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Novel method based on chromogenic media for discrimination and selective enumeration of lactic acid bacteria in fermented milk products

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ABSTRACT

Microbial analyses of fermented milk products require selective methods to discriminate between close species simultaneously present in high amounts. A culture-based method combining novel chromogenic agar media and appropriate incubation conditions was developed to enumerate lactic acid bacteria (LAB) strains in fermented milk. M1 agar, containing two chromogenic substrates, allowed selective enumeration of *Lactobacillus rhamnosus*, two strains of *Lactobacillus paracasei* subsp. *paracasei* and *Streptococcus salivarius* subsp. *thermophilus* based on differential β-galactosidase and β-glucosidase activities. Depending on the presence of some or all of the above strains, M1 agar was supplemented with L-rhamnose or vancomycin and incubations were carried out at 37 °C or 44 °C to increase selectivity. A second agar medium, M2, containing one chromogenic substrates was used to selectively enumerate β-galactosidase producing *Lactobacillus delbrueckii* subsp. *bulgaricus* at 47 °C. By contrast with the usual culture media, the chromogenic method allowed unambiguous enumeration of each species, including discrimination between the two *L. paracasei*, up to 10^9 CFU/g of fermented milk. In addition, the relevance of the method was approved by enumerating reference ATCC strains in pure cultures and fermented milk product. The method could also be used for enumerations on non-Danone commercial fermented milk products containing strains different from those used in this study, showing versatility of the method. To our knowledge, this is the first description of a chromogenic culture method applied to selective enumeration of LAB.

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1. Introduction

Yoghurt has a long history of consumption as dietary product. It presents interesting nutritional and organoleptic properties (Astrup, 2014; El-Abbadi et al., 2014; Rizzoli, 2014) conferred by milk fermentation by starter bacteria, the most commonly employed being *Streptococcus salivarius* subsp. *thermophilus* and *Lactobacillus delbrueckii subsp. bulgaricus*. During the past three decades, yoghurt has also received considerable attention as vehicle for probiotic bacteria and more and more probiotic yoghurts are now available on the market (Boyer and Geng, 2014). Probiotics are defined as "live microorganisms which when administered in adequate amounts confer a health benefit on the host" (FAO, 2006). Members of the genera *Lactobacillus* and *Bifidobacterium* are mainly employed as probiotics, and among lactic acid bacteria (LAB) *Lactobacillus rhamnosus* and *Lactobacillus casei/paracasei* are frequently used and therefore, well studied (Bron et al., 2012). The suggested minimal dose is generally agreed to be around 10^6–10^7 colony forming units CFU/g (Ashraf and Shah, 2011; Karimi et al., 2012; Vasiljevic and Shah, 2008; Vinderola and Reinheimer, 2000). In order to ensure microbiological and nutritional quality and product compliance, manufacturers have to be able to discriminate and selectively quantify each strain. This is

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generally achieved using culture-based and/or molecular methods. Traditional culture-based methods are routinely used in analytical microbiology laboratories to identify and quantify viable microbes in dairy products (Sohier et al., 2014). Many agar plate media and protocols have been proposed to selectively enumerate LAB and bifidobacteria in yoghurt (Ashraf and Shah, 2011; Saccaro et al., 2011; Talwalkar and Kailasapathy, 2004; Tharmaraj and Shah, 2003; Van de Casteele et al., 2006; Vinderola and Reinheimer, 2000). Most of the methods were based on the use of standard non selective LAB culture media, with variations in NaCl concentration, carbon sources, pH, inclusion of antibiotics and different incubation temperatures in order to improve selectivity. However, discriminating power generally remained limited to the genus, and at best to the species (Saccaro et al., 2011; Tharmaraj and Shah, 2003; Van de Casteele et al., 2006). Moreover, in mixed matrices such as yoghurt, it is often difficult to selectively enumerate each individual strain in the presence of the others (Ashraf and Shah, 2011). Despite the profusion of culture-based methods described in the literature, many have been evaluated on isolated cultures only, while evaluation on mixed fermented products is necessary to assess their real performances. Currently, a few internationally validated methods are available for enumeration of bifidobacteria (ISO, 2010), *Lactobacillus acidophilus* (ISO, 2006a), citrate-fermenting bacteria (ISO, 2006a,b) and the characteristic yoghurt fermenters *Lactobacillus bulgaricus and Streptococcus thermophilus* (ISO, 2003) in milk or yogurt products, but their discriminating power towards other LABs present in mixtures is sometimes low (Boyer and Combrisson, 2013). Today, there is a clear need for specific and reliable selective methods, and when the available culture methods are employed, it is generally recommended to use additional molecular methods (mostly PCR-based methods) to confirm identification of the analyzed strains. A recent review has summarized the existing methods as well as those with interesting potentialities for dairy analytical purposes (Sohier et al., 2014). Among the molecular methods, PCR-based methods, flow cytometry and other fluorescent labeling-based methods are the most promising. However, as a drawback of their high sensitivity and specificity, these methods are very sensitive to small experimental variations that may lead to inaccurate results. They require a strong standardization and performances evaluation, and few of them like Flow cytometry method, have been validated yet for quality control use in analytical facilities. In addition, molecular methods are more expensive and often necessitate a more qualified staff than culture-based methods. Thus, culture methods remain very valuable as they are simpler to use, cheaper and provide visual results.

The reference medium for enumeration of *L. paracasei* and *L. rhamnosus* from milk products is MRS (de Man, Rogosa and Sharpe) containing 1 μg/ml vancomycin (MRS-V) (Ashraf and Shah, 2011; Colombo et al., 2014; Tharmaraj and Shah, 2003). The fermenting strains *L. bulgaricus* and *S. thermophilus* can be enumerated on acidified MRS (MRSa) at pH 5.4 ± 0.1 and M17, respectively, according to ISO 7889:2003 (ISO, 2003b). However, when these species are all present in milk products a combination of the above media and variations of incubation conditions have to be employed to deduce the CFU numbers for each species. Even then, individual strains of the same species/subspecies cannot be distinguished and enumerated from mixed samples.

