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Selective enumeration of propionibacteria in Emmental-type cheese using Petrifilm™ Aerobic Count plates added to Lithium Glycerol broth

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Propionibacteria derived from dairy products are relevant starter cultures for the production of Swiss and Emmental-type cheeses, and the monitoring of which is mandatory for proper quality control. This study aimed to evaluate an alternative procedure to enumerate propionibacteria, in order to develop a reliable and practical methodology to be employed by dairy industries. 2,3,5-triphenyltetrazolium chloride (TTC) inhibitory activity was tested against five reference strains (CIRM 09, 38, 39, 40 and 116); TTC at 0·0025% (w/v) was not inhibitory, with the exception of one strain (CIRM 116). Subsequently, the four TTC-resistant strains, three commercial starter cultures (PS-1, PB-I, and CHOO) and twelve Emmental-type cheese samples were subjected to propionibacteria enumeration using Lithium Glycerol (LG) agar, and Petrifilm™ Aerobic Count (AC) plates added to LG broth (anaerobic incubation at 30 °C for 7 d). Petrifilm™ AC added to LG broth presented high counts than LG agar ($P<0·05$) for only two reference strains (CIRM 39, and 40) and for all commercial starter cultures. Cheese sample counts obtained by both procedures did not show significant differences ($P<0·05$). Significant correlation indexes were observed between the counts recorded by both methods ($P<0·05$). These results demonstrate the reliability of Petrifilm™ AC plates added to LG broth in enumerating select *Propionibacterium* spp., despite some limitations observed for specific commercial starter cultures.

Keywords: Propionic bacteria, Petrifilm™ AC, cheese, Emmental.

Microorganisms belonging to the genus *Propionibacterium* are Gram-positive, usually pleomorphic, non-spore-forming, non-motile, range from anaerobic to aerotolerant, generally catalase-positive and are able to ferment lactate to short-chain fatty acids, mainly propionate and acetate (Carvalho et al. 1995; Jan et al. 2002; Turgay et al. 2011). *Propionibacterium* spp. have been isolated from the skin or dairy products, and examples of their species are *Prop. acidipropionici, Prop. freudenreichii, Prop. jensenii* and *Prop. thoenii* (Tilsala-Timisjarvi & Alatossava, 2001; Jenkins et al. 2002).

Propionibacteria originating from milk play an important role in the production of Swiss and Emmental-type cheeses: during maturation, lactic acid bacteria produce lactate, which is converted to propionic acid, acetic acid and carbon dioxide, and contribute to the flavour and the emergence of eyes in these cheeses (Piveteau, 1999; Thierry et al. 2004). Additionally, propionibacteria are able to synthesise biopreservative compounds, such as bacteriocins, as well as folic acid, proline and vitamin B12, as shown by several studies that have assessed the probiotic potential of this genus (Mantere-Alhonen, 1995; Meile et al. 2008; Cousin et al. 2011).

Propionibacterium spp. growth in dairy products must be controlled because it is necessary to ensure minimal concentrations in end products to allow for the development of typical characteristics and probiotic activity (Cousin et al. 2011). For monitoring in the dairy products industry, Yeast Extract Lactate (YEL) culture medium is usually employed to enumerate propionibacteria, after incubation at 30 °C for 5 d; however, this protocol does not provide adequate selectivity because enterococci and lactobacilli can also form

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visible colonies (Thierry et al. 1994). Madec et al. (1994) developed a selective medium named Lithium Glycerol (LG) based on the resistance of propionibacteria to lithium and their ability to ferment glycerol under anaerobiosis. LG agar has shown higher selectivity for propionibacteria when compared with YEL; this selectivity is considered adequate for the enumeration of this group in mixed cultures or foods containing different lactic cultures (Thierry et al. 1994; Thierry & Madec, 1995).

