Bacterial communities and antimicrobial resistance profiling in on-farm dairy processing plants based on a metagenomic approach

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Bacterial communities and antimicrobial resistance profiling in on-farm dairy processing plants based on a metagenomic approach

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Abstract

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On-farm dairy processing plants have a structure in which the farm and the dairy processing plants are in proximity, so that environmental hygiene of farm and dairy plants may affect the dairy processing stages. Unlike general dairy processing plants, these on-farm dairy processing plants are generally operated on a small scale and produce dairy products through LTLT pasteurization. Attributed to these characteristics, microorganisms can enter the dairy supply chain in on-farm processing plants through direct and indirect contact between farms and dairy processing plants. In particular, antimicrobial-resistant bacteria have been reported in dairy farms and their introduction to dairy products can pose a risk to human health

as they can transfer the resistance to their gut microbiota. The objective of this study is to investigate the microbial distribution and antimicrobial resistance of bacterial community at the four dairy processing stages (farm, pre-pasteurization, post-pasteurization, and dairy processing environments stages) of on-farm dairy processing plants. Microbial distribution was investigated by quantification of indicator bacteria contamination and metagenomic analysis. Moreover, the antimicrobial resistance of bacteria communities in on-farm dairy processing plants was investigated by identifying the resistance phenotype of bacterial isolates and detecting antimicrobial resistance genes in bacterial isolates and metagenome.

In culture-dependent approach, indicator bacteria were distributed at all stages of on-farm dairy processing plants. The contamination level of aerobic microbes ranged from 0.70 to 5.90 log CFU/mL at the farm stage, 2.17 to 8.89 log CFU/mL at pre-pasteurization stage, and significantly decreased to 0.20 to 3.97 log CFU/mL after pasteurization. In addition, the contamination level of coliforms ranged from 0.30 to 4.60 log CFU/mL at the farm stage, 0.60 to 5.39 log CFU/mL at pre-pasteurization stage, and significantly decreased to 0.40 to 0.90 log CFU/mL after pasteurization. However, the contamination level of aerobic microbes and coliforms in the final dairy product increased significantly to 0.18 to 8.54 log CFU/mL and 0.18 to 5.23 log CFU/mL, respectively, compared to pasteurized milk, indicating the possibility of cross-contamination with the dairy plant environment at the post-pasteurization stage.

In culture-independent approach based on metagenomic analysis, the relative abundance of *Pseudomonas*, the representative psychrotrophic bacteria, was identified the most at both the farm stage (24.1%) and pre-pasteurization stage (65.9%), indicating the possibility of microbial introduction from farm to dairy

processing plants. *Pseudomonas* and other psychrotrophic bacteria such as *Acinetobacter* and Enterobacteriaceae were still dominant at the post-pasteurization stage, indicating their survival from pasteurization. In core microbiota analysis, 126 genera were distributed at farm stage, while 132 and 105 genera were distributing at pre-pasteurization stage and post-pasteurization, respectively. Among them, 74 genera were distributed at all stages and 13 of them were psychrotrophic bacteria.

In culture-dependent analysis, 59 strains were isolated from on-farm dairy processing plants and 49 of them including *Pseudomonas spp.*, *Acinetobacter spp.*, and *Enterobacter* spp., *Citrobacter* spp., *Kluyvera* spp., *Hafnia* spp., *Buttiauxella* spp., *Raoultella* spp., *Serratia* spp., *Lelliottia* spp., *Klebsiella* spp., *Pantoea* spp., were identified as psychrotrophic bacteria. In results of antimicrobial resistance analysis, 44 of 59 (74.6%) were resistant to at least one antimicrobial agents. FOX-, AMP-, AMC-, and TIC-resistant bacteria were distributed at all stages of on-farm dairy processing plants. In particular, bacterial isolates showing the same AMR pattern were distributed at serial stages of same farms and seasons, indicating the transmission of antimicrobial-resistant bacteria according to processing stages. Moreover, 16 of 59 (27.1%) isolates carried plasmid mediated-antimicrobial resistance genes ($bla_{CTX-M-1}$, bla_{SHV}, bla_{TEM}, *aac(3)-II*, *aac(3)-IV*, and *tet A*), which can be potentially transferred to other bacteria. These genes were also detected in the metagenome of samples from which the corresponding isolates was not isolated, indicating the distribution of antimicrobial resistance genes in uncultured bacteria as well. The distribution of these antimicrobial resistance gene was found at all stages of on-farm dairy processing plants. These results suggest that antimicrobial-resistant psychrotrophic bacteria may spread and persist in entire on-farm dairy processing plants. They could potentially enter final dairy products and pose a threat to human

health. Additionally, this suggests the importance of applying both culture-dependent and culture-independent approaches to identify the distribution of antimicrobial-resistant psychrotrophic bacteria.

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1. Introduction

Dairy products contain nutrients in which it is easy for microorganisms to proliferate, so there is a possibility of contamination with harmful bacteria, such as pathogens and spoilage bacteria, during dairy processes [1]. Moreover, there is a lack of management of non-pathogenic but harmful bacteria such as psychrotrophic and spore-forming bacteria, which affect the quality of dairy products [2]. On-farm dairy processing plants have a structure in which the farm and the dairy processing plants are in proximity, unlike in general dairy processing plants. In these on-farm dairy processing plants, since the farm and dairy processing plants are located adjacent to each other, there are no restrictions on the workers' movements between the farm and dairy plants, and the hygiene conditions of the farm can affect the processing stages [3]. Thus, bacteria from cow feces and farm soil can be easily transmitted to dairy plant environments through the clothes, shoes, and hands of workers, and cross-contamination of dairy processing lines has also been reported [4,5]. These on-farm dairy processing plants are generally operated on a small scale and produce dairy products by low temperature long time (LTLT) pasteurization, which is suitable for the small-scale production of milk and is economically efficient [6]. LTLT pasteurization is a method of pasteurizing raw milk for a long time at a low temperature (63 ℃, 30 mins), and is known as a traditional raw milk pasteurization method for maintaining the taste and nutrition by minimizing the loss of effective nutrients and microorganisms [7]. Thus, unlike in general dairy processing plants, a greater variety of microorganisms would remain after pasteurization. Owing to these characteristics, the farm environments and quality of milk in on-farm dairy processing plants are easily affected by external factors such as season, resulting in

changes to the bacterial community in on-farm dairy processing plants. Therefore, it is crucial to identify the microbial distribution at each stage of the on-farm dairy processing plants to ensure the safety of dairy products produced there.

Antimicrobial resistance (AMR) is a growing threat to global public health and exists in humans, animals, plants, foods, and the environment [8]. In terms of food safety, the spread of AMR can occur through the consumption of animal products because livestock is known to be a reservoir of AMR [9]. Although several studies have indicated that pig and poultry farms are potential transmission routes for AMR [10,11], studies on dairy farms are relatively sparse [12]. Recent studies have shown that antimicrobial-resistant bacteria are distributed in dairy farms and processing systems [13], raising concerns regarding the risk of transferring antimicrobial-resistant bacteria to humans through the consumption of contaminated dairy products [9]. Therefore, a comprehensive study investigating the overall antimicrobial resistance in dairy supply chains, from dairy farms to final dairy products, is needed to better understand the public health risks associated with AMR.

Traditionally, research on the safety of dairy products has relied primarily on culture-dependent methods [2]. This approach has focused mainly on identifying contamination by pathogens, food-poisoning bacteria, and coliforms in dairy products [14–17]. Consequently, many selective culture methods have been developed for isolating and identifying these bacteria. However, the selective culture methods for spoilage bacteria, particularly psychrotrophic bacteria, have not been much developed yet. Thus, it is difficult to identify overall bacterial distribution using culture-dependent methods [2]. To overcome these limitations, studies using culture-independent methods, such as metagenomic analysis, are being conducted in the field of food science [18,19]. Such an approach allows the identification of the distribution of

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not only targeted bacteria but also other bacteria that are not targeted and hard to isolate, thus overcoming the limitations of culture-dependent methods. However, it is challenging to determine the absolute abundance of microorganisms using this approach, and research on the characteristics of isolates such as antimicrobial resistance and virulence poses limitations [20]. Consequently, it is crucial to analyze both culture-dependent and culture-independent approaches to overcome the limitations of each approach and to gain a comprehensive understanding of the bacterial community in dairy products.

The present study aimed to investigate the microbial distribution in on-farm dairy processing plants and the antimicrobial resistance of bacterial communities that remained after pasteurization. To this end, sampling was conducted at on-farm dairy processing plants at various stages of on-farm dairy processing plants, including farm, pre-pasteurization, post-pasteurization, and dairy processing environment stages. The microbial distribution in on-farm dairy processing plants was investigated by quantification of indicator bacteria contamination and 16S rRNA sequencing. Furthermore, antimicrobial resistance profiling was evaluated by antimicrobial susceptibility tests and the detection of antimicrobial resistance genes.

2. Materials and methods

2.1 Sample collection

The dairy samples (n=120) were collected from October 2020 to September 2021 from four on-farm dairy processing plants in different geographical locations, Gyeonggi-do, Chungcheong-nam-do, Gyeongsang-nam-do, and Jeolla-nam-do in South Korea. The sampling was done once per season to identify seasonal differences on microbial distribution in on-farm dairy processing plants. All on-farm dairy processing plants were operated on a small scale, and samples were collected from the farm, pre-pasteurization, post-pasteurization, and dairy processing environments stages. Cow teat skins (n = 16) were selected as representative for the farm stage and collected using a 3M pipette swab kit. Raw milk (n=16) was selected as representative for the pre-pasteurization stage and sampled in a 50 mL conical tube using a sterile syringe. Pasteurized milk (n=16), halloumi cheese (n=8), and mozzarella cheese (n=8) were selected as representative for the post-pasteurization stage. Pasteurized milk was sampled in a 50 mL conical tube using a sterile syringe while halloumi cheese and mozzarella cheese were collected as packaged final products. For dairy processing environments, cheese vats (n=8), curd cutters (n=8), cheese gloves (n=8), dairy plant floors (n=16), and rinsing water of dairy equipment (n=16) were selected. Figure 1 and Table 1 present a schematic of the sampling points and demographics of the on-farm dairy processing plants. The dairy samples were kept cold while being transported to the laboratory.

2.2 Quantification of indicator bacteria contamination in on-farm dairy processing plants

Contamination levels of indicator bacteria, such as aerobic microbes and

coliforms, from dairy samples (n=120), were identified using the aerobic count (AC) and coliform count (EC) 3M Petrifilm plate (3 M, Maplewood, MN, USA). 1 mL of diluted sample was loaded on the lower film and counted after incubation at 37 °C for 24 h.

2.3 Metagenomic DNA extraction and 16S rRNA sequencing

To investigate the shift in microbial composition in the farm and processing stages that could not be revealed by the culture-dependent method, metagenomic analyses were performed on cow teat skin, raw milk, and pasteurized milk samples. Metagenomic DNA was extracted from dairy samples using a Fast DNA Soil kit (MP Biomedicals, Santa Ana, CA, USA), according to the manufacturer's instructions in our previous study [21]. During milk sample treatment, 30 mL of milk were centrifuged at 5000 x g for 30 min at 4 °C. The fat was removed, and the supernatant was discarded. The pellets were washed with PBS and centrifuged at 13000 x g for 1 min. The supernatant was discarded, and the pellet was resuspended in 5 mL PBS, followed by modified kit protocol [22,23]. A swab of the cow teat skin was resuspended in 5 mL PBS, and the kit protocol was followed. The V3–V4 variable region of the 16S rRNA genes was amplified using the primers 341F and 805R. Library sequencing was performed on an Illumina MiSeq platform (Illumina, San Diego, CA, USA) at Macrogen (n=32). (Seoul, South Korea).

