Research Article

DOI: 10.22146/ifnp.83841 https://journal.ugm.ac.id/ifnp
ISSN 2597-9388

Journal of Indonesian Food and Nutrition Progress

Recovery and Molecular Detection of *Lactobacillus plantarum* Dad-13 from The Feces of Healthy Indonesian Volunteers After Intake of Fermented Milk

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Submitted: April 12th, 2023; Revised: January 2nd, 2025; Revised: March 15th, 2025; Accepted: May 28th, 2025; Published: May 29th, 2025

ABSTRACT: *Lactobacillus plantarum* Dad-13 is a promising probiotic candidate that can be developed as a starter culture in the production of fermented milk and is commonly isolated from *dadih*. However, for the bacteria to be considered a probiotic strain, they must survive and thrive in the human digestive tract. This study focused on investigating the recovery level of *L. plantarum* Dad-13 from fecal samples from healthy volunteers following the consumption of fermented milk. Healthy Indonesian adults (n = 30) were instructed to consume fermented milk containing *L. plantarum* Dad-13 for 20 days. For a duration of 20 days, 30 healthy Indonesian adults were instructed to consume fermented milk containing *L. plantarum* Dad-13. Fecal samples were collected at four distinct time intervals: 10 days before ingestion (F1); on day 10 and day 20 while consuming (F2 and F3, respectively); and 10 days after consumption (F4). The viable count of L. plantarum was evaluated by dilution and plating on *Lactobacillus plantarum* Selective Medium (LPSM). The molecular typing approach utilized repetitive sequence polymerase chain reaction (REP PCR) with the primer BOX A1R. The results indicated that consuming fermented milk led to an increase in the number of lactobacilli and lactic acid bacteria. A total of fifteen (15) isolates of lactic acid bacteria were selected from F1, F2, and F4 periods. The samples obtained from the stool of healthy adults during consumption showed over 99% similarity to *L. plantarum* Dad-13. Thus, the results showed that *L. plantarum* Dad-13, as a probiotic candidate, survives in the gut and can be recovered in fecal material.

Keywords: lactic acid bacteria, dadih, fecal sample, repetitive PCR, primer BOX A1R

INTRODUCTION

Probiotic foods are defined as foods containing bacteria in sufficient quantities and kept alive until the time of consumption, and can be beneficial in maintaining human body health (Anonymous, 2001). Bacteria belonging to natural microflora from fermented foods are stated as safe bacteria, because it has been consumed for thousands of years. The benefits conferred by probiotic bacteria range from treating and preventing diarrhea (McFarland, 2006; Hempel *et al*., 2012), exhibiting antipathogenic properties (Tejero-Sariñena *et al*., 2013; Coman *et al*., 2014) to alleviate constipation symptoms (Indrio *et al*., 2014; Barichella *et al*., 2016; Miller *et al*., 2017). Researchers in Indonesia have conducted studies on the isolation and identification of lactic acid bacteria from various fermented foods, as well as other sources. (Rahayu, 2003; Pramono *et al*., 2008; Lawalata *et al*., 2011; Antara *et al*., 2012; Wikandari *et al*., 2012; Suhartatik *et al*., 2014). The lactic acid bacteria found in fermented foods in Indonesia are predominantly *Lactobacillus plantarum*, with *Pediococcus pentosaceus* and *Streptococcus thermophiles* following in abundance (Rahayu, 2003). Certain indigenous lactic acid bacteria have been studied as potential probiotic agents in Indonesia. (Ngatirah *et al*., 2000; Purwandhani and Rahayu, 2003; Utami *et al*., 2009; Rahayu *et al*., 2011). One of the lactic acid bacteria isolated from *dadih* (fermented milk), which has been studied as a possible candidate for a probiotic agent, is *Lactobacillus plantarum* Dad-13 (Rahayu *et al*., 2016).

