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Invitro preselection criteria for probiotic *Weissella Confusa* and *Bifidobacterium bifidum* strains of fermented cereals origin

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Abstract :

Probiotic traits of *Weissella confusa* and *Bifidobacterium bifidum* strains previously isolated from fermented cereals were evaluated. Strains of *Weissella confusa* and *Bifidobacterium bifidum* were tested for their *in vitro* tolerance to bile, resistance to low pH, cell adhesion, antagonistic and hemolytic activity. Among the two organisms, *Bifidobacterium bifidum* showed a better growth at pH 3.5 than *Weissella confusa* which increased with incubation time. Increase in the concentration of bile did not affect the multiplication of viable cells up to 0.8%, beyond which the multiplication was slow in the case of both *Weissella confusa and Bifidobacterium bifidum. Bifidobacterium bifidum* showed good adherence to the substratum than *Weissella confusa*. Both the organisms were non hemolytic and antagonistic against common enteric pathogens, which is an ideal characteristic of a probiotic.

Keywords : Probiotic, acid and bile tolerance, cell adhesion, hemolysis, antagonism.

INTRODUCTION:

The original observation of the positive role played by certain bacteria was first introduced by Russian scientist and Nobel laureate Élie Metchnikoff. The term "Probiotics" was first introduced in 1953 by Werner Kollath (Hamilton-Miller *et al.,* 2003). Contrasting antibiotics, probiotics were defined as microbial derived factors that stimulate the growth of other microorganisms. In 1989 Fuller defined 'probiotics' as "*A live microbial feed supplement which beneficially affects the host animal by improving its intestinal microbial balance"*, which is being widely used. Probiotics were thought to beneficially affect the host by improving its intestinal microbial balance, thus inhibiting pathogens and toxin producing bacteria (Metchnikoff, 1907). Today specific health promoting properties have been investigated and documented which include alleviation of chronic intestinal inflammatory diseases (Mach, 2006), and prevention and treatment of pathogen-induced diarrhea (Yan and Polk, 2006), urino- genital infections, and atopic diseases. The ability of lactobacilli and Bifidobacteria to survive in and colonize the gastrointestinal track has been associated with various health promoting properties and it has been found that the colonization of probiotic bacteria decreased with the increase in the age of the host (Ballongue, 2004). In the recent years there has been interest in incorporating these bacteria in live form (called probiotics) into food especially fermented milk to counteract harmful bacteria in the gastrointestinal track and to promote health effect (Schillinger *et al*., 2005, Tamime *et al.,* 2007). The criteria attributed for the selection of probiotic strains include acid and bile tolerance, survival through the gastrointestinal tract, ability to adhere to intestinal surfaces, exhibiting antimicrobial activity against potential pathogenic bacteria (Ouwehand *et al.,* 2004).He present article deals with probiotic potentiality of the strains of *Weissella confusa and Bifidobacterium bifidum* isolated from various fermented cereals.

MATERIAL AND METHODS

Selection of strains for potential Probiotic use

The organisms isolated from fermented cereals were evaluated for potential use as probiotics. Among them two different lactic acid bacteria present in the different fermented foods, viz. *Weissella confusa referred as* (S2) and *Bifidobacterium bifidum* as (S3) were selected for further study. They were maintained as a frozen stock at - 20°C in distilled water plus 20 % (v/v) glycerol and propagated twice in Man Rogosa Sharpe (MRS) broth (Oxoid Ltd., UK) (De Man *et al.,* 1960) at 30°C before use.

In vitro selection criteria

The selected probiotic organisms were subjected to various selection criteria, which include, acid tolerance, bile tolerance, cell adhesion, hemolytic and antimicrobial activity.

Media and culture conditions (Khalil *et al.,* 2007)

Prior to use, strains were sub cultured (1% v/v) in MRS broth. *Weissella Confusa* and *Bifidobacterium bifidum* were incubated at 37ÚC for 24-48h to obtain a concentration of approximately 10^7 cfu/ml.

Acid tolerance test (Khalil *et al.,* 2007):

Overnight cultures of the test isolates were inoculated into MRS broth previously adjusted to pH 2- 3.5 and 7 with 1 N NAOH/ or HCL. The cultures were incubated aerobically at 37°C for 4 h and the turbidity was

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Table 1. *In vitro* adhesion Assay

A1	Sample (S2)	Absor bance	A2	Sample (S3)	Absor bance
B1	10^3	0.13	B2	10^3	0.15
C1	10^4	0.18	C2	10^4	0.20
D1	10^5	0.25	D2	10^5	0.27
E1	10^6	0.46	E2	10^6	0.49
F1	10^7	0.90	F2	10^7	0.96

A1 – F1 – Wells in column 1, A2 – F2 – Wells in column 2, S2 – *Weissella confusa*, S3 – *Bifidobacterium bifidum.*