2.4 Bioinformatics and statistical analyses

The contamination levels of aerobic microbes and coliforms in all dairy samples were analyzed using one-way ANOVA and unpaired *t-tests*. All sequence data were analyzed using the CLC Microbial Genomics Module as part of the CLC Genomics Workbench 22.0 (QIAGEN Digital Insights, Aarhus, Denmark) [24].

Paired-end reads were imported into the CLC environment and processed by quality filtering based on the quality score. Operational taxonomic units (OTUs) were picked and assigned against the SILVA 16S v132 database at 99% similarity of OTUs [25]. Read counts under 10 were filtered and removed before analysis. Mann–Whitney tests and Kruskal–Wallis tests were used to compare the differences in the relative abundance of microbial composition. Alpha diversity was measured using the number of observed OTUs, Shannon's index, Simpson's index, and Chao1 index, and the significance of the differences was determined using the Mann–Whitney test and Kruskal–Wallis tests. Beta diversity was measured using Bray–Curtis principal coordinate analysis (PCoA). The significance of the differences in beta diversity was determined using permutational multivariate analysis of variance (PERMANOVA).

2.5 Isolation and identification of bacterial strain

Bacteria were isolated from all dairy samples (n=120) of on-farm dairy processing plants to analyze antimicrobial resistance. 5 ml of all dairy samples were inoculated in 45 ml of modified tryptic soy broth (mTSB) (BD, Sparks, MD, USA) with novobiocin and incubated at 37 °C for 24 h. Then, the culture aliquots (50 µL) were streaked on Eosin methylene blue (EMB) agar (BD, Sparks, MD, USA) and incubated at 37 °C for 24 h. Up to 4 colonies per sample, differing in morphology, were selected and streaked on Mueller–Hinton (MH) agar (BD, Sparks, MD, USA) for the pure culture, then incubated at 37 °C for 24 h. A single colony was selected and identified using matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) as per the manufacturer's instructions (Bruker, Billerica, MA, USA).

2.6 Antimicrobial resistance analyses of bacterial isolates

Antimicrobial susceptibility tests were conducted on 59 bacterial isolates (Enterobacteriaceae spp., *Pseudomonas* spp., and *Acinetobacter* spp.) obtained from dairy samples using the disk diffusion (Kirby–Bauer) method. The 16 antimicrobial agents used in the antimicrobial susceptibility test were cefoxitin (FOX, 30 μg), cefotaxime (CTX, 30 μg), ceftazidime (CAZ, 30 μg), cefepime (FEP, 30 μg), ampicillin (AMP, 10 μg), amoxicillin-clavulanic acid (AMC, 20/10 μg), ticarcillin (TIC, 75 μg), tetracycline (TE, 30 μg), sulfamethoxazole-trimethoprim (SXT, 23.75/1.25 μg), gentamicin (CN, 10 μg), aztreonam (ATM, 30 μg), amikacin (AK, 30 μg), imipenem (IPM, 10 μg), meropenem (MEM, 10 μg), nalidixic acid (NA, 30 μg), ciprofloxacin (CIP, 5 μg). The results of the antimicrobial susceptibility tests were interpreted according to the Clinical and Laboratory Standards Institute (CLSI) guidelines [26] and the European Committee on Antimicrobial Susceptibility Testing (EUCAST) guidelines [27].

The presence of plasmid-mediated antimicrobial resistance genes conferring resistance to β-lactam (*bla*CTX-M-1 group, *bla*CTX-M-2 group, *bla*CTX-M-8 group, *bla*CTX-M-9 group, *bla*CTX-M-25 group, *bla*CMY, *bla*SHV, *bla*TEM, *bla*NDM, *bla*KPC, and *bla*OXA), aminoglycoside [*aac(3)-Ⅰ,aac(3)-II, aac(3)-IV,* and *aac(6)-Ib-cr*], tetracycline (*tetA, tetB, tetC,* and *tetD)*, quinolone (*qnrA, qnrB, qnrC,* and *qnrS*), and sulfonamide (*dfrIa, dfrIb, dfr Ⅱ, dfr Ⅶ, dfrXII, sul1,* and *sul2*) was determined using PCR in 59 bacteria isolates. The primer sequences and reaction conditions have been described in the previous study [28] and are summarized in Table 2.

2.7 Detection of antimicrobial resistance genes in metagenome of dairy samples

The presence of plasmid-mediated antimicrobial resistance genes in metagenome of dairy samples (n=62) was determined to identify antimicrobial

resistance of bacteria that were not isolated by the culture method. The list of antimicrobial resistance genes confirmed is same as those described in 2.6. Metagenomic DNA extracted from cow teat skin, raw milk, pasteurized milk, and dairy processing environments was analyzed. Representative dairy processing environment samples from each dairy farm were selected and analyzed. The primer sequences and reaction conditions used in the PCR were the same as those described in 2.6.

3. Results

3.1 Microbial contamination level of indicator bacteria in on-farm dairy processing plants

In total, 120 dairy samples were collected from the farm, processing, consumption, and dairy processing environments of on-farm dairy processing plants over one year and cultured on AC and EC plates (3M Petrifilm plates) to identify the microbial contamination level at each stage. The total aerobic microbial and coliform counts are presented in Figure 2. Both aerobic microbes and coliforms were detected at all stages in on-farm dairy processing plants. The contamination level of aerobic microbes ranged from 0.70 to 5.90 log CFU/mL at the farm stage, 2.17 to 8.89 log CFU/mL at pre-pasteurization stage, and significantly decreased to 0.20 to 3.97 log CFU/mL after pasteurization (p<0.0001). In addition, the contamination level of coliforms ranged from 0.30 to 4.60 log CFU/mL at the farm stage, 0.60 to 5.39 log CFU/mL at pre-pasteurization stage, and significantly decreased to 0.40 to 0.90 log CFU/mL after pasteurization (p<0.001). However, the contamination level of aerobic microbes and coliforms in halloumi cheese, which is the final dairy product, increased significantly to 0.18 to8.54 log CFU/mL (p<0.05) and 0.18~5.23 log CFU/mL (p<0.05), respectively, compared to pasteurized milk. Moreover, aerobic microbes and coliforms were detected in dairy environment samples such as dairy plant floors and rinsing water of equipment.

3.2 Taxonomic composition of dairy microbiota in on-farm dairy processing plants

The taxonomic compositions of farm stage, pre-pasteurization stage, and post-pasteurization stage were analyzed to identify the microbial composition shift according to the different processing stages. The taxonomic compositions of each

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stage at the phylum and genus levels are shown in Figure 3. Taxonomic assignment of OTUs at the phylum level showed that Firmicutes and Proteobacteria were the most abundant phylum in farm stage samples, accounting for 48.2% and 39.1% of the total sample sequences, respectively. Proteobacteria were the most abundant in pre-pasteurization and post-pasteurization stage samples, accounting for 92.3% and 87.1%, respectively. At the genus level, various genera were distributed on the farm stage compared to pre- and post-pasteurization stages. However, *Pseudomonas*, a psychrotrophic bacterium, was predominantly distributed among farm stage, pre-pasteurization stage, and post-pasteurization stage. *Pseudomona*s was the most dominant in farm stage samples (24.1%), and *Bacillus*, *Ralstonia*, *and Romboutsia* were relatively dominant (8.7%, 8.6%, and 8.0%, respectively). Among the pre-pasteurization stage samples, *Pseudomonas* was dominant (65.9%), followed by *Acinetobacter*, *Ralstonia*, and *Kluyvera* (8.9%, 6.4%, and 5.4%, respectively). *Ralstonia* and *Pseudomonas* were the most enriched genera (33.2% and 26.9%, respectively) in post-pasteurization stage samples. The relative abundance of representative psychrotrophic bacteria such as *Pseudomonas*, *Acinetobacter*, and Enterobacteriaceae did not changed significantly after pasteurization, whereas that of *Ralstonia*, an environmental bacterium, significantly increased ($p<0.05$) (Figure 4).

3.3 Shifts in the diversity of dairy microbiota according to the different stages in on-farm dairy processing plants

Alpha diversity was evaluated using the number of observed OTUs, Shannon index, Simpson's index, and Chao1 to determine the richness and diversity of dairy microbiota from farm stage, pre-pasteurization stage, and post-pasteurization stage in on-farm dairy processing plants. Box plots show the

distribution of alpha diversity in dairy samples (Figure 5). All indices showed significant differences between farm stage and pre-pasteurization stage, whereas there were no significant differences between raw milk and pasteurized milk for all indices.

Beta diversity was evaluated to compare variations in microbial composition during these processes (Figure 5). Differences in beta diversity were compared using Bray–Curtis principal coordinate analysis (PCoA) and permutational multivariate analysis of variance (PERMANOVA). Regarding beta diversity, there was a significant difference in the microbial composition between farm sample (farm stage) and dairy plant samples (pre-pasteurization and post-pasteurization stages) ($p<0.05$). However, there was no significant difference in microbial composition between pre-pasteurization stage and post-pasteurization stage.

3.4 Distribution of core microbiota in on-farm dairy processing plants

Figure 6 shows a Venn diagram demonstrating how the genera in the on-farm dairy processing plants were distributed across processes. There were 126, 132, and 105 genera in the farm stage, pre-pasteurization stage, and post-pasteurization stage samples, respectively. A list of the genera found in each process is shown in Supplementary Table 1. Of the 132 genera at pre-pasteurization stage, 109 (82.6%) were commonly distributed at farm stage, and 74 genera, including psychrotrophic bacteria, such as *Pseudomonas*, *Acinetobacter*, and Enterobacteriaceae, were also distributed in post-pasteurization stage. Forty of the 132 genera in pre-pasteurization stage disappeared after pasteurization while 92 genera survived pasteurization. However, 13 genera, including environmental microbiota such as *Geobacillus* were not found in pre-pasteurization stage but appeared at post-pasteurization stage.

3.6 Antimicrobial resistance analyses of bacterial isolates

Fifty-nine bacterial isolates were isolated from dairy samples (n=120) from on-farm dairy processing plants (Figure 7, Table 4). Most isolates belonged to Enterobacteriaceae (89.83%), of which *Enterobacter* spp., *Citrobacter* spp., and *E. coli* were the most common, followed by *Pseudomonas* spp. (6.78%) and *Acinetobacter* spp. (3.39%). Enterobacteriaceae spp. were isolated at all stages, whereas *Pseudomonas* spp. and *Acinetobacter* spp. were isolated from post-pasteurization stages and dairy processing environments. Although most strains were isolated from pre-pasteurization stage (40.7%), they were also isolated in the post-pasteurization stages and dairy processing environments (27.12% and 15.25%, respectively).

