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Comparative Evaluation of Culture Media for Quantification of Lactic Acid Bacteria in Various Dairy Products

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Dairy products are extensively used as carriers of probiotic strains that have potential health benefits. Assessment of the viability of probiotic strains during manufacturing is important to ensure that products meet recommended levels. Hence, the method for accurately quantifying lactic acid bacteria (LAB) in probiotic or dairy products is required. The present study aims to examine the performance of de-Man Rogosa Sharpe (MRS), plate count agar with bromocresol purple (PCA with BCP), and glucose blood liver (BL) agars recommended in the Korea Food Code guidelines for counting LAB. Analysis of the performance of culture media containing 19 lactic acid bacterial species commonly encountered in probiotic and dairy products showed no statistically significant difference between 18 reference strains and three culture media ($p > 0.01$). Furthermore, the suitability of three culture media was verified for the quantitative assessment of LAB in 25 probiotic and dairy products. The number of LAB in three culture media was determined to be more than 10^7 colony-forming unit (CFU)/ml for fermented milk products and 10^8 CFU/ml for condensed fermented milk and probiotic products, indicating that they all satisfied the Korea Food Code guidelines. Moreover, there was no statistically significant difference in the amount of LAB counted in all three culture media, suggesting that they can be used to isolate or enumerate LAB in commercial products. Finally, three culture media will be useful for isolating and enumerating LAB from fermented foods as well as gut microflora.

Keywords: **Lactic acid bacteria, fermented milk product, quantitative medium, statistical analysis**

Introduction

Lactic acid bacteria (LAB) are crucial in the food industry, agriculture, and in clinical fields. They play an important role in fermented foods and are also used as probiotics because they have beneficial effects on humans and animals [1]. Several genera, such as *Lactobacillus*, *Leuconostoc*, *Pediococcus*, and *Weissella*, belong to LAB. Furthermore, *Lactobacillus*, *Lactococcus*, and *Streptococcus* are employed as commercial starters, while *Lactobacillus* and *Bifidobacterium* are used as probiotics [1]. Starters like *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Bifidobacterium animalis* are also used in the production of fermented milk or cheese to obtain the health benefits from dairy products [2–4]. Moreover, bacterial strains in probiotics have health-promoting effects, such as stimulation of the immune system, proliferation of beneficial bacteria, and decreases in pathogen growth and cholesterol levels [5]. The benefits of LAB have led to an expansion of the

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global market for probiotic products. It is anticipated that the global probiotics market will grow by 8.3% from $ 61.1 billion in 2021 to $ 91.1 billion in 2026 [6].

The recommended concentration of probiotic strains in products is greater than 10^8 colony-forming units (CFU)/ml. However, several studies have shown the low viability of probiotic strains in commercial products [7, 8]. These studies show that the need to monitor the survival of probiotic strains in fermented products has often been disregarded, resulting in a large number of products in the market containing low concentrations of viable bacteria [9]. Furthermore, the viability of the LAB strains must be monitored in order to assess the quality of probiotic or dairy products. This low viability issue of probiotics necessitates a routine method for the selective enumeration of LAB strains in products. Consequently, LAB enumeration technologies have been continually investigated and improved as the probiotics and dairy market has expanded and the diversity of probiotic products has increased [10]. The International Organization for Standardization (ISO) has been using culture media to quantify and isolate viable LAB, while other methods, such as fluorescence *in situ* hybridization (FISH), real-time quantitative polymerase chain reaction (PCR), and flow cytometry, or fluorescence cell sorting, have been employed for non-culturing LAB [11, 12].

