Characterization and Technological Properties of *Bifidobacterium* Strains Isolated from Breast-fed Infants

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Abstract: *Bifidobacteria* represent the largest group of human intestinal bacteria. They have an important place in human health and represent the dominant group microflora of the newborn breast-fed. Following the behavior of strains of *Bifidobacteria* isolated from the breast-fed infants and from saline rehydration solution was considered in order to develop therapeutic fermented milk. Samples from newborn infants aged 10 months, or from a saline rehydration solution (Celia/Develop ORS) containing *Bifidobacteria* sold was used and isolated strains belonged to breve and longum species. Those strains showed preferences to neutral pH. They are mesophilic and tolerate high temperatures (42 °C). Glucose was commonly carbohydrate used in selective media for *Bifidobacteria*. Production of titratable acidity and therefore lowering the pH varies from one strain to another.

Key words: Ecology, *Bifidobacterium*, antibiotic resistance, kinetics of growth, acidity.

1. Introduction

Bifidobacteria form the largest group of human intestinal bacteria, especially in children [1]. They occupy an important place in human health. The first species of this genus isolated in 1899 from a healthy infant breast-fed by Henry Tissier which was classified later as *Bifidobacterium bifidum*. They settle in a short time after birth and become the dominant group of bacteria, 92% of the microflora of the newborn breast-fed consists of *Bifidobacteria*. However, the rate of these bacteria is reduced in favor of *Lactobacilli*, *Enterobacteriaceae*, *Streptococci* and *Clostridia* throughout life [2].

Some properties of *Bifidobacteria* have promoted their use in food products called probiotics [3] such as fermented milks, cheese and milk powder [4].

The effect of probiotic *Bifidobacteria* depends on their survival not only in food but also in the gastrointestinal tract [5]. For this reason, it becomes

necessary to identify and assess the population of *Bifidobacteria* in fermented products to ensure a sufficient intake of probiotics for the expected benefits.

2. Materials and Methods

2.1 Origin of Samples

Samples were obtained from newborn infants aged 10 months, or from a saline rehydration solution (Celia/Develop ORS) containing *Bifidobacteria* sold, citrate, lactodextrane, sucrose, minerals, and traces of milk and banana aroma.

2.2 Culture Media

The isolation of *Bifidobacteria* was performed on MRS supplemented with 0.05% cysteine-chloride and 2 mg/L nalidixic acid at pH 6.8. Isolation requires strict anaerobic conditions (anaerobic jars with gas-packs).

The purification is performed by successive transplanting from Petri plates containing selective medium (MRS medium supplemented with 0.05% cysteine-chloride and 2 mg/L nalidixic acid). For

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storage, the strains were frozen at -20 °C in skim milk containing 30% glycerol, 10% of yeast extract and 0.2% cysteine-HCl [6].

2.2.1 Biochemical and Physiological Testing

The pre-identification begins with the observation of colonies on MRS medium supplemented with 0.05% cysteine-chloride and 2 mg/L nalidixic acid. A catalase test is carried out. Research the type fermentation, production of indole, citrate on middle Kempler and Mc Kay, the demonstration of urease are performed. The test of proteolysis of gelatin and by a growth test on bile which is an important criterion for the selection of probiotics was followed. The influence of pH was tested on strains selected at different pH (4, 5, 6.5, 8.5 and 8) and the influence of incubation temperature (25 °C, 30 °C, 45 °C) followed by a growth test in hyper saline environment at 4%, 6.5% NaCl.

2.3 Fermentation of Carbohydrates

The fermentation of sugar is examined on MRS medium containing bromocresol purple as pH indicator. 2% of various sugars (arabinose, glucose, fructose, galactose, lactose, maltose, mannitol, rhamnose, sucrose, xylose, esculin and sorbitol) are added. The preparations were covered with 1 mL of sterile paraffin oil for anaerobiosis. Incubation is carried out at 37 °C from 24 to 48 h

The results of various tests morphological, physiological and biochemical are compared to those described by different authors [7].

2.4 Antibigram

Antimicrobial susceptibility of *Bifidobacteria* strains is determined by the standardized technique of diffusion on MRS agar supplemented with 0.05% cysteine chloride and 2 mg/L nalidixic acid. The discs are tested: Amikacin, Amoxycillin + Clavulanic Acid, Ampicillin, Bacitracin, Cefazolin, Cefotaxime, Cefoxitin, cefsulodin, Ceftazidime, Cephalothin, Ciprofloxacin, Clindamycin, Colistin, Erythromycin, Fusidic Acid, Imipenem, Lincomycin, Nalidixic Acid, Netilmicin, Nitrofurantoin,

Ofloxacin, Oxacillin, pefloxacin, Penicillin, Piperacillin, Pristinamycin, Rifampin, Spiramycin, Erythromycin, Ticarcillin, Tobramycin, Trimethoprim-sulfamethoxazole (co-trimoxazole).