Table 3. shows the antimicrobial-resistance phenotypes of 59 bacterial isolates. Overall, 74.6% (44/59) of bacterial isolates were resistant to at least one antimicrobial agent. In addition, the antimicrobial resistance rate was high, decreasing in the following order: ampicillin (30/59, 50.9%), amoxicillin (29/59, 49.2%), cefoxitin (23/59, 40.0%), ticarcillin (13/59, 22.0%), and cefotaxime (9/59, 15.3%). Almost half of the Enterobacteriaceae spp. were resistant to cefoxitin, ampicillin, and amoxicillin (40.0%, 50.8%, and 49.2%, respectively). All *Pseudomonas* spp. were resistant to cefotaxime, cefepime, ticarcillin, and aztreonam, whereas all *Acinetobacter* spp. were resistant to ceftazidime and cefepime.

The antimicrobial-resistance phenotypes were compared at each stage to investigate their dynamics along the stages (Figure 8). Cefoxitin-, ampicillin-, amoxicillin-, and ticarcillin-resistant bacteria were distributed in all stages of the on-farm dairy processing plants while tetracycline-, sulfamethoxazole/trimethoprim-, gentamicin-resistant bacteria were detected at only pre-pasteurization stage.

Ceftazidime-, cefepime-, aztreonam-resistant bacteria were not found at pre-pasteurization stage but detected after pasteurization.

On comparing the antimicrobial resistance (AMR) pattern of bacterial isolates from same farm and season, the distribution of isolates with same AMR pattern were identified at serial stages in Farms A, B, and C (Figure 9). In addition, isolates from the dairy processing line (pre- and post-pasteurization stages) showed the same AMR pattern as those from dairy processing environments in Farm B. Bacterial isolates with TIC-CTX-FEP-ATM resistance were only found at Farm D.

Plasmid-mediated antimicrobial resistance genes were detected in bacterial isolates, which identified $bla_{CTX-M-1}$ and bla_{SHV} genes in 4 out of 59 isolates (6.8%) and the bla_{TEM} gene in 2 out of 59 isolates (3.9%). Moreover, the *aac(3)-II* gene was identified in 1 out of 59 isolates (1.7%), whereas the *aac(3)-IV* gene was present in 6 out of 59 isolates (10.2%). The *tetA* gene was identified in 9 out of 59 isolates (15.3%). Moreover, the type of β-lactamases was identified. *Enterobacter cloacae* and *Enterobacter asburiae* isolated from cow teat skin carried $bla_{CTX-M-1}$ and bla_{SHV}, which were identified as CTX-M-88, CTX-M-216, SHV-70, and SHV-78 β-lactamases producing genes. *Lelliottia amnigena* and *Buttiauxella noackiae* isolated from raw milk were carrying the bla_{TEM} gene which was identified as the TEM-1 β-lactamase producing gene. *Klebsiella pneumoniae* and *Pantoea agglomerans* isolated from dairy processing environments (cheese vat, dairy plant floor) carried $bla_{CTX-M-1}$ and bla_{SHV}. These were identified as the CTX-M-88 and SHV-70 β-lactamase producing genes, respectively. The list of bacterial isolates and the results of antimicrobial resistance profiling are shown in Table 4.

3.7 Detection of antimicrobial resistance genes in metagenome of dairy samples

Plasmid-mediated antimicrobial resistance genes, including those

conferring resistance against β-lactams, aminoglycosides, and tetracyline were detected in 31 out of 59 metagenome (Figure 12). The bla_{TEM} gene was detected in 15 samples (1 farm, 5 pre-pasteurization, 2 pasteurization, and 7 dairy processing environment stage samples), and the bla_{SHV} gene was detected in 5 samples (2 farm, 2 pre-pasteurization, 1 dairy processing environment stage samples). Among these, bla_{TEM} and bla_{SHV} genes were both detected in one dairy processing environment sample. The *aac(3)-II* gene was detected in 12 sample (3 farm, 9 pasteurization stage samples) whereas *aac(3)-IV* gene was detected in 3 samples (1 pre-pasteurization, 2 post-pasteurization stage samples). The *tetA* gene was detected in 8 samples (3 farm, 3 pre-pasteurization, 1 post-pasteurization, 1 dairy processing environment stage samples).

Comparing the distribution of antimicrobial resistance genes in bacterial isolates and metagenome, the $bla_{TEM,}$ $bla_{SHV,}$ *aac(3)-II*, *aac(3)-IV*, *tetA* genes were detected in the metagenome of the samples from which strains identified as having those genes were isolated. However, the $bla_{CTX-M-1}$ group gene was not detected in the metagenome of the samples from which the strains identified as harboring that gene were isolated. In a total of 24 samples, the antimicrobial resistance genes were detected in the metagenome of samples from which bacteria were not isolated or from which bacteria carrying no resistance genes were isolated (Figure 10).

4. Discussion

On-farm dairy processing plants are characterized by the proximity of farms and dairy plants, leading to the possibility of bacterial transfer from the farm environment to dairy processing plants [29–31]. Dairy products in on-farm dairy processing plants are generally processed in small scale and produced by LTLT pasteurization. Antimicrobial agents are frequently administered to treat diseases of dairy cows, which can lead to the emergence of antimicrobial- resistant bacteria on dairy farms [32,33]. These antimicrobial-resistant bacteria may survive the pasteurization and be transmitted to final dairy products. Therefore, it is important to investigate microbial distribution and their antimicrobial resistance traits in on-farm dairy processing plants. Previous studies on microbial contamination in dairy products have relied on culture-dependent approaches, which have limitations in identifying the composition of untargeted and nonculturable bacteria in dairy products. Therefore, culture-independent approaches, such as metagenomic analysis, are required to understand the overall composition of microorganisms and their antimicrobial resistance traits.

In the present study, the aerobic microbes and coliforms, known as indicator bacteria in food, were distributed at all stages in on-farm dairy processing plants. In particular, the coliforms were detected at the post-pasteurization stage and dairy processing environments. Coliforms are generally known as thermolabile bacteria that do not survive pasteurization in dairy processing lines [34,35]. In the United States and Europe, the detection of coliforms in the post-pasteurization stages of dairy plants is used as an indicator of post-pasteurization contamination (PPC) and unsanitary conditions in the dairy plant environments [34,36]. Moreover, the PPCs of these coliforms have been reported to reduce the shelf life of pasteurized milk and

dairy products [37]. Thus, it is crucial to keep controlling microbial contamination after pasteurization while processing dairy products in on-farm dairy processing plants.

The present study demonstrated the dominance of *Pseudomonas* at farm stage, pre-pasteurization stage, and post-pasteurization stages. *Pseudomonas*, which was dominantly distributed in raw milk at pre-pasteurization stage, was also dominantly distributed in cow teat skin at the farm stage. Previous studies suggested that cow teat skin is a bacterial community reservoir that affects the microbiota of dairy products [31,38]. Therefore, these findings suggest that *Pseudomonas* and other psychrotrophic bacteria in raw milk may have been transmitted from the cow teat skin during the milking process. *Pseudomonas* was widely distributed not only in raw milk but also in pasteurized milk at post-pasteurization stage. Moreover, *Acinetobacter* and Enterobacteriaceae, the other representative psychrotrophic bacteria, also did not differ in relative abundance before and after pasteurization. The abundant distribution of these psychrotrophic bacteria at post-pasteurization stage of on-farm dairy processing plants may be due to their survival on LTLT pasteurization. It is known that the psychrotrophic bacteria have high capacity for heat resistance and biofilm formation in dairy processing pipelines, therefore they may have survived well in the pasteurization process [39,40].

Core microbiota analysis in present study showed the distribution of microbiota sharing across farm, pre-pasteurization, and post-pasteurization stages in on-farm dairy processing plants in genus level. Out of the 132 genera in raw milk, 92 genera (69.7%) survived pasteurization and distributed in pasteurized milk. As they survived and were predominantly distributed after pasteurization, alpha diversity of raw and pasteurized milk did not change significantly and therefore clustered together. Moreover, 74 genera including psychrotrophic bacteria were

distributing in all stages, indicating their persistence over all stages in on-farm dairy processing plants.

Based on the results of predominant distribution of psychtrotrophic bacteria at all stages in on-farm dairy processing plants, the possibility of PPCs occurrence in on-farm dairy processing plants can be inferred. The present study demonstrated that the relative abundance of *Ralstonia*, which is distributed in various environments, significantly increased after pasteurization. This may be attributed to the re-contamination of *Ralstonia* after pasteurization. *Ralstonia* is widely distributed in water, and because dairy processes such as pasteurization and cooling are carried out using a large amount of water, *Ralstonia* may have been introduced into the post-pasteurization stages from these dairy processes [41–43]. Additionally, core microbiota analysis revealed that 13 genera, which were not present in raw milk, appeared after pasteurization. These genera are thermophilic and are commonly found in environments such as water and soil [44,45]. One of them was *Geobacillus* which is known to form spores and biofilms in dairy equipment and pipelines, making it difficult to control once contaminated in dairy processing environments [23]. Since the introduction of these environmental bacteria may occur at the post-pasteurization stages, it is important to ensure hygiene monitoring to prevent cross-contamination from the environment after pasteurization.

Many antimicrobial-resistant bacteria have been reported on farms and dairy plants because of the use of antimicrobial agents on dairy farms [9,13]. In the present study, bacterial isolates from on-farm dairy processing plants were mainly identified as psychrotrophic bacteria, which are involved in the spoilage of milk and dairy products. This result is consistent with the result of a dominant distribution of psychrotrophic bacteria at all stages of the on-farm dairy processing

plants in the metagenome analysis. Moreover, of the 59 bacterial isolates, 44 (74.6%) were resistant to at least one antimicrobial agent and belonged to psychrotrophic bacteria. The antimicrobial resistance rates were high for β-lactams including ampicillin, amoxicillin, cefoxitin, ticarcillin, and cefotaxime.

In particular, by comparing the antimicrobial-resistant pattern of isolates from the same farm and at the same season, bacteria with the same antimicrobial-resistant pattern were distributes at serial stages. This phenomenon may be attributed to antimicrobial-resistant bacteria present in the preceding stage transmitting their antimicrobial resistance to surrounding bacteria leading to an increase in the distribution of antimicrobial-resistant bacteria that subsequently enter the next stage. Alternatively, the phenomenon may be due to the antimicrobial resistant bacteria of the preceding stage surviving and remaining until the next stage. Antimicrobial-resistant bacteria with same AMR pattern belonged to psychrotrophic bacteria and were distributed even after pasteurization. The LTLT pasteurization used in on-farm dairy processing plants might not have been enough to control these antimicrobial-resistant psychrotrophic bacteria due to their characteristics of heat resistance and biofilm formation[40,46]. Psychrotrophic bacteria, such as *Pseudomonas*, *Acinetobacter*, and Enterobacteriaceae, form robust biofilms within the pipelines of milk processing plants; thus they could have remained in the dairy processing environment [40,46].

