

Formulation and Evaluation of a Selective Medium for Lactic Acid Bacteria-Validation on Some Dairy Products

¹Baida Djeghri-Hocine, ¹Messaouda Boukhemis and ^{2,3}Abdeltif Amrane

¹Biochemistry and Applied Microbiology Laboratory, Department of Biochemistry, Faculty of Sciences, Badji-Mokhtar University, BP 12, 23000 Annaba, Algeria

²National School of Chemistry of Rennes, CNRS, UMR 6226, General Leclerc Avenue, CS 50837, 35708 Rennes Cedex 7, France

³European University of Brittany, France

Abstract: Problem statement: Lactic Acid Bacteria (LAB) is characterized by fastidious nutritional requirements leading to the need for complex and rich media to allow growth. Owing to their complex requirements which also varied significantly with species, the formulation of selective media for LAB is difficult. **Approach:** A culture medium for LAB was previously developed based on deproteinated whey supplemented with yeast autolysate and de-lipidated egg yolk. Based on this previously developed medium, some selective media were formulated and their potential for LAB selection and enumeration was examined. **Results:** In this study, it was considered as a basis to define a selective medium, by adding a pH indicator and some inhibitory compounds to select the LAB flora of various dairy products including milk, disinfected or not, fermented milk and yoghurt. The addition of sodium azide to inhibit the contamination flora, including Gram negative flora and purple bromocresol allowed a direct selection of the Gram + and lactose + flora. An acidic pH (5.0) can also be helpfully considered after protein hydrolysis to avoid protein denaturation. **Conclusion:** To complete this study, additional work is needed concerning the improvement of culture medium selectivity by considering other inhibitors, of the Gram negative flora (like lithium chloride for instance), of fungi (like actidione), of the Gram positive flora (excepting the LAB flora) and an association of these inhibitors.

Key words: Characterization, inhibitory compounds, lactic acid bacteria, selective media

INTRODUCTION

Lactic Acid Bacteria (LAB) are characterized by fastidious nutritional requirements leading to an important biosynthesis deficiency (Cogan *et al.*, 1997; Loubiere *et al.*, 1996; Monnet and Gripon, 1994; Stackebrandt and Teuber, 1988). LAB growth required complex and rich media, containing complex nitrogen sources (peptides), carbon sources, vitamins and minerals (to supply for trace elements) (Amouzou *et al.*, 1985; Juillard *et al.*, 1995; Raccach, 1985). These nutrients should be supplied at optimal concentrations (Benthin and Villadsen, 1996; Cocaign-Bousquet *et al.*, 1995; Desmazeaud, 1994).

Several specific and selective culture media are available for the isolation and selection of LAB (De Mann *et al.*, 1960; Rogosa *et al.*, 1951; Talwalkar and Kailasapathy, 2004; Terzaghi and Sandine, 1975; Vinderola and Reinheimer, 1999). These media allow

selecting the different LAB genera. However, the formulation of selective media is very difficult, owing to the high requirements of LAB in growth factors and since they are intolerant to inhibitors (Chamba *et al.*, 1994). Moreover, the variability in the nutritional requirements varied significantly with the strain used (Chamba *et al.*, 1994). Consequently, a selective media cannot allow the numeration of all LAB genera and species.

The available selective media are based on the tolerance of LAB to acidity, to inhibitory compounds or the replacement of glucose by other sugars (Coeuret *et al.*, 2003; Dave and Shah, 1996; Roy, 2001).

A culture medium for LAB growth was previously developed based on deproteinated whey supplemented with yeast autolysis and de-lipidated egg yolk (Djeghri-Hocine *et al.*, 2007). Indeed, egg yolk, an economically attractive local resource

Corresponding Author: Baida Djeghri-Hocine, Biochemistry and Applied Microbiology Laboratory, Department of Biochemistry, Faculty of Sciences, Badji-Mokhtar University, BP 12, 23000 Annaba, Algeria Tel: (+33) 223238155 Fax: (+33) 223238120

(Algeria), contains about 160 g kg⁻¹ proteins, mainly lipoproteins, as well as a high number of vitamins and was found to be efficient for LAB supplementation (Djehri-Hocine *et al.*, 2007). It should also be noted that its protein efficiency ratio, namely the ratio of body weight gain to net protein intake, was higher than that of milk casein for rat nutrition (Sakanaka *et al.*, 2000). Pure cultures of some LAB strains were considered to evaluate its potential for LAB growth. Based on this previously developed medium, some selective media were formulated and their potential for LAB selection and enumeration was examined in this research.

MATERIALS AND METHODS

Media: Deproteinized sweet cheese whey was used as a medium basis. The following components were added in each culture medium: MgSO₄.7H₂O, 0.2 g L⁻¹; MnSO₄.4H₂O, 0.05 g L⁻¹; Tween 80, 1 mL L⁻¹ and agar 15 g L⁻¹. Media were also supplemented with yeast autolysate (100 mL L⁻¹) and de-lipidated egg yolk (4 g L⁻¹).