Sumaryati (2012) reported that *L. plantarum* Dad-13 has the ability to impede the growth of *E. coli*, the bacteria responsible for diarrhea, in vivo, and it can also affect the fecal microflora of mice that were given *L. plantarum* Dad-13. The combination of *L. plantarum* Dad-13 and

Table 1. Characteristics of the subjects

Aspect	Status
Sex, n	30
• Male	9
• Female	21
Age (years)	18.1 – 22.4
Weight (kg)	45.0 – 72.0
Height (cm)	140.0 – 174.5
BMI (kg/m^2)	18.6 – 22.7
Subjects under treatment (%)	0
Subjects with illness history (%)	0

inulin in the animal diet resulted in increased levels of short-chain fatty acids and lactobacilli populations in the feces, while also decreasing the numbers of coliforms and *E. coli* in the feces of Wistar rats. (Utami *et al*., 2011). The combination of inulin and *L. plantarum* in the animal food potentially prevented diarrhea caused by *E. coli* enteropathogenic-stable toxin in Wistar rats (Utami *et al*., 2010).

Lactobacillus plantarum Dad-13 has been demonstrated to serve as a culture starter in producing fermented drinks that consumers prefer (Rahayu *et al*., 2016). However, the resistance of *Lactobacillus plantarum* Dad-13 in passing through the intestines has not been investigated and proved in detail, molecular identification is therefore needed. The use of molecular techniques for the identification of certain organisms has several advantages, such as being more accurate and faster, and for microbes, it is able to cover the entire microbes. Nowadays, some fingerprinting techniques based on repetitive DNA sequences that occur in the entire bacterial genomes have been developed. The techniques are collectively known as repetitive sequence-based PCR (rep-PCR). Currently, there are three generally used Rep–PCR techniques, which are based on different repetitive elements in the bacteria's chromosomes. The three techniques are repetitive extragenic palindromic (REP-PCR), enterobacterial repetitive intergenic consensus sequence (ERIC-PCR) and BOXA1R Element (BOX-PCR) (Versalovic *et al*., 1994). In this paper, the writers report the recovery of *Lactobacillus plantarum* Dad-13 and molecular detection using BOX - BOX-PCR technique, which generates genomic fingerprints that allow the identification of *Lactobacillus plantarum* Dad-13 in the intestines of healthy adults after consuming fermented milk.

MATERIALS AND METHODS

This research utilized fermented milk containing *L. plantarum* Dad-13, approximately 10^9 CFU (PT. Yummi Food Utama, Indonesia), which was then referred to as a fermented milk drink. The fermented milk drink products were stored in a refrigerator at a temperature of less than 4 °C before consumption. The medium used for enumerating population fecal bacteria was *Lactobacillus plantarum* Selective Medium/LPSM (Merck, Burlington, USA) and MRS (Merck, Burlington, USA), both of which are media suitable for *Lactobacillus*.

Subjects of the Research

Thirty subjects including 9 men and 21 women who were healthy and aged 18-25 and avoided certain types of foods (due to food intolerances or allergies, constipation and intestinal cramps that required a specific diet), did not consume antibiotics/antimycotics or certain drugs and did not use anti-diarrhea and laxative during the 40-day study period involved in this study.

This study was carried out following the clinical research guidelines (Good Clinical Practice/GCP) set forth by the International Conference of Harmonization (ICH) and in compliance with the ethics committee of the Indonesian National Agency for Drug and Food Control (BPOM), Faculty of Medicine, Universitas Gadjah Mada, Indonesia.

Characteristics of the Subjects

Data of the characteristics of the subjects are presented in Table 1. The proportion of female subjects exceeded that of male subjects, with a distribution of 70% female and 30% male. No subject was out or stop until the study was completed. The subjects were adults aged 18.1 to 22.4 years old and weight 45.0 to 72.0 kg. Every subject had a normal BMI between 18.6 to 22.7 kg/m^2.

Research Design

The research included a preparatory phase of 10 days during which consuming fermented milk drink was