The most prevalent method for the enumeration or isolation of viable LAB cells employs the culture-dependent method using specific culture media. ISO and the International Dairy Federation recommend using de-Man Rogosa Sharpe (MRS)-clindamycin-ciprofloxacin agar and transgalactose oligosaccharides-mupirocin lithium salt (TOS-MUP) agar to grow *Lactobacillus acidophilus* and *Bifidobacterium* selectively in milk and probiotic products [13]. M17 agar and MRS agar acidified with acetic acid (pH 5.4) are recommended by IDF the for enumeration of *Streptococcus thermophilus* and *L. delbrueckii* subsp. *bulgaricus*, respectively [9, 14]. Plate count agar with bromocresol purple (PCA with BCP) or TOS-MUP medium was used to selectively isolate or count only *Lactobacillus* and *Lactococcus* or *Bifidobacterium* [15]. Moreover, several researchers have used a variety of strategies to isolate specific species from environments with a mixture of different LAB. Furthermore, some researchers have used antibiotics such as vancomycin to isolate *Limosilactobacillus fermentum*, while others have used specific media containing sugar for the isolation of *Lactiplantibacillus plantarum* [16–18]. These media may be used to isolate particular species; however, it is challenging to include all LAB genera that are utilized in probiotic products. Culture media such as MRS-based agar, tryptose proteose peptone yeast extract, reinforced clostridial prussian blue, and glucose blood liver (BL) agar were most widely used to enumerate or isolate various LAB genera such as *Lactobacillus*, *Weissella*, *Pediococcus*, *Lactococcus*, *Bifidobacterium*, and *Streptococcus*. The Korean Food and Drug Administration suggests using MRS, PCA with BCP, and BL agars for counting the number of LAB in probiotic or dairy products.

The objective of the present study is to compare the three culture media (MRS, PCA with BCP, and BL) recommended by the Korea Food Code for the quantification of LAB species. The performance of the media was evaluated by counting LAB in commercial probiotic or dairy products.

Materials and Methods

Bacterial strains

In this study, 19 strains (15 type strains and 4 reference strains) were used: *Bifidobacterium bifidum* KACC 20601^T, *B. animalis* KACC 16638^T, *Bifidobacterium breve* KACC 16639^T, *Bifidobacterium longum* subsp. *longum* KCTC 3128^T, *Lacticaseibacillus casei* KCTC 3109^T, *Lacticaseibacillus paracasei* KCTC 3165, *Lacticaseibacillus rhamnosus* KCTC 13088, *L. plantarum* KCTC 3108^T, *L. acidophilus* KCTC 3164^T, *L. delbrueckii* subsp. *bulgaricus* KACC 12420^T, *L. fermentum* KACC 11441^T, *Lactobacillus gasseri* KACC 12424^T, *Lactobacillus helveticus* KACC 12418^T, *Ligilactobacillus salivarius* KCTC 3600, *Limosilactobacillus reuteri* KCTC 3594^T, *Enterococcus faecalis* KCTC 5290, *Enterococcus faecium* KACC 11954^T, *Lactococcus lactis* KCTC 3769^T, *S. thermophilus* KACC 11857^T (Table 1). Reference strains were obtained from the Korean Collection for Type Cultures (KCTC, Korea) and the Korean Agricultural Culture Collection (KACC, Korea). MRS agar (Difco, SUSA) was used to culture all reference strains except *Bifidobacterium* strains, and *Bifidobacterium* species were cultured on Bifidobacterium agar (Difco). The well-formed colonies were then selected and activated in

Table 1. Statistical analysis results of the number of LAB using quantification media in the reference strains.

