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RESEARCH ARTICLE

PROBIOTIC ATTRIBUTES OF TWO STRAINS OF *Bifidobacterium* animalis subsp. *lactis* –AN *IN –VITRO* ASSESSMENT

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ABSTRACT

Strains of Bifidobacterium animalis subsp. *lactis* are extensively incorporated in commercial probiotic preparations due to a number of technological and physiological advantages attributed to them over other members of the same genus. High strain specificity is being reported among microorganisms in their probiotic attributes making it practically difficult to extrapolate the results obtained for one organism to another at the species or even at the strain level. In this context a study was conducted to assess the probiotic properties of two strains of *Bifidobacterium animalis* subsp. *lactis*, namely *Bifidobacterium animalis* subsp. *lactis* B420 and Bifidobacterium animalis subsp. *lactis* Bb-12 in terms of their acid, bile salt, lysozyme tolerances, cell surface hydrophobicity and antimicrobial activity**.** On assessing their tolerance to various stresses, both the strains exhibited remarkably high acid, bile and lysozyme tolerance to the extent that the survivability at pH 2 after 3 hours of exposure was more than 98% and after 12h of exposure at 3% bile more than 80% of the cells were viable. The cell surface hydrophobicity in terms of adhesion to n-hexadecane was found to be more than 85% for both the strains .The antagonistic activity exhibited by the tested bifidobacterial strains were also at par with each other. On statistically analysing the data no significant difference was observed between the two bifidobacterial cultures for any of the tested probiotic attributes substantiating their wide use and popularity as probiotic cultures.

Key Words: *Bifidobacterium animalis* subsp. *lactis*, Acid, Bile, Lysozyme tolerance, Cell surface hydrophobicity**.**

INTRODUCTION

Bifidobacterium, a genus identified as one of the dominant anaerobic population of the colonic microbiota is constituted by Gram-positive, non-spore-forming, non-motile and catalase-negative anaerobic microorganisms (Sgorbati *et al*., 1995). Members of this genus are considered as one of the most important group of intestinal organisms due to the vital role they play in human health and they are also widely recognized for their probiotic attributes (Roy, 2005). A number of parameters like the ability to survive the gastrointestinal transit, adherence to intestinal cells and anti-microbial properties are being considered as the essential attributes to be tested while screening potential probiotic strains. Many of the previous studies have reported a high degree of variability between strains in their tolerance to acid and bile salt (Pereira and Gibson 2002, Vernazza *et al*., 2006). It is also being observed that the probiotic attributes of different bacterial strains are found to vary even within the species (Soccol *et al*., 2010). The bifidobacterial strains commonly incorporated in commercial probiotic products are *B. animalis* subsp. *lactis, B. bifidum, B. breve, B. longum* subsp. *infantis* and *B. longum* (Holzapfel *et al*., 2001). Among these, the species *B. animalis* is the most widely used one due to its high tolerance to oxygen and acids (Palaria *et al*. 2012). In this context a study was conducted to assess two commercially available strains of *B animalis* subsp. *lactis* namely *Bifidobacterium animalis* subsp. *lactis* B420 and *Bifidobacterium animalis* subsp. *lactis* Bb-12 for various probiotic attributes like acid, bile, lysozyme tolerances, cell surface hydrophobicity and antimicrobial activity.