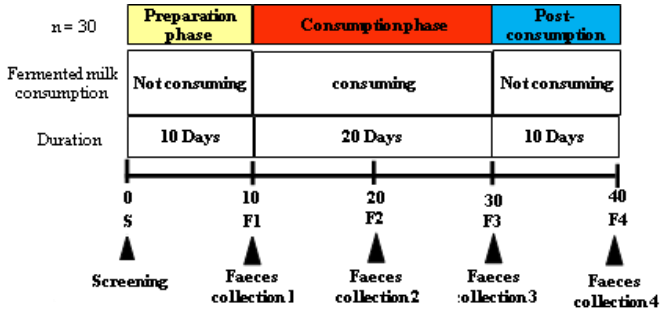

Figure 1. Research Design

abstained, followed by a 20-day phase of consuming the fermented milk drink, and concluded with a 10-day post-consumption period without the fermented milk (Figure 1). During the preparation stage, subjects adhere to their typical dietary practices, with the sole exception of excluding foods containing probiotics. The objective of this stage was to eliminate any potential influences of probiotic intake before the consumption phase. Feces samples were collected on the morning of the 11th day. Subjects were instructed to consume one bottle (185 ml) of a fermented milk beverage daily for a period of 20 days during the consumption phase. Additionally, subjects shared lunch, which consisted of foods and menus provided by the researchers. To ensure the integrity of the study, subjects were instructed to refrain from consuming any other types of fermented milk during this period. At day 21 and 31, the subjects were instructed to collect feces. In the post-consumption phase, the subjects continued to consume their usual foods but were prohibited from consuming any other fermented milk products. On day 41, the subjects were instructed to collect feces. All feces samples underwent analysis, focusing particularly on the content of lactic acid bacteria, the *Lactobacillus,* and *L. plantarum*. Subjects were asked to maintain a daily diary throughout the 40-day study duration. The diary captures information on fermented milk intake (during the consumption phase), other foods eaten, frequency of bowel movements, quality of stools (regarding consistency and color), treatments given, and any reported issues such as diarrhea, constipation, bloating, vomiting, or pain.

Feces Collection

Fecal samples were collected using sterile tubes equipped with a small spoon. The samples were obtained in the home environment of each subject to ensure a natural collection process. The subjects were asked to put the feces on soft paper, and the feces should not be exposed to urine. Then, six spoons of feces were promptly placed into a sterile tube. These samples were then transported to the laboratory in a cooler, maintaining a temperature below 10 °C, and were delivered within a maximum time frame of one hour. Instructions and materials for sample collection were provided to subjects prior to the collection of feces samples. Feces samples, used for molecular detection, were obtained from the subjects number 2, 12, 14, 18, and 30, excluding the third collection (F3) because it was considered the same as F2.

Calculation of the number of lactic acid bacteria (LAB), Lactobacillus, and L. plantarum

Lactic acid bacteria and *Lactobacillus* were enumerated using the dilution and pour plate technique with MRS agar medium and Rogosa agar, while *L. plantarum* was enumerated by the dilution and spread plate method employing LPSM medium. Feces samples were mixed using PBS, 9 times the weight of wet feces. A series of feces suspension dilutions was done using PBS. Each fecal suspension was inoculated into jell medium and incubated at 37 ºC for 48 hours (Harahap *et al*., 2021).

DNA extraction

The isolation of bacterial DNA was purified using the phenol-chloroform method (Harahap *et al*., 2021). A solution of 100 mL of DNA in TE buffer was mixed with 100 mL of phenol-chloroform in a 1:1 ratio, then homogenized and centrifuged at 16,050 x g for 10 minutes. The upper layer obtained was transferred into a new microtube, where it was supplemented with 0.1x the volume of 3M Na-acetate and 2x the volume of absolute ethanol, then incubated at -20 °C overnight. The incubated mixture was then centrifuged at 16,050 x g for 5 minutes. Then, the supernatant was discarded, and the resulting pellet was dry-aired and then added with 500 mL of TE buffer liquid (Harahap *et al*., 2021).

PCR analysis

Repetitive PCR was done for 6.5 hours, 1 mL of genomic DNA with the GoTaq® and Ready To Go (RTG) qPCR kits (Promega, Madison, USA) were mixed in 23 mL of nuclease free water and 1 mL primary BOX A1R (5

'CTACGGCAAGGCGACGCTGACGCTGACG-3') so that the total reaction volume was 25 mL. PCR cycles ran on under specific condition: initial denaturation at a temperature of 95 °C for 4 minutes. This was followed by 30 cycles consisting of denaturation at a temperature of 92 °C for 1 minute, primer attachment at a temperature of 50 °C for 1.5 minute, and polymerization at a temperature of 68 °C for 8 minutes. The polymerization process was continued at a temperature of 65 °C for 10 minutes (Harahap *et al*., 2021).

Fingerprint Analysis

Clustered analysis of repetitive PCR obtained was performed by the NTSYS (Numerical Taxonomy and Multivariate Analysis System) program (Harahap *et al*., 2021). Each isolate having no similarity was identified by 16S rDNA sequence. The process of determining the DNA base sequence was conducted by 1st BASE, Singapore. The sequence similarity of each sample was assessed through BLAST comparisons with the GenBank database. The base sequence data taken from the NCBI international database was used to create the phylogenetic tree using MEGA5 program (version 5.10) (Tamura *et al*., 2011).

