

Research Article

Authenticity of Commercial Probiotics and Taxonomic Strategies of *Bifidobacteria* spp

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The article focuses on two major probiotic-related issues, the first one being the quality control and authenticity of commercially available probiotic products, the second one is various taxonomic strategies to be followed for *Bifidobacteria* spp. *Bifidobacteria*, lactic acid bacteria, and a few *Enterococcal* spp are probiotic microbes. The isolation and identification of probiotic microbes, other than *Bifidobacterium*, are simple and straightforward. PCR, reverse transcription-PCR, and many more techniques have been developed, and are in extensive use. However, they are irreproducible, laborious, and unreliable for *Bifidobacterium*. Almost all the problems involve the intrinsic nature of anaerobic or microaerophilic bacteria. Therefore, researchers have concentrated on developing protein-based assays for the simple and rapid identification of members of *Bifidobacterium*. In this review, I discuss the different methods available for the isolation and identification of this genus. The emphasis is on the F6PPK assay because it is unique to *Bifidobacterium* spp.

Keywords: *Bifidobacteria*; F6PPK, Probiotics

1. Probiotics

Probiotics are microscopic organisms, either bacteria or yeasts that are found in the human gut and gastrointestinal system. Therefore, they are domestic in nature, omnipresent, symbiotic, and are called “beneficial bacteria” or “friendly” because their major role is in the prevention of illness [1]. The term ‘probiotic’ derives from the Greek ‘pro’ (‘for’) and Greek ‘bios’ (‘life’) [2]. The World Health Organization (WHO) defines probiotics as “live microorganisms which, upon their consumption, induce good health”. According to the reports, the most extensively used probiotic microbes are in the genera *Lactobacillus*, *Bifidobacterium*, *Enterococcus*, and *Saccharomyces*. Most probiotic microbes can resist bile, low pH, alkaline environments, and gastric juices. In the gut, the adherence of probiotic bacteria to host cells allows them to replace pathogenic bacteria. They play a significant role in the maintenance of good health.

A few probiotic organisms were found in fermented foods, and some are even found in the milk that is transferred from mothers to offspring. The progeny thus inherit useful bacteria from their mothers, which prevent pathogenic infection. Therefore, cesarean-born children do not acquire useful bacteria, so they are susceptible to infection by pathogens. Although many useful bacteria are present in the human gut, the content of *Bifidobacterium* is highest in infants and decreases as an individual grows to adulthood. This may be attributable to continually changing food habits, or to the consumption of antibiotics and drugs that are harmful to the gut micro biome.

Recent reports have even indicated that the content of *Bifidobacterium* is almost negligible in diabetic patients, predominantly because the gut pH is altered by the consumption of metformin. It has been reported that members of *Bifidobacterium* produce vitamins, postbiotics, and bioactive molecules. However, although they confer good health, there has been very little research into the nature of bifidobacteria. Unusually, the isolation, identification, and growth of bifidobacteria are difficult, and expensive. Therefore, it is essential to review the methods available and the very recent reports in this field. In this review, we discuss the various methods used for the isolation, identification, and growth of *Bifidobacterium*.

2. History

Probiotics have come into the limelight and drawn the attention of modern science quite recently, although they have been recognized since the Roman and Greek eras. The Roman naturalist Pliny recommended the consumption of fermented milk for intestinal problems. Therefore, the roles of these bacteria as probiotics have been known for centuries, and many traditional products are still in use.

In 1899, Henry Tissier, a French researcher, first identified and reported ‘Y-shaped’ bacteria in the breastfed infants gut. Élie Metchnikoff, a Russian scientist, suggested that the gut micro biota could be manipulated by exchanging beneficial microbes for pathogenic ones. Subsequently, he validated the phenomenon by consuming fermented sour milk, which he included in his diet, hypothesizing that it would extend his life. The bacterium, which he called ‘Bulgarian bacillus’, now ‘*Lactobacillus delbrueckii* subsp. *bulgaricus*’, protects against proteolytic bacteria and maintains the pH of the stomach. He received the Nobel Prize in 1908 [3].

