A non-yeast kefir-like fermented milk development with *Lactobacillus acidophilus* KCNU and *Lactobacillus brevis* Bmb6.

Bomee Lee[1]*, Cheng-Chung Yong[2]*, Hae-Chang Yi[1], Saehun Kim[3†], Sejong Oh[2†].

[1] Samik Dairy & Food Co. Ltd, 453 Yeoksam-ro, Seoul, Republic of Korea

[2] Division of Animal Science, Chonnam National University, Republic of Korea

[3] College of Life Sciences and Biotechnology, Korea University, Seoul, Republic of Korea

* Both authors contributed equally

Running title: Non-yeast kefir-like fermented milk by functional starter

[†] Corresponding authors

Sae Hun Kim (ORCID iD: 0000-0002-0990-2268)

College of Life Sciences and Biotechnology, Korea University, Seoul, 02841, Korea

Email: saehkim@korea.ac.kr

Sejong Oh

Division of Animal Science, Chonnam National University, Gwangju, 61186, Korea

Email: soh@chonnam.ac.kr

Contact: +82-62-530-2116

Abstract

The use of yeast assist kefir fermentation, but also can cause food spoilage if uncontrolled. Hence, in this study, the microbial composition of an existing commercial kefir starter was modified to produce a functional starter, where *Lactobacillus acidophilus* KCNU and *Lactobacillus brevis* Bmb6 were used to replace yeast in the original starter to produce non-yeast kefir-like fermented milk. The functional starter containing *L. acidophilus* KCNU and *L. brevis* Bmb6 demonstrated excellent stability with 10^{10} CFU/g of total viable cells throughout the 12 weeks low-temperature storage. The newly developed functional starter also displayed a similar fermentation efficacy as the yeast-containing control starter, by completing the milk fermentation within 12 h, with a comparable total number of viable cells (10^8 CFU/mL) in the final products, as in control. Sensory evaluation revealed that the functional starter-fermented milk highly resembled the flavor of the control kefir, with enhanced sourness. Furthermore, oral administration of functional starter-fermented milk significantly improved the disease activity index score by preventing drastic weight-loss and further deterioration of disease symptoms in DSS-induced mice. Altogether, *L. acidophilus* KCNU and *L. brevis* Bmb6 have successfully replaced yeast in a commercial starter pack to produce a kefir-like fermented milk beverage with additional health benefits. The outcome of this study provides an insight that the specific role of yeast in the fermentation process could be replaced with suitable probiotic candidates.

Keywords: yeast; kefir; starter culture; fermentation; *Lactobacillus*

Introduction

Kefir, an acidic-alcoholic fermented milk product with an acidic taste and a creamy consistency, is produced by fermenting milk with a complex mixture of microorganisms, consisting of acetic acid bacteria, lactic acid bacteria, and yeast, at 25–28°C. Among the complex microbial composition, *Lactobacillus* spp., is the dominant species in the kefir microbial population, accounting for up to 80% of all microorganisms, while the rest is represented by *Bifidobacterium* sp., *Lactococcus* sp., and yeast (Miguel et al., 2010; Witthuhn et al., 2004). Blooming reports on the health-promoting effects of kefir resulting in an emerging trend for the use of kefir as a healthy and rehydrating beverage (de LeBlanc et al., 2007; Hertzler & Clancy, 2003; Huseini et al., 2012; Liu et al., 2005, 2006; Lopitz-Otsoa et al., 2006; Matsuu et al., 2003).

Yeast in kefir can play a double role. The production of carbon dioxide (CO_2) and ethanol by yeast during alcoholic fermentation are responsible for the unique flavor in kefir (Adriana & Socaciu, 2008). However, uncontrolled growth and the excess production of CO_2 and ethanol through secondary alcoholic fermentation in yeast could lead to off-flavor, accumulation of CO_2, leading to swollen containers during storage and packaging, eventually blowing off the package (Kwak et al., 1996; O'Brien et al., 2016). As food safety has become a primary global concern, food spoilage as in the accumulation of CO_2 at the headspace of kefir has become an obstacle for the rapid economic growth and industry development (Danilović et al., 2018).

Hence, strain selection is essential to the production of a unique starter culture that could maintain the traditional attributes of the product while improving its aroma, safety, shelf-life, and functional benefits of the product. In this study, a new functional starter was developed by replacing yeast in a commercial starter with two functional probiotic strains, the bacteriocin-

producing *L. acidophilus* KCNU and the colitis-ameliorating *L. brevis* Bmb6, for the production of kefir-like fermented milk with additional health benefits.

