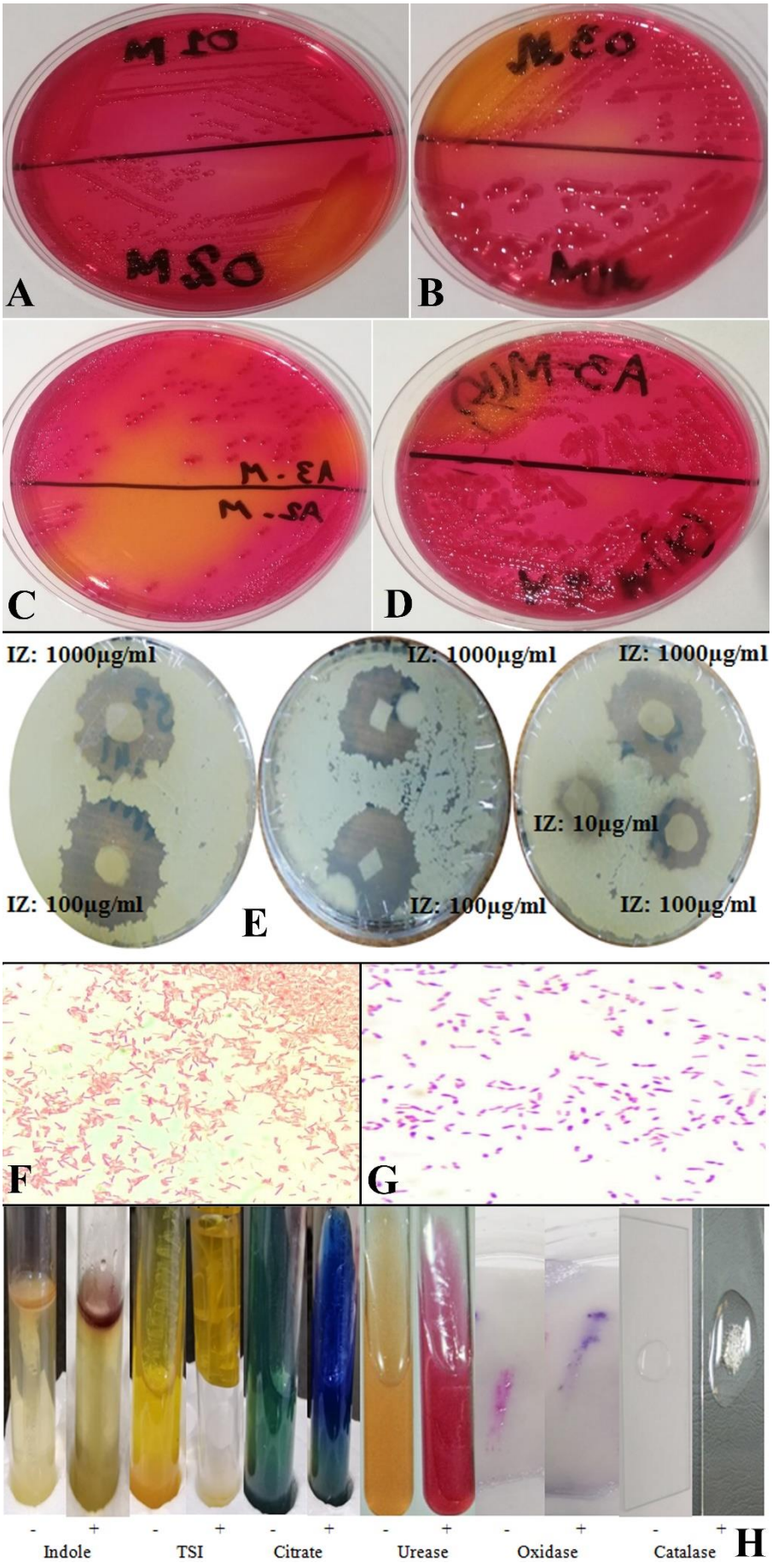


Figure 2. Culture plates showing the viable colonies.


A
B
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Citrate
Urease
Oxidase
Catalase
H

Figure 3. Morphological characteristics exhibited by subcultures on MacConkey agar. **A)** Petri plate streaked with sample id O1M and O2M. **B)** Petri plate streaked with sample id O3M and A1M. **C)** Petri plate streaked with sample id A2M-E and A3M-E. **D)** Petri plate streaked with sample id A2M-K and A3M-K. (E) Inhibition zones created by antibiotics**.** The left and middle Petri plates suggested that bacteria were resistant at 3 concentrations i.e. 0.1, 1, and 10 (µg/ml) while inhibition zones (IZ) were created at 100 and 1000 (µg/ml). Whereas the right Petri plate indicated that bacteria were resistant at 2 concentrations of 0.1 and 1 (µg/ml) while IZs were created at 10, 100 and 1000 (µg/ml). All Petri plates depict the most trivial case where there was no overlapping of inhibition zones. **(F-G)** Gram stain testing showing gram-negative (left) in red and gram-positive (right) bacteria in purple under a microscope. **(H)** Different biochemical tests used in the identification of various bacterial isolates.

