



## Enumeration Of *Bifidobacterium* Spp., *Lactobacillus Acidophilus* and Starter Cultures from Commercial Probiotic Yogurts and Freeze-Dried Yogurt Starter Mixes

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### ABSTRACT

Probiotic yogurt is a popular functional food to deliver of probiotic cells for the health-enhancing effects worldwide. The viability of probiotics in yogurt before consumption is the most important factor to providing desired effects, however, probiotic microorganisms have occasionally inadequate viability in marketable food products. In this current study, *Bifidobacterium* spp., *L. acidophilus* and yoghurt starter bacteria enumerations were made in commercial probiotic yoghurt and freeze-dried yogurt mixes. RCA 5.3 and MRS 5.2 media were used for *L. delbrueckii* subsp. *bulgaricus* counting, ST Agar and M17 Agar were used for *Streptococcus thermophilus* counts. While using MRS-Bile Agar and RCA-Clindamycin Agar for *L. acidophilus* enumeration, *Bifidobacterium* spp. counts were performed using MRS-NNLP medium. 5 out of yoghurt samples (A, C, D, and E) did not reveal satisfactory recovery (< 5 log CFU/g) for *L. delbrueckii* subsp. *bulgaricus* colonies on MRS 5.2 Agar while *L. delbrueckii* subsp. *bulgaricus* colony counts on RCA 5.3 Agar below 5 log CFU/g for same tested 4 samples (A, C, D, and E). The recovery rates over 9 log CFU/g were obtained in the enumerations made for all yogurt samples on both ST and M17 media. The problem of insufficient recovery rates that occurred for *L. delbrueckii* subsp. *bulgaricus* in some yogurt samples was not valid for *S. thermophilus*. This work indicated that high amounts of *L. acidophilus* were detected on both media in both of the two yoghurt samples declared as *L. acidophilus* on the label (F and G). On the other hand, bifidobacteria was determined above 5 log CFU/g in only 1 yoghurt sample (B) out of 7 probiotic yoghurts claimed to be *Bifidobacterium* spp. This study reveals relevant information on probiotic and starter counts of commercial probiotic yogurts in Turkey and discusses in detail the possible reasons for the results obtained.

### 1. Introduction

Probiotics are defined as “live microorganisms that, when administered in adequate amounts, confer a health benefit on the host” by Hill et al. (2014). The probiotic microorganisms that occur in products as a single or mixed cultures have been generally sourced from the gut or from artisanal fermented foods, such as pickles, yoghurts, and cheeses. The majority of the probiotics used in commercial probiotic preparations, are mainly from the *Lactobacillus* genera like *Lactobacillus acidophilus*, *Lactobacillus casei*, *Lactobacillus johnsonii*, *Lactobacillus amylovorus* species and *Bifidobacterium* genera like *Bifidobacterium animalis*, *Bifidobacterium bifidum*, *Bifidobacterium infantis*, and *Bifidobacterium adolescentis* species which are accepted as Generally Regarded

as Safe (GRAS) status in the United States <http://access-data.fda.gov> or granting Qualified Presumption of Safety status by the European Food Safety Authority (EFSA) (O’Toole, Marchesi et al. 2017). Various therapeutic and health promoting effects have been attributed to probiotic microorganisms including antagonistic effects to pathogens in human gut, immune system booster effects, accelerating the growth of desirable microorganisms and strengthening the body’s defence mechanisms etc. (Meybodi et al., 2020).

