

Method for differentiated enumeration of mixed cultures of thermophilic lactic acid bacteria and bifidobacteria by using only one culture medium

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Abstract – The dairy industry has recently prepared probiotic products containing, in addition to the specific yoghurt cultures, *Lactobacillus acidophilus* and bifidobacteria. Identification of the species and enumeration of the micro-organisms are easier for yoghurt cultures than for probiotic products containing *Lactobacillus delbrueckii* subsp. *bulgaricus*, *Streptococcus thermophilus*, bifidobacteria and *Lactobacillus acidophilus*. A new method has been developed using the commercially available HHD agar, which provides a morphological differentiation of colonies and a counting of single species on the same plate. Results obtained with HHD are very close to those obtained by the use of media specific for each strain (MRS, MRS at pH 5.4, MRS + dicloxacillin, M17). Instead of using many selective culture media, only one culture medium was used with saving of material and performance rapidity allowing the method to be applied to the routine controls of the probiotic products.
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dairy product / lactic acid bacteria / bifidobacteria / HHD culture medium / separate enumeration

Résumé – Méthode pour le dénombrement différencié de cultures mixtes de bactéries lactiques thermophiles et de bifidobactéries en utilisant un seul milieu de culture. L'industrie laitière a récemment mis au point des produits probiotiques qui contiennent, en plus des cultures pour yaourt, *Lactobacillus acidophilus* et des bifidobactéries. L'identification des espèces et le dénombrement microbien sont plus faciles pour les cultures de yaourt que pour les produits probiotiques qui contiennent *Lactobacillus delbrueckii* subsp. *bulgaricus*, *Streptococcus thermophilus*, bifidobactéries et *Lactobacillus acidophilus*. Ce travail décrit une nouvelle méthode qui utilise seulement le milieu de culture HHD agar afin de différencier la morphologie des colonies et de dénombrer chaque espèce sur la même boîte. Les résultats du dénombrement microbien sur le milieu HHD agar sont très simi-

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laire à ceux qui ont été obtenus avec des milieux spécifiques pour chaque espèce (MRS, MRS à pH 5,4, MRS + dicloxacillin, M17). L'usage de l'agar HHD en remplacement de plusieurs milieux offre des avantages tels qu'une économie de matériels et une rapidité de dénombrement, et peut s'appliquer aux contrôles de routine des produits probiotiques. © Inra/Elsevier, Paris

produit laitier / bactérie lactique / bifidobactérie / milieu de culture HHD / dénombrement différencié

1. INTRODUCTION

In the dairy industry, starters of mixed cultures are used, among which the well-known yoghurt culture, mixture of *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Streptococcus thermophilus*. The introduction of probiotic micro-organisms is concerned with the manufacturing of new products containing, in addition to specific yoghurt cultures, *Lactobacillus acidophilus* and/or bifidobacteria [2, 4, 11–14].

Since the products are prepared from many strains, they require a selective enumeration of different microbial species. While specific yoghurt microflora is easily identified by an officially recognized IDF method [8], for cultures different from yoghurt bacteria, the identification is quite difficult.

For counting *L. acidophilus*, the ECA medium (Esculin-Cellobiose Agar), as described by Hunger [6] and Lapierre [15], can not be applied because some strains are esculin-negative. Hence, several culture media based on modifications of MRS Agar [3], replacement of glucose with trehalose (MRS Agar with trehalose) [15] and use of maltose (modified MRS Agar) [5], have been tested but in these media, bifidobacteria also grow. Pedersen [18] describes a MRS Agar supplemented with bile that is able to inhibit the growth of yoghurt microflora and bifidobacteria. LA Agar [12] medium is specifically used for products containing *L. acidophilus* and bifidobacteria but it has been shown not to be selective for high contents of streptococci and other lactobacilli [18].