Materials and methods

Cultivation of microorganisms

Twelve starter microorganisms (*Bifidobacterium longum, Lactobacillus casei, Lactobacillus plantarum, Lactobacillus acidophilus, Lactobacillus bulgaricus, Lactobacillus fermentum, Lactobacillus reuteri, Streptococcus thermophilus,* three *Lactococcus lactis* spp., and *Saccharomyces cerevisiae*) were provided by Samik Dairy & Food Co. Ltd, Gangnam-gu, Seoul, Korea. The bacteriocin-producing *Lactobacillus acidophilus* KCNU was obtained from the Korean Culture Center of Microorganisms, Seodaemun-gu, Seoul, Korea. *Lactobacillus brevis* Bmb6 with prominent anti-inflammatory effects was isolated from kimchi (Shin, 2017). All *Lactobacillus* strains were cultivated and maintained in MRS broth at 37°C; *Lactococcus* strains were cultivated and maintained in M17-lactose broth at 32°C; *Streptococcus* strains were cultivated and maintained in M17 broth 43°C; *Bifidobacterium longum* was cultivated and maintained in BL broth at 37°C under anaerobic condition; Yeast (*S. cerevisiae*) was cultivated and maintained in YGC broth at 28°C.

Stability assessment of bacteriocin from *L. acidophilus* KCNU

The crude bacteriocin was extracted from *L. acidophilus* KCNU, as described previously (Oh et al., 2008). *L. acidophilus* KCNU was cultured in MRS broth at 37°C for 18

h, followed by centrifugation at 6000×g for 30 min at 4°C. Cell-free supernatant was collected as crude bacteriocin, and the pH was adjusted to 7.0 using 10 N NaOH. The crude protein was subjected to filtration through a 0.45 μm filter, heat-treated, and vacuum-dried or freeze-dried before being evaluated for its antimicrobial activity by the spot-on-lawn method (Ahn & Stiles, 1990). The antimicrobial activity of the crude bacteriocin was expressed as arbitrary units (AU) per mL of crude bacteriocin.

Stability of the functional starters

The microbial composition of the control starter and the non-yeast functional starter were tabulated in Table 1. All strains were cultured for 1-2 days to reach 10^{10} CFU/mL. Cell pellets were collected via centrifugation at 3000×g for 30 min. Cell pellet from each strain was mixed and resuspended in 40% (v/v) glycerol in reconstituted skim milk as cryoprotectant. The mixture was then subjected to freeze-drying for 72 h. The freeze-dried starter powder was vacuum packed in an aluminum-coated vinyl pack. The starter was either stored at –20°C (frozen storage) or 5°C (cold storage) for 12 weeks, and the total number of viable cells was determined by at every four weeks interval. The total number of viable cells was the average of the viable count of *Lactobacillus, Lactococcus, Bifidobacterium, Streptococcus*, and yeast. *Lactobacillus* strains were cultivated in MRS-lactose agar at 37°C under anaerobic condition for 48h; *Lactococcus* strains were cultivated in M17-lactose agar at 32°C under anaerobic condition for 24h; *Bifidobacterium longum* was cultivated in BL agar at 37°C under anaerobic condition for 48h; *Saccharomyces cerevisiae* was cultivated in PDA agar at 28°C under anaerobic condition for 72h.

Preparation of functional starter-fermented milk

Sterilized milk was inoculated using 0.02% (w/w) of the functional starter pack (10^{10} CFU/g) and fermented at 25°C under normal atmospheric conditions for approximately 24 h. The control consisted of sterilized milk inoculated with 2% (w/w) of original commercial starter (10^7 CFU/g) and fermented at 25°C under normal atmospheric conditions until the pH 4.5 was reached. The pH and the total number of viable cells of fermented milk were evaluated and recorded at every four h interval. The total number of viable cells was the average of the viable count of *Lactobacillus, Lactococcus, Bifidobacterium, Streptococcus*, and yeast cultivated in different media and culture conditions. The final products were evaluated by 50 regular fermented milk consumers, consisting of university students and staff, to determine the general public acceptability. The fermented milk was scored 1 (worst) to 7 (best) according to taste, texture, and sourness of the beverages.

Colitis-ameliorating assessment of functional starter-fermented milk

Seven-weeks old female ICR mice were obtained from the Daehan Lab (Daejeon, Korea) and acclimated for one week in the Animal Housing Unit (room temperature of 22-25°C, 50-60% humidity, and 12 h light/dark cycle; standard mouse chow-diet and water were provided ad libitum), according to the guidelines provided by the Institutional Animal Care and Use Committee of the Chonnam National University (CNU-IACUC-YB-2016-47; Chonnam National University, Gwangju, Korea).

