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Characterizing Lactic Acid Bacteria and Thermotolerant Coliforms in Spoiled Dairy Products from Bosnia and Herzegovina

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ABSTRACT

Introduction: The rising foodborne disease outbreaks poses significant challenges to key objectives in food microbiology. This trend is primarily attributed to global population growth and intensified food production. A thorough microbiological assessment of end products is therefore crucial. **Methods:** We evaluated the bacterial presence and abundance in various dairy products (sour cream, cottage cheese, buttercream, cream cheese, pasteurized milk, protein-rich milk, and yogurt) sourced from a local supermarket in Bosnia and Herzegovina. Two enumeration methods (pour plating and most probable number) were employed alongside morphological, biochemical, and molecular analyses (Gram staining, oxidase test, catalase test, indole test, lipolytic activity assay, and RT-qPCR). Our focus was on spoilage-causing lactic acid bacteria (LAB), hygiene indicator thermotolerant coliforms (TC), and the foodborne pathogen *Salmonella* spp. **Results:** Six out of seven dairy products harbored high levels of LAB, suggesting potential spoilage, with the exception of cottage cheese. Additionally, both TC and *Escherichia coli* exceeded acceptable microbial limits, particularly in pasteurized milk. Furthermore, initial tests detected presumptive *Salmonella* spp. in cream cheese, protein-rich milk, and yogurt. **Conclusion:** These results highlight the need for stringent sanitary practices during dairy production to extend product shelf-life and prevent premature spoilage from unwanted bacterial presence. Moreover, eliminating pathogen contamination during manufacturing is crucial to mitigate serious food safety risks, including potential food poisoning.

INTRODUCTION

Microbial examination is pivotal in food microbiology, addressing safety and quality concerns. This broad field leverages tools from molecular biology, genetics, biostatistics, and legal regulations to, for example, identify spoilage microorganisms, detect foodborne pathogens, and develop predictive models for shelf-life. Despite its diverse applications, the core objective of food microbiology remains unchanged: ensuring consumer safety. Ensuring food safety amidst rising global population presents a significant challenge. The escalating demand for food necessitates increased production, potentially compromising rigorous safety protocols. In this context, the need for an expedited food production to meet the requirements of a growing population may lead to reduced focus on safety of the end product [1-5].

Bacteria play diverse roles in food, both beneficial (*e.g.*, fermentation) and harmful (*e.g.*, spoilage) [6, 8]. Careful monitoring and identification of these microorganisms are crucial, as some can significantly impact food quality and even pose health risks [9]. Spoilage-causing bacteria indicate potential issues in the manufacturing process, while indicator species, belonging to the same family as known pathogens, can signal potential contamination.

Recent research has identified connections between different types of bacteria involved in food spoilage, including spoilage-causing bacteria like lactic acid bacteria (LAB), pathogenic indicator bacteria, and general quality indicators [10]. LAB can spoil food by fermenting carbohydrates, leading to undesirable changes in smell, texture, and color. These spoilage bacteria can serve as

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indicators of manufacturing quality, alongside specific pathogenic indicator organisms [3].

Thermotolerant coliforms, often referred to fecal coliforms, belong to the *Enterobacteriaceae* family. Their presence in food serves as a valuable indicator of hygiene practices during processing. Detecting fecal coliforms suggests potential fecal contamination and raises concerns about sanitary conditions, potentially increasing the risk of foodborne illness outbreaks. The presence of thermotolerant coliforms in food may also indicate the presence of specific pathogens like *E. coli*, as they are a subgroup of thermotolerant coliforms. Furthermore, this may suggest the presence of potentially pathogenic bacteria, increasing the risk of foodborne illness and potentially leading to severe human diseases such as diarrheal illness and hemolytic uremic syndrome [8, 10].

This study hypothesizes that elevated levels of LAB and thermotolerant coliforms TC in dairy products might suggest suboptimal sanitary conditions and raise concerns about potential contributions to foodborne outbreaks [11].

This study investigates the microbial communities of dairy, specifically targeting spoilage-causing lactic acid bacteria and indicator thermotolerant coliforms, alongside the presence of the foodborne pathogen *Salmonella* spp. (an index organism from *Enterobacteriaceae*). By quantitatively and qualitatively analyzing these organisms, we aim to gain valuable insights into the safety and quality of dairy products and identify potential shortcomings in associated manufacturing processes.

MATERIAL AND METHODS

Preparation of samples for analysis

Acquisition and transport of the samples. Dairy products constitute a valuable source for food safety investigations due to their consumption by diverse population groups, ranging from infants to elderly, necessitating careful monitoring of consumer safety. As known harborers of LAB, dairy products also offer advantages in terms of sample homogenization. Seven different commercially available dairy product brands were chosen from a single supermarket chain in Sarajevo city. These included: pasteurized milk, sour cream, protein milk, cottage cheese, buttercream, yogurt, and cream cheese. For each product type, five individual products from the same batch were obtained. Products were transported to the laboratory within 15 min using insulated coolers or refrigerated transport boxes equipped with ice packs (-20 °C) or equivalent cooling agents maintaining temperatures below 5 °C to ensure appropriate temperature control. Upon arrival at the lab, samples were immediately transferred to a 4 °C refrigerator. On the product expiry date as labeled on the packaging, samples were analyzed.

Preparation and homogenization of sample for analysis. For analysis, 10 g of sample weight was used for cream-like products (sour cream, cottage cheese, buttercream, cream cheese) and 10 ml of sample volume was used for liquid products (pasteurized milk, protein milk, and yogurt) in each case. Samples were homogenized to ensure a uniform suspension of microorganisms before further analysis [12]. The homogenization procedure depended on the characteristics of the product, including liquid products such as milk, yogurt, and protein milk, as well as cream-like products like sour cream, cottage cheese, cream cheese, and buttercream. For liquid products, homogenization was achieved by vigorous shaking with sterile diluent for a standardized duration of 10-15 strokes. For cream-like products, samples were thoroughly scraped from the bottom of the package using a sterile spoon and then mixed using continuous figure-eight stirring motions for 5 min.

