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Chapter 6

Protocol to select bifidobacteria from fecal and environmental samples.

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Abstract

Bifidobacteria are commensal microorganisms able to colonize several ecological niches. Since their discovery, culture-dependent methods combined with the most modern Next-Generation Sequencing techniques have contributed to shed light on the ecological, functional and genomic features of bifidobacterial, electing them to microorganisms with probiotic traits. Thanks to their health-promoting effects, several members of the *Bifidobacterium* genus have been included in a variety of functional foods and drugs. In this context, the functional relevance of bifidobacteria in the gut explains ongoing efforts to isolate novel and potentially beneficial strains. For this purpose, development of effective and selective isolation protocols in concert with knowledge on the physiological characteristics of bifidobacterial are fundamental requirements for their recovery and discovery from their natural environments, in particular from fecal samples.

Key words: *Bifidobacterium*, isolation, culture media

1. Introduction

Bifidobacteria are Gram-positive commensal microorganisms belonging to the *Bifidobacterium* genus, which forms a deep-branching lineage within the Actinobacteria phylum. The first specimen of this microbial genus was isolated from a fecal sample of a breast-fed infant by Tissier in 1899. Since then bifidobacteria have been isolated from several ecological niches including the oral cavity, sewage, human blood, rumen liquid, fermented milk and water kefir, the insect gut and the mammalian gastrointestinal tract. In recent decades, particular interest has focused on the *Bifidobacterium* genus because of the probiotic features attributed to certain bifidobacterial members. Thanks to their ability to exert such asserted beneficial and health-promoting effects, which encompass stimulation of the immune system, protection against pathogens and nutrient provision through the breakdown of non-digestible carbohydrates, several bifidobacterial strains have been incorporated into a variety of functional foods and drug formulations (1, 2). Based on their importance, the scientific community has dedicated and continues to devote concerted efforts to isolate and characterize novel bifidobacterial strains. However, the effective isolation of microorganisms, including that of bifidobacteria, from their natural environment is not a straightforward procedure, since it requires a thorough knowledge of their nutritional needs and metabolic capacities coupled with the ability to *in vitro* reproduce their specific ecological niche. In this context, the introduction of culture-independent approaches based on Next Generation Sequencing (NGS) techniques associated with the more traditional culture-dependent approaches has contributed to broaden our knowledge concerning the *Bifidobacterium* genus by means of comparative and functional investigations, allowing the reformulation and improvement of culture media for specific selection of bifidobacterial species (3).

Collectively, bifidobacteria are fastidious microorganisms that are not easy to manipulate under standard laboratory conditions. Indeed, being deprived of an efficient oxygen detoxification system, they require an oxygen-free environment and the addition of reducing agents in their cultivation

medium for optimal growth. Moreover, the ability of these commensal microorganisms to degrade a wide variety of complex carbohydrates, typical of the gastrointestinal ecosystem of their host, is another key feature to consider for the formulation of selective culture media. Specifically, bifidobacteria are able to degrade diet-derived sugars, for example glucans, pectins, fructans, xylans and/or resistant starch, as well as host-derived glycans, such as human milk oligosaccharides and mucins (4). In this context, NGS-based studies have revealed that most bifidobacterial genomes contain a substantial arsenal of genes involved in the degradation and subsequent utilization of aforementioned carbohydrates. Although the ability to metabolize particular human- and/or diet-derived glycans is strain-dependent, all bifidobacterial genomes contain glycosyl hydrolase (GH)-encoding genes coupled with gene sequences responsible for the assembly of carbohydrate-specific ATP-binding cassette transporters, proton symporters, permeases and phosphoenolpyruvate-phosphotransferase systems. The cooperation of these enzymes and transporter systems allows saccharides to be shuttled into the main bifidobacterial metabolic pathway, the so-called 'bifid shunt', were simple sugars converge for energy production. At the end of this metabolic route, 2.5 Mol ATP, 1 Mol of lactate and 1.5 Mol of acetate per 1 Mol of glucose are generated. All together, these characteristics/needs should be considered for the correct formulation of (selective) cultivation media suitable for the isolation of bifidobacterial species (5, 6).