The control and treatment groups were administered with PBS for the first seven days. Drinking water was then replaced with 4% (w/v) dextran sulfate sodium (DSS) water from day-7 to day-14 to induce colitis in mice. The control group was administered with PBS

through oral gavage from the beginning until the end day-14, while the samples group was administered with 0.1 g of functional starter-fermented milk through oral gavage from day-7 to day-14. Bodyweight, fecal condition, and disease activity index (DAI) was assessed daily based on a scoring system, as shown in Table 2 (Herias et al., 2005).

Statistical analysis

All data were expressed as mean ± standard deviation. Paired-sample *t*-test was performed using SPSS© version 20 (IBM© SPSS© Statistics, USA), with a $p < 0.05$ indicating statistical significance.

Results

Stability of bacteriocin from *L. acidophilus* KCNU

The antimicrobial activity of crude bacteriocin produced by *L. acidophilus* KCNU was not affected by heat treatment, vacuum concentration, or freeze-drying process was maintained at 6400 AU/mL, regardless of the treatment process (Table 3), indicating the ability of crude bacteriocin to withstand downstream processes for industrial application.

Stability of starters

Upon the freeze-drying process, the functional starter achieved total viability at 10^{10} CFU/g, and the number fluctuated within the range of 10^{10} CFU/g throughout the study (Figure

1). At the end of 12 weeks storage period, functional starter stored at –20°C contained 10.66 ± 0.11^{10} CFU/g of total viable cells and 10.38 ± 0.06^{10} CFU/g of total viable cells for functional starter stored at 5°C. indicating their stability over long-term storage at low temperatures.

Characteristics of the functional starter-fermented milk

The fermentation efficacy of the functional starter was comparable to the control starter, both achieved pH 4.5, the optimal pH of kefir at a time near 12 h (Figure 2a). Moreover, at 12 h, both fermented milks have a similar number of viable *Lactobacillus* count, with 8.94 ± 0.50^{10} CFU/mL in control and 8.99 ± 0.35^{10} CFU/mL in functional starter-fermented milk (Figure 2b). Also, the sensory evaluation revealed the acceptance of the functional starter-fermented milk by the panels, with a similar texture, sourness, and taste as the control kefir beverage (Figure 3).

Mitigation effects of the functional fermented milk on colitis model

In this experiment, treatment group mice were administered with functional starter-fermented milk, which contained the bacteriocin-producing *L. acidophilus* KCNU and the anti-inflammatory *L. brevis* Bmb6 strains. Upon DSS induction, the control group mice exhibited a drastic decrease in body weight on day-9 and continued until the end of the day-14 (Figure 4a). In contrast, the body weight of the treatment group mice was decreased gradually throughout the study, but the reduction rate was lower, as compared to the control group. The control group had a DAI score of 0.4 on day-8, which showed a gradual increase in the DAI score to 7.6 at day-14 (Figure 4b). Meanwhile, the DAI score of treatment group mice begun

to increase from 2.0 on day-10 and fluctuated between 4.3 to 4.6 until day-14. Whereas, the DAI score of control group mice continued to increase with DAI score of 7.6 at the end of the study. These results were showing that the ability of functional starter-fermented milk to prevent further deterioration of colitis-symptoms in treatment group mice, as compared to the control.

Discussion

The microbial composition of a starter greatly affects the flavor, nutritional value, health-promoting effects, and shelf life of a fermented dairy product. The use of yeast as a member of the starter culture plays a double role. Yeast could either positively or negatively affect the quality of dairy products, depending on their interaction with other starter strains and the fermentation conditions (Viljoen, 2001). For instance, formation and accumulation of CO_2 in the containers, leading to swollen and bloated containers during storage, had shortened the shelf-life of the products and causing economic loss to the food manufacturer as well (Danilović et al., 2018; Foschino et al., 1993; Kwak et al., 1996; O'Brien et al., 2016; Sarkar, 2008). Hence, in the present study, the use of yeast in starter was replaced by two functional *Lactobacillus* strains, *L. acidophilus* KCNU and *L. brevis* Bmb6, for production of a health-promoting kefir-like fermented milk.

L. acidophilus KCNU, a bacteriocin-producing strain, isolated from the porcine small intestine, has been reported to be effective against various pathogens, including *Bacillus cereus, Enterococcus aerogenes, Escherichia coli, Listeria monocytogenes, Staphylococcus aureus, Pseudomonas aeruginosa,* and *Yersinia enterocolitica*. In addition, *L. acidophilus* KCNU also reported exerting prominent acid and bile tolerance and cholesterol-lowering ability, which

197 suggests its use for food fermentation or pharmaceutical (Oh et al., 2008). Hence, the use of
198 *L. acidophilus* KCNU as a member of the starter could greatly improve the safety by reducing
199 the risk of contamination of the starter culture and spoilage on the end products, while assisting
200 host to a maintain balance gut homeostasis through suppressing the growth of gut pathogens.
201 It is crucial for the bacteriocin from *L. acidophilus* to resist the harsh downstream dehydration
202 processes, including heat-treatment, vacuum drying, or freeze-drying process. In this study,
203 upon the dehydration treatments, the crude bacteriocin from *L. acidophilus* KCNU able to
204 maintain a stable antimicrobial activity at 6400 AU/mL, similar to the antimicrobial activity of
205 the unprocessed crude bacteriocin, suggesting its suitability to be used as a starter culture.