Raw milk is a reservoir of antimicrobial resistance genes [1,47]. Some antimicrobial resistance genes can be transmitted horizontally to other bacteria mediated by plasmids [28,48]. The present study revealed the presence of antimicrobial resistance genes, including *bla*CTX-M-1, *bla*SHV, *bla*TEM, *aac(3)-II*, *aac(3)-IV*, and *tetA*, in bacterial isolates from on-farm dairy processing plants. In metagenome,

antimicrobial resistance genes were also detected at all stages of on-farm dairy processing plants. Notably, they were also detected in the metagenome of samples from which no bacterial strains were isolated or from which bacterial strain not carrying those genes were isolated. This indicates that the strains possessing the antimicrobial resistance genes were not isolated by culture method, which suggest that antimicrobial resistance genes distributed in the bacterial community of on-farm dairy processing plants are difficult to identify by the culture-dependent approach alone, emphasizing the importance of culture-independent antimicrobial resistance studies. These antimicrobial resistance genes can be transmitted through horizontal transfer, and there is the potential to spread resistance genes within the same genus or between different genera of bacteria [47]. If these antimicrobial-resistant bacteria are transmitted to immunocompromised individuals, including patients, or the elderly, through food consumption, they may pose a significant threat to their health [49]. Therefore, indiscriminate use of antimicrobial agents on farms should be avoided and substituted to alternatives such as bacteriophage and natural compounds[50–52].

5. Conclusion

The present study investigated the microbial distribution and antimicrobial resistance profiles at the farm stage, pre-pasteurization stage, post-pasteurization stage, and dairy processing environment stages of on-farm dairy processing plants using both culture-dependent and culture-independent approaches. The dominance of psychrotrophic bacteria at all stages of on-farm dairy processing plants were identified by metagenomic analysis. Additionally, bacterial isolates from on-farm dairy processing plants were mainly identified as psychrotrophic bacteria. Most of them were antimicrobial-resistant bacteria and isolates with the same AMR pattern were distributed at serial stages of same farms and seasons. Moreover, the distribution of antimicrobial resistance genes, which can be transferred to other bacteria, were identified at all stages of on-farm dairy processing plants. This suggests that antimicrobial-resistant psychrotrophic bacteria spread and persist in entire on-farm dairy processing plants and may enter final dairy products, potentially transmitting antimicrobial resistance to humans through food intake. Furthermore, microbial distribution and antimicrobial-resistance of uncultured bacteria, which could not be identified with culture-dependent methods, was identified through culture-independent approach, suggesting the need for controlling antimicrobial-resistant psychrotrophic bacteria in on-farm dairy processing plants based on culture-dependent and culture-independent methods. The findings of this study showed the possibility of contamination of antimicrobial-resistant psychrotrophic bacteria during dairy processing in on-farm dairy processing plants, providing valuable insights for controlling such bacteria effectively to improve the quality of dairy products and safeguard human health. There should be an effort to reduce the proliferation of antimicrobial-resistant

psychrotrophic bacteria and the transmission of their antimicrobial resistance during the cold chain of dairy products, from raw milk production to the distribution of dairy products to consumers.

Table 1. Demographics of on-farm dairy processing plants and sampling information.

Farm ID	On-farm dairy processing plant A	On-farm dairy processing plant B	On-farm dairy processing plant C	On-farm dairy processing plant D
Province	Gyeonggi-do	Chungcheong-nam-do	Gyeongsang-nam-do	Jeolla-nam-do
Number of dairy cows	n=60	n=70	n=100	n=1200
Production of raw milk per day (ton)	1 ton	1.5 ton	1.4 ton	1.7 ton
Farm stage	n=4	n=4	n=4	n=4
Pre-pasteurization stage	n=4	n=4	n=4	n=4
Post-pasteurization stage	n=4	n=4	n=12	n=12
Dairy processing environments	n=8	n=8	n=20	n=20

Table 2. Oligonucleotide sequence of primers to detect antimicrobial resistance genes.

Antimicrobial resistance	Genes		Nucleotide sequence	Amplicon size (bp)	Annealing temp. (°C)	Reference
β-lactam	$bla_{CTX-M-1}$ group	F	GTTACAATGTGTGAG AAGCAG	1,041	60	(Jouini et al., 2007)
		R	CCGTTTCCGCTATTA CAAAC			
	$bla_{CTX-M-2}$ group	F	CGACGCTACCCCTGC TATT	832	60	(Jouini et al., 2007)
		R	CAGAAACCGTGGGT TACGAT			
	$bla_{CTX-M-8}$ group	F	GGCGCTGGAGAAAA GCAG	862	60	(Jouini et al., 2007)
		R	GGTTTTATCCCCGAC AACC			
	$bla_{CTX-M-9}$ group	F	GTGACAAAGAGAGT GCAACGG	857	60	(Jouini et al., 2007)
		R	ATGATTCTCGCCGCT GAAGCC			
	$bla_{CTX-M-25}$ group	F	GCACGATGACATTC GGG	327	60	(Jouini et al., 2007)
		R	AACCCACGATGTGG GTAGC			
	bla_{CMY}	F	AACACACTGATTGC GTCTGAC	1,226	60	(Jouini et al., 2007)
		R	CTGGGCCTCATCGTC AGTTA			
	bla_{SHV}	F	TCGCCTGTGTATTAT CTCCC	768	54	(Jouini et al., 2007)
		R	CGCAGATAAATCAC CACAATG			
	bla_{TEM}	F	TCCGCTCATGAGACA ATAACC	1,057	58	(Jouini et al., 2007)
		R	ACGCTCAGTGGAAC GAAAAC			
	bla_{OXA}	F	ACACAATACATATC AACTTCGC	813	60	(Jouini et al., 2007)
		R	AGTGTGTTTAGAATG GTGATC			
aminoglycoside	aac(3)- I	F	ACCTACTCCCAACAT CAGCC	169	60	(Saenz et al., 2007)
		R	ATATAGATCTCACTA CGCGC			
	aac(3)-II	F	ACTGTGATGGGATA CGCGTC	237	60	(Saenz et al., 2007)
		R	CTCCGTCAGCGTTTC AGCTA			
	aac(3)-IV	F	CTTCAGGATGGCAA GTTGGT	286	60	(Saenz et al., 2007)
		R	TCATCTCGTTCTCCG CTCAT			
	aac(6)-Ib-cr	F	TTGCGATGCTCTATG AGTGGCTA	482	50	(Liao et al., 2007)
		R	CTCGAATGCCTGGCG TGTTT			

tetracycline	tetA	F	GCTACATCCTGCTTG CCTTC	210	58	(Saenz et al., 2007)
		R	CATAGATCGCCGTG AAGAG			
	tetB	F	TTGGTTAGGGGCAA GTTTTG	659	56	(Saenz et al., 2007)
		R	GTAATGGGCCAATA ACACCG			
	tetD	F	AAACCATTACGGCA TTCTGC	787	60	(Saenz et al., 2007)
		R	GACCGGATACACCA TCCATC			
quinolone	qnrA	F	ATTTCTCA CGCCAGGATTTG	516	53	(Liao et al., 2007)
		R	GATCGGCAAAGGTT AGGTCA			
	qnrB	F	GATCGTGAAAGCCA GAAAGG	469	53	(Liao et al., 2007)
		R	ACGATGCCTGGTAGT TGTCC			
	qnrC	F	GGGTTGTACATTTAT TGAATC	447	50	(Liao et al., 2007)
		R	TCCACTTTACGAGGT TCT			
	qnrS	F	ACGACATTCGTCAAC TGCAA	417	53	(Liao et al., 2007)
		R	TAAATTGGCACCCTG TAGGC			
sulfonamide	dfrIa	F	GTGAAACTATCACTA ATGG	474	55	(Saenz et al., 2004)
		R	TTAACCCTTTTGCCA GATTT			
	dfrIb	F	GAGCAGCTICTITTIA AAGC	393	60	(Saenz et al., 2004)
		R	TTAGCCCTTTIICCAA TTTT			
	dfrII	F	GATCACGTGCGCAA GAAATC	141	50	(Saenz et al., 2004)
		R	AAGCGCAGCCACAG GATAAAT			
	dfrVII	F	TTGAAAATTTCATTG ATT	474	55	(Saenz et al., 2004)
		R	TTAGCCTTTTTTTCCA AATCT			
	dfrXII	F	GGTGSGCAGAAGAT TTTTCGC	319	60	(Saenz et al., 2004)
		R	TGGGAAGAAGGCGT CACCCTC			
	sul1	F	TGGTGACGGTGTTCG GCATTC	789	63	(Jouini et al., 2007)
		R	GCGAGGGTTTCCGA GAAGGTG			
	sul2	F	CGGCATCGTCAACAT AACC	722	50	(Jouini et al., 2007)
		R	GTGTGCGGATGAAG TCAG			

Table 3. Antimicrobial-resistance phenotype of bacteria isolated from dairy samples of on-farm dairy processing plants.

Antimicrobial agents	No. (%) of resistant flora						
	Farm A	Farm B		Farm C	Farm D		
	Enterobacteriaceae spp. (n=10)	Enterobacteriaceae spp. (n=20)	*Acinetobacter* spp. (n=2)	Enterobacteriaceae spp. (n=12)	Enterobactericaceae spp. (n=11)	*Pseudomonas* spp. (n=4)	Total (n=59)
FOX	4 (40)	6 (30)	0 (0)	7 (58.3)	6 (54.5)	0 (0)	23 (40.0)
CTX	0 (0)	3 (15)	0 (0)	2 (16.7)	0 (0)	4 (100)	9 (15.3)
CAZ	0 (0)	0 (0)	2 (100)	1 (8.3)	0 (0)	0 (0)	3 (5.1)
FEP	0 (0)	0 (0)	2 (100)	0 (0)	0 (0)	4 (100)	6 (10.2)
AMP	4 (40)	13 (65)	0 (0)	9 (75)	4 (36.4)	0 (0)	30 (50.8)
AMC	7 (70)	10 (50)	0 (0)	7 (58.3)	5 (45.5)	0 (0)	29 (49.2)
TIC	2 (20)	3 (23.1)	0 (0)	3 (25)	1 (9.1)	4 (100)	13 (22.0)
TE	1 (10)	0 (0)	0 (0)	1 (8.3)	0 (0)	0 (0)	2 (3.4)
SXT	1 (10)	0 (0)	0 (0)	1 (8.3)	0 (0)	0 (0)	2 (3.4)
CN	1 (10)	0 (0)	0 (0)	1 (8.3)	0 (0)	0 (0)	2 (3.4)
ATM	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	4 (100)	4 (6.8)
AK	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
IPM	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
MEM	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
NA	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
CIP	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)

* Abbreviations: FOX, Cefoxitin; CTX, Cefotaxime; CAZ, Ceftazidime; FEP, Cefepime; AMP, Ampicillin; AMC, Amoxicillin-clavulanic acid; TIC, Ticarcillin; TE, Tetracycline; SXT, Sulfamethoxazole-trimethoprim; CN, Gentamicin; ATM, Aztreonam; AK, Amikacin; IPM, Imipenem; MEM, Meropenem; NA, Nalidixic acid; CIP, Ciprofloxacin

Table 4. List of bacteria isolates from dairy samples of on-farm dairy processing plants and their antimicrobial resistance analysis results.

