Frontiers in Life Sciences and Related Technologies 6(1) (2025) 9-14

Contents lists available at Dergipark

Frontiers in Life Sciences and Related Technologies

Journal homepage: http://dergipark.org.tr/en/pub/flsrt

Research article

Microbiological changes of kefir traditionally produced from different milks according to storage time

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Abstract

Kefir is a fermented dairy product known for its positive effects on health. It has been reported to have positive effects on gastrointestinal diseases, hypertension, metabolic disorders, and the immune system due to its lactic acid bacteria, yeasts, and various bioactive compounds. The study aimed to investigate the microbiological properties of kefirs produced from different milk types and pasteurization processes. Different types of milk were used in kefir production, such as UHT cow, pasteurized cow, open cow, and pasteurized goat. For each milk, kefir was produced with a 24-hour incubation period followed by microbial analysis on days 1, 7, 14, and 21. The microbial flora was assessed based on total bacterial counts, as well as specific enumeration of *Lactobacillus* sp., *Lactococcus* sp., coliform bacteria, molds, and yeasts. The pH levels of the kefir samples were also measured. The analysis showed that pH values decreased with increasing storage time in all kefir types. Especially the *Lactobacillus* sp. count of kefirs produced from goat's milk was lower than other milk types and decreased until day 21. It was also observed that the number of coliform bacteria decreased faster in kefir produced with UHT milk, while it was not detected in other kefirs after the 14th day. The study revealed that the microbial structure of kefirs varied significantly according to milk type, pasteurization method, and storage time. Open cow's milk kefirs produced by traditional methods were found to be richer in probiotic bacteria but at risk of contamination.

Keywords: *Diet; kefir; kefir grain; traditional food*

1. Introduction

Fermented products show positive effects on health thanks to various microorganisms and the compounds produced by these microorganisms. Microbial fermentation of kefir produces various bioactive compounds, vitamins, and minerals. Thanks to these compounds, kefir is known to have various health benefits such as antimicrobial, hypocholesterolemic, immunostimulant, and antitumor effects (Gokirmakli and Guzel-Seydim, 2022; Bozkir et al., 2024). Kefir is an acidic-alcoholic fermented milk beverage with unique characteristics such as its slightly sour and yeasty taste and viscous and creamy density. It is a food characterized by its high nutritional, biological, and dietetic value and is recommended as an alternative option for gastrointestinal, metabolic, cancer, hypertension, cardiac, and allergic diseases (Aydin, 2023; Saleem et al., 2023; Cheng et al., 2024). It is especially recommended to be consumed by patients, the elderly, pregnant women, lactating women, infants, lactose intolerant individuals, and healthy people. Kefir consumption has increased worldwide in recent years, and the global kefir market is expected to at least double by 2030 (Tavsanli et al., 2024).

Kefir grains are white to yellowish, irregularly shaped cauliflower-like grains traditionally used in kefir production

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https://doi.org/10.51753/flsrt.1561917 Author contributions
Received 05 October 2024; Accepted 23 January 2025
Available online 30 April 2025

(Yousefvand et al., 2022). The starter of kefir grain consists of lactic acid bacteria and yeast that produce lactic acid and alcohol. The quality of kefir is greatly influenced by the type of milk used, the amount of kefir grains, kefir grain microorganisms, and the incubation period (Arslan, 2015; Sulmiyati et al., 2019; de Souza et al., 2024). Kefir has a complex mixture of more than 50 bacteria and yeasts. Kefir grains contain *Lb. Kefir, Lb. Casei, Lb. Fermentum, Lb. Acidophilus, Lactococcus lactis, Streptococcus thermophilus,* and yeast species such as *Candida albicans, Saccharomyces cerevisiae, Kluyeromyces marxianus, and Pichia caribbica* (Sulmiyati et al., 2019; Saleem et al., 2023; Kalamaki et al., 2024).

Various kefir grains and kefir drinks have been evaluated in terms of microbiota and sensory composition. However, detailed microbial characterization studies examining kefirs produced under home conditions and made with different milk types (different milk types such as goat, cow, and different pasteurization processes) have not yet been reported. The aim of this study was to investigate and compare the microbiological characteristics of kefir grains and traditional kefirs made with different milk types and different pasteurization processes.