206 The development of starter culture is crucial for the production of fermented foods as
207 it allows a consistent production of fermented foods. It is of utmost importance to maintain the
208 viability of the bacteria and prolong the shelf-life of commercial starter culture (Taskila, 2017).
209 The newly developed *L. acidophilus* KCNU and *L. brevis* Bmb6 containing functional starter
210 exhibited excellent stability by maintaining the total number of viable cells at 10^{10} CFU/g
211 throughout the 12 weeks storage period, at – 20°C and 5°C. Freeze-drying is one of the most
212 commonly practiced preservation techniques for commercial starter culture production, owing
213 to its capability to maintain a high number of viable bacterial cells and to prolong the shelf-life
214 of the product (Taskila, 2017). Based on our results, frozen storage is preferable to cold storage
215 when considering storing the product for a more extended period (> 12 weeks).

216 Ideally, industrial kefir fermentation should be completed within 8 h, with the final pH
217 of 4.5, to meet the smooth and continuous downstream procedures such as the cooling and
218 packaging process (Lee et al., 2018). Certain yeast strains were capable of assimilating lactate,
219 producing alkaline end products that could neutralize the acids, thereby prolong the
220 fermentation period (Potter & Hotchkiss, 1995; Soulides, 1955). However, the replacement of

yeast with *L. acidophilus* KCNU and *L. brevis* Bmb6 did not improve the fermentation efficacy of the functional starter. Also, the absence of yeast in the functional starter did not alter the total number of the viable count, with 10^8 CFU/mL upon completion of the milk fermentation, indicating the use of *L. acidophilus* KCNU and *L. brevis* Bmb6 did not affect the total number of viable cells in the fermented milk as in the control kefir. Meanwhile, sensory evaluation by regular fermented milk consumers revealed that the functional starter-fermented high resembled the control yeast-kefir in terms of the taste and texture of the fermented milk, with enhanced sourness taste

Previously, the administration of *L. brevis* Bmb6 has been reported to effectively improve the symptoms of bowel inflammation in DSS-induced colitis mice (Shin, 2017). In this study, the colitis-ameliorating property of *L. brevis* Bmb6 remained in the end product, fermented milk after a series of industrial manufacturing processes. Administration of the functional starter-fermented milk prevented a drastic decrease in body weight of DSS-induced mice, thereby rendering further deterioration of colitis-symptoms in DSS-induced mice at the later stages (Day-13 and -14), suggesting that *L. brevis* Bmb6-containing fermented milk can mitigate the symptom of gastrointestinal disorders. This colitis ameliorating effects of *L. brevis* Bmb6 was attributed to enhance gut epithelium integrity, promote the recovery of epithelial cells, and suppress the pro-inflammatory cytokines, TNF-α, and IFN-γ (Shin, 2017).

In summary, a non-yeast functional starter has been developed by substituting yeast in a commercial starter with *L. acidophilus* KCNU and *L. brevis* Bmb6. The new functional starter demonstrated excellent storage stability and the functional starter-fermented milk high resembling the control yeast-kefir in terms of fermentation efficacy, total viable cells, taste, and texture of the fermented milk, with enhanced sourness. Moreover, the functional starter-fermented milk retained the ability *L. brevis* Bmb6 in relieving intestinal inflammation. The

outcome of this study provides an insight into the development of non-yeast starter for fermented dairy products through the use of other functional lactic acid bacteria.

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Author Contributions

Conceptualization: Oh S, Kim S. Data curation and formal analysis: Lee B, Yong C-C, Yi H-C. Methodology: Lee B, Yi H-C. Software: Lee B, Yi H-C. Validation: Lee B, Yi H-C. Investigation: Lee B, Yi H-C. Writing - original draft: Yong C-C. Writing - review & editing: Lee B, Yong C-C, Yi H-C, Kim S, Oh S.

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334 **Tables**

335 **Table 1** Composition of LAB strains in the control and functional starter cultures used in this
336 study.