For the selective identification of bifidobacteria, a large number of media are also used [1, 17, 19, 20], such as MRS + dicloxacillin [22], Modified Selective Rogosa Agar [21], MGLP Agar [24], TOS Agar Medium [25], NPNL Medium [23] according to the IDF [9]. It was observed that the selectivity of these media is generally antibiotic-related and cell recoveries are low. Furthermore, many of them are not commercially available.

A method already developed for the differential enumeration of homofermentative and heterofermentative lactic acid bacteria provides the morphological differentiation of colonies in HHD Agar medium [16]. The differentiation of species is based on the use of a limited amount of fructose, as the only source of carbohydrates, and on the Bromocresol green, as an indicator. The species are recognizable from the different colour, as it was observed by MacDonald [16], and from the shape, appearance and sizes of the colonies.

The aim of this study is to determine the suitability of the HHD Agar medium for the detection and the specific enumeration of *S. thermophilus*, *L. delbrueckii* subsp. *bulgaricus*, *L. acidophilus* and bifidobacteria on the same plate.

2. MATERIALS AND METHODS

2.1. Pure cultures

Lactobacillus acidophilus LA1 and K8, *Lactobacillus delbrueckii* subsp. *bulgaricus* Lb170, Lb187 and Lb1, *Streptococcus thermophilus* S62

and YS18 were obtained from Centro Sperimentale del Latte, Zelo B. Persico (MI) Italy.

Bifidobacterium infantis Bi1, *Bifidobacterium longum* B110, *Bifidobacterium breve* Bbr8 were obtained from Prof. V. Scardovi, Ist. Microbiologia, Univ. di Bologna, Italy.

All strains were stored freeze-dried in a refrigerated room. *S. thermophilus* was cultured in sterilized milk + 0.1 % yeast extract, inoculated at 1 %, incubated for 3 h at 42 °C, *L. delbrueckii* subsp. *bulgaricus* in sterilized milk + 0.1 % yeast extract, inoculated at 1 %, incubated for 5 h at 42 °C, and *L. acidophilus* and bifidobacteria cultures in sterilized milk + 0.5 % glucose + 1 % yeast extract, inoculated at 1 % and incubated for 15 h at 37 °C. Pure cultures were analysed once or several times starting from a fresh culture.

2.2. Mixed cultures

Yoghurt: 10 samples.

Probiotic fermented milk containing *S. thermophilus*, *L. delbrueckii* subsp. *bulgaricus*, *L. acidophilus* and bifidobacteria spp. or some of them: 15 samples.

Freeze-dried probiotic products containing *S. thermophilus*, *L. delbrueckii* subsp. *bulgaricus*, *L. acidophilus* and bifidobacteria spp. or some of them: 19 samples.

2.3. Media

HHD (dehydrated: Lactic Acid Bacteria Differential Broth, Cod M1086 from HI Media Laboratories Pvt Limited, Bombay - 400 086, India) from MacDonald et al. [16].

All ingredients, except for Tween 80 were dissolved by heating, then Tween 80 was added; the medium was sterilized in an autoclave at 121 °C for 15 min. Just before use, the medium was melted and distributed in plates of 90 mm diam (12 mL/plate) and left to solidify. The plates were maintained in laminar flows for drying for 20 min.

The following commercially available media were used as reference: MRS Agar (DIFCO) for *L. acidophilus* [3]; MRS Agar (DIFCO) at pH 5.4 for *L. delbrueckii* subsp. *bulgaricus* (IDF Standard 117A [8]); MRS Agar (DIFCO) Dicloxacillin (Sigma) for bifidobacteria [22]; M17 Agar (DIFCO) for *S. thermophilus* [8].

2.4. Procedure

Forty samples were analysed for each microbial species. To perform the analysis, samples were diluted 1:10 with peptone/saline solution and homogenized in Stomacher for 1 min 30 s; then decimal dilutions were prepared [10]. Inoculation in HHD was carried out by spreading 0.1 mL of the decimal dilutions to the surface of each plate. Incubation occurred anaerobically at 37 °C for 3 days in Gas Pak with CO₂ and H₂ atmosphere.