Strain	P-value	No. of LAB (log CFU/ml) [1]		
		MRS	PCA with BCP	BL
Bifidobacterium bifidum KACC 20601^T	4.61E-06	8.79 ± 0.04 a	7.53 ± 0.11 b	8.74 ± 0.11 a
Bifidobacterium animalis KACC 16638^T	0.949	8.22 ± 0.13 a	8.24 ± 0.16 a	8.26 ± 0.16 a
Bifidobacterium breve KACC 16639^T	0.0353	8.02 ± 0.06 a	7.91 ± 0.28 a	7.49 ± 0.18 a
Bifidobacterium longum subsp. *longum* KCTC 3128^T	0.0751	8.55 ± 0.08 a	8.51 ± 0.11 a	8.79 ± 0.17 a
Lacticaseibacillus casei KCTC 3109^T	0.829	8.34 ± 0.04 a	8.34 ± 0.03 a	8.32 ± 0.09 a
Lacticaseibacillus paracasei KCTC 3165	0.453	8.80 ± 0.01 a	8.79 ± 0.11 a	8.88 ± 0.10 a
Lacticaseibacillus rhamnosus KCTC 13088	0.423	8.07 ± 0.05 a	8.11 ± 0.02 a	8.18 ± 0.16 a
Lactiplantibacillus plantarum KCTC 3108^T	0.0189	8.26 ± 0.04 a	8.26 ± 0.01 a	8.19 ± 0.00 a
Lactobacillus acidophilus KCTC 3164^T	0.49	7.92 ± 0.08 a	7.87 ± 0.13 a	7.77 ± 0.21 a
Lactobacillus delbrueckii subsp. *bulgaricus* KACC 12420^T	0.103	8.07 ± 0.02 a	8.18 ± 0.01 a	8.08 ± 0.10 a
Lactobacillus fermentum KACC 11441^T	0.0673	8.58 ± 0.08 a	8.74 ± 0.04 a	8.69 ± 0.08 a
Lactobacillus gasseri KACC 12424^T	0.0667	8.37 ± 0.02 a	8.41 ± 0.04 a	8.32 ± 0.05 a
Lactobacillus helveticus KACC 12418^T	0.435	8.19 ± 0.09 a	8.20 ± 0.14 a	8.08 ± 0.13 a
Ligilactobacillus salivarius KCTC 3600	0.109	7.89 ± 0.61 a	8.32 ± 0.10 a	7.57 ± 0.11 a
Limosilactobacillus reuteri KCTC 3594^T	0.958	8.03 ± 0.26 a	8.02 ± 0.22 a	8.07 ± 0.20 a
Enterococcus faecalis KCTC 5290	0.258	8.47 ± 0.10 a	8.52 ± 0.09 a	8.61 ± 0.09 a
Enterococcus faecium KACC 11954^T	0.882	8.72 ± 0.04 a	8.73 ± 0.02 a	8.71 ± 0.08 a
Lactococcus lactis KCTC 3769^T	0.394	8.33 ± 0.05 a	8.33 ± 0.02 a	8.42 ± 0.14 a
Streptococcus thermophilus KACC 11857^T	0.184	8.38 ± 0.03 a	8.32 ± 0.11 a	8.50 ± 0.14 a

[1] Data values are indicated as the mean ± standard deviation. The different letters between culture media (a-b) indicate significant differences at $p < 0.01$, Duncan's multiple range test. ^{T}, type strain

broth medium for further studies.

Preparation of MRS, PCA with PCP, and BL media

The MRS medium powder (Difco) was dissolved in 1 L of distilled water. The PCA with BCP medium powder (MB Cell, Korea) was dissolved in 1 L of distilled water. The BL medium powder (MB cell) was dissolved in 950 ml distilled water. All prepared media were heated with stirring and boiled for 2 min to completely dissolve the solids, and then autoclaved. Defibrinated horse blood (5%) was added aseptically after cooling to 50℃ for BL medium.

Enumeration of reference strains on three culture media

Reference strains were cultured in MRS or Bifidobacterium broth. The cell suspensions were centrifugated at 16,000 ×g for 3 min and the obtained cell pellet washed with sterile saline (0.85% NaCl, w/v) solution. The washed pellet was resuspended in a sterile saline solution to give an optical density of 1.0 at 600 nm. The LAB strains were serially diluted using sterile saline solution, and an aliquot of the cells was inoculated with MRS, PCA with BCP, and BL agar using pouring or spreading methods. For the pouring method, 1 ml of the cells was added to a sterilized petri dish, and about 15 ml of cooled culture medium was added and mixed while swirling. Then, 5 ml of the same culture medium was overlaid to prevent colony spread after solidification. For the spreading method, 0.1 ml of the diluted cells were spread onto BL agar. Plate inoculated each reference strain except *L. delbrueckii* subsp. *bulgaricus* and *S. thermophilus* was incubated at 35℃ for 72 h under anaerobic conditions. Plates inoculated with *L. delbrueckii* subsp. *bulgaricus* and *S. thermophilus* were incubated at 42℃ for 72 h under anaerobic conditions. A colony count was performed after the completion of the incubation period.