RESULTS AND DISCUSSION

Effects of L. plantarum Dad-13 consumption to the number of Lactobacillus plantarum, lactic acid bacteria, and Lactobacillus in the subjects' feces

The growth of *L. plantarum* in LPSM selective media was characterized by milky-white, round-shaped colonies surrounded by a clear yellow zone. Before consuming fermented milk beverages containing *L. plantarum* Dad-13, seven individuals were confirmed to have no detectable *L. plantarum* in their feces, while the remaining thirteen subjects had a small presence of *L. plantarum* in their feces, as shown in Table 2. Following a 10-day period of consuming fermented milk, all subjects exhibited an elevated count of *L. plantarum* in their feces, reaching 7.27 ± 0.5 log CFU/g, which remained consistent after 20 days of consumption.

The number of *L. plantarum* colonies was seen to be increased after 10 and 20 days of consuming fermented milk (Table 2). The result indicated that *L. plantarum* is capable of surviving and growing in the digestive tract, which is a requirement for a bacterial strain to become a probiotic strain. Previous studies reported that the probiotic strain of *Lactobacillus casei* Shirota (LcS) was detected in the feces of healthy subjects who consumed 10.1 log LcS living cells over a span of 4 days, approximately 6.79 ± 0.56 log cells/g feces (Yuki *et al*., 1999). Three times daily consumption of a 100 ml probiotic drink containing *Lactobacillus casei* 10^9 CFU per ml could increase the viability of the probiotics in feces up to 10^7 CFU / g of feces of healthy individuals (Spanhaak *et al*., 1998). The concentration of LcS in feces could attain 8.1 ± 0.9 log CFU/g in subjects who consumed fermented milk with 10^{11} CFU of LcS daily for a period of 7 days (Fujimoto *et al*., 2008). Tuohy *et al*. (2007) indicated that the quantity of LcS in the feces of each subject remained fairly consistent throughout the consumption period, lasting up to 21 days. Similar results were presented by a previous study (Goossens *et al*., 2003)., where after a week of consuming fermented oatmeal with *L. plantarum* 229v, the median number of lactobacilli rose significantly from 4.2 to 8.2 log CFU / g of feces, then became relatively stable over the 4 weeks period of consumption. After one week not consuming fermented oatmeal containing *L. plantarum* average

Table 2. The number of *L. plantarum*, lactic acid bacteria and *Lactobacillus* in the feces sample after *L. plantarum* Dad-13 consumption

Microorganism	Before consumption	After 10 days consumption	After 20 days consumption	After 10 days no consumption
L. plantarum (log CFU/g)	3.08 ± 2.00	7.27 ± 0.53*	6.90 ± 0.79*	3.81 ± 1.37*
Lactic acid bacteria (log CFU/g)	7.56 ± 0.77	8.64 ± 0.59*	8.57 ± 0.54*	8.09 ± 0.71*
Lactobacillus (log CFU/g)	6.25 ± 0.78	7.30 ± 0.50*	6.96 ± 1.41*	5.99 ± 1.20*

* Mean significant differences were analyzed using Wilcoxon paired t-test with $p < 0.05$.

number of *L. plantarum* decreased significantly up to 4.4 log CFU / g (from 2.2 to 6.5 log CFU / g).

After 10-day period without drinking fermented milk, the number of *L. plantarum* decreased (Table 2), yet it was still found in higher numbers compared to the baseline period. The results of this research indicated that *L. plantarum* Dad-13 has the ability to withstand the digestive tract and survive in the feces. It was believed that the rise in the *L. plantarum* population during consumption was due to the presence of the *L. plantarum* Dad-13 colony. Following the discontinuation of consumption, *L. plantarum* was still identified in the feces, which was assumed to be either the indigenous *L. Plantarum* of the subject or that *L. plantarum* Dad-13 remained viable and multiplied in the digestive tract until the 10th day after the consumption had ended. This was supported by the fact that some feces contained higher levels of *L. plantarum* than before fermented drink consumption. However, re-confirmation was still needed molecularly to ensure whether the *L. plantarum* isolate was indeed *L. plantarum* Dad-13.