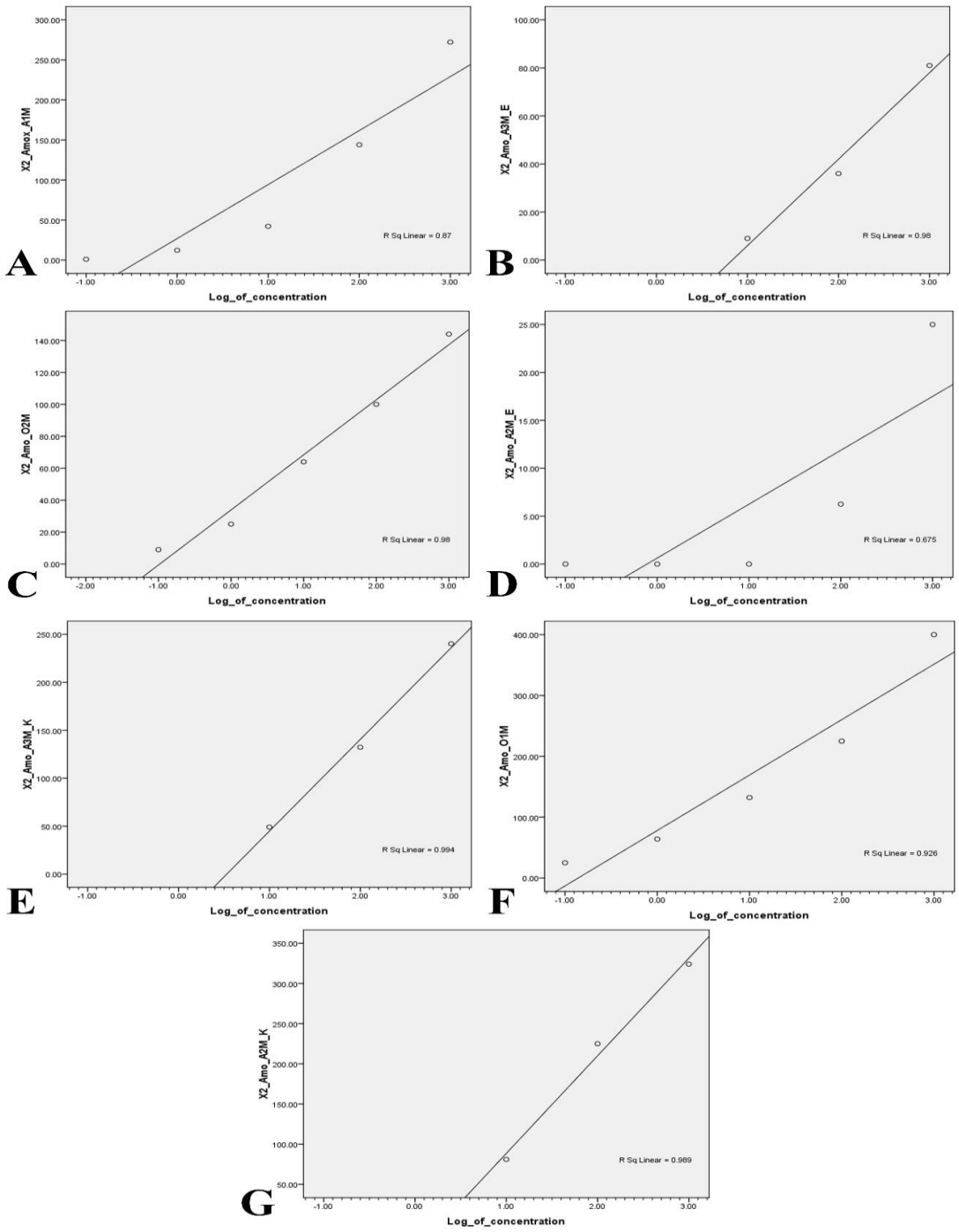

Figure 4. Scatter plot depicting X-intercept for the evaluation of MIC against Amoxicillin.

