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Identification of *Bifidobacterium* Strains Isolated from Kashk-e Zard: A Traditional Iranian Fermented Cereal-Dairy Based Food

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Abstract

Objectives: The genus *Bifidobactrium* enjoys considerable significance among the probiotic bacteria for having appropriately adapted to the human gastrointestinal tract. As the properties of *Bifidobacteria* are strain-oriented and niche-dependent, there is growing interest in studying the different sources of these probiotics. Kashk-e Zard, a traditional fermented food produced from wheat and yogurt through a two-week, two-step fermentation process, is rich in probiotics and is worthy of study in this regard. The present study aimed to identify *Bifidobacterium* spp. in Kashk-e Zard.

Methods: Twenty-three samples of Kashk-e Zard were collected and subjected to *Bifidobacterium* identification experiments. Polymerase chain reaction (PCR) and sequencing methods were applied for bacterial identification.

Results: Twelve of the isolates obtained were G +, rod-shaped, and catalase-, whereas only three of them identified positive for fructose 6-phosphate phosphoketolase (F6PPK a *Bifidobacterium* specific test) and mupirocin resistance. These three isolates were then considered for further identification using the 16SrDNA sequencing technique.

Conclusions: Although carbohydrate fermentation patterns specified these three isolates as *B. infantis*, *B. bifidum*, and *B. longum*, the molecular results did not confirm *B. longum*, which is still also controversial in the literature. Overall, our results demonstrated that Kashk-e Zard is a rich potential source of probiotic bacteria and further investigations should be undertaken.

Keywords: Kashk-e Zard, Bifidobacterium Strains, PCR, Bacterial Identification

1. Background

Bifidobacterium is a genus of G+, non-motile, often branched anaerobic bacteria that are ubiquitous, endosymbiotic inhabitants of the gastrointestinal tract and vagina (1, 2). They colonize the intestine of newborns within the first few days after birth and they represent up to 95% of the intestinal microflora of breast-fed infants (3, 4). Some *Bifidobacterium* strains are considered to be important probiotics and are widely used as cell suspensions or as freeze-dried additives in the food industry for the production of cultured milk, beverages, cheese products, cookies, and powdered milk (5, 6). Several health-promoting effects of *Bifidobacteria* have been reported including immunomodulation, the elimination of procarcinogens, production of vitamins, prevention of diarrhea and intestinal infections, alleviation of constipation, production of antimicrobials against harmful intestinal bacteria, and protection of the mucosal epithelium against pathogenic bacteria invasion (7-10).

The properties of *Bifidobacterium* species are strictly strain-dependent, and therefore there has been growing interest in the detailed characterization of newly isolated strains with potentially probiotic properties (11-13). Therefore, since the first isolation from human breast-fed infants (14), Bifidobacteria have been the objects of numerous nutritional, biochemical, ecological, taxonomical, and genetic studies (10). Until now, this genus has contained 42 species, but this number is growing. Interest concerning the probiotic potential of specific *Bifidobacterium* strains may induce the exploration of uninvestigated habitats and new species (15). Moreover, further investigations should focus on the selection of human Bifidobacterial isolates that are able to survive in food for extended periods of time (16).

Kashk-e Zard is a popular traditional product in the southeastern part of Iran (Sistan-o Balouchestan province). It is produced by mixing wheat flour (35%), yogurt (65%), and a variety of vegetables, salt, and spices followed by a two-week, two-step fermentation process. First, wheat flour in dough form is mixed with yogurt and salt and fermented for one week in a closed container placed in a warm environment. Yogurt is subsequently added again, and the dough is kneaded and fermented for another 7 - 10 days. Finally, after drying, the product is milled and granu-

lated to an average granule size of 1 - 3 mm (17).

2. Objectives

In this study, the isolation and identification of possible *Bifidobacterium* strains from Kashk-e Zard was considered using biochemical and molecular techniques.

