Effect of Enumeration Method on *Bifidobacterium* Cell Counts in Commercial Powder Products

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Received April 5, 2010; Accepted May 20, 2010

In this study, the effects of enumeration methods on viable bifidobacterial counts in powder products were examined to determine the most appropriate enumeration conditions. The bifidobacterial counts of nine commercial powder products containing *Bifidobacterium* species were determined by a pour plate method using different diluents including Mitsuoka's buffer and Ringer's solution. Mitsuoka's buffer gave significantly higher bifidobacterial counts than Ringer's solution when used as diluent for eight of nine commercial powder products containing various *Bifidobacterium* species. The counts obtained with Ringer's solution were on average 51.6% of those obtained using Mitsuoka's buffer. Addition of Tween 80 or phosphate buffer to Ringer's solution increased the bifidobacterial counts. Also, the diluent had a significantly greater impact in the suspension step than in the subsequent dilution step. In conclusion, the diluent has great effect on the enumeration of bifidobacteria when using the plate count method. Also, the composition of diluents influences bifidobacterial counts.

Key words: *Bifidobacterium*; enumeration; probiotics; infant formula; Mitsuoka's buffer

INTRODUCTION

Bifidobacterium has been extensively studied since its first discovery by Tisser about one hundred years ago. Research conducted by many scientists over the years has shown that bifidobacteria have various physiological effects on consumers (16, 17, 24). Currently, bifidobacteria have been used in various bifidobacterial foods including dairy products (yogurt, drink and cheese), powdered formula for infants, and health foods (1, 6, 19). In particular, bifidobacteria-containing powdered formula for infants or children have recently become popular worldwide. Some possible reasons for this are that many scientists have demonstrated the importance of bifidobacteria for the health of infants (21) and that stable bifidobacteria powders have been developed for use in commercial powder products (2). With a wide variety of probiotics-containing products available in the food market, consumers are faced with the difficulty of deciding which products to choose. To help consumers with selection, one judgment criteria is the viable bacterial count labeled on the products. As probiotics are defined by the FAO/WHO Working Group (9), the advantage of displaying the viable bacterial counts derives from the concept that live probiotic bacteria may improve the intestinal environment (7, 20).

Several techniques such as culture methods (5, 18, 20), real-time PCR methods (13, 23) and flow cytometric methods (10, 11) have been used to determine viable bifidobacterial counts. Real-time PCR methods and flow cytometric methods have rapidly gained popularity, but are not yet in routine use, while the quantitative RT-PCR method (12) requires further investigation.

Enumeration by the culture method is a relatively easy technique and because of this its applicability to qualitative investigations (8, 18) will continue to be important for many years to come. Recently, Abe et al. (3) reported that in the plate counting method, the suspension-dilution medium and plating medium affected the viable counts of various *Bifidobacterium* species, and that the viable counts differed greatly depending on the diluents or plating media used in the plate count. In particular, they highlighted the importance of choosing the diluent and recommended Mitsuoka's buffer (14), which is composed of phosphate buffer, cysteine, Tween 80 and agar, as one of the best diluents for bifidobacteria. However, they did not provide any information on why Mitsuoka's buffer is better than the other diluents or how commercial powder products are affected by it. Determining the correct viable counts of probiotics in commercial powder products is very important for evaluating the quality of the products. Therefore, further investigation of the effects of Mitsuoka's buffer on bifidobacterial counts is necessary to enable the correct determination of bifidobacterial counts in commercial powdered products.

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Table 1. List of commercial powder products containing bifidobacteria and the bifidobacterial counts