In parallel, an antibiotic reference strains *Staphylococcus aureus* ATCC 25923, *Staphylococcus aureus* ATCC 29213, *Staphylococcus aureus* ATCC 43300 is directed by the same technique on both media and Muller Hinton MRSV ancomycin. All discs from Bio-Rad (Marnes-la-Coquette, France), except Vancomycin of Oxoid (Basingstoke, Hampshire, England).

2.5 Kinetics of Growth

In pure culture, the kinetics growth of *Bifidobacteria* strains is followed by counting on MRS medium supplemented with 0.05% cysteine-chloride and 2 mg/L nalidixic acid) solid, and at different time (0 h, 2 h, 4 h, 6 h ...up to 72 h).

2.5.1 Determination of Titratable Acidity

The determination of acidity during growth in milk was performed as described by Accolas et al. [8], using NaOH (N/9) in the presence of phenolphthalein indicator (1% in alcohol).

pH monitoring during the growth.

The acidity produced in the milk is also followed by measuring the pH using a pH meter.

3. Results and Discussion

The colonies of *Bifidobacteria* developed on MRS medium supplemented with 0.05% cysteine-chloride and 2 mg/L nalidixic acid are Gram positive appearance varies. They are whitish and cream, regular contour, and of varying diameter. This macroscopic appearance is often found in *Bifidobacteria*. The purified strains were all catalase negative, they do not possess urease, do not produce indole and do not liquefy gelatin but they are resistant to 2% of bile. These characteristics are typical of the genus *Bifidobacterium*. The results showed that all strains ferment some sugars (glucose, fructose, maltose and

lactose). Strains BL1, BL2 ferment arabinose. This property seems to distinguish the species from other species longum. Moreover, strains BR1, BR2 do not ferment arabinose, xylose or the cons they ferment sorbitol, all these characteristics were showed in Table 1.

With reference to the literature, the strains isolated from fecal of newborn infants belong to two species of *Bifidobacterium*. The two strains BL1 and BL2 belonged to the species longum and BR1 and BR2 (isolated from saline rehydration solution) to breve.

Table 1 Morphological, biochemical and physiological strain BR1, BL1 and BL2 isolated from the fecal newborn infants and BR2 strain isolated from the saline rehydration solution.

Characteristics	The strains			
	BR1	BR2	BL1	BL2
Macroscopic aspect	Cream colonies, Punctiform, white and regular contour cream, regular contour			
Microscopic aspect	Rods Rods and formebifide (V, Y)			
Gram stainreaction	+	+	+	+
Catalase	-	-	-	-
Citrate permease	+	+	+	+
Indole production	-	-	-	-
Urease	-	-	-	-
Gelatin Hydrolysis	-	-	-	-
Bile resistance (2%)	+	+	+	+
CO ₂	-	-	-	-
Glucose	+	+	+	+
Lactose	+	+	+	+
Fructose	+	+	+	+
Arabinose	-	-	+	+
Xylose	-	-	+/-	+
Maltose	+	+	+/-	+
Mannitol	+	+	-	-
Esculine	+	+	-	-
Sucrose	+	+	+	+
Rhamnose	-	-	-	-
Sorbitol	+	+/-	-	-
Galactose	+	+	+/-	+/-
pH 4, 5, 8.5	-	-	-	-
pH 6.5, 8	+	+	+	+
T 4 °C, 5 °C, 25 °C	-	-	-	-
T 45 °C	-	+	-	-
NaCl 4%	+	+	+	+
NaCl 6.5%	-	-	-	-

The results of sensitivity or the resistance of the strains isolated from fecal or from saline rehydration solution to the different antibiotics were regrouped in the Table 2. The growth kinetics results of the strains BL1, BL2, BR1 and BR2 in pure culture are illustrated in the Fig. 1A, the evolution of pH Fig. 1B and titrableacidity Fig. 1C.

Bifidobacteria are commensal bacteria of humans, they are also found in animals [9]. The isolation of

Table 2 Susceptibility (sensitivity and resistance) strains BR1, BL1 and BL2 isolated from the fecal newborn infants and BR2 strain isolated from the saline rehydration solution.