3. Methods

3.1. Sampling, Cultivation, and Identification

Twenty-three samples of Kashk-e Zard were collected from different regions of the Sistan-o Balouchestan province of Iran and were transferred to the laboratory in aseptic containers. Ten grams of each sample were homogenized with 90 mL of peptone water in sterile bags using a Stomacher® food blender and then serially diluted ten-fold to obtain 10 - 7 dilutions. The dilutions were surface-plate cultured on de Man Rogosa and Sharpe (MRS) agar (Merck, Germany) and incubated at 37°C for 72 hours in an atmosphere of 5 - 10% CO_2. After incubation, colony forming units (CFU) were quantified using a colony counter, and representative morphological types were isolated. Selected colonies were then cultured on MRS agar in order to obtain pure cultures. After initial identification, further diagnostic and evaluation tests including gram-staining for microscopic and morphologic inspection, catalase activity, oxidase test, motility test in sulphide indole motility (SIM) medium (Merck, Germany), indole production from tryptophan, and carbohydrate fermentation patterns in triple sugar iron (TSI) medium were performed to confirm the presence of the genus Bifidobacterium in the samples. Fructose 6-phosphate phosphoketolase (F6PPK), mupirocin tolerance, and skimmed milk coagulation tests were also implemented in order to obtain further confirmation. Identification of the isolates at the species level was conducted through biochemical testing. Carbohydrate fermentation profiles including amygdalin, L-arabinose, cellobiose, esculin, fructose, galactose, glucose, gluconate, lactose, maltose, mannitol, mannose, melesitose, melibiose, raffinose, rhamnose, D-ribose, salicin, sorbitol, sucrose, trehalose, xylose, starch, and inulin, were compared against a standard table. The isolates identified based on carbohydrate fermentation patterns were subjected to molecular methods in order to obtain more precise identifications.

3.2. DNA Extraction

DNA was extracted according to the protocol reported by Marmur (1961) (18) and modified by Kurzak et al. (1998) (19), and then suspended in 50 μL of TE buffer (10 mM Tris-HCl (Merck, Germany) and 1 Mm EDTA (Merck, Germany), pH 8). Five microliters of this template DNA were added directly to the PCR tube. The amount of DNA obtained quantified a UV spectrum (260 nm) and its integrity was visualized using agarose gel electrophoresis at 1% w/v, by staining with ethidium bromide and subsequent visualization under UV light.

3.3. 16S rDNA Amplification

From 16S rRNA, a gene A fragment of 1kb was amplified using the primers 7-f (5'-AGAGTTTGATCCTGGCTCAG-3') and 261-r (5'-AGGAGGTGATCCAGCCGCA-3'). Each PCR tube (50 μL) contained a reaction mixture of 36 μL of sterile water, 5 μL of 10 $\times$ PCR buffer, 1.5 μL of $MgCl_2$ (10 mM), 1 μL of dNTP, 2 μL of each primer, 0.2 μL of Taq polymerase, and 3 μL of template DNA. The of carried as program was: 94°C for 5 minutes; 30 cycles of 94°C for 1 minutes, 55°C for 1 minute and 72°C for 1 minute; and a final extension step at 72°C for 7 minute, respectively. After cycling, the PCR products were visualized using electrophoresis on a 1% w/v agarose gel (1 hour 100 V), by staining with ethidium bromide (0.5 μg/mL), visualizing under UV light, and then comparing them to a standard sample.

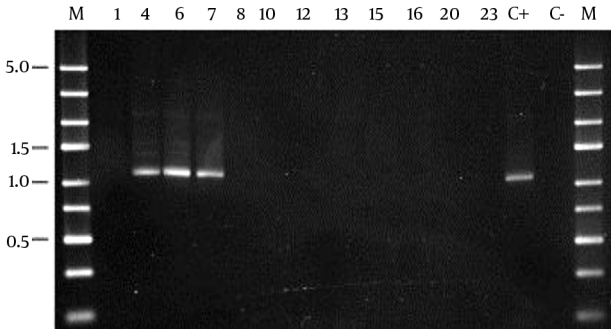
3.4. DNA Sequencing

The PCR products of the representative isolates were purified and the amplified 16S rRNA gene was sequenced. The results were compared with the sequences deposited in the Gen Bank database using the basic local alignment search tool (BLAST) algorithm (http://www.ncbi.nlm.nih.gov/BLAST/). The obtained sequences were deposited in the Gen Bank database using the web-based data submission tool, Bank It (http://www.ncbi.nlm.nih.gov/BankIt). Finally, a phylogenic tree for the isolated Lactobacillus strains was depicted using the MEGA5 version 3.1 program (cut off = 50, bootstrap = 1000).

