VNU Journal of Science: Natural Sciences and Technology, Vol. 32, No. 1S (2016) 269-276

An Efficient Method for Isolation of Bifidobacteria from Infant Gut

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Received 15 August 2016
Revised 25 August 2016; Accepted 09 September 2016

Abstract: An efficient method was established for isolation of bifidobacteria from fecal samples of breast-fed infants. The method was based on the combination of Hungate technique applied on anoxic semi-liquid agar tubes, instead of double layer agar plates. Four growth media including BFM, BIM25, MRS and 385 were compared for the isolation efficiency on the basis of Hungate technique. Thus, among 30 isolates obtained from fecal samples of 14 breast fed infants of different ages under 1 year only eight bifidobacteria-like isolates were selected based on cell morphology and fermentation motif. It is revealed that Hungate technique with the use of anaerobic MRS and BFM media was more efficient for isolation of bifidobacteria than that with BIM25 and 385 media. The difference of isolation efficiency in MRS and BFM medium was not obvious. It is therefore recommended that the BFM medium would be applied for isolation of bifidobacteria generally, and in this case, from gut, whereas the MRS medium should be suitable for cultivation and maintaining of pure cultures. In addition, isolation efficiency would also depend on infant's ages and the way how fecal samples have been stored before isolation.

Keywords: Bifidobacteria, infant gut, Hungate technique, anaerobic growth media.

1. Introduction

Bifidobacteria are the first bacterial groups colonizing the intestinal tract of human infants since they are activated by glycoprotein of K-casein which is abundant in colostrums and to a lesser extent, human milk [1]. Number of bifidobacteria in the gut microflora however reduces with the age, in adults the group contributes for 25% of total microflora, representing the third most abundant group after the Bacteroides and Eubacterium [2].

Isolation of bifidobacteria is a challenging process since (i) the bacteria grow under strictly anaerobic condition and (ii) isolation on common growth media such as De Man Rogosa Sharpe (MRS) and Reinforced Clostridial Agar (RCA) usually misleads to other genera of lactic acid bacteria [3]. To avoid such undesired isolation, attempts have been given to develop selective growth media and at the same time, anaerobic cultivation techniques effective for bifidobacteria.

Most of studies on bifidobacteria used the double layer agar plate method for the cultivation. However this technique might not be suitable for many species of bifidobacteria since a strict anaerobic condition would not be established. In a more advanced way, the plates are prepared inside an anaerobic airlock chamber and incubated in anaerobic jars under controlled oxygen-limited atmosphere [4]. The Hungate technique as an alternative method, which has been originally designed for isolation and cultivation of obligate anaerobes such as sulfate-reducing bacteria or methanogens [5], and even for human gut microorganisms such as Clostridia [6, 7], however

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has not been used extensively for bifidobacteria. The technique employs inert gas like nitrogen or argon to flush away oxygen from the media and cultivating vessels (tubes or serum bottles), leading to a well anoxic condition in the vessels.

Concerning culturing media, selective and differential media have been developed. Some media such as YN-6 [8], BIM25 [9] contain mixture of antibiotics like nalidixic acid, polymixin B sulfate, kanamycin sulfate, neomycin sulfate to inhibit other LAB but not bifidobacteria. Other media such as BFM use inhibitory agents other than antibiotics (i.e. methylene blue, lithium chloride, propionic acid…) to inhibit growth of lactobacilli [10]. The YN-17 medium [11], on the other hand, contains sorbitol as fermentation substrate to differentiate bifidobacteria of human and animal origins in environmental samples. However, from the large number of selective media available, it can be concluded that there is no standard medium for detection of bifidobacteria from all environments [3].

In Vietnam, there is little understanding of the practice work with bifidobacteria due to inappropriate laboratory conditions for the anaerobes. Hence, the research and application dealing with this bacterial group still remain limited, despite of large practical demand. The present study aimed to (i) investigate the effectiveness of Hungate technique, and (ii) compare the efficiency of different cultivation media for the isolation of bifidobacteria from intestinal tract of breast-fed infants in Vietnam in order to establish an effective method to obtain pure cultures for research and application.

2. Materials and methods

2.1. Sampling technique

Fresh fecal samples were collected from breast feed infants of the ages 1 to 12 months. Before being transferred to laboratory, the samples were stored in two different ways, i.e. (i) put in falcon tubes and kept at 4°C or (ii) put in glass tubes with previously prepared anoxic medium and kept at room temperature.