Antibiotics	Charge of discs	Symbol	BL1	BL2	BR1	BR2
Amikacin	30 µg	AN	S	R	R	R
Amoxycillin + Clavulanic acid	20 µg + 10 µg	AMC	S	S	S	S
Ampicillin	10 µg	AM	R	S	S	S
Bacitracin	20-40 µg	BAC				
Cefazolin	30 µg	CZ	S	S	S	S
Cefotaxime	30 µg	CTX	S	S	R	S
Cefoxitin	30 µg	FOX	S	S	S	S
Cefsulodin	30 µg	CFS	R	R	R	R
Ceftazidime	30 µg	CAZ	R	S	S	S
Cephalothin	30 µg	CF	S	S	S	S
Ciprofloxacin	5 µg	CIP	R	R	R	S
Clindamycin	2 µg	CM	S	S	R	S
Colistin	50 µg	CS 50	R	R	S	R
Erythromycin	15 µg	E	S	S	R	S
Imipenem	10 µg	IPM	S	S	S	S
Lincomycin	15 µg	L	S	S	R	S
Nalidixic Acid	30 µg	NA	R	R	S	R
Netilmicin	30 µg	NET	R	S	S	S
Nitrofurantoin	300 µg	FT	S	S	S	S
Ofloxacin	5 µg	OFX	R	R	R	S
Oxacillin	1 µg	OX1	R	R	R	R
Pefloxacin	5 µg	PEF	R	R	R	S
Penicillin	6 µg/10 IU	P	S	S	R	S
Piperacillin	75 µg	PIP 75	S	S	S	S
Pristinamycin	15 µg	PT	S	S	S	S
Rifampin	30 µg	RA 30	S	S	S	S
Spiramycin	100 µg	SP	S	S	R	S
Tetracycline	30 µg	TE	S	S	S	S
Ticarcillin	75 µg	TIC	S	S	R	S
Tobramycin	10 µg	TM	R	R	S	R
Trimethoprim-sulfamethoxazole (co-trimoxazole)	1.25 µg + 23.75 µg	SXT	R	R	R	R
Vancomycin	30 µg	VA	S	S	S	S

Fig. 1 Kinetics of growth (A), the evolution of pH (B) and the evolution of titratable acidity (C) strains: *Bifidobacterium longum* BL1, BL2 and *Bifidobacterium breve* BR1, BR2 in skim milk medium without yeast extract.

Bifidobacteria requires quite specific conditions, which involve systems such as anaerobic Anaerocult or gas pack and rich culture media such as TPY, Beerens medium, MRS cysteine, Columbia medium modified [7, 10, 11]. The pre-identification of strains of bifidobacteria on MRS medium supplemented with 0.05% cysteine chloride and 2 mg/L nalidixic acid showed an appearance of small colonies regular outline and variable aspect.

Cells forming colonies are Gram positive, characterized by varying forms, but often bifid forms that are typical for *Bifidobacteria*. Pleomorphism observed in *Bifidobacteria* is often associated with the composition of culture medium. Studies have shown that the cellular morphology of *Bifidobacteria* is influenced by the nature of the carbon source present in

the culture medium [2, 13].

All strains belonging to the genus *Bifidobacterium* are catalase negative, which does not form indole, does not have a urease activity and does not liquefy gelatin. These biochemical characteristics are consistent with additional features specific to gender, reported by Mitsuoka [14].

All *Bifidobacteria* strains showed good growth on medium supplemented with 2% bile salts. Also it is found that the *Bifidobacteria* degrade bile salts, this criterion probiotic is due to an enzyme that hydrolyzes bile salts (BHS) [15], this enzyme was isolated from the stem *Bifidobacterium longum* BB536 [16], and in strain *Bifidobacterium longum* SBT2928 [17]. Adaptive mechanisms of tolerance to bile salts could lead to better adaptation to the environment and colonic

available carbon sources and a persistent increase in the viability of *Bifidobacterium* in the intestinal environment [18, 19].

The strains isolated from fecal of newborn infants are resistant to nalidixic acid, trimethoprim-sulfamethoxazole (cotrimoxazole). These antibiotics are used as selective agents in synthetic media for the isolation and enumeration of *Bifidobacteria* [20]. Differentiation of *Bifidobacterial* species is based on the fermentation of carbohydrates. Indeed, *Bifidobacterium longum* NCC 2705 has genes coding for fumarase, oxoglutarate dehydrogenase in, and malate dehydrogenase. These enzymes allow the degradation of several sugars (arabinose, xylose, ribose, cellobiose, melibiose, maltose, raffinose and mannose) [21].

