Microbiological analysis of commercial calf milk replacer and antibiotic resistance in isolated *Enterococcus* spp.

ABSTRACT

One of the reasons why calf milk replacer is preferred over unpasteurized bulk tank milk or waste milk with antibiotics on farms is that it prevents epidemic diseases and antibiotic resistance that may occur on the farm. In this study analyzed commercial calf milk replacer products (n = 12) obtained from dairy farms around Türkiye by microbiological culture and polymerase chain reaction (PCR). In order to evaluate the microbiological quality of calf milk replacer, total bacteria count, coliform *E. coli* and *E. coli* O157-H7, *Salmonella* spp., *Staphylococcus* spp., *Streptococcus* spp., *Enterococcus* spp. analyses were performed according to microbiological analysis methods determined according to ISO standards. *Enterococcus* spp. was isolated from all 12 calf milk replacer samples analyzed and molecularly confirmed by PCR with the presence of the gross-Es gene. *Salmonella* spp., *E. coli*, *Staphylococcus* spp. and *Streptococcus* spp. were not isolated from the samples. Additionally, in the bacterial counts, an average of $5.3x10^7$ Enterococci were counted from all samples in 1 g of calf milk replacer. Antimicrobial analysis of the isolated bacteria was completed according to CLSI 2022 data, and 11 isolates were defined as multi drug resistance, and one isolate was defined as extensive drug resistance. It was also determined that the isolate defined as extensive drug resistance was resistant to Vancomycin and carried the Van A resistance gene. Many proteins used in the preparation of calf milk replacers are of animal origin and may contain pathogenic bacteria. It is known that milk replacers affect microbiota. It was shown in this study that if calf milk replacers are not prepared under the regulations, they may cause harm rather than benefit to on-farm biosecurity factors. It is concerning that calves are given calf milk replacers containing antibiotic-resistant *Enterococcus* spp. to sustain their lives when they are most vulnerable to disease during the window of susceptibility. When using calf milk replacer in calf feeding, veterinarians should be informed about the microbiological certification of the product and provide information about pasteurization and presentation for consumption.

Keywords: Antibiotic resistance, calf milk replacer, vancomycin resistance Enterococci

INTRODUCTION

Calves can only digest milk or protein in liquid form. A study conducted by USDA 2014 determined that 63.9% of medium-sized dairy cattle farms fed calves with milk replacers (USDA, 2016). Calf milk replacers are usually prepared from whole milk powder, skim milk powder, industrial residues such as casein, whey, and whey protein, plant oil, animal plasma, soybean protein, essential amino acids Lecithin, L-Lysine, DL-Methionine, vitamin and mineral supplements (USDA, 2018). Dairy calves are fed milk replacer until weaning time, especially for economic reasons (when milk is more expensive than milk replacer) (Wood, 2022). Also, farms can use milk replacers in calf feeding to protect against pathogens such as *Salmonella* spp., *Mycoplasma* spp., *Brucella abortus*, *Mycobacterium avium* ssp. *Paratuberculosis* and bovine viral diarrhea virus (BVDV), which are shed with milk (Maunsell

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Research Article

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and Donovan, 2008). *E. coli* and *Enterococcus* spp. found in the flora of the gastrointestinal tract of cattle are opportunistic pathogens (Silva et al., 2012). Especially in bulk-tank milk with high fecal contamination, these agents are more likely to cause infection in calves (Aust et al., 2013). Enterococci are considered non-infectious, however can cause nosocomial infections in humans due to the development of antibiotic resistance mechanisms (Ben Braiek and Smaoui 2019).

Before being placed on the market, milk replacer products must document the results of the microbiological analysis in terms of pathogenic bacteria, and total mesophilic bacteria count, and coliforms (Scheid, 2012). However, the animal-derived products contained in milk replacers also bring a bacterial load (Cooper and Watson, 2013). Soy-derived products, whey, and animal feed containing animal-derived proteins contaminated with *Salmonella* spp. may cause disease in animals that consume the feed (McGuirk, 2008). Calf milk replacers not prepared under the regulations may pose a biosafety threat. Calves are sensitive to pathogens close to the weaning period because of the immunological perspective called the window of susceptibility (Chase et al., 2008). For this reason, some products use antibiotic calf milk replacer in specific proportions, but this solution comes with antibiotic resistance (Langford et al., 2003).