2. Materials and methods

2.1. Preparation of kefir samples

Kefir grains required for kefir production were obtained and activated at home using traditional methods. Sterile ultra-heat treated (UHT) cow milk, pasteurized cow milk, open cow milk, and pasteurized goat milk were used for kefir production. The kefir production process was carried out in the Karabuk University Gastronomy and Culinary Arts Application Kitchen and the samples were transferred to the Karabuk University Microbiology Laboratory for microbial analysis without breaking the cold chain. The kefirs were coded as kefir produced with open cow's milk (K100), kefir produced with pasteurized cow's milk (K200), kefir produced with UHT cow's milk (K300), kefir produced with pasteurized goat's milk (K400), kefir produced with half pasteurized goat's milk and cow's milk (K500). In order to activate kefir grains, 5% kefir grains were added to cow's milk (300 ml), which was heat-treated at 90°C for 10 minutes to kefir to be produced with open cow's milk (K100). Then, it was left for incubation for 24 hours at room temperature. After incubation, kefir grains were filtered under hygienic conditions, and the activation of the grains was completed by repeating the same process one more time. This time, the activated grains were added to the milk, which was heat treated at 90°C for 10 minutes for kefir production and kept under the same incubation conditions. After incubation, the grains were separated from kefir under hygienic conditions, and the drinkable kefir was bottled for ripening and stored at +4°C for 24 hours. The production processes of K200, K300, K400, and K500 kefir samples were carried out in the same way. Inoculated samples were incubated at room temperature for 24 hours. After incubation, the grains were collected by passing through a sterile plastic strainer, and the same process was repeated once more to complete the activation of the grains. The activated grains were added to 5% kefir grains into 300 ml milk to ferment kefir and kept under the same incubation conditions. After incubation, the grains were separated from the kefir under hygienic conditions and the kefir in drinkable form was bottled and stored at +4°C for 24 hours for ripening.

2.2. Microbiological and physiological analyses

Microbiological analysis of kefir samples coded K100, K200, K300, K400, and K500 was carried out in the Microbiology laboratory of Karabuk University Faculty of Medicine. In order to determine the number of bacteria forming the microbial flora of kefirs and their contamination status, each kefir sample was divided into four separate sterile tubes by paying attention to sterile conditions and kept at 4°C, which is the recommended condition for storage, and analyzed on the 1st day, 7th day, 14th day and 21st day. The pH values of kefir samples were determined using a pH meter (Thermo Orion Model-420A′).

2.3. Preparation of media and dilution solution

In the samples obtained from kefirs produced in all codes, 1 ml kefir was used for each analysis, and conventional methods were used in microbiological analyses. For this purpose, Milk Plate Count Agar (MCA) (Merck Millipore, Germany) was used for the determination of total viable bacteria count, M17 Agar (Merck Millipore, Germany) for the determination of Lactobacillus species and MRS Agar (Merck Millipore, Germany) for the determination of Bifidobacterium species. For the detection of contaminated microorganisms, VRB Agar (Merck Millipore, Germany) was used for the detection of coliform group bacteria, and Potato Dextrose Agar (Merck Millipore, Germany) was used for the detection of mold and yeast. Commercial powder/granule media were used in culture procedures and prepared with attention to sterility. The powder/granular media were stored in the original clamshell packaging in the dark, at ambient temperature, and in the refrigerator (MRS Agar) according to the manufacturer's instructions. They were sterilized in an autoclave at 121°C for 15 min and kept in a water bath at 47°C until use. Buffered Peptone Water was used as a dilution solution. 1 L of distilled water was added to 1 gram of solution and homogenized with a magnetic stirrer. It was divided into tubes at 9 ml per tube and sterilized. It was used after it was brought to ambient temperature.

