JOURNAL OF PURE AND APPLIED MICROBIOLOGY, Dec. 2014. Vol. **8**(6), p. 4273-4280

In vitro and *In vivo* Safety Assessment of *Bifidobacterium longum* BBMN68, A Potential Probiotic Isolated from Healthy Centenarians

Ming Zhang[1#], Ai-ping Liu [2,3,#], Jingli Jiang [2,3], Lu Jiang [2] and Fazheng Ren[2]*

[1] School of Food and Chemical Engineering, Beijing Technology and Business University, Beijing - 100048, China.
[2] Key Laboratory of Functional Dairy, College of Food Science and Nutritional Engineering, China Agricultural University, Beijing 100083, China.
[3] MengNiu Diary (Beijing) Co. Ltd., Tongzhou, Beijing 101107, China.

(Received: 23 July 2014; accepted: 10 September 2014)

***Bidifobacterium longum* BBMN68 (BBMN68) is a potential probiotic, which was selected from centenarians feces isolates in Bama. To evaluate the strain's safety, antimicrobial susceptibility, mucin degradation activity, bacterial translocation activity and acute toxicity were investigated. The results of the distribution of inhibition zone diameters showed that BBMN68 were susceptible to most of the antibiotics except for aminoglycoside. Based on recovery of mucin carbohydrate and SDS–PAGE analysis of mucin glycoprotein in liquid medium, BBMN68 had no mucinolytic activity. No viable bacteria were recovered from blood and tissue samples of mice, and no treatment-associated illness or death was observed. Acute oral toxicity study showed that the strain had no toxic reaction with mice, and the mice maximum tolerable dose of BBMN68 frozen cultures was greater than 4.6×10^{11} cfu/kg bw/day. The results obtained in this study suggest that consumption of BBMN68 at a normal dose should be safe for human, BBMN68 may be exploited as a probiotic in food and dairy industry.**

Key words: Probiotic, *Bidifobacterium longum* BBMN68, Safety, Antimicrobial susceptibility, Toxicity.

Probiotics have a variety of beneficial health impacts including intestinal microbiota regulation, immunomodulation and anticarcinogenic effects[1]. The health benefits described for probiotics make them good candidates for noval functional foods[2]. As a result, new bacterial strains with specific probiotic activities are being identified and introduced into the food and pharmaceutical markets.

* To whom all correspondence should be addressed.
Tel.: +86 10 62736344; Fax: +86 10 62736344;
E-mail: 36103805@qq.com

Bifidobacterium is the most frequently used probiotics for human consumption, and due to their long history of safe use, some species have the "Qualified Presumption of Safety" (QPS) status[3]. However, since the newly isolated bifidobacterial strains often have no previous history of use in food products and do not necessarily share the GRAS (Generally Recognized As Safe) status of traditional lactic acid bacteria strains. Furthermore, the safety isssues of bifidobacterium has been questioned recently[4-6]. Some bifidobacterial strains used as probiotic are common members of the human intestinal microflora, they may be regarded as opportunistic pathogens like other commensal bacteria[7]. Commensal bifidobacteria have been related to certain dental infections, pulmonary infections,

bacteremia, abscesses and bloodstream infections[7]. Antibiotic resistance of probiotic strains is another risk since the potential antibiotic resistance gene transfer to pathogenic ones. So the safety assessment of a newly isolated bifidobacteria strain is an important prerequisite for its approval as a probiotic[8-10].

Bifidobacterium longum BBMN68 (BBMN68) was newly isolated from Bama centenarians (Guangxi, China, one of the five well-known longevity regions). In an initial assay, BBMN68 could remarkably promote immunostimulation, improve intestinal digestion ability and enhance immune barrier function [11,12]. The objctive of this study was to evaluate the safety of BBMN68 by antimicrobial susceptibility tests, mucin degradation tests, bacterial translocation and acute toxicity test in Kunming (KM) mouse.

MATERIALS AND METHODS

Bacterial strains and culture conditions

BBMN68 (CGMCC 2265) was isolated from fecal samples of healthy centenarians. The strain was identified based on the sequences of 16S rRNA gene and the sugar fermentation pattern, using the API 50 CH kit (BioMérieux, Montalieu Vercie, France) and a computer-aided identification program (version 4.0, BioMérieux)[11]. The strain grew for 24 h at 37 °C Man Rogosa and Sharpe broth (MRS; Beijing Land Bridge Technology Co., Ltd, China) and was serially transferred at least three times prior to use in this study.