Initial dilution procedure. After homogenization, 10 g (or 10 mL) of sample was transferred aseptically into sterile containers using sterile pipettes for liquid products and sterile spoons for cream-like products. The initial dilution was prepared by homogenizing 10 g or mL of sample in 90 mL of BPW, which was the starting point. Following the addition of diluent, the sample was homogenized again by vigorous shaking for 2-3 min. For samples where high microbial loads were anticipated, a subsequent serial dilution series was prepared (10^{-1}, 10^{-2}, and10^{-3}). [13]. Following dilution, flasks were incubated overnight in an orbital shaker at 37 °C and 170 rpm under microaerophilic conditions (less than 21% oxygen) to facilitate sufficient growth and even distribution of the targeted microbial groups for further analysis.

Growth media. To enumerate LAB, complex medium de Man Rogosa & Sharpe (MRS) was used (Sigma Aldrich, St. Louis, MO, USA), Lauryl Sulphate (LST) broth for coliforms (tryptose 40g, lactose 10g, sodium chloride 10g, sodium lauryl sulfate 0.2 g, dipotassium hydrogen phosphate 5.5g, potassium dihydrogen phosphate 5.5g, Milli-Q water 1X), *E. coli* (EC) broth (tryptose 20g, potassium phosphate monobasic 1.5g, dipotassium hydrogen phosphate 4g, lactose 5g, sodium chloride 5g, bile salts 1.5g, reagent-grade water 1l) for thermotolerant coliforms, and Tryptone Broth for *E. coli* (tryptone 10g, sodium chloride 5g, reagent-grade water 1l) [14, 15]. For lipolytic activity test, agar plates containing Tween 20 and Tween 80 were used (peptone 10g, sodium chloride 5g, calcium chloride 0.1g, agar 15g, reagent-grade water 900 ml, supplemented with 1% Tween 20 or Tween 80) [16].

Enumeration of bacteria. This study required an accurate method to quantify viable bacteria per gram of food sample. Numerous viable count techniques exist, each with advantages and limitations depending on the specific objectives of the study (*e.g.*, targeting specific microbes, quantitative *vs*. qualitative assessment). Factors influencing method selection include microorganism type (aerobic, anaerobic), and the properties of culture media used (enrichment, selective, differential-selective) [17,

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19]. This study employed two enumeration techniques: the pour plate and the most probable number (MNP). For LAB enumeration, the pour plate technique was used. An aliquot of 0.1 mL from each dilution was pipetted onto sterile Petri dishes. The plates were then poured with MRS agar and allowed to solidify. To create microaerophilic conditions for LAB, a second thin layer (2-3 mm) of MRS agar was carefully poured over the first layer after solidification. Plates were incubated at 37 °C for 48 h. Following incubation, only plates containing between 25 and 250 colonies were selected for further analysis. To enumerate thermotolerant coliforms and *E. coli*, the three-tube Most Probable Number (MPN) method was employed. This method comprises three consecutive tests: presumptive test in LST broth (35 ºC, 24 h), thermotolerant confirmative test in EC broth (45.5 ºC, 24 h) and *E. coli* confirmation test in Tryptone broth (35 °C, 24 h). Each tube was prepared with a 1:10 dilution of the sample in the specific medium appropriate for the corresponding test.

Qualitative methods

Morphological identification

Gram staining. Gram staining was performed according to Hucker's Crystal Violet method [20] on five randomly selected bacterial colonies from the culture plate. Staining was initiated by flooding the smear with Hucker's crystal violet dye for 60 seconds. Following crystal violet staining, the slide was rinsed gently with sterile water to remove excess dye. Lugol's iodine solution (Gram's iodine) was then applied to the slide for 1 min and allowed to cover the entire smear. The slide was again rinsed gently with sterile water to remove excess Lugol's iodine. Decolorization was then performed by applying acetone-alcohol solution for 10-15 sec, using a gentle swishing motion or dipping the slide into a coplin jar containing the solution. Stop decolorization when no further purple dye runs off the smear. Procedure was continued by flooding the slide with safranin solution (safranin and 95% ethyl alcohol mixture) for 30 sec. The slide was then rinsed thoroughly with sterile water to remove excess counterstain. Microscopic examination was performed to observe the morphological characteristics and Gram staining reaction (Gram-positive: purple; Gram-negative: pink) of each isolate.

Oxidase test. Five representative isolates, randomly selected from MRS agar plates for LABand MacConkey agar plates for presumptive TC suspected to be *E. coli*, were tested for oxidase enzyme activity. As both LAB and presumptive TC can exhibit negative oxidase reactions, this test was employed to aid in differentiating between these genera and refine the presumptive identifications. The test was performed by placing a sterile filter paper disc onto a Petri dish containing Oxidase Kovacs Reagent. The filter paper was then moistened with a few drops of the reagent. Using glass rod, five representative isolates were transferred on the five different filter papers placed making a smear in the area where oxidase specific reagent was applied. The positive oxidase reaction involved the oxidized cytochrome oxidizing tetramethyl-p-phenylenediamine (TMPD), resulting in a characteristic purplish-blue product [21] (Figure 1). This positive reaction was observed as a dark blue to violet color change within the first 15 sec. Conversely, a negative reaction displayed no color change within the same timeframe. Following the oxidase assay, positive reactions were indicated by a violet or dark blue coloration within the smear area, signifying the presence of oxidase enzyme. Conversely, negative reactions displayed no color change, suggesting the absence of the enzyme.