2. Requirements for the isolation of members of the *Bifidobacterium* genus

2.1. Maintenance of an anaerobic environment

With the exception of some facultative aerobic/microaerophilic species, bifidobacteria are generally strictly anaerobic, thus requiring oxygen-free conditions for growth. The maintenance of an oxygen-deprived environment is achieved by the use of special devices that allow anaerobic incubation of cultures/plates such as traditional anaerobic jars or anaerobic chambers/incubators. In case of hermetic anaerobic jars, an oxygen-free atmosphere can be obtained by inserting, in the jar itself, commercially available chemical packs containing certain reagents that are able to remove oxygen

and release carbon dioxide, thus creating an anaerobic environment suitable for bifidobacterial growth. These peculiar devices have the advantage of ensuing secure transport of a bacterial culture by avoiding or minimizing oxygen exposure. However, anaerobic jars generally offer the accommodation of just a limited number of microbial cultures/plates. For this reason, in the modern era, microbiological laboratories have replaced anaerobic jars with anaerobic cabinets that guarantee the incubation of bacterial cultures under static conditions and strictly controlled atmosphere consisting of a gas mixture of CO_2, H_2 and N_2.

Despite their anaerobic nature, unlike certain highly oxygen-intolerant, strictly anaerobic microorganisms, bifidobacteria are able to withstand limited exposure to (low levels of) oxygen, which permits their manipulation under standard atmospheric conditions, but with the foresight to take some precautionary measures to avoid injury of bifidobacterial cells. Firstly, in order to create a favourable environment for bifidobacterial growth, appropriate reducing agents are commonly added to their cultivation media. L-cysteine hydrochloride is arguably the most frequently used reducing agent for bifidobacterial culture media. Indeed, cysteine not only reduces the potential redox, but also provides better anaerobic conditions for bifidobacterial growth as well as an essential nitrogen source (7). Sodium thioglycolate, sodium pyruvate and ascorbic acid represent other widely employed reducing agents (8). Secondly, cultures and plates should be placed under anaerobic conditions immediately following inoculation and plating, respectively.

2.2 pH

Bifidobacteria are moderately acid-tolerant microorganisms. The optimum pH is between 6.5 and 7.0. However, these commensal microbes are able to grow in a pH range between 4.5 and 8.5 (9), though care should be taken not to leave overnight cultures too long in their own acidified medium as this may negatively affect viability.

2.1.3 Temperature

Bifidobacteria are mesophilic microorganisms with an optimum growth temperature between 36 and 38 °C. No growth occurs below 20 °C or above 46 °C. Growth at 45 °C seems to discriminate animal from human strains; indeed, bifidobacterial species isolated from the animal gut commonly display a slightly higher optimum growth temperature of 41-43 °C (10).

3. Cultivation media preparation for bifidobacterial isolation (see also Chapter 1 of this book for additional information on cultivation media)

3.1 Selective and counter-selective agents for the recovery of bifidobacteria

Several commercially available cultivation media are routinely employed for the isolation of bifidobacterial strains. However, most represent complex cultivation media with a chemically undefined composition and high nutrient content. For this reason, they allow growth of a wide range of bacteria that can prevent or complicate growth and subsequent isolation of bifidobacteria. In order to overcome this limitation and make a complex culture media selective for bifidobacteria, the addition of selective or counter-selective agents is recommended. Specifically, selective agents act by promoting the growth of target bacteria, while counter-selective agents operate by inhibiting the growth of competitor microorganisms. In this context, mupirocin (100 mg/L), an antibiotic against which bifidobacteria are intrinsically resistant, is the most widely used counter-selective agent (11). However, starting from complex matrices with a huge amount of different microbial genera such as fecal samples, the selectivity of mupirocin-based media is limited, since multiple anaerobic bacteria, especially clostridia, are resistant to this antibiotic. A recent study revealed that the antibiotic norfloxacin (200 mg/L) inhibits growth of clostridia, although certain bifidobacterial species are also sensitive to norfloxacin. Therefore, this counter-selective agent may actually prevent the isolation of certain bifidobacterial strains. Other studies included cycloheximide (0.01 mg/L), kanamycin sulphate (0.05 g/L) or amphotericin B (0.005 g/L) as counter-selective agents to allow bifidobacterial growth while inhibiting growth of other microbes, including moulds (12, 13). In several cases,

