Suitability of MRS-bile agar for the selective enumeration of mixed probiotic bacteria in presence of mesophilic lactic acid cultures and yoghurt bacteria

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Abstract
Measuring the viability of probiotic microorganisms in food products using plate count methodology is a common practice due to the simplicity (ease of performance), inexpensive and routine testing characters of this method. In present study, the suitability of de man rogosa and sharpe agar (MRS) bile agar medium for the selective enumeration of mixed probiotic bacteria (*Lactobacillus acidophilus* LA-5, *L. casei* 431 and *Bifidobacterium lactis* BB-12) in presence of mesophilic lactic cultures (*Lactococcus lactis* ssp. *lactis* and *Lactococcus lactis* ssp. *cremoris*) and yoghurt bacteria (*Streptococcus thermophilus* and *Lactobacillus delbrueckii* ssp. *bulgaricus*) was investigated. Yoghurt bacteria did not grow neither in presence of 0.15% nor 0.30% of bile salts, as was expected. Mesophilic lactic starters could grow at both concentrations of bile salts at all incubation temperatures except 37°C. According to these results, MRS-bile agar (0.15 bile salts) could be successfully used for selective enumeration of mixed probiotic cultures in presence of mesophilic culture and/or yoghurt bacteria when plates were incubated at 37°C for 72 h.

Keywords**:** Enumeration; mesophilic; MRS-bile agar; probiotic

INTRODUCTION

Viability of probiotic bacteria, the number of viable and active cells per g or mL of probiotic food products at the moment of consumption is the most critical value for these products, as it determines their efficacy Therefore, in order to maintain consumer confidence in probiotic products, it is important to ensure a high survival rate of the bacteria both during production and over the product's shelf life (Mortazavian and Sohrabvandi, 2006).

Plate count methodology for measuring the viability of probiotic microorganisms comprises advantages of simplicity (ease of performance), availability, inexpensive and routine testing characters. However, this methodology suffers from poor reproducibility and discriminatory power especially in simultaneous counting of mixed cultures, different media performance from cell recovery rate (colony forming ability) point of view when a certain culture is cultivated in several relevant culture media or when various strains of a species are cultivated in a culture medium (overestimation or underestimation), and probability of emergence of atypical colonies in plates (Mortazavian *et al*., 2007; Mortazavian and Sohrabvand, 2006; McCartney, 2005). Numerous types of culture media have been proposed for differential and selective enumeration of probiotic bacteria, alone or in presence of yoghurt bacteria such as MRS-maltose agar (Talwalkar and Kailasapthy, 2004; Lim *et al*., 1995), MRS-salicin agar and MRS-sorbitol agar (Mortazavian *et al*., 2007; Dave and Shah, 1996; Lankapuhra and Shah, 1996), MRS-LP agar (Vinderola and Reinheimer, 1999), MRS-NNLP agar (Talwalkar and Kailasapthy, 2004;

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Dave and Shah, 1996), MRS-bile agar (Mortazavian *et al*., 2007; Vinderola and Reinheimer,1999 ; Dave and Shah, 1996), MRS-*L. casei* agar (Talwalkar and Kailasapthy, 2004), MRS-IM agar (Anon, 2005), RCPB-agar/pH5 (Rybka and Kailasapathy, 1996), bifidobacteria agar (Charteris *et al*., 1997), AMC-agar (Munoa and Pares, 1988), DP-agar (Bonaparte *et al*., 2001), MBG-agar (Davidson *et al*., 2000), BL-OG agar (Lim *et al*., 1995), TPPY-E agar (Ghoddusi and Robinson, 1996), TOS-agar, TOS-NNLP agar and *L*-arabinose agar (Wijsman *et al*., 1989), and BIM-25 agar (Munoa and Pares, 1988). Mortazavian *et al*. (2007) reported that MRS-bile agar as a cheap, available and simple-performing culture medium, could be successfully used for selective enumeration of mixed probiotic bacteria (*L. acidophilus*, *L. casei* and bifidobacteria) in presence of yoghurt bacteria (*Streptococcus thermophilus* and *Lactobacillus delbrueckii* ssp. *bulgaricus*). The aim of this study was to investigate the suitability of MRS-bile agar for the selective enumeration of mixed probiotic bacteria (*Lactobacillus acidophilus*, *L. casei* and bifidobacteria) in presence of mesophilic lactic cultures (*Lactococcus lactis* ssp. *lactis* and *Lactococcus lactis* spp. *cremoris*) and yoghurt bacteria. Mentioned mesophilic culture (with or without yoghurt bacteria) is commonly used in production of fermented cheese, fermented cream and drinks based on fermented milk.