The study aims to investigate microbiological analysis and the antimicrobial resistance of bacteria isolated from commercially available milk replacers in Türkiye.

MATERIALS AND METHODS

Microbiological analysis

Twelve different calf milk replacers available in the market in Türkiye were collected, and the products were numbered 1-12 due to commercial concerns. Calf milk replacer samples were collected according to the sample collection procedures described in ISO 6579-1:2017 (ISO, 2017). Twenty-five g of calf milk replacer was enriched in 225 mL peptone water (CM1049, Lab M) at 37°C for 18 h. As a result of pre-enrichment, 1 mL of enriched culture was passaged into 9 mL of Rappaport Vassiliadis Soy Broth (HP007, LabM). It was incubated at 42°C for 24 h. On the third day, a loopful of this culture was passaged onto XLT-4 agar (CM1061, Thermo Scientific), incubated at 37°C for 24 h and black colonies were tried to be identified (Hadimli et al., 2017). The enriched culture was also passaged onto blood agar for general mesophilic pathogenic bacteria analysis and incubated for 48 hours at 5% CO_2 (Jinneman, 1998). The number of coliform bacteria was detected according to ISO 4832:2013 guidelines (ISO 4832, 2013). MacConkey agar (70143, Sigma Aldrich), Eosin-Methylene Blue agar (70186, Sigma Aldrich) were used for the detection of coliform bacteria and sorbitol MacConkey agar for the detection of *E. coli* O157-H7. Tryptic Soy Agar %5 sheep blood (22091, Sigma Aldrich) was used determine the bacterial load in the gram calves milk replacer, which was determined using the 10-fold dilution method according to ISO 4833-1:2013 guideline (ISO, 2013). Modified Edwards medium (CM0027B, Thermo Scientific) was used to detect *Enterococcus* spp. The isolates were differentiated from *Streptococcus* spp. by the bile-esculin test (Aun et al., 2021).

Antimicrobial susceptibility test

The antibiotic susceptibility of the isolates was determined by the Kirby-Bauer disk diffusion method (Bauer et al., 1966). For the antimicrobial susceptibility test, antimicrobial agents were selected for gastrointestinal system infections of calves. Antibiotic disc used in this study list with abbreviation: VA: Vancomycin, S: Streptomycin, P: Penicillin, AMC: Amoxacillin, RA Rifampicin, L: Lincomycin, TE: Tetracycline, IPM: Imipenem, SXT: Trimethoprim / sulfamethoxazole, CN: Gentamicin, ENR: Enrofloxacin, E:

Erythromycin. Zone diameters were recorded in mm, and antibiotic resistance was determined according to the clinical breaking point reference values prepared for veterinary isolates by the Clinical and Laboratory Standards Institute (CLSI, 2022).

Identification of Enterococcus and AMR with molecular methods

DNA was isolated from the *Enterococcus* spp., according to the Wizard® Genomic DNA Purification Kit Gram-Positive bacteria protocol. The concentrations of the isolated DNA were determined by spectrophotometer (Nanodrop 2000, Thermo Scientific). All *Enterococcus* isolates were PCR confirmed with the forward primer ACAGTTGTTGCAGTCGGTGA and the reverse primer ACATAACTTGGTCGCCTGCT, which was prepared to specifically give an 85 bp PCR product according to the NCBI accession number AY328542 1 for the groES gene. In addition, isolates were screened by PCR for the presence of the vancomycin resistance gene van A (667 Bp), CP036247 1, and NCBI accession number forward primer GTTGCAATACTGTTTGGGGGT reverse primer CAACTAACGCGGCACTGTTT. The primer sets were designed by NCBI primer blast for this study. For the PCR mixture, five µL master mix (5x) (Solis Biodyne, Estonia), one µL forward primer from 10 µM working stock, one µL reverse primer from 10 µM working stock, four µL DNA (25 ng/ µL) 15.9 µL sterile nuclease-free water were added for a total volume of 25 µL. The thermal cycle (T100, Bio-Rad) was repeated 34 times with a pre-denaturation step at 94°C for 10 min, followed by 94°C denaturation for 1 min, 60°C binding for 1 min, 72°C extension for 1 min, and final extension at 72°C for 10 min. A 1% agarose gel was prepared for electrophoresis (maxicell-minicell, EC Apparatus Corporation) of PCR products. Ethidium bromide was added to the gel to a final concentration of 0.5 µg/ mL. Gel wells were loaded with five µL each of PCR products and 100 bp DNA ladder (Solis Biodyne, Estonia). The results were visualized by a gel imaging device (212 Pro, Gel-Logic).