The conventional procedures for the enumeration of microbial groups in foods, including starter and probiotic cultures, are very time-consuming and require a variety of apparatus to ensure ideal conditions of microbial growth (as example, anaerobiosis generators and chambers). Thus, reliable alternative methods for enumerating microorganisms are needed for food industries, enhancing laboratory work and shortening the time required to obtain final results (Brichta-Harhay et al. 2008; Bremser et al. 2011). The Petrifilm™ system (3M Microbiology, St. Paul, MN, USA) is an example of such an alternative method that increases the speed of enumeration (McGregor et al. 1995; Bulte et al. 1998; Beloti et al. 2002). As a ready-to-use system, Petrifilm™ represents an improvement in speed and efficiency in conducting analyses, as well as a reduction in the laboratory work required to obtain results (Senyk et al. 1987). Petrifilm™ Aerobic Count (AC) plates have been used in combination with selective culture media for the enumeration of lactic acid bacteria (LAB), with reliable results that justify their use in the monitoring of these microorganisms in fermented foods (Champagne et al. 1994; Pattison et al. 1998; Nero et al. 2006, 2008; Ortolani et al. 2007; Gonçalves et al. 2009; Miranda et al. 2011). However, the performance of this system for the enumeration of propionic acid bacteria has not been evaluated until now.

The aim of this study was to evaluate the performance of Petrifilm™ AC associated with LG broth as an alternative method for enumerating *Propionibacterium* spp. strains and commercial starter cultures, and also propionibacteria from Emmental-type cheeses.

Materials and methods

Microbial cultures and cheese samples

Five reference strains of *Propionibacterium* spp. (*Prop. freudenreichii* subsp. *shermanii* CIRM 01, *Prop. acidipropionici* CIRM 38, *Prop. jensenii* CIRM 39, *Prop. thoenii* CIRM 40, and *Prop. freudenreichii* subsp. *freudenreichii* CIRM 116) were obtained from the Centre International de Ressources Microbiennes – Bactéries d'Intérêt Alimentaire (CIRM-BIA, INRA, Rennes, France) and stored at −20 °C in YEL agar. A loop of each stock culture was added to LG broth and incubated at 30 °C for 24–48 h; the obtained cultures were then streaked on LG agar plates and incubated under the same conditions. Isolated colonies were then transferred to LG broth and incubated at 30 °C to obtain cultures with turbidity similar to McFarland tube 1, which approximately corresponds to 3×10^8 colony forming units per ml (CFU/ml).

Three commercial lyophilised cultures used in Emmental cheese production were included in this study: PS-1 (Chr. Hansen A/S, Horsholm, Denmark), PB-I (Sacco, Cadorago, Italy), and CHOO (Zit™ Eyes, Danisco, Niebüll, Germany). All stock cultures were kept at −20 °C, and upon use, approximately 1 g each culture was transferred to LG broth and incubated at 30 °C for 24 h. Then, colonies isolation and culture preparation steps were performed as described previously for reference strains.

Emmental-type cheeses produced by three different dairy companies (one French and two Brazilian) were purchased from various retail stores and kept under refrigeration until microbiological analysis.

Culture media

LG broth was previously prepared as described by Madec et al. (1994) with the following composition (in one litre): 10 g lithium lactate, 10 g peptone, 10 g yeast extract, 6 g glycerol, 1 g powdered milk, 50 mg bromocresol purple, 328 mg K_2HPO_4 and 56 mg $MnSO_4$ (all chemicals from Sigma Aldrich, St. Louis, MO, USA). LG agar was prepared by adding bacteriological agar at 1·5% (w/v) (Merck KGaA, Darmstadt, Germany) in LG broth. After autoclaving, a cocktail of commercial antibiotics (obtained from the kit Pal Probiobac™, Laboratoires Standa, Caen, France) was added to the LG broth and agar to confer selectivity for propionibacteria.

YEL broth was prepared according Malik et al. (1968) with the following composition (in one litre): 10 g peptone, 20 ml sodium lactate, 10 g yeast extract, 328 mg K_2HPO_4 and 56 mg $MnSO_4$ (all chemicals from Sigma-Aldrich). YEL agar was prepared by adding bacteriological agar at 1·5% (w/v) (Merck) in YEL broth. YEL broth and agar were used as non-selective culture media for propionibacteria.