2.4. Total viable bacteria, Lactobacillus sp. *and Bifidobacterium* sp. *count (cfu/mL)*

For the determination of the total number of viable bacteria; 1 ml of kefir samples was transferred to test tubes containing 9 ml of 0.1% Buffered Peptone Water. It was mixed in a vortex for 5-7 seconds. Inoculations of 1 ml were made from the tubes diluted at ratios of 1/1, 1/10, 1/100, 1/100, 1/1000, 1/10000, 1/100000, 1/1000000. 10-12 ml Milk Plate Count Agar (MCA) (Merck Millipore, Germany) medium was added to the Petri dishes and allowed to solidify. Petri dishes were incubated at 37°C for 72 hours in an aerobic environment. At the end of incubation, colonies were counted and the total number of bacteria in 1 ml was determined. For *Lactobacillus* sp. analysis; 1 ml of kefir samples were transferred to test tubes containing 9 ml of 0.1% Buffered Peptone Water. It was mixed in a vortex for 5-7 seconds. The tubes were diluted at ratios of 1/1, 1/10, 1/100, 1/100, 1/1000, 1/10000, 1/100000, 1/1000000 and inoculated with 1 ml. 20 ml of M17 Agar (Merck Millipore, Germany) medium was added to the Petri dishes and allowed to solidify. Petri dishes were incubated at 37°C for 72 hours in an aerobic environment. At the end of incubation, colonies were

counted and the number of *Lactobacillus* sp. bacteria in 1 ml was determined. For *Bifidobacterium* sp. analysis; 1 ml of kefir samples were transferred to test tubes containing 9 ml of 0.1% Buffered Peptone Water. After mixing for 5-7 seconds in the vortex, 1 ml was inoculated into tubes diluted at ratios of 1/1, 1/10, 1/100, 1/100, 1/1000, 1/10000, 1/100000, 1/1000000. After 20 ml of MRSA (Merck Millipore, Germany) medium was added to the Petri dishes and allowed to solidify, the Petri dishes inoculated with the medium were placed in anaerobic jars, and anaerogenic kit was added and placed in an incubator and anaerobic incubation was performed at 37°C for 72 hours. At the end of incubation, the number of *Bifidobacterium* sp. in 1 ml was determined by counting the colonies formed. For the enumeration of *Lactobacillus* sp. and *Bifidobacterium* sp., 1 mL of kefir sample was transferred into test tubes containing 9 mL of 0.1% Buffered Peptone Water and vortexed for 5–7 seconds. Serial dilutions were prepared at 1/1, 1/10, 1/100, 1/1000, 1/10000, 1/100000, and 1/1000000, and 1 mL of each dilution was inoculated onto selective media. For *Lactobacillus* sp., M17 agar (Merck Millipore, Germany) was used, and plates were incubated aerobically at 37°C for 72 hours. For *Bifidobacterium* sp., MRSA medium (Merck Millipore, Germany) was used; the inoculated Petri dishes were placed in anaerobic jars with an anaerogenic kit and incubated anaerobically at 37°C for 72 hours. After incubation, colony counts were performed, and the number of viable *Lactobacillus* sp. and *Bifidobacterium* sp. per milliliter was determined.

2.5. Coliform group bacteria and mold/yeast count (cfu/mL)

For the detection of coliform group bacteria as a contamination indicator, 1 ml of kefir samples were inoculated without dilution. 20 ml VRB Agar (Merck Millipore, Germany) medium was added to the Petri dishes and allowed to solidify. In order to create a microaerophilic environment, 5-6 ml of medium was added again after the first layer of the medium solidified. Petri dishes were incubated at 37°C for 72 hours in an aerobic environment and the number of coliform group bacteria in 1 ml was determined by counting the colonies formed.

For mold and yeast detection, 1 ml of kefir samples were inoculated without dilution. 20 ml of Potato Dextrose Agar (Merck Millipore, Germany) medium was added to the Petri dishes and the Petri dishes were incubated at 25°C for 72 hours in an aerobic environment. At the end of incubation, colonies were counted and the number of mold yeast in 1 ml was determined.

2.6. Statistical analysis

The data obtained were evaluated with the SPSS for Windows (Version 20.0, Statistical Package for Social Sciences) program. Descriptive statistics of continuous variables in the study were shown with mean and standard deviation values, and descriptive statistics of categorical variables were shown with frequency and percentage. The Pearson correlation coefficient was used to examine the relationship between dependent variables. In statistical analyses, measurements with a *p*-value below 0.05 ($p<0.05$) were accepted as significant.

3. Results

The distribution of total bacterial count values of K100, K200, K300, K400, and K500 coded products according to storage periods under the same temperature and storage conditions are shown in Table 1. According to the results of the analysis, the total bacterial counts in kefir types showed a rapid decrease in K100 and K300 as the storage period prolonged compared to day 1, while this was not observed in K200, K400, and K500.