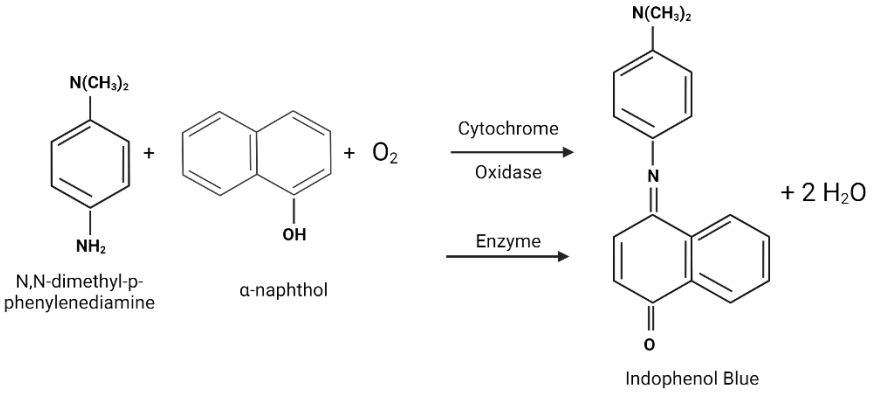


Fig. 1. Mode of action of cytochrome oxidase [21]

Catalase test. The catalase test detects the presence of catalase enzyme in bacteria, which aids in species identification and understanding their hydrogen peroxide tolerance. Catalase is a unique class of enzyme, distinct from other hydroperoxidases like peroxidase. Its primary role is to detoxify hydrogen peroxide (H_2O_2), which can otherwise accumulate and create a toxic environment for bacteria. This ability contributes to the bacteria's tolerance to oxidative stress. Several methods can be used to detect catalase in bacteria, including the slide catalase test, the

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tube method, the capillary tube and cover slip method, and others. In this study, we employed the slide (drop) catalase test due to its advantages of speed, reliability, and the ability to directly use bacteria from agar plates. For the purpose of testing, a commercially available 3% hydrogen peroxide solution was utilized for the catalase test. This concentration facilitates visualization of the positive reaction, characterized by the formation of white bubbles. A sterile glass slide served as an ideal platform for observing this reaction due to its smooth, transparent surface. A sterile inoculation loop was used to transfer a small amount of inoculum from a single, previously isolated colony on an agar plate onto a sterile glass slide. To ensure safety and accommodate potential gas production, the glass slide was placed within an empty, sterile Petri dish. Two drops of 3% hydrogen peroxide were then added onto the bacterial smear on the slide. Observing rapid gas formation (white bubbles) within the first 5 seconds indicated a positive catalase reaction. Conversely, no gas evolution at any time signified a negative result [22]. For each bacterial isolate, three independent catalase tests were performed to verify the results and enhance reliability.

Indole test. The indole test is a valuable biochemical tool for confirming *E. coli* identification. This test detects the ability of bacteria to break down tryptophan, producing indole as a byproduct. *E. coli* strains typically possess the enzyme tryptophanase, enabling them to perform this conversion, while many other bacterial species lack this enzymatic activity. The indole test relies on the enzyme tryptophanase, which catalyzes the breakdown of the amino acid tryptophan. This reaction produces indole, a compound that reacts with the added reagent to form a characteristic "cherry-red ring" at the top of the broth tube within the first 5 sec [23]. The characteristic "cherry-red" color observed in a positive indole test arises from the reaction between Indole Kovacs Reagent (containing p-dimethylaminobenzaldehyde) and indoxyl. Indoxyl is an intermediate compound formed during the enzymatic breakdown of tryptophan by tryptophanase [24].

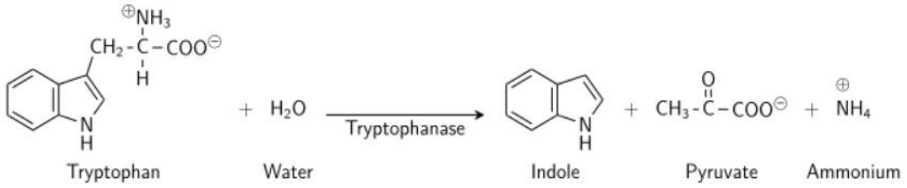


Fig. 2. Mode of action of Tryptophanase enzyme [24]

This study employed the indole test to detect the presence of *E. coli* in samples based on its ability to break down tryptophan and produce indole. Bacterial isolates were incubated in Tryptophan Broth (10 g/L) at 35 °C for 24-48 h. Afterwards, 0.5 mL of commercially available Kovacs Reagent was added to each culture tube. A positive indole test, indicating the presence of the tryptophanase enzyme, was characterized by a distinct cherry-red ring forming at the top of the broth within the first 10 sec after adding the reagent. Conversely, a negative result, signifying the absence of tryptophanase, appeared as a yellow, cloudy ring. Both positive and negative reactions were visually assessed and documented before interpreting the results in conjunction with the Most Probable Number (MPN) enumeration method [25].

Lipolytic activity assay. The lipolytic activity assay was employed to assess the ability of isolated colonies to produce enzymes capable of causing spoilage in fermented dairy products [26]. This assay is becoming increasingly relevant in the biotechnology industry, particularly for applications involving lipases [27]. Lipases are enzymes known for their ability to break down triacylglycerols (fats) into simpler molecules like diglycerides and fatty acids, as illustrated in Figure 3.

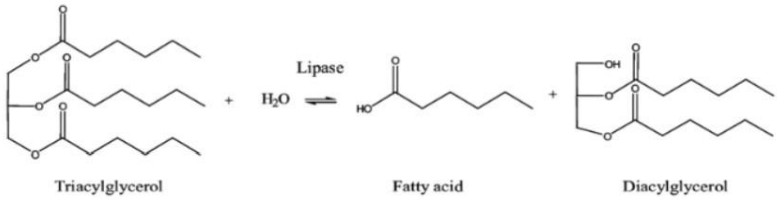


Fig. 3. Mode of action of Lipase enzyme [27].

Isolated colonies were subcultured aseptically in nutrient broth (NB; 3 g/L beef extract, 5 g/L peptone) overnight, incubating for at least 12 h at their optimal growth temperatures [28]. 0.1 mL of each isolate culture was aseptically transferred onto separate plates containing 1% Tween 20 agar and 1% Tween 80 agar. The plates

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were then incubated for at least 12 h at 35 °C under sterile conditions. Lipolytic activity was observed as the formation of non-transparent, circular zones with Ca^{2+} crystals surrounding the colonies [22]. Lipolytic activity was assessed after 5 days of incubation at 35 °C. The diameter of the newly formed non-transparent zone surrounding each colony was measured and used to calculate an enzymatic index (EI) using the following formula:

$$\mathrm{EI} = \frac{\text{diameter of hydrolysis zone (mm)}}{\text{diameter of colony (mm)}}$$

Lipolytic activity was categorized as positive if crystals were observed forming around the colonies, indicating enzymatic breakdown of the agar substrate. Conversely, colonies lacking surrounding crystals were categorized as negative, suggesting no lipolytic activity.