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MATERIALS AND METHODS

Bacterial cultures

Two *Bifidobacterium* cultures namely, *Bifidobacterium animalis* subsp. *lactis* Bb-12 (Bb-12, Chr. Hansen, Denmark), *Bifidobacterium animalis* subsp. *lactis* B420 (B-420, Danisco, Germany) were used in this study. The bifidobacterial cultures were maintained in modified MRS broth (mMRS broth, MRS broth + 0.05% L-cysteine hydrochloride, Arroyo *et al*., 1994) with weekly sub-culturing. Active cultures were prepared by 2 to 3 transfers in mMRS broth followed by anaerobic incubation (Anaero Hi Gas Pack, HiMedia Laboratories Ltd. Mumbai) at 37°C for 24 h. Indicator organisms used were *Escherichia coli* NCDC247, *Salmonella typhimurium* NCDC113, *Staphylococcus aureus* NCDC109, *Shigella dysenteriae* NCDC107, *Enterococcus faecalis* NCDC116 (National Collection of Dairy Cultures, DM Division, NDRI, Karnal). They were maintained in nutrient agar slants and the sub-culturing was done after every 14 days. The cultures were activated by 2-3 transfers in nutrient broth followed by incubation at 37°C for 24 h.

Acid, bile salt and lysozyme tolerance

24 h old active cultures of *Bifidobacterium* strains were inoculated at 2 percent level into sterile distilled water adjusted

to various pH levels (1.5, 2.0, 2.5) as well as to mMRS broth with varying concentrations of bile salt (0, 2, 2.5 and 3%) or lysozyme (100 ppm, 0.1mg/ml, 100mg/L) and incubated anaerobically at 37°C. For assessing the acid tolerance one milliliter from each tube was taken immediately (0 h), after 1, 2, 3 h and appropriate dilutions were plated on Bifidobacterium agar (HiMedia Laboratories Ltd., Mumbai) (Clark *et al.*, 1993). For determining the bile salt resistance, samples were taken immediately (0 h), after 3 and 12 h and proceeded as mentioned in the case of acid tolerance (Gilliland and Walker, 1990). The lysozyme tolerance was determined by plating appropriate dilutions at 0 and 24 h (Brennan *et al.*, 1986). In all the cases the colonies developed were counted after 48 h of anaerobic incubation at 37°C. The percentage survival of the organism was determined as per the equation given below (FAO/WHO, 2001).

$$\text{Percentage survival} = \frac{\text{Log number of viable cells after treatment}}{\text{Log number of viable organisms before treatment}} \times 100$$

Cell Surface Hydrophobicity

The cell surface hydrophobicity of bifidobacterial cultures was assessed by the BATH (bacterial adhesion to hydrocarbons) assay using n-Hexadecane as per Rosenberg *et al*. (1980) with slight modifications. *Bifidobacterium* cells, grown anaerobically at 37°C for 18 h, were harvested by centrifuging at 12000 rpm for 10 min at 4°C. The pellet obtained was resuspended in Phosphate urea magnesium sulphate (PUM) buffer ($K_2HPO_4.3H_2O$-22.2g/l, KH_2PO_4- 7.26g/L, Urea-1.8g/L, $MgSO_4$ -0.2g/L, Ph- 7.1) and washed twice by centrifuging as mentioned above. The washed pellet was resuspended in PUM buffer and the absorbance was adjusted to 0.8 to 0.9 at 610 nm. To 4.8 ml of this bacterial suspension 0.8 ml of n-Hexadecane was added and incubated at 37°C for 10 min for temperature equilibration. After this, the two phases were mixed using a vortex mixer for 2 min at full speed. The mixed solution was kept at 37°C for 1 h for phase separation. The lower aqueous phase was separated with the help of glass pipette and the light absorbance of the aqueous phase was determined at 610 nm. The fraction of adherent cells was taken as percent decrease in absorbance of the aqueous phase after mixing and phase separation as compared to that of original suspension. The cell surface hydrophobicity was calculated as follows:

$$\text{Cell surface hydrophobicity (\%)} = \frac{\text{Initial O.D} - \text{Final O.D.}}{\text{Initial O.D.}} \times 100$$