RESULTS

The isolates were negative for *Salmonella* spp., coliform *E. coli*, and *E. coli* O157-H7. However, as a result of selective media analysis and identification, *Enterococcus* spp. was isolated from all milk replacer samples. In the total bacterial count analysis, a calf average of 5.3 x 10^7 CFU *Enterococcus* was counted in 1 gram of calf milk replacer (Table 1).

Table 1. Bacteria isolated from calf milk replacers, total number of bacteria per gram

Calf Milk Replacer Product Number	Isolated bacteria	gr/CFU
1	*Enterococcus* spp.	$7x10^7$
2	*Enterococcus* spp.	$4.2x10^7$
3	*Enterococcus* spp.	$3.6x10^7$
4	*Enterococcus* spp.	$8.2\ x10^7$
5	*Enterococcus* spp.	$4.2\ x10^7$
6	*Enterococcus* spp.	$5x10^7$
7	*Enterococcus* spp.	$3x10^7$
8	*Enterococcus* spp.	$7.6x10^7$
9	*Enterococcus* spp.	$6x10^7$
10	*Enterococcus* spp.	$4.7x10^7$
11	*Enterococcus* spp.	$5x10^7$
12	*Enterococcus* spp.	$5.5x10^7$

According to the antimicrobial susceptibility test result, vancomycin resistance was detected in one isolate. One of the isolates (sample 12) was detected as extensive drug (XDR) resistance, and 11 were seen as multidrug resistance (MDR). Although the isolates were found to be sensitive to penicillin and enrofloxacin, imipenem, rifampicin, and vancomycin (only isolate number 12), which can be used in emergencies for humans, were resistant (Table 2). The isolates were confirmed to be *Enterococcus* spp. molecularly by PCR in terms of the presence of the Gros-ES gene Figure 1.

Table 2. Antimicrobial susceptibility test results of *Enterococcus* spp. isolated from calf milk replacers

Calf Milk Replacer Product Number	VA-30	S-10	CN-30	AMC-30	P-10	E-15	TE-30	RA-5	IPM-10	L-2	SXT-25	ENR-10
1	S	R	R	R	S	R	R	R	R	R	R	S
2	S	R	R	R	S	I	R	R	R	R	R	S
3	S	R	R	R	S	I	R	R	R	R	R	S
4	S	R	R	R	S	I	R	R	R	R	R	S
5	S	R	R	R	S	I	R	R	R	R	R	S
6	S	R	R	R	S	I	R	R	R	R	R	S
7	S	R	R	R	S	S	R	R	R	R	R	S
8	S	R	R	R	S	R	R	R	R	R	R	S
9	S	R	R	R	S	R	R	R	R	R	R	S
10	S	R	R	R	S	R	R	R	R	R	R	S
11	S	R	R	R	S	I	R	R	R	R	R	S
12	R	R	R	R	S	R	R	R	R	R	R	S

VA: Vancomycin, S: Streptomycin, CN: Gentamicin, AMC: Amoxacillin, P: Penicillin, E: Erythromycin, TE: Tetracycline, RA: Rifampicin, IPM: Imipenem, L: Lincomycin, SXT: Trimethoprim/sulfamethoxazole, ENR: Enrofloxacin.