Control	Functional starter
*Bifidobacterium longum**	*Bifidobacterium longum**
*Lactobacillus casei**	*Lactobacillus casei**
*Lactobacillus plantarum**	*Lactobacillus plantarum**
*Lactobacillus acidophilus**	*Lactobacillus acidophilus**
*Lactobacillus bulgaricus**	*Lactobacillus bulgaricus**
*Lactobacillus fermentum**	*Lactobacillus fermentum**
*Lactobacillus reuteri**	*Lactobacillus reuteri**
*Streptococcus thermophilus**	*Streptococcus thermophilus**
Lactococcus lactis ssp. *lactis**	*Lactococcus lactis* ssp. *lactis**
Lactococcus lactis ssp. *cremoris**	*Lactococcus lactis* ssp. *cremoris**
Lactococcus lactis ssp. *lactis biovar diacethylactis**	*Lactococcus lactis* ssp. *lactis biovar diacethylactis **
*Saccharomyces cerevisiae**	*Lactobacillus acidophilus* KCNU†
	Lactobacillus brevis Bmb6‡

337 * Strains were provided by Samik Dairy & Food Co. Ltd, Seoul, Korea.

338 † *Lactobacillus acidophilus* KCNU was obtained from Korean Culture Collection of
339 Microorganisms.

340 ‡ *Lactobacillus brevis* Bmb6 was obtained from Chonnam National University (Shin, 2017).

Table 2 Scoring system for disease activity index*

score	Weight loss (%)	Stool consistency†	Gross bleeding
0	None	Normal	Negative
1	1-5	Loose	Negative
2	5-10	Loose	Hemoccult positive
3	11-15	Diarrhoea	Hemoccult positive
4	>15	Diarrhoea	Bleeding

* Disease activity index, DAI = (score of weight loss + stool consistency + gross bleeding)/3

† Normal stool = well-formed pellets; loose = pasty stool that does not stick to the anus; diarrhoea = liquid stool that sticks to anus.

354 **Table 3** Antimicrobial activity of crude bacteriocin produced by *Lactobacillus acidophilus*
355 KCNU.

Sample	Treatment condition	Antimicrobial activity, AU/mL*
Filtration	Filtration through a 0.45 μm membrane	6400
Heat-treatment	Heat treatment (65°C for 20 min) followed by 0.45 μm membrane filtration	6400
Vacuum concentration	Vacuum evaporation at 55°C	6400
Freeze-drying	Heat treatment (65°C for 20 min) followed by freeze-drying (–50°C, 6 torr)	6400

356 ***** Antimicrobial activity was expressed as arbitrary unit per milliliter (AU/mL) using the
357 formula (1000 μL/ 10μL) × reciprocal of the highest dilution showing visible inhibitory activity.