One strategy to improve selectivity of the existing culture media and methods is the development of chromogenic media (Manafi, 2000; Druggan and Iversen, 2014). They are based on the cleavage of a colorless substrate (chromogen) by target bacteria's enzymatic activity, which releases a chromophore resulting, usually after oxidation, in colored colonies on agar plates. While chromogenic media have been regularly developed, validated and commercialized for analyses of pathogenic and spoilage bacteria starting from the 80s, they have been totally neglected for analyses of LABs and probiotics (Sohier et al., 2014). The development of chromogenic media relies on the knowledge of bacterial metabolism, and thus, is particularly appropriate to the case of LABs and probiotics that are generally well characterized. Moreover, chromogenic media represent an innovative alternative to selective media using antibiotics, as LABs are generally selected to be the least possible antibiotic-resistant due to food safety considerations (EFSA, 2012). These media are also well adapted to selective enumerations from mixed samples, as demonstrated for instance in clinical and environmental microbiology (Akter et al., 2014; Noble and Weisberg, 2005; Perry and Freydiere, 2007).

This study describes a method based on new chromogenic media to selectively enumerate LABs in fermented milk products. The objective was to discriminate between *L. rhamnosus*, two close strains of *L. paracasei*, *L. bulgaricus* and *S. thermophilus*. The media and culture conditions were evaluated on pure cultures but also on mixtures from fermented milk products. The results were compared to those obtained with reference media, when applicable, in order to establish the new medias' performances.

2. Materials and methods

2.1. Bacterial strains and cultures

L. rhamnosus CNCM (Collection Nationale de Cultures de Microorganismes, Institut Pasteur, Paris, France) I-3690, *L. rhamnosus* ATCC (American Type Culture Collection) 53103, *L. paracasei* subsp. *paracasei* CNCM I-1518 (hereafter named *L. paracasei* 1, for simplification), *L. paracasei* subsp. *paracasei* CNCM I-3689 (hereafter named *L. paracasei* 2), *L. casei* subsp. *casei* ATCC 393, *L. delbrueckii* subsp. *bulgaricus* CNCM I-2787, *L. delbrueckii* subsp. *bulgaricus* ATCC 11842, *S. salivarius* subsp. *thermophilus* CNCM I-2773, CNCM I-2835, CNCM I-2778 and *S. salivarius* subsp. *thermophilus* ATCC 19258 were used in this study. All strains were stored at −80 °C in MRS broth (BD Difco, New Jersey, USA) for *Lactobacillus* strains or Elliker (BD Difco) for *S. thermophilus*, supplemented with 5.3% DMSO (v/v). *Lactobacillus* pure cultures were grown from frozen stocks inoculated in MRS broth and *S. thermophilus* was grown on M17 + 0.5% lactose.

2.2. Characterization of bacterial enzymatic activities

Phenotypical comparison of the strains was carried out at the Laboratory Veterinary Agency (London, UK) using Phenotype Microarrays (Biolog, Hayward CA, USA), according to the manufacturer's specifications. Based on the use of different carbon sources, nitrogen, phosphate, sulfur, on the inclusion of nutritional additives or stress-inducing agents (salts, pH, antimicrobial agents), about 2000 phenotypes were compared. This resulted in strain-specific phenotypical profiles for each targeted strain. Differential carbohydrate fermentation patterns were observed and selected for preliminary testing of the corresponding enzymatic activities. USB chromogenic agar (bioMérieux, Marcy l'Etoile, France) was used as non-selective medium to distinguish the strains based on their β-galactosidase and/or β-glucosidase activities. Pure cultures from MRS or M17 broth were plated on USB non selective agar medium and incubated aerobically, microaerobically (using GasPak EZ Container Systems, Becton Dickinson) or anaerobically (using Oxoid™ Anaerogen™, Thermo Scientific) at 37 °C. Colony color was observed after 48 h incubation.

2.3. Standard and chromogenic agar media (Patent application number WO2015092258)

Agar medium used as reference to enumerate *L. rhamnosus* and *L. paracasei* in fermented milk products was MRS + vancomycin 1 μg/ml (MRS-V). *L. bulgaricus* and *S. thermophilus* were enumerated on MRS acidified to pH 5.4 (MRSa) and M17 respectively. MRS and M17 were purchased from bioMérieux and vancomycin was from Sigma Aldrich, Saint Louis, USA. The compositions of M1 and M2 chromogenic media set up in this study are described in Table 1.

