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Research Article

Biochemical Identification and Qualitative determination of *Bifidobacteria* directly on Petri plate

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Abstract:

The amplification of the xylulose-5-phosphate/fructose-6-phosphate phosphoketolase (*xfp*) gene and a biochemical assay to detect fructose-6-phosphate phosphoketolase (F6PPK) have been extensively used to identify *Bifidobacterium* spp. In conventional assays, after isolation and harvesting of the stationary phase, cultures were treated with different reagents sequentially, finally leading to the formation of ferric hydroxamate (reddish-violet color) which was measured spectrophotometrically. The existing methods (F6PPK based) for identifying *Bifidobacterium* spp. were developed based on the availability of pure bacterial cultures and were not suitable for mass screening. Therefore, the simultaneous isolation and identification of *Bifidobacterium* spp. directly from agar plates is needed. Here, we have developed an on-plate method for the rapid, simultaneous isolation and identification of *Bifidobacterium* spp. from a variety of cultures, in which the isolation, harvesting and washing of pure cultures have been bypassed. Assay begins with making test plate and a master plate. This process assists in obtaining the F6PPK positive, viable colonies from the master plate. The effects of the addition of minimal concentrations of cetyltrimethylammonium bromide (0.20–0.45 mg/mL) on cell lysis, and the subsequent color development in the plate assays, were assessed. Mixed cultures of E. coli. Lactobacillus, and *Bifidobacterium* spp. were used. No phosphoketolase activity was observed when the plates contained *E. coli, and Lactobacillus* spp, and activity were observed only with disrupted *Bifidobacterium* spp. The on-plate F6PPK assay developed here was an efficient alternative to the currently available (F6PPK based) methods that was only used after the isolation of pure *Bifidobacterium* spp. The detection of target bacteria using this on-plate F6PPK assay will aid future studies looking at the composition and dynamics of intestinal microbiota.

Keywords: Bifidobacterium, cetyltrimethylammonium bromide, fructose-6-phosphate phosphoketolase, 16S rRNA

Introduction

The genus *Bifidobacterium* differs from other bacteria, such as lactobacilli, *Corynebacterium*, and actinomycetes, because of its unique metabolic pathway, the bifid shunt, in which the key enzyme is fructose-6-phosphate phosphoketolase (F6PPK). Scardovi [1] (1986) was the first to report that F6PPK can be used to identify Bifidobacteria and was the first to introduce F6PPK for identification purposes. Since then, Biblioni et al. [2] (2000) used an enzymatic colorimetric assay, and Orban and Patterson (2000) [3], used an exclusive F6PPK based assay to identify Bifidobacteria. There are few reports of media based selective isolation of non-fermenting Bifidobacteria [4, 5]. The first enzyme-based method for the identification of Bifidobacterial was reported by Vlkova et al. (2005) [6]. Bunisova (2014) [7] exploited the xylulose-5-phosphate (xfp) gene, 16S rRNA, HSP 60 and Fus (elongation factor EF-G) for the identification of Bifidobacteria. A genome-based identification approach using multiplex PCR was reported and was considered to be one of the most advanced techniques developed for Bifidobacterial identification [8]. However, in the majority of cases, the measurement of the 16s rRNA gene and F6PPK protein levels for identification and characterization of Bifidobacteria is beyond the technical capacity of small-scale industries or clinics. In the past, it was advised that isolates were sent to appropriate national repositories or reference centers, such as the Microbial Type Culture Collection (MTCC, Chandigarh, India), or the German Collection of Microorganisms and Cell Culture (DSMZ, Brunswick, Germany). However, this process is not cost-effective, and is laborious, time-consuming, and runs the risk of contamination. Although microbial identification is a normal process in microbiology laboratories, the specific isolation and identification of Bifidobacteria is costlier as one needs anaerobic conditions to maintain their survival. The best way of overcoming these constraints is to produce a simple, single-plate method that allows the simultaneous isolation and identification of individual species from mixed cultures.

Current methods for the identification of *Bifidobacteria* begins with the isolation of the target organism, and its identification takes place in a second step. The most accurate bioassay for identifying *Bifidobacteria* is the F6PPK assay [9, 10]. During conventional *Bifidobacterium* identification, isolates are lysed by sonication or by cetyltrimethylammonium bromide (CTAB) treatment to extract the cellular factors (F6PPK as a major component) that are used in the assay. In the next step, the cellular factors are identified to determine whether the organism belongs to the genus *Bifidobacterium*. Currently, there are four different methods to do this such as the F6PPK-test, the F6PPK-test with Triton X-100, the F6PPK-test with CTAB (F6PPK-CTAB-test), and PCR using genus specific primers for the specific detection of *Bifidobacteria*. All of the above are based on small modifications of the F6PPK method developed by Orban and Pattersons (2000) [3]. In brief, in one method, the F6PPK test was modified by the introduction of Triton X-100. The other method used the addition of CTAB. A final method used genus-specific primers to amplify the *xfp* gene that encodes F6PPK. All the above methods are reported to be reliable, although they are time-consuming, require skilled manpower to execute and are costly [11, 12, 13].