Yogurt is one of the popular fermented dairy product which is fermented by *Lactobacillus bulgaricus* and *Streptococcus thermophilus* and it has a long history for health-promoting effects by means of being nutritionally rich in protein, calcium, riboflavin, vitamin B6, and vitamin B12 (Ashraf and Shah 2011). Yogurt bacteria could not survive through the gastric passage and colonise

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within the gastrointestinal tract owing to acid and bile sensitivity, hence, the bacteria do not play a role for human gut health (Ashraf and Shah 2011; Meybodi et al., 2020). For a long time researchers and manufacturers have increasingly added probiotic microorganisms to improve the functional characteristics of yoghurt besides existing nutritional benefits. Indeed, probiotic yogurts regarded as one of the most popular functional foods worldwide, in a way that confirms probiotic yoghurt sales have grown at a Compound Annual Growth rate (CAGR) of 5.1 % between 2016 and 2020 according to market analysis <http://futuremarketinsights.com>. While it is predicted that bacteria that are very sensitive to oxygen and contain various technological barriers, defined as new generation probiotics such as *Akkermansia muciniphila* and *Faecalibacterium prausnitzii*, will dominate the probiotic marketplace in the coming years, it seems that *Lactobacillus* and *Bifidobacterium* species, which are traditional probiotics, will continue to be used in probiotic yoghurts for a long time.

On the other hand, according to recommendations, there should be minimum  $10^6$  CFU mL<sup>-1</sup> of viable probiotic bacteria at the time of consumption to provide expected health benefits. However, many previous studies have revealed that probiotic bacteria are often below the recommended viability level in the products on the market (Meybodi et al., 2020; Shah 2000; Shah et al., 1995; Shima et al., 2012). Accordingly, it is important to observe that the viability and survival of the added probiotic microorganisms and interactions amongst probiotics and starter cultures in yogurt throughout the storage to ensure that it can provide the expected health-promoting benefits to the consumers. It is noteworthy that presence of both starter cultures and probiotic cultures in a same product matrix, can make it difficult to achieve a differential or selective colony count of probiotic bacteria (Van de Castele et al., 2006). Nevertheless, various culture media have been developed for the selective enumeration and differentiation of concomitant probiotic and starter bacteria in yoghurt or other probiotic products (Ashraf and Shah 2011).

Based on all this information, the aim of this study was to determine survivability of starter and probiotic bacteria in local and global commercial probiotic yogurts and assess some previously suggested selective media.

## 2. Materials and Methods

### 2.1. Yogurt samples

Seven commercial probiotic yoghurt and two probiotic freeze-dried yogurt mixes were purchased from Turkish supermarkets. While five of these yoghurts were produced by local manufacturers, two of them were in the brands of global producers, and both freeze-

dried probiotic culture mixes were products from local producers. Probiotic yogurt A, C, D, E, and I contained only bifidobacteria as a probiotic microorganism and apricot, date-chia, fig-oat, strawberry and apricot, respectively. Probiotic yogurt B possessed bifidobacteria and *Lactobacillus rhamnosus* without any ingredients whilst F freeze-dried and G liquid probiotic yogurt mixes included *L.acidophilus* as a probiotic microorganism. Overall, probiotic yogurt H contained only bifidobacteria without any ingredients. While these analyzes were performed 4 days before the expiration date of A, C and D yogurts, the analysis day of B yogurt was the last day of the expiration date. While the expiration date of E yogurt was 10 days, the expiration date of H and I yogurts were 20 and 22 days, respectively. As of the day of analysis, while the expiration date of F freeze-dried probiotic yogurt mix was 143 days, it was the last day of the expiry date of G liquid probiotic yogurt mix.

### 2.2. Selective and differential media

The media used for bifidobacteria enumeration was deMan Rogosa Sharpe (MRS) Agar supplemented with neomycin sulphate (100 mg/l), nalidixic acid (15 mg/l), paramomycin (200 mg/l), lithium chloride (3 g/l) (NNPL) according to Dave and Shah (1996). MRS Agar with Ox-bile (0.15 %, w/v) and Reinforced Clostridial Agar (RCA) supplemented with bromocresol green solution (0.2 %, w/v, autoclaved at 121 °C for 15 min, 20 ml/l) and clindamycin (5 mg/100 ml, filter-sterilized, 2 ml/l) were chosen for selective and differential enumeration of *L. acidophilus* according to Darukaradhyia et al., (2006). MRS 5.2 Agar (adjusted to 5.2 with filter-sterilized acetic acid) and RCA 5.3 Agar (adjusted to 5.3 with filter-sterilized acetic acid) were used for counting *L. delbrueckii* subsp. *bulgaricus* according to recommendation of Van de Castele et al. (2006). M17 Agar and *Streptococcus thermophilus* (ST) Agar were used for enumeration of *S. thermophilus* according to recommendation of Ashraf and Shah (2011). The incubation conditions applied for each media are listed in Table 1.