Sweet whey preparation: Sweet whey resulted from Edough Camembert production (Edough, Annaba, Algeria). Whey proteins were removed after thermal shock at acidic pH, namely addition of HCl 5 N (pH 4.5) followed by sterilization at 120°C for 15 min. After centrifugation at 4000 r.p.m. for 15 min (Beckman TJ6; Beckman Coulter, Fullerton, CA, USA), the supernatant free of whey proteins was harvested and used for media preparation.

Yeast Autolysate (YA) preparation: Baker's yeast was used for the preparation of yeast autolysate. It was solubilised in distilled water (1 kg L⁻¹) and then heated at 50°C for 24 h, before boiled for 5 min. After rapid cooling, the solution was homogenized by means of Ultra Turrax (Rhema Labortechnik, Hofstein, Germany) for 30 sec and then filtered (0.45 µm filters-Sartorius, Palaiseau, France). The pH of permeate was then adjusted to 6.8, before it was sterilized at 120°C for 20 min.

De-lipidated Egg Yolk (DEY) preparation: Commercial and local Hen's eggs were used. Each egg weighed 62 g on average, containing 8.5 g of egg yolk on average. Each egg yolk was mixed with 50 mL acetone and then manually stirred during 5 min. After 30 min settling, 30 mL (800 mL L⁻¹) alcohol was added to the mixture, which was then manually stirred during 10 min. After removing the solvents, the precipitate was washed twice with distilled water. The de-lipidated egg yolks were dried at 40°C until reaching a constant weight (the final water content was 340 g kg⁻¹).

Table 1: Composition of the various media used

Medium composition ^a	M1	M2	M3	M4	M5	M6
Purple bromocresol	-	+	-	+	+	-
Sodium azide	-	-	-	+	-	-
Thallium acetate	-	-	-	-	+	-
Fungizone	-	-	-	-	-	+
pH	6.4	6.4	5.0	6.4	6.4	6.4

^a: All media contained the following basis: Deproteinized sweet cheese whey supplemented with 0.2 g L⁻¹ MgSO₄.7H₂O, 0.05 g L⁻¹ MnSO₄.4H₂O, 1 mL L⁻¹ Tween 80, 15 g L⁻¹ agar, 100 mL L⁻¹ yeast autolysate and 4 g L⁻¹ de-lipidated egg yolk. As indicated, 0.25 g L⁻¹ sodium azide, 0.5 g L⁻¹ thallium acetate, 20 mg L⁻¹ fungizone and 0.17 g L⁻¹ purple bromocresol were also added to the media

Table 2: Nature of the considered dairy products, biotope and conditions of sampling

Product	Sampling		pH	T (°C)
	location	Conditions		
Crude cow milk	Farm (Annaba)	Manual milking without disinfection ^a	6.6	4
		Manual milking after disinfection ^b	6.6	4
Traditional fermented cow milk (L' ben)	Traditional dairy	With a sterile ladle in an open tank	4.4	4
Yoghurt (iprolait)	Commercial	Conditioned pot of 125 mL	4.6	4

^a: Manual milking without nipples disinfection and without removing the first milk jets; ^b: Manual milking after nipples were washed with soapy water, rinsed with sterilized water and wiped with sterile compresses and after removing the first milk jets

Six media were prepared (M1-M6). Medium M1 corresponded to the reference medium, namely without addition of any inhibitory component or pH indicator. Media M2, M4 and M5 contained bromocresol purple to differentiate between the lactose positive and lactose negative strains. Sodium azide or thallium acetate, which inhibit the Gram negative flora were added in media M4 and M5 and fungi zone was added in medium M6 to inhibit yeast flora. These inhibitory compounds were chosen based on previous microbiological analysis of milks, sampled in similar conditions (personal communication). All media were homogenized by means of Ultra Turrax (Rhema Labortechnik) for 30 sec and then filtered (0.45 µm filters-Sartorius). Medium pH was then adjusted to 6.4, except medium M3 whose pH was adjusted to 5.0. Media were solidified by adding 15 g L⁻¹ agar, before sterilised at 120°C for 20 min.

Media composition was collected in Table 1.

Microbiological analysis of dairy products: To test the potential of the various formulated media Table 1 for LAB growth and selection, they were inoculated with four dairy products of various microbiological quality and characteristics Table 2, in view of LAB flora isolation.