2.4. Enumerations on two Danone fermented dairy products and fermented product with ATCC strains

Two Danone fermented dairy products (FDP) were used to test and improve efficiency of M1 and M2 agar media and to set up the method's growth conditions leading to selective enumeration of each constituent strain from the mixture. The strain composition of FDP-1 and FDP-2 is detailed in Table 2. Serial (factor-10) dilutions of the fermented products were made in tryptone salt (bioMérieux). One ml of the appropriate dilutions (three consecutive dilutions in the range of the expected colony counts) was then deposited in duplicate Petri dishes, and 15 ml of M1 or M2 agar media were poured into Petri plates as follows. For FDP-1, two series of plates was prepared, one containing M1, and the other containing M2. For FDP-2, four series of plates containing either M1, M1 + L-rhamnose 10 g/L (M1-R), M1 + vancomycin 1 μg/ml (M1-V) or M2 were made. After careful mixing and solidification, the plates were incubated for 48 h at the appropriate temperature: the M1 series for FDP-1 enumeration was incubated at 37 °C, the M2 series (for both FDP-1 and FDP-2) was incubated at 47 °C, the M1-R and M1-V series were incubated at 37 °C, and M1 for FDP-2 enumeration was incubated at 44 °C. All M1 and M1-supplemented plates were incubated under microaerobiosis using GasPak EZ Container Systems (Becton Dickinson) and M2 plates were incubated under anaerobiosis obtained with Oxoid™ Anaerogen™ (Thermo Scientific). In parallel, enumerations were also carried out using the reference agar media described above. Samples from the same serial dilutions as the ones prepared for enumeration with the chromogenic method were used. MRS-V plates were incubated for 48 h at 37 °C (for FDP-1) or at 37 °C and 44 °C (for FDP-2) under microaerobiosis (using GasPak EZ Container Systems, Becton Dickinson), M17 plates were incubated for 48 h at 37 °C (for FDP-1) or 44 °C (for FDP-2) under aerobiosis and MRSa was incubated for 48 h at 44 °C (for FDP-1) and 50 °C (for FDP-2) under anaerobiosis. Three different bottles of each FDP were analyzed, on three consecutive days.

In a similar manner, enumerations tests with a fermented dairy product containing the following reference strains from ATCC collection: *L. rhamnosus* ATCC 53103, *L. casei* subsp. *casei* ATCC 393, *L. delbrueckii* subsp. *bulgaricus* ATCC 11842 and *S. salivarius* subsp. *thermophilus* ATCC 19258 in combination with the *L. paracasei* 2 (to maintain a similar product with two *L. casei*/*L. paracasei* strains) was performed to confirm the relevance of the method.

Table 2
Strain composition of FDP-1 and FDP-2.

Product	Strain
FDP-1	- *L. paracasei* subsp. *paracasei* CNCM I-1518 (*L. paracasei* 1)
	- *L. delbrueckii* subsp. *bulgaricus* CNCM I-2787
	- *S. thermophilus* CNCM I-2773, CNCM I-2835 and CNCM I-2778
FDP-2	- *L. paracasei* subsp. *paracasei* CNCM I-1518 (*L. paracasei* 1)
	- *L. paracasei* subsp. *paracasei* CNCM I-3689 (*L. paracasei* 2)
	- *Lactobacillus rhamnosus* CNCM I-3690
	- *L. delbrueckii* subsp. *bulgaricus* CNCM I-2787
	- *S. thermophilus* CNCM I-2773, CNCM I-2835 and CNCM I-2778

2.5. Enumerations on various commercial products (non-Danone)

Eight commercial products sold in Europe and USA between 2012 and 2014 and containing strains of the same species as the ones targeted in this study were analyzed. The best combination of chromogenic media to be used for each product was determined based on the available labeling. The products were diluted, petri dishes were inoculated, prepared with the appropriate M1 and M2

Table 1
Composition of M1 and M2 agar.

Constituent	Concentration ($g.L^{-1}$)
M1[a]	
Tryptic digest of casein	2.5
Peptic digest of meat	2.5
Papaic digest of soy	5
Sodium glycerophosphate	19
Lactose	5
Yeast extract	2.5
Meat extract	5
Magnesium sulfate	0.25
Ascorbic acid	0.5
Agar	15
6-chloro-3-indoxyl-β-D-galactopyranoside (Salmon-gal)	0.2
5-bromo-4-chloro-3-indolyl-β-D-glucopyranoside (X-glc)	0.1
M2[b]	
Peptone	10
Yeast extract	5
Beef extract	10
Potassium phosphate dibasic	2
Sodium acetate	5
Ammonium citrate dibasic	2
Magnesium sulfate	0.2
Manganese sulfate monohydrate	0.05
Agar	15
5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal)	0.15

[a] The final pH is 7.2 ± 0.2.
[b] The final pH is 5.4 ± 0.2.

agar media and incubated as described above for FDP-1 and FDP-2. Duplicate plates were prepared for each dilution, and three bottles of each FDP were tested for each product. Identification of the different species was confirmed by 16S rDNA sequencing. Colonies from each type were re-isolated, DNA was isolated with FTA/FTA Elute Sample Collection Cards and Kits (GE Healthcare, Buckinghamshire, United Kingdom) and 16S rDNA genes were identified using MicroSeq® 500 16S rDNA Sequencing Kit (Life Technologies, Carlsbad, USA) according to the manufacturers' instructions. Sequences of at least 500 bp were compared using Microbial identification system (Microseq® Analysis Software, Life Technologies).

2.6. Robustness of the method

Statistical analyses to determine repeatability, reproducibility and uncertainty of the method were carried out following interlaboratory tests (two laboratories). For each laboratory and each incubation condition, three different batches from FDP-1 and FDP-2 were analyzed, and for each batch three different bottles of each FDP were enumerated on three consecutive days. The repeatability limit r ($\log_{10}$ CFU/g) was calculated as the value less than or equal to which the absolute difference between two tests results obtained under repeatability conditions (same method, material, operator, laboratory, short time interval) is expected with a probability of 95%. The reproducibility limit R ($\log_{10}$ CFU/g) was calculated as the value less than or equal to which the absolute difference between two test results obtained under reproducibility conditions (same method, different material, operator, laboratory) is expected with a probability of 95%. Calculations of repeatability and reproducibility following interlaboratory tests are described in NF V 03-110 (NF, 2010). A three-way ANOVA with nested effects for each strain and laboratory was used. Uncertainty (in $\log_{10}$ CFU/g) corresponding to a 95% confidence interval of the measurements was determined for the analysis of one or three bottles. Statistical analyses were performed using SAS® v9.3 software (SAS Institute Inc., Cary, USA).