### 2.3. Microbiological enumerations

Ten grams of each probiotic yogurt samples were suspended in 90 ml of sterile ringer solution and homogenized in a stomacher for 2 minutes. For freeze-dried probiotic yogurt mix, 1gr mix were suspended in 9 ml of sterile ringer solution. The homogenized suspension (1ml) was serially diluted in sterile 9 ml of ringer solution and 0.1 ml of the appropriate dilution was spread into the above-mentioned selective and differential media in triplicate. After incubation time, plates containing 25 to 250 colonies were enumerated and colony forming units (CFU) per gram of the yogurt samples was calculated. These numbers are described as viable counts ( $\log_{10}$  CFU/g) (Talwalkar and Kailasapathy,2004).

Table 1  
Selective and differential agar media used for probiotic and starter bacteria in yoghurt

Media	Incubation			Presumptive bacteria target	Reference
	Temp.	Time	Oxygen		
MRS-NNLP	45 °C	72 h	anaerobic	Bifidobacteria	Dave and Shah (1996)
RCA-Clindamycin	37 °C	72 h	microaerophilic	<i>L.acidophilus</i>	Darukaradhya et al. (2006)
MRS-Bile	37 °C	72 h	microaerophilic	<i>L.acidophilus</i>	Darukaradhya et al. (2006)
MRS 5.2	45°C	72 h	anaerobic	<i>L.delbrueckii</i> subsp. <i>bulgaricus</i>	Van de Castele et al. (2006)
RCA 5.3	45°C	72 h	anaerobic	<i>L.delbrueckii</i> subsp. <i>bulgaricus</i>	Van de Castele et al. (2006)
M17	45°C	24 h	aerobic	<i>S.thermophilus</i>	Ashraf and Shah (2011)
ST	37 °C	24 h	aerobic	<i>S.thermophilus</i>	Ashraf and Shah (2011)

#### 2.4. Statistical analysis

Statistical analysis of the data was performed by ANOVA and Tukey's mean comparison tests using the Minitab statistical package (version 18; Minitab Inc., State College, PA) to determine significant differences between the assessed responses.

### 3. Results and Discussion

#### 3.1. Enumeration of *Lactobacillus delbrueckii* subsp. *bulgaricus* in commercial probiotic yogurt samples

In this current study, MRS Agar at pH 5.2 (MRS 5.2 Agar) and RCA Agar at pH 5.3 (RCA 5.3 Agar) were used for enumeration of *L. delbrueckii* subsp. *bulgaricus* when the incubation is carried out at 45°C for 72 h under anaerobic incubation. These media were previously recommended by various researchers for *L. delbrueckii* subsp. *bulgaricus* (Ashraf & Shah, 2011; Lankaputhra & Shah, 1996; Van de Castele et al., 2006). While Van de Castele et al. (2006) stated that MRS 5.2 Agar was the most suitable media for enumeration of *L. delbrueckii* subsp. *bulgaricus*, RCA 5.3 Agar with anaerobic incubation at 45°C for 72 h was found suitable media for selective recovery and enumeration by Dave and Shah (1996). On the other hand, some of the bifidobacteria strains also grew in these media, however, *L. delbrueckii* subsp. *bulgaricus* colonies were easily differentiated from those of bifidobacteria (Ashraf and Shah, 2011; Ashraf and Smith, 2015). Interestingly, in this present study 5 yoghurt samples (A, B, C, D, and E) did not reveal satisfactory recovery (< 5 log CFU/g) for *L. delbrueckii* subsp. *bulgaricus* colonies on MRS 5.2 Agar while *L. delbrueckii* subsp. *bulgaricus* colony counts on RCA 5.3 Agar below 5 log CFU/g for same tested 4 samples (A, C, D, and E).