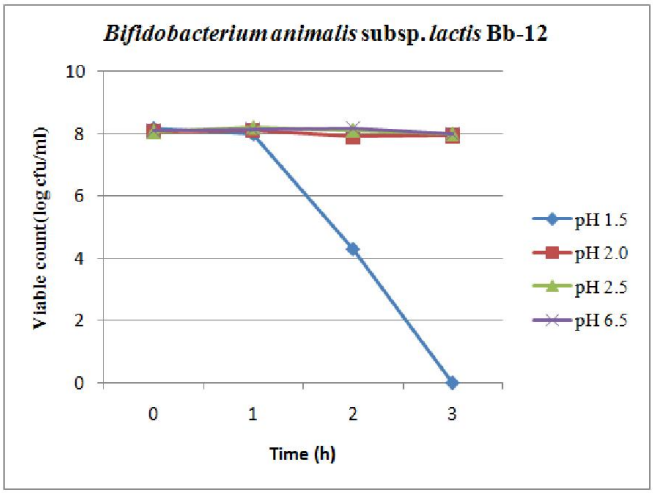
Antagonistic Activity against Enteric Organisms

The antimicrobial activity was determined by the Agar well method (Anand *et al*., 1984). For this nutrient agar containing 0.1 percent Tween-80 was seeded with the test culture (*E. coli* NCDC247, *S. typhimurium* NCDC113, *S. dysenteriae* NCDC107, *E. faecalis* NCDC116, *S. aureus* NCDC109) at the rate of one percent and poured into the plates. Wells of 0.7 cm were made on solidified seeded agar and hundred µl of the 24 h old culture of bifidobacterial strains was poured into wells. Plates were incubated at 37°C for 48 h and the diameter of the zone of inhibition formed was measured. Data was statistically analyzed using ANOVA according to the General Linear Models procedure of Systat Version 6.0.1 (1996, SPSS Inc.). When significant (1 and 5% levels) differences were observed individual values were compared by Fisher's Least Significant difference.

RESULTS AND DISCUSSION

Acid, bile salt and lysozyme tolerance of bifidobacterial cultures

The ability of *Bifidobacterium animalis* subsp. *lactis* Bb-12 and *Bifidobacterium animalis* subsp. *lactis* Bb-420 to survive at different levels of pH, bile salt or lysozyme is depicted in Figure 1. Both the strains exhibited somewhat similar pattern in their ability to survive their exposure to different stress conditions. They were found to be highly tolerant to pH levels of 2.0 and 2.5, as there was no marked reduction in their cell numbers even after 3 h of incubation at these pH levels. At the lowest pH tested (pH 1.5) also, good survivability was shown by both the strains even after 2 hours of exposure. However after these 2 hours of exposure, a marked reduction in viability occurred as the count after three hours of exposure to this pH was drastically lower.



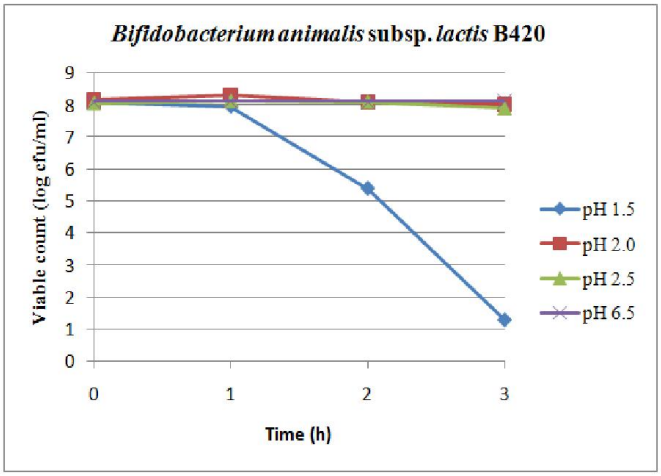
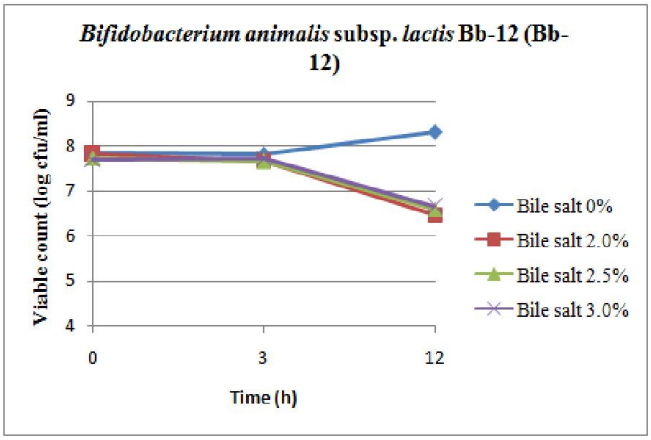