Product	Product Type	Species [‡]	Country	Bifidobacterial count (CFU g ⁻¹)		Relative value
				Mitsuoka's buffer	Ringer's solution	
A	PI	<i>B. longum</i>	Japan	1.2 ± 0.17 × 10 ^{11†}	6.7 ± 1.4 × 10 ¹⁰	55.8%
B	SS	<i>B. breve</i>	Japan	4.0 ± 0.39 × 10 ^{9†}	1.4 ± 1.4 × 10 ⁹	35.0%
C	PF	<i>B. animalis</i>	Europe	5.9 ± 0.37 × 10 ^{6†}	1.6 ± 0.36 × 10 ⁶	27.1%
D	PF	<i>B. longum</i>	Indonesia	8.3 ± 0.48 × 10 ^{6†}	6.0 ± 0.98 × 10 ⁶	72.3%
E	PF	<i>B. animalis</i>	China	6.0 ± 2.4 × 10 ^{5†}	2.2 ± 0.94 × 10 ⁵	36.7%
F	PF	<i>B. animalis</i>	Europe	2.7 ± 0.09 × 10 ^{6†}	7.0 ± 0.67 × 10 ⁵	25.9%
G	PF	<i>B. animalis</i>	Europe	6.8 ± 0.66 × 10 ⁶	6.1 ± 0.18 × 10 ⁶	89.7%
H	PF	<i>B. longum</i>	Indonesia	5.2 ± 0.12 × 10 ^{6†}	2.4 ± 0.96 × 10 ⁶	46.2%
I	PF	Mix [#]	Indonesia	1.9 ± 0.1 × 10 ^{7†}	1.4 ± 0.15 × 10 ⁷	73.7%

Bifidobacterial counts are the mean ± S.D., which were obtained from three repeated tests except for Product A (12 repeated tests). Relative values are the ratios of bifidobacterial counts using Ringer's solution versus bifidobacterial counts using Mitsuoka's buffer for the same product. Agar was omitted from Mitsuoka's buffer during the dilution step.

PI; Probiotic ingredient powder, SS; Stick type supplement, PF; Powdered formula.

[‡]: Each species isolated from each product was identified by the PCR method using the specific primers for each species as reported previously (Ventura et al., 2001; Matsuki et al., 2004).

[#]: Contained a mixture of *B. longum* and *B. breve*

[†]: Significantly different from counts obtained Ringer's solution ($p < 0.05$).

The aim of this study was to confirm the effect of Mitsuoka's buffer on bifidobacterial counts and to elucidate the factors affecting viable bifidobacterial count determination.

MATERIALS AND METHODS

Commercial powder products

Nine bifidobacteria-containing commercial powder products sold in the global market (Product A to Product I) were used in the bifidobacteria enumeration tests. The product types and bifidobacteria species used are shown in Table 1. Product A was a commercial powder ingredient containing *Bifidobacterium longum* supplemented with cornstarch. Product B was a commercial powder food containing *Bifidobacterium breve* packed in sticks. Products C, D, E, F, G, H, and I were powdered formulas for infants or children, which are sold in various countries. These powdered formulas contain *B. longum*, *B. animalis* subsp. *lactis* or both *B. longum* and *B. breve*. The *Bifidobacterium* species isolated from each product were identified by the PCR method using specific primers for each species as described in previous reports (13, 22). All products were stored under appropriate conditions and tested before the end of their shelf lives.

Diluents for enumeration

Four diluents comprising Mitsuoka's buffer (4.5 g potassium dihydrogen phosphate, 6.0 g disodium hydrogen phosphate, 0.5 g L-cysteine HCl, 0.5 g Tween-80, and 1.0 g BactoTM agar per 1,000 ml water), quarter

strength Ringer's solution (1 tablet per 500 ml) (Ringer's Solution; Merck), peptone water (1.0 g casein peptone per 1,000 ml), and buffered peptone water (10 g BactopectonTM, 5.0 g sodium chloride, 9.0 g disodium hydrogen phosphate dodecahydrate, and 1.5 g potassium dihydrogen phosphate per 1,000 ml water) were used for the comparison of the effects of diluents on bifidobacterial counts in commercial powder products. Except Ringer's solution, all ingredients were purchased from Becton Dickinson Japan (Tokyo, Japan), Kanto Chemical (Hyogo, Japan) or Nacalai Tesque Inc. (Kyoto, Japan).

To observe the effects of the constituents of Mitsuoka's buffer on the bifidobacterial count of powder products, Ringer's solution and Mitsuoka's buffer were modified. Specifically, phosphate salts (potassium dihydrogen phosphate and disodium hydrogen phosphate), L-cysteine or Tween 80 was added to Ringer's solution at the same concentration as used in Mitsuoka's buffer. Also, Tween 80, L-cysteine or both were omitted in preparing Mitsuoka's buffer.