This inadequate recovery of the *L. delbrueckii* subsp. *bulgaricus* in some yogurt samples may be attributed to declining in pH values towards the end of the storage period. Indeed, it was observed that the pH values of the samples with low numbers of *L. delbrueckii* subsp. *bulgaricus* were lower compared to the other yoghurt samples (data not shown). For example, pH values of A and E yoghurt samples were determined as 4.17 and 4.20, respectively, whilst mean pH values for B and H yoghurt samples were measured as 4.30 and 4.35, respectively, hence, it is estimated that this pH decrease has a negative impact on the number of *L. delbrueckii* subsp. *bulgaricus*. Similarly, in a previous study conducted by Mani-

López et al. (2014), the researchers observed that the decrease in pH in probiotic yogurts during refrigeration storage caused a reduction in the viability of bacteria. In parallel with results obtained from this current work, Mani-López et al. (2014) observed the low recovery rates (about 5 log CFU/g) of *L. delbrueckii* subsp. *bulgaricus* per contra *S. thermophilus* recovery (about 7-8 log CFU/g) at the end of the 35 days storage in probiotic yogurt samples which separately produced with *L. casei* and *L. reuteri*. It is a fact that the post-acidification phenomenon in yoghurt increases with the addition of probiotic microorganisms and prebiotics, and the presence of *L. delbrueckii* subsp. *bulgaricus* is known to be one of the biggest causes of post-acidification in yoghurt, however, *L. delbrueckii* subsp. *bulgaricus* is expected to be more resistant to post acidification conditions than *S. thermophilus* (Deshwal et al. 2021). On the other hand, starter culture mixes with a high cocci/bacilli ratio or without *L. delbrueckii* subsp. *bulgaricus* are recommended so that post-acidification does not adversely affect the quality and consumer acceptability of yogurt taking into account that some of the basic flavor components of yogurt are lower (Deshwal et al., 2021; Pinto et al., 2009). In this present study, in probiotic yoghurt samples detected low recovery of *L. delbrueckii* subsp. *bulgaricus* (below 5 log CFU/g), this starter lactobacilli might be used in low amounts in the mix to prevent post acidification or the *L. delbrueckii* subsp. *bulgaricus* in these mixes may have been selected from strains that could not provide sufficient resistance in the face of decreasing pH. The same situation was previously revealed by Coeuret et al. (2004) who stated that some probiotic products included fewer lactobacilli numbers than claimed or none, however, these researchers attributed this to disruption of the cold-chain or strain-dependent loss of viability. Again, the low recovery of *L. delbrueckii* subsp. *bulgaricus* in some probiotic yogurt samples can be explained by study of Temmerman et al. (2003) in which they stated that *L. delbrueckii* subsp. *bulgaricus* rapidly overgrown by the other bacteria in dairy products -especially in the presence of other lactobacilli- and its isolation becomes difficult. As for the comparison between the media, no statistical difference was observed between the numbers of *L. delbrueckii* subsp. *bulgaricus* obtained in MRS and RCA media. Previously, Van de Castele et al. (2006) reported that higher recovery was obtained in MRS 5.2 medium compared to RCA medium in terms of *L. delbrueckii* subsp. *bulgaricus* LMG 6901 numbers when trying different media for enumeration of starter and probiotic bacteria

in probiotic dairy products. In addition, study of Ashraf and Smith (2015) demonstrated that a higher recovery rate of *L. delbrueckii* subsp. *bulgaricus* 11842 strain achieved in MRS 5.2 media ( $8.95 \pm 0.05$ ) compared to RCA 6.1 and 6.8 media ( $8.56$  and  $8.26 \pm 0.06$ , respectively) when anaerobic incubations were carried out at

$37^\circ\text{C}$  for 72 h. It is noteworthy that the *L. delbrueckii* subsp. *bulgaricus* numbers obtained from both media for 5 yogurt samples (B, F, G, H, and I) varied from 6.19 log CFU/g to 8.82 log CFU/g and these counts are consistent with the literature (Lankaputhra and Shah, 1996; Van de Castele et al., 2006).

