

Insights in the Human Bifidobacterial Flora Through Culture-Dependent and Independent Techniques

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Bifidobacteria belong to the so-called beneficial intestinal flora. Before attempting to raise their intestinal levels to improve the health status of the host, it is of importance to know about physiological variations in the bifidobacterial colonisation of the human intestine. This paper reports on three studies held on premature, full-term infants and young adults. The intestinal colonisation with bifidobacteria was addressed using both a cultural approach and PCR detection/identification of species belonging to the genus *Bifidobacterium*. Cultivable bifidobacteria could not be retrieved from fecal samples obtained from premature babies. However, PCR detection showed a large majority of premature harboured bifidobacteria, possibly under the cultural detection threshold. Full-term infants readily harboured cultivable bifidobacteria with *B. longum-infantis*, *B. breve* and *B. bifidum* as dominant species while most young adults also had cultivable faecal bifidobacteria but this time with *B. adolescentis* and *B. catenulatum-pseudocatenulatum* as dominant species. This work also puts the emphasis on good sampling and preservation conditions for an optimised recovery of cultivable bifidobacteria.

Keywords: *Bifidobacterium*, premature, neonate, adult, intestinal flora

Abbreviations: CFU : Colony Forming Unit; PCR : Polymerase Chain Reaction; SD : Standard Deviation

1. Introduction

Bifidobacteria are non spore-forming, non-motile, Gram positive, strictly anaerobic rods. They were discovered at the turn of the twentieth century by Tissier [1] in stools of breast-fed infants. In the fifties bifidobacteria were named after their peculiar Y-shape (Fig. 1) when the genus was created to differentiate these bacteria from lactobacilli [2]. Currently, around 30 species belong to the genus *Bifidobacterium* but only 12 are isolated from human sources. Since bifidobacteria were found in large numbers in the stools of healthy neonates and used in fermented milks drunk by European populations with a great life expectancy, these bacteria have conveyed an image of wholesomeness and healthiness [3]. Nowadays, an increasing number of strains of bifidobacteria are used in probiotic products. This rising interest in feeding people with live bifidobacteria through various dairy products and/or alimentary complements to improve their health status puts forward the importance of knowing about physiological variations in the bifidobacterial colonisation of the human intestine, prior to any ingestion of probiotic products. Some previous reports have shown the colonisation of the intestine by bifidobacteria was age-related. Neonates harbour high counts in intestinal bifidobacteria while these numbers seem to decrease through adulthood and reach their lowest level in older people [4, 5]. Species of the genus *Bifidobacterium* colonising the human gut also seem to evolve with age : *B. longum-B. infantis*, *B. breve* and *B. bifidum* dominate the infant flora while *B. adolescentis* and *B. catenulatum* become more prevalent in the adult intestinal flora [4, 6, 7]. Geographical-dependent variations in proportions of bifidobacteria in the gut have also been reported [8]. One of the impediments in evaluating the intestinal

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bifidobacterial flora is the sensitivity of bifidobacteria to oxygen and the use of selective media [9]. Therefore, molecular biology methods have been developed to help unravelling the diversity of the global intestinal flora and that of intestinal bifidobacteria through culture-independent techniques [8, 10, 11]. Nevertheless, very few comparative studies on results obtained with culture-dependent and -independent methods are available. However, when bifidobacteria are concerned, it seems that discrepancies mostly occur in the analysis of adult intestinal floras while 80% of bifidobacterial signal detected signals can usually be matched to a cultural isolate [12].

The purpose of this paper is to describe our experience in cultural techniques allowing for an optimal recovery of bifidobacteria, in the identification to the species level of isolated strains through PCR multiplex as well as direct detection of bifidobacterial species in stools using this latter method. These experiments have been conducted on various human populations such as prematures, neonates and adults.

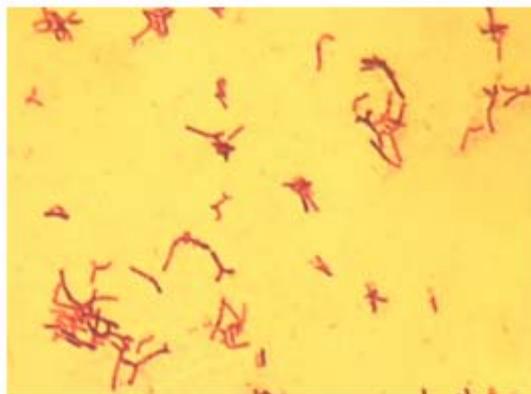


Fig. 1 Gram-stained bifidobacteria with typical Y-shaped rods ($\times 1000$).