Assessment of antibiotic susceptibility

Antibiotic susceptibility of strains was determined on MRS Agar using antibiotic discs (Tiantan Biological Products Co. Ltd, Beijing, China). After drying the surface, antibiotic discs were placed on the agar plate and incubated at 37 °C. Zone diameters were recorded after 24 h incubation. 23 antibiotics are listed in Table 1. The precision and accuracy of antibiotic susceptibility testing is monitored by using *Escherischia coli* ATCC 25922. Antibiotic susceptibility levels were reported as resistant (R), moderate susceptibility (M) or susceptibility (S)[13].

Mucin degradation assay

Mucinolytic activity was determined as described by Fernandez with slight modifications[14] (Fernandez *et al.*, 2005). Partially purified hog gastric mucin (HGM) from a commercial source (type III, Sigma–Aldrich, Inc., MO, USA) and a basic anaerobic culture medium were used. The composition of this medium includes (g/L): tryptone 7.5; casitone 7.5; yeast extract 3.0, meat extract 5.0; NaCl 5.0; $K_2HPO_4 \cdot 3H_2O$ 3.0; KH_2PO_4 0.5; $MgSO_4 \cdot 7H_2O$ 0.5; cysteine HCl 0.5. The pH of the medium was adjusted to 7.2±0.2. 200 µl of 24 h bacterial cultrue was inoculated into 10ml of basal medium and basal medium containing 0.5% HGM with or without 1% glucose, cultured at 37 °C for 48 h under anaerobic condition (Anaero Pack). *Clostridium perfringens* C01 and commercial *Bifidobacterium longum* BBL were used as positive or negative control respectively. The bacterial growth was monitored by changes in turbidity (absorbance at 600 nm, Unicon, ShangHai) and pH value.

At the end of incubation, 10 ml of culture was centrifuged (10,000 g, 4°C, 30 min) to obtain a supernatant liquid. The supernatant was mixed with 15 ml of 99% chilled ethanol and recentrifuged. The pellet was collected and suspended in 6 ml of 0.1 M NaCl. Ethanol precipitation and recentrifugation were repeated, and resuspended in 0.5 ml 10 mM Tris–HCl buffer. Total carbohydrate (CHO) concentration in ethanol-precipitated mucin residues was determined by the phenol-sulfuric acid assay. Percent recovery of mucin carbohydrate was determined using the following formula: % Recovery = [1 " (concentration in test samples/ concentration in control sample)] × 100, where no inoculated samples were used as control. Each sample was assayed in triplicate.

To demonstrate any change in the composition of mucin after incubation in liquid medium, the electrophoretic patterns of ethanol-precipitated mucin samples were analyzed by SDS-PAGE using 12.5 % polyacrylamide as the separating gel. Gels were stained with Coomassie blue (Bio-Safe Coomassie, Bio-Rad Laboratories, Inc., USA)

Bacterial translocation

The animals used in this study were cared for in accordance with the Guide for the Care and Use of Laboratory Animals and the protocol was approved by the Animal Ethics Committee of China Agricultural University.20 SPF KM mice (10 male 18.1-20.8 g and 10 female 18.0-21.0 g) were divided

randomly into two groups, each group consisted of 5 male and 5 female mice. After 7 days of acclimation, BBMM68 suspension (1×1012 cfu/kg bw) or skim milk was orally administered once each day for 1 week. Mice were killed by intraperitoneal administration of sodium pentothal (50 mg/kg), and blood was collected into EDTA-containing tubes by cardiac puncture in sterile conditions. The liver, spleen and kidney were removed in sterile conditions and weighed.

Bacterial translocation was analyzed in blood, liver, kidney and spleen. 50 ul of blood were cultured in BBL and BHI agar medium, incubated in MRS anaerobically or in BHI aerobically at 37 °C for 48 h. Tissue samples were homogenized in buffered peptone water (1 g/ml) and 100 ml of the resulting homogenates were cultured in MRS and BHI agar as previously mentioned. The colonies were enumerated and the results were expressed as incidence of translocation (number of mice where colonies were detected/total number of mice). Positive growth on agar plates was defined by the presence of even a single colony of any microorganisms.