3. Results

3.1. Preliminary characterization of enzymatic activities

Following a comparative analysis with Phenotype Microarrays technology on the starter and probiotic strains used in this study (data not shown), two potentially interesting enzymatic activities were selected. Beta-galactosidase was expressed by *S. thermophilus* and *L. bulgaricus*, but not by *L. paracasei*. Interestingly, β-glucosidase was expressed by *L. paracasei* 1 but not *L. paracasei* 2. *L. rhamnosus* expressed both β-galactosidase and β-glucosidase. Preliminary assays on non-selective USB agar plates confirmed these activities and the possibility to use them to phenotypically distinguish the strains (Table 3). The starters *S. thermophilus* and *L. bulgaricus* formed pink colonies (β-gal+). It should be mentioned that the three *S. thermophilus* strains could not be distinguished from each other based on the targeted enzymatic activities. Discrimination between the three strains was out of the scope of this study, as from a quality control point of view it is not important to be able to selectively enumerate each individual strain. *L. paracasei* 1 and 2 (blue and colorless, respectively) could be distinguished from each other and from *L. rhamnosus* (blue to purple colonies due to the double enzymatic activity). Thus, these two enzymatic activities were potentially interesting to be exploited into more specific chromogenic media.

Table 3
Screening of enzymatic activities on USB agar.

Strain	Enzymatic activity		Colony color on USB
L. rhamnosus	β-gal+	β-gluc+	blue/purple
L. paracasei 1	β-gal-	β-gluc+	blue
L. paracasei 2	β-gal-	β-gluc-	colorless
S. thermophilus 1	β-gal+	β-gluc-	pink
S. thermophilus 2	β-gal+	β-gluc-	pink
S. thermophilus 3	β-gal+	β-gluc-	pink
L. bulgaricus	β-gal+	β-gluc-	pink

β-gal+, β-galactosidase positive; β-gal-, β-galactosidase negative.
β-gluc+, β-glucosidase positive; β-gluc-, β-glucosidase negative.

3.2. Chromogenic media set up and assessment

Two chromogenic media adapted to the growth requirements of LABs and allowing good expression and visualization of β-galactosidase and/or β-glucosidase were designed (Table 1). They were first tested with pure cultures of each strain, and then in combination. The M1 medium was dedicated to discrimination between *L. paracasei* 1, *L. paracasei* 2, *L. rhamnosus* and *S. thermophilus* (Table 4). Growth of *L. bulgaricus* was not optimal on M1, giving small colonies and satellites. As this species displays optimal growth in acidic conditions and higher incubation temperature, the M2 medium was set up to selectively and solely enumerate *L. bulgaricus*, and incubation was performed at 47 °C under anaerobiosis. *L. rhamnosus* could also grow on M2 at 47 °C but it produced colorless colonies that were unequivocally distinguishable from *L. bulgaricus*.

Assays performed on FDP-1 and FDP-2 allowed overall good discrimination (data not shown). However, distinction between *L. rhamnosus* and *L. paracasei 1* on M1 remained difficult due to close colony colors (blue and turquoise, respectively). Moreover, enumeration of *L. paracasei* 2 was not optimal due to a lower charge in the products, slower growth and small colony size. In order to improve distinction between *L. rhamnosus* and *L. paracasei 1,* M1 was supplemented with L-rhamnose. In these conditions, growth and color of *L. paracasei* 1 remained unchanged but *L. rhamnosus* colonies were colorless (Table 4). Thus, M1-R allowed clear distinction between the two species. In order to unambiguously discriminate *L. rhamnosus* from *L. paracasei* 2, both displaying colorless colonies on M1-R, this medium was combined with incubation at 37 °C and was dedicated to enumeration of *L. paracasei* 1, while *L. rhamnosus* was enumerated on M1 medium incubated at 44 °C (no growth of *L. paracasei* 2). In order to improve selective enumeration of *L. paracasei* 2, vancomycin was added to M1, and incubation was performed at 37 °C. The growth of *S. thermophilus* was inhibited on M1-V, allowing clear-cut visualization of *L. paracasei* 2 small colorless colonies (Table 4).

3.3. Selective enumeration on Danone fermented milk products

FDP-1 and FDP-2 were analyzed using the method set up in this study as well as reference media. For FDP-1, which contains only *L. paracasei* 1 and the four fermenting strains (three *S. thermophilus* and one *L. bulgaricus*), M1 plates incubated at 37 °C allowed selective enumeration of *L. paracasei* 1 and *S. thermophilus*, and M2 plates incubated at 47 °C were used to selectively count *L. bulgaricus.* Similar CFU numbers of *L. paracasei* 1 and *S. thermophilus* were found on M1 agar and on reference agar media (Table 5). It must be mentioned that, by contrast with the high selectivity of *S. thermophilus* counts on M1, results from M17 reference agar were only approximate due to the important noise from other species present.

FDP-2 contains the four fermenting strains (three *S. thermophilus* and one *L. bulgaricus*), two *L. paracasei* and one *L. rhamnosus* strain. Thus, to clearly discriminate all the strains, *L. paracasei* 1 was

Table 4
Growth and colony characteristics on M1 and M2 agar.

Strain	Media and incubation conditions				
	M1	M2	M1	M1-R	M1-V
	37 °C, microaerobiosis	47 °C, anaerobiosis	44 °C, microaerobiosis	37 °C, microaerobiosis	37 °C, microaerobiosis
L. rhamnosus	Blue	Colorless	Blue	Colorless	Blue
L. paracasei 1	Turquoise	NG	NG	Turquoise	Turquoise
L. paracasei 2	Colorless	NG	NG	Colorless	Colorless
S. thermophilus 1, 2, 3	Magenta	NG	Magenta	Magenta	NG
L. bulgaricus	NG	Green	NG	NG	NG

NG, no growth.