Table 2

Viable counts of yogurt starter bacteria, *Bifidobacterium* spp. and *L. acidophilus* on different media (log CFU/g)

SAMPLE	M17	ST	RCA-Clindamycin	MRS-Bile	MRS 5.2	RCA 5.3	MRS-NNLP
A	$9.97 \pm 0.22^a$	$10.07 \pm 0.08^a$	-	-	<5	<5	<5
B	$9.87 \pm 0.05^{ab}$	$10.19 \pm 0.65^a$	-	-	$7.88 \pm 0.01^a$	$8.05 \pm 0.06^a$	$7.85 \pm 0.07$
C	$9.54 \pm 0.10^{bcd}$	$9.45 \pm 0.05^a$	-	-	<5	<5	<5
D	$9.72 \pm 0.04^{abc}$	$9.73 \pm 0.02^a$	-	-	<5	<5	<5
E	$9.82 \pm 0.01^{ab}$	$9.81 \pm 0.13^a$	-	-	<5	<5	<5
F	$9.16 \pm 0.04^e$	$9.29 \pm 0.17^a$	$8.12 \pm 0.02^b$	$8.21 \pm 0.07^b$	$7.84 \pm 0.10^a$	$7.88 \pm 0.48^a$	-
G	$9.48 \pm 0.03^{cde}$	$9.44 \pm 0.18^a$	$10.08 \pm 0.02^a$	$9.40 \pm 0.35^a$	$8.82 \pm 0.12^a$	$8.44 \pm 0.52^a$	-
H	$9.27 \pm 0.01^{de}$	$9.34 \pm 0.00^a$	-	-	$8.22 \pm 0.11^a$	$7.60 \pm 0.17^a$	<5
I	$9.42 \pm 0.03^{cde}$	$9.83 \pm 0.02^a$	-	-	$6.19 \pm 0.34^b$	$6.41 \pm 0.21^b$	<5

-: yogurt did not claim to possess those probiotic strains

<sup>a,b,c,d,e</sup> Values in same column having different superscripts differ significantly ( $p < 0.05$ ), means in media used for same targeted bacteria do not differ significantly

### 3.2. Enumeration of *Streptococcus thermophilus* in commercial probiotic yogurt samples

In this current study, M17 Agar and ST Agar were used for enumeration of *S. thermophilus* when the incubation is carried out at  $45^\circ\text{C}$  and  $37^\circ\text{C}$  for 72 h under aerobic incubation, respectively. M17 media were previously recommended by International Dairy Federation (IDF, 1981) for selective enumeration of *S. thermophilus* from yogurt and also various researchers reported that M17 medium was the most suitable media for enumeration and isolation of lactic streptococci (Ashraf and Shah, 2011; Van de Castele et al., 2006). On the other hand, ST Agar at  $37^\circ\text{C}$  and aerobic conditions were recommended by Tharmaraj and Shah (2003) for *S. thermophilus* amongst the media tested. In this present work, the recovery rates over  $9 \log \text{CFU/g}$  were obtained in the enumerations made for all yogurt samples on both ST and M17 media. The problem of insufficient recovery rates that occurred for *L. delbrueckii* subsp. *bulgaricus* in some yogurt samples was not valid for *S. thermophilus*. These numbers from the present study were very similar to results reported by Mani-López et al. (2014) who reported that *S. thermophilus* counts from the commercial probiotic yogurts stored for 35 days varied from  $9.48 \log \text{CFU/g}$  to  $10.34 \log \text{CFU/g}$ . Moreover, in consistent with our results these researchers also revealed that *S. thermophilus* numbers in probiotic yogurts produced individually with *L. casei*, *L. reuteri* and *L. acidophilus* remained higher than *L. delbrueckii* subsp. *bulgaricus* and probiotic bacteria at the end of the storage. Similar results were obtained in the study of Gueimonde et al. (2004) where *S. thermophilus* counts from  $10^7$  to  $10^9 \text{CFU/ml}$  obtained after 30 d of cold storage. In fact, in a previous study Mani-López et al. (2014) four culture mixtures were prepared to produce yogurts as follows: (1) *S. thermophilus* and *L. delbrueckii* subsp. *bulgaricus*, (2) *S. thermophilus*, *L. delbrueckii* subsp. *bulgaricus* and probiotic *L. acidophilus*, (3) *S. thermophilus*, *L. delbrueckii* subsp. *bulgaricus* and probiotic *L. casei*, (4) *S. thermophilus*, *L. delbrueckii* subsp. *bulgaricus* and probiotic *L. reuteri*. The researchers examining the viability of these bacteria during storage determined that when