2. Material and Methods

2. 1. Human subjects and faecal sampling

Three independent studies are reported here. The first one was performed on 11 healthy male human volunteers included in a clinical trial from January to November 1998 after they had given a written informed consent. Their mean age was 23.2 ± 2.62 years (range 20-28 years). They underwent two test periods of ten days each, separated by an interval of at least 6 weeks. They were fed a standardised diet throughout the two test periods, free from prebiotic and/or probiotic products. In addition to this standard diet, they drank a daily 30mL-dose of a concentrated whey obtained from the fermentation of milk by either *Bifidobacterium breve* C50 or *Bifidobacterium breve* C7 strain, to try to raise their intestinal proportion of bifidobacteria. Fresh stool samples were collected on days 0, 3 and 10 of each test period in a sterile container directly placed in a Genbag Anaer[®] device (Biomérieux, Marcy l'Etoile, France)(Fig. 2, on the right). This device allows the sample to remain in an anaerobic environment until it is processed at the laboratory. All faecal samples were thereafter carried to the microbiology laboratory and processed within 2 hours after collection.

The second clinical study included 21 healthy full-term newborns born between August 1999 and January 2000 at the maternité Pavillon de la Sainte Famille (Clinique du Bois, Lille, France). Inclusion criteria were vaginal delivery, gestational age between 38 and 42 weeks, birth weight >2500 g, bottle-feeding previously decided by parents and written informed consent of the two parents or the legal guardian. Stool sampling was performed using Potagerm Amies + agar[®] swabs (Fig. 2, on the left). These devices allow for the preservation of anaerobiosis for 48 hours. Samples were collected at 1, 2, 3

and 4 months. They were transported to the laboratory of Microbiology within 24 hours and directly processed as described below.

The third and last study included 27 premature babies (14 males and 13 females) born either vaginally or by caesarean section between November 2003 and June 2004 at Amiens Central Hospital. Their mean gestational age was 29 ± 2.2 weeks and their mean birth weight was 1319 ± 457.7 g. The mean age at sampling was 25 ± 14.5 weeks. Stools were collected in a sterile container and frozen at -80°C until culture was undertaken as described below.

2. 2. Cultural procedures

Bifidobacteria are usually considered as fastidious microorganisms because of their weak tolerance to oxygen. However, the sampling and cultural procedures described for adults and healthy full-term infants allow for a maximal recovery of cultivable bifidobacteria. Swabs obtained from healthy neonates were homogenised in 9 mL pre-reduced (15 min at 100°C) $\frac{1}{4}$ Ringer solution supplemented with 0.3% cysteine hydrochloride. Ten-fold dilutions were performed using the same diluant. Appropriate dilutions were then plated onto selective and non-selective media. Typically, dilutions were 10^{-2} to 10^{-4} for selective media such as Beerens [13] or MRS (Difco, Saint Sauveur, France) and 10^{-4} to 10^{-6} for non-selective media such as horse blood (5%, V/V) Columbia agar (Difco). For stool sample dilutions, approximately 1g was homogenised in 9 mL of pre-reduced $\frac{1}{4}$ Ringer solution. Ten-fold dilutions were performed as previously, 10^{-4} to 10^{-6} dilutions plated onto selective media and 10^{-6} to 10^{-8} onto non-selective ones. Aerobic incubation was carried out at 37°C for 48 hours. Anaerobic incubation was carried out for 5 days at 37°C using an anaerobic chamber (Fig. 3). The inner atmosphere was composed of the following gas mix: $\text{N}_2/\text{CO}_2/\text{H}_2$ (85%/5%/10%). Remaining stool samples and undiluted suspensions obtained from swabs were kept frozen at -20°C for further analysis using molecular biology techniques.

After incubation, each type of colony was described, enumerated and subjected to Gram staining. Catalase activity test was performed using H_2O_2 . Each colony exhibiting cultural and phenotypical characteristics compatible with those of bifidobacteria was subcultured on Rosenow broth (Biorad, France) and kept frozen at -20°C until further identification using the multiplex PCR described below.



Fig. 2 Portagerm (on the left and) Genbag anaer devices.



Fig. 3 Bactron II anaerobic chamber.