Species	Farm	Source	Antimicrobial resistance phenotype	Antimicrobial resistance genes
Kluyvera cryocresecens	Farm A	Cow teat skin	TIC	aac(3)-Ⅱ
Citrobacter braakii	Farm A	Cow teat skin	AMP, AMC, FOX	-
Eshcherichia coli	Farm A	Cow teat skin	-	-
Citrobacter braakii	Farm A	Cow teat skin	AMP, AMC, FOX	-
Citrobacter braakii	Farm A	Cow teat skin	AMC, FOX	-
Citrobacter braakii	Farm A	Cow teat skin	AMC, FOX	-
Eshcherichia coli	Farm A	Raw milk	-	-
Hafnia alvei	Farm A	Raw milk	AMP, AMC	-
Hafnia alvei	Farm A	Raw milk	AMC, FOX	-
Buttiauxella noackiae	Farm A	Raw milk	TIC, AMP, AMC, SXT, CN, TE	TEM-1
Serratia liquefaciens	Farm B	Raw milk	-	tetA
Eshcherichia coli	Farm B	Raw milk	-	-
Eshcherichia coli	Farm B	Raw milk	-	-
Serratia liquefaciens	Farm B	Raw milk	AMP, AMC	-
Serratia liquefaciens	Farm B	Raw milk	AMP, AMC	-
Eshcherichia coli	Farm B	Raw milk	-	-
Citrobacter freundii	Farm B	Raw milk	AMP, AMC, FOX, CTX	-
Hafnia alvei	Farm B	Raw milk	TIC, AMP, AMC	tetA, aac(3)-Ⅳ
Enterbacter cloacae	Farm B	Raw milk	AMP, AMC, FOX	tetA
Buttiauxella gaviniae	Farm B	Pasteurized milk	-	-

Species	Farm	Source	Antimicrobial resistance phenotype	Antimicrobial resistance genes
Enterobacter asburiae	Farm B	Pasteurized milk	AMP, AMC, FOX, CTX	tetA, aac(3)-IV
Buttiauxella gaviniae	Farm B	Pasteurized milk	AMP	-
Buttiauxella gaviniae	Farm B	Pasteurized milk	AMP, AMC	-
Acinetobacter ursingii	Farm B	Pasteurized milk	CAZ, FEP	-
Enterobacter cloacae	Farm B	Dairy plant floor	AMP, AMC, FOX	-
Kluyvera cryocresecens	Farm B	Rinsing water of dairy eqipment	TIC, AMP	-
Enterbacter kobei	Farm B	Dairy plant floor	AMP, AMC, FOX	tetA
Pantoea agglomerans	Farm B	Dairy plant floor	-	-
Pantoea agglomerans	Farm B	Dairy plant floor	AMP, FOX, CTX	CTX-M-88, SHV-70, tetA
Lelliottia amnigena	Farm B	Dairy plant floor	-	-
Raoultella terrigena	Farm B	Rinsing water of dairy eqipment	TIC, AMP, AMC	-
Acinetobacter ursingii	Farm B	Rinsing water of dairy eqipment	CAZ, FEP	-
Enterobacter cloacae	Farm C	Cow teat skin	TIC, AMC, FOX	CTX-M-88, SHV-70, SHV-78

Species	Farm	Source	Antimicrobial resistance phenotype	Antimicrobial resistance genes
Enterobacter asburiae	Farm C	Cow teat skin	AMP, AMC, FOX	CTX-M-216, SHV-70, aac(3)-IV
Enterobacter kobei	Farm C	Cow teat skin	AMP, AMC, FOX	aac(3)-IV
Eshcherichia coli	Farm C	Cow teat skin	-	-
Citrobacter braakii	Farm C	Raw milk	AMP, AMC, FOX	-
Enterobacter cloacae	Farm C	Raw milk	AMP, AMC, FOX	-
Serratia liquefaciens	Farm C	Raw milk	AMP	tetA
Lelliottia amnigena	Farm C	Raw milk	TIC, AMP, SXT, CN, TE	TEM-1
Citrobacter freundii	Farm C	Raw milk	AMP, AMC, FOX	-
Hafnia alvei	Farm C	Halloumi cheese	-	tetA, aac(3)-IV
Enterbacter asburiae	Farm C	Mozzarella cheese	AMP, AMC, FOX, CTX	tetA, aac(3)-IV
Klebsiella pneumoniae	Farm C	Cheese vat	TIC, AMP, CTX, CAZ	CTX-M-88, SHV-70
Eshcherichia coli	Farm D	Cow teat skin	-	-
Eshcherichia coli	Farm D	Raw milk	-	-
Eshcherichia coli	Farm D	Raw milk	-	-
Enterobacter cloacae	Farm D	Raw milk	AMP, AMC, FOX	-
Citrobacter freundii	Farm D	Raw milk	FOX	-
Eshcherichia coli	Farm D	Raw milk	-	-
Raoultella ornithinolytica	Farm D	Pasteurized milk	TIC, AMP	-
Pseudomonas koreensis	Farm D	Pasteurized milk	TIC, ATM, CTX, FEP	-
Enterobacter aerogenes	Farm D	Halloumi cheese	AMP, AMC, FOX	-

Species	Farm	Source	Antimicrobial resistance phenotype	Antimicrobial resistance genes
Enterobacter cloacae	Farm D	Halloumi cheese	AMC, FOX	-
Enterobacter cloacae	Farm D	Halloumi cheese	AMC, FOX	-
Enterobacter cloacae	Farm D	Halloumi cheese	AMC, FOX	-
Enterobacter cloacae	Farm D	Mozzarella cheese	AMP, AMC, FOX	-
Pseudomonas chlororaphis	Farm D	Mozzarella cheese	TIC, ATM, CTX, FEP	-
Pseudomonas koreensis	Farm D	Halloumi cheese	TIC, ATM, CTX, FEP	-
Pseudomonas koreensis	Farm D	Mozzarella cheese	TIC, ATM, CTX, FEP	-

* Abbreviations: FOX, Cefoxitin; CTX, Cefotaxime; CAZ, Ceftazidime; FEP, Cefepime; AMP, Ampicillin; AMC, Amoxicillin-clavulanic acid; TIC, Ticarcillin; TE, Tetracycline; SXT, Sulfamethoxazole-trimethoprim; CN, Gentamicin; ATM, Aztreonam

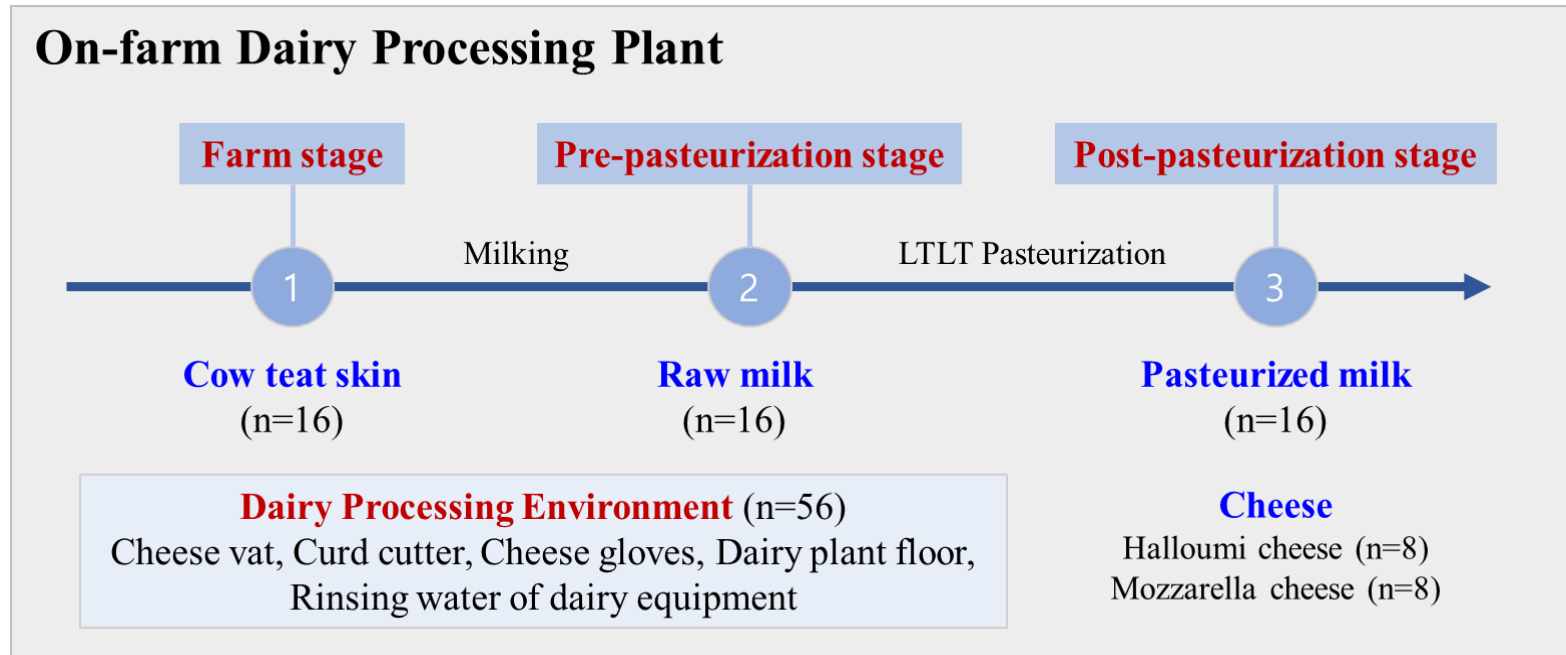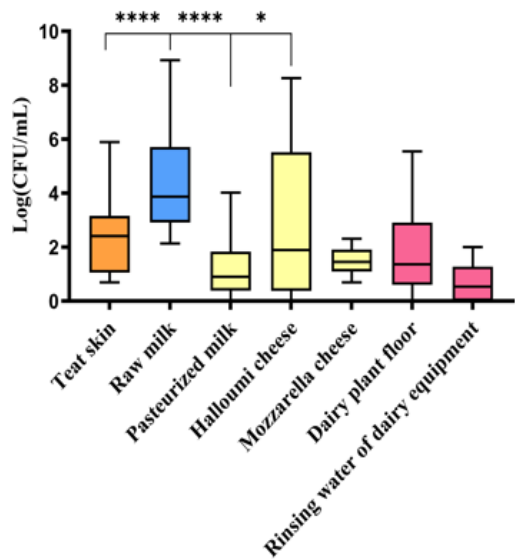**Figure 1.** Sampling points schematic.