Enumerations in control medium were carried out by the pour plate method of 1 mL of dilution and incubated at 37 °C for 48 h in M17 Agar for *S. thermophilus* and anaerobically in GasPak at 37 °C for 72 h for other culture media.

After incubation, colonies were counted in all media and in HHD by separating the species through the different morphology of the colonies both with the naked eye and at stereoscopic microscopy Wild-M8 (Leitz) (oculars 10 ×, lens 5-50 ×) and expressed as CFU (Colony-Forming Units)·g⁻¹.

The results were confirmed by examining under the microscope (Zeiss oculars 10 ×, lens 100 ×) the square root of colonies on the plates.

2.5. Photos

Photos of colonies were made with stereoscopic microscopy in a dark room with a Minolta X-300S camera, with film for artificial light 160 ASA at the tungsten with microscopic magnification 120 ×.

2.6. Statistical analysis

After logarithmic transformation of the results to normalize the distribution, the statistical analyses were performed according to the IDF [7] using the software Lotus 1-2-3. The regression equation $y = ax + b$ was calculated, where y refers to counts on reference medium and x to those on HHD medium. The accuracy of y was estimated by $s_{y,x}$, the residual standard deviation of the regression. Counts of both media were compared using Student's t -test applied to $d_m / (s_d / n^{1/2})$, where d_m is the mean of $d = y - x$, s_d is the standard deviation of d and n the number of samples.

3. RESULTS AND DISCUSSION

3.1. Colony morphology on reference media

The colonies of bifidobacteria in MRS Agar, MRS Agar at pH 5.4 and MRS Agar Dicloxacillin were of white-cream colour, of lenticular shape with regular sides. Colonies of *L. delbrueckii* subsp. *bulgaricus* in MRS Agar or in MRS Agar at pH 5.4 were circular or trilobate, of white-cream colour with an external fluffy ring which may be examined under the stereoscopic microscope or with a magnifier. This ring was not found in the colonies of *L. acidophilus* that are much more irregular shaped than those of *L. delbrueckii* subsp. *bulgaricus*.

Colonies of *S. thermophilus* in M17 Agar as well as in MRS Agar were white-cream coloured, lenticular, with regular sides. The colonies were recognizable from those of lactobacilli and bifidobacteria since they were very small in this second culture medium.

For the enumeration of specific yoghurt cultures, these media allow a good selectivity and are generally applied without problems as reference test.

3.2. Colony morphology on HHD Agar medium

The different strains of the same species showed a similar morphology on HHD, while the 4 species under examination had a clearly different morphology. Only *S. thermophilus* exhibited 2 different morphologies which were both well recognizable.

Colony morphology of the 4 species on HHD Agar medium is shown in figures 1–5. Colonies of *S. thermophilus* appeared as follows: type 1) generally small, circular, smooth and almost transparent (figure 1); type 2) circular, convex and dark green coloured (figure 2).

Colonies of bifidobacteria were similar in size to those of *S. thermophilus*, but they

appeared translucent, convex like a drop of water (figure 3). Colonies of *L. delbrueckii* subsp. *bulgaricus* were large, of irregular shape, with flat surface, regular bright green with internal undulatory streaking (figure 4). Colonies of *L. acidophilus* were large, of irregular shape, convex and pyramid shaped surface, light brown with a small central spot of dark green colour (figure 5).

Mixed cultures of different species are shown in figure 6. It was observed that the morphology of the colonies was typical for each species and was still well recognizable when cultures developed together.

The medium did not maintain its original colour but changed from blue to green, green-yellow and yellow depending on the development of acidifying colonies on the plate. This is due to the bromcresol green that has a pK value of 4.6.