The original Orban and Pattersons (2000) F6PPK assay has few disadvantages, but is not designed to process large numbers of samples and requires the isolation of 5–10 mL of pure culture. In brief, their method involves growing, harvesting and washing the cells with phosphate buffer (0.05 M phosphate buffer, pH 6.5, plus cysteine, 500 mg/L) and then incubation in buffer, followed by sonication to obtain the lysate. The lysate is mixed with sodium fluoride (NaF), Na Iodoacetate, and fructose-6-phosphate sequentially. It is then incubated at 37°C for 30 min, followed by the addition of hydroxylamine HCl and incubated for 10 min at room temperature to stop the reaction. After this, trichloroacetic acid (TCA) and HCl is added and finally, $FeCl_3$ is added for color development. The development of a reddish-violet color indicates the presence of F6PPK and a yellow color shows a negative result.

All the same, the primary concern associated with this assay is its non-specificity, because all *Bifidobacteria* does not make sufficient amounts of F6PPK. This is the primary disadvantage as the lowest F6PPK producers may not be identified. Most importantly, a test should be easy to conduct, and it should be able to be performed in the field. The assay should be able to bypass the use of isolated cultures, cell disruption, centrifugation, and large amounts of costly reagents, such as fructose-6-phosphate. The present method is miniaturized and feasible in the field, hence, the quantity of reagents required are minimal. Therefore, we have developed a bioassay system that is efficient, simple, rapid, and reliable for the simultaneous isolation and identification of *Bifidobacterium* spp.

Materials and Methods

In this study, F6PPK activity was measured using the reagents and processes described by Orban and Patterson (2000), Scardovi (1965, 1969), and Scardovi (1981, 1986) [14, 15]. All reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA). Fructose-6-phosphate and TCA solutions were made 30 min before the start of the assay.

Bacterial strains and growth conditions

The bacterial cultures evaluated in this study included strains of *Bifidobacterium* spp. obtained from DSMZ, ATCC, NEB and various other sources (Table 1), as well as *Lactobacillus* spp. isolated from commercial sources, *E. coli* (laboratory strain), *Enterococcus raffinosus* strains (novel isolates from a human subject) [16], and two strains of *Bifidobacterium* that were isolated from human subjects. Both fecal (mixed as well as individual samples) and standard cultures were analyzed in the assay. The experiments were validated by combining the isolates before identifying the *Bifidobacteria*, samples from cow's milk, fecal material, poultry excreta and commercial probiotics.

Conventional phosphoketolase assay

The Orban (2000) gold standard method is briefly described below. The isolated pure *Bifidobacterial* cultures were grown in MRS broth, (Hi-Media, Mumbai, India) under anaerobic conditions at 37°C to obtain a stationary phase culture. The cells were harvested by centrifugation at 8000 RPM, at 4°C for 10-12 min (Remi centrifuge, Process plant and Machinery Ltd, Mumbai, India) and washed with phosphate buffer twice. After this, the cells were treated with CTAB (180 µg) for efficient lysis, and then incubated for 10–12 min at room temperature. The second step involved the addition of a solution containing NaF, fructose-6-phosphate and potassium acetate (750 µg, 20 µg, and 1.25 µg, respectively), and then incubated for 5–10 min. Finally, samples were treated with H_3NO: HCl, (195 mg), and incubated at room temperature for 10 min. The contents of the reaction along with the cell lysis components were precipitated by the addition of TCA (150 mg), 4 N HCl (1 mL) and for color development, ferric chloride (50 mg) was also introduced at this step. An incubation step followed for 10 min at room temperature. The appearance of a reddish-violet color indicates the presence of *Bifidobacteria*.