Figure 2. Microbial contamination level of farm stage, pre-pasteurization stage, post-pasteurization stages, and dairy processing environment stage in on-farm dairy processing plants. Microbial contamination level was evaluated by colony count of indicator bacteria. Colonies were counted using aerobic count (AC) plate and coliforms count (EC) 3M petrifilm plate. (a) Counts of aerobic microbes in each stage, (b) Counts of coliforms in each stage. Significant differences were analyzed using ordinary one-way ANOVA and unpaired t-test. **** $p<0.0001$, *** $p<0.001$, * $p<0.05$.
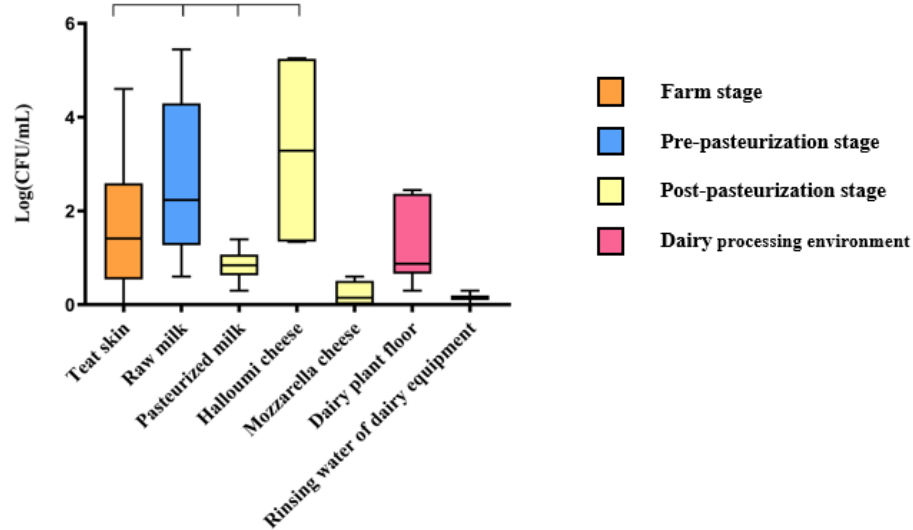
Aerobic microbes

(a)

Y-axis: Log(CFU/mL)

X-axis categories: Teat skin, Raw milk, Pasteurized milk, Halloumi cheese, Mozzarella cheese, Dairy plant floor, Rinsing water of dairy equipment

Significance markers: ****, ****, *

Coliforms

(b)

Y-axis: Log(CFU/mL)

X-axis categories: Teat skin, Raw milk, Pasteurized milk, Halloumi cheese, Mozzarella cheese, Dairy plant floor, Rinsing water of dairy equipment

Significance markers: *, ***, *

Legend:
- Farm stage
- Pre-pasteurization stage
- Post-pasteurization stage
- Dairy processing environment

3 2

Figure 3. Taxonomic composition of farm stage, pre-pasteurization stage, and post-pasteurization stage samples collected from on-farm dairy processing plants. (a) Taxonomic composition at phylum level. (c) Taxonomic composition at genus level. Only top 20 genera were shown in (c and d). Merged bar plot of taxonomic composition in farm stage, pre-pasteurization stage, and post-pasteurization stage samples (b) at the phylum level and (d) genus level. Top 15 genera in each process are shown in (e) in descending order.
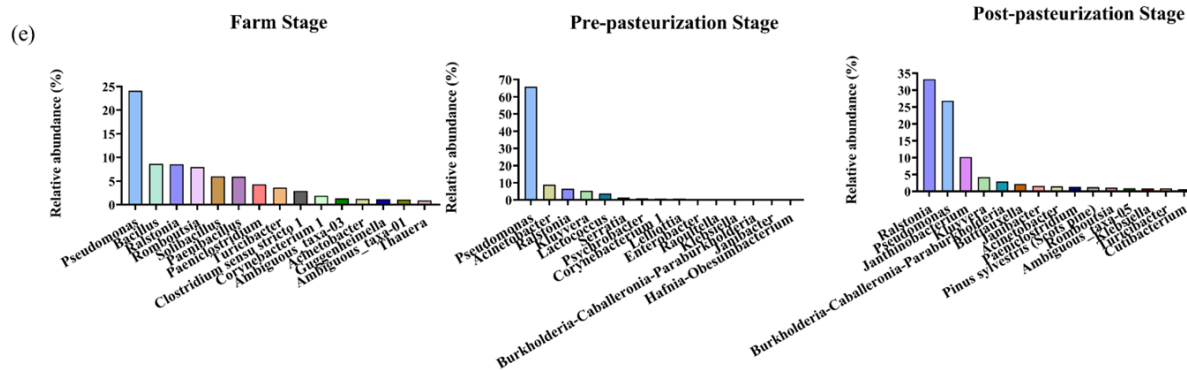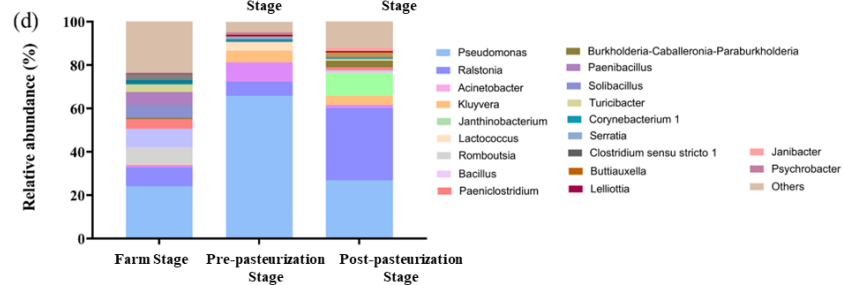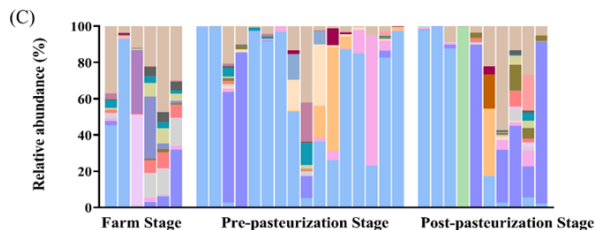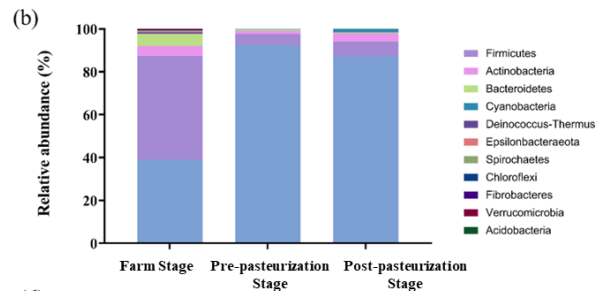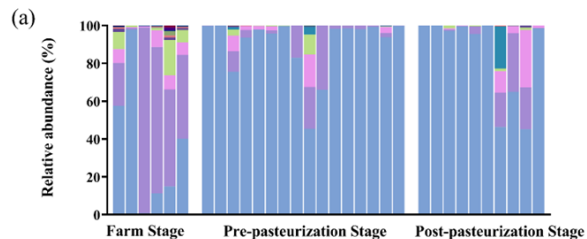
Figure 4. (a-c) Relative abundance of representative psychrotrophic bacteria (*Pseudomonas*, *Acinetobacter*, Enterobacteriaceae) in farm stage, pre-pasteurization stage, and post-pasteurization stage samples collected from on-farm dairy processing plants. (d) Genus with significant differences in relative abundance between raw milk and pasteurized milk. Nonparametric test based on unpaired Kruskal-Wallis test and Man-Whitney test was performed to analyze significant difference among relative abundance of psychrotrophic bacteria. * $p<0.05$.
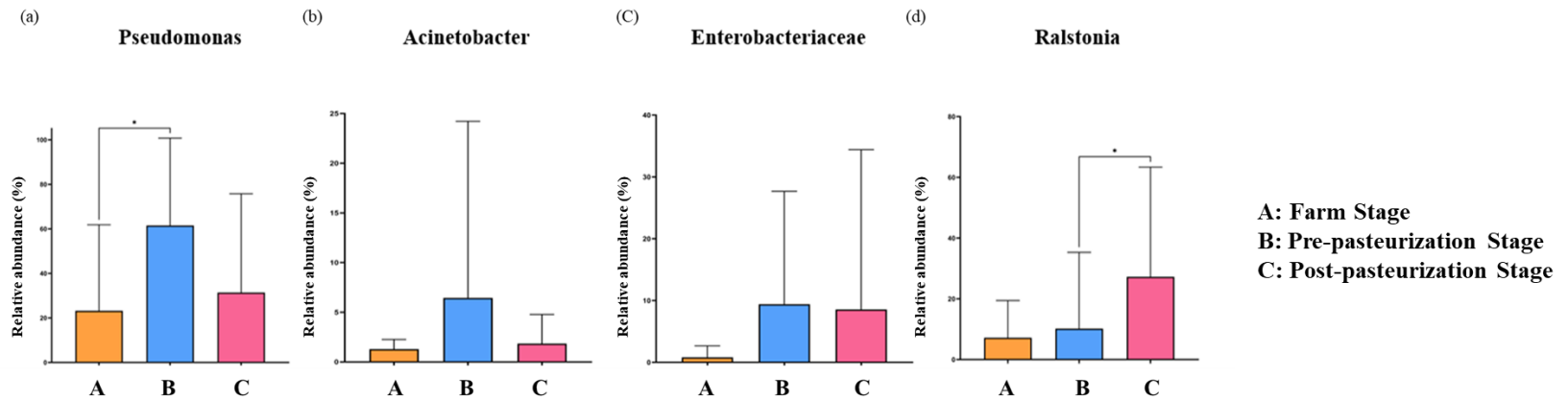
Figure 5. Diversity of farm stage, pre-pasteurization stage, and post-pasteurization stage in on-farm dairy processing plants. (a-d) shows alpha diversity measured in 4 different indices; (a) the number of observed OTUs, (b) Shannon's index, (c) Simpson's index, and (d) Chao1 index. (e) shows beta diversity of farm stage, pre-pasteurization stage, and post-pasteurization stage in on-farm dairy processing plants in Bray-Curtis principal coordinate analysis (PcoA). (*$p<0.05$, **$p<0.01$, ***$p<0.0001$)

(a) **(b)**

(c) **(d)**

(e)
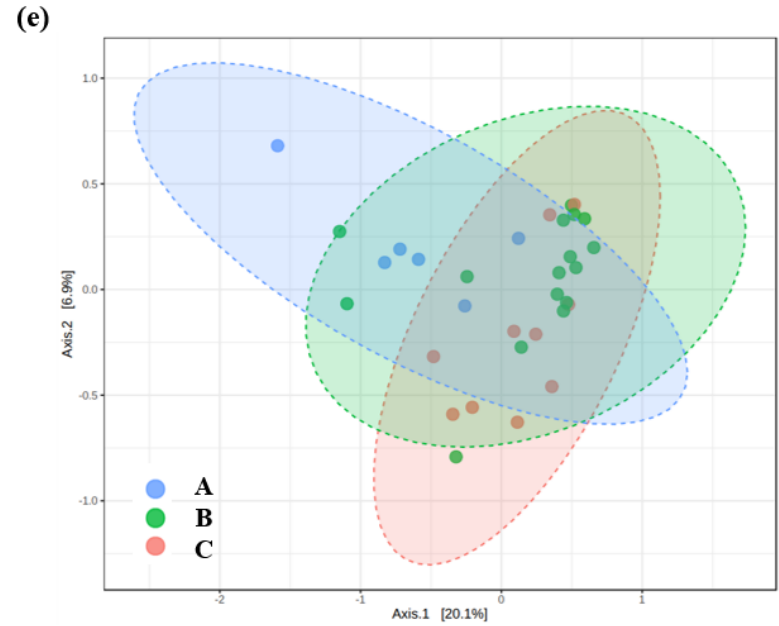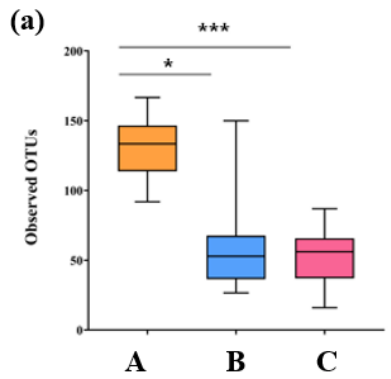
A: Farm Stage
B: Pre-pasteurization Stage
C: Post-pasteurization Stage