About 100 mL of a given product were introduced in a sterile vial, quickly cooled at 4°C and maintained at this temperature until analysis (Chamba *et al.*, 1994). Successive decimal dilutions of each sample were carried out in peptone water (15 g L⁻¹ proteose peptone (Difco, Franklin Lakes, NJ, USA) and 5 g L⁻¹ NaCl). Dilutions 4-6 (10⁻⁴-10⁻⁶) were considered and were used to inoculate the considered medium. Inoculation was carried out by the method of the double layer, in order to promote anaerobic conditions, to favor growth of the LAB flora, which is micro-aerophilic. The Petri dishes were incubated for 72 h in an enriched CO₂ atmosphere at 30 and 45°C to allow growth of mesophilic and thermophilic species. Examination of the morphologic characteristics of the colonies, their enumeration and their radial growth rate were considered to evaluate culture growth.

Characterization of the various colonies: In addition to the morphological characteristics, the catalase test and the microscopic observation (including Gram and cell morphology), the nature of the flora found in the various samples was checked by cultivation on different culture media. Before each culture, the purity of the considered species was microscopically checked.

Enterococcus and *Lactococcus* displayed similar morphological characteristics, namely short cocci chains (mainly double cocci cells). Moreover, both are Gram positive, catalase negative and facultative anaerobes, optimal growth was recorded at 37°C (Leyral and Vierling, 2007). To differentiate between both genera, Gram + and catalase – cocci were cultivated on solid Mossel medium and incubated at 37°C for 48 h. The development of *Enterococcus* species on this medium leads to black colonies (Stackebrandt and Teuber, 1988); while *Lactococcus*

species were generally inhibited on this medium (they only lead in some cases to very weak growth illustrated by small transparent colonies).

Gram negative and lactose positive bacilli, which are supposed to be coliforms, were transferred on brilliant-green bile lactose broth and incubated under Durham bell jar for 24 h at 30 and 44°C for the total and faecal coliforms, respectively.

To search for yeast cells, big and nearly oval cells were transferred on Sabouraud agar and incubated for 5 days at 22°C.

Gram+, catalase – and lactose + cocci were transferred on M17 medium (Terzaghi and Sandine, 1975), incubated at 10, 30 and 45°C, while Gram +, catalase – and lactose + bacilli were transferred on MRS medium (De Mann *et al.*, 1960) and incubated at 15, 30 and 45°C.

RESULTS

On non-disinfected milk (Table 2) LAB species gave small colonies of 1-2 mm and grew slower than the contamination flora, which colonize the medium after approximately 72 h. Medium M2 also allowed the development of the bacterial flora present in the considered dairy products (Table 3 and 4). However, the presence of bromocresol purple allowed differentiating between the lactose positive and negative colonies. Indeed, the pH decrease after lactose fermentation resulted in a yellow contour of the lactose + colonies due to the color change of the pH indicator.

For all samples medium M3 appeared to be the most appropriate for growth of Gram + and catalase – bacilli, owing to the high growth rates and the colony diameters (1-2 mm) (Table 3).

Table 3: Growth of the micro-flora of the various dairy products analyzed cultivated on the different media tested at 30 and 45°C

Products			M1	M2	M3	M4 ^a	M5 ^a	M6
No	Growth	Diameter	++	++	+	+	+ ^b	NA
Disinfected Milk	Colonies	Color	1-3 mm W, Y or O ^c	1-3 mm W, Y or O ^c	1-2 mm White	1-2 mm W, Y or O ^c	1-2 mm W, Y or O ^c	
Disinfected Milk	Growth	Diameter	++	++	+	+	+ ^b	NA
Disinfected Milk	Colonies	Color	1-3 mm W, Y or O ^c	1-3 mm W, Y or O ^c	1-2 mm White	1-2 mm W, Y or O ^c	1-2 mm W, Y or O ^c	
Fermented Milk	Growth	Diameter	+	+ ^d	+	+	NA	+
Fermented Milk	Colonies	Color	1-4 mm White	1-2 mm T and B ^e				
Yoghurt ^f	Growth	Diameter	+	+	+	+	NA	NA
Yoghurt ^f	Colonies	Color	1-2 mm W or T and B ^e	NA				

^a: Presence of lactose positive and negative colonies in case of growth on these media; ^b: Lower growth rates than those recorded on medium M4; ^c: White, yellow and orange; ^d: Presence of a high number of lactose positive colonies; ^e: Transparent and brilliant; ^f: Incubation temperature, 45°C; ^g: White or transparent and brilliant; NA: Not available

Table 4: Microscopic observation and catalase test of the micro-flora of the various dairy products cultivated on the different media tested

Products	M1	M2	M3	M4	M5	M6
No disinfected milk	1, 2, 3 ^a and 4	1, 2, 3 ^a and 4	1 and 3	1 ^b and 3	1 ^b and 3	NA
Disinfected milk	1, 3 and some G-colonies	1, 3 and some G-colonies	1 and 3, but no G-colonies	1 and 3, but no G-colonies	1 and 3, but no G-colonies	NA
Fermented milk	1 and 3, but no G-colonies ^c	1 and 3, but no G-colonies ^c	1 and 3, but no G-colonies ^c	NA	NA	1 and 3, no yeast cells
Yoghurt	1 and 3 ^d	1 and 3 ^d	1 and 3 ^d	NA	NA	NA