Table 1: Strains used in this study

Bacterial strains	Strain No.	Reference
B. animalis Subspp lactis	DSMZ 10140	Meile et al. 1997
B. thermacidophilum Subspp thermacidophilum	DSMZ 15837	Dong et al. 2000
B. adolescentis	DSMZ 20083	Reuter 1963
B. longum Subspp infantis	DSMZ 20088	Reuter 1963
B. longum Subspp longum	DSMZ 20219	Reuter 1963
B. asteroids	DSMZ 20089	Scardovi and Trovatelli 1969
B. animalis	DSMZ 20105	Mitsuoka 1969
B. breve	DSMZ 20213	Reuter 1963
B. indicum	DSMZ 20214	Scardovi and Trovatelli 1969
B. catenulatum	Fecal isolate 14-month-old baby	This study
E. coli (DH5- α)	NEB C2987 H	This study
E. raffinosus	Novel isolate	Kammara 2013
L. rhamnosus	Isolated from commercial sources	This study
L. acidophilus	ATCC 4356	Hansen and Mocquot 1970
L. shirota	Isolated from Yakult probiotic drink	This study
L. bulgaricus	ATCC 11842	Weiss et al. 1983
L. salivarius	ATCC 11741	Rogosa et al. 1974

Table 2: Comparison of F6PPK assay methods

Methods		Buffers and Media				Chemicals and Reagents			
Broth (ml)	Harvest & Wash step	CTAB (µg)	NaF (µg)	KI Ace (µg)	F6p (mg)	$H_3NO.HCl$ (Mg)	TCA (Mg)	4N HCl (µl)	$Fecl_3.6\ H_2o$ (mg)
1. Existing 10	Yes	180	750	1.25	20	195	150	1000	50
2. On plate No	No	6.75	45	0.075	1.2	1.95	2.25	15	0.75

Colony preparation

Samples of cow's milk, fecal material from breastfed infants, commercial probiotics and poultry excreta were diluted in De Man, Rogosa and Sharpe (MRS) medium (Hi-media, Mumbai, India) or *Bifidobacterial* broth (special peptone, 23 g/L; sodium chloride, 5 g/L; glucose, 5 g/L; soluble starch, 1 g/L; L-cysteine hydrochloride, 0.30 g/L), and then plated on MRS or *Bifidobacteria* agar media (termed the main plate) (MRS/Bifido media obtained from Hi-Media laboratories, Mumbai, India). Once bacteria are observed a master plate is produced and stored at 4°C. The original plate is subjected to the F6PPK assay. Then, the samples were cultured under anaerobic conditions for 3–4 d. At the same time, the plates containing the isolated colonies were subjected to an on-plate phosphoketolase assay (the present method). In brief, *Bifidobacterium* were cultured anaerobically on *Bifidobacteria* agar plates for 3–4 d to obtain single, isolated colonies. Next, 0, 5, 10, 15, 20 µL of a 450 µg/mL CTAB solution was added to the surface of the colonies to give a final CTAB concentration ranging from 2.25–9 µg (each colony was exposed to a single CTAB concentration). Then, the samples were incubated for 5 min, after which they were evaluated for the establishment of a reddish-violet color (indicative of ferric hydroxamate production used to determine the level of CTAB that would be needed for the identification of individual strains following on-plate cell disruption). At the same time, E. coli, Lactobacillus, and different *Bifidobacterium* spp. (DSMZ) isolates (Table 1) were mixed, plated, and the resulting isolated colonies were treated with CTAB to conduct the on-plate F6PPK assay (ferric hydroxamate is the F6PPK assay final product that can be quantified or used to state the presence of *Bifidobacteria*). Our developed method was further validated by subjecting different novel *Bifidobacterial* isolates, milk samples, fecal samples, commercially available probiotics and poultry excreta to the test.

Phosphoketolase assay on agar plates

After pretreatment of the cells with 9 µg of CTAB [17], 15 µL of a solution containing NaF, 3 mg/mL) and potassium or sodium iodoacetate (5 mg/mL) in H_2O was added to the surface of each colony. Then, the samples were further incubated for 10 min at 37°C. After this, 15 µL of sodium fructose-6-phosphate (80 mg/mL) was added, and the samples were incubated at 37°C for 30 min. Following the incubation, approximately 15 µL of hydroxylamine hydrochloride (13 g/100 mL) was added, and the samples were further incubated at room temperature for 10 min to stop the reaction. Next, 15 µL of each of the following solutions were added sequentially: TCA (15% w/v), 4 N HCl, and ferric chloride hexahydrate (5% w/v) in 0.1 N HCl. Then, the samples were incubated at room temperature for 10 min, and the formation of a reddish-violet color (indicative of ferric hydroxamate production) was observed and then transferred onto Whatman filter paper by either soaking the assay plate or blotting the colonies onto the paper (by placing the paper on a lysed colony). The method does not require a centrifugation step, or anaerobic chamber for the bacterial growth.