In 1917, the German scientist Alfred Nissle isolated *Escherichia coli* from the feces of a World War I soldier. The soldier was infected with *Shigella*, to which he was resistant, and did not develop diarrhea during an outbreak of shigellosis. The new bacterial strain, named ‘*Escherichia coli* Nissle 1917’ was subsequently used to treat intestinal diseases, and this probiotic is still in use today. In 1920, the development of probiotics encountered a major setback in experiments performed by Leo F. Rettger. He showed that Metchnikoff’s *L. bulgaricus* is highly susceptible to stomach acids, and therefore cannot survive in the intestine. Later it was demonstrated that the bacterium naturally exists in our gut, where it acts as a probiotic and confers many health benefits when reintroduced. Another such bacterium is *L. acidophilus*, which has been shown to be effective against constipation [4].

3. In popular culture

Today, probiotics have a wide array of applications and benefits, attracting the attention of the scientific community throughout the world. In 2001, the WHO Food and Agriculture Organization (FAO) defined probiotics as “live micro-organisms which, when administered in adequate amounts, confer a health benefit on the host”. However, the European Food Safety Authority (EFSA) does not accept the WHO definition because such health claims are not measurable. A group of probiotic researchers in Europe assembled in London in 2013 to discuss the scope and development of probiotics since 2001. The conclusions of that meeting were published in June 2014 [5] [6].

4. Array of applications

Probiotics are widely used to enhance animal immunity and digestion, and to maintain gut homeostasis. Probiotics are effective in treating diarrhea, urological infections, and inflammatory bowel syndrome (IBS). The control of diseases such as IBS and eczema, lactose intolerance, weight gain, high cholesterol, *Helicobacter pylori* infection, necrotizing enterocolitis, and vitamin deficiency [7] [8] are other benefits of probiotics.

5. Commercial products

Yakult (Yakult Danone India PVT LTD) is the first commercially available dairy-based probiotic, marketed by Nestle in 1935, and New Belly is the latest. Many dairy-based probiotics have entered the market, non-dairy and un-fermented probiotics produced recently, including breakfast cereals, snack bars, etc. Kefir, yogurt, kombucha, kimchi, and sauerkraut are among other countless commercially available probiotics on the market. Probiotics are even available for animals, including pets, and have become a multi-million-dollar industry [9]. Probiotics have become prominent in the field of research, especially in health care they have tested for their contribution to the field of immunology. Probiotic technology has created a niche for itself in the fields of science and technology.

6. Common problems and ambiguities in commercial probiotics

Poor standards and lack of quality control criteria for many probiotic supplements have recently become a public concern. Low bacterial contents and a lack of specific bacteria have been reported in some products, as well as

contamination and misleading claims pertaining to the number of viable bacteria and the identities of the probiotic strains in products in the USA [10-16]. The concept of probiotics is very old in India. Most normally used “mother’s recipes”, such as homemade curd, which is routinely consumed, are probiotic products that mainly contain *Lactobacillus*. The word “probiotics” has just arrived in India, like old wine in a new bottle. Many multinational companies are investing in and formulating probiotic products in India, including Swiss Garnier Life Sciences, Aristo Pharma, Wallace Pharma, and many more. Probiotics may be bacteria, molds, or yeast, but most of them are bacteria, and the most prominent and accepted probiotics are lactic acid bacteria. *Lactobacillus casei*, *L. lactis*, *L. helveticus*, *L. salivarius*, *L. bulgaricus*, *L. johnsonii*, *B. bifidum*, *B. brevis*, *B. longum*, and some *Saccharomyces* strains. The latest additions to the list of commercial probiotics in India are Biozora, Bioclin, Probiza, EuBioz, Darolac, Eugi, and Bifilin, and most probiotic supplements contain *L. acidophilus*, *L. rhamnosus*, *B. longum*, *B. bifidum*, *S. Boulardi*, *Streptococcus thermophilus*, etc. However, it has become very difficult to obtain a reliable, effective, and health-benefiting probiotic supplement. The probiotic organism must be nonpathogenic, nontoxic, and gastric-acid resistant, and must be able to produce postbiotics. The basic elements of probiotics and their authenticity as efficient probiotic supplements must be analyzed, and interpreted.