Enumeration of commercial probiotic and dairy products

A total of 25 commercial products (8 fermented milk

products, 10 condensed fermented milk products, and 7 probiotic products) were purchased at the local markets in Korea. The number of LAB in commercial probiotic or dairy products was counted according to the Korea Food Code. Briefly, each sample was shaken vigorously, and 25 g of each sample was mixed with 225 ml of sterile saline solution and homogenized at 230 rpm for 2 min using a stomacher (Circulator stomacher 400; Seward Limited, UK). The homogenate was serially diluted, and the diluted sample was inoculated into each culture medium as described above. The pouring method was followed for organisms growing in MRS and PCA with BCP agars. On the BL agar plate, 0.1 ml of each diluted sample was spread. All plates were incubated at 35℃ for 72 h under anaerobic conditions. The colonies appearing on each culture medium were counted after the end of the incubation period.

Statistical analysis

The experiments were performed in triplicate and repeated three times. All of the data were analyzed using R version 4.1.0 to determine significant differences ($p < 0.01$) between the mean value by one-way analysis of variance (ANOVA) analysis and Duncan's multiple range test. The p value (probability value) less than 0.01 indicated statistically significant differences [11, 19].

Results and Discussion

Effect of culture media on the growth of reference strains

Several studies have developed a variety of culture media to isolate or enumerate LAB, including MRS for lactobacilli; MRS-salicin agar for *L. casei*, *L. paracasei*, and *L. rhamnosus* [20]; MRS-clindamycin for *L. acidophilus* group species [21]; reinforced clostridial 5.3 agar for *L. delbrueckii* subsp. *bulgaricus* [20]; and M17 agar for lactococci [22]. Furthermore, MRS, PCA with BCP, and BL agars are recommended for the detection and enumeration of several LAB species present in food sources [12].

In Korea, 19 LAB species, including *B. bifidum*, *B. animalis* subsp. *lactis*, *B. breve*, *B. longum*, *L. casei*, *L. paracasei*, *L. rhamnosus*, *L. plantarum*, *L. acidophilus*, *L. delbrueckii* subsp. *bulgaricus*, *L. fermentum*, *L. gasseri*, *L. helveticus*, *L. salivarius*, *L. reuteri*, *E. faecalis*, *E. faecium*, *Lc. lactis*, and *S. thermophilus* are allowed as probiotics and are publicly notified ingredients that can be applied to food products. Furthermore, although LAB are detected in food samples by detection methods such as PCR and metagenome sequencing, they are only occasionally isolated using culture media with anaerobic conditions designed for LAB [7, 8, 23]. However, the efficacy of commercially available culture media for the enumeration of nineteen LAB species using the culture-dependent method has not been evaluated. Therefore, in this study, we compared the culture media for representative LAB species, evaluating three basal media, including MRS, PCA with BCP, and BL agars.

The growth performance of 19 species of representative probiotics was examined in three culture media. The number of colonies of each reference strain that appeared on the three different culture media under anaerobic conditions is shown in Table 1. The mean for the number of LAB ranged between 7.49 ± 0.18 and 8.88 ± 0.10 log CFU/ml. Further, the mean number of LAB was 8.32 ± 0.31 log CFU/ml on MRS agar, whereas it was 8.28 ± 0.33 and 8.30 ± 0.41 log CFU/ml on PCA with BCP and BL agars, respectively (Fig. 1A). Moreover, the significance of the correlation between the number of species and the three culture media was determined using ANOVA analysis. The analysis of variance showed no statistical difference ($p < 0.01$) between the three media for all species, except *B. bifidum* KACC 20601^T. This result suggests that the three culture media were effective in growing all species except *B. bifidum* KACC 20601^T.