2. 3. Multiplex PCR identification of isolated strains

Multiplex PCR identification of putative isolated strains of bifidobacteria was carried out as described previously [7]. Briefly, for each strain, DNA extraction was performed on a fresh subculture carried out anaerobically at 37°C (24 to 48h) on Columbia broth. Centrifugation of 1.5 mL of this culture was carried out at $4500g$ (5 min, 4°C). The bacterial pellet was retained and subjected to DNA extraction using Nucleospin Tissue™ kit (Macherey-Nagel, Hoerd, France) according to the supplier's instructions

(support protocol for Gram positive bacteria). Incubation with lysozyme (20 mg/ml) was carried out at 37°C for 1 h. It was followed by a second incubation with proteinase K (30 mg/ml) at 56°C for 3 h. RNase A (Sigma, Saint Quentin-Fallavier, France) was then added to the mixture to eliminate contaminating RNA (incubation for 5 min at room temperature). A further 10-min incubation at 70°C with B3 buffer containing chaotropic salts (Nucleospin Tissue™ kit) was performed to obtain a thorough lysis of the bacteria. The resulting DNA suspension was then purified on a minicolumn provided in the kit.

The PCR reaction mixture (50 µL) was composed of 5 µL of 10×PCR buffer II (Six pack Ampli Taq Gold DNA polymerase with buffer II, Applied Biosystem, Courtaboeuf, France) ; 2 mM MgCl₂ ; 400 µM of each dNTP; 0.4 µM of each species-specific primer required; 2.5 U of Taq DNA polymerase (Applied Biosystem); and 10 µL of template DNA. Amplification was carried out in a PTC-200 thermal cycler (PTC-200, MJ Research). The amplification consisted of one cycle at 94°C for 5 min, 35 cycles of 94°C for 30 s, 59°C for 30 s, and 72°C for 1 min to 2 min 42 s (elongation gradient of 3 s for each additional cycle), and a final cycle of 72°C for 5 min. Amplified products were run on a 1.5% agarose gel, stained with ethidium bromide and visualised under UV transillumination.

2. 4. Direct detection of bifidobacterial species in stool samples using multiplex PCR

Centrifugation was applied to 1.35 mL of undiluted fecal suspensions obtained from swab homogenisation (30 000g, 5 min, 4°C) and the pellet subjected to the same extraction procedure as the bacterial pellet from cultures. PCR amplification was performed on these DNA extracts as mentioned above.

3. Results and Discussion

3. 1. Study on healthy adults

Counts in bifidobacteria are reported in table 1, according to the whey taken by healthy volunteers. Cultivable bifidobacteria were detected in 60 samples out of 66 (90.9%). When detected, bifidobacterial counts ranged from 6.5 to 11.9 log CFU/g of stool. When means are compared, no significant statistical difference was observed, whatever the day of sampling or the whey taken by the volunteers. This was unfortunate because the clinical trial was attempted to show an increase in faecal intestinal bifidobacteria after consumption of *B. breve* C50 whey, which had previously shown such a bifidogenic effect on gnotobiotic animals and humans as opposed to *B. breve* C7 whey, which had not [14-16]. This may partly be explained by the high bifidobacterial counts exhibited by subjects at the beginning of our study. Hence, a rise in these already nearly maximal counts would have been difficult to witness.

As variations in the total cultivable flora could to some extent explain some of the variations in bifidobacterial counts, we also expressed the intestinal colonisation level with bifidobacteria through percentage of the total cultivable flora (Table 1, figures between brackets). Proportions of detectable bifidobacteria varied from 0.01 to 96.3% of the total cultivable flora. Some authors have proposed the threshold of 1% of the total cultivable flora to assign bacteria to the dominant flora [17]. When such a criterion is applied to our results, bifidobacteria are part of the dominant cultivable flora in 43 out of 66 samples (65.1%). Subject N°4 was peculiar because he was never found to harbour dominant bifidobacteria in his faecal flora. All other subjects had at least half of their samples with dominant bifidobacteria. Interestingly, this subject reported a stool frequency well above all others (mean frequency of 3 stools per day). Nevertheless, there was no significant variation in the proportions of bifidobacteria throughout the study.

A representative sample (50 strains) of isolated bifidobacteria that could be subcultured were further subjected to multiplex PCR identification. Two subjects harboured strains that could not be identified through multiplex PCR. Species frequencies for adult subjects are reported in Table 2. The number of identified species for a given subject ranged from 1 to 4. *B. adolescentis* and *B. catenulatum/B.*

pseudocatenulatum were the most frequently identified species in adults, as previously reported [6, 18]. However, *B. longum* was not as frequently found as in earlier reports.