Figure 1a. Survival of Bifidobacterial strains after 1, 2, 3 hours of exposure to different pH levels (1.5, 2.0, 2.5,6.5)



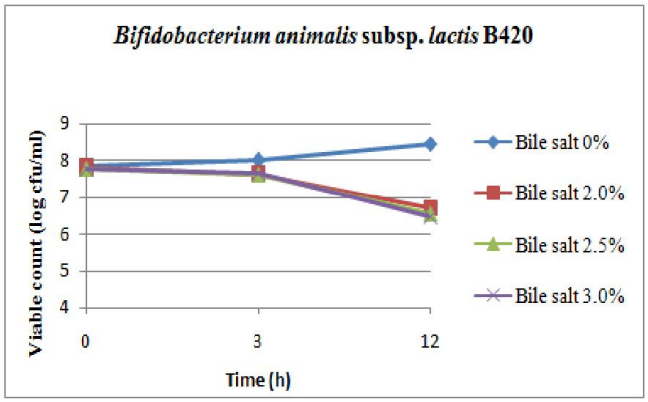
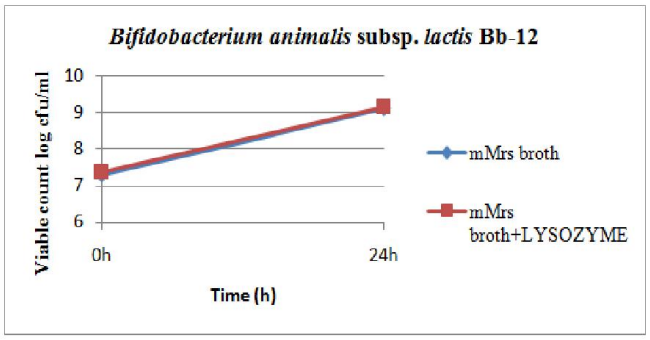


Figure 1b. Survival of Bifidobacterial strains after 3 and 12 hours of exposure to different bile salt concentrations levels (2.0, 2.5,3.0 %)



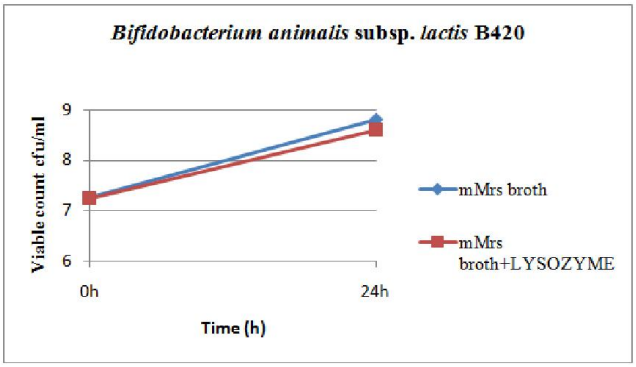


Figure 1c. Survival of Bifidobacterial strains after 24 hours of exposure to 100ppm Lysozyme