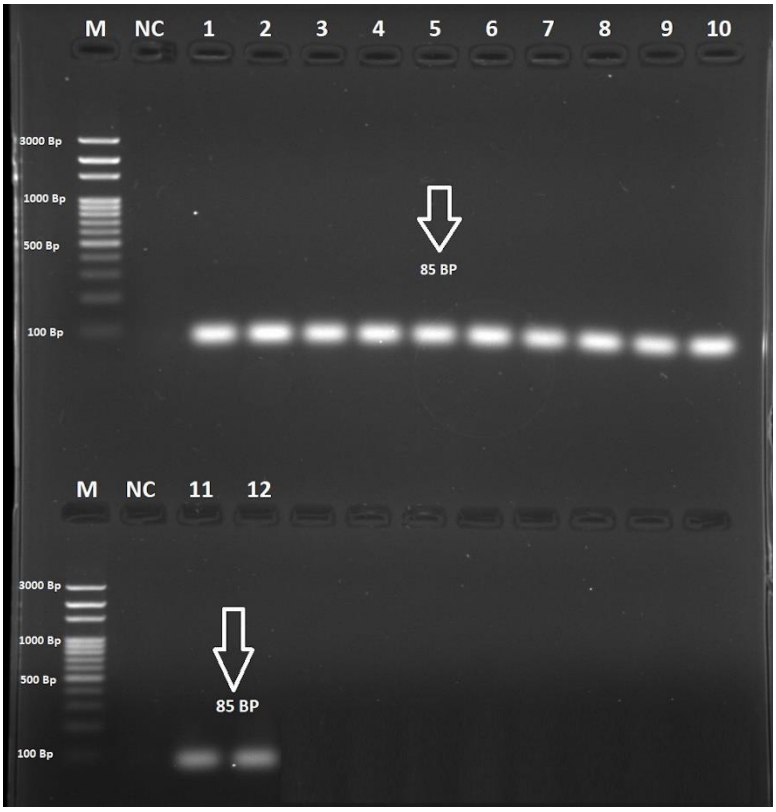

Figure 1. PCR gel image of Enterococcus spp. isolated from calf milk replacers in terms of Gros-ES gene. M: Marker (100 bp), NC: Negative control, Line 1-12: Samples.

The isolates were also screened by PCR for the Vancomycin resistance gene Van A, which is one of the most critical parameters for Enterococcus, and the presence of this resistance gene was detected only in isolate number 12 (Figure 2).

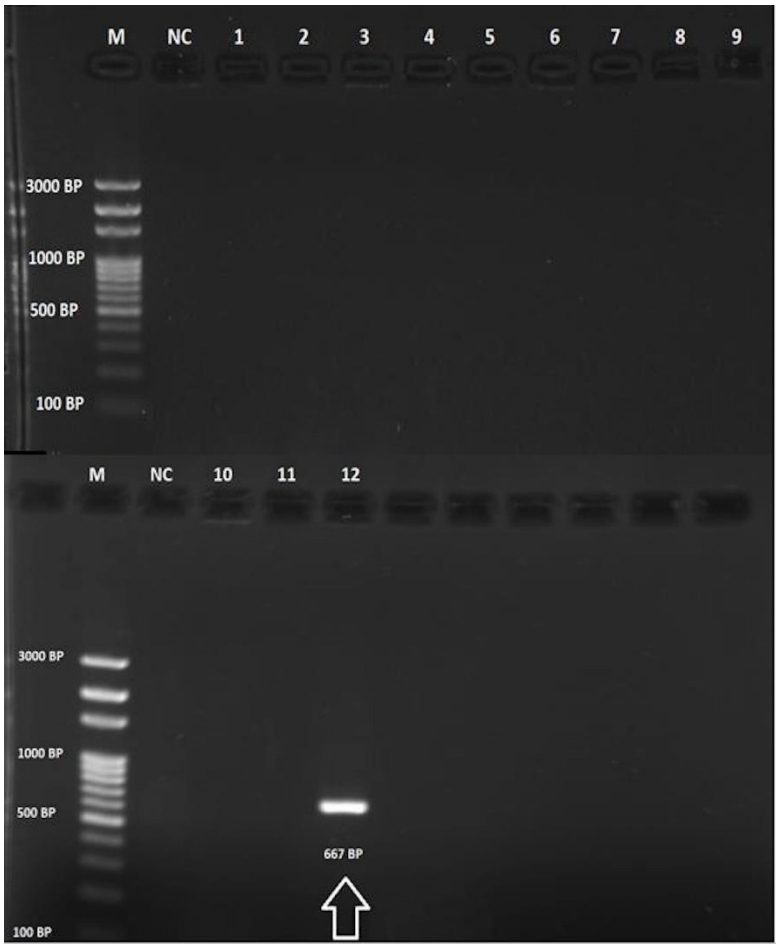

Figure 2. PCR gel image for the Van A gene. M: Marker (100 bp), NC: Negative control, Line 1-12: Samples.