2.2. Isolation of bifidobacteria

Isolation of bifidobacteria was carried out by using Hungate technique applied for semi-liquid (1 %) agar tubes. Thus, the fecal samples were homogenized and subjected for serial dilutions in anoxic 0.9 % NaCl solution. Afterward, 0.1 ml aliquotes of the sample suspension were inoculated in anaerobic tubes containing warm (~40°C) 9 ml sterile anoxic semi-liquid medium (1 % agar) by using 1 ml sterile syringers. The tubes were then transferred to water bath for agar solidification, flushed with N_2 gas (Messer Vietnam) for 30 second and incubated upside down at 37°C in the dark (Fig. 1A). Single colonies in deep agar layers in the tubes (Fig 1B) were selectively picked by means of glass capillaries and transferred to anaerobic tubes containing liquid growth medium. Growth of the bacteria was examined via pH decrease in the medium and microscopic observation for specific cell morphology.

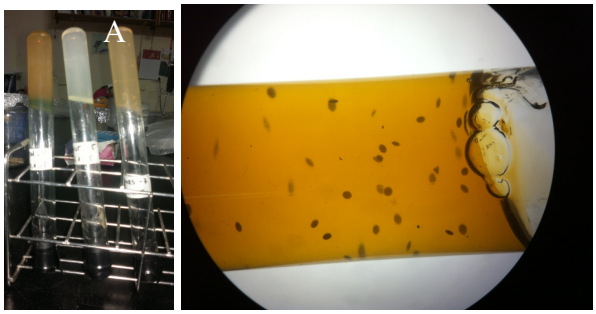

Figure 1. Isolation of bifidobacteria in semi-liquid agar tubes by using Hungate technique.
A - The semi-liquid agar tubes with different media incubated in upside-down position; B - Bacterial colonies in deep agar observed under stereoscopic microscope Zeiss Stemi 2000-C.

Table 1. Growth media for isolation of bifidobacteria used in this study (per liter)[#]

Chemicals	**MRS** (DSMZ, Germany)	**BIM25** (Munoa & Pares, 1988) [9]	**BFM** (Nebra & Blanch, 1999) [10]	**385** (NBRC, Japan)
Pancreatic digest of casein	–	–	–	10 g
Peptone	10 g	5 g	5 g	–
Tryptone	–	5 g	2 g	–
Meat extract	–	–	–	5 g
Beef extract	10 g	8 g	2 g	–
Yeast extract	5 g	1 g	7 g	5 g
Glucose	20 g	1 g	–	10 g
Starch	–	1 g	2 g	–
Lactulose	–	–	5 g	–
L-cysteine*	0.5 g	0.5 g	0,5 g	0.5 g
NaCl	–	–	5 g	–
Riboflavin*	–	–	1 mg	–
Thiamine*	–	–	1 mg	–
Tween 80	1 g	–	–	1 ml
KH_2PO_4	2 g	–	–	3 g
Sodium-acetate	5 g	5 g	–	–
$(NH_4)_2$-H-citrate	2 g	0.5 g	–	–
$MgSO_4.7H_2O$	0.2 g	–	–	–
$MnSO_4.nH_2O$	0.05 g	–	–	–
Sodium-bisulfite	–	0.5 g	–	–
Sodium-ascorbate*	–	–	–	10 g
Methylene blue	–	–	16 mg	–
Lithium chloride	–	–	2 g	–
Propionic acid 99%*	–	–	5 ml	–
Nalidixic acid*	–	–	0.02 g	–
Polymyxin B sulphate*	–	–	0.0085 g	–
Kanamycin sulphate*	–	–	0.05 g	–
Sodium-iodoacetate *	–	–	0.025 g	–
2,3,5-triphenyl-tetrazolium chloride*	–	–	0.025 g	–
Distilled water	1 liter	1 liter	1 liter	1 liter
Bacto agar (for semiliquid medium)	10 g	10 g	10 g	10 g
pH	6 – 6.5	6.8	5.5	6.8

[#] After autoclaving, the media were flushed with nitrogen (Messer Vietnam) to remove oxygen.
*Heat sensitive compounds were prepared in stock solution, membrane sterilized (pore size 0.2 μm) and added to the medium after autoclaving and cooling.

Four different growth media, including MRS, BFM, BIM25 and 358 were used for the isolation of bifidobacteria from the fecal samples (Tab. 1). Of the four media, BFM and BIM25 are specific for bifidobacteria whereas MRS and 385 are unspecific and suitable for all lactic acid bacteria.