Oral toxicity study of BBMN68 fresh culture in mice

BBMN68 cells were incubated in MRS broth at 37 °C for 48 h (vialbe count, 2×10^8 cfu/ml), the culture and 5-fold concentration culture were used as the test material. 60 SPF KM mice were assigned randomly to three groups, each group consisted of 10 male and 10 female mice. Initial body weights of the male mice ranged from 18.4-21.6 g and that of female range from 18.6-21.6 g. The treatment group were administered skim milk, BBMN68 fresh culture and 5-fold concentration culture at a dose of 4×10^9 cfu/kg bw and 2×10^{10} cfu/kg bw by oral gavage for continious three days. The general appearances and toxicity reaction were observed daily for 7 days and bodyweight was record at the end of the experiment.

Acute oral toxicity study of BBMN68 frozen culture in mice

BBMN68 frozen culture was prepared by adding cryoprotectant (8% skim milk and 3% glycerol) to harvested bacteria (viable count, 3×10^{10} cfu/g). Initial body weights of 20 SPF KM mice, including 10 male and 10 female, ranged from 18-21 g, detailed clinical observations were made prior to exposure of the animals to the test material. The animals were fasted for 18h and administered a dose of 15.45 g/kg bw of test material twice in 24 h by oral gavage. Following administration, the animals' general appearances were observed for three continious hours, and daily thereafter for 14 days. Body weights were record on day 15, and then all mice were euthanized by exsanguination under ether anesthesia and macroscopic necropsied.

Statistical analysis

Results were expressed as mean ± SD for each group. *P* values less than 0.05 were regarded as significant difference between means using a two tailed Student's t-test.

RESULTS

Antimicrobial susceptibility tests

The results of the antimicrobial disc-diffusion susceptibility tests on BBMN68 were summarized in Table 1. BBMN68 was found to be sensitive to all the tested antibiotics except for aminoglycosides, the strain was moderate susceptibility to low-level neomycin (30 µg), gentamycin (10 µg) and streptomycin (10 µg) with the disc-diffusion method. However, it was sensitive to high-level gentamycin (120 µg) or streptomycin (300 µg).

Mucin degradation assay

The growth of bacteria after incubation in four kinds of media was indicated by the changes in culture absorbance at 600 nm and pH values. The results were showed in Table 2. No growth occurred in the blank control, similar growth was also detected in BBMN68 and the reference Bifidobacterium species, *B.longum* BBL. All bifidobacterial strains showed higher OD_{600nm} in the medium supplemented with 1% glucose or with both 1% glucose and 0.5% HGM than in the medium without glucose. The pH of the medium containing glucose dropped significantly ($P<0.05$) and the medium without glucose had no significant change, which was in accordance with the results of OD_{600nm}. The positive culture, incubated with *C.perfringens* showed high OD_{600nm} values than bifidobacterial strains and blank culture in all the media, and the pH value in medium without glucose decline slightly.

At the end of incubation, the mucin in the basal medium with 0.5% HGM was recovery

by ethanol precipitation method, and the decrease in total CHO content in mucin residues was used as one of the indicators of oligosaccharide chain degradation. The recovery percentages of mucin CHO in samples incubated BBMN68 and BBL range were 95.17±1.16% and 94.44±1.28% respective. The recovery of CHO in samples incubated with *C.perfringens* strain dropped to a low level 26.49±2.68 % (Table 3).

Mucin residues were also analyzed by SDS-PAGE stained with Coomassie blue for protein residues (Figure 1). The sample incubated with bifidobacterium strains exhibited the same SDS-PAGE patterns in comparison to the blank. However, SDS-PAGE of *C.perfringens* yielded patterns of degraded mucin. These observations indicated that BBMN68 had no mucin degradation activity.

Bacterial translocation

No noticeable behavioral or activity changes were observed in the mice after treatment with BBMN68 and no treatment-related illness or death occurred during the experimental period. There was no significant difference in body weight between the control group and BBMN68 treatment group (Table 4, $P>0.05$), and there were no significant differences in food intake throughout the experiment (Data not shown, $P>0.05$). Measurement of tissue weights showed that there were no significant differences in liver, spleen and kidney weights between the control group and the groups treated orally (Table 4, $P>0.05$).