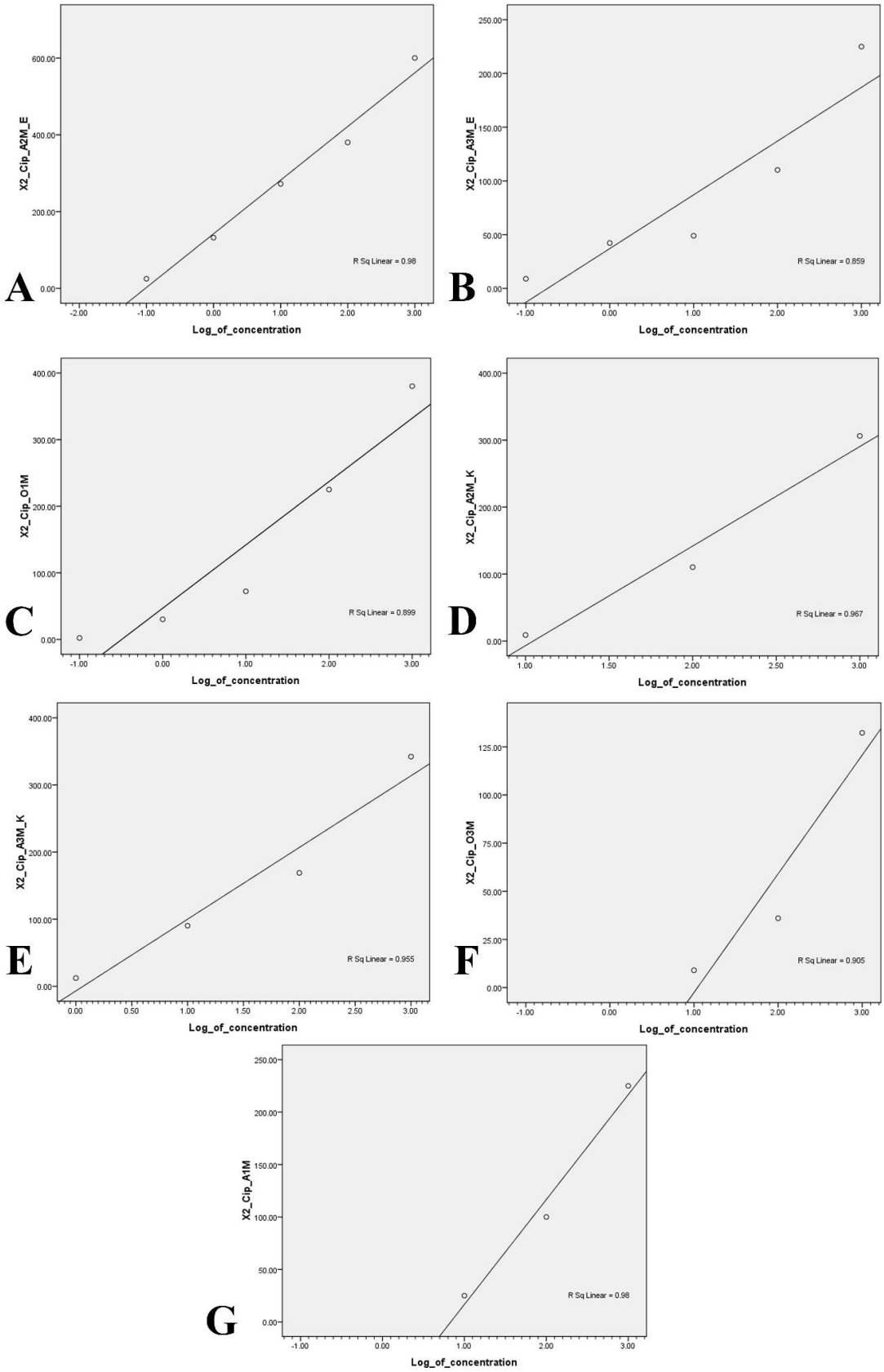

Figure 5. Scatter plot depicting X-intercept for the evaluation of MIC against Ciprofloxacin.

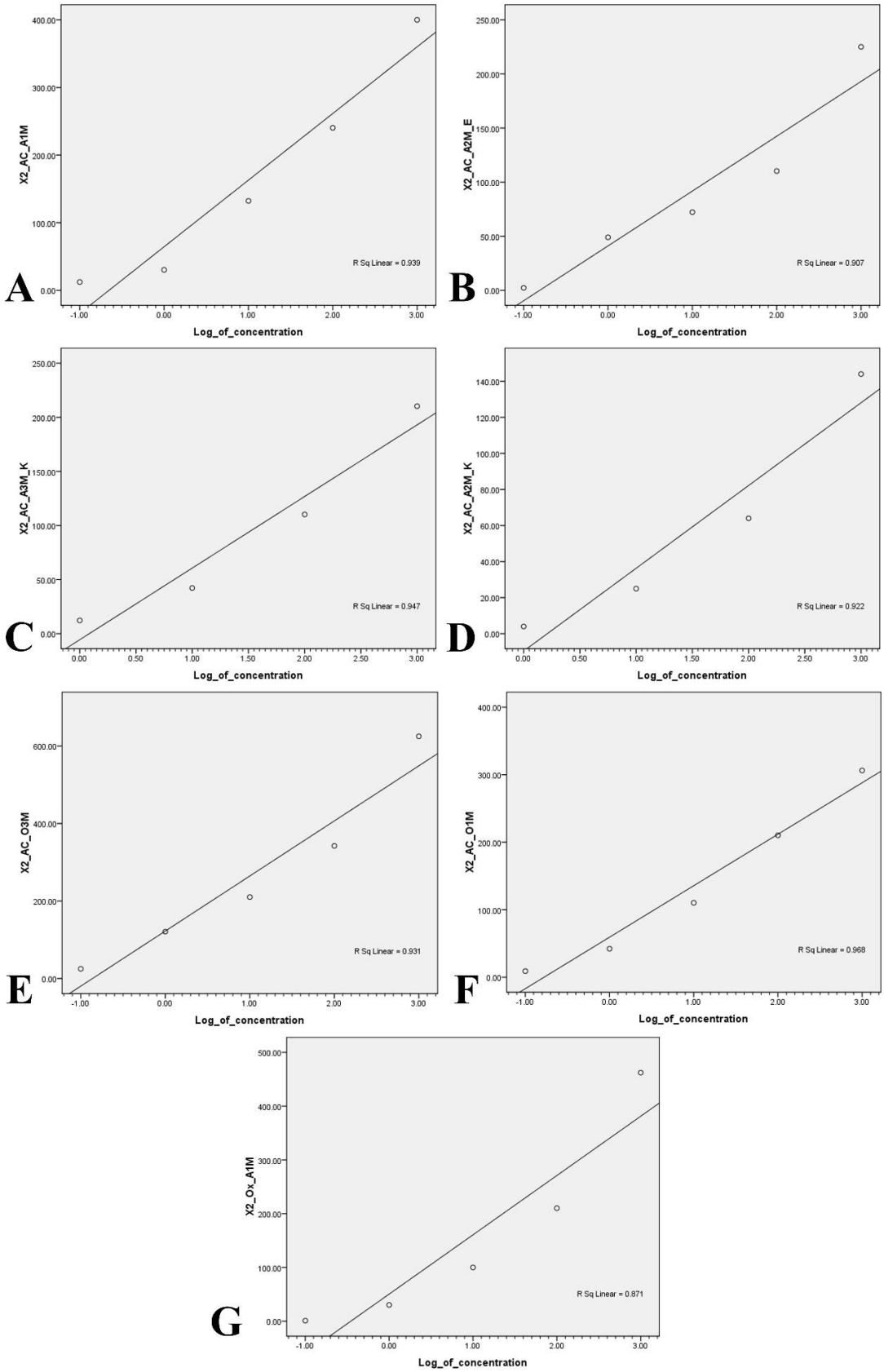

Figure 6. Scatter plot depicting X-intercept for the evaluation of MIC against ampicillin + Cloxacillin and Oxytetracycline.