MATERIAL AND MEHODS

Starter cultures: The direct-in vat-set (DVS) pouches of commercial lyophilized cultures including several cultures of yoghurt bacteria (mixed culture of *Streptococcus thermophilus* and *Lactobacillus delbrueckii* ssp. *bulgaricus*: CH-1, X11 and X16), several mesophilic cultures (*Lactococcus lactis* spp. *lactis* and *Lactococcus lactis* spp. *cremoris*: R-704, R-705 and R-707), and probiotic cultures including *Lactobacillus acidophilus* La-5, *Lactobacillus casei* 431 and *Bifidobacterium lactis* BB-12 were supplied by Chr-Hansen (Horsholm, Denmark). These starter cultures are widely used by dairy industry to produce fermented milk products (Korbekandi *et al*., 2011). The cultures were maintained according to manufacturer's instructions, until further use.

Experimental design: The DVS lyophilized pouches of different strains of yoghurt bacteria (*S. thermophilus* and *L. delbrueckii* ssp. *bulgaricus*) and different strains of mesophilic cultures (*Lac. lactis* ssp. *lactis* and *Lac. lactis* spp. *cremoris*) were separately dissolved in 1 l of low-fat sterilized milk. In each experiment term, the powder suspension was gently stirred for 5 min till the homogenized mixture was obtained. Then, immediately, 1 ml of starter solution was applied to make serial decimal dilutions with ringer solution. Finally, the samples from suitable dilution rates were cultivated and enumerated using MRS-bile agar medium (MRS agar: Merck, Darmstadp, Germany, and Bile: Sigma, Reyde, USA) applying pour plate technique. Bile was added into the MRS medium in concentrations of 0.15% (Vinderola and Reinheimer, 1999) or 0.30% and the medium was autoclaved at 121ºC for 15 min. All the samples were incubated at 25°C or 31°C or 37°C for 72 h under both aerobic and anaerobic conditions. Anaerobic condition was generated by using the GasPak system (Merck, Darmstadt, Germany). The numbers of viable cells were expressed in terms of cfu/g of starter powders.
After considering the growth results of yoghurt bacteria and mesophilic culture under mentioned conditions, the procedure was carried out again with simultaneous addition of three probiotic strains (*L. acidophilus* LA-5, *L. casei* 431 and *B. lactis* BB-12) with the two adjunct cultures. The samples were incubated at 37°C for 72 h under both aerobic and anaerobic conditions (0.15% or 0.30% bile salts). Morphological characteristics of the colonies of these probiotics were analyzed and scanned using stereo-digital microscopy (SDM) (SV6, Zisse, Germany) in a comparative approach with the results reported by Mortazavian *et al*. (2007).

Statistical analysis: Experiments were performed in quadruples in different working days and the significant differences among the means were analyzed using ANOVA test from Minitab software (version 13, 2002).

RESULTS

Growth of yoghurt bacteria: Table 1 shows the viable counts of the sum of yoghurt bacteria and the sum of mesophilic culture per gram of lyophilized starter culture powders. As was expected, yoghurt bacteria did not growth neither in presence of 0.15% nor 0.30% of bile salts, at all incubation temperatures (25°C or 31°C or 37°C after 72 h). This characteristic has been reported previously (Mortazavian *et al*.,

2007; Vinderola and Reinheimer, 1999).