2.3. DNA extraction, xpf gene fragment amplification and sequencing

Being different from other lactic acid bacteria (LAB), bifidobacteria possess bifid-shunt pathway while grow with hexoses, leading to production of acetic acid together with lactic acid

[12]. The key enzyme fructose-6-phosphoketolase (F-6-PPK) involving in the bifid-shunt pathway is unique for bifidobacteria. Therefore, the enzyme and its coding gene are used as molecular markers for identifying these bacteria in different environments [12].

Genomic DNA of the isolates was extracted following Marmur's method with some modifications [13]. Fragments of *xfp* gene coding for the fructose - 6 - phosphoketolase (unique for the bifidobacteria) were obtained via PCR with the specific primer pair U1R (5'ACCTGCCCGAAGTACATCGAC 3') and U2L (5'GAGCTCCAGATGCCGTGACG 3') [12]. The thermocycling reactions were carried out according to the authors, i.e. starting with denaturation step for 4 min at 94°C, followed by 35 cycles of 94°C for 30 s, 60°C for 30 s, 72°C for 60 s, and a final extension step at 72°C for 10 min before ending at 4°C. The PCR products were then analyzed by agarose gel electrophoresis to confirm for PCR products of ~ 520 bp. Representative gene fragments were purified with AccuPrep PCR Purification Kit (Bioneer, Korea) and subjected to sequencing with ABI Prism BigDye Terminator cycler sequencing Kit on automatic sequencer 3110 Avant Applied Biosystems (ABI). The obtained sequences were then aligned with corresponding sequences available in the GenBank database by using Blast Search tool.

3. Results and discussion

3.1. Isolation of bifidobacteria from infant fecal samples

During 2014 - 2015, a total of 14 fecal samples from breast fed infants of different ages under one year were collected for the isolation of bifidobacteria (Tab. 2). The samples were selected as representatives for three groups of ages, i.e. (i) under three months (G1, G2, G3, G5, G6, G7, B1), (ii) from 3 - 6 months (G4, B2, B4) and (iii) from 7 - 11 months (G8, G9, B3, B5).

Table 2. Infant fecal samples collected during 2014 - 2015 in Hanoi and surrounding areas

No	Sample name	Gender	Age (months)	Sample storage	Number of strains isolated
Group 1: infants under 3 months					
1	G1	Girl	1	In anoxic medium, RT	3
2	G2	Girl	1	4°C	2
3	G3	Girl	2	4°C	2
4	B1	Boy	2	In anoxic medium, RT	2
5	G5	Girl	2	In anoxic medium, RT	2
6	G6	Girl	2	4°C	2
7	G7	Girl	1.5	4°C	2
Group 2: infants from 3 - 6 months					
8	G4	Girl	3	4°C	2
9	B2	Boy	3	4°C	2
10	B4	Boy	5	In anoxic medium, RT	2
Group 3: infants from 8 - 11 months					
11	G8	Girl	9	In anoxic medium, RT	2
12	G9	Girl	8	4°C	2
13	B3	Boy	10	In anoxic medium, RT	3
14	B5	Boy	11	In anoxic medium, RT	2

Nevertheless, isolation efficiency for bifidobacteria depends to a large extent upon (i) the sampling procedure and (ii) the isolation media (Tab. 3).

By using Hungate technique for serial dilutions in anoxic semi-liquid agar tubes with four different growth media MRS, 385, BIM25 and BFM, 30 bacterial strains were isolated from the collected fecal samples. These isolates were selected based on two categories, (i) the representativeness (i.e. being represent for the sample origin, the isolation medium and colony morphology), and (ii) the abundance (i.e. being present at higher dilution levels, reflecting the abundance in the original samples). Thus, 6 to 8 isolates were obtained from semi-liquid agar tubes of each growth medium used. However, based on the specificity of bifidobacteria cell morphology and fermentation motif, only 8 strains were selected from these 30 isolates to make a bifidobacteria-like group (Tab. 3).

The bifidobacteria-like isolates should have cells of rod to irregular rod shapes, occasionally show V or Y cell types, occur single or in groups (Fig. 2). Physiologically, these strains should grow fermentatively on sugar substrates and produce organic acids, lowering pH of the growth medium whereas no gas (CO_2) should be formed (Figure 2).

3.2. Analyzing the presence of xfp gene in the selected isolates

In this study, the presence of *xfp* gene was used as molecular indicator for detecting strains of the genus *Bifidobacterium*. Thus, 593 bp fragments of the *xfp* gene were amplified from genome of the 8 selected isolates of bifidobacteria-like group (Tab. 3) in PCR reactions using the specific primer pair U1R/U2L and the obtained products were analyzed by electrophoresis on 2% agarose gel (Fig. 3).