For enumeration of *Bifidobacterium*, BL and *Bifidobacterium* agar media are used [24]. However, *Bifidobacterium* medium is more suitable for isolating *Bifidobacterium* strains. A previous study compared *Bifidobacterium* agar medium to BL medium to confirm their performance for enumerating *Bifidobacterium*, and similar colony numbers were obtained on both media [24, 25]. However, lower colony numbers on *Bifidobacterium* agar medium compared to BL medium were observed for some *Bifidobacterium* species. Therefore, we counted *Bifidobacterium* strains using BL medium.

B. bifidum KACC 20601^T produced significantly different cell numbers on PCA with BCP agar (7.53 ± 0.11 log CFU/ml) from the cell numbers on MRS and BL agars (8.79 ± 0.04 and 8.74 ± 0.11 log CFU/ml, respectively). The number of colonies on PCA with BCP agar was approximately 16 times less than the number of

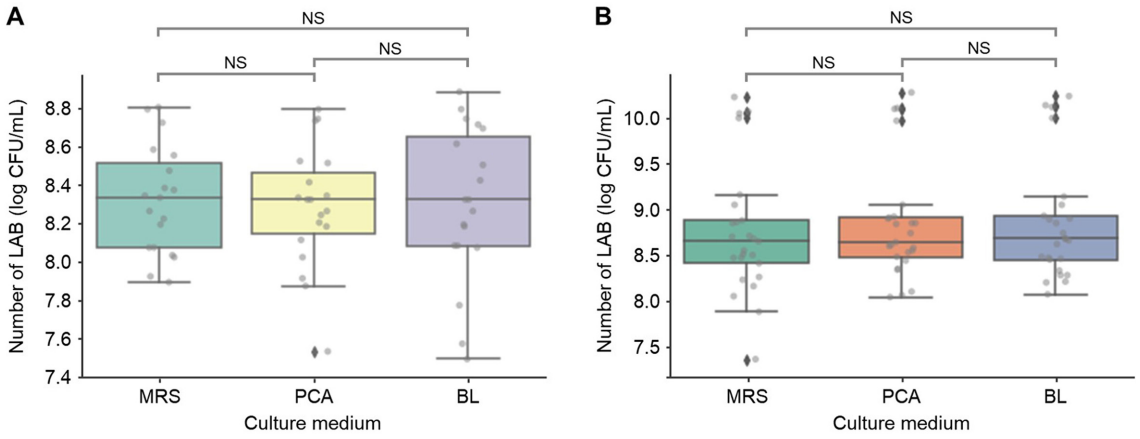

Fig. 1. (A) Average number of reference strains cultured on MRS, PCA with BCP, and BL agar, (B) Average number of LAB strains in probiotic and dairy products on three culture media. NS, not significant ($p \geq 0.05$).

cells produced on other media. PCA with BCP media (PCA supplemented with bromocresol purple) is one of the most common culture media used for the enumeration of lactobacilli [26, 27]. Lactobacilli form easily distinguishable, yellow-colored colonies in the depth or on the surface of medium. Although it is the media routinely used for quality control in the manufacture of dairy products, in this study, *B. bifidum* KACC 20601^T strain grew poorly compared to MRS and BL agars. Furthermore, a previous study has reported that *B. bifidum* does not grow well on PCA with BCP medium even under anaerobic conditions, which is consistent with our study [26]. Another previous study has shown that *B. bifidum* strain grew poorly on Bifidobacterium medium (BFM) compared to other *Bifidobacterium* species [25]. BFM contains propionic acid, lithium chloride, and methylene blue, which inhibit the growth of some LAB species. Similarly, PCA with BCP agar contains bromocresol purple to selectively isolate LAB, which may inhibit the growth of *B. bifidum* strain as well as non-LAB strains. These results suggest that PCA with BCP is not suitable for the enumeration of *B. bifidum*.

