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Safety and Properties of *Bifidobacteria* Isolated from Traditional Dairy Products from Iran

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In current study the probiotic properties of five strains registered in NCBI database out of 33 isolated *Bifidobacteria* from traditional dairy products taken from west provinces of Iran were evaluated. The *Bifidobacteria* had been isolated using specific culture media, identified by a unique enzymatic method followed by amplification of 16 srDNA sequence. The five strains which were used in this study were primarily identified by species-specific primers and characterized using carbohydrate fermentation profile. The PCR results showed that four strains out of five were *Bifidobacterium bifidum* and one of those was *Bifidobacterium lactis.* The probiotic safety and efficacy of these isolates including antibacterial activity, tolerance to bile, acid, high concentrations of NaCl, as well as bile salt hydrolysis activity and growth in various temperatures were investigated. All of the tested strains had significant probiotic properties, including hydrolysis of bile salts, tolerance to bile and low acidity and have shown notable antibacterial activity against *sal. Typhymurium*, *S. aureus* and *E.coli,* while none of them had antifungal activity against *Candida albicans*. The results showed that the strains isolated from native dairy products have good viability in simulated gastric and bile conditions and also considerable probiotic activities and due to lack of the hemolytic reaction and toxic metabolites are safe for human consumption and can be used as probiotic in foods and pharmaceuticals.

Key words: Probiotic, Bifidobacterium, assessment, Isolated, properties.

Probiotics are microbial cell preparations or components of microbial cells that have beneficial effects on the health and well-being of the host (Joint FAO/WHO Working Group 2002). The effects of probiotic microorganisms on human health and prevention of some diseases have been proved by lots of studies (Mattila *et al*., 1999, Salminen *et al*., 2010). One of the most important bacteria considered as probiotic is *Bifidobacterium* genus, which has been firstly isolated by Tisser from infants fed by breast milk (Picard *et al*., 2005). *Bifidobacterium* genus is one of the first groups colonizing in gastrointestinal tract. There are various claims on its health beneficial properties (Gomes *et al*., 1999, Schrezenmeir, 2008) for which it is being used in dairy products, infant milk formula, feed and supplements. Currently there is a great trend to employ new strains isolated from different sources as Probiotics.

Besides the benefits of probiotics, producing toxic metabolites, inducing microbial infections and resistance to vital antibiotics and transferring microbial genes are some of the problems which may be caused by consuming live probiotic microorganisms (Morelli, 2000, Lahtinen *et al*., 2009). Therefore, safety, viability and activity of new strains employed in food industries should be assessed (Ishibashi *et al*., 2001). The safety of these bacteria is usually assessed by criteria suggested by FAO/WHO guideline (Joint FAO/WHO Working Group 2002).

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Fermented dairy products, especially the traditional ones are known as good sources for lactic bacteria. Traditional fermented products are widely produced and used in all parts of Iran. It is argued that the mentioned fermented products are good candidates for isolating local unique strains of *Bifidobacteria*. A number of certain fermented products produced in western areas of Iran (Kordestan, Kermanshah, Ilam and Lorestan) have been chosen because of their good aromatic properties and their specific tastes.

In the current study, the key factors which have been considered by previous studies have been used for evaluating the safety, activity and efficiency of 5 isolated strains containing new genes registered as EU7594329 (IR007-15), EU746405 (IR007-93), EU746406(IR007-94), EU7594328 (IR007-108) and EU746407 (IR007- 113) at Nucleotide/NCBI (Heidarpour *et al*., 2008) have been assessed.

MATERIALS AND METHODS

All of the assessment procedures have been carried out on the selected strains duplicated by three replicates. For quantitative data, mean of the results were reported.