combination of two or more counter-selective agents allows the formulation of a suitable cultivation medium for the selective isolation of bifidobacteria (14).

Concerning selective agents, glacial acetic acid (1.0 mL/L) may be supplemented to commercial formulations in order to enhance the selectivity towards bifidobacteria, since this chemical compound inhibits growth of certain non-bifidobacterial species. For the same purpose, sodium propionate (3.0 g/L) and lithium chloride (2.0 g/L) can be added to culture media. Indeed, being able to inhibit growth of lactic acid bacteria and moulds, the above-mentioned supplements have been used for selective isolation of bifidobacteria from dairy products (14, 15).

In addition to antibiotics and mineral salts, some complex polysaccharides may also be counted among the selective agents for bifidobacterial isolation of. In this context, carbohydrate sources such as galacto-oligosaccharides (GOS), fructo-oligosaccharides (FOS), xylo-oligosaccharides (XOS) have been demonstrated to allow selective bifidobacterial growth. These selective and counter-selective agents when used on their own or in combination, and when employed with the correct cultivation media, facilitate the isolation of bifidobacteria from complex matrices, such as fecal samples.

3.2 Validated cultivation media for the isolation of bifidobacterial (see also Chapter 1)

No single standard medium for the isolation of bifidobacteria has emerged from the large number of published studies. Despite this, several cultivation media can be used for the isolation of bifidobacteria, with the addition of appropriate supplements (listed above).

3.2.1 De Man Rogosa Sharpe (MRS) medium

MRS is probably the most frequently recommended chemically undefined culture medium for the isolation of bifidobacteria. Originally, it was formulated for the isolation and growth of *Lactobacillus* species. However, the supplementation of L-cysteine to the MRS (MRSc) medium contributes to transform it into a suitable media for bifidobacterial isolation. From its formulations, several recipes of modified MRS (mMRSc) have been used for the isolation of the target microorganisms. In this

regard, several water-soluble vitamins have be supplemented to MRS, including pantothenic acid, riboflavin and thiamine as well as bovine whey-derived protein such as α-lactalbumin or β-lactoglobulin for improved bifidobacterial recovery (16). In the same way, mMRSc was formulated by substituting glucose in the original MRS formulation with an alternative carbohydrate source. Glucose can be metabolized by a very wide variety of bacteria, which then will compete with growth of bifidobacteria, thus complicating the isolation of these microorganisms. To overcome this limitation and enhance bifidobacterial proliferation, MRSc may be prepared starting from its singular ingredients excluding glucose, but including another carbon source (at a final concentration of 0.5 % or 1 % w/v) that can be degraded and utilized by (certain) bifidobacteria, based on their genetic repertoire features.

Media composition: peptone (10.0 g/L), beef extract (10.0 g/L), yeast extract (4.0 g/L), glucose (20.0 g/L), sodium acetate trihydrate (5.0 g/L), K_2HPO_4 (2.0 g/L), triammonium citrate (2.0 g/L), $MgSO_4.7H_2O$ (0.2 g/L), $MnSO_4.4H_2O$ (0.05 g/L), Tween 80 (1.0 ml/L).

3.2.2 Tryptone, Phytone and Yeast extract (TPY) medium

TPY is a commercially available culture medium that is commonly used for the isolation of novel bifidobacterial species (17). As MRS, TPY is a chemically undefined medium, thus requiring the supplementation of counter-selective agents, especially mupirocin, to selectively grow bifidobacteria and prevent the proliferation of other microbial genera.