there was a probiotic bacteria in the mix, the numbers of *S. thermophilus* remained very high at the end of storage, but the viability of *L. delbrueckii* subsp. *bulgaricus* was affected very negatively, however, the number of *L. delbrueckii* subsp. *bulgaricus* in yogurts prepared without probiotic bacteria was around  $10 \log \text{CFU/ml}$  at the end of the storage period and it was higher than *S. thermophilus*. Hence, the result of the aforementioned study supports the findings obtained from this present study and it is seen that *S. thermophilus* is not suppressed in the presence of probiotic bacteria, but *L. delbrueckii* subsp. *bulgaricus* is negatively affected. Again, in consistent with this current findings Rutella et al. (2016) reported that whilst *L. delbrueckii* subsp. *bulgaricus* declined from  $8.52 \pm 0.16$  to  $6.50 \log \text{CFU/ml}$  at the end of the 27 d. cold storage, there were minimal differences over storage time in the viability of *S. thermophilus* numbers. This situation has been attributed by the various researchers to the fact that *S. thermophilus* is more resistant to cold stress than *L. delbrueckii* subsp. *bulgaricus* (Donkor et al., 2007; Rutella et al., 2016).

### 3.3. Enumeration of *Bifidobacterium* spp. and *L. acidophilus* in commercial probiotic yogurt samples

In this current study, MRS-Bile Agar and RCA-Clindamycin Agar were used for enumeration of *L. acidophilus* when the incubation is carried out at  $37^\circ\text{C}$  for 72 h under aerobic incubation. Besides, MRS-NNLP media was used for enumeration of *Bifidobacterium* spp. with anaerobic incubation at  $45^\circ\text{C}$  for 72 h. MRS-Bile Agar were previously recommended by de Carvalho Lima et al. (2009) for *L. acidophilus*, while RCA-Clindamycin Agar was recommended by International Organization for Standardization (Organisation, 2006) for the enumeration of presumptive *L. acidophilus* in dairy products. MRS-NNLP Agar was found as suitable media for selective enumeration of bifidobacteria from probiotic yogurts (Ashraf and Shah, 2011; Van de Castele et al., 2006). Actually, although there are studies suggesting the addition of 0.05% L-cysteine to this medium, de Carvalho Lima et al. (2009) could not detect a difference in performance between MRS-NNLP media containing cysteine and those without. Therefore, in this