Table 1
Total bacterial count (cfu/ml) of kefir types according to temperature and storage periods.

Types of kefir	Day 1	Day 7	Day 14	Day 21
K100	63.10^7	60.10^7	15.10^6	12.10^6
K200	40.10^7	60.10^7	22.10^6	24.10^6
K300	57.10^7	53.10^7	37.10^6	18.10^6
K400	32.10^7	26.10^7	62.10^6	20.10^6
K500	35.10^7	22.10^7	47.10^6	20.10^6

When the correlation between the total bacterial counts of kefir types according to the storage periods was analyzed, it was observed that there was a significant difference at day 7 and a significant decrease in the total bacterial count at day 14 ($p<0.05$) (Table 2).

Table 2
Correlation between total bacteria values of kefir types at different storage days.

		1	2	3	4	5
1	Total bacteria count day 1	1				
2	Total bacteria count day 7	0.745	1			
3	Total bacteria count day 14	-0.713	-.890*	1		
4	Total bacteria count day 21	-0.768	-0.246	0.342	1	
5	Kefir type	-0.734	-.929*	0.869	0.433	1

** $p<0.05$*

The effect of temperature and storage conditions on coliform group bacteria count changes of kefir types are shown in Table 3. According to the results of the analysis, there was a positive change in the number of coliform group bacteria in K100, K200, K300, and K400 kefir types between the 1st day and the 7th, 14th, and 21st days. It was determined that this situation developed due to the decrease in the pH level of the samples due to the prolongation of the storage period.

Table 3
Coliform group bacteria count (cfu/ml) of kefir types according to temperature and storage periods.

Types of kefir	Day 1	Day 7	Day 14	Day 21
K100	20.10^1	10.10^1	1.10^1	0
K200	58.10^1	2.10^1	0	0
K300	7.10^1	0	0	0
K400	3.10^1	3.10^1	0	0
K500	2.10^2	6.10^1	0	0

The effects of temperature and storage conditions on the Lactococcus sp. bacteria count changes of kefir types are shown in Table 4. According to the analysis results, there were differences between kefir samples and storage periods. It was observed that *Lactococcus* sp. bacteria content reached the highest levels in K300 and K500 on the 1st day and in K100, K200, and K400 on the 7th day during fermentation; *Lactococcus* sp. bacteria count showed a significant decrease in K300 and K500 on the 14th and 21st days compared to the 1st day.

When the correlation between the total *Lactococcus* sp. bacteria species according to the storage times of kefir types

were examined, it was observed that there was a significant negative difference in the 1st day values according to kefir types, and there was a significant positive difference between the 1st day and the 7th day for all kefir types (p<0.05) (Table 5).

Table 4
Lactococcus sp. bacteria count (cfu/ml) of kefir types according to temperature and storage periods.

Types of kefir	Day 1	Day 7	Day 14	Day 21
K100	37.10^7	50.10^7	10.10^6	3.10^6
K200	37.10^7	50.10^7	12.10^6	7.10^6
K300	30.10^7	25.10^7	2.10^6	1.10^6
K400	21.10^7	72.10^6	15.10^6	2.10^6
K500	20.10^7	18.10^7	12.10^6	1.10^6

Table 5
Correlation between total *Lactococcus* spp. bacteria counts of kefir types depending on storage periods.

		1	2	3	4	5
1	Kefir type	1				
2	*Lactococcus* sp. bacteria count day 1	-.955*	1			
3	*Lactococcus* sp. bacteria count day 7	-0.875	.948*	1		
4	*Lactococcus* sp. bacteria count day 14	0.225	-0.307	-0.153	1	
5	*Lactococcus* sp. bacteria count day 21	-0.572	0.679	0.712	0.31	1

** p<0.05*

The effects of temperature and storage conditions on the *Lactobacillus* sp. bacteria count changes of kefir types are shown in Table 6. According to the results of the analysis of the products stored and analyzed in the same environment, it is seen that the highest level of Lactobacillus sp. species bacteria counts in the product coded K100 reached the highest level on the 7th day and the highest level on the 1st day in kefir samples produced with other milk types. It is seen that the prolongation of the storage period causes the number of *Lactobacillus* sp. bacteria to decrease.

Table 6
Lactobacillus sp. count (cfu/ml) of kefir types according to temperature and storage periods.