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Figure Legends

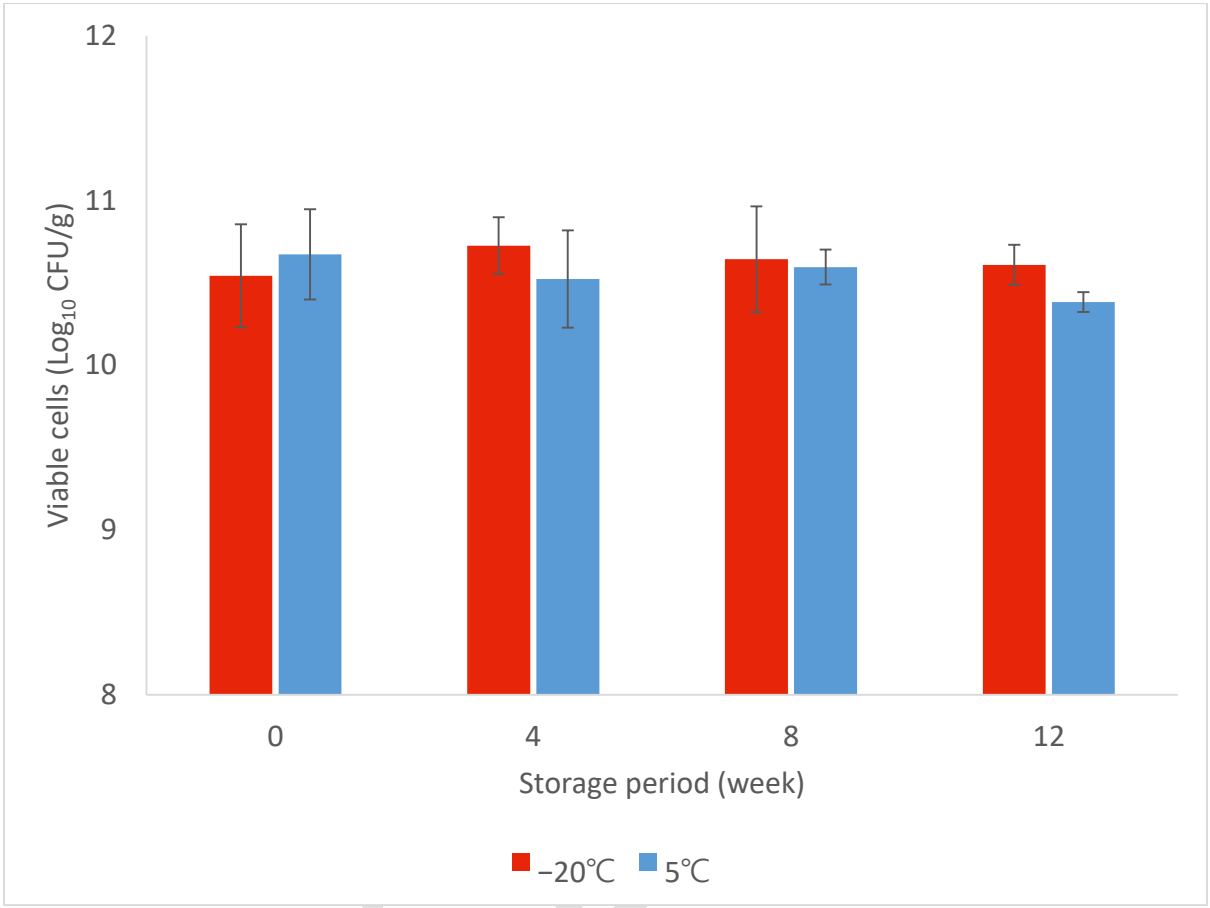

Fig. 1. The total number of viable cells in starter cultures during storage at –20°C and 5°C. Results are expressed as the mean ± standard deviation of three independent experiments (n = 3). Paired *t*-test was performed with no significant differences between the sample ($p > 0.05$).