The viability of the cultures at pH 1.5 was significantly lower than their viability at other pH levels used in this study and no significant difference in the viability of cultures was observed at pH 2.0, 2.5 and 6.5. Both the strains exhibited high acid tolerance and on statistical analysis no significant difference was observed between the cultures in terms of their pH tolerance ($P<0.05$). In the case of bile tolerance also, both the strains performed extremely well. At all the bile salt concentrations tested (2, 2.5 and 3%), the viable count of both the strains remained as that of the initial levels and almost similar to that of control (0% bile salt concentration) even after three hours of exposure. However, after 12 hours of exposure a marked reduction was observed in the viable count of treated samples. Despite this drastic reduction in viable count, remarkably high survivability (more than 83% of initial count) was shown by both strains even at the highest bile concentration tested i.e. 3%. As in the case of acid tolerance, no significant difference was observed between Bb-12 and B-420 in terms of bile tolerance also. It was also observed that both the strains could tolerate and even grow in the presence of lysozyme at 100 ppm levels reaching to a population similar to that obtained in the medium devoid of lysozyme. In the case of lysozyme tolerance also, no significant difference was observed between the two cultures.

On determining the percentage survivability of the strains at the highest stress levels they could tolerate (pH 2, bile concentration of 3%, Table 1), it was found that both the strains exhibited more than 98% and 83% survivability on exposure to this high acidity and bile salt concentration respectively. The more than 98% survivability observed at pH 2 after 3 hours of exposure by these two bifidobacterial strains is profoundly higher than the 44.05 +_ 1.70 % viability reported by Sanchez *et al*., (2007) for the acid-pH-resistant mutant *B. longum* biotype longum 8809dpH. The high tolerance exhibited by both the strains indicates that they are equally competent in terms of acid and bile tolerance, substantiating their wide use as probiotic bifidobacterial strains for incorporation in fermented milks.

During their gastrointestinal transit microorganisms have to face a number of natural defence mechanisms like the presence of lysozyme in saliva, the acidic environment of the stomach, and the bile secretions in the small intestine. These protection mechanisms are equally effective against beneficial organisms also. So their ability to withstand these constraints is an important criteria while selecting potential probiotic organisms, as it is essential that they should reach their site of action, i.e., the colon in a physiologically active form to elicit many of their beneficial effects. In the present study, pH 1.5 was observed as the most lethal among all the pH levels used. The results are in accordance with several studies, which reported a substantial reduction in the viability of cells at pH 2.0 or below (Clark *et al.*, 1993, Lankaputhra and Shah, 1995). In general, with the exception of *Bifidobacterium animalis*, all Bifidobacteria are reported to have weak acid tolerance (Maus and Ingham, 2003). The high acid and bile salt resistance of *Bifidobacterium animalis* subsp. *lactis* Bb-12 is being reported in a number of previous studies (Vernazza *et al*., 2006, Jungersen *et al*., 2014). In agreement with these we also observed very high tolerance by both the tested strains of *Bifidobacterium animalis* subsp. *lactis*. The results are in accordance with that of Alander *et al*. (2001) who observed comparable pH and bile tolerance between *B. lactis* Bb-12 and *Bifidobacterium* species 420. The high acid tolerance of *Bifidobacterium animalis* subsp. *lactis* Bb-12 is attributed

Table 1. Percentage survivability of *Bifidobacterium* strains at pH 2 and bile salt concentration of 3%

Probiotic strain	Viable cell count at pH 2(log cfu/ml)		% survivability	Viable cell count at 3% bile salt (log cfu/ml)		% survivability
	0h	3h		0h	12h	
B-420	8.146	8.021	98.5%	7.785	6.477	83.2%
Bb-12	8.08	7.95	98.4%	7.699	6.634	86.2%

Table 2. Cell surface hydrophobicity and Antimicrobial activity of *Bifidobacterium* strains

Culture Name	Percent Hydrophobicity	Zone of Inhibition (*diameter, mm, exclusive of well diameter 7 mm)*			
		E.coli CDC247	*S. typhimurium* NCDC113	*S.dysenterie* NCDC107	*S.aureus* NCDC10
Bifidobacterium animalis subsp. lactis Bb-12	88.77	15.00	9.50	11.50	9.50
Bifidobacterium animalis subsp. lactis B420	94.51	14.50	11.25	11.50	11.00

partly to the low pH induction of H^+-ATPase activity, an enzyme complex involved in maintaining intracellular pH homeostasis in bacteria (Matsumoto *et al*., 2004). Studies of Takahashi *et al*., (2004) also suggested that the acid tolerance response of bifidobacterial strains may be attributed to their cytoplasmic pH homeostasis system. Sa´nchez *et al*., (2007) reported the involvement of a number of changes in the levels of different proteins jointly capable of controlling the intracellular pH in the acid adaptation and response to acid pH shown by a bifidobacterial strain.