We did not detect viable BBMN68 in samples of blood, kidney, spleen or liver from animals in any group. There was no bacterial growth on agar plates, which indicates that the viscera were not contaminated, and there was no translocation of bacteria from the gut into different tissues.

Oral toxicity study of BBMN68

In this study, oral toxicity test of BBMN68 culture (with livable cell 4×10^9 cfu/kg bw) and its 5-fold concentration (2×10^{10} cfu/kg bw) incubation culture were preformed. Mean bodyweight gain in

Table 1. Antimicrobial susceptibility profiles of BBMN68

Antibiotic	Concentration (µg/disc)	Inhibition Zone (mm)	Antibiotic susceptibility profiles[a]
Penicillin G	10	34.47±2.84	S
Ampicillin	10	31.00±0.39	S
Piperacillin	100	21.64±0.58	S
Amoxicillin/Clavulanic Acid	20/10	35.13±1.00	S
Cephalothin	30	29.62±1.19	S
Ceftazidime	30	33.51±0.43	S
Cefepime	30	42.81±0.24	S
Cefotaxime	30	44.84±0.28	S
Norfloxacin	10	19.85±1.36	S
Gatifloxacin	5	22.46±0.82	S
Ciprofloxacin	5	27.26±0.68	S
Erythromycin	15	38.08±1.26	S
Neomycin	30	17.41±0.31	M
Kanamycin	30	23.47±1.74	S
Gentamycin	120	19.15±0.75	S
Gentamycin	10	12.84±0.16	M
Streptomycin	300	21.48±1.24	S
Streptomycin	10	13.04±0.68	M
Tetracycline	30	38.38±0.58	S
Vancomycin	30	24.71±0.94	S
Rifampicin	5	31.01±0.93	S
Clindamycin	2	23.93±0.15	S
Chloramphenicol	30	39.73±0.35	S

[a]R: resistant, M: moderately susceptible, S: susceptible

Table 2. Growth of BBMN68 and control strain in different culture media

	Basal medium		0.5%HGM		1%glucose		0.5%HGM+1%glucose	
	OD_{600}	pH	OD_{600}	pH	OD_{600}	pH	OD_{600}	pH
BLANK[b]	0.055±0.002	7.19±0.01	0.083±0.006	7.18±0.01	0.051±0.003	7.18±0.02	0.093±0.008	7.16±0.02
BBMN68	0.061±0.010	7.13±0.01	0.095±0.004	7.17±0.01	1.678±0.008*	3.99±0.01*	1.559±0.007*	4.02±0.01*
BBL	0.052±0.001	7.14±0.01	0.096±0.001	7.11±0.02	1.804±0.002*	4.03±0.01*	1.694±0.003*	3.99±0.02*
C. perfringens	0.589±0.030	6.91±0.14	1.134±0.012*	6.34±0.01*	1.976±0.010*	4.42±0.05*	1.766±0.009*	4.31±0.01*

* $P < 0.05$ compared to basal medium

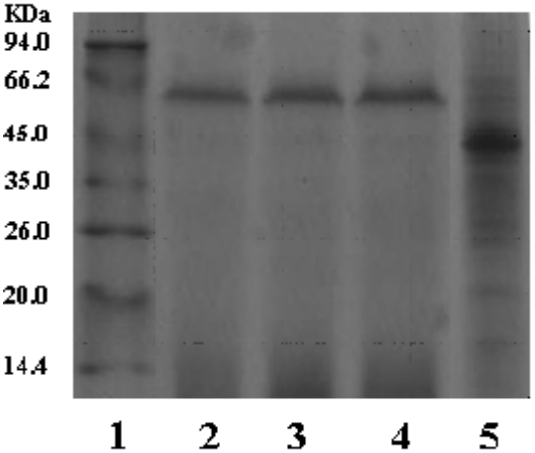
the BBMN68 culture groups (male: 29.0±1.1 g; female: 27.4±1.1 g) were similar to the control groups (male: 29.0±0.9 g; female: 27.2±0.9 g; $P>0.05$), and that in the 5-fold concentration culture groups (male: 28.3±1.0 g; female: 28.3±1. 2g) also did not differ significantly from the control group (male: 29.0±1.5 g; female: 27.7±1.1 g; $P>0.05$). No mortality or adverse effects occurred in control group or in both groups which administered BBMN68 culture.