Table 5
Enumerations from two Danone products (data from internal validation).

Product and species	Reference medium				Chromogenic medium				Difference vs reference medium
	Method	CFU/g	log_{10} CFU/g	SD[a]	Method	CFU/g	log_{10} CFU/g	SD[a]	log_{10} CFU/g
FDP-1									
L. paracasei 1	MRS-V, 37 °C	$5.11.10^8$	8.71	0.07	M1, 37 °C	$5.02 \cdot 10^8$	8.70	0.05	0.01
S. thermophilus	M17, 37 °C	$7.22.10^8$	8.86	0.07	M1, 37 °C	$6.55 \cdot 10^8$	8.82	0.06	0.04
L. bulgaricus	MRSa, 44 °C	$3.04.10^6$	6.48	0.07	M2, 47 °C	$2.56 \cdot 10^6$	6.41	0.05	0.07
FDP-2									
L. paracasei 1	MRS-V, 37 °C[b]	$4.21.10^8$	8.62	0.11	M1-R, 37 °C	$1.45 \cdot 10^8$	8.16	0.06	NA
L. paracasei 2					M1-V, 37 °C	$4.32 \cdot 10^7$	7.64	0.06	NA
L. rhamnosus	MRS-V, 44 °C	$1.76.10^8$	8.24	0.12	M1, 44 °C	$1.87 \cdot 10^8$	8.27	0.07	0.03
S. thermophilus	M17, 44 °C[c]	NA	NA	NA	M1, 44 °C	$6.75 \cdot 10^8$	8.83	0.13	NA
L. bulgaricus	MRSa, 50 °C	$2.59.10^5$	5.41	0.18	M2, 47 °C	$3.00 \cdot 10^5$	5.48	0.15	0.07

NA, not applicable.
[a] For reference medium the SD was calculated on 9 values (enumeration results: 3 cups of product per day/3 different days); for chromogenic medium the SD was calculated on 27 values (enumeration results: 3 cups of product per day/3 different productions of medium/3 different days).
[b] This condition also allows growth of *L. rhamnosus*. Therefore, *L. paracasei* [1 + 2] counts are obtained by subtraction of *L. rhamnosus* counts at 44 °C from total counts of [*L. rhamnosus* + *L. paracasei* 1 + 2] at 37 °C.
[c] The impossibility to determine *S. thermophilus* counts on reference medium does not allow comparison with counts on chromogenic agar.

enumerated on M1-R and *L. paracasei* 2 was enumerated on M1-V (Fig. 1B and C, respectively). The presence of high *S. thermophilus* load was not an issue, as these vancomycin-sensitive strains did not grow on M1-V, and presented a distinctive magenta color on M1-R (Fig. 1B). *L. rhamnosus* and *S. thermophilus* were both enumerated on M1 agar incubated at 44 °C (Fig. 1A), while M2 incubated at 47 °C was used to determine *L. bulgaricus* counts (Fig. 1D). *L. rhamnosus* counts were similar on M1 agar and usual culture media (Table 5). Using standard MRS-V agar, not only *L. paracasei* 1 and 2 could not be distinguished from each other, but they also could not be distinguished from *L. rhamnosus*. Thus, only global *L. paracasei* counts were determined by subtracting total *L. rhamnosus* + *L. paracasei* (obtained from incubation at 37 °C) from *L. rhamnosus* counts (obtained from growth at 44 °C). By contrast, M1-R or M1-V allowed selective enumeration of each strain. *L. bulgaricus* enumerated on M2 agar provided consistent results with MRSa. For both FDP-1 and FDP-2, due to results imprecisions on reference media and the fact that outcomes on reference and chromogenic media were different, statistical comparison of colony counts between reference and chromogenic media could not be carried out. The limits of repeatability and reproducibility of the method were calculated, as well as the uncertainty for analysis of one bottle of each FDP or for the mean of measurements on three bottles (Table 6). Repeatability and reproducibility give an indication of the acceptable values to be obtained for reliable results. Limit of reproducibility results indicate satisfactory precision of the method, and were in agreement with the usually accepted threshold value (close to 0.5 log CFU/g) (ISO, 2010; ISO, 2006a).

3.4. Suitability of the method for ATCC strains enumeration

Individual ATCC reference strains (*L. rhamnosus* ATCC 53103, *L. casei* subsp. *casei* ATCC 393, *L. delbrueckii* subsp. *bulgaricus* ATCC 11842 and *S. salivarius* subsp. *thermophilus* ATCC 19258) and a fermented dairy product with both the mix of ATCC strains and Danone *L. paracasei* 2 strain were analyzed (Table 7). For individual strain cultures, all colony colors were in conformity with the expected species/subspecies colors on their respective chromogenic specific media, except for *L. delbrueckii* subsp. *bulgaricus* ATCC 11842 probably due to a growth issue at 47 °C (growth temperature recommended by ATCC: 37 °C). Enumeration levels were similar to those obtained with the MRS and M17 reference media. For the fermented milk product, the relevance of the method was confirmed except for *L. delbrueckii* subsp. *bulgaricus* ATCC 11842.

3.5. Suitability of the method for enumerations on other commercial products

Eight fermented milk products with various protein and flavor (fruits) content were analyzed (Table 8). For products containing only *L. rhamnosus* and *S. thermophilus*, M1 plates incubated at 44 °C were used to discriminate and enumerate both strains. *L. paracasei/casei* could be enumerated on M1-R or M1-V, depending on the presence of other strains in the mixture. *L. bulgaricus* was enumerated on M2 plates incubated at 47 °C. All colony colors were in conformity with the expected species/subspecies colors. Moreover, analyses of 16S rDNA sequences confirmed that all the species were correctly identified based on colony colors.