Types of kefir	Day 1	Day 7	Day 14	Day 21
K100	24.10^7	26.10^7	12.10^6	11.10^6
K200	16.10^7	10.10^7	9.10^6	15.10^6
K300	14.10^7	22.10^6	10.10^6	6.10^6
K400	11.10^7	20.10^6	5.10^6	45.10^5
K500	10.10^7	3.10^7	4.10^6	35.10^5

Table 7
Correlation between *Lactobacillus* sp. bacteria counts of kefir types during storage periods.

		1	2	3	4	5
1	Kefir type	1				
2	*Lactobacillus* sp. bacteria count day 1	-.937*	1			
3	*Lactobacillus* sp. bacteria count day 7	-0.833	.958*	1		
4	*Lactobacillus* sp. bacteria count day 14	-.933*	.887*	0.722	1	
5	*Lactobacillus* sp. bacteria count day 21	-0.83	0.67	0.617	0.66	1

** p<0.05*

The correlation between *Lactobacillus* sp. bacteria counts of kefir types in different storage periods is shown in Table 7. Day 1 *Lactobacillus* sp. bacteria counts showed a significant difference between kefir types. There was a significant negative correlation in the number of *Lactobacillus* sp. bacteria from kefir K100 to kefir K500. A significant negative relationship was also observed between kefir types on days 7 and 14 (p<0.05).

According to the results of the analysis of the changes in mold and yeast bacteria counts of kefir types due to temperature and storage conditions, intense mold and yeast bacteria counts were observed in all kefir types between the 1st day and the 7th, 14th, and 21st-day counts. The prolongation of the storage period caused intense mold and yeast bacteria to be found in all kefir types.

The pH changes of kefir types due to temperature and storage conditions are shown in Table 8. According to the results of the analysis, a decrease in pH values was observed in all kefir types between the 1st day and the 7th, 14th, and 21st days.

Table 8
pH change of kefir types according to temperature and storage periods.

Types of kefir	Day 1	Day 7	Day 14	Day 21
K100	4.53	4.49	4.46	4.43
K200	4.5	4.47	4.42	4.38
K300	4.45	4.42	4.35	4.32
K400	4.5	4.48	4.44	4.43
K500	4.5	4.48	4.45	4.43

The correlation between the pH changes of kefir types depending on the storage periods is shown in Table 9. A significant correlation was observed in the pH values of all kefir types according to storage times (p<0.05, p<0.001).

Table 9
Correlation between pH changes of kefir types at different storage periods.

		1	2	3	4	5
1	Kefir type	1				
2	pH day 1	-0.329	1			
3	pH day 7	-0.057	.957*	1		
4	pH day 14	0	.944*	.993**	1	
5	pH day 21	0.162	0.866	.959*	.975**	1

**p<0.05 *p<0.001*

4. Discussion

The pH value, which is important in determining the quality of food, determines the acidity level by reflecting the activity of hydrogen ions. In kefir production, the fermentation process is usually completed in the pH range of 4.5-4.6. Changes in pH values during the storage of kefir are an indication of the shelf life of the product and the slow fermentation that occurs during the storage process (Putri et al., 2020; Acar, 2023; Li et al., 2024). In this study, microbiological analysis of traditional kefirs prepared with cow and goat milk with different pasteurization processes was evaluated. The pH measurements of kefir samples showed a statistically significant difference depending on the storage periods (p<0.05). It was observed that the general average pH value was 4.49 on the 1st day of the research, and pH values decreased statistically significantly in all kefir types on the 7th, 14th, and 21st days of the research. It was understood that there was no significant difference in pH values between kefir samples obtained from cow or goat milk (p>0.05). In the study conducted by Buran (2020), the pH values of kefir samples from cow's milk ranged between 4.39 and 4.47, while the pH values of samples from goat's milk ranged between 4.18 and 4.42 (Buran, 2020). In the study conducted by Acik et al., pH values in kefir samples were found in the range of 4.13-4.55 (Acik et al., 2020). Yousefvand et al. (2022) reported that the pH values of kefir samples measured after 1, 7, 14, and 21 days of storage at 4°C ranged between 4.49 and 4.53 on day 1

and gradually decreased throughout the storage period. The findings of the current study are consistent with this result. The differences in pH values are thought to be related to factors such as fermentation time and the type of milk used (Ektik, 2022; Li et al., 2024).