Enumeration of bifidobacteria

For the enumeration of bifidobacteria in the various powder products, 1 g (for Product A and B) or 5 g (for Product C to I) of each sample were suspended thoroughly in 99 ml or 95 ml of diluent at room temperature and serially diluted with the same diluent to an appropriate concentration range, as reported previously (3). During the dilution step, agar was omitted from Mitsuoka's buffer to improve the fluidity. The

Table 2. Effect of adding substances to Ringer's solution on bifidobacterial counts

Diluent	Bifidobacterial count (CFU g ⁻¹) (Relative value)	
	Product A	Product I
Mitsuoka's buffer	1.1 ± 0.19 × 10 ¹¹ a (100.0 %)	1.9 ± 0.1 × 10 ⁷ a (100 %)
Ringer's solution	5.8 ± 1.9 × 10 ¹⁰ b (52.7 %)	1.4 ± 0.15 × 10 ⁷ b (73.7%)
Ringer's solution + Phosphate*	8.8 ± 1.3 × 10 ¹⁰ c (80.0 %)	1.3 ± 0.21 × 10 ⁷ b (68.4%)
Ringer's solution + Cysteine*	6.6 ± 2.2 × 10 ¹⁰ b (60.0%)	1.3 ± 0.12 × 10 ⁷ b (68.4%)
Ringer's solution + Tween 80*	7.5 ± 3.0 × 10 ¹⁰ c (68.2%)	1.3 ± 0.21 × 10 ⁷ b (68.4%)

Bifidobacterial counts are the mean ± S.D. obtained from six (Product A) or three (Product I) repeated tests. The bifidobacterial count obtained with Mitsuoka's buffer was set as 100% for each product, and the counts obtained from the other diluents were calculated as relative values. Agar was omitted from Mitsuoka's buffer during the dilution step. Different superscript letters (a–c) denote significant difference within each product.

*: Each ingredient, phosphate (potassium dihydrogen phosphate and disodium hydrogen phosphate), L-cysteine hydrochloride or Tween-80, was added to Ringer's solution at the same concentration as that used in Mitsuoka's buffer.

viable bifidobacteria in each diluted suspension were enumerated by the pour plate method. For the analyses of Products A and B, Reinforced Clostridial Agar medium (Oxoid Ltd., Hampshire, England) was used as the plating medium. To determine bifidobacterial counts of the commercial powdered formulas (Product C to I), transoligosaccharide (TOS) propionate agar medium (Yakult Pharmaceutical Industry Co., Ltd, Tokyo, Japan), in which most lactobacilli cannot form colonies, was used as the plating medium because some products contained not only bifidobacteria but also lactobacilli. After three days incubation at 37°C under an anaerobic condition (Anaerobox, N₂: 80%, H₂: 10%, CO₂: 10%), the colonies formed on each plate were counted after confirmation of bifidobacterial cells by microscopic observation.

Effect of diluents in suspension and dilution steps

To observe the effects of Mitsuoka's buffer and Ringer's solution in the suspension or dilution step, the two diluents were used in four combinations as follows: 1) Mitsuoka's buffer was used in both suspension and dilution steps, 2) Mitsuoka's buffer was used in only the suspension step and Ringer's solution was used in the subsequent dilution step, 3) Ringer's solution was used in only the suspension step and Mitsuoka's buffer was used in the subsequent dilution step, and 4) Ringer's solution was used in both suspension and dilution steps.

Statistical Analysis

All tests were repeated more than three times. A paired

or an unpaired Student's t-test was performed to evaluate the differences in bifidobacterial viable count. A *p* value less than 0.05 was considered significant.

RESULTS

Bifidobacterial cell counts in commercial powder products

Table 1 shows the bifidobacterial counts of the nine commercial powder products. Although three different species (*B. longum*, *B. breve* and *B. animalis*) were used in the products, significantly (*p*<0.05) higher cell counts were observed when Mitsuoka's buffer was used as the diluent rather than Ringer's solution for all products except Product G. The cell counts obtained using Ringer's solution as diluent ranged from 25.9% to 89.7% (average: 51.6%) of the counts obtained with Mitsuoka's buffer. Also, when product C was enumerated using peptone water and buffered peptone saline as diluents, the bifidobacterial counts were 3.1 ± 0.27 × 10⁶/g and 2.4 ± 0.09 × 10⁶/g, respectively, which were significantly lower (*p*<0.01) than with Mitsuoka's buffer, but significantly higher than with Ringer's solution (*p*<0.05).