4. Discussion

Most fermented milk products contain complex mixtures of strains (Ashraf and Shah, 2011), which is of concern for analytical purposes in the dairy industry. Moreover, due to often close

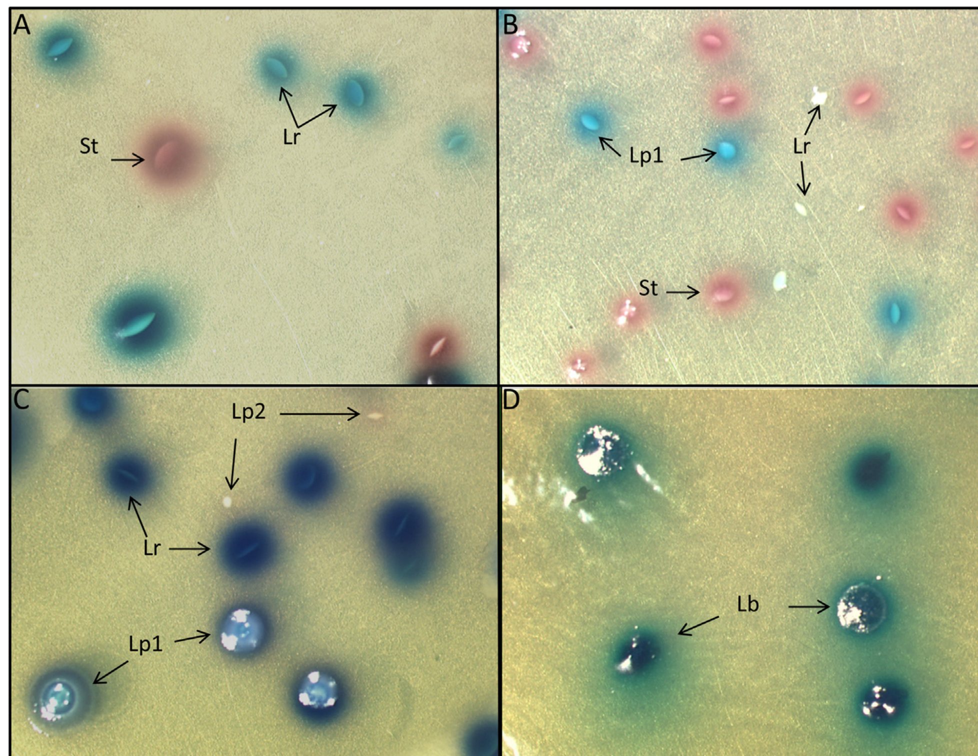

Fig. 1. Selective enumerations from FDP-2 on chromogenic agar. A, colonies of *S. thermophilus* (St, magenta) and *L. rhamnosus* (Lr, blue) grown on M1 agar at 44 °C. B, colonies of *S. thermophilus* (St, magenta), *L. paracasei* 1 (Lp1, turquoise) and *L. rhamnosus* (Lr, colorless) grown on M1-R at 37 °C. C, colonies of *L. paracasei* 1 (Lp1, turquoise) and *L. rhamnosus* (Lr, blue) and *L. paracasei* 2 (Lp2, colorless) grown on M1-V at 37 °C. D, colonies of *L. bulgaricus* (Lb, green) grown on M2 at 47 °C. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

phylogenetic relationships between probiotic and starter strains of LAB, standard enumeration media generally offer limited discrimination abilities. Selective enumeration on antibiotic-containing media can sometimes be used due to intrinsic antibiotic resistance of some strains. However, as this character is generally unwanted and therefore not used to screen for interesting strains, it cannot be routinely used in selective media. The growing interest for culture-independent methods may have impeded the development of new selective media, although culture-based methods remain highly suitable for dairy industry quality control analyses. Since the review from Talwalkar and Kailasapathy published eleven years ago (2004) in which the "urgent need" for selective media to enumerate probiotics in yogurts was pointed out, several media have been proposed (see for instance Ashraf and Shah, 2011). Yet, no significant improvement towards better discrimination was made, and some of these methods still necessitate confirming the results, usually through the additional use of a molecular method. Some agar media using a pH indicator have been proposed, where discrimination was made based on colony coloration after a change in pH due to metabolization of a substrate (Lee and Lee, 2008; Sakai et al., 2010). For instance, based on the differential ability of *L. rhamnosus* and *L. casei*/*L. paracasei* to ferment L-rhamnose, a MRS-derived medium incorporating L-rhamnose and a pH indicator (M-RTLV agar) was set up to selectively enumerate *L. rhamnosus* recovered from faeces after consumption of a probiotic product. The resulting *L. rhamnosus* colonies presented a characteristic color and could be distinguished by spread plate technique from the other colonies (Sakai et al., 2010). M-RTLV agar was not developed for analytical purposes and concerned *L. paracasei and L. rhamnosus* discrimination. Moreover, confirmation by ELISA was recommended. Nevertheless, this study illustrates well the use of specific colony color concept to increase selectivity. A step forward into the development of innovative solutions is the use of chromogenic media. The first chromogenic agar media were set up in the 80s and

Table 6
Precision of chromogenic media (data from interlaboratory validation).