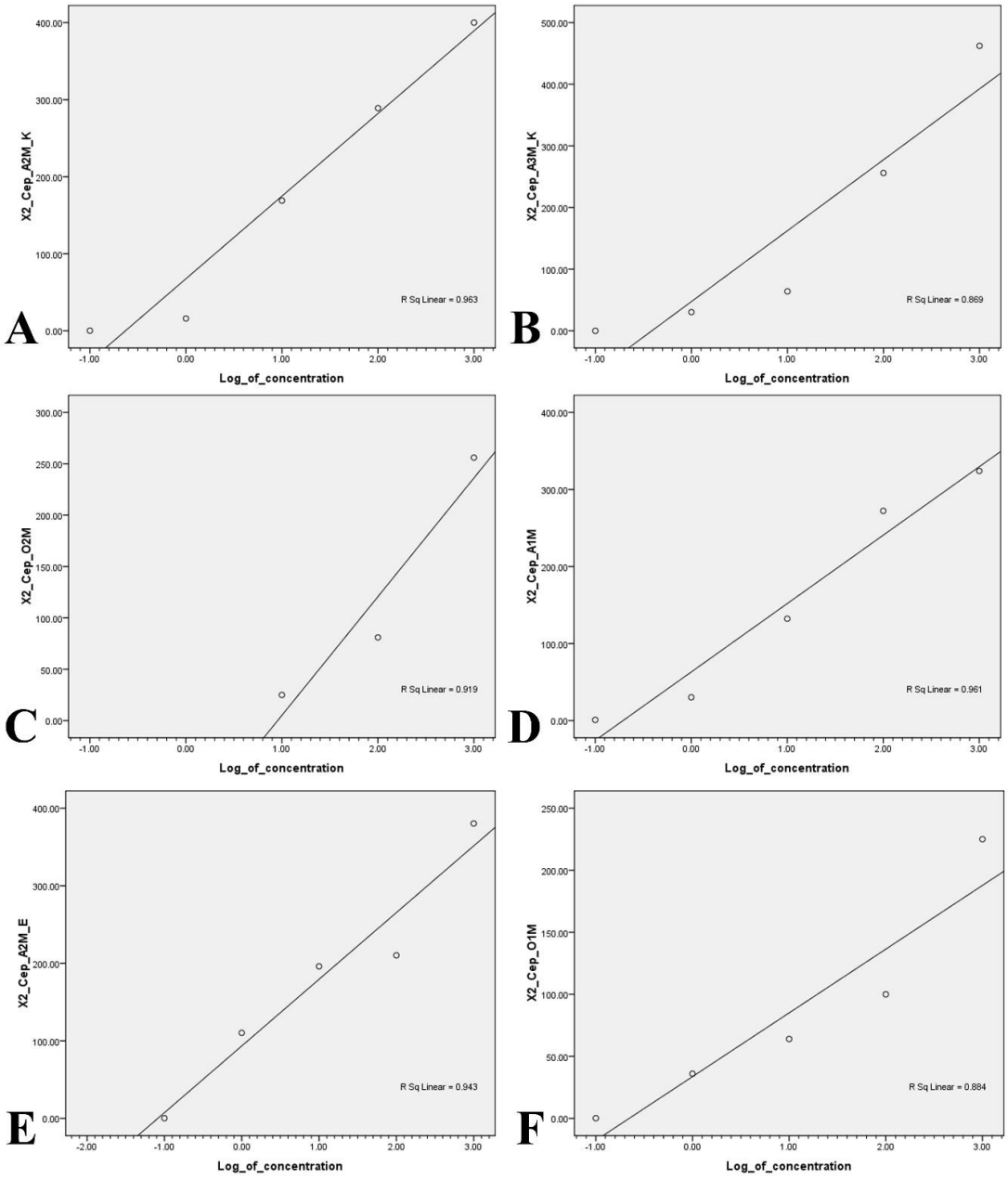

Figure 7**.** Scatter plot depicting X-intercept for the evaluation of MIC against Cephradine.

Table 1. Antibiotics and their concentrations.

S/No.	Antibiotics	Concentration (µg/ml)				
		1	2	3	4	5
1	Amoxicillin	0.1	1	10	100	1000
2	Ampicillin + Cloxacillin	0.1	1	10	100	1000
3	Cephradine	0.1	1	10	100	1000
4	Ciprofloxacin	0.1	1	10	100	1000
5	Oxytetracycline	0.1	1	10	100	1000

Table 2. Colony-forming unit of each bacterial isolate.

Sample ID	Dilution Factor	Number of Colonies	CFU (cu/ml)
A1M	10^3	230	230,000 or 2.3×10^6
A2M	10^3	215	215,000 or 2.15×10^5
A3M	10^3	210	210,000 or 2.1×10^5
O1M	10^3	195	195,000 or 1.95×10^5
O2M	10^3	201	201,000 or 2.01×10^5
O3M	10^3	206	206,000 or 2.06×10^5

Table 3. Morphological characteristics exhibited by bacterial isolates.