In this study, we assayed 9–10 probiotic supplements purchased from drug stores and retail pharmaceutical outlets in India. Only two products were acceptable, with 30 % viable counts (relative to the total count claimed). They contained only the species stated, but three of the products contained < 10 % – 20 % of the claimed count. Of the commercial probiotic products tested, only four products were grossly deficient in one or more attributes; the remaining had acceptable viable counts, albeit < 0.01 % of the counts claimed. A few commercial probiotic products claimed to contain milligrams of colony-forming units rather than the number of live bacteria, making it impossible to verify the claimed viable count. In some products, no *Lactobacillus* or bifidobacterial strains were found. Some probiotic supplements contained *L. acidophilus* instead of *L. rhamnosus*; some had a larger number of *E. faecium* (not stated on the label). No probiotic supplement contained all the claimed species. These findings, together with published examples [16, 17] [3, 15], indicate that > 70 % of Indian products are below standard and do not meet international standards.

Three major concerns arise from these facts. First, and foremost the high temperatures in India facilitate rapid, frequent, and easy contamination. Therefore, contamination and the presence of potentially pathogenic species are principal concerns. The most frequent and potentially pathogenic contaminants observed in probiotic supplements are *E. faecium* [9] and *E. paediococci* [3], which are vancomycin-resistant.

Moreover, several probiotic strains are known to acquire virulence factors from wild-type strains (Lund and Edland 2001). Second, serious problems have been reported with the quality control and labeling of probiotic products, not only in India but in many developed countries, reducing the efficacy of probiotics [3]. It is difficult to rectify microbiological shortcomings involving contamination, long-term preservation, processing, and so on. The problem of contamination in India is mainly attributable to the engagement of under qualified youth as cheap labor in the industry. Another reason may be the lack of hygiene and clean manufacturing facilities in the industries concerned

and a lack of safe laboratory practices. Yet, another major and very common problem is the deterioration of raw material during both processing and distribution, largely because of the very high temperatures ambient during the tableting or encapsulation stage or improper storage in retail outlets. Quality control in the final stages of product sale could resolve all these problems, rendering these probiotics safe for consumption.

India lacks strict regulatory bodies to ensure the quality of probiotic supplements that is standard in the USA and Europe. Therefore, it may be important for the federal government to continuously monitor stringent quality control rules and regulations. According to the report, commercial probiotic products carry bifidobacteria as the major component. Most claims pertaining to the numbers and viability of these bacteria and their beneficial effects after consumption are questionable. Therefore, it is essential to understand the isolation, identification, and growth parameters of these bacteria. The main aim of the present review is to clarify the bifidobacterial system as a whole, and on the contrary, its taxonomy.

7. Bifidobacteria as a probiotic

Probiotics have been a fascinating subject since 1900. Henry Tissier first observed and documented that children with diarrhea had low bifidobacterial (bifid) counts in the gut. On the contrary, these bacteria are abundant in healthy children [10]. Members of the genus *Bifidobacterium* are Gram-positive, and most species are probiotic, so their presence in the animal system is safe and beneficial to the organism. They are non-spore-forming, non-motile, branched (usually 'Y-shaped') anaerobic bacteria that inhabit the mouth, guts, and vaginas of animals, including humans [11]. They form a major microflora in the colon. As probiotics, they are a wide variety of uses.

8. Uses of bifidobacteria

The beneficial effects and safe uses of members of *Bifidobacterium* are evident in the historical consumption of fermented milk. It has been reported, that lactic-acid-producing bacteria in foods are commensal microorganisms that are safely consumed. A recent report on the safety of *Bifidobacterium* excluded it as a potential pathogen and concluded that bifidobacteria do not pose a health risk [12], but are beneficial.