Enterococcus spp. obtained from calf milk replacer number 12 was found to be resistant to vancomycin both with its antimicrobial susceptibility and molecular Van A gene.

DISCUSSION

According to USDA regulations, calf milk replacer must be negative for *Salmonella* (Scheid, 2012). In the study hypothesis, the main isolated was thought to be *Salmonella* because it is known to be abundant in soy-based products, whey. However, in the study, no *Salmonella* was isolated from the calf milk replacers found in the market. In another study conducted in the USA in 2014, *Salmonella* was detected in 5.5% of 55 milk powders and dried whey facilities (Hayman et al., 2020). In another research conducted due to *Salmonella* cases in salmonella-free pig farms in Sweden, 28 different plant-derived *Salmonella* were isolated in samples taken from feed made from imported soybeans in pig feed products (Wierup and Häggblom, 2010).

Enterococcus spp. was isolated from all samples in the study. Although it does not act as the primary pathogen and can be found in the flora, it is thought that it has the power to change the intestinal microbiota and may cause diarrhea and productivity losses. A study investigating how the fecal microbiota of postpartum calves changes within the first week reported that *Enterococcus* spp. was isolated in

the flora starting from the first 6 hours of the birth of a healthy calf (Schwaiger et al., 2020). In microbiota studies conducted on newborn calves, it has been reported that calf milk replacer allows more microflora development compared to the cow milk diet group due to its prebiotic effect and nutritional diversity (Badman et al., 2019; Kumar et al., 2021). Although it does not act as the primary pathogen and can be found in the flora, it is thought that it has the power to change the intestinal microbiota and may cause diarrhea and productivity losses. The slightest change in the GUT microbiota affects the entire flora. In the other study, an increase in the number of *E. coli, Enterococcus spp.*, and a decrease in the number of other beneficial bacteria were detected, depending on the type of antibiotic used (Amin and Seifert, 2021).

Although it is not known how *Enterococcus* spp. contaminates the calf milk replacer, it is thought that it may originate from residues of dairy products such as whey. In their studies, other researchers isolated *Enterococcus* spp. from dairy products and whey (de Sousa et al., 2020; Muguerza et al., 2006). Another study reported that it is included in the starter culture for many cheeses, such as Mozzarella, and can be found in large amounts in whey because it is a thermo-resistant structure (Giraffa, 2003). It was thought that the reason why the number of *Enterococcus* spp. isolated in the study was so high that it was related to the method of obtaining this cheese and whey.

It is a known fact that the multitude of antibiotics used in farm animals indirectly threatens antibiotic resistance in humans. For economic reasons, some farms offer waste antibiotic milk to calves. However, this situation brings with it antibiotic resistance. In a study conducted on 114 calves, it was determined that antibiotic resistance increased in the *E. coli* shed by the feces of calves fed with antibiotic waste milk for 52 days (Aust et al., 2013). To prevent this, farms are recommended to use calf milk replacer (Firth et al., 2021). However, the antibiotic resistance of Enterococcus bacteria isolated from calf milk replacer in the study is thought-provoking. To date, there are nine different types of vancomycin resistance in enterococci. Among these, the three most common variants are types of van A, B and C. Vancomycin is an antimicrobial effective against most Gram-positive bacteria (Cetinkaya et al., 2000). It is considered a 'drug of last resort' and is classified as critically important to human medicine. Therefore, the presence of this resistance gene in enterococcus is considered alarming, and there are many studies on it in farm animals (Nilsson, 2012). Although Enterococci seems to be a commensal agent, its presence in calf milk replacer should be reconsidered by the authorities when considered together with antibiotic resistance.

CONCLUSION

Although *Enterococcus* spp. can be found in the flora of newborn calves, it is thought that it may have a pathogenic effect by changing the microflora structure when given to calves in high numbers under inappropriate conditions. Also, as a result of the study conducted, it was observed that calf milk replacers may pose a biosecurity and antimicrobial resistance threat. It is recommended to use this type of calf milk replacer by pasteurizing it before feeding the calf. In addition to such products, additional probiotic supplements can be recommended to encourage bacterial competition and exclusion for proper rumen and microbiota development.

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