study, MRS-NNLP media was used without the addition of cysteine. This work indicated that high amounts of *L. acidophilus* were detected on both media in both of the two yoghurt samples declared as *L. acidophilus* on the label (F and G). On the other hand, bifidobacteria was determined above 5 log CFU/g in only 1 yoghurt sample (B) out of 7 probiotic yoghurts claimed to be *Bifidobacterium* spp. In consistent with our results, Mani-López et al. (2014) observed that the numbers of *L. acidophilus* decreased 1-1.5 log during storage but remained at the level recommended by FAO/WHO in yogurt and fermented milk samples that contained *L. acidophilus*. Although the number of bifidobacteria was found to be above the minimum recommended number in this present study, Coeuret et al. (2004) stated that some commercial probiotic products contained fewer lactobacilli than claimed, or none, due to the disruption of the cold chain or strain-dependent loss of viability. It is not a very surprising finding that bifidobacteria were found to be above 5 log CFU/g in only 1 sample tested, because when the literature is examined, it has been seen that there are many similar results. Accordingly, inhibited probiotic contents (*Bifidobacterium lactis*, *L. rhamnosus* etc.) of the end product were detected when co-cultured with the fast-acidifying strain *S. thermophilus* (Oliveira et al. 2009). Indeed, many researchers have reached results reporting that the number of bifidobacteria in probiotic dairy products or supplements is much lower than it should be, or that it cannot be detected at all (De Vecchi et al., 2008; Lewis et al., 2016; Temmerman et al., 2003). In this regard, Marinova et al. (2019) reported that viable bacteria were not detected in 11.53 % of the tested probiotic dietary supplements in Bulgarian market and 7.69 % contained a minimal amount (about 10<sup>2</sup> CFU/g) while Zawistowska-Rojek et al. (2016) observed that only one medicinal product, two dietary supplement and two foods of all analyzed 25 different products revealed good quality in regard to number of probiotic cells. Previously, it is highlighted that the usage of some fruit juice or their pulps may negatively influence the viability of probiotic bacteria in yoghurt probably due to the high acidity or the antibacterial components (Meybodi et al., 2020; Shori, 2015). In this current study, the fact that 6 out of the 7 probiotic yogurt samples in which the bifidobacteria count was below 5 log CFU/g were fruit-supplemented, actually shows that one of the reasons for this result may be the effect of fruit additives. On the other hand, Van de Castele et al. (2006) stated that the plating technique used whilst counting bifidobacteria from dairy products also affected the results, and the pour-plate method was more appropriate since these bacteria were oxygen sensitive. In fact, researchers who have encountered similar findings about absence or low recovery of bifidobacteria in probiotic commercial products have previously expressed many ideas about possible reasons for this: it might be due to (1) the lack of optimal selective/elective/differential enumeration media and MRS-NNLP agar not being a suitable medium for reliably counting this bacterial group (Talwalkar and Kailasapathy, 2004; Temmerman et al., 2003), the lack of differentiation and

recovery amongst the strains of different LAB species and (2) possible antagonism between all strains used in producing the probiotic product (Oberget al. 2011), (3) drawbacks of plating methods, which is still the most used approach, such as labor intensive, revealing variable results and not determining the viable but not culturable (VBNC) bifidobacteria cells (Di Lena et al., 2015; Fusco et al., 2021; Huys et al., 2013), (4) the dilution of the sample (the problem that these probiotic bacteria start to disappear as the dilutions increase) (Talwalkar and Kailasapathy 2004), (5) inability of some probiotic cells to form colony on solid medium through bacterial stress (Talwalkar and Kailasapathy 2004), (6) negatively influence of freeze-drying and encapsulation processes on probiotic bacteria recovery and/or freeze-dried probiotic products (Masco et al. 2005) (7) decrease of the bifidobacteria cells's survival rate over inappropriate transportation and storage conditions (Di Lena et al. 2015), and (8) voluntary or involuntary mislabeling of the bifidobacteria counts (Fusco et al. 2021). As a result, on the occasion of this study, it is worth remembering that whatever the reason - from the producer, from the culture supplier or from the impropriety of the analysis- in probiotic products the number of probiotic bacteria should be constantly monitored at the beginning of production and during storage in order to give the desired health benefits. Whatever the factors are, these should be tried to be corrected and new generation technologies such as molecular and omics approaches, flow cytometry etc. should be rapidly integrated into the applications for probiotic enumeration and monitoring in order to get rid of the disadvantages of the plate counting methods in solid media. It should also be noted that recently there have been studies with the concepts of 'postbiotic' and 'paraprobiotic' that these microorganisms are beneficial even if they are not alive and that VBNC probiotic cells also have health benefits (Aguilar-Toalá et al., 2018; Fiore et al., 2020; Fusco et al., 2021; Taverniti and Guglielmetti 2011), however, this does not change the minimum number that should be in a product manufactured with the claim of being a probiotic food or supplement. Hence, all these concepts should be evaluated differently.

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