2,3,5-Triphenyltetrazolium chloride (TTC) reduction by propionibacteria

All reference strains were diluted ten-fold in 0·85% NaCl (w/v) and plated (in duplicate) by pouring using YEL agar added to TTC (Sigma-Aldrich), in order to achieve end concentrations of 0·0025 and 0·0050% (w/v) (Beloti et al. 1999). As controls, the same dilutions were plated on YEL agar without TTC. All plates were incubated at 30 °C for 7 d under anaerobiosis (Anaerocult, Merck); afterwards, the resulting colonies were enumerated and the counts expressed in CFU/ml. During enumeration, the ability of the *Propionibacterium* spp. strains to reduce TTC, thus forming red colonies, was recorded. This experiment was conducted in triplicate.

Propionibacteria enumeration

Immediately after recovering of *Propionibacterium* spp. strains and propionibacteria commercial starter cultures,

Table 1. Mean counts (±SD) of *Propionibacterium* spp. reference cultures plated on Yeast Extract Lactate (YEL) agar with 2,3,5-Triphenyltetrazolium chloride (TTC) at 0·0025 and 0·0050% (w/v)

Propionibacterium spp.	YEL added to TTC		Control†	Statistics‡
Cultures	0·0025%	0·0050%		
CIRM 01	9·70 ± 0·18[a]	9·29 ± 0·91[a]	9·67 ± 0·19[a]	$F_{(2,6)}=0·520$, $P=0·619$
CIRM 38	8·37 ± 0·35[a]	8·31 ± 0·44[a]	8·30 ± 0·34[a]	$F_{(2,6)}=0·025$, $P=0·976$
CIRM 39	9·34 ± 1·01[a]	nr	9·67 ± 0·29[a]	$F_{(1,4)}=0·298$, $P=0·614$
CIRM 40	8·36 ± 1·14[a]	nr	8·60 ± 0·55[a]	$F_{(1,4)}=0·111$, $P=0·756$
CIRM 116	nr	nr	9·77 ± 0·12	—

† Control: *Propionibacterium* spp. cultures were plated in YEAL agar without TTC, to show their real counts without inhibitory agent (TTC); ‡Analysis of Variance (ANOVA), *F*: ANOVA value, *P*: level of significance. Mean values in a same row followed by distinct superscript letters are significantly different ($P<0·05$). nr: not recorded

the obtained cultures were subjected to ten-fold dilution using LG broth. For cheese samples, 25 g of each cheese was obtained with a sterile knife and transferred to individual sterile bags containing 225 ml of a 0·85% NaCl (w/v) solution, homogenised, and subjected to ten-fold dilution using LG broth. Four dilutions of each culture and cheese sample were selected and pour plated on LG agar (duplicate) and in Petrifilm™ AC plates. The plates were incubated at 30 °C for 7 d at anaerobic conditions (Anaerocult, Merck). After incubation, the colonies formed on the plates were counted and the results expressed in CFU/ml (cultures) or CFU/g (cheeses). Reference strains and commercial cultures were tested in triplicate, and twelve cheese samples were evaluated (four from each dairy company).

Molecular identification of Propionibacterium spp.