Sample ID	Color	Elevation	Form	Margins	Texture	Lactose Fermentation
A1M	Red	Convex	Circular	Entire	Moist	Positive
A2M-E	Dark pink	Convex	Circular	Entire	Smooth	Positive
A2M-K	Red	Raised	Circular	Entire	Mucoid	Positive
A3M-E	Dark pink	Convex	Circular	Entire	Smooth	Positive
A3M-K	Red	Raised	Circular	Entire	Mucoid	Positive
O1M	Red	Convex	Irregular	Undulate	Smooth	Positive
O2M	Red	Convex	Irregular	Undulate	Smooth	Positive
O3M	Red	Convex	Circular	Entire	Mucoid	Positive

Table 4. Biochemical testing of bacterial isolates shows probable bacterial species.

Sample ID	O3M	O2M	O1M	A3M-K	A3M-E	A2M-K	A2M-E	A1M
Gram Stain	+	+	+	+	+	+	+	+
Catalase	-	-	-	-	-	-	-	-
Oxidase	-	-	-	-	-	-	-	-
Indole	-	-	-	-	-	-	-	-
Urease	-	+	+	-	-	-	-	-
Citrate	+	+	+	-	-	-	-	+
Gas	-	-	-	-	-	-	-	-
Motility	-	-	-	+	-	+	-	-
Probable Bacterial Species	*Streptococcus spp.*	*Bifidobacteria spp.*	*Bifidobacteria spp.*	*Lactobacillus spp.*	*Enterococcus spp.*	*Lactobacillus spp.*	*Enterococcus spp.*	*Streptococcus spp.*

Table 5. Calculation of X^2 value for the evaluation of MIC.

Antibiotic	**Amoxicillin against A1M**					**Amoxicillin against A2M-E**				
Log of Conc. (µg/ml)	-1	0	1	2	3	-1	0	1	2	3
Inhibition zone (mm)	7	12	18	29	38	R	R	R	13	15
X	1	3.5	6.5	12	16.5	R	R	R	2.5	5
X2	1	12.25	42.25	144	272.25	R	R	R	6.25	25
Antibiotic	Ampicillin + Cloxacillin against A1M					Ampicillin + Cloxacillin against A2M-E				
Log of Conc. (µg/ml)	-1	0	1	2	3	-1	0	1	2	3
Inhibition zone (mm)	12	16	28	36	45	8	19	22	26	35
X	3.5	5.5	11.5	15.5	20	1.5	7	8.5	10.5	15
X2	12.25	30.25	132.25	240.25	400	2.25	49	72.25	110.25	225
Antibiotic	Cephradine against A1M					Cephradine against A2M-E				
Log of Conc. (µg/ml)	-1	0	1	2	3	-1	0	1	2	3
Inhibition zone (mm)	7	16	28	38	41	6	21	28	34	44
X	1	5.5	11.5	16.5	18	0.5	10.5	14	14.5	19.5
X2	1	30.25	132.25	272.25	324	0.25	110.25	196	210.25	380.25
Antibiotic	Ciprofloxacin against A1M					Ciprofloxacin against A2M-E				
Log of Conc. (µg/ml)	-1	0	1	2	3	-1	0	1	2	3
Inhibition zone (mm)	0	0	15	25	35	15	28	38	44	54
X	R	R	5	10	15	5	11.5	16.5	19.5	24.5
X2	R	R	25	100	225	25	132.25	272.25	380.25	600.25
Antibiotic	Oxytetracycline against A1M					Oxytetracycline against A2M-E				

Log of Conc. (µg/ml)	-1	0	1	2	3	-1	0	1	2	3
Inhibition zone (mm)	7	16	25	34	48	9	14	25	37	48
X	1	5.5	10	14.5	21.5	2	4.5	10	16	21.5
X2	1	30.25	100	210.25	462.25	4	20.25	100	256	462.25

Continued

Antibiotic	**Amoxicillin against A2M-K**					**Amoxicillin against A3M-E**				
Log of Conc. (µg/ml)	-1	0	1	2	3	-1	0	1	2	3
Inhibition zone (mm)	R	R	23	35	41	R	R	11	17	23
X	R	R	9	15	18	R	R	3	6	9
X2	R	R	81	225	324	R	R	9	36	81
Antibiotic	Ampicillin + Cloxacillin against A2M-K					Ampicillin + Cloxacillin against A3M-E				
Log of Conc. (µg/ml)	-1	0	1	2	3	-1	0	1	2	3
Inhibition zone (mm)	R	9	15	21	29	R	R	R	R	R
X	R	2	5	8	12	R	R	R	R	R
X2	R	4	25	64	144	R	R	R	R	R
Antibiotic	Cephradine against A2M-K					Cephradine against A3M-E				
Log of Conc. (µg/ml)	-1	0	1	2	3	-1	0	1	2	3
Inhibition zone (mm)	4	13	31	39	45	R	R	R	R	R
X	-0.5	4	13	17	20	R	R	R	R	R
X2	0.25	16	169	289	400	R	R	R	R	R
Antibiotic	Ciprofloxacin against A2M-K					Ciprofloxacin against A3M-E				
Log of Conc. (µg/ml)	-1	0	1	2	3	-1	0	1	2	3
Inhibition zone (mm)	R	R	11	26	40	11	18	19	26	35

X	R	R	3	10.5	17.5	3	6.5	7	10.5	15
X2	R	R	9	110.25	306.25	9	42.25	49	110.25	225
Antibiotic		Oxytetracycline against A2M-K					Oxytetracycline against A3M-E			