It is known that the "Bifid shunt" is a phosphoketolase reaction by which D-fructose-6-phosphate is converted to erythrose-4 -phosphate and acetyl-1-phosphate. This reaction has been utilized to test/identify *Bifidobacterium* spp. Hence, the same cultures, including positive and negative controls, were also subjected to the standard F6PPK assay. The intensity of the reddish-violet color formed by different *Bifidobacterium* strains was determined using a spectrophotometer to measure the absorbance at 505 nm. The plates with no *Bifidobacteria*, but with *Lactobacillus* plus all reagents except for F6PPK, were used as negative controls.

PCR amplification of *xfp* and 16S Rrna

Polymerase chain reaction amplification was used to validate the results obtained by the on-plate phosphoketolase assay, and is essential if the absorbance produced by the ferric hydroxamate/reddish-violet color is <0.15 (determined by using the traditional F6PPK with various bacterial to produce the reddish-violet color). In brief, PCR amplification for further validation of *Bifidobacteria* was followed by the amplification of the 16S rRNA and *xfp* genes. Colonies that showed a reddish -violet color were chosen for further validation; the colonies were inoculated into the liquid medium and grown until they reached the stationary phase. A genomic DNA isolation kit (MBI Fermentas, Burlington, ON, Canada) was used to isolate genomic DNA from the cultures as directed by the manufacturer. The PCRs were performed with an Eppendorf Master cycler gradient PCR apparatus (Eppendorf India PVT LTD, Ambattur, Chennai). The PCRs were carried out in 100 µL with 50–100 ng of genomic DNA as template, 2.5 U of recombinant *Pfu* DNA polymerase (MBI Fermentas), 1× reaction buffer, 200 µM dNTPs, and 20 picomole of forward and reverse primers $^{5'}$-CTC CTG GAA ACG GGT GG-$^{3'}$ (from the 65th nucleotide of the 16S sequence) and a reverse primer $^{5'}$-GGT GTT CTT CCC GAT ATC TAC A-$^{3'}$ (from nucleotide 613 of the 16S sequence). Similarly, conserved regions of the *xfp* gene, as well as the full-length *xfp* gene, were amplified using the same reaction parameters. The *xfp* forward and reverse primers corresponded to the conserved regions of the gene (amplified products of 500–600 bp). The primers used were, ready-made universal 16S rRNA (Eurofins Genomics Germany, GMBH, Germany) and *xfp* conserved and full-length gene forward and reverse primers. Gene amplification was performed with the 16S Bifido forward primer $^{5'}$-CTC CTG GAA ACG GGT GG-$^{3'}$ (from the 65th nucleotide number of the 16S sequences) and a reverse primer $^{5'}$-GGT GTT CTT CCC GAT ATC TAC A-$^{3'}$ (from nucleotide 613 of 16S sequence, approx. MW of the amplified product was predicted to be 1,500 bp, all the 16S rRNA primers were exclusively determined in this study.

Gene amplification was carried out under the following conditions: hot start at 95°C for 5 min, followed by 25 cycles of denaturation at 94°C for 45 s, annealing at 55°C for 1 min, and extension at 72°C for 1 min for 16S rRNA amplification; and hot start at 95°C for 5 min, followed by 25 cycles of denaturation at 94°C for 45 s, annealing at 61°C for 60 s, and extension at 72°C for 2 min, followed by a final extension of 72°C for 4 min, for amplification of the conserved regions of the *xfp* gene. To amplify the full-length, (2.5-kb) *xfp* gene, the following parameters were used: hot start at 94°C for 5 min, followed by 20 cycles of denaturation at 94°C for 45 s, annealing at 64°C for 1 min, extension at 72°C for 2.5 min, followed by a final extension at 72°C for 10 min. *Bifidobacteria* isolates were identified by PCR as described by Kok et al. (1996). For *Bifidobacteria* identification, we included two universal 16S rRNA gene primers, in addition to the genus-specific primers in the PCR amplification to generate approximately 1,500 bp and 595 bp fragments. The following primers were used: F6PPK forward $^{5'}$-CCA TGA ACC TGC TCG TCG TCT CCT CCC ACG TGT-$^{3'}$ (from nucleotide 1601 of F6PPK) and reverse primer $^{5'}$-GAC AAG CCG GTG CTG TTC GCT TAC-$^{3'}$ (from nucleotide 2127). Primers were synthesized from Eurofins Inc, Eurofins Genomics, (Ebersberg, Germany). Primers were derived based on the NCBI nucleotide database accession numbers JF340474 and FN600543 for *xfp*. The following parameters were used: hot start 94°C for 5 min, followed by 25 cycles of denaturation at 94°C for 1 min, annealing at 61°C for 1 min, and extension at 72°C for 1.5 min, followed by a final extension at 72°C for 5 min. The full-length *xfp* gene and conserved regions of the *xfp* gene were also amplified with different primers.