Growth of mesophilic bacteria: Mesophilic lactic acid bacteria were able to grow in MRS-bile agar in presence of bile salts (0.15 or 0.30%) except when the plats were incubated at 37°C for 72 h (Table 1). At 37°C, independent of bile salt concentration (0.15 or 0.30%), none of mesophilic starter strains were grown. This result is a good promising approach because when cultures of MRS-bile agar are incubated at 37°C, neither yoghurt bacteria nor mesophilic lactic bacteria are able to grow. Therefore, selective/differential enumeration of probiotic cultures in presence of mentioned adjunct cultures would be simply possible. It seems that mesophilic cultures could only growth at their optimum temperature (about 25°C) in presence of bile salts as a stress factor. In other incubation temperatures (rather than 37°C), growth of mesophilic starter strains was significantly restricted at plates with higher concentrations of bile salts (0.30% compared to 0.15%, Table 1).

Growth of mixed probiotic culture in presence of mesophilic culture and yoghurt bacteria: Table 2 represents the viable counts of each probiotic bacteria (*L. acidophilus* LA-5, *L. casei* 431 and *B. lactis* BB-12) per gram of lyophilized starter culture powders in presence of both mesophilic and yoghurt bacteria. Because on one hand, yoghurt bacteria were unable to growth in presence of bile salts (0.15 or 0.30%) and on the other hand, mesophilic bacteria were unable to grow in presence of bile salts (0.15 or 0.30%) at incubation temperature of 37°C for 72 h (not other incubation temperutre), by using MRS-bile agar (0.15% bile salts), selective or differential enumeration of probiotic bacteria in culture compositions containing thermophilic (yoghurt bacteria) and/or mesophilic adjunct cultures can be achieved. With respect to our previous study (Mortazavian *et al*., 2007) regarding selective or differential enumeration of probiotics in presence of yoghurt bacteria, MRS-bile agar (0.15% bile salts) was suitable for differential enumeration of ACY culture composition (*L. acidophilus*, *L. casei* and yoghurt bacteria) when plates incubated aerobically or anaerobically, selective enumeration (for *L. casei*) of BCY culture composition (*L. casei*, bifidobacteria and yoghurt bacteria) at aerobic condition, selective enumeration (for *L. acidophilus*) of ABY culture composition (*L. acidophilus*, bifidobacteria and yoghurt bacteria) at aerobic condition, and differential enumeration of ABCY culture composition (*L. acidophilus* and *L. casei*, bifidobacteria and yoghurt bacteria) at aerobic conditions. Also, in the ABY and ABCY culture compositions, viable counts of bifidobacteria could be selectively achieved using the subtractive enumeration method (SEM). These results obtained from several principles as following: 1) *Bifidobacterium* spp. are not able to grow under aerobic condition; 2) there was no significant difference between the viable counts of *L. acidophilus* and *L. casei* under aerobic and anaerobic conditions after 72 h incubation; 3) bifidobacteria and *L. casei* colonies were differentiated at anaerobio-

Table 1. The viable counts of the sum of yoghurt and mesophilic bacteria per gram of lyophilized starter culture powders[1].

Bile Concentration (%)	Incubation Temperature (ºC)	Atmosphere conditions	Sum of yoghurt bacteria (log cfu/ml)			Sum of mesophilic bacteria (log cfu/ml)		
			Y_1[3]	Y^2	Y^3	M_1	M_2	M_3
0.15	25	+2	NG[4]	NG	NG	10.91±0.02	10.65±0.01	10.86±0.05
0.15	25	-	NG	NG	NG	10.95±0.03	10.68±0.03	10.92±0.03
0.15	31	+	NG	NG	NG	10.72±0.02	10.50±0.04	10.74±0.06
0.15	31	-	NG	NG	NG	10.75±0.05	10.58±0.02	10.78±0.04
0.15	37	+	NG	NG	NG	NG	NG	NG
0.15	37	-	NG	NG	NG	NG	NG	NG
0.30	25	+	NG	NG	NG	10.33±0.04	10.11±0.01	10.60±0.05
0.30	25	-	NG	NG	NG	10.37±0.03	10.27±0.04	10.64±0.08
0.30	31	+	NG	NG	NG	9.60±0.05	9.48±0.06	10.01±0.01
0.30	31	-	NG	NG	NG	10.02±0.04	9.82±0.08	10.33±0.04
0.30	37	+	NG	NG	NG	NG	NG	NG
0.30	37	-	NG	NG	NG	NG	NG	NG

[1]The means in a column shown with different letters are significantly different (p<0.05). [2]The signs "+" and "-" represent aerobic and anaerobic conditions, respectively. [3]Y_1 = yoghurt bacteria: CH-1, Y_2 = yoghurt bacteria: X11, Y_3 = yoghurt bacteria: X16, M_1= Mesophilic culture: R-704, M_2= Mesophilic culture: R-705, M_3= Mesophilic culture: 707. [4]NG = No growth.