Acute oral toxicity study of BBMN68

In this study, BBMN68 frozen culture (viable count, 3×10^{10} cfu/g) was used test materials to evaluate its toxicity by an acute oral toxicity study. Mortalities did not occur in mice administrated a dose of 15.45 g/kg bw (4.6×10^{11} cfu/kg bw) of test material throughout 14 days observation period, necropsy result of both the male and female animal dose groups showed no anomalous findings. There was no evidence of any toxicity of BBMN68 culture or vialbe cell in the acute toxicity study.

Table 3. Recovery rate of mucin CHO in basal medium with 0.5%HGM

	Recovery(%)
BBMN68	95.17±1.16
BBL	94.44±1.28
C. perfringens	26.49±2.68



1: protein standard ladder; 2:blank; 3: BBMN68; 4: BBL; 5 *C. perfringens*

Fig. 1. SDS-PAGE analysis of mucin residues

Table 4. Impact of BBMN68 on body and organ weights of mice

Treatment	Female/Male	Body (g)	Liver (g)	Kidney (g)	Spleen (g)
Control	Female	27.33±1.00	1.11±0.14	0.33±0.05	0.11±0.03
	Male	22.53±1.14	0.86±0.10	0.29±0.04	0.09±0.01
BBMN68	Female	26.87±0.98	0.92±0.16	0.34±0.05	0.11±0.02
	Male	23.69±1.29	0.91±0.06	0.32±0.02	0.11±0.04

DISCUSSION

Although Bifidobacteria is generally considered as safe for dietary use, this safety status should be carefully assessed in each new potential probiotic bacterium, since some of the properties that confer toxicity or infectivity are strain-specific. Our previous study showed that BBMN68 had good ability to immunostimulate, improve intestinal digestion and enhance immune barrier function[11,12]. In this study, we assessed the safety of BBMN68 by analyzing antibiotic susceptibility and mucin degradation activity, bacterial translocation ability, oral toxicity and acute oral toxicity.

As regards the interaction with intestinal microbiota, there is a risk involved in the use of probiotic bacteria resistant to some antibiotics, particularly when this resistance can be transferred to other strains, species and even genera, including potential human or animal pathogens[2]. Compared with lactobacilli, strains usually used as probiotics, bifidobacteria appear to be more susceptible to antibiotics. In this study, the disc assay showed that BBMN68 was sensitive to Penicillins, Beta-lactamase inhibitors, cephalosporin, quinolones, macrolides, tetracyclines, Glycopeptides, except for low-level aminoglycosides (Neomycin, Gentamycin & Streptomycin). This was consistent with a previous study on antimicrobial susceptibility of bifidobacteria[14,15]. The phenomenon might be explained by the fact that anaerobic bacteria was generally lack of cytochrome-mediated drug transport[16]. Moubareck reported potentially acquired resistance of tetracycline and minocycline was observed in a proportion of 14% of tested bifidobacterial strains[15]. Our study showed that BBMN68 strain was sensitive to tetracycline. Our results suggested that the strain was resistant to the tested antibiotics, and seemed to be suitable for administration during antibiotic treatment. However, since the spreading of antibiotic resistance genes by probiotic strains is clearly undesirable, the strains should be tested for the presence of transferable resistance genes before being used as probiotic cultures in the further study.

The mucus layer (mucin) coating the surface of the gastrointestinal tract plays an important role in the mucosal barrier system. Any damage or disturbance of this mucin layer will compromise the host's mucosal defence function[17]. Therefore, the ability to degrade mucin has been recommended as an important indicator of potential pathogenicity and local toxicity of lumen bacteria[4]. Although many bifidobacteria strains were regarded to be safe, Vankerckhoven reported that some Bifidobacterium species such as *B. bifidum* and a few strains of *B. breve* and *B. longum* were able to degrade intestinal mucin *in vitro* to different extents[18]. In the present study, no significant change in the optical density at 600 nm of the culture was found following incubation with the test strains in the medium presence of 0.5% HGM without glucose after 48 h, no mucin fragments were derived from the mucin suspension incubated with test strains, these results demonstrated that the test bifidobacteria strains were unable to degrade gastrointestinal mucin *in vitro*, which suggests the novel probiotic candidate, BBMN68 strain is likely to be non-invasive at the mucosal interface.