Molecular conformation of isolates. RT-qPCR assays offer versatile applications, including identifying specific bacteria, quantifying their presence, or simply detecting their existence within a target group. However, DNA extraction from food samples is a crucial first step before performing these assays. To enhance the efficiency of DNA extraction kits and improve bacterial detection sensitivity, food samples were enriched in 0.1% Buffered Peptone Water (BPW) prior to DNA extraction. The enrichment step involved static incubation (without shaking) at 37 °C for 16 h (overnight) [27, 28]. Following the protocol outlined in Table 1, a 5 µL aliquot of the enriched sample was mixed with 200 µL of the lysis solution containing lysis buffer, proteinase K, and nuclease-free water.

Table 1. Thermal cycler settings for preparing lysate from the samples.

Temperature	Time
55°C	30 min
95°C	10 min
4°C	∞

Following DNA extraction from the enriched samples, the next step was to prepare qPCR reactions for the 96-well plate. We opted for the lyophilized assay bead method over the liquid assay. Lyophilized assay beads offer several advantages for preparing qPCR reactions. These kits pre-assemble all necessary components directly within the PCR tube, simplifying setup and reducing potential errors. Additionally, they utilize conserved bacterial DNA regions, such as *16s* and *23s rRNA* genes, ensuring broader target coverage within the chosen species. As an example, we employed the TaqMan™ *E. coli* spp Assay Beads and MicroSEQ™ *Salmonella* spp. Detection Kit in this study. Each qPCR reaction required 30 µL of the previously prepared DNA material. This volume was carefully added to individual reaction tubes within the 96-well plate base. Following addition, the plate was vortexed briefly and centrifuged to ensure thorough mixing and eliminate air bubbles. Finally, the tubes were securely capped and loaded onto the RT-qPCR machine, programmed with the specific settings outlined in Table 2.

Table 2. Thermal cycler settings used in the process of specific bacteria detection.

Stage	Stage 1 (enzyme activation)	Stage 2 (PCR)	
Repetition	1 (Hold)	40 cycles	
Temperature	95 °C	95 °C	60 °C
Time	2 min	3 sec	30 sec

The RT-qPCR molecular method was employed as a confirmatory technique to verify the presence of specific bacteria identified through previous methods. The Ct values obtained from the qPCR reactions served as the basis for interpreting the results. Samples with Ct values below 35 were considered positive for the target bacteria, aligning with the manufacturers' instructions for the utilized kits.

Statistical analysis. Student t-tests were employed in all statistical analyses. The level of significance (*p*-value) was set at 0.05, with $P<0.05$ considered statistically significant, indicating evidence against the null hypothesis.

Mean values used in calculations represent the averages derived from three independent experiments. Each individual experiment batch additionally contained three technical replicates, ensuring robustness and reliability in the data collection enhancing the reproducibility of the study's analyses.

RESULTS

Enumeration of viable LAB. The total viable LAB in food samples were enumerated using direct colony counting on MRS agar plates. LAB abundance varied considerably, ranging from 2.5E+02 CFU/g to 2.4E+06 CFU/g. The distribution of LAB across different dairy products is illustrated in Figure 4, with values expressed

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in log_{10}. Amongst the dairy products analyzed, pasteurized milk exhibited the highest LAB count (1.69E+06 CFU/g, P<0.05), significantly exceeding the recommended optimal level for non-spoiled food (>1E+03 CFU/g). Similarly, elevated LAB levels were observed in buttercream (1.4E+05 CFU/g), surpassing the established safe limit (<1E+03 CFU/g). Cottage cheese displayed lower LAB (6.1E+02 CFU/g), fitting uncontaminated food's range (<1E+03 CFU/g) for this product. Student's t-tests was used in all statistical analysis.

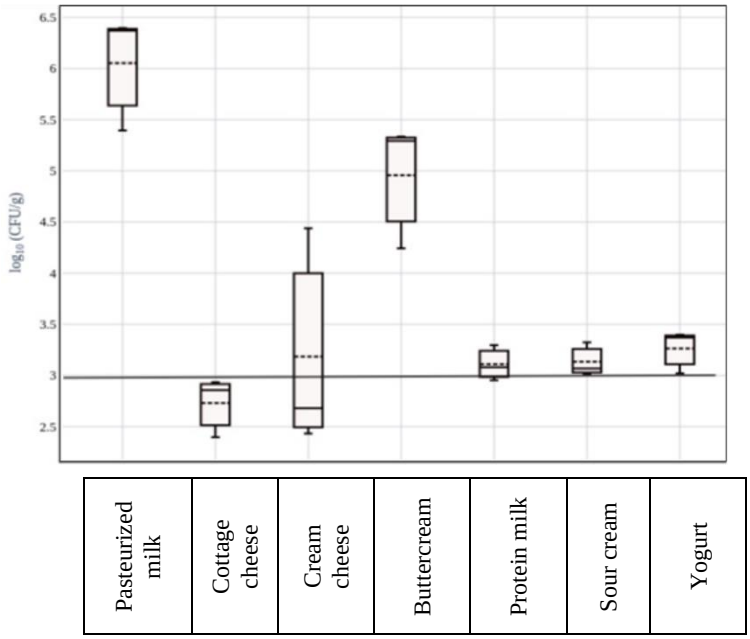


Fig. 4. Total viable count of LAB in dairy food samples. The box plot shows the total viable count of LAB across seven different dairy products with the average value for each sample (dash line). The upper and lower whiskers depict the maximum and minimum log_{10} CFU/g number calculated for LAB isolates.