Table 2. The viable counts of probiotic bacteria per gram of lyophilized starter culture powders, in presence of mesophilic cultures and yoghurt bacteria[1].

Bile Concentration (%)	Incubation Temperature (ºC)	Atmosphere conditions	Probiotic bacteria		
			L. acidophilus LA-5	*L. casei* 431	*B. lactis* BB-12
0.15	37	+[2]	10.04±0.05	10.46±0.08	NG3
0.15	37	-	10.02±0.04	10.47±0.07	10.68±0.03
0.30	37	+	10.01±0.06	10.44±0.05	NG
0.30	37	-	10.02±0.03	10.45±0.06	10.66±0.05

[1]The means in a column shown with different letters are significantly different (p<0.005)/mesophilic and thermophilic cultures did not growth. [2]The signs "+" and "-" represents aerobic and anaerobic conditions, respectively. [3]NG = No growth.

sis as well as *L. acidophilus* and *L. casei* colonies under aerobiosis or anaerobiosis according to their own colony characteristics. Suitability of the MRS-bile agar for the enumeration of ACY culture composition had been confirmed by Vinderola and Reinheimer (2000). Also, selective enumeration of *L. acidophilus* at aerobic conditions in ABY culture compositions had been already proposed by Lankaputhra and Shah (1996) and Vinderola and Reinheimer (1999).

In this study, *L. acidophilus* formed two types of creamy colonies under aerobic atmosphere; the first type consisting of round and large ones (1.5-3.5 mm in diameter) with a transparent zone around similar to sun-shine Figure, and the second type constituting of colonies which appeared in small (0.5-2.5 mm in diameter) various irregular shapes. Under anaerobic atmosphere, two types of colonies were identified; the first one was characterized by round and relatively big colonies with a thorny shrub-like network appearance