Probiotics are orally consumed for various reasons, including the treatment of IBS. They are the best supplements for the treatment of traveler's diarrhea and replacing the pathogens in the gut. Bifidobacteria are consumed as a probiotic because their metabolism produces lactic acid and vitamins. By lowering the pH of the gut, they indirectly inhibit the growth of Gram-negative bacteria, maintaining the health of the microflora. The probiotic effects of the bifidobacteria are also attributable to their adherence to the intestinal epithelial cells. This property also fits them for a wide range of applications in medical science. For instance, genetically modified bifidobacteria are used to suppress tumor cells in the treatment of cancer, extending the options for the treatment of cancer and circumventing the painful, expensive, and time-consuming procedures currently in use.

9. Distribution of bifidobacteria in the gut

To understand the distribution of *Bifidobacterium spp* in the gut, viability tests and 16S rRNA PCR analyses have been used. DNA was purified from fecal samples obtained from different sources. The subsequent PCR analysis

concluded that *B. catenulatum* was the most commonly found taxon, followed by *B. longum* and *B. adolescentis*. Other *Bifidobacterium* species, such as *B. breve*, *B. infantis*, and *B. longum* were found in both infant and adult guts [13].

10. Recent studies of *Bifidobacterium*

It has been reported that probiotics are beneficial, and are very exciting and preferred research nowadays. Our growing knowledge of the genus *Bifidobacterium* and its genome clarifies how these species work and the ways they can be manipulated to extend their benefits. *Bifidobacteria* utilize a broad range of carbohydrates, including plant-derived oligosaccharides and polysaccharides that escape digestion in the upper parts of the alimentary tract. *Bifidobacterial* gene mapping and genomic analyses have shown that they have adapted to the carbohydrate-rich gastrointestinal tract and they encode a larger number of carbohydrate-metabolizing enzymes.

The metabolic pathways of carbohydrate metabolism differ in each member of the *Bifidobacterium*, so different *bifidobacterial* strains may have different carbohydrate-utilizing abilities [13]. The influence of certain indigestible carbohydrates (prebiotics) on the growth and metabolic activities of *bifidobacteria* varies with the transcriptional regulation of these bacterial strains [14]. It has been reported that these bacteria survive the acidic conditions and bile secretion in the stomach. To consider *Bifidobacterium* a probiotic, it must survive harsh intestinal conditions. Survival is not enough; it must also multiply and colonize the alimentary tract. Uraipan and Hong Pattarakere (2015) [15] showed that *Bifidobacterium* not only survives and multiplies but acts antagonistically against food-borne pathogenic bacteria.

11. *Bifidobacteria* as therapeutic agents

The *bifidobacteria* in the gut has been associated with the regulation of intestinal inflammation. *Bifidobacterium infantis* grown on human milk oligosaccharides showed significant adherence to the Caco-2 cell line. These experiments suggested that the expression of inflammation-related genes [15] was down regulated in the presence of *B. infantis*.

12. *Bifidobacterial* identification methods

The earliest method by which *Bifidobacterium* was differentiated from morphologically similar bacteria was PCR, with genus-specific primers or oligonucleotide probes [18]. The techniques applied to the detection and identification of *Bifidobacterium* include modern molecular methods such as amplified ribosomal DNA restriction analysis (ARDRA), pulsed-field gel electrophoresis (PFGE), randomly amplified polymorphic DNA (RAPD), and other ribotyping and community profiling techniques, such as PCR coupled to temperature gradient gel electrophoresis (TGGE) and denaturing gradient gel electrophoresis (DGGE)-PCR [19]. A 16S-rRNA-targeted species-specific PCR was developed to confirm the distribution of *Bifidobacterium spp* in the human gut. Various PCR primers were constructed to find various *Bifidobacterium spp*, which have been isolated from the human gut. This has emerged as an effective method of analyzing the *Bifidobacterium spp* that inhabit the gut [20].

Another method of identifying *Bifidobacterium* is with a hybridization probe. A *Bifidobacterium*-genus-specific primer that binds a variable region of 16S rRNA (V9) has been extensively studied and used to construct a hybridization probe. The probe is designated '1m3' (sequence 5¹-CGGGTGCTICCCACTTTCATG-3¹) and is used to identify all known bifidobacterial types. It was used to differentiate them from other bacteria [21]. Two different tools as Genus-specific PCR and DGGE were used extensively to determine bifidobacterial diversity of human feces. A PCR was performed with *Bifidobacterium* 16S rDNA as the template and genus-specific primers. The resulting 520-bp DNA fragment was separated in a sequence-specific fashion with DGGE [22].