Sizes of colonies varied depending upon the number of colonies developed on the plate: bifidobacteria and *S. thermophilus* generally were of similar sizes, while *L. delbrueckii* subsp. *bulgaricus* as well as *L. acidophilus* were larger than the others.

The use of HHD medium allowed the 4 different species to be distinguished even though the colonies showed a difference of 4–5 orders of magnitude: the colonies of the most dominant species built a carpet from which the subdominant colonies stand out and could be easily distinguished.

3.3. Comparison of counts on HHD agar and reference media

Counts on HHD and reference media were very well correlated for the 4 species (table I). Moreover, values of s_d were very close to the corresponding values of $s_{y,x}$ (standard deviation from the regression), which shows the similarity of results with both methods [7]. However, counts on HHD were slightly, but significantly higher than those on reference media for the 4 species, as indicated by the *t* values (table I). HHD medium,

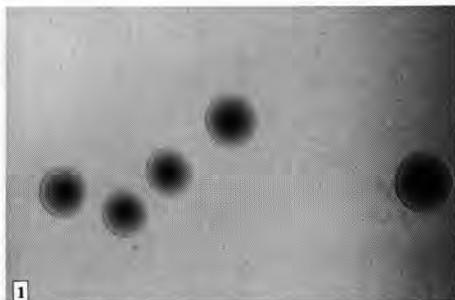


Figure 1. Morphology of colonies of *Streptococcus thermophilus* – type 1, made with stereoscopic microscopy (120 × magnification).

Figure 1. Morphologie des colonies de *Streptococcus thermophilus* – type 1, faites par microscopie stéréoscopique (grossissement × 120).

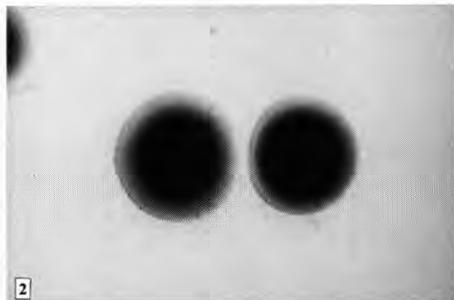


Figure 2. Morphology of colonies of *Streptococcus thermophilus* – type 2, made with stereoscopic microscopy (120 × magnification).

Figure 2. Morphologie des colonies de *Streptococcus thermophilus* – type 2, faites par microscopie stéréoscopique (grossissement × 120).

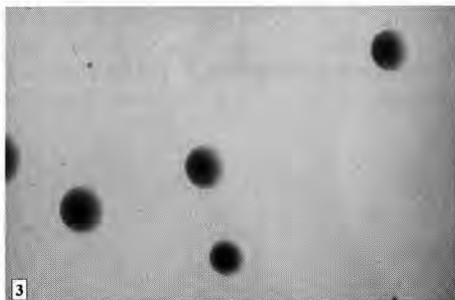


Figure 3. Morphology of colonies of bifidobacteria, made with stereoscopic microscopy (120 × magnification).

Figure 3. Morphologie des colonies de bifidobactéries, faites par microscopie stéréoscopique (grossissement × 120).

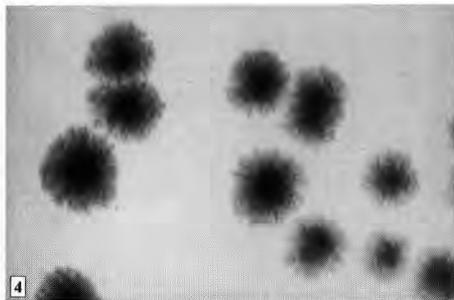


Figure 4. Morphology of colonies of *Lactobacillus delbrueckii* subsp. *bulgaricus*, made with stereoscopic microscopy (120 × magnification).

Figure 4. Morphologie des colonies de *Lactobacillus delbrueckii* subsp. *bulgaricus*, faites par microscopie stéréoscopique (grossissement × 120).