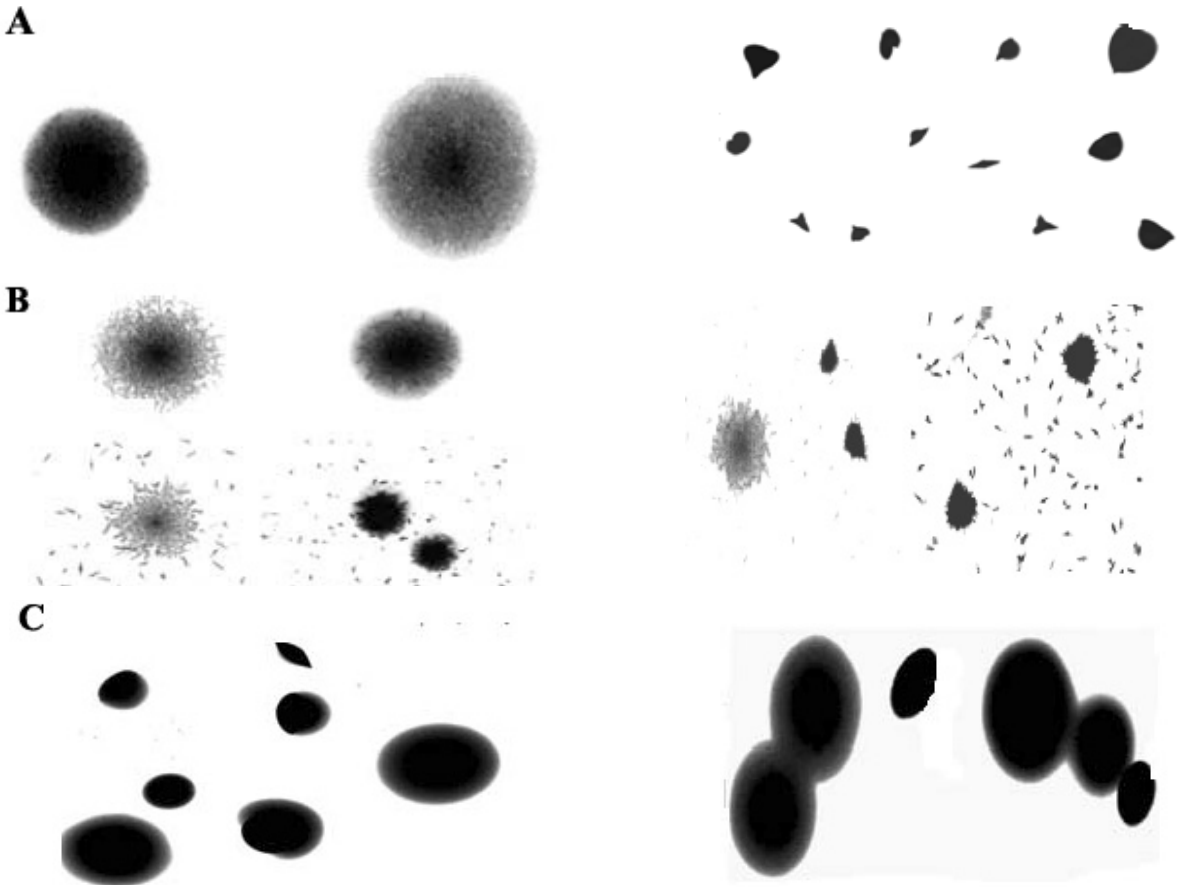

Figure 1. Morphology of probiotic colonies (×100). A: Two types of *L. acidophilus* colonies under aerobic conditions; B: Two types of *L. acidophilus* or bifidobacteria colonies under anaerobic conditions; C: *L. casei* colonies under aerobic or anaerobic conditions.

(1.0 to 2.0 mm in diameter), and the second was distinguished by small irregularly-shaped colonies with thorn-shaped accessories looked like a hedgehog (0.45-0.75 mm in diameter). The shapes of the colonies are shown in Figure 1. The results are in agreement with those obtained by Mortazavian *et al*. (2007). Bifidobacteria colonies characteristics under anaerobic condition were just similar to *L. acidophilus* at the same atmosphere. *L. casei* yielded round-white creamy colonies with even circumference (similar to a lentil-0.5 to 3.0 mm in diameter). Roundness and even circumference of the *L. casei* colonies were their most significant attributes. In differential enumeration of *L. acidophilus* and *L. casei*, the colonies of the former could be easily differentiated by their thorn-shrub-like area from those of the latter with their lentil-shape and even circumference and color. Therefore, by using MRS-bile agar (0.15% bile salts-incubation temperature of 37°C for 72 h), selective or differential enumeration of probiotic bacteria in different probiotic culture compositions such as ABY and ABYM-types (M=mesophilic culture), ACY and ACYM-types and BCY and BCYM-types as well as ABCY or ABCYM-types would be possible. According to Table 2, growth of probiotic bacteria was not significantly affected by the concentrations of bile salts (0.30% compared to 0.15%), because probiotic bacteria are relatively tolerant to bile salts (Mortazavian and Sohrabvandi, 2006). However, generally, the colony forming ability of mesophilic bacteria was significantly lower in presence of 0.3% bile salts compared to 0.15%. Furthermore, mesophilic cultures grew significantly higher at 25°C compared to 31°C, at the same bile concentration (0.15% or 0.3%) as well as under anaerobiosis compared to aerobiosis at the same incubation temperature (25°C or 31°C).