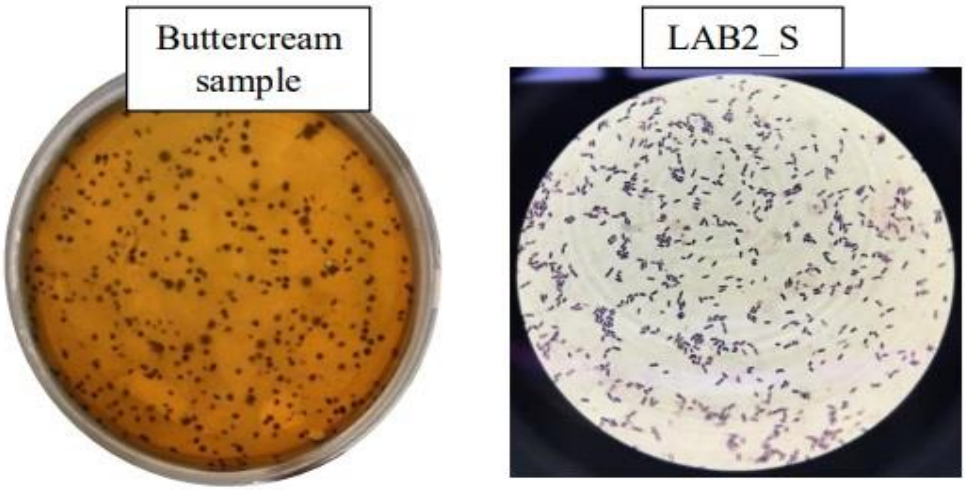


Fig. 5. Representation of LAB colonies on MRS agar plate (right) and microscopic image of a representative isolate after Gram Staining (left). Colonies appeared to have a dark green color, approximately 3-5 mm in diameter size. An optical microscopy image of one isolated from the same sample represented here was recorded using 40x magnification.

Morphological identification of LAB. Gram staining was assessed for isolates grown on MRS agar plates, following previously described methods. Colony sizes varied across the different samples, ranging from 0.5 to 2 cm in diameter. Gram staining was performed on a selection of colonies from each sample, and all seven samples yielded positive (+) reactions. Microscopic examination of Gram-stained colonies revealed a characteristically violet coloration (Figure 5). Table 3 summarizes detailed information for one representative

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isolate from each sample, including their consistent Gram-positive staining results. Notably, despite minor variations such as the presence of short chains, most isolates displayed similar morphological features under 40x magnification, appearing predominantly rod-shaped.

Table 3. Overview of representative isolates of LAB after Gram staining procedure.

	ID number of isolate	Food sample	Medium	Color	Gram stain reaction
Total: 63 isolates	LAB1_S1	Sample 1	MRS	Purple	+
	LAB3_S2	Sample 2	MRS	Purple	+
	LAB2_S3	Sample 3	MRS	Purple	+
	LAB2_S4	Sample 4	MRS	Purple	+
	LAB2_S5	Sample 5	MRS	Purple	+
	LAB2_S6	Sample 6	MRS	Purple	+
	LAB1_S7	Sample 7	MRS	Purple	+

Biochemical identification of LAB. Sixty-three isolated LAB underwent screening with oxidase and catalase tests. Additionally, their growth ability at 35 °C ± 2°C and pH 5.5 ± 0.2 was assessed. All isolates displayed negative reactions (-) in both oxidase and catalase tests, indicating their likely non-aerobic and non-hydrogen peroxide-producing nature.

Table 4. Results obtained from catalase and oxidase biochemical identification tests

	Catalase	Oxidase	Growth at 35 ± 2.0	Growth at pH 5.5 ± 0.2
LAB1_S1	-	-	+	+
LAB3_S2	-	-	+	+
LAB2_S3	-	-	+	+
LAB2_S4	-	-	+	+
LAB2_S5	-	-	+	+
LAB2_S6	-	-	+	+
LAB1_S7	-	-	+	+

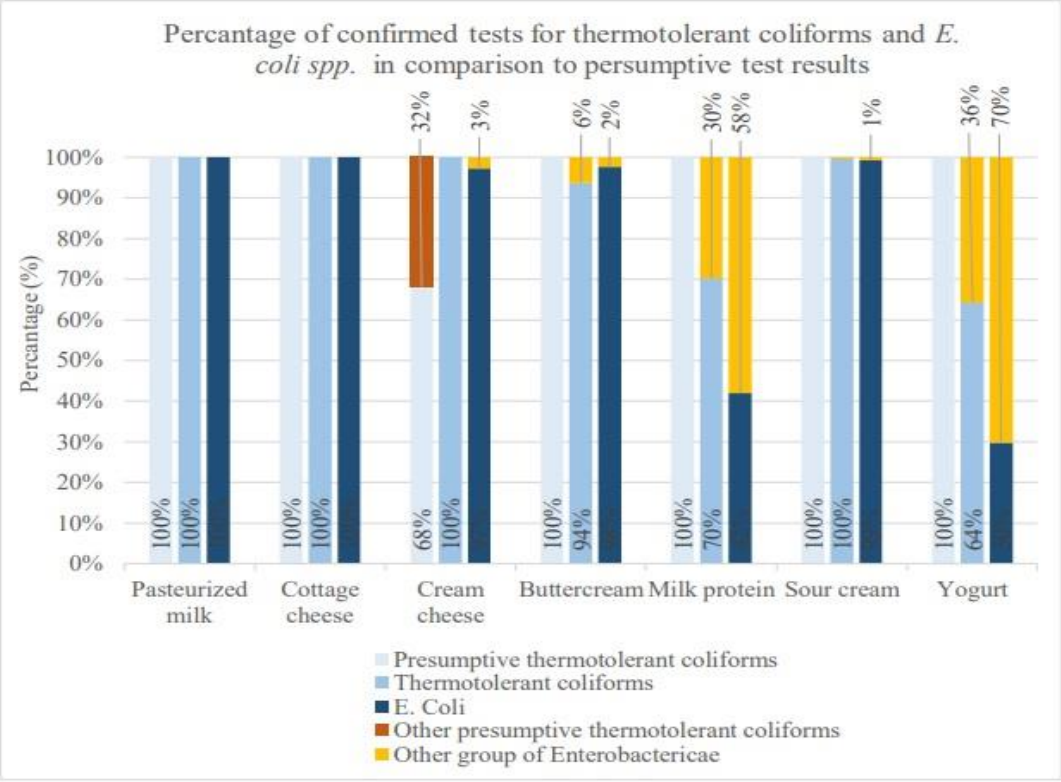


Fig. 6. Overall representation of percentage for confirmed thermotolerant coliforms and *E. coli spp*. tests in comparison to presumptive test results. Blue bars represent the percentage of positive tubes recorded by gas production and growth observed. The yellow color of the bars indicates the percentage of tubes in which turbidity was observed due to the growth of the bacteria rather than gas production.