To verify the adequate selectivity of the tested culture media, a total of 70 colonies recorded from cheese samples plated on LG agar and Petrifilm™ AC added to LG broth were subjected to morphological characterisation by Gram staining and genus-specific PCR (Dasen et al. 1998). The selected colonies were streaked on YEL agar (incubation at anaerobic conditions, Anaerocult, Merck, at 30 °C for 48 h), and isolated colonies were transferred to YEL broth (incubated at 30 °C for 48 h). The purified cultures were subjected to DNA extraction using the Wizard Genomic DNA Purification Kit (Promega Corporation, Madison, WI, USA). The multiplex PCR reactions contained 12,5 µl of 2 × Go Taq Green Master Mix (Promega), 1 µl of the primer bak4 (AGGAGGTGATCCARCCGCA, reverse, at 100 µM), 1 µl of the primer bak11w (AGTTTGATCMTGGCTCAG, forward, at 100 µM), 0,5 µl of the primer gd1 (TGCTTTCGATACGGGTTGAC, forward, at 100 µM), 2 µl of extracted DNA (in a minimal concentration of 15 ng/µl), and ultra-pure PCR water (Promega) to a final volume of 50 µl. The PCR conditions were initial denaturation at 95 °C for 3 min; 35 cycles at 95 °C for 30 s, 58 °C for 30 s and 72 °C for 30 s; and a final extension step at 72 °C for 10 min. Amplification products were mixed with 20x GelRed stain (Biotium Inc., Hayward, CA, USA) at a ratio of 5 : 1, and the products were submitted to electrophoresis in a 1% agarose gel made with 0·5 × TBE. PCR products of 889 bp (primers bak4 and gd1) were recorded as typical for *Propionibacterium* spp., and PCR products of 1508 bp (primers bak11w and bak4) were recorded as positive controls for the reactions.

Statistical analysis of results

The counts of reference strains plated on YEL agar with and without added TTC were compared to verify the inhibition of this substance over the propionibacteria. In addition, the TTC reduction capacity of the cultures was observed.

The counts of reference strains, commercial cultures and cheese samples obtained on LG agar and Petrifilm™ AC added to LG broth were converted to $\log_{10}$, and the mean counts were compared by ANOVA and Tukey tests ($P<0·05$). In addition, the $\log_{10}$ counts were compared by linear regression ($P<0·05$). All analyses were performed using software Statistica 7.0 (StatSoft Inc., Tulsa, OK, USA).

Results and discussion

All tested strains were capable of reducing TTC, forming red colonies in YEL agar, and inhibitory activity of TTC was observed only in specific situations (Table 1): CIRM 39, CIRM 40, and CIRM 116 presented very small colonies when plated on YEL agar added to TTC at 0·0050% (w/v), making proper and reliable enumeration difficult and indicating an inhibitory activity of TTC. However, 0·0025% (w/v) TTC did not inhibit the growth of all reference strains, except CIRM 116 TTC (Table 1). TTC at 0·0050% (w/v) determined an inhibitory activity that resulted in small sized colonies of some *Propionibacterium* spp. strains; however, when the colonies enumeration was possible, the final counts did not differ from the controls in YEL agar without TTC ($P>0·05$, Table 1). This dye is used as a colour indicator in a number of culture media for the enumeration of bacteria (Senyk et al. 1987), as in some Petrifilm™ plate systems (e.g., AC), because it facilitates the visualisation of the resulting colonies by its reduction and the appearance of red pigment (Kenner et al. 1961). Based on these results, CIRM 116 was not considered in the following methods conducted to evaluate the performance of Petrifilm™ AC plates added to LG broth to enumerate propionibacteria.

Table 2. Mean counts (± SD) of Propionibacterium spp. reference strains, commercial cultures, and from Emmenthal cheese type samples subjected to enumeration by Lithium Glycerol (LG) agar and Petrifilm™ AC plates added to LG broth