Bifidobacteria strains

The Bifidobacteria isolated from Iranian dairies recognized as Bifidobacteria by microbiological methods and amplified by 16 srDNA primers Bif164/Bif662 (Kaufmann, 1997) registered as EU7594329 (IR007-15), EU746405 (IR007-93), EU746406 (IR007-94), EU7594328 (IR007-108) and EU746407 (IR007- 113) in Nucleotide/NCBI (Heidarpour *et al*., 2008). According to the results of identification by species-specific PCR primers stated in table 1 of Matsuki (Matsuki *et al*., 2003), IR0015 was identified as *Bifidobacterium animalis* and the 4 other isolated ones were *Bifidobacterium bifidum.* The strains had been kept in skim milk containing 10% glycerol in -70 °C. In order to get single colonies the bacterial stocks were cultured in MRS broth followed by streaking on TOS agar containing Li-mupirucine (50 mg/L) (ISO/IDF 29981 2011 and Simpsona *et al*., 2004).

Heamolysis and L- Arginine hydrolysis activity

Heamolysis ability of all strains was tested on blood agar medium incubating at 37 °C for 24 h (Ishibashi *et al*., 2001).

Table 1. The probiotic properties and safety criteria of five Bifidobacteria isolated strains

No. of isolates	Isolated strain	NCBI	Strain ID	Heamolysis	L- Arg Hydrolysis	Skim milk Agglutination	Gas production	Growthat p CNaCl			Growthin	
								15	37	43	4%	6.5%
1	IR007-15	EU 594329	*Bifidobacterium animalis*	-	–	–	–	+	+	+	+	+
2	IR007-93	EU 746405	*Bifidobacterium bifidum*	-	–	+	–	–	+	+	+	+
3	IR007-94	EU 746406	*Bifidobacterium bifidum*	-	–	+	–	+	+	+	+	+
4	IR007-108	EU 594328	*Bifidobacterium bifidum*	-	–	+	–	–	+	+	+	+
5	IR007-113	EU 746407	*Bifidobacterium bifidum*	-	–	+	–	–	+	+	+	+

L- Arginine hydrolysis activity was also assessed by MRS broth containing 0.3% L-Arg (Ishibashi *et al*., 2001 and Simpsona *et al*., 2004). Each tube was inoculated by a colony of each of the isolated strains, and incubated anaerobically at 37 °C for 24 h along with a same medium without any bacteria as negative control. Hydrolysis was tested by Nessler's reagent. 100 ml of the cultures and the same amount of reagent were pipetted on a wattman paper and color change was observed. Bright orange to red interpreted as positive reaction while yellow was considered as negative (Borriello *et al*., 2003).

Agglutination and gas production

The ability of Bifidobacteria strains which are intended to be used in dairy industry to producing gas and agglutinating the peptides is an undesirable trait. One colony of 24 hours culture of each isolate was inoculated in 10% skim milk and 0.5% yeast extract incubated at 37 °C for 24 h and agglutination was evaluated by inverting the tubes to test the clot.

Gas production was tested by Durham tubes in modified MRS broth incubated at 37 °C for 24 h.

Tolerance to low pH and Gastric juice

In order to investigating the ability of the isolated strains to live in the acidic products and gastric conditions, the isolated strains were subjected to acidified MRS broth adjusted to pH 2.5 and 4.5 with 1 N HCl compared to MRS broth with normal pH 6.5, incubated an overnight at 37 °C. Survival of bacteria was tested by single streaking on MRS agar plates, incubated at 37 °C for 24-48 h in anaerobic incubation. The quantification of the survived bacteria was performed by determination of turbidity of the three MRS broths at 620 nm, followed by enumeration of the survived bacteria by serial dilutions (Klayraung *et al*., 2008 and Saarela *et al*., 2000, Yanyan *et al*., 2010 and Harun-ur-Rashid *et al*., 2007).

Table 2. Survival of five Bifidobacteria isolated strains at different pH and 0.3% bile concentration, growth in gastric enzymes