4. Results

The colony counts for the assumed *Bifidobacterium* isolates in this study ranged from 2.37 to 7.17 log CFU/mL. The lower limit recommended by the international dairy federation (IDF) for bifidobacterial counts in dairy products is 106 CFU per milliliter. Fifty-four of the isolates that appeared as white and round or spindly colonies on mMRS agar were considered to be *Bifidobacterium*. Among these isolates, only 12 were identified as G+, rod-shaped, and catalase-. For further confirmation at the genus level, the suspected isolates were tested by cultivation on TSI and SIM

Figure 1. PCR Products of 12 Assumed *Bifidobacterium* isolates



M, Ladder; 4, 6, and 7, positive samples; 1, 8, 10, 12, 13, 15, 16, 20, and 23,negative samples; C+, Positive control; and C-, Negative control.

media, F6PPK, mupirocin susceptibility, and milk coagulation tests (Table 1). Mupirocin susceptibility tests were then performed to differentiate *Bifidobacteria* from *lactobacilli* because the cultural and biochemical properties of both genera overlap. *Bifidobacteria* are resistant to mupirocin, whereas lactobacilli are susceptible to it. Phosphokctolase assays using F6PPK were performed on all of the isolates that were considered to belong to the genus *Bifidobacterium* this test is considered a unique and specific non-molecular test for *Bifidobacteria* and is commonly used for their definitive identification. Of the 12 isolates, only isolates 4, 6, and 7 were both mupirocin resistant and $F6PP^{+}$.

Isolates 4, 6, and 7, which passed all the previous tests successfully, were then subjected to specific biochemical and genetic tests in order to precisely determine their species (Tables 2 and 3). According to the fermentation patterns, isolates 4, 6, and 7 were respectively identified as *B. infantis*, *B. bifidum*, and *B. longum* (Table 2). The amplified DNA fragments for isolates 4 (966 bp), 6 (1057 bp), and 7 (1032 bp) were sequenced and compared to sequences deposited in the NCBI database using the BLAST algorithm (Figure 1, Table 3). The biochemical results were confirmed using molecular techniques; however, isolate 7 was not recognized at the species level (Table 3).

5. Discussion

In the present study, Kashk-e Zard samples were evaluated for the presence of strains of *Bifidobacterium*. As previously mentioned, the IDF's recommended lower limit for Bifidobacterial counts in dairy products is 106 CFU per milliliter. In Japan, this recommendation is 107 viable probiotic cells per gram or milliliter (20). Generally, *Bifidobacteria* exhibit poor viability in fermented dairy products and various studies have indicated that not all probiotic products contain the recommended levels of viable microorganisms (21, 22). It is also important that high counts of *Bifidobacteria* are necessary to provide beneficial effects on human health. However, *Bifidobacterium* spp. are susceptible to acids and oxygen, so *Bifidobacteria* counts in supplemented yogurts may easily decrease during storage (23). Nonetheless, as acceptable *Bifidobacteria* counts were observed in all the samples in this study, Kashk-e Zard seems to be a promising source of probiotic bacteria.

Differences in the nucleotide sequences of the 16S rRNA gene are commonly used for the development of genus, species, and subspecies-specific PCR primers for Bifidobacterial identification (24-27).