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Enumeration of thermotolerant coliforms isolates. The MPN method [31] revealed significant *E. coli* presence in all tested food samples. Using LST broth, a presumptive test indicated 95% gas production across samples, suggestive of thermotolerant coliforms. Subsequent confirmation with EC broth yielded positive results in 90% of cases. Protein-rich milk exhibited a statistically non-significant reduction in gas production (70% decrease) compared to the control ($P > 0.05$). In contrast, yogurt showed a significant reduction (64%) in gas production ($P < 0.05$). Further confirmation using EC broth successfully identified *Enterobacteriaceae* and displayed a strong correlation between confirmed *Enterobacteriaceae* counts and true-positive *E. coli*. Both protein-rich milk and yogurt showed significantly lower counts of presumptive *E. coli* in Tryptophan tubes compared to the control, suggesting the presence of other *Enterobacteriaceae* species (Figure 6). However, confirmation of true *E. coli* using additional tests revealed good overlap with the initial presumptive results, with 81% confirmed as true *E. coli*.

Quantitative analysis of both total coliforms (TC) and *E. coli* in dairy products revealed levels exceeding established microbial criteria limits ($P < 0.05$). Pasteurized milk exhibited the highest prevalence, with a most probable number (MPN) of 1100 per milliliter and a standard error of the mean (SE) of ± 0.29 (Figure 7). This value was significantly higher than those observed in other samples ($P < 0.05$). Both cream cheese and buttercream also showed high density, with average of 2 log_{10} MPN ± 0.96 SE and 2.42 log_{10} MPN ± 0.9 SE. TC and *E. coli* in these samples fit within 4800 MPN/g to 4.3 MPN/g range for upper and lower 95% confidence limits, or 3.68 to 0.63 MPN/g on log_{10} scale.

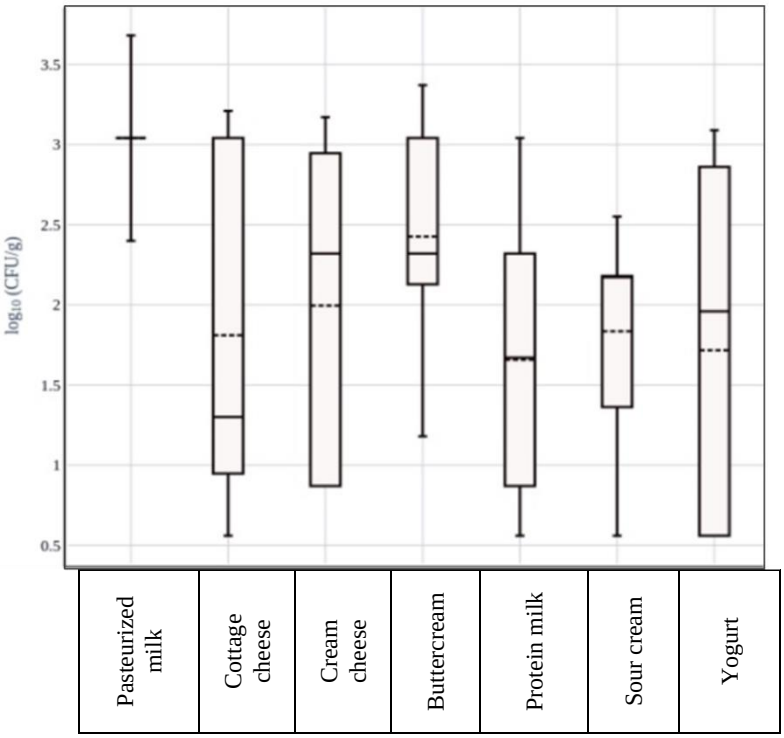


Fig. 7. Box plot representation of overall density distribution of thermotolerant coliforms and *E. coli spp.* in food samples. Whiskers depict the upper and lower 95% confidence interval expressed in log_{10} MPN/g number.

The presence of *E. coli* spp. in food samples was investigated using Tryptophan broth medium. This analysis included pasteurized milk, protein milk, and yogurt (Figure 8). Results revealed the pasteurized milk sample to have the highest density distribution of *E. coli spp.* at 3.04 log_{10} MPN ± 0.43 SE, compared to the other samples as well as the average number for the allowed amount of *E. coli spp*. throughout different dairy food samples. In contrast, protein milk and yogurt samples displayed a lower density of *E. coli* spp., with average values falling within the marginal range allowed for this bacterial group in food samples; the respective mean values for protein milk and yogurt were 0.78 log_{10} MPN ± 0.49 SE and 0.62 log_{10} MPN ± 0.51 SE.

Morphological identification of thermotolerant coliforms isolates. Further identification of the thermotolerant coliforms involved isolating pure cultures from EC broth onto MacConkey agar. Visual inspection revealed two distinct colony morphologies suggestive of *Enterobacteriaceae*: confirmed *E. coli* (3mm diameter, pink colonies) and potential *Salmonella* spp. (1mm diameter, white colonies). *E. coli* ferments lactose present

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in the agar, resulting in the characteristic pink coloration, while presumed *Salmonella* spp. do not ferment lactose, leading to white colonies. Both colony types displayed Gram-negative staining (pink coloration), confirming their membership within the *Enterobacteriaceae* family. Microscopic examination further confirmed the presence of rod-shaped bacteria (Figure 9).