Figure 6. Distribution of core microbiota across the farm stage, pre-pasteurization stage, and post-pasteurization stage of on-farm dairy 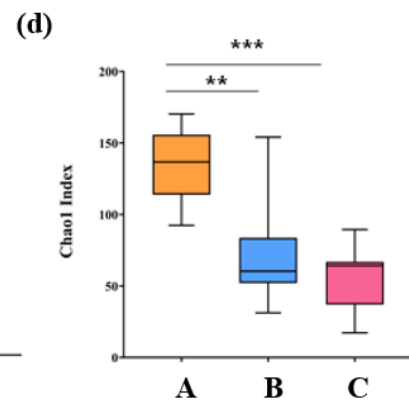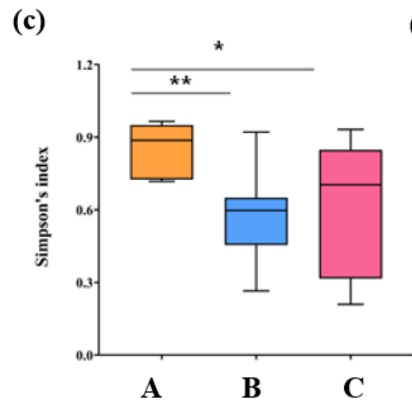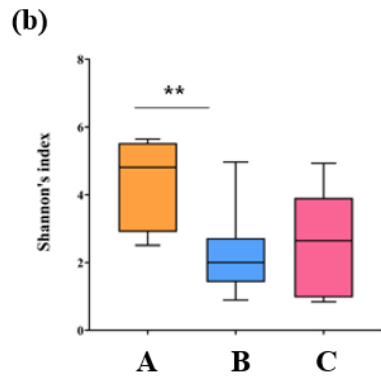processing plants. Venn diagram shows the number of shared genera in metagenome of farm stage, pre-pasteurization stage, and post-pasteurization stage. Numbers in bracket indicates the number of total genera in each stage. Blue circled part indicates the genera distributing in all stages. Red circled part indicates the genera appeared newly after pasteurization. 13 genera in red circle were listed in descending order of distribution.

Farm Stage
(126)

Geobacillus
Hydrogeneophaga
Undibacterium
Thermomonas
Diaphorobacter
Hydrogenophilus
Macellibacteroides
Curvibacter
Dermacoccus
Sphingobium
Acidovorax
Treponema
Actinotalea

Psychrotrophic
bacteria
- *Pseudomonas*
- *Acinetobacter*
- *Klebsiella*
- *Enterobacter*
⋮
Environmental
bacteria
- *Kluyvera*
- *Ralstonia*
- *Romboutsia*
⋮

13

35

4

74

5

18

9

Pre-pasteurization Stage
(132)

Post-pasteurization Stage
(105)

Figure 7. The distribution of bacterial isolates at each sampling point. Isolates were distinguished at the genus level.
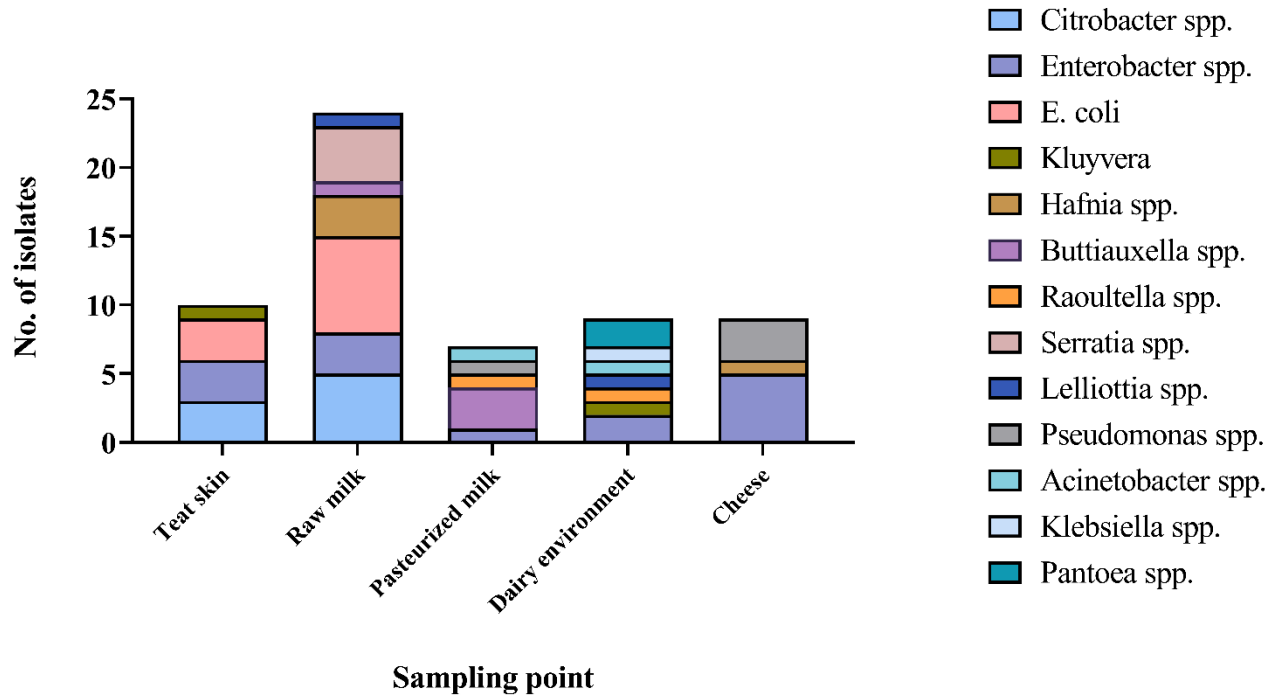
Figure 8. Shifts in antimicrobial resistance phenotype of bacterial isolates according to the stages in on-farm dairy processing plants. Interleaved bars show the antimicrobial resistance rate against the 16 antimicrobial agents used in our study at each stage in on-farm dairy processing plants. Antimicrobial agents which all bacterial isolates were susceptible are not shown on the figure (Ciprofloxacin, Imipenem, Meropenem, Nalidicic acid). The abbreviation of antimicrobials is as follow: FOX, Cefoxitin; CTX, Cefotaxime; CAZ, Ceftazidime; FEP, Cefepime; AMP, Ampicillin; AMC, Amoxicillin-clavulanic acid; TIC, Ticarcillin; TE, Tetracycline; SXT, Sulfamethoxazole-trimethoprim; CN, Gentamicin; ATM, Aztreonam

Figure 9. Antimicrobial resistance (AMR) pattern of bacterial isolates distributed at farm stage, pre-pasteurization stage, post-pasteurization stage, and dairy processing environment stage in on-farm dairy processing plants. The abbreviation of antimicrobials is as follow: FOX, Cefoxitin; CTX, Cefotaxime; CAZ, Ceftazidime; FEP, Cefepime; AMP, Ampicillin; AMC, Amoxicillin-clavulanic acid; TIC, Ticarcillin; TE, Tetracycline; SXT, Sulfamethoxazole-trimethoprim; CN, Gentamicin; ATM, Aztreonam
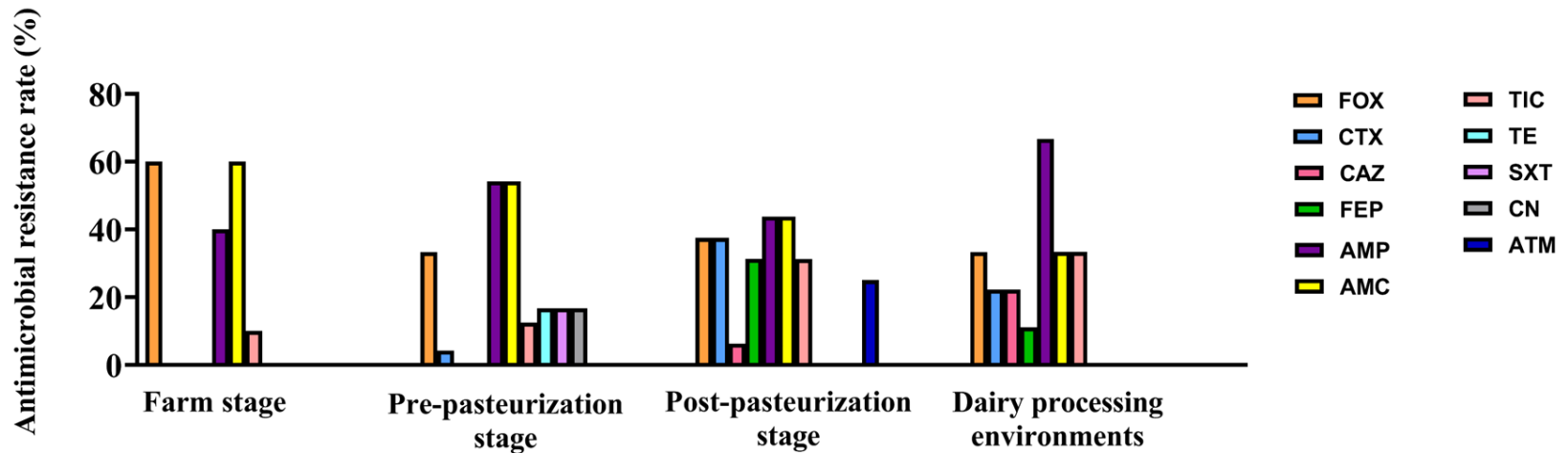
Farm A	Farm Stage (n=5)	Pre-pasteurization Stage (n=5)	Post-pasteurization Stage (n=0)	Dairy processing environments (n=0)
Spring	AMP-AMC-FOX (n=1)			
Summer	TIC (n=1)	AMC (n=1) AMP-AMC-TIC-SXT-CN-TE (n=1)		
Fall	AMP-AMC (n=1)	AMP-AMC (n=1)		
Winter	AMP-AMC-FOX (n=1)			

Farm B	Farm Stage	Pre-pasteurization Stage (n=9)	Post-pasteurization Stage (n=5)	Dairy processing environments (n=8)
Spring			AMP-AMC-FOX-CTX (n=1)	
Summer		AMP-AMC-FOX (n=1)	CAZ-FEP (n=1)	AMP-AMC-FOX (n=2) CAZ-FEP (n=1)
Fall		AMP-AMC (n=1) AMP-AMC-TIC (n=1) AMP-AMC-FOX-CTX (n=1)	AMP (n=1) AMP-AMC (n=1)	AMP-TIC (n=1) AMP-AMC-TIC (n=1) AMP-FOX-CTX (n=1)
Winter		AMP-AMC (n=1)		

Farm C	Farm Stage (n=4)	Pre-pasteurization Stage (n=5)	Post-pasteurization Stage (n=2)	Dairy processing environments (n=1)
Spring		AMP-TIC-SXT-CN-TE (n=1)	AMP-AMC-FOX-CTX (n=1)	AMP-TIC-CTX-CAZ (n=1)
Summer		AMP (n=1)		
Fall		AMP-AMC-FOX (n=1)		
Winter	AMP-AMC-FOX (n=2) AMP-TIC-FOX (n=1)	AMP-AMC-FOX (n=2)		

Farm D	Farm Stage (n=1)	Pre-pasteurization Stage (n=5)	Post-pasteurization Stage (n=9)	Dairy processing environments (n=0)
Spring			TIC-CTX-FEP-ATM (n=3)	
Summer		FOX (n=1) AMP-AMC-FOX (n=1)	AMP-TIC (n=1)	
Fall			AMC-FOX (n=1) AMP-AMC-FOX (n=1) TIC-CTX-FEP-ATM (n=1)	
Winter			AMP-AMC-FOX (n=1)	

4 3

Figure 10. Detection in presence of 27 antimicrobial resistance genes inferring resistance to β-lactams (*bla*$_{CTX-M-1}$ group, *bla*$_{CTX-M-2}$ group, *bla*$_{CTX-M-8}$ group, *bla*$_{CTX-M-9}$ group, *bla*$_{CTX-M-25}$ group, *bla*$_{CMY}$, *bla*$_{SHV}$, *bla*$_{TEM}$, and *bla*$_{OXA}$), aminoglycosides (*aac(3)-I*, *aac(3)-II*, *aac(3)-IV*, *aac(6)-Ib-cr*), tetracyclines (*tetA*, t*etB*, *tetD*), and quinolone (*qnrA*, *qnrB*, *qnrC*, *qnrS*), and sulfonamide (*dfrIa*, *dfrIb*, *dfrII*, *dfrVII*, *dfrXII*, *sul1*, *sul2*) in bacteria isolates and metagenome from dairy samples in on-farm dairy processing plants utilizing PCR. The box in white color indicates the absence of antimicrobial resistance genes and the box in blue color indicates the presence of antimicrobial resistance genes. Boxes in the same column indicate the same sample, with the boxes aligned above and below indicating the isolates and metagenome from the same samples, respectively.