		Control (MRS broth pH=6.5, without bile)		pH 4.5 0.3% Bile2.5				MRS broth containing		Gastric juice	
										MRS broth+ 2.5% Pepsin	MRS broth+ 2% Trypsin
No. of isolates	Isolated strain	OD	Cells/ml	OD	Cell/ml	OD	Cell/ml	OD	Cells/ml	Growth	Growth
1	IR007-15	2.33	4.9×10^{9}	2.14	3.1×10^{7}	1.98	2×10^{5}	2.10	2.7×10^{7}	+	+
2	IR007-93	2.52	7.3×10^{9}	2.18	2.9×10^{8}	1.92	3.2×10^{5}	2.14	1.6×10^{8}	+	+
3	IR007-94	2.74	1.4×10^{10}	2.24	8.2×10^{8}	2.06	8×10^{5}	2.22	4.2×10^{8}	+	+
4	IR007-108	2.28	3×10^{9}	2.08	2×10^{8}	1.78	6.2×10^{4}	1.96	3.4×10^{7}	+	+
5	IR007-113	2.25	4.2×10^{9}	2.16	1×10^{8}	1.66	2.4×10^{4}	2.00	4×10^{8}	+	+

Table 3. The diameters of inhibition zones and their mean of antagonistic activity of isolates against *S. thyphimurium, S. aureus , E.coli* and *Candida albicans*

Microorganism	*Salmonella*		*S. aureus*		*E.coli*		*Candida*	
Test Strain	Spot test	CSF	Spot test	CSF	Spot test	CSF	Spot test	CSF
15	30	25	27	25	23	20	5	-
93	36	28	32	30	27	25	8	6
94	28	23	25	19	23	19	5	3
108	24	22	23	20	23	21	6	6
113	40	36	32	30	26	26	10	7
Mean	31.6	27.75	27.8	24.8	24.4	22.2	6.8	5.5

Fig. 1. The agglutination ability of skim milk by isolated strains left to right strains # IR007-15, IR007-93, IR007-94, IR007-108, IR007-113 and two commercial strains

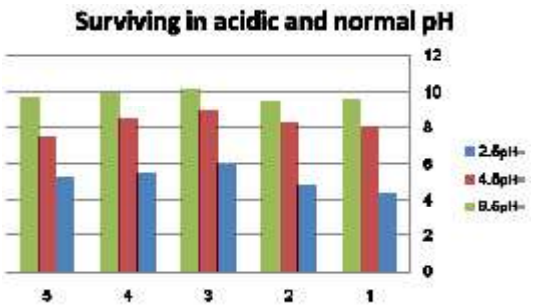

Fig. 2. Comparison between survived isolated Bifidobacterial strains (log of counted numbers) in acidic and normal pH

Fig. 3-4. Inhibition zones resulted from antimicrobial activity of test strains against *S. aureus* (3) and *S. thyphimurium* (4)

Subsequently the resistance of test strains to gastric enzymes of stomach was evaluated by inoculating them in an aqueous solution containing NaCl and 2.5% pepsin and 2% trypsin for 4 hours followed by streaking on MRS agar in order to assess the survival of bacteria (Klayraung *et al*., 2008).

Hydrolysis activity and bile salt resistance

The isolates were subjected to 0.3% bile salts (Bile Oxalat, Merck) in MRS broth along with MRS broth without bile salts as control media. After incubation at 37°C, in anaerobic condition, turbidity of the broths was determined at 620 nm. The survival rate of each strain was expressed as the number of viable cells in the presence of bile salt compared to control media (Simpsona *et al*., 2004).

In order to assess the hydrolysis of bile salts a loopful of an overnight MRS broth culture of each strain was streak on MRS agar containing 5 g/l thioglycolate and 5 g/l sodium salt of taurodeoxycholate acid (TDCA) (bile salt) along with MRS agar without bile salt as control, incubated at 37 °C for 72 h. The agar plates were dried at 37 °C for 24 h, before testing (Valdez *et al*., 2001, Begley *et al*., 2006).

Surviving in different salt and temperature conditions

The isolated strains were cultured in MRS broth containing 2.5%, 4% and 6.5% NaCl and their viability in the mentioned conditions and at different temperatures (15 °C, 37 °C and 43 °C) were evaluated by streaking the incubated broths on MRS agar (Daoudou *et al*., 2011, Yavuzdurmaz *et al*., 2007).

In addition the important parameters for industrialization such as Agglutination in skim milk and gas production were assessed.