Quantification of LAB in commercial products

The majority of probiotic or dairy products contain one type of LAB as well as mixed cultures with various genera of LAB species. In 2017, 85 probiotic products in Korea were investigated, and *L. acidophilus* was the most frequently used LAB species (81.2%), followed by *B. animalis* subsp. *lactis* (77.6%), *L. rhamnosus* (75.3%), *B. bifidum* (72.9%), *L. plantarum* (68.2%), and *B. longum* (65.9%) [19]. In addition, the case of products mixed with seven different species accounted for 24.7%, followed by nineteen (11.8%) and six (11.8%) LAB mixtures, with an average of nine LAB combined in the products [19]. In the Korea Food Code, the standard for the number of LAB in fermented milk products is more than 10^7 CFU/ml, while the level for condensed fermented milk products and probiotic products is more than 10^8 CFU/ml [28].

The enumeration of LAB in 25 commercial products was performed to evaluate the use of three different culture media. As a result, the number of LAB was found to be greater than 10^7 CFU/ml for fermented milk products (7.35 ± 0.45 to 8.92 ± 0.05 log CFU/ml), 10^8 CFU/ml for condensed fermented milk products (8.04 ± 0.08 to 9.15 ± 0.11 log CFU/ml), and 10^8 CFU/ml for probiotic products (8.22 ± 0.28 to 10.27 ± 0.02 log CFU/ml) in three culture media. Hence, all three media were suitable for the Korea Food Code standards (Table 2). The average number of LAB on MRS, PCA with BCP, and BL agars were 8.74 ± 0.71, 8.82 ± 0.63, and 8.82 ± 0.66 log CFU/ml, respectively (Fig. 1B). The quantity of LAB was largest in BL agar, followed by PCA with BCP and MRS agars. In a similar study, Oh *et al.* (2015) compared culture media (BCP plate count agar, PCA with BCP, and MRS) to quantify LAB in yogurt, and the number of colonies was highest on BCP plate count agar, followed by MRS and PCA with BCP agars [28]. One probiotic product

Table 2. The number of LAB using three culture media in commercial products.