Product and species	Method	r ($\log_{10}$ CFU/g)	R ($\log_{10}$ CFU/g)	Uncertainty ($\log_{10}$ CFU/g) 1 bottle/3 bottles
FDP-1				
L. paracasei 1	M1, 37 °C	0.13	0.20	0.16/0.13
S. thermophilus	M1, 37 °C	0.22	0.63	0.46/0.44
L. bulgaricus	M2, 47 °C	0.12	0.18	0.15/0.13
FDP-2				
L. paracasei 1	M1-R, 37 °C	0.16	0.18	0.17/0.13
L. paracasei 2	M1-V, 37 °C	0.17	0.48	0.36/0.32
L. rhamnosus	M1, 44 °C	0.13	0.20	0.17/0.12
S. thermophilus	M1, 44 °C	0.18	0.30	0.24/0.17
L. bulgaricus	M2, 47 °C	0.40	0.26	0.33/0.22

r, limit of repeatability, R, limit of reproducibility.

Table 7
Enumerations of reference ATCC strains in pure culture and fermented dairy product.

Species	Strain references	Method	CFU/g	log_{10} CFU/g	Colony color
Individual strains					
L. rhamnosus	ATCC 53103	M1, 44 °C	$5.60 \cdot 10^9$	9.75	Blue
L. casei	ATCC 393	M1, 37 °C	$1.80 \cdot 10^9$	9.25	Turquoise
L. casei	ATCC 393	M1-V, 37 °C	$1.90 \cdot 10^9$	9.27	Turquoise
L. casei	ATCC 393	M1-R, 37 °C	$1.90 \cdot 10^9$	9.28	Turquoise
L. bulgaricus	ATCC 11842	M2, 47 °C	ND[a]	ND[a]	ND[a]
Fermented product					
L. rhamnosus	ATCC 53103	M1, 44 °C	$1.60 \cdot 10^8$	8.20	Blue
L. casei	ATCC 393	M1-R, 37 °C	$1.50 \cdot 10^8$	8.18	Turquoise
S. thermophilus	ATCC 19258	M1, 44 °C	$2.40 \cdot 10^8$	8.38	Magenta
L. bulgaricus	ATCC 11842	M2, 47 °C	ND[a]	ND[a]	ND[a]

[a] *L. bulgaricus* ATCC 11842 strain doesn't grow at 47 °C.

Table 8
Enumerations from non-Danone commercial products.

Year	Country	Product category	Plain/flavors	Species	Method	CFU/g	log_{10} CFU/g	SD[a]	Colony color
2012	USA	High protein dairy product	Plain	*L. rhamnosus*	M1, 44 °C	$1.20 \cdot 10^8$	8.08	0.00	Blue
				S. thermophilus		$1.40 \cdot 10^8$	8.16	0.02	Magenta
2013	UK	High protein dairy product	Plain	*L. rhamnosus*	M1, 44 °C	$1.00 \cdot 10^8$	7.99	0.09	Blue
				S. thermophilus		$6.70 \cdot 10^7$	7.82	0.08	Magenta
				L. paracasei/casei	M1-R, 37 °C	$1.10 \cdot 10^8$	8.04	0.09	Turquoise
2013	UK	High protein dairy product	Plain	*L. paracasei/casei*	M1-V, 37 °C	$8.60 \cdot 10^2$	2.93	0.02	Turquoise
2014	UK	Non-dairy product – coco milk 98%	Plain	*L. rhamnosus*	M1, 44 °C	$3.20 \cdot 10^6$	6.50	0.02	Blue
				S. thermophilus		$1.30 \cdot 10^7$	7.11	0.08	Magenta
				L. bulgaricus	M2, 47 °C	$1.30 \cdot 10^7$	7.10	0.12	Green
2014	France	High protein dairy product	With strawberry	*S. thermophilus*	M1, 37 °C	$3.60 \cdot 10^9$	9.54	0.11	Magenta
2014	France	High protein dairy product	With strawberry	*S. thermophilus*	M1, 37 °C	$9.90 \cdot 10^9$	9.00	0.04	Magenta
				L. bulgaricus	M2, 47 °C	$1.50 \cdot 10^3$	3.18	0.03	Green
2014	Finland	High protein lactose free quark	With strawberry	*S. thermophilus*	M1, 44 °C	$1.87 \cdot 10^8$	8.27	0.08	Magenta
				L. bulgaricus	M2, 47 °C	$2.23 \cdot 10^5$	5.34	0.10	Green
2014	Finland	High protein traditional skyr dairy	With strawberry	*L. rhamnosus*	M1, 44 °C	$4.37 \cdot 10^7$	7.63	0.10	Blue
				S. thermophilus		$2.27 \cdot 10^8$	8.35	0.03	Magenta

[a] SD was calculated on 3 enumeration values (3 cups).

commercialized starting from the 90s for clinical, environmental and food (pathogenic and spoilage bacteria) analytical microbiology (Akter et al., 2014; Alonso et al., 1999; Manafi, 2000; Perry and Freydiere, 2007). Surprisingly, very little attention has been given to the development of such selective methods for LABs and probiotics (Sohier et al., 2014). Chromogenic media are user-friendly and require less technical knowledge from analytical teams than molecular methods. Nevertheless, their set up necessitates a good knowledge of the physiological properties of the strains to be enumerated. With the huge amount of data generated by the application of –omics technologies, most fermenting LABs and probiotics included in commercial products are usually very well characterized. This knowledge can serve to find specific selection targets, up to the strain level.