Antimicrobial activity

The antimicrobial activities of the isolated strains were assessed by spot test and CFS (Cell Free Supernatant) method (Magdalena *et al*., 2005, Suskovic *et al*., 2010) against *Salmonella thyphimurium* (ATCC 14028), *Staph. aureus* (ATCC 25923), *E.coli* (ATCC 25922) and *Candida albicans* (ATCC 10231). For spot test the isolated Bifidobacteria were cultured in MRS broth and after an overnight incubation, a loopful of the cultures containing 10^7 and 10^{12} cells were spot on MRS agar plates. The plates were left in room temperature in order to absorption, and incubated in anaerobic condition at 37 °C for 24 h, for allowing the bacteria to grow and release their metabolites in the MRS agar. Then the agars were overlaid by TSA media containing 10^7-10^{10} cells/ ml of each of the mentioned bacteria incubated at 37 °C for 24 h. After incubation the inhibition zones were assessed (Gagnon *et al*., 2004, Kaboosi *et al*., 2011).

For CFS method the 24 hours bacterial suspension in MRS broth were centrifuged at 10000 g and the supernatants were used for their antibacterial activity. 100 ml of each supernatant was put in wells made in MRS agar left in room temperature for 1 hour to release the active metabolites in agar. The agars were overlaid by TSA media containing 10^7-10^{10} cells/ ml of each of the mentioned bacteria incubated at 37 °C for 24 h. After incubation the inhibition zones were assessed (Gagnon *et al*., 2004).

RESULTS

The results of heamolysis ability, L-Arginine hydrolysis activity and agglutination of skim milk, as well as gas production and surviving in different salt and temperature conditions are shown in table 1.

The number of viable bacteria in pH 2.5 and 4.5 compared to pH 6.5 and 0.3% bile salt concentration have been calculated and along with the results to simulated gastric enzymes are shown in table 2.

Bile salt hydrolysis

The ability of isolates to hydrolyze bile salt was assessed by the precipitation zone around the colonies, which were positive in all strains.

Antimicrobial activity

In the antimicrobial tests the diameter of inhibition zones were 5-40 mm, revealed that all Bfidobacteria strains showed inhibition effects against *S. thyphimurium*, *S. aureus, E.coli,* and *Candida albicans* (Figs. 3 & 4).

DISCUSSION

According to the results all of the 5 isolated *Bifidobacteria* met safety criteria including lack of heamolysis and L-Arginine hydrolysis activity which are important for safety of consumers. All strains showed considerable probiotic properties as well. The significant properties such as being survived in high salt concentrations, different temperatures and resistance to gastric enzymes make them suitable candidates for using in probiotic foods and pharmaceuticals.

According to table 1 and Fig. 1 all strains except IR0015 showed suitable agglutination ability that can make suitable viscosity in yoghurt. The IR0015 can be considered as a good candidate for using in fermented milks.

According to the results all of the five strains exhibited relatively high viability in low pH (2.5 and 4.5) after an overnight incubation. While the preceded studies have tested the viability and survival rate of Bifidobacterial strains in 4 hours incubation (Klayraung *et al*., 2008), our isolates have exposed to the same conditions during an overnight (i.e. 16 hours) and showed good viability.

Based on Fig. 2 the maximum decrease of log of number of viable bacteria in pH 4.5 was 2, which shows the ability of the isolated strains for using them in acidic products such as yoghurt. In addition the isolated strains showed not more than 5 log decreases in pH 2.5 which suggests that the mentioned isolates can tolerate the acidic conditions of consumers' gastric juice.

Resistance of the isolated strains in the presence of pepsin and trypsin revealed their good viability in gastrointestinal conditions so can be used as probiotic cultures in food industry and pharmaceutical supplements which provide health benefits for consumers.

The antibacterial test showed that all

isolates have a minor to significant antimicrobial activity by both two Spot test and CFS method. The largest inhibition zone diameter against all tested microorganisms was belonged to IR00113. The comparison of means of antagonistic activity was observed against *Salmonella thyphimurium, S. aureus* and *E.coli* respectively. The mentioned bacteria are risk factors and health indicators of foods and according to the results all isolates can be applied as probiotic microorganisms for prevention and treatment of gastrointestinal diseases.

No inhibiting activity against *Candida albicans* was seen, that shows none of the isolates are suitable candidates for using in vaginal products.

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