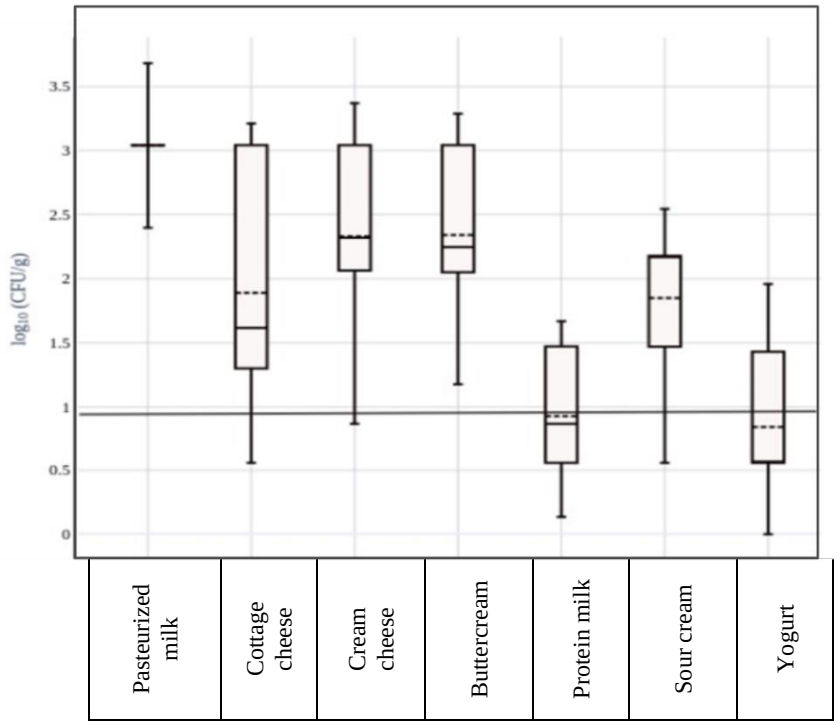


Fig. 8. Prevalence of *E. coli spp.* in dairy food products using the confirmative testing from Tryptophan broth medium.

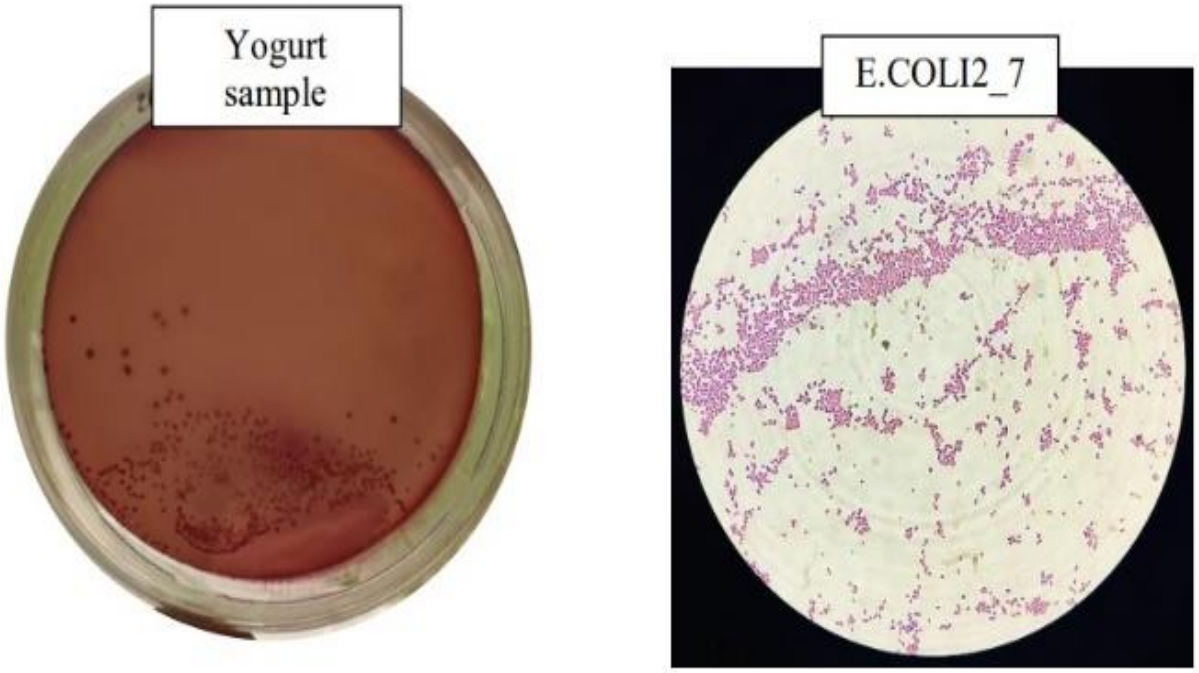


Fig. 9. Isolated *E. coli* pure colonies on MacConkey agar (left) as a confirmed thermotolerant coliform and member of the *Enterobacteriaceae* family. Optical microscopy picture of rod-shaped *E. coli* isolates (right), showing Gram-negative reaction (40x).

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Table 5. Type of agar medium. Representation of positive (+) or negative (-) lipolytic reaction in respective agar medium for *E. coli* spp. and *Salmonella* spp. isolates. Lipolytic activity is additionally expressed as an enzymatic activity (EI) value.

		Tween 20 Lipolytic activity							EI	Tween 80 Lipolytic activity							EI
Type of isolate	*E. coli*	+	+	+	+	+	+	+	0.4	+	+	+	+	+	+	+	0.8
	Presumptive *Salmonella* spp.	-	-	-	-	-	-	-	0.9	-	-	-	-	+	-	+	1.2

Biochemical identification of thermotolerant coliforms isolates and presumptive pathogen. Biochemical tests confirmed the presence of both *E. coli* and presumptive *Salmonella* spp. Representative *E. coli* colonies tested negative for both catalase and oxidase enzymes. Similarly, presumed *Salmonella* spp. displayed negative results for these tests. Further analysis using Tryptophan broth and indole testing yielded positive results for *E. coli* in 81% of cases, indicated by a distinct red layer (1 cm thick) at the top of the broth. The remaining 19% of isolates, while showing growth, lacked gas production and exhibited a yellow layer (1 cm thick) upon indole testing, suggesting further investigation is needed.

Lipolytic activity of *E. coli spp*. and presumptive *Salmonella spp*. The lipolytic activity of isolated TC *E. coli* and presumptive *Salmonella* spp. was assessed by evaluating the formation and size of calcium opaque zones around their colonies. Following subculture in nutrient broth, isolates from MacConkey agar were screened on both Tween 20 and Tween 80 agar plates. While most *E. coli* isolates exhibited positive lipolytic reactions on both media, the zones they formed were smaller (<1 cm) and lacked transparency, resulting in a relatively low average Enzyme Index (EI) of 0.6 across all *E. coli* samples.