Results & Discussions

The results of the present study demonstrated that the simultaneous on-plate isolation and identification of *Bifidobacterium* spp. was rapid, reliable, cost-effective, and was utilized to process many samples simultaneously. A reddish-violet color was formed by *Bifidobacterial* cells, but not E. coli, *Lactobacillus acidophilus* (ATCC 4356: Hansen and Mocquot et al.,), or *E. raffinosus* cells, when treated with CTAB and then subjected to the on-plate assay. This was independent of the cell disruption procedure used (Fig. 1A). Additionally, *Bifidobacterial* cultures that were not treated with CTAB did not react (Figs. 1A, B). DSMZ cultures (Table 1) novel fecal isolates, and commercial probiotics were all subjected to the F6PPK plate assay, which resulted in the formation of a reddish-violet color (of different intensities) with an absence of non-specific or background color. In the case of the conventional test, a slight reddish-violet color was observed, even in controls in the absence of CTAB or containing *Bifidobacterium* spp. and in those which were not subjected to sonication, thereby demonstrating the non-specificity/background of the assay. These results may have occurred as a result of cell lysis during the harvesting or washing of the cells.

Color formation and the capture of the reddish-violet color on the filter paper occurred even in response to the low levels of CTAB used (2.25 µg; colony no. 1, Fig. 1A). Conversely, the conventional phosphoketolase test described by Orban and Patterson (2000), required at least 180 µg (0.45 mg/mL stock solution) of CTAB for optimal cell lysis. Thus, the present plate assay requires tenfold less CTAB (2.25–9 µg) than the conventional assay. This may be because the added solution stays on the cell surface for a sufficiently long time. Additionally, the method developed here required a single colony. Similarly, as shown in Table 2, the method described here also minimized the use of other reaction constituents, such as NaF (45 µg), sodium iodoacetate (75 µg), sodium-fructose-6-phosphate (1,200 µg), hydroxylamine hydrochloride (1.95 mg), TCA (2.25 mg), HCl (4N, 15 µL), and ferric chloride hexahydrate (0.7 mg). Most importantly, the method bypassed the necessity for pure *Bifidobacteria* cultures.

Figure 1

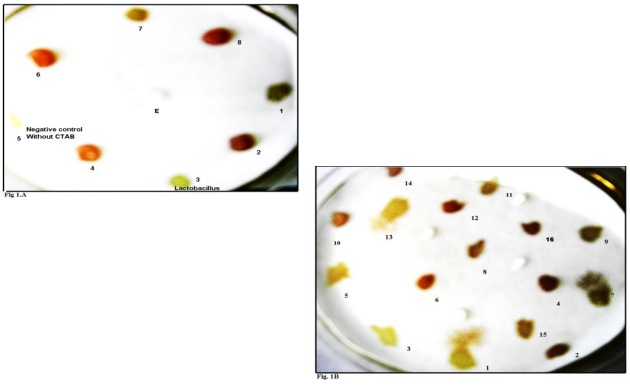

Figure legends

Fig. 1A. The F6PPK assay was conducted as described in the Materials and methods section. Briefly, *Bifidobacterium* was grown on *Bifidobacterium* agar plates for 2–3 d at 37°C under anaerobic conditions. Then, the samples were treated with 0 (colony no. 5), 2.25 µg (colony no. 1), 3.0 µg (colony no. 2), 4.5 µg (colony no. 4), 6.75 µg (colony no. 6), 7.5 µg (colony no. 7) or 9 µg (colony nos. 3, 8, and Escherichia coli) of CTAB, after which they were subjected to the F6PPK assay. Sample 3 was a Lactobacillus strain subjected to the same treatment as the *Bifidobacterium* samples. Sample 5 was a *Bifidobacterium* strain that was not treated with CTAB. E is an E. coli strain that was subjected to the same treatment as the *Bifidobacterium* samples.

Fig. 1B. On-plate F6PPK assay for isolation of *Bifidobacterium* from mixed cultures.