In the present study both the bifidobacterial strains exhibited remarkably high resistance to bile salt also, retaining more than 80% viability even after 12 hours of exposure to 3% bile. It is being reported that bifidobacterial strains adapted to high bile salt concentrations develops cross resistance to other bile salts, and an increase in the survival at low pH (Noriega *et al*., 2004). Another study reported an increase in membrane-bound H_-ATPase activity, an attribute associated with maintenance of intracellular pH homeostasis, in response to exposure to bile in a strain of *Bifidobacterium animalis* (Sa´nchez *et al*., 2006).These studies suggest the existence of common mechanisms imparting both acid and bile salt resistance. Such an association between the acquisition of resistance to acid pH and bile salt tolerance, has been postulated for a number of *Bifidobacterium* strains (Saarela *et al*., 2004, Sanchez *et al*., 2007). Such a mechanism can be hypothesized for the bifidobacterial strains of the current study also as both of them exhibited remarkably high acid as well as bile resistance.

Considering the fact that lysozyme is naturally present in saliva and other body fluids, it is inevitable that the probiotic bacterium has to interact with lysozyme during its gastrointestinal transit. Resistance to a lysozyme concentration of 25-35mg/l is being recommended as a criterion for the selection of lactic acid bacterial strain for use in dairy industry (Guglielmonti *et al*., 2007). In the current study, both the bifidobacterial strains were unaffected by the presence of lysozyme at a concentration of 100mg/l demonstrating their suitability for use in dairy industry. Lysozyme resistance exhibited by bifidobacteria is reported to be species and/or strain specific (Rada *et al*., 2010). The same study also reported *Bifidobacterium animalis* as the most susceptible species at a lysozyme concentration of 400mg/l and also inhibition of its growth in human milk samples containing 15 to 58mg/l lysozyme. However in the present study both the tested strains of Bifidobacterium animalis subsp. lactis could grow well at a lysozyme concentration of 100mg/l. Ability of some bifidobacterial strains to tolerate lysozyme concentration as high as 500mg/l is also being reported (Zinedine and Faid, 2007).As the lysozyme concentration used in the present study is at a lower level, further studies are to be carried to assess the extent of lysozyme tolerance exhibited by the bifidobacterial strains used in this study.

Cell surface hydrophobicity and Antimicrobial activity of *Bifidobacterium* strains

Adhesion of probiotic organisms to the intestinal mucosa is considered as an important factor for the elicitation of many of the probiotic health effects. As variations are observed in the adhesion properties of probiotic strains depending on their cell surface properties like hydrophobicity and extracellular protein profiles (Botes *et al*. 2008), determination of cell surface hydrophobicity is considered as an indirect method for the *in vitro* assessment of the ability of cells to attach to the intestinal mucosa. In the present study both the strains showed very high cell surface hydrophobicity; 88.77% by *Bifidobacterium animalis* subsp. *lactis* Bb-12 and 94.51% by *Bifidobacterium animalis* subsp. *lactis B420*. However there was no significant difference between the two strains in terms of cell surface hydrophobicity. The percentage cell surface hydrophobicity observed in the present study is markedly higher than the less than 5% level reported for *B. animalis* subsp. *lactis* DSMZ 10140 (the type strain of this subspecies) by Bevilacqua *et al*. (2012). The high cell surface hydrophobicity exhibited` by both the strains suggests their ability to adhere to intestinal epithelial cells, a feature contributory towards better possibilities of colonization of this organism and thereby improvement in their beneficial effects such as competitive exclusion of pathogenic organisms.