Most of the isolates of the bifidobacteria-like group yielded PCR products of expected size of ~520 bp., except strain NG17, indicating that they likely belong to the genus *Bifidobacterium*. To confirm this, representative PCR products of the *xfp* gene fragments from strains NG3, NG6, NG15 and NG21 were subjected to sequencing and aligning to related sequences in the GenBank. For those samples which yielded unspecific PCR products such as NG3 and NG5, the interested bands (marked on figure 3) were excised from the agarose gel, then the DNA was extracted from the gel and used as template for sequencing reaction. The results showed that these gene fragments indeed were most closely related to *xfp* gene sequences of *Bifidobacterium* species, i.e. *B. bifidum* (NG3, NG6) and *B. animalis* (NG15, NG21), respectively (100% sequence homology).

Table 3. Bifidobacterium-like isolates from infant fecal samples

No	Isolate	Sample origin	Sample storage	Isolation medium	Colony morphology	Cell morphology	pH after 48 h
Group 1: infants under 3 months							
1	NG21	G1	In anoxic medium, RT	MRS	Small, round	Irregular rods, occur in groups	4.5
2	NG25			BFM	Small, round	Short rods	4.5
Group 2: infants from 3 - 6 months							
3	NG4	B3	In anoxic medium, RT	MRS	Ellipse	Short rods	5.5
4	NG6			BFM	Ellipse	Rods, variable sizes	5.5
5	NG8	B4	In anoxic medium, RT	MRS	Star fruit	Short rods	4.5
6	NG15			BFM	Small ellipse	Irregular rods	5.5
Group 3: infants from 7 - 11 months							
7	NG3	B5	In anoxic medium, RT	MRS	Rough round to oval	Irregular rods	4.0
8	NG17	G8	4°C	MRS	Small white round	Long rods	3.5 - 4

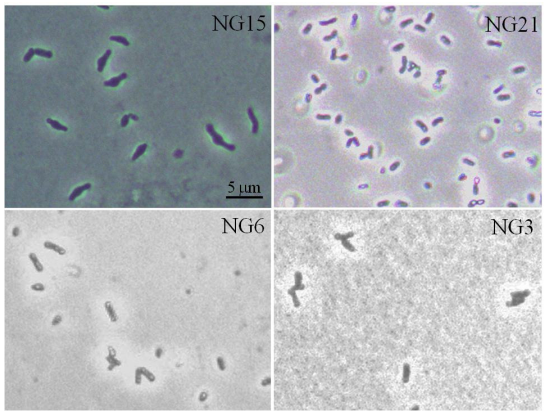

Figure 2. Cell morphology of representative isolates of the bifidobacteria-like group observed under a phase contrast microscope. Bar 5 µm, applied to all pictures.

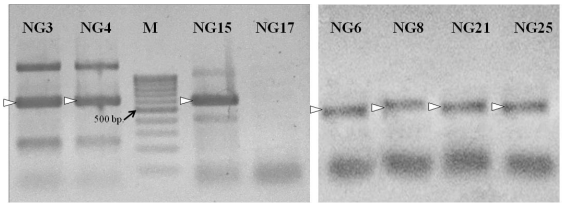

Figure 3. Electrophoretic agarose gels showing *xfp* gene fragments from 8 selected isolates obtained via PCR with the specific primer pair U1R/U2L. M - DNA marker, the marked band is 500 bp long.

3.3. Discussion

The human intestinal tract contains more than 100 trillion (10^{14}) microbial cells, phylogenetically affiliate to at least 1000 different species [14]. However, over 70–80% of the total number of gut bacterial species have not been cultivated despite of the development of culture-dependent and molecular techniques [2]. Such a large amount of gut bacteria remains uncultivated might due to (i) the high sensitivity to oxygen of most species and (ii) the existence of multiple intercellular communications in the gut microbiota [4].

We demonstrated here the development of an efficient method for isolation of bifidobacteria from infant fecal samples. Thus, instead of the double layer agar plate technique which is difficult to get anoxic outside an anaerobic chamber, the Hungate technique could be efficiently applied for the isolation of this bacterial group from human gut in laboratory. The technique has been reported in studies on enumeration of bifidobacteria from other animals [15].