The DSMZ isolates and pure isolates of *Bifidobacterium* mixed with *Escherichia coli*, *Enterococcus raffinosus*, or *Lactobacillus* strains were cultured on agar plates for 3–4 d. Then, the samples were treated with 9.0 µg of CTAB, and subjected to the procedure described in the Materials and methods. Briefly, the cells were exposed to 15 µL of a solution containing NaF (3 mg/mL) and sodium iodoacetate (5 mg/mL), after which 15 µL of sodium fructose-6-phosphate (80 mg/mL) was added, and the plates were incubated at 37°C for 30 min. Following incubation, approximately 15 µL of hydroxylamine hydrochloride (13 g/100 mL) was added, and the samples were incubated at room temperature for 10 min. Next, the following reagents (15 µL of each) were sequentially added: TCA (15% w/v), 4 N HCl, and ferric chloride hexahydrate (5% w/v) in 0.1 N HCl. Then, the samples were incubated at room temperature for 10 min, and the color was observed and captured on Whatmann filter paper or normal paper. Samples 2, 4, 6, 8, 10, 12, 14, and 16 all produced a reddish-violet color, confirming the presence of F6PPK; hence, they are bifidobacteria (as F6PPK is considered to be an identifying marker for bifidobacteria), while samples 1, 3, 5, 7, 9, 11, and 13 produced a yellow color (indicating the absence of F6PPK) and are, thus, considered to be F6PPK-negative and hence, they are not Bifidobacteria. The names of each sample used in the plate assay are: 1. *Bifidobacterium breve*; 2. *Bifidobacterium adolescentis*; 3. *Bifidobacterium* isolate, 4. *Bifidobacterium catenulatum*; 5. *Bifidobacterium indicum*, 6. *Bifidobacterium animalis*; 7. *Bifidobacterium asteroides*; 8. *Bifidobacterium animalis*, subsp. *lactis*; 9. *Bifidobacterium thermacidophilum*; 10. *Bifidobacterium longum* subsp. *infantis*; and 11. *B. longum* subsp. *longum*.

Figure 2

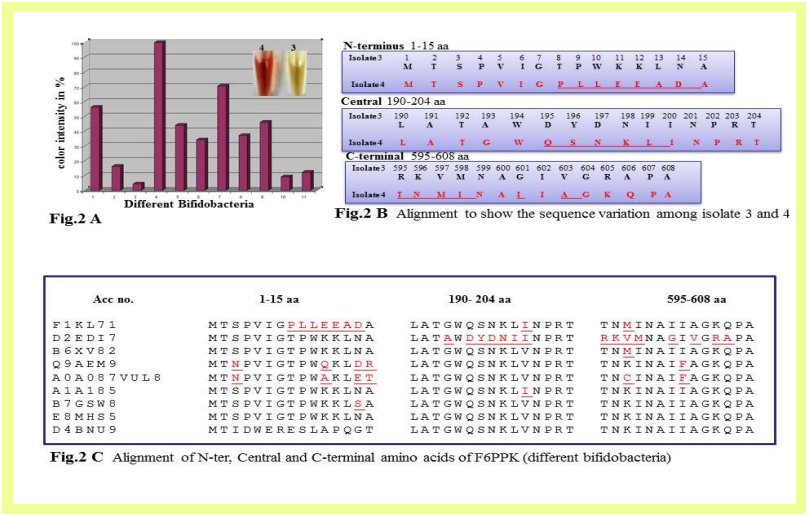

Fig. 2. Conventional F6PPK assay (Orban and Patterson, 2002). The resulting reddish-violet color was spectrophotometrically measured for the different bifidobacterial strains. The method followed has been described in the Materials and methods. The bifidobacterial strains used are: 1. Bifidobacterium breve; 2. Bifidobacterium adolescentis; 3. Bifidobacterium isolate; 4. Bifidobacterium catenulatum; 5. Bifidobacterium indicum, 6. Bifidobacterium animalis; 7. Bifidobacterium asteroids; 8. Bifidobacterium animalis subsp. lactis; 9. Bifidobacterium thermacidophilum; 10. Bifidobacterium longum subsp. infantis; and 11. B. longum subsp. longum. The inset shows the intensity of the reddish-violet color formed by subjecting isolates 3 and 4 (as stated in Fig. 2) to the assay. Here, isolate 4 corresponds to B. catenulatum (a novel isolate).

The amplification of the xylulose-5-phosphate/fructose-6-phosphate phosphoketolase (xfp) gene and the fructose-6-phosphate phosphoketolase (F6PPK) biochemical assay has been extensively used to identify Bifidobacterium spp. In the conventional assay, after isolation and harvesting of the stationary phase cultures are treated with different reagents sequentially finally leading to the formation of ferrous hydroxamate (reddish-violet color) which is measured spectrophotometrically. The existing methods for identifying Bifidobacterium spp. were developed based on the availability of pure bacterial cultures.