DISCUSSION

In the majority of culture compositions used for the production of commercial fermented milk products, yogurt bacteria (*S. thermophilus* and *L. delbrueckii* ssp. *bulgaricus*) are incorporated as adducted cultures to probiotic cultures. Therefore, they could interfere in selective enumeration of probiotic strains in final products. Addtion of bile salts into culture media (0.15% w/w) is of the simplest and the most efficient method avoiding growth of yogurt bacteria after incubation at 37°C for at least 72 h (Mortazavian *et al*., 2007; Vinderola and Reinheimer, 1999). In this study, it was shown that mentioned bacteria did not growth even at 25°C or 31°C. In presence of bile salts, the cell wall of yogurt bacteria is deteriorated, while probiotics are commonly resistant to bile (Mortazavian and Sohrabvandi, 2006).

Lc. lactis ssp. *lactis* and *Lc. lactis* ssp. *cremoris* are the most commonly used lactic strains as mesophilic cultures in production of fermented milks such as fermented cheese, fermented cream and drinks based on fermented milk. They could be added with/without yogurt bacteria. Therefore, these strains could also interfere in selective enumeration of probiotic strains especially when they are employed with yogurt bacteria. The results of this study revealed that *Lc. lactis* ssp. *lactis* and *Lc. lactis* ssp. *cremoris* are able to growth in presence of bile salts (0.15 or 0.30%) when the plates were incubated at 25 or 31°C. In contrast, non of mesophilic strans were able to growth at different concentrations of bile (0.15 or 0.30%) when the plates were incubated at 37°C. The optimum growth temperature for lactic mesophilic bacteria is about 25-30°C (Korbekandi *et al*., 2011) and reasonably, their ability to growth at higher temperatures is considerably limited by presence of other stress factors such as nutritive substances in media and bile salts (as antimicrobial agents). According to these results, incubation of cultures at 37°C would inhibit the growth of mesophilic lactic strains even at bile concentration of 0.15%. Changing incubation temperature far from optimum growth temperatures of starter cultures is an efficient way to increase selectivity of culture media for enumeration of target strain. According to what mentioned, employing two screening factors (presence of bile and changing incubation temperature) to the support cultures (the two strains of yogurt bacteria and the two strains of mesophilic lactic bacteria) could easily make the MRS agar medium fully selective for enumeration of each probiotic strain (*L. acidophilus* LA-5, *L. casei* 431 and *B. lactis* BB-12). On the other words, MRS-bile agar (0.15% bile) incubated at 37°C (aerobically or anaerobically) is an effective medium for selective enumeration of *L. acidophilus* or *L. casei* or *B. lactis*. It means that all for adjuncts strains are screened by applying mentioned conditions. However, the other important problem for selective enumeration of probiotic strains is emerged when different probiotic strains are cultivated simultaneously. Because *Bifidobacterium* spp. are obligatory anaerobic microorganisms, anaerobic incubation of cultures will result in selective enumeration of *L. acidophilus* or *L. casei* (in ABYM or CBYM culture compositions)

(Mortazavian *et al*., 2007; Vinderola and Reinheimer, 2000; 1999; Lankaputhra and Shah, 1996). Also, because the colonies of the two mentioned bacteria can be morphologically differentiated on plates (Mortazavian *et al*., 2007 as well as the results of this study), selective enumeration of *L. acidophilus* and *L. casei* is achievable. The main trouble is faced for selective enumeration of bifidobacteria when these strains are cocultured with *L. acidophilus* due to the same morphological characteristics of their colonies under aerobiosis. This problem has been overcome by applying SEM methodology (Mortazavian *et al*., 2007).

CONCLUSIONS

MRS-bile agar (0.15% bile salts) could be successfully used for selective or differential enumeration of probiotic cultures (*L. acidophilus*, *L. casei* and bifidobacteria) in presence of adjunct lactic acid cultures (thermophilic and/or mesophilic cultures) when the plates were incubated at 37°C for 72 h (aerobically or anaerobically). Four main practical conclusion could be rendered as follows by using MRS-bile agar with mentioned conditions: 1) Differential enumeration of ACY culture composition when plates incubated aerobically or anaerobically; 2) selective enumeration (for *L. casei*) of BCY culture composition at aerobic condition; 3) selective enumeration of ABY culture composition at aerobic condition; and 4) differential enumeration of ABCY culture composition at aerobic condition. Also, in the ABY and ABCY culture compositions, viable counts of bifidobacteria might be selectively achieved using the subtractive enumeration method (SEM).

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