To identify strains isolated from human samples, a multiplex PCR based on three clusters of species was developed. This is a convenient method, and the number of PCR cycles and the time required are drastically reduced. After specific extraction techniques, it was directly applied to DNA isolated from swabs or stool samples, without prior bacterial culture. Therefore, it is useful in differentiating human bifidobacterial isolates from related species [23]. The forward and reverse primers for the bifidobacterial 16S rRNA genes were constructed for the specific detection of *B. lactis*. The specificity of this technique was subsequently, verified with various DNA samples that were isolated from single and mixed *Bifidobacterium* and *Lactobacillus* samples. A multiplex PCR was developed with genus-specific primers that bind a conserved bacterial 16S rDNA sequence, generating reproducible results, and this may be the best approach ever established [24].

A new technique was developed for the species-level identification of *Bifidobacterium*. A PCR with two *Bifidobacterium*-specific primers directed against the 16S ribosomal genes (Bif164 and Bif662) generated a PCR product from twelve *Bifidobacterium* type strains that were isolated from the human alimentary canal. The PCR products were then purified digested with five different restriction enzymes. The resulting restriction fragments were used as fingerprints for the identification of various *Bifidobacterium spp.* This method was used to differentiate *Bifidobacterium spp.* that originated from natural and artificial sources [25].

These modern methods have been adapted for the detection, identification, and enumeration of *Bifidobacterium spp.* with PCR. Later, the *trans*-aldolase gene was used to identify bifidobacteria from various sources. *Bifidobacterium spp.* isolated from the feces of human adults and babies were identified with the PCR amplification of 301-bp *trans*-aldolase gene fragments and a comparison of their relative migration on DGGE [26]. A PFGE-based method of bifidobacterial detection was developed in which the extracted genomic DNAs of commercially important *Bifidobacterium spp.* were subjected to *Xba*I and *Spe*I digestion, which generated several different genomic DNA fingerprints that distinguished bifidobacterial spp [27].

The patterns of the restriction fragment bands play a crucial role in this method. Each bifidobacterial DNA generates a unique digestion pattern, so this method is reliable. In another development, 5' Nuclease assays were developed using the 16S–23S rRNA gene intergenic sequence instead of the 16S rRNA gene sequence. This method is used for phylogenetic analysis and the specific detection of bacteria. However, it is difficult to develop highly specific PCR primers and probes for different *Bifidobacterium spp.* because of the strong sequence similarities among the

bifidobacterial 16S rRNAs. The intergenic spacer of the 16S–23S rRNA genes was subsequently used for a more detailed analysis of *Bifidobacterium* spp.

These sequences are less conserved than the 16S rDNA sequences, with more variations [28]. This intergenic region is frequently used for bifidobacterial identification. The RAPD technique is a rapid and reliable method for the characterization of bifidobacteria, and allows the rapid identification of isolates from commercial dairy products [29]. There are four main types of culture medium for *Bifidobacterium*: basal, elective, differential, and selective media. Neomycin–paramomycin–nalidixic acid–lithium chloride (NPNL) medium is comprised of glucose-containing blood–liver agar together with neomycin sulfate, nalidixic acid, paramomycin sulfate, and lithium chloride. This is a universal reference medium for the isolation of *Bifidobacterium* from fermented dairy products. Other recommended media for the selective enumeration of *Bifidobacterium* include Columbia agar with propionic acid (5.0 mL) and dicloxacillin (2.0 mg/L) as additives; and de Man, Rogosa, and Sharpe (MRS) medium supplemented with neomycin, paramomycin, nalidixic acid, and lithium chloride 278 [30].