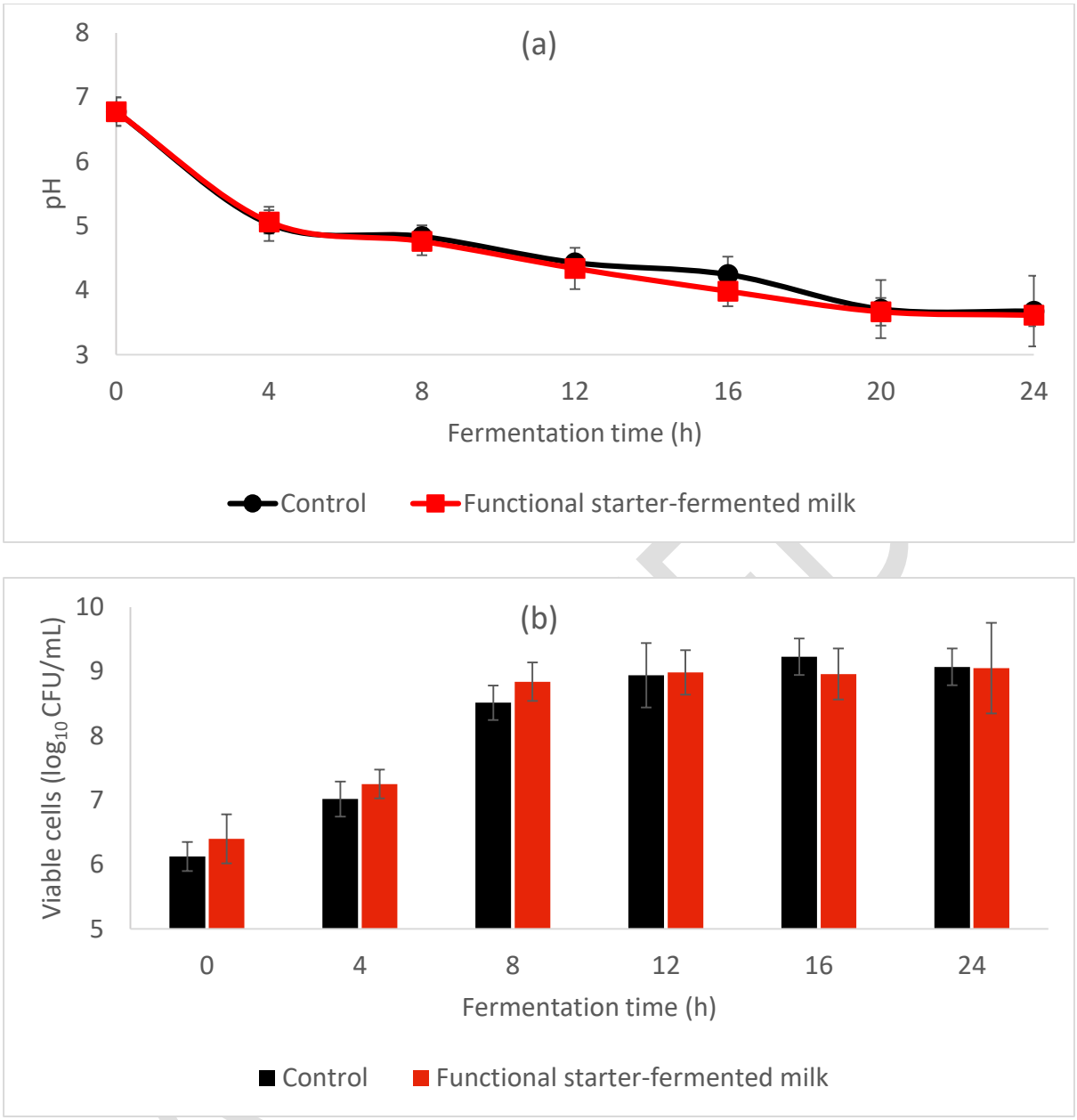

Fig. 2. Change in the (a) pH and (b) the total number of viable count during milk fermentation with different starters, at 25°C for 24 h. Results are expressed as the mean ± standard deviation of three independent experiments (n = 3).

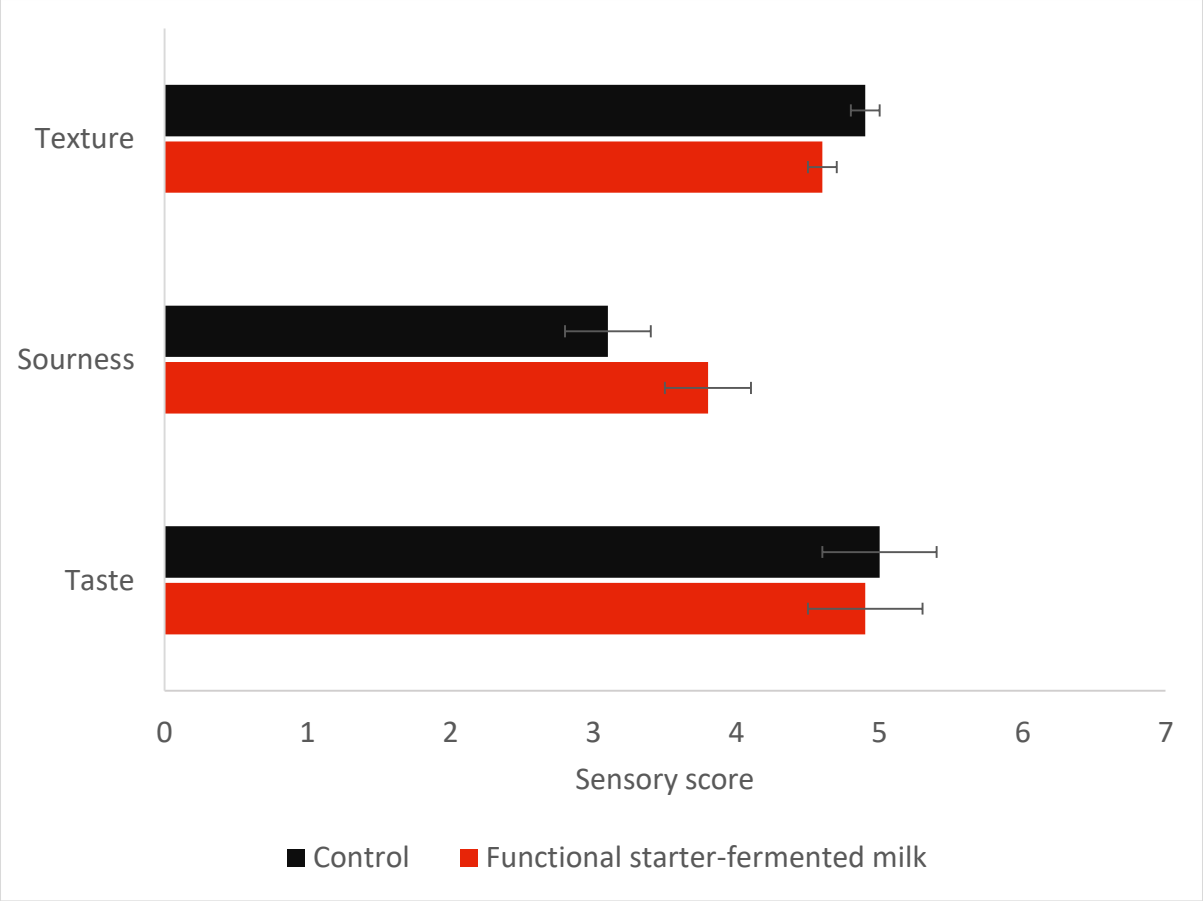

Fig. 3. Sensory evaluation of fermented milk with different starters. Results are expressed as the mean ± standard deviation of 50 individual subjects (n = 50). Paired *t*-test was performed with no significant differences between the sample ($p > 0.05$).