Bacterial translocation is the passage of bacteria from the intestinal tract to extra-intestinal sites and organs and is considered as the first step in the developing bacterial infections from intestinal pathogens[19]. Therefore, it constitutes an important safety parameter for potential probiotics that has been examined so far in the literature. Although very scarce, some cases of

bifidobacterium translocation have been recently reported. Yamazaki observed that *B. longum* BB536 translocated into liver, kidney and mesenteric lymph nodes at one or two weeks after administration in germ-free mice, although no illness or death occurred[20]. On the other hand, BBMN68 was not detected in samples of blood, kidney, spleen or liver from animals in any group, which implied the orally administered BBMN68 does not appear to possess the potential to translocate to the examined tissues.

Conventional oral toxicology evaluation has been advocated as a fundamental test for assessing safety, and was widely applied in safety assessment for probiotic studies[5,6,21]. In this study, viable BBMN68 strain was administrated in oral toxicity study in mice. Either the fresh incubated culture (4×10^9 cfu/kg bw) or 5-fold concertration incubated culture (2×10^{10} cfu/kg bw) was well-tolerated in mice. In acute oral toxicity test, no treatment-related systemic or local toxicity was detected, the mice maximum tolerable dose of BBMN68 frozen cultures (viable count, 3×10^{10} cfu/g) was greater than 15g/kg bw/day (4.6×10^{11} cfu/kg bw). Based on the results of this work, The acceptable daily intake for a 70 kg person would be 10.5g frozen cultures per day (considering the safety factor of 100), which is hundred times the amount of probiotic normally recommended for human consumption[2].

In conclusion, the studies described in this paper were conducted as a comprehensive safety assessment of BBMN68. Based on the results form this study, we can conduct that the potential probiotic strain was antibiotic susceptibility, have no mucin degrading and translocation activity. Acute oral toxicity study also showed that the mice maximum tolerable dose of BBMN68 frozen cultures was greater than 4.6×10^{11} cfu/kg bw/day. The result suggests that consumption of BBMN68 at a normal dose should be safe for human, BBMN68 may be exploited as a probiotic and culture in food and dairy industry.

ACKNOWLEDGMENTS

We gratefully acknowledge the financial support from the Ministry of Science and Technology of China (2012BAD12B08), International Science and Technology Cooperation Program of China (2011DFA32550), Research Foundation for Youth Scholars of Beijing Technology and Business University and Beijing Municipal Commission of Education Co-constructed Program.

REFERENCES

1. Parvez, S., Malik, K.A., Kang, S.A., Kim, H.Y. Probiotics and their fermented food products are beneficial for health. *J. Appl. Microbiol.*, 2006; **100**(6):1171-85.
2. Vasijevic, T., Shah, N.P. Probiotics - From Metchnikoff to bioactives. *Int. Dairy J.*, 2008;**18**(7):714-28.
3. ESFA. Introduction of a Qualified Presumption of Safety (QPS) approach for assessment of selected microorganisms referred to EFSA - Opinion of the Scientific Committee. *EFSA J.*, 2007; **587**:1-16.
4. Fernandez, M.F., Boris, S., Barbes, C. Safety evaluation of *Lactobacillus delbrueckii subsp. lactis* UO 004, a probiotic bacterium. *Res. Microbiol.*, 2005; **156**(2):154-60.
5. Yakabe, T., Moore, E.L., Yokota, S., Sui, H., Nobuta, Y., Fukao, M., Palmer, H., Yajima, N. Safety assessment of Lactobacillus brevis KB290 as a probiotic strain. *Food Chem. Toxicol.* , 2009; **47**(10): 2450-3.
6. Zhou, J.S., Shu, Q., Rutherfurd, K.J., Prasad, J., Birtles, M.J., Gopal, P.K., Gill, H.S. Safety assessment of potential probiotic lactic acid bacterial strains *Lactobacillus rhamnosus* HN001, *Lb. acidophilus* HN017, and *Bifidobacterium lactis* HN019 in BALB/c mice. *Int. J. Food Microbiol.*, 2000; **56**(1):87-96.
7. Saarela, M., Matto, J., Mattila-Sandholm, T. Safety aspects of Lactobacillus and Bifidobacterium species originating from human oro-gastrointestinal tract or from probiotic products. *Microbial Ecol. Health Dis.*, 2002; **14**: 233-40.
8. Abe, F., Muto, M., Yaeshima, T., Iwatsuki, K., Aihara, H., Ohashi, Y., Fujisawa, T. Safety evaluation of probiotic bifidobacteria by analysis of mucin degradation activity and translocation ability. *Anaerobe* , 2010; **16**(2):131-6.
9. Szabo, N.J., Dolan, L.C., Burdock, G.A., Shibano, T., Sato, S., Suzuki, H., Uesugi, T., Yamahira, S., Toba, M., Ueno, H. Safety evaluation of *Lactobacillus pentosus* strain b240. *Food Chem. Toxicol.*, 2011; **49**(1):251-8.
10. Zhou, J.S., Shu, Q., Rutherfurd, K.J., Prasad, J., Gopal, P.K., Gill, H.S. Acute oral toxicity