Inhibition of pathogens is considered as one of the major mechanism of action of probiotics. On assessing antibacterial activity by well diffusion method, both the strains exhibited a broad spectrum of antimicrobial activity, against both Gram negative organisms *Shigella dysenteriae* NCDC107, *Salmonella typhimurium* NCDC113, *Escherichia coli* NCDC24 and the Gram positive *Staphylococcus aureus* NCDC109, showing zones of clearance ranging from 9.50 to 15.0 mm (Table 2). Both the strains showed highest antagonistic potential against *Escherichia coli* NCDC24 (15.0 mm) followed by *Shigella dysenteriae* NCDC107 (11.50 mm). Both of them did not show any inhibitory activity against *Enterococcus faecalis* NCDC116 and no significant differences were observed between the bifidobacterial strains in their antibacterial activity ($P<0.05$). A number of mechanisms like production of inhibitory substances (organic acids, H_2O_2,

bacteriocins), competition for nutrients and sites of adherence (mucus, cell receptors), toxin removal/degradation and induction of host immune responses are being suggested for the inhibition of pathogens by probiotic microorganisms (Lievin *et al*., 2000). Production of organic acids as part of their normal metabolic processes is identified as one of the mechanisms for the bifidobacterial inhibitory activity against pathogens. Jungersen *et al*., (2014) suggested the direct effect of lactate and acetates rather than the lowering of pH caused by these acids as the reason for the antagonistic effect exhibited by *Bifidobacterium animalis* subsp. *lactis* Bb-12 against *E coli* and *C jejuni*.

In the present study no significant difference was observed between the two strains of *Bifidobacterium animalis* subsp. *lactis*, the Bb-12 and B-420 for any of the probiotic attributes tested suggesting that both of these strains are competent enough to survive the constraints during their gastrointestinal transit and elicit beneficial effects upon consumption as probiotics. Genome sequencing studies of different strains of *Bifidobacterium animalis* subsp. *lactis* including that of Bb-12 and B-420 has reported high sequence similarity among the strains of this subspecies (Milani *et al*., 2013). This finding is supportive to the observations of this study that despite the wide strain specificity reported for the probiotic attributes these two *Bifidobacterium animalis* subsp. *lactis* strains are found to be equally competent/similar in terms of their probiotic potential.

Conclusions

Parameters like survival during the gastrointestinal transit, adherence to mucus, exhibition of anti-microbial and immunostimulatory properties are being mentioned as the criteria for the *in vitro* evaluation and selection of probiotics in the guidelines generated by The Food and Agriculture Organization/World Health Organization (FAO/WHO). In this perspective, the present study could be considered as a comprehensive one in that it included the assessment of a number of probiotic parameters, i.e. acid, bile salt, lysozyme tolerances, cell surface hydrophobicity and antimicrobial attributes. Validating their widely acclaimed probiotic potential, both the tested strains, *Bifidobacterium animalis* subsp. *lactis* Bb-12 and *Bifidobacterium animalis* subsp. *lactis* B420 exhibited remarkably high acid, bile and lysozyme tolerance. The cell surface hydrophobicity, a feature considered as an indirect measure of the adhesion potential, of both the strains was found to be high with *Bifidobacterium animalis* subsp. *lactis* B420 showing 94.51% and *Bifidobacterium animalis* subsp. *lactis* Bb-12 exhibiting 88.77%. Both the strains were also found to be equally effective in their antagonistic activity exhibiting a broad spectrum of antimicrobial activity. Although high strain specificity is attributed to most of the probiotic properties, this study could not establish any significant difference between the two tested strains for any of the probiotic attributes assessed. This study also provided data on various probiotic attributes of two commercially available probiotic cultures which could effectively be utilized as a reference while screening and selecting potential probiotic candidates.

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