Medium composition: Trypticase peptone (10.0 g/L), Phytone peptone (5.0 g/L), Glucose (15.0 g/L), yeast extract (2.5 g/L), cysteine (0.5 g/L), K_2HPO_4 (2.0 g/L), $MgCl_2$ (0.5 g/L), $ZnSO_4$ (0.25 g/L), $FeCl_3$ (0.002 g/L).

3.2.3 Wilchins-Chalgren (WC) medium

WC medium has been exploited for the routine growth and isolation of novel bifidobacterial species. The selectivity of WC towards bifidobacteria may be improved by the addition of certain supplements such soya peptone (5.0 g/L), L-cysteine (0.5 g/L), Tween 80 (1.0 ml/L) and glacial acetic acid (1.0

ml/L) together with mupirocin. Specifically, soya peptone, being a source of raffinose-based oligosaccharides, stimulates growth of non-glucose fermenting bacteria, thus favouring the proliferation of bifidobacteria (18). Moreover, recently, supplementation of 8-hydroxyquinoline (90.0 mg/L) to WC has provided a more selective medium in favour of bifidobacteria due to the ability of this compound to prevent clostridial growth (19).

Medium composition: casein enzymatic hydrolysate (10.0 g/L), peptic digest of animal tissue (10.0 g/L), yeast extract (5.0 g/L), glucose (1.0 g/L), sodium chloride (5.0 g/L), L-arginine (1.0 g/L), sodium pyruvate (1.0 g/L), hemin (0.005 g/L) and menadione (0.0005 g/L).

3.2.4 Transgalactosylated Oligosaccharides Propionate mupirocin (TOS-Propionate-MUP) medium

TOS-propionate-MUP combines both selective and counter-selective agents for bifidobacterial isolation. Indeed, it contains the antibiotic mupirocin as well as the transgalactosylated oligosaccharides (TOS), complex carbohydrates obtained by the β-galactosidase enzyme-based transformation of lactose, which bifidobacteria are able to degrade and utilize as a sole energy and carbon source. In 2010, the International Organization for Standardization elected the TOS-propionate-MUP as the more suitable culture medium for the enumeration of bifidobacteria in fermented milk (20). More recently, a variant of the TOS medium has been proposed by replacing TOS with raffinose (5.0 g/L) (21).

TOS-propionate composition: peptone from casein (10.0 g/L), yeast extract (1.0 g/L), KH_2PO_4 (3.0 g/L), K_2HPO_4 (4.8 g/L), ammonium sulphate (3.0 g/L), $MgSO_4.7H_2O$ (0.2 g/L), L-cysteine (0.5 g/L), sodium propionate (15.0 g/L), (trans-)galactooligosaccharides (TOS) (10.0 g/L).

3.2.5 Other complex generic cultivation media

Other commercially available cultivation media have been employed for the isolation and growth of bifidobacterial, being based on several complex components: Reinforced Clostridial Medium (RCM), Brain Heart Infusion (BHI), Gifu Anaerobic Medium (GAM) and Columbia agar base medium (CAB) (7, 22). Addition of selective or counter-selective agents has enhanced the selectivity of these culture

media towards bifidobacteria. Specifically, supplementation of L-cysteine is required to lower the redox potential of these culture media and favouring growth of bifidobacterial species.

3.2.6 Chemically defined medium (CDM)

Recently, NGS technologies have favoured the development of new protocols for the isolation of bacteria. Notably, whole metagenome shotgun (WMGS) sequencing provides insights into the genetic composition of microorganisms that form a complex microbial consortium. Specifically, WMGS methodology allows the reconstruction of genomes of as yet uncultured bacteria, including novel species, and to predict their metabolic properties. Once the associated metabolic activities of a novel hypothetical species of interest are revealed, its isolation may be facilitated by employing specific carbohydrates/substrates for the formulation of the cultivation medium. In this context, a chemically defined medium has been used for the isolation of novel gut bifidobacterial species based on their metabolic features (3). CDM was supplemented with mupirocin, L-cysteine and a specific carbohydrate.