Effects of constituents of Mitsuoka's buffer on bifidobacterial cell counts

Using Products A and I, the effects of adding phosphate, cysteine or Tween 80, which are major constituents of Mitsuoka's buffer, into Ringer's solution on bifidobacterial counts were examined (Table 2). In Product A, adding phosphate or Tween 80 produced

Table 3. Effect of omitting constituents from Mitsuoka's buffer on bifidobacterial counts

Diluent	Bifidobacterial count (CFU g ⁻¹) (Relative value)	
	Product A	Product I
Mitsuoka's buffer	1.1 ± 0.11 × 10 ¹¹ a (100%)	1.9 ± 0.1 × 10 ⁷ a (100%)
-Tween 80*	9.9 ± 1.0 × 10 ¹⁰ b (90.0%)	1.2 ± 0.1 × 10 ⁷ b (63.2%)
-Cysteine*	1.0 ± 0.13 × 10 ¹¹ ab (90.9%)	1.8 ± 0.26 × 10 ⁷ ab (94.7%)
-Tween 80, -Cysteine*	9.0 ± 0.2 × 10 ¹⁰ b (81.8%)	5.9 ± 0.26 × 10 ⁶ c (31.1%)

Bifidobacterial counts are the mean ± S.D. obtained from five (Product A) or three (Product I) repeated tests. The bifidobacterial count enumerated from using Mitsuoka's buffer was set as 100% for each product, and the counts obtained from the other diluents were calculated as relative values. Agar was omitted from Mitsuoka's buffer during the dilution step.

Different superscript letters (a–c) denote significant difference within each product.

*: Tween 80, cysteine, or both Tween 80 and cysteine were omitted from Mitsuoka's buffer.

Table 4. Effects of using Mitsuoka's buffer and Ringer's solution in the suspension and dilution steps on bifidobacteria cell counts of Product A.

Suspension step	Dilution step	Bifidobacterial count (CFU g ⁻¹) (Relative value)
Mitsuoka's buffer	Mitsuoka's buffer	1.3 ± 0.18 × 10 ¹¹ a (100%)
Mitsuoka's buffer	Ringer's solution	1.1 ± 0.13 × 10 ¹¹ a (84.6%)
Ringer's solution	Mitsuoka's buffer	9.0 ± 1.7 × 10 ¹⁰ b (69.2%)
Ringer's solution	Ringer's solution	8.9 ± 1.4 × 10 ¹⁰ b (68.5%)

Bifidobacterial counts are the mean ± S.D. calculated from 6 repeated tests. The bifidobacterial count obtained with Mitsuoka's buffer in both the suspension and dilution steps was set as 100%, and the counts obtained from the other combinations were calculated as relative values. Agar was omitted from Mitsuoka's buffer during the dilution step.

Different superscript letters (a, b) denote significant difference.

significantly higher counts in comparison with Ringer's solution, although the addition of cysteine had no effect. However, even with the addition of phosphate or Tween 80, the counts did not reach those obtained with Mitsuoka's buffer. In Product I, none of the added constituents improved the bifidobacterial count.

In the study examining the effects of omitting constituents from Mitsuoka's buffer, omitting Tween 80 from Mitsuoka's buffer significantly decreased the bifidobacterial counts of both Products A and I, whereas omitting cysteine had no effect (Table 3). Further decrease of cell counts was observed dramatically in Product I when both Tween 80 and cysteine were omitted.

Effects of buffer in suspension and dilution step on cell counts

The effects of using Mitsuoka's buffer and Ringer's solution in the suspension and dilution steps on bifidobacteria cell counts were tested using Product A (Table 4). When Ringer's solution was used in the suspension step, the bifidobacterial count obtained was significantly lower than when Mitsuoka's buffer was used, even when Mitsuoka's buffer was used in the subsequent dilution step. On the other hand, when Mitsuoka's buffer was used in the suspension step, the counts were significantly higher than when Ringer's solution was used, even when Ringer's solution was used in the subsequent dilution step.

DISCUSSION

Recently, many bifidobacteria-containing powder products such as powder infant formulas have gained popularity, possibly because many studies of the beneficial effects of bifidobacteria on infant health have been reported. However, in order that the probiotic products produce the expected effects, the powder products need to maintain a large number of viable probiotic cells during their shelf life. To evaluate products containing probiotic bifidobacteria, an appropriate method to analyze the exact number of viable bifidobacteria is necessary. However as shown in previous reports (3, 8, 18), the counts are affected by the enumeration conditions, especially the diluent.