and bacterial translocation studies on potentially probiotic strains of lactic acid bacteria. *Food Chem. Toxicol.*, 2000; **38**(2-3):153-61.

11. Yang, H., Liu, A., Zhang, M., Ibrahim, S.A., Pang, Z., Leng, X., Ren, F. Oral administration of live Bifidobacterium substrains isolated from centenarians enhances intestinal function in mice. *Curr. Microbiol.*, 2009; **59**(4):439-45.
12. Yang, H.Y., Liu, S.L., Ibrahim, S.A., Zhao, L., Jiang, J.L., Sun, W.F., Ren, F.Z. Oral administration of live Bifidobacterium substrains isolated from healthy centenarians enhanced immune function in BALB/c mice. *Nutr. Res.*, 2009; **29**(4):281-9.
13. Wikler, M.A. Performance standards for antimicrobial susceptibility testing: fifteenth informational supplement. Clinical and Laboratory Standards Institute. 2009.
14. Masco, L., Van Hoorde, K., De Brandt, E., Swings, J., Huys, G. Antimicrobial susceptibility of Bifidobacterium strains from humans, animals and probiotic products. *J. Antimicrob. Chemother.*, 2006; **58**(1):85-94.
15. Moubareck, C., Gavini, F., Vaugien, L., Butel, M.J., Doucet-Populaire, F. Antimicrobial susceptibility of bifidobacteria. *J. Antimicrob. Chemother.*, 2005; **55**(6):38-44.
16. Bryan, L.E., Kowand, S.K., Van Den Elzen, H.M. Mechanism of aminoglycoside antibiotic resistance in anaerobic bacteria: *Clostridium perfringens* and *Bacteroides fragilis*. *Antimicrob. Agents Chemother.*, 1979; **15**(1):7-13.
17. Zhou, J.S., Gopal, P.K., Gill, H.S. Potential probiotic lactic acid bacteria *Lactobacillus rhamnosus* (HN001), *Lactobacillus acidophilus* (HN017) and *Bifidobacterium lactis* (HN019) do not degrade gastric mucin *in vitro*. *Int. J. Food Microbiol.*, 2001; **63**(1-2):81-90.
18. Vankerckhoven, V., Huys, G., Vancanneyt, M., Vael, C., Klare, I., Romond, M.B., Entenza, J.M., Moreillon, P., Wind, R.D., Knol, J., Wiertz, E., Pot, B., Vaughan, E.E., Kahlmeter, G., Goossens, H. Biosafety assessment of probiotics used for human consumption: recommendations from the EU-PROSAFE project. *Trends Food Sci.Technol.*, 2008;**19**:102-14.
19. Liong, M.T. Safety of probiotics: translocation and infection. *Nutr. Rev.*, 2008; **66**(4):192-202.
20. Yamazaki, S., Machii, K., Tsuyuki, S., Momose, H., Kawashima, T., Ueda, K. Immunological responses to monoassociated *Bifidobacterium longum* and their relation to prevention of bacterial invasion. *Immunology*, 1985; **56**(1):43-50.
21. Endres, J.R., Clewell, A., Jade, K.A., Farber, T., Hauswirth, J., Schauss, A.G. Safety assessment of a proprietary preparation of a novel Probiotic, *Bacillus coagulans*, as a food ingredient. *Food Chem. Toxicol.*, 2009; **47**(6):1231-8.