CDM composition: sodium acetate (4.0 g/L), tri-ammonium citrate (1.0 g/L), KH_2PO_4 (2.0 g/L), K_2HPO_4 (2.0 g/L), $MgSO_4$ $7H_2O$ (0.5 g/L), $MnSO_4.H_2O$ (0.05 g/L), $FeSO_4$ $7H_2O$ (0.02 g/L), $CaCl_2$ (0.2 g/L), adenine (20 mg/L), xanthine (40 mg/L), cysteine (0.4 g/L), aspartic acid (0.3 g/L), glutamic acid (0.3 g/L), orotic acid (0.5 g/L), *p*-aminobenzoic acid (0.5 mg/L), folic acid (0.5 mg/L), nicotinic acid (2.0 mg/L), calcium-pantothenate (2.0 mg/L), biotin (1.0 mg/L), pyridoxal (2.0 mg/L), riboflavin (2.0 mg/L), vitamin B_{12} (1.0 mg/L) and 0.2 g/L of the following amino acids: alanine, arginine, glycine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, proline, serine, threonine, tryptophan, tyrosine, and valine. This medium must be sterilized by filtration (filter pore size 0.20 μm).

3.2.7 Notes for the preparation of culture media

- Culture media are generally sterilized by autoclaving (121 °C for 15 min). If the medium contains heat-sensitive components that cannot be added after sterilization by autoclaving, the medium should be sterilized by filtration (filter pore size 0.20 μm).

- Antibiotics should be resuspended in the appropriate solvent prior to their addition to the culture medium.
- Notably, antibiotics and vitamins are thermo-sensitive. For this reason, the stock solutions of these supplements should be filter-sterilized and added to the medium only when the latter has reached a temperature between 50-55 °C.
- None of the abovementioned media allows exclusive growth of bifidobacteria, despite the addition of selective and counter-selective agents. For this reason, isolated strains should be subjected to identification by 16S rRNA gene or ITS sequencing.
- As indicated in the previous paragraph, there is no universal, highly selective medium for the isolation of bifidobacteria. Moreover, bifidobacterial species possess different metabolic requirements. For these reasons, the use of different cultivation media offers the possibility to isolate different bifidobacterial taxa from the same sample.
- In order to obtain solid culture media, bacteriological agar (1.5%) should be added prior to autoclaving.

4. Protocol for isolation of bifidobacterial from fecal or other environmental samples

The first step to ensure successful isolation of bifidobacteria is to properly store and treat fecal or environmental samples immediately after collection. In order to avoid bifidobacterial exposure to oxygen, samples should be placed under anaerobic conditions and ideally be processed as soon as possible following collection. For this purpose, collected samples should be suspended immediately in a pre-reduced saline solution or peptone water and shipped to the laboratory under frozen conditions in an anaerobic jar. Promptly upon receipt, one gram of sample should be mixed with nine ml of an isotonic pre-reduced saline solution and serially diluted. Serial dilutions should be spread onto plates and covered by the selected agar-based medium. The number of plated dilutions depends on the complexity of the sample; the higher the bacterial concentration of a sample is, the more it may have to be diluted to avoid confluent growth of the colonies. After medium solidification, plates should be incubated under anaerobic conditions for at least 48h at 37°C. Morphologically distinct

colonies should be randomly picked and re-streaked to isolate purified bacterial strains. Once the isolated strains are obtained, the DNA should be extracted and subjected to partial 16S rRNA gene-based sequencing to identify the isolated species.

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Figure legend

Figure 1. Schematic procedure for the isolation of bifidobacterial species. Panel a displays the classical method for the recovery of bifidobacteria, while panel b highlights the protocol for the isolation of bifidobacterial species through a custom-made culture medium based on WMGS data.