Continued

Antibiotic		**Amoxicillin against A3M-K**					**Amoxicillin against O1M**			
Log of Conc. (µg/ml)	-1	0	1	2	3	-1	0	1	2	3
Inhibition zone (mm)	R	R	19	28	36	15	21	28	35	45
X	R	R	7	11.5	15.5	5	8	11.5	15	20
X2	R	R	49	132.25	240.25	25	64	132.25	225	400
Antibiotic		Ampicillin + Cloxacillin against A3M-K					Ampicillin + Cloxacillin against O1M			
Log of Conc. (µg/ml)	-1	0	1	2	3	-1	0	1	2	3
Inhibition zone (mm)	R	12	18	26	34	11	18	26	34	40
X	R	3.5	6.5	10.5	14.5	3	6.5	10.5	15.4	17.5
X2	R	12.25	42.25	110.25	210.25	9	42.25	110.25	210.25	306.25
Antibiotic		Cephradine against A3M-K					Cephradine against O1M			
Log of Conc. (µg/ml)	-1	0	1	2	3	-1	0	1	2	3
Inhibition zone (mm)	5	16	21	37	48	3	17	21	25	35
X	0	5.5	8	16	21.5	-0.4	6	8	10	15
X2	0	30.25	64	256	462.25	0.16	36	64	100	225
Antibiotic		Ciprofloxacin against A3M-K					Ciprofloxacin against O1M			
Log of Conc. (µg/ml)	-1	0	1	2	3	-1	0	1	2	3
Inhibition zone (mm)	R	12	24	31	42	8	16	22	35	44
X	R	3.5	9.5	13	18.5	1.5	5.5	8.5	15	19.5

X2	R	12.25	90.25	169	342.25	2.25	30.25	72.25	225	380.25
Antibiotic		Oxytetracycline against A3M-K						Oxytetracycline against O1M		
Log of Conc. (µg/ml)	-1	0	1	2	3	-1	0	1	2	3
Inhibition zone (mm)	12	19	21	36	47	R	R	R	15	21
X	3.5	7	8	15.5	21	R	R	R	5	8
X2	12.25	49	64	240.25	441	R	R	R	25	64

Continued

Antibiotic		**Amoxicillin against O2M**						**Amoxicillin against O3M**		
Log of Conc. (µg/ml)	-1	0	1	2	3	-1	0	1	2	3
Inhibition zone (mm)	11	15	21	25	29	R	R	R	R	R
X	3	5	8	10	12	R	R	R	R	R
X2	9	25	64	100	144	R	R	R	R	R
Antibiotic		Ampicillin + Cloxacillin against O2M						Ampicillin + Cloxacillin against O3M		
Log of Conc. (µg/ml)	-1	0	1	2	3	-1	0	1	2	3
Inhibition zone (mm)	R	R	R	R	R	15	27	34	42	55
X	R	R	R	R	R	5	11	14.5	18.5	25
X2	R	R	R	R	R	25	121	210.25	342.25	625
Antibiotic		Cephradine against O2M						Cephradine against O3M		
Log of Conc. (µg/ml)	-1	0	1	2	3	-1	0	1	2	3
Inhibition zone (mm)	R	R	15	23	37	R	R	R	R	R
X	R	R	5	9	16	R	R	R	R	R
X2	R	R	25	81	256	R	R	R	R	R
Antibiotic		Ciprofloxacin against O2M						Ciprofloxacin against O3M		

Log of Conc. (µg/ml)	-1	0	1	2	3	-1	0	1	2	3
Inhibition zone (mm)	R	R	R	R	R	R	R	11	17	28
X	R	R	R	R	R	R	R	3	6	11.5
X2	R	R	R	R	R	R	R	9	36	132.25
Antibiotic	Oxytetracycline against O2M					Oxytetracycline against O3M				
Log of Conc. (µg/ml)	-1	0	1	2	3	-1	0	1	2	3
Inhibition zone (mm)	R	R	R	R	R	R	8	17	22	34
X	R	R	R	R	R	R	1.5	6	8.5	14.5
X2	R	R	R	R	R	R	2.25	36	72.25	210.25

Table 6. MIC value of Antibiotics at five different concentrations against bacterial isolates.

Antibiotics	Sample ID	Concentrations (µg/ml)					R^2	MIC
		0.1	1	10	100	1000		
Amoxicillin	A1M	7	12	18	29	38	0.87	0.2238
	A2M-E	R	R	R	13	15	0.675	0.4466
	A2M-K	R	R	23	35	41	0.989	3.5481
	A3M-E	R	R	11	17	23	0.98	6.3095
	A3M-K	R	R	19	28	36	0.994	2.4547
	O1M	15	21	28	35	45	0.926	0.0794
	O2M	11	15	21	25	29	0.98	0.0602
	O3M	R	R	R	R	R	-	-
Ampicillin + Cloxacillin	A1M	12	16	28	36	45	0.939	0.1318
	A2M-E	8	19	22	26	35	0.907	0.083
	A2M-K	R	9	15	21	29	0.922	1.1481
	A3M-E	R	R	R	R	R	-	-
	A3M-K	R	12	18	26	34	0.947	0.575
	O1M	11	18	26	34	40	0.968	0.0199
	O2M	R	R	R	R	R	-	-
	O3M	15	27	34	42	55	0.931	0.724
Cephradine	A1M	7	16	28	38	41	0.961	0.1096
	A2M-E	6	21	28	34	44	0.943	0.0457
	A2M-K	4	13	31	39	45	0.963	0.1479
	A3M-E	R	R	R	R	R	-	-
	A3M-K	5	16	21	37	48	0.869	0.2238
	O1M	3	17	21	25	35	0.884	0.1174
	O2M	R	R	15	23	37	0.919	6.4565
	O3M	R	R	R	R	R	-	-