According to Spanhaak *et al.* (1998), a few days after no longer consuming a probiotic drink, the number of probiotics was likely to return to the initial number of the condition before consuming probiotic drinks. According to Touhy *et al.* (2007), *L. casei* could continue to survive for one week after consuming probiotic fermented drinks was discontinued. It revealed that *L. casei* can grow and double after several days in the digestive tract. *L. casei* in the feces of the subjects was detected after 9, 11, and 14 days of discontinuously consuming fermented drink, as much as 6.1 ± 1.3 $\log_{10}$ CFU/g, 4.7 ± 0.9 $\log_{10}$ CFU/g, and 3.1 LcS±0.5 $\log_{10}$ CFU/g, respectively (Fujimoto *et al*., 2008). Research indicated that probiotic bacteria may have the ability to survive and grow within the digestive system; however, stopping the intake of probiotic beverages for an extended period is likely to decrease the quantity of bacteria present in stool samples. The effect of probiotics on the gut microbiota seems to be associated with their ability to survive and proliferate.

The intake of fermented drinks also increased the amount of lactic acid bacteria and *Lactobacillus* in each feces (Table 2). After consuming fermented drinks for 10 days, there was a notable decline in the amount of lactic acid bacteria known as *Lactobacillus*, although the count remained greater than it was initially. However, it could not simply be explained that the increase in the number of this lactic acid bacteria was due to the consumption of *L. plantarum* Dad-13, or that it could encourage the growth of lactic acid bacteria and the existing indigenous lactobacilli in the digestive tract. Research conducted by Goossens *et al*. (2003) also revealed that the amount of lactic acid bacteria, lactobacilli, and clostridia in feces increased following the consumption of *L. plantarum* 299v.

Identification of L. plantarum isolates

The results of the morphological identification of fifteen isolates obtained from five volunteers with the consumption period F1, F2, and F4 will be analyzed in more detail using a molecular method with repetitive PCR. Macroscopically, the fifteen isolates were found to have a circular shape, varied in size, colored in white and yellow-white, with LPSM media showing yellow color around the colony. Microscopically, the result of Gram staining was purple, which meant that the bacteria isolated was a Gram-positive, with a basil cell shape.

Molecular identification of isolate by repetitive PCR methods using primary BOX A1R

The results of molecular detection of the fifteen isolates taken from the feces of the volunteers were shown in Figure 2. The indigenous isolates taken in the period before consumption had different DNA band patterns among isolates visualized in 2% agarose by UV-VIS analysis. The DNA patterns of the isolates taken at the time of consumption of fermented milk showed similar results to *L. plantarum* Dad-13. From five isolates taken from feces during consumption (F2), three isolates (number 8, 9, and 11) were classified into the same group as *L. plantarum* Dad-13 (number 6). It showed that *L. plantarum* Dad-13, which was contained in the fermented milk, could endure in the digestive tract and could be recovered at the time of consumption.

Repetitive PCR used in research by O. Abaci *et al*, 2011 could separate *C. albicans* strain 109 and six reference strains. In research by Rahayu *et al*., 2011 (Rahayu *et al*., 2011) identification of species of indigenous strains (isolated from several Indonesian fermented foods) has been done using Multiplex Primers but has not been able to distinguish *L. plantarum* Dad-13 with other *L. plantarum* (T-3, Mut-7, Mut-13) so that this study used Repetitive PCR method with primary BOX A1R for molecular detection of the fifteen *L. plantarum* isolates. Repetitive PCR with primary BOX A1R could distinguish *L. plantarum* Dad-13, *L. plantarum* Mut-7, and *L. plantarum* Mut-13 as shown in Figure 2.