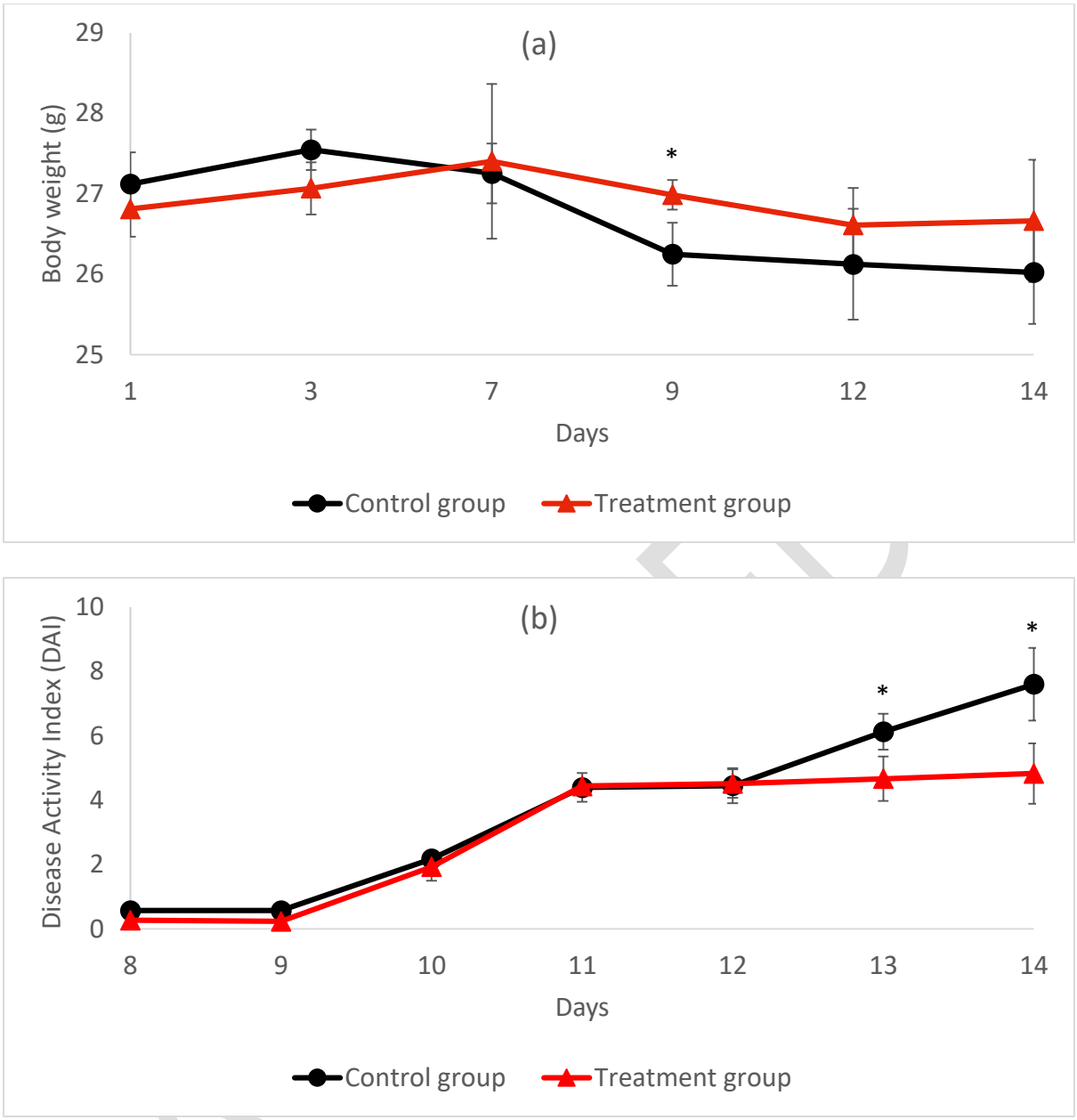

Fig. 4. Changes in the (a) body weight and (b) disease activity index of dextran sulfate sodium-induced mice. Results are expressed as the mean ± standard deviation of five independent experiments (n = 5). Paired t-test was performed. The asterisk (*) indicates significant differences between the control and kefir group ($p < 0.05$). Treatment group: Functional starter-fermented milk-fed DSS-mice; Control group: yeast-starter kefir-fed DSS-mice