Presumptive *Salmonella* spp. isolates from protein-rich milk and yogurt samples displayed positive lipolytic activity. Interestingly, they showed higher EI values on Tween 80 agar plates compared to Tween 20. The highest EI, reaching 1.2, was obtained for the yogurt sample, suggesting more pronounced lipolytic activity in that isolate. Conversely, isolates demonstrated lower EI values on Tween 20 agar plates.

***E. coli spp.* and presumptive *Salmonella spp*. conformation by RT-qPCR.** RT-qPCR was used to confirm the presence of indicator bacteria detected through culture-based methods, specifically *E. coli* spp. and *Salmonella* spp. Employing a commercially available kit for *E. coli* detection in food, all samples yielded Ct values ranging from 17.8 to 20.8, falling within the established cut-off for positive detection (+). Applying the commercially available kit for *Salmonella* spp. detection in food yielded mostly negative results (>35 Ct or "undetermined"), signifying the absence of presumptive *Salmonella* spp. However, three out of seven dairy samples (cream cheese, protein milk, and yogurt) exhibited detectable Ct values (Table 6). Successful detection (+) was assigned based on averaging Ct values from three independent experiments for each positive sample.

Table 6. Molecular detection of indicator bacteria from dairy food samples. Successful detection is denoted as a plus sign (Ct<35) whereas not detected bacteria from food samples was interpreted as a minus sign (Ct>35).

		Sample name						
		Pasteurized milk	Cottage cheese	Cream cheese	Butter-cream	Milk protein	Sour cream	Yogurt
Type of detection assay kit	*E. coli*	+	+	+	+	+	+	+
	Salmonella spp.	-	-	+	-	+	-	+

DISCUSSION

This study investigated the bacterial composition and food safety of dairy products using both qualitative and quantitative methods. Our primary focus was to assess the abundance of lactic acid bacteria (LAB) as well as thermotolerant coliforms and *E. coli*, which serve as indicators of spoilage and contamination, respectively.

Six out of seven dairy product samples displayed elevated levels of LAB compared to typical ranges for these products (>1E+03 CFU/g). Only cottage cheese and, to a lesser extent, cream cheese, fell within the expected range. Notably, the milk sample possessed the highest LAB count, suggesting potential contamination from cows or insufficient starter culture incorporation during processing. Elevated LAB levels can pose food safety concerns, depending on the specific LAB species present and their associated metabolic activities.

Biochemical identification and morphological analysis of the isolated LAB identified their potential role in dairy spoilage [7]. The isolates displayed typical characteristics of LAB, being Gram-positive, oxidase-negative, catalase-negative, and rod-shaped.

The growth and distribution of microorganisms in food are heavily influenced by water activity (Aw). Bacteria typically thrive in environments with Aw values above 0.95, where water is readily available for their needs. Consequently, reducing the water content of dairy products is a potential strategy for limiting or controlling the spoilage rate. This approach targets both pathogenic and spoilage organisms by restricting their access to the

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water they require for growth and proliferation. *Lactobacillus* spp., often used in fermentation, are typically found in high-water-content products like liquid samples, where they can easily distribute and metabolize. However, our study observed permissible levels of LAB in butter, despite its lower water content.

LAB found in dairy products are versatile organisms, able to survive with or without oxygen (facultative anaerobes). Their primary metabolic pathway ferments glucose into lactic acid (homofermentative), producing two molecules per molecule of glucose. This acidic environment, with a pH below 62-65 °C, effectively inhibits the growth of many harmful bacteria, including pathogens [32]. However, their thermoduric nature allows them to survive pasteurization at these temperatures, potentially leading to spoilage reactions [32].

Morphological and biochemical analysis suggested the presence of pathogenic bacteria, including presumptive *Salmonella* spp. This aligns with research indicating that such indicator organisms within the *Enterobacteriaceae* family often signal the potential coexistence of true pathogens [15, 33].

While this study primarily investigated broader relationships between established microbial indicators, indices, and spoilage bacteria, it is noteworthy that pathogenic species like presumptive *Salmonella* spp. require further confirmation. This highlights the need for more comprehensive research using methods like serotyping to provide definitive identification.

While the detected levels of presumptive *Salmonella* spp. may not pose an immediate health threat, their presence highlights the need for potential adjustments in food manufacturing practices [34]. The presence of these bacteria often indicates less-than-optimal sanitation or hygiene during dairy production, which should be addressed to ensure product safety and consumer confidence. While most processing equipment in dairy production is made of stainless steel, the presence of rubber components introduces a crucial risk factor for contamination [15]. Even a single bacterium remaining in these susceptible zones can lead to significant spoilage and indicator-positive results in final products [15].

As previously discussed, LAB can create a highly acidic environment in food due to their homofermentative metabolism. This acidic environment inhibits the growth of undesirable microorganisms like thermotolerant coliforms, *E. coli*, and *Salmonella* spp., acting as a natural antagonist against them. Despite the expectation that LAB would suppress thermotolerant coliforms like *E. coli* and *Salmonella* spp. [35, 36], this study observed limited inhibitory effects. This could be attributed to complex interactions between microbial communities within the food ecosystem, the presence of components buffering the acidic environment, and the potential adaptation of pathogenic strains to acidic conditions [35, 37-40].

While our study aimed to provide a broad overview of unique dairy products from Bosnia and Herzegovina sourced from a single supermarket, we acknowledge its scope as a limitation. For a more comprehensive analysis, incorporating products from various market chains and a larger sample size would be imperative.

In conclusion, this study has provided a systematic examination of the bacterial composition as well as the food safety of dairy products. Results gained from this study imply that the dairy food products possess a possible food safety risk and raise concerns regarding hygiene in dairy production, requiring further confirmation and readjustments in manufacturing practices. In addition to these findings, this study serves as a good starting point for further research regarding the safety of dairy products in Bosnia and Herzegovina.

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CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest associated with this manuscript.

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