13. Fructose-6-phosphate phosphoketolase (F6PPK)/xylulose 5-phosphate (XFP)

F6ppk assay is the standard method followed for the identification of *Bifidobacteria*, which is extremely important in distinguishing colonies of these bacteria from those of other genera [31]. The unique mechanism by which bifidobacteria degrade hexose sugars via the bifid shunt, with the help of the F6PPK enzyme, is a crucial marker for the taxonomic identification of the family Bifidobacteriaceae [32]. Species-specific oligonucleotide probes directed against f6ppk were used to rapidly identify *Bifidobacterium* spp. were not successful [33, 34]. Meile et al. reported that a degenerate oligonucleotide probe designed to detect the common N-terminal sequence detected the gene encoding F6PPK on the chromosome of *B. lactis* [35]. The conventional method, used since 1969, is based on the spectrophotometric measurement of the reddish violet color developed by the reagents added to disrupted bifidobacterial cells. The protocol requires the following reagents: 0.05 M phosphate-buffered saline containing 500 mg/L cysteine (solution 1); 6.0 mg/mL NaF, 10 mg/mL Na-iodoacetate, and 80 mg/L fructose-6-phosphate in distilled water (solution 2); 13.9 g hydroxylamine HCl/100 mL of water (solution 3); 15 % trichloroacetic acid (w/v) in water (solution 4); 4.0 M HCl and 0.5 % FeCl₃· 6H₂O in 0.1 M HCl (solution 5).

14. Conventional method in brief

An aerobically grown culture was harvested washed repeatedly with solution 1 and re-suspended in the same buffer. The cells lysed by sonication, mixed with 0.25 mL of solution 2, and incubated at 37 °C for 30 min. The reaction terminated by the addition of solution 3. After incubation for 10 min at room temperature, 1.0 mL of solutions 4.0 and 5.0 added, and the color developer solution is finally added. The reddish-violet color intensity is measured spectroscopically at 435 nm, which indicates a positive result. Orban et al. comparatively analyzed the conventional protocol and a modified protocol in which Triton X-100 with sonication or CTAB without sonication is used to lyse the bacterial cells directly. The method to disrupt the cells based on CTAB was shown to be the best for the F6PPK analysis [31, 36]. An assay using membrane vesicles or cell-free extracts of *Bifidobacterium* rather than whole-cell extracts showed that the F6PPK enzyme is situated on the bacterial cell surface. This study may stimulate further

research in the field [37]. Since 2001, no further nucleotide probe method for the detection of *Bifidobacterium* has been developed and although all these methods are still used, only the modified conventional bioassay has received attention for a long time. Bifidobacterial taxonomic issues All prokaryotes are usually subjected to 16S rRNA sequencing to determine their exact taxonomic positions. Sequence similarity of $\geq 98\%$ directs its specific taxonomic orientation.

This is not always possible for many reasons, such as physical parameters or ambiguities in the generated DNA sequences. Importantly, the bifidobacterial genome is GC-rich, so the amplification of certain genes with PCR is difficult. Therefore, new methods that are specific for these bacteria must be developed for their identification. Fortunately, the bifidobacterial system has a unique gene/protein, known as XFP/F6PPK. Several specific tests are available for bifidobacterial identification based on this enzyme, both PCR-based and biochemical assay. The PCR-based assay is straightforward, but it does not specifically identify the exact bifidobacterial species. Therefore, a very sensitive biochemical assay may be necessary. The recently reported method of Choyem et al. may be suitable for classifying bifidobacteria to the species level. It focuses on the intensity of the reddish-brown color formed in the F6PPK assay, which is spectrophotometrically measured. The intensity of the final compound depends on the affinity levels of the protein for the substrate, which results in the formation of the final product. The bifidobacterial F6PPK sequences vary between strains and between species, and *B. catanulatum* is one of the highest producers of F6PPK. Therefore, it is used as a control, against which other species that produce lower levels of the reddish-brown hydroxide can be compared. Although this process is easily executed in the laboratory, it cannot be used for classification under field conditions. Therefore, the methods developed are still not easily applicable, and the classification of *Bifidobacterium* is in warrants further investigation. Acknowledgements We acknowledge and appreciate the facilities provided by CSIR-CFTRI, and acknowledge CSIR-UGC for AC and TWAS fellowship for ZEBA. KR acknowledges the DST-SERB for funding.

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