Knowing that the tested strains belong to the same species, they should possess more than 97% 16S rRNA gene sequence identity (28). The analyses of the 16S rRNA sequences of *B. longum*, *B. infantis*, and *B. Bifidum* isolated from Kashk-e Zard did not allow the clear separation of taxonomic entities at the species level for isolate 7. The taxonomical position of these aforementioned subspecies has been controversial for quite some time, and differentiation between *B. longum* ssp. longum and *B. longum* subsp. infantis is still complex due to more than 97% 16S rRNA gene sequence similarity (29). Sakata et al. (2002) (30) concluded that *B. infantis* and *B. suis* should be unified as *B. longum* and the latter species divided into three biotypes, the infantis type, the longum type, and the suis type. Compiling all published information, Mattarelli et al. (2008) (31) proposed the descriptions of B. *longum* subsp. longum, *B. longum* subsp. infantis, and *B. longum* subsp. suis.

Four *Bifidobacterium* species have been generally reported in the literature for their use as probiotic cultures in dairy products *B. longum*, *B. infantis*, *B. breve*, and *B. bifidum* playing important roles in the gut (7, 32, 33). The most common *Bifidobacterium* species found in the intestines of human neonates and breast-fed infants are *B. breve*, *B. infantis*, and *B. bifidum* (15, 24), while those in the intestines of adults are *B. adolescentis* and *B. longum* (23, 24, 34).

The isolation of *Bifidobacterium* from Kashk-e Zard demonstrated that this traditional product is a promising source of these probiotic strains. In particular, the high degree of *Bifidobacterium*'s viability over a relatively long period may attract researchers' attention for further studies on Kashk-e Zard. In the future, investigating indigenous strains may lead to the introduction of regional starters that are more compatible with the gastrointestinal microflora of the local population. However, there is a long way to go from the perspective of probiotic approval and commercialization.

Table 1. Identification Test Results for the 12 Assumed *Bifidobacterium* Isolates

Sample No	Gram Staining	Oxidase	Catalase	Coagulation	H_2S Production	F6PPK	Mupirocin Susceptibility
1	+	-	-	+	-	-	-
4	+	-	-	+	+	+	+
6	+	-	-	+	+	+	+
7	+	-	-	+	+	+	+
8	+	-	-	+	-	-	-
10	+	-	-	-	-	-	-
12	+	-	-	-	-	-	-
13	+	-	-	-	-	-	-
15	+	-	-	+	-	-	-
16	+	-	-	+	-	-	-
20	+	-	-	-	-	-	-
23	+	-	-	-	-	-	-

Table 2. Carbohydrate Assimilation Patterns of the Confirmed Isolates

Sample No.	**4**	**6**	**7**
Culture temperature	**37°C**	**37°C**	**37°C**
O_2	**Anaerobic**	**Anaerobic**	**Anaerobic**
L-arabinose	-	-	+
Cellobiose	-	-	-
Fructose	+	+	+
Galactose	+	+	+
Glucose			
Gluconate	-	-	-
Lactose	+	+	+
Maltose	+	-	+
Mannitol	-	-	-
Mannose	D	-	D
Melesitose	-	-	+
Melibiose	+	D	+
Raffinose	+	-	+
Rhamnose			
D-ribose	+	-	+
Salicin	-	-	-
Sorbitol	-	-	-
Sucrose	+	D	+
Trehalose	-	-	-
Xylose	D	-	D
Starch	-	-	-
Inulin	D	-	-
Species	*B. infantis*	*B. bifidum*	*B. longum*

Table 3. Comparison of the Sequenced PCR Products with the Deposited Standards Using the BLAST Algorithm

Sample No.	Description	Max Score	Total Score	Query Coverage	E Value	Max Ident	Links
4	gi|148361514|EF589113.1	*Bifidobacterium infantis* strain IDCC 4201, 16s ribosomal RNA gene	762	762	98%	0.0	98%
6	gi|125584403|EF370998.1	*Bifidobacterium bifidum* strain THT 010101, 16s ribosomal RNA gene	762	762	98%	0.0	98%
7	gi|65307104|AY987032.1	Uncultured bacterium clone G011 16s ribosomal RNA gene	762	762	98%	0.0	98%

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