Group	Sample	*n*	LG agar	Petrifilm™ added to LG broth	Statistics†
Reference strains	CIRM 01	3	$9{\cdot}86 \pm 0{\cdot}10^{a}$	$9{\cdot}82 \pm 0{\cdot}08^{a}$	$F_{(1,4)}=0{\cdot}21$, $P=0{\cdot}668$
	CIRM 38	3	$8{\cdot}02 \pm 0{\cdot}17^{a}$	$8{\cdot}13 \pm 0{\cdot}20^{a}$	$F_{(1,4)}=0{\cdot}61$, $P=0{\cdot}479$
	CIRM 39	3	$6{\cdot}93 \pm 0{\cdot}82^{a}$	$8{\cdot}49 \pm 0{\cdot}30^{b}$	$F_{(1,4)}=9{\cdot}44$, $P=0{\cdot}037$
	CIRM 40	3	$8{\cdot}24 \pm 0{\cdot}30^{a}$	$9{\cdot}11 \pm 0{\cdot}35^{b}$	$F_{(1,4)}=10{\cdot}69$, $P=0{\cdot}031$
	all strains	12	$8{\cdot}26 \pm 1{\cdot}16^{a}$	$8{\cdot}89 \pm 0{\cdot}71^{a}$	$F_{(1,22)}=2{\cdot}57$, $P=0{\cdot}123$
Commercial cultures	PS-1	3	$8{\cdot}24 \pm 0{\cdot}28^{a}$	$9{\cdot}29 \pm 0{\cdot}18^{b}$	$F_{(1,4)}=96{\cdot}15$, $P=0{\cdot}001$
	PB-I	3	$8{\cdot}82 \pm 0{\cdot}06^{a}$	$9{\cdot}54 \pm 0{\cdot}11^{b}$	$F_{(1,4)}=9{\cdot}59$, $P=0{\cdot}036$
	CHOO	3	$5{\cdot}91 \pm 0{\cdot}79^{a}$	$8{\cdot}01 \pm 1{\cdot}08^{b}$	$F_{(1,4)}=30{\cdot}49$, $P=0{\cdot}005$
	All cultures	9	$7{\cdot}56 \pm 1{\cdot}54^{a}$	$8{\cdot}94 \pm 0{\cdot}9^{b}$	$F_{(1,16)}=5{\cdot}44$, $P=0{\cdot}033$
Cheese samples	Company 1	4	$5{\cdot}78 \pm 0{\cdot}41^{a}$	$6{\cdot}73 \pm 0{\cdot}40^{b}$	$F_{(1,6)}=10{\cdot}78$, $P=0{\cdot}017$
	Company 2	4	$7{\cdot}97 \pm 1{\cdot}25^{a}$	$8{\cdot}90 \pm 1{\cdot}11^{a}$	$F_{(1,6)}=1{\cdot}26$, $P=0{\cdot}304$
	Company 3	4	$8{\cdot}41 \pm 0{\cdot}55^{a}$	$8{\cdot}56 \pm 0{\cdot}58^{a}$	$F_{(1,6)}=0{\cdot}14$, $P=0{\cdot}725$
	All companies	12	$7{\cdot}39 \pm 1{\cdot}41^{a}$	$8{\cdot}06 \pm 0{\cdot}21^{a}$	$F_{(1,22)}=1{\cdot}58$, $P=0{\cdot}221$

†Analysis of Variance (ANOVA), *F*: ANOVA value, *P*: level of significance. Mean values in a same row followed by distinct superscript letters are significantly different ($P<0{\cdot}05$)