In this study, we investigated the effects of diluent on bifidobacterial counts of commercial powder products in an attempt to find the best bifidobacteria enumerating method that can be used on a commercial basis. When a powdered sample is reconstituted and suspended in a solution, a phenomenon called 'rehydration' occurs (15). Bacteria that fail to reconstitute the cell membranes during rehydration cannot grow on plating medium upon subsequent incubation (4). Also, because bifidobacteria are anaerobic bacteria, oxygen toxicity during suspension in the diluent may affect the cell activity. Therefore, the choice of a suitable diluent is important to obtain accurate cell counts.

As shown in Table 1, Mitsuoka's buffer yielded much higher counts than Ringer's solution for most products containing *B. longum*, *B. breve*, or *B. animalis*, demonstrating the importance of selecting the appropriate diluents in enumerating bifidobacteria in commercial probiotic products. These results are comparable to those of a previous report (3). Also, the effects of the constituents of Mitsuoka's buffer were investigated to identify why Mitsuoka's buffer was best. Adding Tween 80 to Ringer's solution and omitting Tween 80 from Mitsuoka's buffer affected the cell counts, although cysteine had no effect (Table 2 and 3). These results suggest that Tween 80 may be one of the important factors in diluents for yielding accurate counts of bifidobacteria in powder products. It was previously reported that Mitsuoka's buffer was the best among nine diluents (3). Mitsuoka's buffer contains both Tween 80 and cysteine while the other diluents contain neither Tween 80 nor cysteine. Tween 80 is a polysorbate and is generally used as a surface acting agent. When enumerating bifidobacteria in powder products, it is necessary to completely suspend and disperse the product in the suspension step because the bacterial cells in powdered products are more likely to aggregate than liquid products.

Tween 80 may facilitate the dispersion of probiotic cells in the diluent. On the other hand, since bifidobacteria are anaerobes and exposure to oxygen during suspension is a concern, the addition of cysteine is assumed to help maintain a reduced state. Although omitting cysteine alone from Mitsuoka's buffer had no effect on the bifidobacterial counts of both Products A and I, omission of both Tween 80 and cysteine yielded lower counts compared with omission of Tween 80 alone, as shown in Table 3. Therefore, cysteine may affect the bifidobacterial count although the effect is smaller than Tween 80.

On the other hand, adding phosphate to Ringer's solution was effective at increasing the bifidobacterial count of Product A, although the count of Product I was not affected (Table 2). As Ringer's solution has no buffering constituents, such as phosphate, this finding indicates that stabilizing the pH during the rehydration process may be important in the suspension step of powder products. This conjecture is supported by the finding that peptone water and buffered peptone saline yielded higher counts than Ringer's solution for Product C. Furthermore, since there are various bifidobacteria-containing products on the market, the pH at the time of reconstitution and suspension may vary depending on the product. Therefore, inclusion of constituents having a buffering effect, such as phosphates, may be useful for preventing damage to bifidobacteria in the suspension step. Investigation of the effect of pH on bifidobacteria enumeration is necessary to find the best method to obtain accurate bifidobacterial counts for commercial products.

Interestingly, use of Mitsuoka's buffer in the suspension step was more effective for yielding higher counts than when used in the dilution step, as shown in Table 4. This result indicates that the initial suspension step is more important for attaining maximum counts. Presumably, Tween 80 in Mitsuoka's buffer facilitates good dispersal of bifidobacteria cells in the suspension step; cysteine protects the bifidobacteria from oxygen toxicity during suspension; and phosphates stabilize the pH. All these contribute to minimizing damage during the rehydration of dried cells. Since the first suspension step involves rehydration of dried bifidobacteria, the diluent may exert greater effects in this step than in the subsequent dilution step. Further study to elucidate the relationship between the diluent used in the rehydration step and the restoration of the growth capability of dried bifidobacterial cells is necessary to understand the usefulness of Mitsuoka's buffer.

In conclusion, Mitsuoka's buffer is superior to Ringer's solution for enumerating bifidobacteria in commercial powder products containing bifidobacteria,

presumably due to its constituents: Tween 80, phosphate buffer and cysteine. Although further investigation is necessary to find the best method for enumerating bifidobacteria in commercial products, at this time, we recommend using Mitsuoka's buffer as the diluent for accurate evaluation of powder products containing bifidobacteria. Hopefully this investigation will contribute to the development of probiotics in the world.

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