Buran (2020) found that *Lactococcus* sp. numbers in kefir produced with cow and goat milk varied between 7.15-7.93 and 7.00-8.30 log cfu/mL; Bakan (2021) found that *Lactococcus* sp. numbers of concentrated kefir types produced by different methods varied between 8.08-8.76 log cfu/g. In this study, it was understood that the Lactococcus sp. bacteria content reached the highest levels on day 1; K300 and K500 during fermentation and decreased as a result of fermentation in the following storage days.

While Bakan (2021) determined that the number of *Lactobacillus* sp. in concentrated kefir types produced by different methods ranged between 8.17 and 9.01 log cfu/g, da Costa et al. (2020) reported that the number of *Lactobacillus* sp. increased until the 14th day of storage in four out of six kefir samples, but then decreased, whereas in the remaining two samples, the decrease occurred on the 7th day. When the literature is examined, the results of some researchers indicate that the number of *Lactobacillus* sp. in kefir decreases during storage, while others indicate that it first increases and then decreases or remains constant (Sendogan et al., 2021; Yousefvand et al., 2022; Acar, 2023; Gulhan, 2023). This is thought to be due to the effects of fermentation metabolites on lactic acid bacteria resulting from microbiota differences in kefir. In this study, the number of *Lactobacillu*s sp. bacteria in kefir samples was generally higher than the literatüre (Egea et al., 2022; Sánchez-Rodríguez et al., 2024; Onat et al., 2025). When the kefir types were evaluated within themselves, it was observed that the number of *Lactobacillus* sp. bacteria in kefirs made with goat milk was lower than the kefirs made with other milk types, and the increase was higher on the 21st day, while the number of *Lactobacillus* sp. bacteria of kefirs made with cow milk decreased gradually with the prolongation of the storage period. Yeasts are effective in the development of the taste and aroma of kefir and in the establishment of symbiosis between microorganisms. Buran (2020) reported that yeast results in kefir produced with cow and goat milk varied between 4.16-5.49 and 4.00-5.18 log cfu/mL, respectively. Ciftci and Oncul (2022) reported that they detected 2.37 and 3.08 log cfu/mL yeast in plain kefir and fruit kefir. In literature studies, the mold count in kefir samples produced by industrial method is below the detectable values (Yilmaz et al., 2022; Salik et al., 2023; Gulhan, 2023). In this study, intense mold and yeast were detected in all kefir types starting from the 7th day.

Ciftci and Oncul (2022) found that the total number of coliform bacteria in the samples was below the detectable value as a result of total coliform bacteria count. In this study, the number of coliform group bacteria was not detected in kefir produced with UHT milk (K300) as of the 7th day, while no coliform group bacteria were detected after the 14th day in the other kefirs except K100, while no coliform group bacteria were detected only on the 21st day in kefir made with open cow's milk (K100). With the prolongation of the storage period, the pH values of kefirs decrease, and these low pH values negatively affect the ability of bacteria to survive. For this reason, all bacterial species may have decreased in number by being affected by the decreasing pH values due to the prolongation of the storage period.

5. Conclusion

When the data of the study are evaluated, it is seen that storage periods and the type of milk used in kefir production, as well as the pasteurization processes applied to the milk affect the microbial load of the kefirs obtained. As a result of the study, goat milk was found to be the most inefficient milk type in terms of probiotic bacteria in kefir production. The prolonged storage period causes a decrease in the number of probiotic bacteria in kefirs. As of day 7, *Lactobacillus* sp. and *Lactococcus* sp. bacteria species of all kefir types show a decrease. This may be due to decreasing pH values. In light of the data obtained, the highest probiotic bacteria species were found in open cow milk produced by traditional methods, while the lowest value was found in kefir produced with pasteurized goat milk. These results indicate that the production of kefir from cow milk with traditional methods for kefir production provides an advantage in terms of probiotic bacteria species, but poses a risk in terms of coliform bacteria species. This study is one of the rare studies that examined the difference between the microbial values of kefirs produced with the use of cow's milk and goat's milk which have undergone different pasteurization processes according to storage periods. It is thought that the study data will make a positive contribution to the literature.

Conflict of interest: The authors declare that they have no conflict of interests.

Informed consent: The authors declare that this manuscript did not involve human or animal participants and informed consent was not collected.

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