Continued

Antibiotics	Sample ID	Concentrations (µg/ml)					R^2	MIC
		0.1	1	10	100	1000		
Ciprofloxacin	A1M	R	R	15	25	35	0.98	5.0118
	A2M-E	15	28	38	44	54	0.98	0.0512
	A2M-K	R	R	11	26	40	0.967	6.3095
	A3M-E	11	18	19	26	35	0.859	0.0977
	A3M-K	R	12	24	31	42	0.955	0.5128
	O1M	8	16	22	35	44	0.899	0.1862
	O2M	R	R	R	R	R	-	-
	O3M	R	R	11	17	28	0.905	8.1283
Oxytetracycline	A1M	7	16	15	34	48	0.871	0.1995
	A2M-E	9	14	25	37	48	0.899	0.1995
	A2M-K	9	14	23	29	43	0.84	0.1949
	A3M-E	8	17	24	38	40	0.921	0.1148
	A3M-K	12	19	21	36	47	0.854	0.1584
	O1M	R	R	R	15	21	1	63.0957
	O2M	R	R	R	R	R	-	-
	O3M	R	8	17	22	34	0.872	1.2022

Inhibition zones of all samples created by different Antibiotics at each concentration (µg/ml) were measured in mm. All inhibition zones created on a particular sample at each concentration were used to calculate MIC for that sample. Higher R^2 values indicated stronger linear associations [52].

Table 7. Antibiotic susceptibility testing against bacterial isolates.

Sample ID			**A1M**					**A2M-E**					**A2M-K**		
Organism			***Streptococcus spp.***					***Enterococcus spp.***					***Lactobacillus spp.***		
Antibiotic			Amoxicillin					Amoxicillin					Amoxicillin		
Conc. (µg/ml)	0.1	1	10	100	1000	0.1	1	10	100	1000	0.1	1	10	100	1000
Inhibition zone (mm)	7	12	18	29	38	0	0	0	13	15	0	0	23	35	41
Interpretation	R	R	I	S	S	R	R	R	R	I	R	R	S	S	S
Antibiotic			Ampicillin + Cloxacillin					Ampicillin + Cloxacillin					Ampicillin + Cloxacillin		
Conc. (µg/ml)	0.1	1	10	100	1000	0.1	1	10	100	1000	0.1	1	10	100	1000
Inhibition zone (mm)	12	16	28	36	45	8	19	22	26	35	0	9	15	21	29
Interpretation	R	I	S	S	S	R	I	S	S	S	R	R	I	S	S
Antibiotic			Cephradine					Cephradine					Cephradine		
Conc. (µg/ml)	0.1	1	10	100	1000	0.1	1	10	100	1000	0.1	1	10	100	1000
Inhibition zone (mm)	12	16	28	36	45	8	19	22	26	35	0	9	15	21	29
Interpretation	R	I	S	S	S	R	I	S	S	S	R	R	I	S	S
Antibiotic			Ciprofloxacin					Ciprofloxacin					Ciprofloxacin		
Conc. (µg/ml)	0.1	1	10	100	1000	0.1	1	10	100	1000	0.1	1	10	100	1000
Inhibition zone (mm)	0	0	15	25	35	15	28	38	44	54	0	0	11	26	40
Interpretation	R	R	I	S	S	I	S	S	S	S	R	R	R	S	S
Antibiotic			Oxytetracycline					Oxytetracycline					Oxytetracycline		
Conc. (µg/ml)	0.1	1	10	100	1000	0.1	1	10	100	1000	0.1	1	10	100	1000
Inhibition zone (mm)	7	16	25	34	48	9	14	25	37	48	9	14	23	29	43
Interpretation	R	I	S	S	S	R	R	S	S	S	R	R	S	S	S
Continued															
Sample ID			**A3M-E**					**A3M-K**					**O1M**		

Organism	***Enterococcus spp.***					***Lactobacillus spp.***					***Bifidobacteria spp.***				
Antibiotic	Amoxicillin					Amoxicillin					Amoxicillin				
Conc. (µg/ml)	0.1	1	10	100	1000	0.1	1	10	100	1000	0.1	1	10	100	1000
Inhibition zone (mm)	0	0	11	17	23	0	0	19	28	36	15	21	28	35	45
Interpretation	R	R	R	I	S	R	R	I	S	S	I	S	S	S	S
Antibiotic	Ampicillin + Cloxacillin					Ampicillin + Cloxacillin					Ampicillin + Cloxacillin				
Conc. (µg/ml)	0.1	1	10	100	1000	0.1	1	10	100	1000	0.1	1	10	100	1000
Inhibition zone (mm)	0	0	0	0	0	0	12	18	26	34	11	18	26	34	40
Interpretation	R	R	R	R	R	R	R	I	S	S	R	I	S	S	S
Antibiotic	Cephradine					Cephradine					Cephradine				
Conc. (µg/ml)	0.1	1	10	100	1000	0.1	1	10	100	1000	0.1	1	10	100	1000
Inhibition zone (mm)	0	0	0	0	0	5	16	21	37	48	3	17	21	25	35
Interpretation	R	R	R	R	R	R	I	S	S	S	R	I	S	S	S
Antibiotic	Ciprofloxacin					Ciprofloxacin					Ciprofloxacin				
Conc. (µg/ml)	0.1	1	10	100	1000	0.1	1	10	100	1000	0.1	1	10	100	1000
Inhibition zone (mm)	11	18	19	26	35	0	12	24	31	42	8	16	22	35	44
Interpretation	R	I	I	S	S	R	R	S	S	S	R	I	S	S	S
Antibiotic	Oxytetracycline					Oxytetracycline					Oxytetracycline				
Conc. (µg/ml)	0.1	1	10	100	1000	0.1	1	10	100	1000	0.1	1	10	100	1000
Inhibition zone (mm)	8	17	24	38	40	12	19	21	36	47	0	0	0	15	21
Interpretation	R	I	S	S	S	R	I	S	S	S	R	R	R	I	S