The comparison with the profile described by fermentation Scardovi [7] and Tamime et al. [22] led to identifying two species of *Bifidobacterium* breve which includes strains BR1, BR2, the species *longum* BL1, BL2.

The degradation of carbon substrates by *Bifidobacteria* leads to the formation of two acids (lactic and acetic) which lead to a lowering of pH of the medium. This shift in pH has no influence on the growth of these bacteria for 24 hours of incubation [23].

The effect of pH on microbial growth affects enzyme activity, cell permeability of certain nutrients which depends on ionic balance. The results showed that at pH 4, 4.5 and 5 no growth was observed. *Bifidobacteria* are generally sensitive to pH values below 4.6 [24]. Matsumoto et al. [25] indicated that tolerance of *Bifidobacterium longum*, *Bifidobacterium adolescentis* and *Bifidobacterium pseudocatenulatum* at acidic pH values is limited, a significant decrease in the viability of strains in a medium at pH 3 after only one hour of incubation. Moderate growth and a significant slowdown in growth of the strains isolated from fecal and saline rehydration solution is obtained in the medium at pH 8 and a total inhibition in the medium at pH 8.5. Optimum growth for different strains is obtained in the medium at pH 6.5.

These results confirm that *Bifidobacteria* preferring neutral or slightly acidic pH was between 5 and 8 as was reported by several authors [26-29].

The incubation temperature is on transport systems through the membrane and therefore the disruption of cellular metabolism. The results of the growth of *Bifidobacterium* strains in MRS medium cysteine, incubated at various temperatures show variability in behavior among species and strains. Indeed at incubation temperature of 4 °C and 25 °C, there is complete inhibition of growth.

Moreover, the growth of all strains is better at the incubation temperature of 30 °C and 37 °C. At a temperature of 45 °C, there is a total absence of growth of three strains of *Bifidobacterium* isolated from fecal newborn infants. This behavior can be explained by the fact that the strains are of human origin and cannot withstand high temperatures. The same results were reported by several authors [7, 9, 30, 31]. While there is moderate growth of the strain BR2 isolated from saline rehydration solution. These observations show firstly that the incubation temperature is an important parameter that can affect the growth of *Bifidobacteria*, which, on the other hand, confirms that *Bifidobacteria* of human origin are mesophilic bacteria growing at optimal temperatures of 30-37 °C, but can adapt to higher temperatures.

If the introduction of *Bifidobacteria* in dairy industry has made there over 20 years in technologically advanced countries, it is against, not yet possible in some other countries such as Algeria. This situation is related to the constraints posed by the genus *Bifidobacterium* which is very sensitive to acidity developed in milk and aerobically on prevailing there.

Production of acidity depends on several parameters: incubation temperature, physiological state of bacteria, the inoculum concentration, and the milk used. Evaluation of titratable acidity, pH and the number of bacteria produced during growth of strains in pure culture as well reveal a significant difference between *Bifidobacterium* strains used (BL1, BL2, BR1, and

BR2) of as much as Martinez-Villaluenga and Gomes [32] observed that the growth rate and generation time of bifidobacteria in the UHT milk vary among strains. After 6 h of incubation it is observed that the strains (BL1, BL2) belonging to *Bifidobacterium longum* are more acidifying than strains belonging to *Bifidobacterium breve* (BR1, BR2). Differences in acid production by strains of bifidobacteria have been reported by several authors [32-34]. These differences in behavior of *Bifidobacterium* strains in the different milks may be due partly to the composition of milk and also to the proteolytic activity that varies from strain to strain.

Survival of *Bifidobacteria* remains low. However, this survival may also be significantly improved by the addition of indigestible substances commonly known as “prebiotics”. Among prebiotics mainly used in the food industry, there are inulin and oligofructose, honey bee [35]

On the other hand, another factor that appears to inhibit the growth of *Bifidobacteria* in milk medium is oxygen; however the degree of tolerance to this factor depends on the species and culture medium; to remedy this problem the adding a reducing agent such as cysteine hydrochloric seems to have its effect.

4. Conclusions

According to the results of the phenotypic characterization the strains (BL1, BL2, BR1 and BR2) were isolated from breast-fed infants and saline rehydration solution belonged to two species of *Bifidobacterium* (*longum*, *breve*). The strains BL1 and BL2 have shown some technological properties such as the tolerance to the high temperature (42 °C), production of acidity, the resistance to the antibiotics and their kinetics of growth in skim milk, which suggests their possible use in the food industry. However, more studies are needed to test these strains for the human health.

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