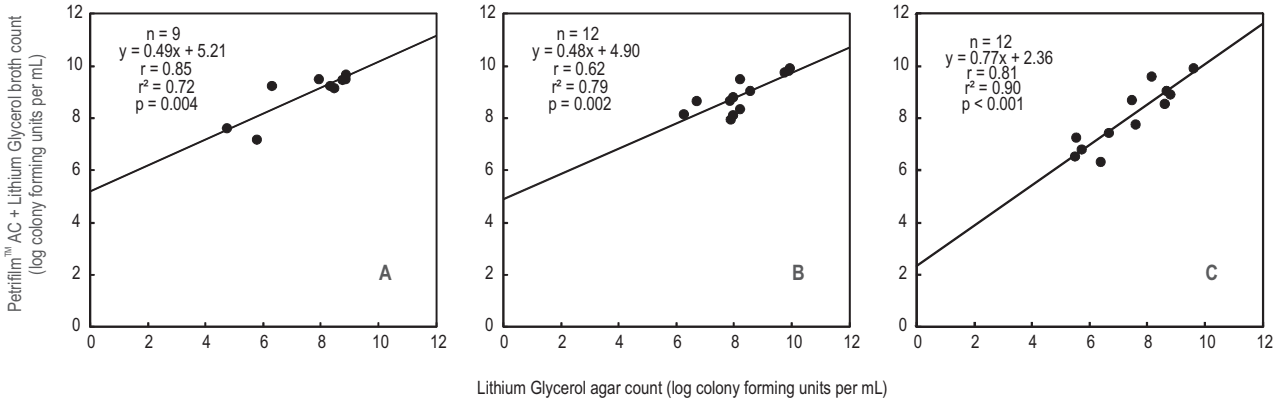

Fig. 1. Linear regression parameters and dispersion of propionibacteria counts (in log of colony forming units per ml) of reference strains (a), and commercial cultures (b), and Emmental-type cheese samples (c) obtained by Lithium Glycerol (LG) agar and Petrifilm™ AC added to LG broth. In each graph: *n*: number of samples; *r*: correlation index; r^2: coefficient of determination; *P*: level of significance.

Figure 1 shows the linear regression parameters and dispersion of propionibacteria counts of reference and commercial cultures and cheese samples obtained by LG agar and Petrifilm™ AC plates associated with LG broth. The correlation coefficients obtained for each data group were significant ($P<0{\cdot}05$), demonstrating the levels of equivalence of both methods. Despite presenting significant correlations (Fig. 1), Petrifilm™ AC added to LG broth trended to present higher colony counts when compared to the conventional plating system, although the differences were not significant difference according to ANOVA ($P>0{\cdot}05$, Table 2). Although the observed differences between the tested methodologies, the results indicate the feasibility of the Petrifilm™ AC for enumerating propionibacteria when compared with the conventional plating system, as observed in previous studies focusing other starter cultures and lactic bacteria, microorganisms that present similar biochemical and technological properties (Champagne et al. 1994; McGregor et al. 1995; Pattison et al. 1998; Nero et al. 2006, 2008; Ortolani et al. 2007; Gonçalves et al. 2009; Miranda et al. 2011).

The morphological analysis of the 70 colonies selected from both LG agar and Petrifilm™ AC plates added to LG broth indicate the adequate selectivity of this culture medium for this genus: all of the colonies presented typical *Propionibacterium* spp. characteristics (Gram positive, pleomorphic). Furthermore, PCR of the 70 isolates resulted in 889 bp products, confirming the identification of *Propionibacterium* spp. (Dasen et al. 1998). Based on the obtained data, the presence of lithium, glycerol, and a cocktail of antimicrobials, associated with incubation under anaerobiosis were sufficient to provide adequate selectivity of the culture medium to enumerate only *Propionibacterium* spp., either by conventional plating procedure or by association with Petrifilm™ AC plates. Although typically employed for propionibacteria enumeration in cheese, LG selective agents can inhibit the growth of some specific strains of this genus (Rossi et al. 2000), which can explain

some of the differences observed in the present study (Table 2). Because commercial cultures usually are composed of several strains of starter cultures (Dworkin et al. 2006), some of them may have been inhibited by the selective agents in LG agar and were not able to form visible colonies (Table 2). In contrast, the Petrifilm™ AC plates added to LG broth may have provided better conditions for strains to form visible colonies, considering the presence of additional nutritive substances of the system, and also the presence of a dye, TTC, that improves the visualisation of colonies.

Propionibacterium spp. were able to reduce TTC properly, and the dye did not show inhibitory activity against the strains, when present at 0·0025% (w/v), except for the CIRM 116 reference strain. When associated with LG broth, the Petrifilm™ AC plates presented adequate selectivity and the proper recovery of *Propionibacterium* spp. reference strains and propionibacteria commercial starter cultures, as well as from the Emmental-type cheese samples. The obtained results indicate the adequacy of Petrifilm™ AC plates added to LG broth for the enumeration of propionibacteria as a monitoring tool in the dairy industry.

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