Source	Farm stage															
Isolates			**CTX-M-1 SHV aac(3)-IV**								**aac(3)-II**					
Metagenomic DNA				**aac(3)-II tetA**		**SHV**	**tetA**			**SHV**	**aac(3)-II**			**aac(3)-II tetA**	**TEM**	
Source	**Pre-pasteurization stage**															
Isolates	**aac(3)-IV tetA**							**TEM**		**tetA**	**TEM**	**tetA**	**tetA**			
Metagenomic DNA	**TEM aac(3)-IV tetA**							**TEM**		**tetA**	**TEM**	**TEM tetA**				**TEM**
Source	**Post-pasteurization stage**															
Isolates																
Metagenomic DNA	**TEM aac(3)-II**		**SHV**	**TEM**	**aac(3)-II**	**aac(3)-II**	**aac(3)-II**			**aac(3)-II aac(3)-IV tetA**		**aac(3)-II**	**aac(3)-II**	**aac(3)-II**		**SHV aac(3)-II aac(3)-IV**
Source	**Dairy processing environments**															
Isolates						**CTX-M-1 SHV tetA**										**Absence**
Metagenomic DNA	**TEM**	**TEM**				**TEM SHV tetA**	**TEM**		**TEM**	**TEM**	**TEM**					**Presence**

Supplementary Tables

Supplementary Table 1. List of genera in the metagenomes of samples collected from farm stage, pre-pasteurization stage, and post-pasteurization stage on-farm dairy processing plants.

List of genera distributed only at farm stage (n=13)	List of genera distributed only at pre-pasteurization stage (n=5)	List of genera distributed only at post-pasteurization stage (n=9)
Lysinibacillus	*Serratia*	*Hydrogenophaga*
Anoxybacillus	*Salmonella*	*Undibacterium*
Fibrobacter	*Leuconostoc*	*Thermomonas*
Puniceicoccus	*Citrobacter*	*Diaphorobacter*
Aeribacillus	*Anaerococcus*	*Hydrogenophilus*
Alistipes		*Macellibacteroides*
Uncultured-09		*Macellibacteroides*
Brevibacillus		*Dermacoccus*
Lachnospiraceae NK4A136 group		*Sphingobium*
Pelagibacterium		*Acidovorax*
Bacterioides		
Ruminococcus 1		
Ambigous_taxa-02		

List of genera distributed commonly at all stages (n=74)

Ralstonia	*Lactococcus*	*Solibacillus*
Pseudomonas	*Caulobacter*	*Enterococcus*
Kluyvera	*Thauera*	*Carya cathayensis*
Burkholderia-Caballeronia-Paraburkholderia	*Raoultella*	*Facklamia*
Buttiauxella	*Halomonas*	*Ornithinicoccus*
Janibacter	*Uncultured-05*	*Uncultured Sphingobacteriia bacterium*
Acinetobacter	*Fastidiosipila*	*Marinobacterium*
Paeniclostridium	*Flavobacterium*	*Hafnia-Obesumbacterium*
Romboutsia	*Chryseobacterium*	*Cellulosilyticum*
Ambiguous_taxa-05	*Dietzia*	*Tessaracoccus*
Klebsiella	*Truepera*	*Georgenia*
Turicibacter	*Mesorhizobium*	*Corynebacterium*
Cutibacterium	*Tectona grandis*	*Family XIII AD3011 group*
Staphylococcus	*Christensenellaceae R-7 group*	*Proteiniclasticum*
Lelliottia	*Stenotrophomonas*	*Uncultured bacterium-02*
Corynebacterium 1	*Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium*	*Fermentimonas*
Clostridium sensu stricto 1	*Tetrasphaera*	*Lachnospiraceae NK3A20 group*
Bradyrhizobium	*Paracoccus*	*Petrimonas*
Pelomonas	*Uncultured bacterium-04*	*Aerococcus*
Bacillus	*Sediminibacterium*	*Psychrobacter*
Guggenheimella	*Variovorax*	*Uncultured-03*
Streptococcus	*W5053*	*Enterobacter*
Ambiguous_taxa-01	*Uncultured-08*	*Atopostipes*
Solanum melongena (eggplant)	*Uncultured-10*	*Pseudaminobacter*
Rhodococcus	*Escherichia-Shigella*	

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국문 초록

메타지노믹스를 활용한 목장형 유가공장에서의 미생물 군집 및 항생제 내성 특성 연구

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목장형 유가공장은 농장과 유가공장이 가까이 위치해 있기 때문에 농장 및 유가공장 환경 위생이 유가공품 가공 과정에 영향을 줄 수 있다. 이러한 목장형 유가공장은 주로 소규모로 운영되고 저온살균 방법을 통해 원유를 가공한다. 이와 같은 특징 때문에 직·간접적인 접촉을 통해 농장에서 유가공장으로 미생물 유입이 일어날 수 있으며 이는 유가공품의 품질 저하에 영향을 미칠 수 있다. 특히 젖소 농장 및 유가공장에서 항생제 내성균이 보고되고 있으며 이는 유가공품에 유입 시 유가공품 섭취를 통해 인체에 전달될 가능성이 있다. 본 연구는 목장형 유가공장의 농장 단계, 살균 전 단계, 살균 후 단계, 유가공장 환경 단계에서의 미생물 분포와 미생물 군집의 항생제 내성을 조사하고자 하였다. 미생물 분포는 지표세균 오염의 정량화 및 메타지놈 분석을 통해 확인하였다. 또한, 목장형 유가공장의 각 단계 샘플에서 분리한 세균의 다양한 항생제에 대한 내성 표현형을 확인하고 분리주와 메타지놈에서의 항생제 내

성 유전자 분포를 확인하여 목장형 유가공장의 미생물 군집에서의 항생제 내성균 분포를 파악하였다.

배양 의존적 방법에 의한 분석 결과 지표세균은 목장형 유가공장의 전 단계에 분포하고 있었다. 호기성 세균은 농장 단계에서 0.70 ~ 5.90 log CFU/mL, 살균 전 단계에 2.17~8.89 log CFU/mL가 분포하고 있었으며 살균 후에는 0.20~3.97 log CFU/mL로 유의하게 감소하였다. 대장균군의 경우 농장 단계에 0.30 ~4.60 log CFU/mL, 살균 전 단계에 0.60 ~ 5.39 log CFU/mL가 분포하고 있었으며 살균 후에 0.40~0.90 log CFU/mL로 유의하게 감소했다. 하지만 최종 유가공품에서 호기성 세균과 대장균군의 오염도가 각각 0.18~8.54 log CFU/mL, 0.18~5.23 log CFU/mL로 증가하였고 이는 살균 후 단계에서 유가공장 환경과의 교차오염이 일어나고 있을 가능성을 의미한다.

메타지놈 분석을 기반으로 한 배양 비의존적 방법에 의한 분석에서는 대표적인 저온성 세균으로 알려진 *Pseudomonas*의 상대적 분포가 농장 단계 (24.1%) 와 살균 전 단계 (65.9%)에서 모두 우세하게 확인되었는데 이는 목장형 유가공장에서 농장에서 유가공장으로 미생물 유입이 일어날 수 있음을 의미한다. 살균 후에도 *Pseudomonas*를 포함한 다른 저온성 세균 (*Acinetobacter*, Enterobacteriaceae)의 분포가 여전히 우세하였고 이는 이들이 살균에 저항하여 생존하였을 가능성을 나타낸다. 핵심 미생물군 분석에서는 농장 단계에서 126개, 살균 전 단계에서 132개, 살균 후 단계에서 105개의 속이 확인되었는데 이 중 74개의 속이 목장형 유가공장 전 단계에 공통적으로 분포하고 있었으며

13개 속이 저온성 세균에 해당하였다. 목장형 유가공장의 각 단계 샘플에서 총 59 균주가 분리되었으며 이 중 *Pseudomonas spp.*, *Acinetobacter spp.*, and *Enterobacter* spp., *Citrobacter* spp., *Kluyvera* spp., *Hafnia* spp., *Buttiauxella* spp., *Raoultella* spp., *Serratia* spp., *Lelliottia* spp., *Klebsiella* spp., *Pantoea* spp.를 포함한 49 균주가 저온성 세균으로 확인되었다. 항생제 내성 분석 결과, 59 균주 중 44 균주 (74.6%)가 한 개 이상의 항생제에 내성 표현형을 보였다. FOX, AMP, AMC, TIC에 내성을 지니는 균주는 목장형 유가공장의 전 단계에 분포하고 있었다. 특히 같은 항생제 내성 패턴을 지니는 균주가 같은 농장, 같은 계절의 연속적인 단계에 분포하는 것이 확인되었다. 나아가 59균주 중 16 균주는 (27.1%) 플라스미드를 매개로 전달되는 항생제 내성 유전자 ($bla_{CTX-M-1}$, bla_{SHV}, bla_{TEM}, *aac(3)-II*, *aac(3)-IV*, and *tet A*)를 지니고 있는 것이 확인되었으며 이는 이들이 잠재적으로 다른 세균에 항생제 내성 유전자를 전달할 가능성이 있음을 의미한다. 항생제 내성 유전자를 보유하는 균주가 분리되지 않은 샘플의 메타지놈에서도 해당 유전자가 검출되었고 이를 통해 분리되지 않은 균도 항생제 내성 유전자를 보유하는 것을 확인하였다. 이러한 항생제 내성 유전자의 분포는 목장형 유가공장의 전 단계에서 모두 확인되었다. 이러한 연구 결과는 목장형 유가공장 전체에 항생제 내성 저온성 세균이 지속적으로 분포 및 확산될 가능성이 있고 잠재적으로 최종 유가공품에 유입되어 인간 건강에 위협이 될 수 있음을 시사한다. 또한, 본 연구 결과는 항생제 내성 저온성 세균의 분포를 파악하는데 분리·배양법과 유전자 검색에 의한 분석법의 적용에 대한 중요성을 제시하였다.