Product	Type	P-value	No. of LAB (log CFU/ml) [1]		
			MRS	PCA with BCP	BL
P1	Probiotic product	0.37	10.22 ± 0.04 a	10.27 ± 0.02 a	10.23 ± 0.05 a
P2	Probiotic product	0.786	8.22 ± 0.28 a	8.43 ± 0.17 a	8.46 ± 0.71 a
P3	Probiotic product	0.896	8.69 ± 0.52 a	8.83 ± 0.56 a	8.61 ± 0.65 a
P4	Probiotic product	0.357	8.50 ± 0.11 a	8.59 ± 0.08 a	8.67 ± 0.17 a
P5	Fermented milk	0.0398	7.35 ± 0.45 a	8.03 ± 0.18 a	8.27 ± 0.35 a
P6	Condensed fermented milk	0.114	8.15 ± 0.36 a	8.60 ± 0.09 a	8.65 ± 0.26 a
P7	Fermented milk	0.125	8.40 ± 0.05 a	8.47 ± 0.18 a	8.20 ± 0.15 a
P8	Condensed fermented milk	0.31	8.49 ± 0.11 a	8.54 ± 0.07 a	8.44 ± 0.02 a
P9	Fermented milk	0.0693	8.46 ± 0.16 a	8.63 ± 0.08 a	8.27 ± 0.18 a
P10	Probiotic product	0.241	10.06 ± 0.02 a	10.10 ± 0.04 a	10.11 ± 0.04 a
P11	Probiotic product	0.829	9.99 ± 0.06 a	9.96 ± 0.12 a	9.99 ± 0.04 a
P12	Probiotic product	0.0863	10.04 ± 0.04 a	10.09 ± 0.06 a	10.13 ± 0.02 a
P13	Fermented milk	0.0882	8.84 ± 0.02 a	8.90 ± 0.03 a	8.92 ± 0.05 a
P14	Fermented milk	0.649	8.46 ± 0.10 a	8.52 ± 0.14 a	8.45 ± 0.02 a
P15	Fermented milk	0.129	8.70 ± 0.04 a	8.73 ± 0.04 a	8.67 ± 0.01 a
P16	Fermented milk	0.924	8.87 ± 0.12 a	8.89 ± 0.03 a	8.89 ± 0.03 a
P17	Fermented milk	0.0292	7.87 ± 0.06 a	8.09 ± 0.03 a	8.19 ± 0.17 a
P18	Condensed fermented milk	0.0326	8.64 ± 0.09 a	8.84 ± 0.09 a	8.84 ± 0.05 a
P19	Condensed fermented milk	0.975	9.04 ± 0.02 a	9.04 ± 0.03 a	9.04 ± 0.03 a
P20	Condensed fermented milk	0.563	8.85 ± 0.09 a	8.91 ± 0.06 a	8.88 ± 0.08 a
P21	Condensed fermented milk	0.0941	8.54 ± 0.08 a	8.33 ± 0.17 a	8.32 ± 0.07 a
P22	Condensed fermented milk	0.0444	9.15 ± 0.11 a	8.84 ± 0.12 a	9.13 ± 0.16 a
P23	Condensed fermented milk	0.0651	8.25 ± 0.07 a	8.34 ± 0.10 a	8.47 ± 0.10 a
P24	Condensed fermented milk	0.916	8.04 ± 0.08 a	8.05 ± 0.03 a	8.06 ± 0.02 a
P25	Condensed fermented milk	0.084	8.67 ± 0.11 a	8.57 ± 0.04 a	8.73 ± 0.04 a

[1] Data values are indicated as the mean ± standard deviation. The same letters between culture media indicate no significant difference, Duncan's multiple range test.

(P11) contained *B. bifidum*, a species that did not grow well on PCA with BCP medium. Unlike pure culture, there was no difference in viability among the three media. In this product, not only *B. bifidum* but also several LAB species were mixed, so it was not possible to evaluate the difference in the viability of *B. bifidum* alone.

The number of LAB in each commercial product that appeared on the three different culture media (MRS, PCA with BCP, and BL) under anaerobic conditions is shown in Table 2. The 25 commercial products (8 fermented milk products, 10 condensed fermented milk products, and 7 probiotic products) contained between 7.35 ± 0.45 to 10.27 ± 0.02 log CFU/ml (Table 2). Furthermore, the p value was ≥ 0.0292 based on the analysis of the variance test. This result indicates that there was no statistically significant difference between the culture media. Therefore, MRS, PCA with BCP, and BL agars were confirmed to be suitable for culturing LAB as recommended by the Korea Food Code.

Conclusions

In this study, the performance of three culture media (MRS, PCA with BCP, and BL) used for counting LAB in the Korea Food Code was compared. Our results demonstrated that LAB can be quantified in a consistent manner regardless of the three types of culture media (i.e.,

MRS, PCA with BCP, and BL agars). However, MRS and BL agars were more effective than PCA with BCP agar in growing *B. bifidum*. The recovery on all three media agars suggests that they can be employed for the enumeration of LAB in routine monitoring of probiotic or fermented dairy products, such as cheese and yogurt. Furthermore, our results indicate improved precision of the quantitative test technique for probiotic products and might even serve as the foundation for implementing and updating the LAB test method. To further increase the efficiency of LAB testing, it is necessary to consider the introduction of the rapid test method for LAB along with the continuous evaluation of the culture medium.

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Conflict of Interest

The authors have no financial conflicts of interest to declare.

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