These methods are inefficient, laborious, and costly. Hence, the existing methods are not suited for mass screening and the simultaneous isolation and identification of Bifidobacterium spp. in mixed cultures directly from agar plates. Here, we developed an on-plate method for the rapid, simultaneous isolation and identification of Bifidobacterium spp. from varied cultures, in which the pure culture isolation, harvesting and washing steps were bypassed. The effect of the addition of minimal concentrations of cetyltrimethylammonium bromide (0.20–0.45 mg/mL) for cell lysis, as well as the subsequent color development in the plate assay, was assessed. Mixed cultures of Escherichia coli, Lactobacillus, and Bifidobacterium spp. were used. No phosphoketolase activity was observed when plates contained disrupted E. coli, and Lactobacillus spp, and activity was observed only with disrupted Bifidobacterium spp. The on-plate F6PPK assay developed here is an efficient alternative to currently available methods that must be conducted after isolating pure Bifidobacterium spp. The identification of target bacteria by the on-plate F6PPK assay will lead to future studies of the composition and dynamics of intestinal microbiota.

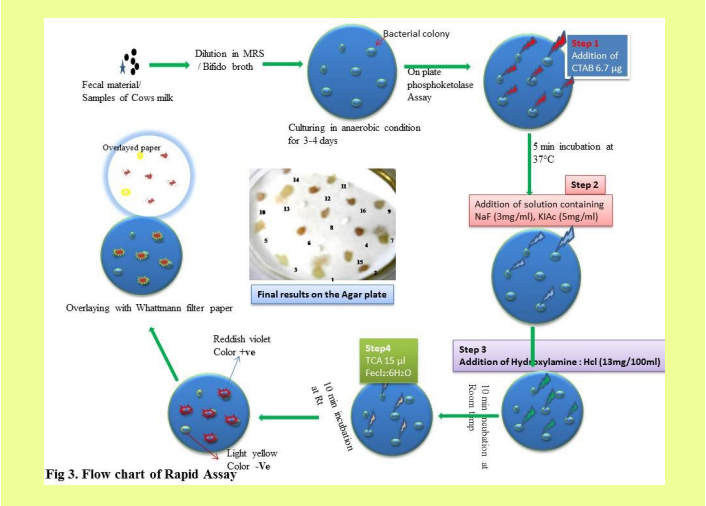

Fig. 3. *Flow chart of the rapid assay.*

Measurable variations in color intensity were observed among the *Bifidobacterial* strains in the plate assay (Figs. 1A, B), indicating that the method could be used to differentiate between *Bifidobacterium* spp. The intensity of the reddish-violet color [Fig. 2 (inset)] shows that the reddish-violet color and a light-yellow color (negative reaction) that formed in the conventional assay also varied among *Bifidobacterial* strains. A maximum value of 1.60 was observed for *B. catenulatum* (isolate four as shown in Fig. 2), indicating that the final product of the F6PPK assay, ferric hydroxamate, could be read at 505 nm, which indicated not only the presence of F6PPK, but also the presence of a High-affinity enzyme for the substrate (or the cells produced a higher level of enzyme). A minimum value of 0.146 was observed for *B. longum* sub spp. *infantis* (Fig.2). The on plate F6PPK assay cannot identify *Bifidobacterium* spp. producing a reddish-violet color intensity <0.150, which was found to be the sole disadvantage of this method. Figure 2 clearly shows the different intensities of the reaction products formed. Therefore, based on the above phenomenon we concluded that this difference in color intensity could be exploited for the differentiation of different *Bifidobacteria*l species. The most important steps that account for the success rate of this test are: always select milky white and transparent colonies (the targeted colonies should therefore be well separated); always keep the assay plate in the horizontal position; use a minimal amount of absorbent paper/Whatman filter paper to obtain the reddish-violet color; and as soon as the color is absorbed, dry the paper at room temperature only. Nevertheless, based on the intensities of the reddish-violet color formed by the different *Bifidobacterial* strains (Figs. 1A, B, and 2); the method developed here could simultaneously isolate and identify many *Bifidobacterium* species.