Application of Hungate technique for the isolation of bifidobacteria from 14 fecal samples by using four different growth media, selective (BFM and BIM25) as well as non-selective (MRS and 385), revealed that two media MRS and BFM were more efficient than BIM25 and 385 media. In the published data, Munoa and Pares [9] proposed that BIM25 medium could serve as selective medium for isolation and enumeration of bifidobacteria from natural aquatic environments. In such habitats the bacteria could be more tolerant to oxygen than in the intestinal tract of human and warm blooded animals. This could be observed through the effect of sampling procedure on the isolation efficiency showed in this study. Of the 14 collected fecal samples, only 5 samples yielded positive isolates and four of them were transferred immediately to anoxic medium before being subjected to isolation in the laboratory (Tab. 2). Such a sampling procedure could have minimized the negative effect of oxygen to the bifidobacteria, and at the same time slightly increased number of this bacterial group, giving more appropriate conditions for the isolation process.

Comparing two media MRS and BFM, the difference in isolation efficiency could not be observed in this study, the reason might be the small number of isolates obtained. Nevertheless, while MRS is a non-selective medium, BFM is highly selective and contains a mixture of antibiotics for inhibiting other lactic acid bacteria such as lactobacilli ad streptococci [10]. It is therefore recommended to use the BFM medium for selective isolation of bifidobacteria from gut system. However, being simpler and also commercially available, MRS medium could be used for cultivation and maintenance of pure cultures after the isolation step.

Bifidobacteria are supposed to be dominant in breast fed infant gut system at early stages of

development. They are therefore expected to be isolated from the samples of the group 1 and 2 (i.e. infants under 6 months) at a higher frequency than from the last group (infants of the ages 7 – 11 months). This is assumed to be related to the changes in the feed conditions from mother's milk to other complex foods for most of infants at the ages of 6th month on ward. In this study, 6 of the 8 isolates in bifidobacteria-like group were obtained from infants under 6 months, whereas only 2 isolates came from infants of the age 7 - 11 months, one of which was identified not belonged to bifidobacteria. Although the number of isolated strains was not big, preliminary results could provide first hints for making strategies in selecting and storing samples, as well as efficient isolation technique for getting pure cultures of bifidobacteria in the laboratory.

4. Conclusion

The present study proposed an effective method for bifidobacterium isolation, the matter is still considered challenging to microbiologists. Using anoxic BFM or MRS medium in combination with Hungate technique could efficiently isolate bifidobacteria from breast-fed infant gut. Besides that, the sampling procedure, i.e. sample selection and sample storing would also have significant effects on the isolation results. It is recommended that fecal samples from infants under 6 months should be selected and stored in anoxic medium at room temperature for higher isolation efficiency.

Acknowledgements

The study was supported by project "Đánh giá nguồn gen vi khuẩn lactic bản địa định hướng ứng dụng trong thực phẩm, dược phẩm và thức ăn chăn nuôi" funded by the Ministry of Science and Technology, Vietnam.

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Phương pháp phân lập bifidobacteria hiệu quả từ đường ruột trẻ sơ sinh

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Tóm tắt: Trong nghiên cứu này, phương pháp phân lập hiệu quả đối với bifidobacteria từ đường ruột trẻ sơ sinh được xây dựng trên cơ sở áp dụng kỹ thuật Hungate cho phương pháp ống thạch bán lỏng kỵ khí thay cho phương pháp thạch đĩa hai lớp truyền thống. Bốn loại môi trường nuôi cấy là BFM, BIM25, MRS và 385 được so sánh về hiệu quả sử dụng trong phân lập bifidobacteria bằng kỹ thuật Hungate. Trong số 30 chủng phân lập từ các mẫu phân của 14 trẻ sơ sinh ở các tháng tuổi khác nhau dưới 1 năm chỉ có 8 chủng được chọn vào nhóm bifidobacteria tiềm năng dựa trên hình thái tế bào và hình thức lên men. Kết quả cho thấy kỹ thuật Hungate kết hợp với sử dụng môi trường MRS hay BFM có hiệu quả cao hơn so với hai môi trường còn lại là BIM25 và 385 trong việc phân lập bifidobacteria. Sự khác biệt giữa hiệu quả phân lập của môi trường BFM và MRS là không rõ rệt. Trên cơ sở những kết quả thu được chúng tôi khuyến cáo ưu tiên dụng môi trường BFM để phân lập bifidobacteria từ đường ruột trẻ sơ sinh, trong khi đó môi trường MRS được sử dụng để nuôi cấy và duy trì chủng thuần khiết sau bước phân lập. Ngoài ra, hiệu quả phân lập còn bị ảnh hưởng bởi tháng tuổi của trẻ cũng như quy trình bảo quản mẫu trước khi phân lập.

Từ khóa: Bifidobacteria, đường ruột trẻ sơ sinh, kỹ thuật Hungate, môi trường nuôi cấy kỵ khí.