Table 1. Counts in faecal bifidobacteria according to whey ingested.

Subject N°	C7 whey			C50 whey		
	Day 0	Day 3	Day 10	Day 0	Day 3	Day 10
1	8.1 ^a (1.18) ^b	6.7 (0.06)	6.5 (0.11)	10.7 (42)	11.1 (42)	9.4 (33.8)
2	8.4 (40.3)	8.6 (6)	8.4 (22.7)	9.3 (5.9)	10.5 (33.7)	11.9 (60.8)
3	9.8 (0.5)	11.4 (36)	11.0 (15)	9.9 (22.6)	9.1 (14.9)	8.9 (21.3)
4	ND ^c (<0.001)	ND (<0.001)	7.1 (0.38)	ND (<0.001)	6.6 (0.06)	ND (<0.001)
5	8 (2)	9.8 (1.6)	9.6 (1.7)	10.7 (13.4)	11.2 (5.9)	10.0 (7.1)
6	8.6 (9.1)	10.1 (5.2)	7.9 (5.3)	ND (<0.001)	8.3 (0.2)	7.7 (0.2)
7	9.1 (11.8)	9.2 (39)	9.1 (17.8)	6.0 (0.04)	8.2 (0.4)	8.6 (0.02)
8	8.7 (1.8)	7.7 (0.3)	7.6 (0.6)	8.7 (8.5)	8.9 (0.8)	10.6 (30.8)
9	10.5 (8.9)	10.5 (1.3)	7.1 (1.9)	8.6 (0.9)	8.6 (40)	9.2 (42.1)
10	6.4 (0.08)	ND (<0.001)	7.3 (0.01)	9.0 (7.1)	9.9 (49.4)	11.4 (6.4)
11	9.9 (8.2)	10.9 (96.3)	11.9 (38)	10.0 (0.55)	10.2 (16.2)	9.7 (17.2)
Mean ± SD	8.7 ± 1.17 (8.4 ± 2.01)	9.4 ± 1.54 (20.6 ± 32.26)	8.5 ± 1.73 (9.4 ± 12.50)	9.2 ± 1.44 (11.2 ± 13.6)	9.3 ± 1.40 (18.5 ± 19.22)	9.7 ± 1.28 (22.0 ± 19.92)

^a: counts expressed as log CFU/g of stool

^b: counts expressed as proportion of the total cultivable faecal flora

^c: Not detected

Table 2. Carriage frequencies of bifidobacterial species for adults (n=11) and full-term infants (n=21)

Species	% adults	% full-term infants
<i>B. adolescentis</i>	45.4 (5) ^a	14.3 (3)
<i>B. angulatum</i>	0 (0)	9.5 (2)
<i>B. bifidum</i>	18.2* (2)	66.7* (14)
<i>B. breve</i>	0** (0)	38.1** (8)
<i>B. catenulatum/pseudocatenulatum</i>	36.4 (4)	14.3 (3)
<i>B. dentium</i>	9.1 (1)	4.8 (1)
<i>B. longum-B. infantis</i>	18.2 (2)	47.6 (10)

^a: Number of carriers

Carriage frequencies significantly different between infants and adults at (Fisher's exact test) :

*: p=0.0233, **: p=0.02924

3. 2. Study on full-term infants

Cultivable bifidobacteria were retrieved from all collected samples (84). Sampling with swabs does not allow for results to be given as log CFU per gram of stool as the exact weight of stool sampled cannot be measured. Therefore, table 3 only gives proportions of bifidobacteria within the total cultivable flora as well as the percentage of carriers of each species of the genus *Bifidobacterium* throughout the study.

Table 3. Proportion of bifidobacteria within the total flora and carriage frequencies of bifidobacterial species from month 1 to month 4.