Several *Bifidobacterial* cultures from the DSMZ, as well as fecal isolate mixed with *E. coli* and *Lactobacillus* strains, milk samples, and commercial probiotics, were subjected to the on-plate F6PPK assay (Fig. 1B). All the *Bifidobacterial* cultures evaluated produced a definitive color reaction with no background or non-specificity signals (Figs. 1A, B). Also, this simple, efficient, on-plate phosphoketolase test was capable of isolating and identifying 20 or more samples simultaneously within an h (including the incubation time). Moreover, this assay did not result in any false positives when compared with the conventional assay. Therefore, many samples can be assayed simultaneously using minimal laboratory reagents and labor. For further validation, the several *Bifidobacteria*l samples were subjected to PCR amplification of *xfp*, and 16S rRNA and approximately 2500 and 520 bp product (the correct predicted amplicon sizes) were seen in five samples (A, B, C, F, and G), and the remaining samples were negative (D, E). Furthermore, an amplicon of 1,500 bp (16S rRNA) was observed in all the samples (data not shown).

Table 3. PCR amplification and F6PPK plate assay results

Commercial probiotic formulation	*Xfp* PCR amplification	F6PPK on plate assay
A	Positive	Negative
B	Positive	Negative
C	Positive	Negative
D	Negative	Negative
E	Negative	Negative
F	Positive	Positive
G	Positive	Negative

Validation

The developed plate assay was further validated to check its efficiency and reproducibility in a subsequent experiment in which mixed cultures of Lactobacillus and *Bifidobacteria* were grown for 3–4 d and subjected to the assay.

In the present study, species-specific PCR and sequence analysis established that *B. catenulatum* was the primary component of commercially available probiotic formulations.

However, when the same products were subjected to the on-plate assay (and the conventional assay), they were F6PPK negative (Table 3). This was primarily because the lower the amount of reddish-violet color produced the lower the chances of finding the *Bifidobacteria*. However, sample F was positive in both events because the commercial probiotic contained *B. catenulatum*, *B. asteroids,* and *B. breve* at high levels and these three bacteria could make larger amounts of ferric hydroxamate (fig. 2). This may have been due to the oxygen toxicity that occurred under the assay conditions (as well as during the processing of the probiotic samples), in which exposure to oxygen toxicity occurred at different levels of commercialization, which resulted in decreased bacterial recovery in the medium. The colonies which showed a reddish-violet color were positive and were taken for further validation. They were subjected to the F6PPK assay, and PCR analysis. The most conventional dehydration methods, spray-drying and freeze-drying, strongly affected the survival rates of the bacteria, thereby resulting in increased toxicity of subsequent processing steps [18].

The plate assay developed in this study was further validated by comparing it with the conventional F6PPK assay for the evaluation of several different *Bifidobacterial* cultures procured from the different sources as mentioned above (Tables 1 and 3). The results obtained using our method were comparable to those obtained using the conventional F6PPK assay, as well as the results obtained using molecular methods such as 16S rRNA and *xfp* amplification. Taken together, the outcome of this study indicated that the on-plate phosphoketolase assay was a novel, rapid, efficient, mass screening method in the absence of non -specificity/background. The present method is well suited to the simultaneous isolation and identification of *Bifidobacteria* from unknown cultures. However, the most significant disadvantage of these assays occurred when the color intensity resulting from the production of ferric hydroxamate was low, i.e., the lower the intensity, the lower the probability of identifying the *Bifidobacteria*. When this occurs, we suggest that genus-specific PCRs should be used for further verification and validation only.

Conclusion:

We have developed a technique for reliable isolation and identification of *Bifidobacteria*. This method bypasses the necessity for pure *Bifidobacteria*, their harvesting, or washing, and uses tenfold less reaction components, and can simultaneously isolate and identify multiple samples. No costly or specialized equipment (such as anaerobic conditions) or skilled technicians are required. The entire procedure for this assay is shown in Fig. 3. Although mutations are noted in isolate number three, which is a *B. catenulatum* strain, it produced lower or negligible amounts of ferric hydroxamate.

For any microbiological method, an essential component is a pure isolate. Most of the present methods require isolated bacteria for further characterization. This may be easier for most bacteria, but not so with *Bifidobacteria*. This is because they are anaerobic in nature and are highly sensitive to oxygen. Therefore, there is a high probability that the bacteria will be lost during the assay.

Therefore, the assay should be very simple, the sample should be spread only once onto the plate, followed by an overlay of reagents and the outcome followed based on the biochemical reaction between F6PPK and the substrate. However, this may not be possible with all of the *Bifidobacterial spp*, as a few of them contain very low levels of F6PPK. If this occurs, a more sensitive method is indispensable and currently, we are in the process of developing this. Currently, PCR-based methods also need bacteria (pure culture) which may be suspected to be *Bifidobacteria*, or there should be some clue. Here we believe that our method may represent the best way to simultaneously isolate and identify *Bifidobacteria*.

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