^a: Gram + and catalase-cocci, bacilli and coccobacilli only chains associated; ^b: Gram + and catalase-cocci, only chains associated; ^cA high number of yeasts was also observed; ^d: Bacilli were observed, but no coccobacilli; NA: Not Available; 1: Gram + and catalase – cocci, chain or pair associated; 2: Gram + and catalase + cocci, cluster or chain associated; 3: Gram + and catalase – bacilli and coccobacilli, isolated or chain associated; 4: Gram – bacilli and coccobacilli

Inhibitory compounds for Gram negative bacilli (Table 4), Sodium azide and thallium acetate, added in media M4 and M5 (Table 2), where not tested in presence of fermented milk and yoghurt (Table 3 and 4). LAB flora grew after 48 h, leading to white colonies of 1-2 mm diameter, with a yellow contour due to lactose fermentation (Table 3). However, the relatively weak growth and the thin and transparent colonies observed in presence of thallium acetate should be noted.

Yeasts were found in the fermented milk. Medium M6 which contained fungizone was therefore tested in presence of fermented milk (Table 3 and 4). The addition of fungizone led to an orange coloration of the medium and very thin, transparent and brilliant colonies were observed.

Complementary analysis showed the apparition of trouble and gas production after growing 24 h on brilliant-green bile lactose broth at 30 and 44°C indicated the presence of total and faecal coliforms (Refai, 1981). Moreover, samples contamination by enterococci was confirmed by the presence of black colonies after growth on Mossel medium (Stackebrandt and Teuber, 1988).

DISCUSSION

Medium M1 shows an interesting potential for growth of the LAB and the contamination flora present on the considered dairy products (Table 3 and 4); in particular, on non-disinfected milk (Table 2). The grainy appearance of medium M3, consequently to the formation of a creamy-white precipitate, was most likely due to protein denaturation, since medium pH (5.0) was lower than other pH media (6.4) (Table 1). However, an acidic pH had improved medium selectivity. Indeed, for all samples medium M3 appeared to be the most appropriate for growth of Gram + and catalase – bacilli (Table 3).

Gram negative bacilli were totally inhibited in presence of inhibitory compounds (Table 4), Sodium azide and thallium acetate, added in media M4 and M5 (Table 2). As expected, yeast growth was inhibited by

the presence of the fungizone added in medium M6 (Table 3 and 4).

Complementary analysis showed an important LAB flora for all examined products, including mesophilic and thermophilic species and confirmed the presence of a contamination flora, including enterococci, coliforms in the non-disinfected milk and yeasts in the fermented milk.

Media M2 and M1 showed an interesting potential for bacterial flora, both LAB and contamination flora, of the considered dairy products. LAB flora showed lower growth rates than contamination flora (coliforms and enterococci). The decrease of the pH to 5.0 can be a helpful tools of bacterial selection, especially for the selection of the more acidophilic, namely the lactobacilli. Sodium azide addition improved medium selectivity, while thallium acetate which inhibited the Gram negative flora also delayed LAB growth. Even if fungizone totally inhibited yeast growth, it modified the morphological characteristics of the LAB flora, in agreement with the intolerance of LAB to inhibitors (Chamba *et al.*, 1994). Purple bromocresol addition was helpful for the selection of the Gram + and lactose + flora, which can be subsequently used to prepare fermented dairy products; it is therefore an interesting tools for LAB flora isolation, which is mainly lactose positive.

CONCLUSION

A medium based on deproteinated whey supplemented with yeast autolysate and de-lipidated egg yolk (Djeghri-Hocine *et al.*, 2007) showed an interesting potential for growth of the bacterial flora of the various tested dairy products. The addition of sodium azide, to inhibit the contamination flora, including Gram negative flora, as well as purple bromocresol allowed a direct selection of the Gram + and lactose + flora, characteristics of the majority of the LAB species. An acidic pH (5.0) appeared also helpful, but should be considered after protein hydrolysis to avoid protein denaturation (or flocculation) and to favor the release of amino acids and especially small

peptides, essential metabolites for LAB growth (Leh and Charles, 1989).

To complete this study, additional work is needed concerning the improvement of culture medium selectivity by considering other inhibitors, of the Gram negative flora (like lithium chloride for instance), of fungi (like actidione), of the Gram positive flora (excepting the LAB flora) and an association of these inhibitors. Optimization of their concentrations should also be considered. Isolation of LAB species by considering the obtained medium should be subsequently examined for medium validation.

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