	Month 1	Month 2	Month 3	Month 4
% bifidobacteria	27 ± 22.3 ^a	35 ± 25.1	42 ± 26.5	43 ± 23.1
<i>B. adolescentis</i>	3 (14.3) ^b	2 (9.5)	2 (9.5)	3 (14.3)
<i>B. angulatum</i>	0 (0)	0 (0)	0 (0)	2 (9.5)
<i>B. bifidum</i>	12 (57.1)	14 (66.7)	18 (85.7)	14 (66.7)
<i>B. breve</i>	12 (57.1)	12 (57.1)	7 (33.3)	7 (33.3)
<i>B. catenulatum/pseudocatenulatum</i>	1 (4.8)	1 (4.8)	3 (14.3)	3 (14.3)
<i>B. dentium</i>	0 (0)	0 (0)	1 (4.8)	0 (0)
<i>B. longum-B. infantis</i>	11 (52.4)	12 (57.2)	10 (47.6)	12 (57.1)

^a: Results given as mean ± SD

^b: Number of carriers (%)

All isolated strains were subjected to multiplex PCR identification. *B. bifidum* and *B. longum-infantis* were the most prevalent species and their carriage remained stable over the 4 months. *B. breve* was also frequently isolated in infants but its carriage frequency began to decrease by month 3. Interestingly, global *B. bifidum* and *B. breve* carriage frequencies were significantly higher in infants than in adults (Table 2). Only two previous studies using phenotypic methods as identification tools attempted to follow up species carriage over time [19, 20]. If *B. breve* and *B. longum-infantis* were also found as dominant and stable, *B. bifidum* was reported as an infrequent and unstable species. Along with the difference in identification techniques, a distinct geographical origin of babies from our study and others could to some extent explain these discrepancies.

Additionally, direct detection of bifidobacterial species on the undiluted faecal suspensions was carried out for infants exhibiting unstable colonisation profiles for a given strain. A few additional positive signal were recovered for some samples, accounting for the presence of bifidobacteria under the cultural detection threshold [12].

3.3. Study on premature infants :

No cultivable bifidobacteria could be retrieved from these 27 samples. Even though it is commonly acknowledged that premature intestine is not as frequently colonised with bifidobacteria as the intestine of full-term infants [21, 22], the quality of sample preservation can be raised for this study. Indeed, stool samples were not placed in devices allowing for preservation of anaerobiosis. Moreover, they were not processed directly on arrival at the microbiology laboratory. Therefore, the lack of recovery of cultivable bifidobacteria could be linked not only to a delayed implantation of bifidobacteria but also to technical impediments.

To overcome these technical drawbacks, direct PCR detection of human bifidobacterial species was performed on undiluted stool suspensions as mentioned above. Although no cultivable bifidobacteria could be retrieve from any stool sample (possibly because of a bad preservation of anaerobiosis), only 6 out of 27 (22%) undiluted suspensions tested by direct multiplex detection did not yield any positive signal for human bifidobacterial species.

As it has been previously described that, in premature infants, intestinal implantation of bifidobacteria is delayed [21, 23], we checked whether PCR negative samples were those with the youngest "age". The mean age of babies with bifidobacteria-negative samples was of 28 ± 13.9 days (range 8-51 days) while the average age of babies with bifidobacteria-positive samples was of 26 ± 13.4 days (4-60 days). Hence, no significant difference in sampling time could be put forward to explain PCR negative samples for bifidobacteria. Moreover, bifidobacteria could be detected directly in samples as soon as on the 4th day after birth, which is an improvement as compared with previous reports. It would be of interest to enlarge our premature population, especially targeting the first week after birth to fully evaluate whether

multiplex PCR is an interesting tool for an early detection of bifidobacteria (before they reach the detection level of cultural techniques).

PCR detection gave 21 (78%) bifidobacteria-positive samples among which the majority exhibited a double signal for *B. bifidum* and *B. breve* (18 over 21 positive samples, 86%). For one infant, only *B. breve* signal was recovered while for another, only *B. bifidum* was detected. The sample for the remaining baby exhibited a double positive signal for *B. longum* and *B. bifidum*. This species distribution is in accordance with another recent study held on premature infants [23].

4. Conclusion

When sampling procedures took care of preserving faecal bifidobacteria from oxygen (i.e. studies held on healthy full-term newborns and adults), isolation of bifidobacteria was achieved in a large majority of samples. These results show that sample preservation is of utmost importance when detection of bifidobacteria is undertaken through cultural techniques. However, direct PCR detection of human species of the genus *Bifidobacterium* is a valuable additional tool when sample preservation has not been optimal. PCR identification of isolated strains provides interesting additional information on the distribution of bifidobacterial species and its variations intra- and interindividually, as this kind of data is still scarce in the literature and could be of importance while evaluating beneficial effects of pre- and/or probiotic products.

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