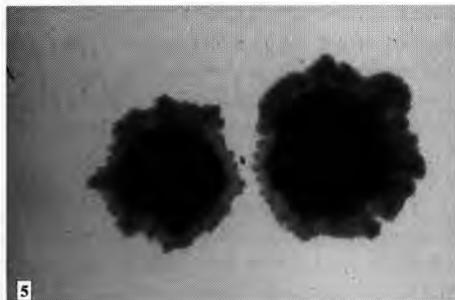


Figure 5. Morphology of colonies of *Lactobacillus acidophilus*, made with stereoscopic microscopy (120 × magnification).

Figure 5. Morphologie des colonies de *Lactobacillus acidophilus*, faites par microscopie stéréoscopique (grossissement × 120).

Table I. Counts and statistical analysis on the reference and HHD media (logCFU.g⁻¹).
Tableau I. Dénombrements et analyse statistique sur les milieux de référence et HHD (logUFC.g⁻¹).

Spécies	<i>S. thermophilus</i>	Bifidobacteria	<i>L. bulgaricus</i>	<i>L. acidophilus</i>
Sample size	40	40	40	40
Min - Max on reference medium y	8.00-11.28	6.00-11.43	5.48-9.67	7.48-9.28
Min - Max on HHD medium x	8.11-11.26	6.23-11.58	5.70-9.72	7.75-9.88
Mean of reference medium	9.58	8.71	7.81	8.45
Mean of HHD medium	9.67	8.86	7.90	8.64
Mean of differences d = y - x	0.09	0.15	0.09	0.19
Standard deviation of differences SD	0.1675	0.3571	0.2443	0.1586
Students t - test	3.83***	2.59*	2.28*	7.84**
Regression equation	y = 1.05x + (-0.61)	y = 0.96x + 0.23	y = 0.94x + 0.35	y = 0.94x + 0.34s
Correlation coefficient r	0.99	0.97	0.96	0.95
Standard deviation from regression S _{y,x}	0.1575	0.3555	0.2418	0.1601

*, ** = mean count on the reference and HHD media differed significantly (*P < 0.05, ** P < 0.01).

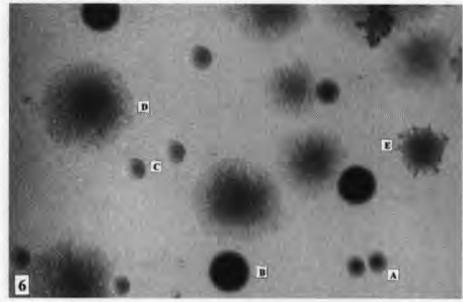


Figure 6. Morphology of colonies of mixed cultures, made with stereoscopic microscopy (120 × magnification). **A.** *Streptococcus thermophilus* type 1. **B.** *Streptococcus thermophilus* type 2. **C.** Bifidobacteria. **D.** *Lactobacillus delbrueckii* subsp. *bulgaricus*. **E.** *Lactobacillus acidophilus*.

Figure 6. Morphologie des colonies des cultures mixtes faites par microscopie stéréoscopique (grossissement × 120). **A.** *Streptococcus thermophilus* type 1. **B.** *Streptococcus thermophilus* type 2. **C.** Bifidobacteria. **D.** *Lactobacillus delbrueckii* subsp. *bulgaricus*. **E.** *Lactobacillus acidophilus*.

which is not a selective medium, allowed in particular a better recovery of *L. acidophilus* (+ 0.19 log) and bifidobacteria (+ 0.15 log).

4. CONCLUSIONS

The main advantage of the method is the use of only one culture medium to enumerate *S. thermophilus*, *L. delbrueckii* subsp. *bulgaricus*, *L. acidophilus* and bifidobacteria in mixed cultures containing these species in different amounts with consequent performance rapidity, savings of material and economic profit.

The commercially available HHD Agar medium can be applied for the enumeration of these 4 species in the routine controls of dairy and probiotic products.

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