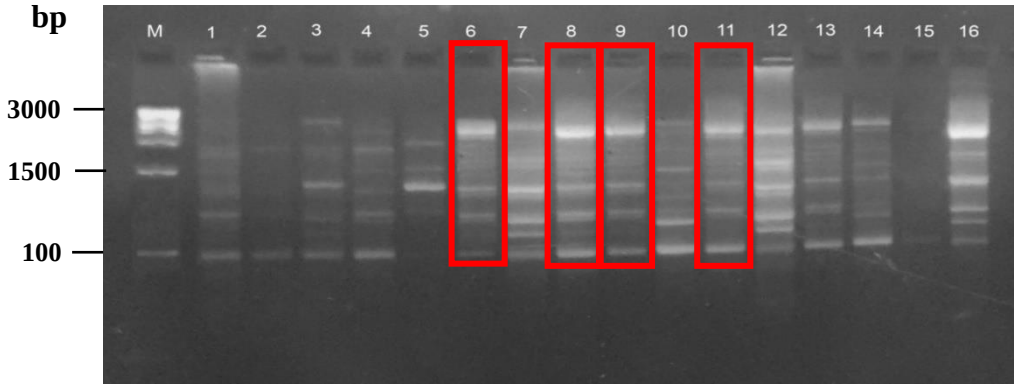

Figure 2. Result of REP-PCR using BOX A1R primers. M: Molecular weight markers (100-3000 base pairs or bp); 1- F1.2; 2- F1.12; 3- F1.14; 4- F1.18; 5- F1.30; 6- *L. plantarum* Dad-13; 7- F2.1; 8- F2.12; 9- F2.14; 10- F2.18; 11- F2.30; 12- F4.2; 13- F4.12; 14- F4.14; 15- F4.18; 16- F4.30. The isolates in red boxes have similar markers.

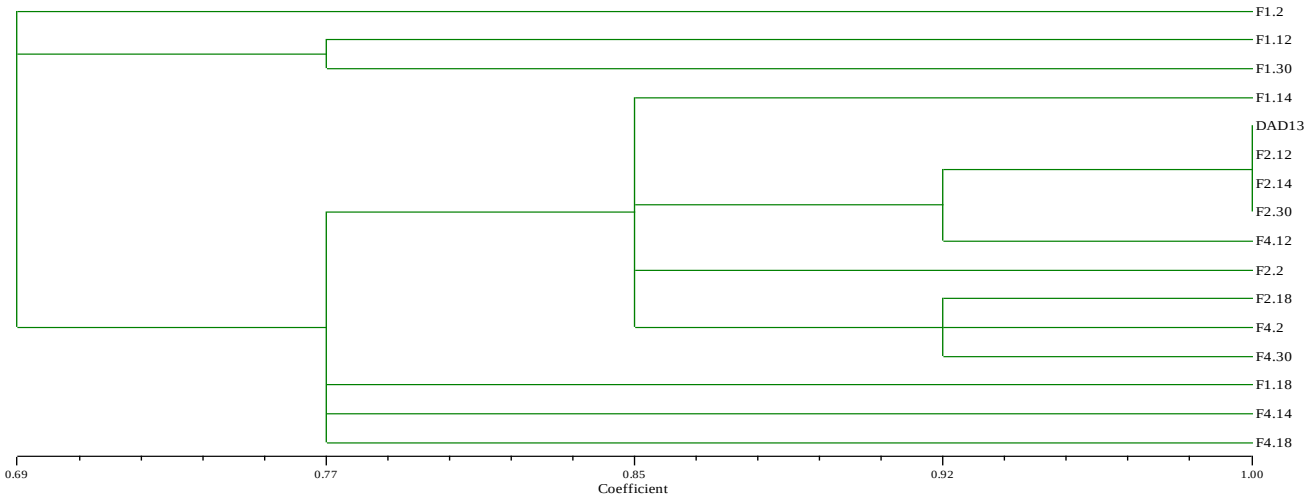

Figure 3. Result of NTSYS program

For isolates taken after the consumption (F4) showed different results with *L. plantarum* Dad-13, but there was closeness between the rep-PCR results taken during the consumption period and after consumption. The closeness (similarity) between the isolates was known by using NTSYS program as shown in Figure 3. The F2.12, F2.14, and F2.30 were the three isolates with strong closeness with the *L. plantarum* Dad-13 (DAD.13) isolate. Thus, the result of NTSYS further supports that *L. plantarum* Dad-13 was found in fecal samples as several isolates have close similarity with the standard isolate of *L. plantarum* Dad-13.

CONCLUSION

The number of *L. plantarum* increased in all subjects' feces after consuming fermented milk containing *L. plantarum* Dad-13 for 10 days and 20 days. After the consumption was halted, the number of *L. plantarum* in the feces decreased, although the presence of *L. plantarum* was still detected. BOX-PCR analysis also revealed the presence of *L. plantarum* Dad-13 in the feces of volunteers during the intake of fermented milk. This suggests that the L. plantarum Dad-13 that had recovered was able to persist in the digestive tract.

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