Continued

Sample ID	**O2M**	**O3M**
Organism	***Enterococcus spp.***	***Lactobacillus spp.***
Antibiotic	Amoxicillin	Amoxicillin

Conc. (µg/ml)	0.1	1	10	100	1000	0.1	1	10	100	1000
Inhibition zone (mm)	11	15	21	25	29	0	0	0	0	0
Interpretation	R	I	S	S	S	R	R	R	R	R
Antibiotic	Ampicillin + Cloxacillin					Ampicillin + Cloxacillin				
Conc. (µg/ml)	0.1	1	10	100	1000	0.1	1	10	100	1000
Inhibition zone (mm)	0	0	0	0	0	15	27	34	42	55
Interpretation	R	R	R	R	R	I	S	S	S	S
Antibiotic	Cephradine					Cephradine				
Conc. (µg/ml)	0.1	1	10	100	1000	0.1	1	10	100	1000
Inhibition zone (mm)	0	0	15	23	37	0	0	0	0	0
Interpretation	R	R	I	S	S	R	R	R	R	R
Antibiotic	Ciprofloxacin					Ciprofloxacin				
Conc. (µg/ml)	0.1	1	10	100	1000	0.1	1	10	100	1000
Inhibition zone (mm)	0	0	0	0	0	0	0	11	17	28
Interpretation	R	R	R	R	R	R	R	R	I	S
Antibiotic	Oxytetracycline					Oxytetracycline				
Conc. (µg/ml)	0.1	1	10	100	1000	0.1	1	10	100	1000
Inhibition zone (mm)	0	0	0	0	0	0	8	17	22	34
Interpretation	R	R	R	R	R	R	R	I	S	S

Table 8. Correlations among antibiotics.

		MIC-AMOX	MIC-AMC	MIC-CED	MIC-CFX	MIC-OT
MIC-AMOX	Pearson Correlation	1	.978**	-.339	-.031	-.419
	Sig. (2-tailed)		.004	.510	.954	.409
	N	7	5	6	6	6
MIC-AMC	Pearson Correlation	.978**	1	.541	.637	-.465
	Sig. (2-tailed)	.004		.347	.174	.353
	N	5	6	5	6	6
MIC-CED	Pearson Correlation	-.339	.541	1	.084	-.100
	Sig. (2-tailed)	.510	.347		.893	.873
	N	6	5	6	5	5
MIC-CFX	Pearson Correlation	-.031	.637	.084	1	-.334
	Sig. (2-tailed)	.954	.174	.893		.464
	N	6	6	5	7	7
MIC-OT	Pearson Correlation	-.419	-.465	-.100	-.334	1
	Sig. (2-tailed)	.409	.353	.873	.464	
	N	6	6	5	7	7

**. Correlation is significant at the 0.01 level (2-tailed). Other values of Pearson Correlation are significant at the 0.05 level (2-tailed).

Table 9. Independent samples test comparing the means of conventional and organic cow's raw milk.

		Independent Samples Test								
		Levene's Test for Equality of Variances		t-test for Equality of Means						
		F	Sig.	T	Df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	95% Confidence Interval of the Difference	
									Lower	Upper
MIC_AMOX	Equal variances assumed	3.256	.131	-1.352	5	.234	-2.5267400	1.8694804	-7.3323924	2.2789124
	Equal variances not assumed			-2.262	4.001	.087	-2.5267400	1.1172654	-5.6285853	.5751053
MIC_AMC	Equal variances assumed	.020	.894	-.262	4	.806	-.1125250	.4291051	-1.3039118	1.0788618
	Equal variances not assumed			-.262	2.063	.817	-.1125250	.4302543	-1.9103620	1.6853120
MIC_CED	Equal variances assumed	10517.144	.000	1.625	4	.180	3.1552000	1.9417481	-2.2359570	8.5463570
	Equal variances not assumed			.995	1.000	.501	3.1552000	3.1697686	-37.0942869	43.4046869
MIC_CFX	Equal variances assumed	5.522	.066	.571	5	.593	1.7606500	3.0859733	-6.1720968	9.6933968
	Equal variances not assumed			.420	1.241	.736	1.7606500	4.1944568	-32.3693509	35.8906509
MIC_OT	Equal variances assumed	5961249.963	.000	1.953	5	.108	31.9755300	16.3755043	-10.1190439	74.0701039
	Equal variances not assumed			1.033	1.000	.490	31.9755300	30.9467544	-361.2397357	425.1907957

Information is provided in two parts: (A) Levene's Test for Equality of Variances and (B) t-test for Equality of Means. Here, F is the test statistic of Levene's test, *t* is the computed test statistic, Sig. is the p-value corresponding to this test statistic, and *df* is the degrees of freedom. *Std. Error Difference* describes the standard error of the