In this study, we developed a method based on the combination of two chromogenic agar media and different incubation conditions to selectively enumerate mixed *L. rhamnosus*, *L. paracasei*, *S. thermophilus* and *L. bulgaricus*. M2 contains the chromogenic substrate X-gal, which is cleaved in galactose and 5-bromo-4-chloro-3-hydroxyindole in the presence of β-galactosidase activity generating blue colonies after oxidation, and allows easy detection of *L. bulgaricus* at 47 °C and anaerobiosis. Here, green colonies were observed rather than blue, which might be related to the acidic pH of M2 agar. M1 contains two chromogenic substrates, Salmon-gal and X-glc, which are cleaved by β-galactosidase and β-glucosidase, to give a pink and turquoise color, respectively. Thus, strains presenting either one of these enzymatic activities or both can be detected. In the case of a strain expressing both β-gal and β-glc, *e.g. L. rhamnosus* in this study, the resulting color is blue. Although it was possible to directly enumerate *L. rhamnosus* on M1 agar, in practice, to undoubtedly distinguish blue *L. rhamnosus* from turquoise *L. paracasei* 1 colonies, we chose to enumerate *L. rhamnosus* on M1 at 44 °C (no growth of *L. paracasei*) and *L. paracasei 1* on M1-R. Indeed, L-rhamnose added to M1 was preferentially used by *L. rhamnosus* and β-gal and β-glc were not expressed, leading to colorless colonies.

Results from enumerations on FDP-1 showed that each targeted species (or subspecies) could be directly discriminated and quantified from a complex mixture. This could not be achieved with standard methods, which have been developed for use on yogurt containing only characteristic starter LAB.

Another major advantage of the chromogenic method was the ability to discriminate at the strain level, as shown from selective enumerations on FDP-2 of both *L. paracasei* 1 and *L. paracasei* 2 with M1-based agar. A few agar media have been proposed for enumeration of *L. paracasei* in yoghurt and cheese and discrimination between closely related LABs. However, their selectivity remained low and varied strongly depending on the other LABs present in the matrix (Colombo et al., 2014; Karimi et al., 2012; Sakai et al., 2010). In the case of FDP-2 analyzed in this study, none of the existing agar media were selective enough to directly enumerate *L. paracasei*, either both strains counted together or separately. With the aim to establish a method that can be routinely used for quality control of fermented milk products, repeatability, reproducibility and uncertainty were calculated. The limits of repeatability and reproducibility (*r* and *R*, respectively) could not be compared with those of the reference methods because the latter did not allow sufficient discrimination, and thus, colonies identity

remained putative on these media. r and R are important for quantitative analyses, as they give some indications about the results reliability. Values above the defined limits may evoke abnormal results and can be used as internal quality controls.

Moreover, we have also shown that the chromogenic method is relevant for enumeration of other strains by using specific known reference ATCC strains, in pure culture and within a fermented mix milk product. We have met a growth issue with the *L. delbrueckii* subsp. *bulgaricus* ATCC 11842 strain at 47 °C, which is probably too high compared to optimal growth conditions for this strain (37 °C, based on ATCC source). This could require an optimization of the experimental growth conditions specifically for this strain on M2 chromogenic medium. Nevertheless, several other Danone *L. delbrueckii* subsp. *bulgaricus* strains were tested on the M2 chromogenic media and showed similar phenotypes than the *L. delbrueckii* subsp. *bulgaricus* CNCM-I-2787 strain (data not shown).

Higher selectivity of culture media is often detrimental to universality of use. Indeed, as exemplified by several authors, most media developed for selective enumerations often display result variability depending on the strain and on the other species present in the mixture (Ashraf and Shah, 2011; Colombo et al., 2014; Talwalkar and Kailasapathy, 2004; Van de Casteele et al., 2006). However, sufficiently selective media are needed to ensure bacterial content and health or nutritional effects of probiotic products. Here, although the M1 and M2 media and enumeration method were developed with the aim to specifically distinguish the strains contained in FDP-1 and FDP-2, we have also shown that selective enumerations on other non-Danone commercial products that probably contain different strains from different ferment suppliers could be successfully carried out with mixtures containing 10^2–10^9 CFU/g of each LAB. Depending on the combinations of strains present in the products, different combinations of M1, with or without vancomycin or L-rhamnose, and M2 associated with different incubation conditions can be chosen. This demonstrates the versatility of the method. As the resulting colony colors depend on β-galactosidase and β-glucosidase activities, an important preliminary condition for the extended use of our method to selective enumerations in other milk products is to verify the presence/absence of these enzymatic activities in the targeted strains.

5. Conclusions

We have shown that it is possible to set up selective culture methods based on the physiological knowledge of strains. This chromogenic method presented several advantages over the available reference methods. It allowed identification and selective enumeration of all the target strains without requiring confirmation with a molecular method. The chromogenic method could be used directly on the milk products and each species or strain could be enumerated at a density up to 10^8–10^9 CFU/g. Based on differential enzymatic activity, each of the two *L. paracasei* subspecies could be efficiently distinguished and enumerated. In addition, they were directly enumerated, while with standard media colony counts were only estimated (subtracted) from the total *L. rhamnosus* + *L. paracasei* counts. For all species, bacterial counts were equivalent to those obtained with reference media. Finally, *S. thermophilus* could be directly enumerated from the mixture. To our knowledge, this is the first description of a method using chromogenic agar media to enumerate LABs from a complex milk mixture. The method is user-friendly and could be adapted to selective enumeration of other strains. The use of chromogenic agar represents an innovative improvement toward more selective culture methods required for quality controls of dairy products. It definitely deserves more attention in order to extend its use to other species, subspecies or strain mixtures. Indeed, the method was validated on two specific Danone fermented dairy products and its proof of concept was confirmed by the analysis of different non-Danone products available on the market. Nevertheless, it should be considered that a strong knowledge on phenotypes of strains of interest is always necessary to adapt the method by defining appropriate cultures conditions (e.g. temperature, selective substrates, etc…). The availability of robust methods that allow simultaneous characterization and enumeration of LAB is also very valuable for the constitution of regulatory files. In addition, the use of chromogenic media could have additional advantages, such as a reduction of time to results (reduction of Petri plates incubation time) or a reduction of the number of media required for enumeration and discrimination of LAB in complex dairy fermented products.

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