Table: 2. Antagonistic activity against food borne pathogens (Zone of inhibition in 100 ìl)

Food borne pathogens	*Weissella confusa* (S2)	*Bifidobacterium bifidum* (S3)
Staphylococcus aureus	14mm	10mm
Escherichia coli	12mm	15mm
Bacillus cereus	10mm	7mm
Salmonella enteritidis	15mm	12mm
Shigella dysentriae	12mm	9mm

Figures: Effect of different intestinal pH and bile on Probiotic Organisms *invitro*

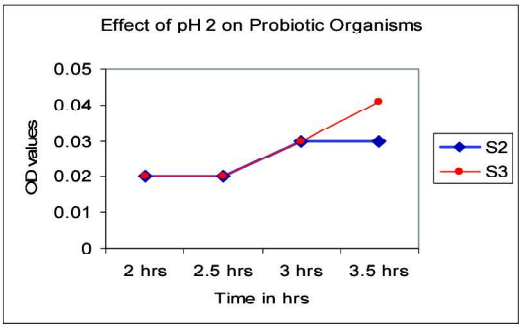

Fig. 1 Effect of pH 2 on Probiotic Organisms

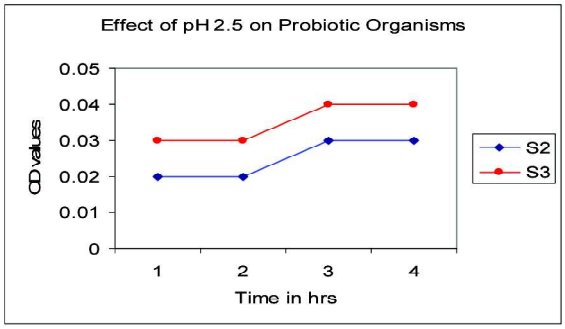

Fig. 2. Effect of pH 2.5 on Probiotic Organisms

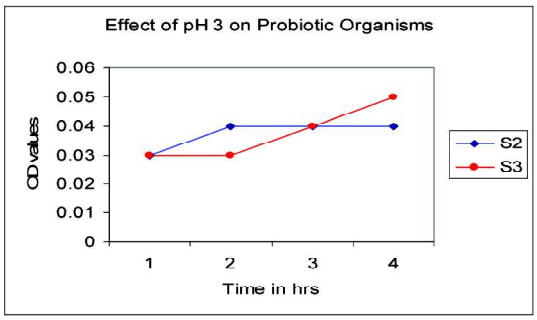

Fig. 3 Effect of pH 3 on Probiotic Organisms

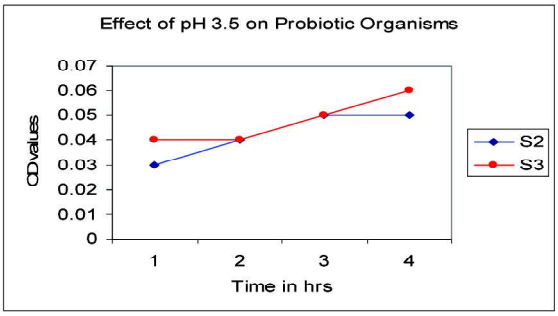

Fig. 4 Effect of pH 3.5 on Probiotic Organisms

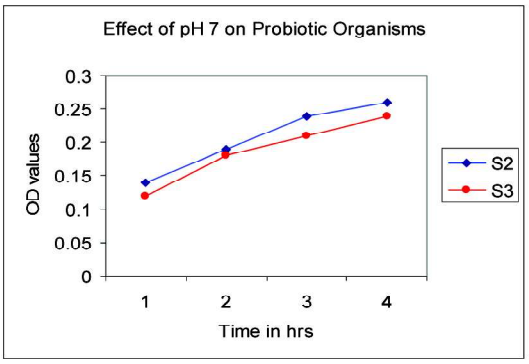

Fig. 5 Effect of pH 7 on Probiotic Organisms

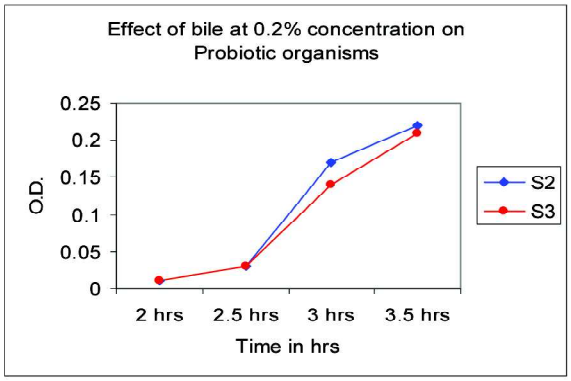

Figure.6 Effect of Bile 0.2% on Probiotic Organisms

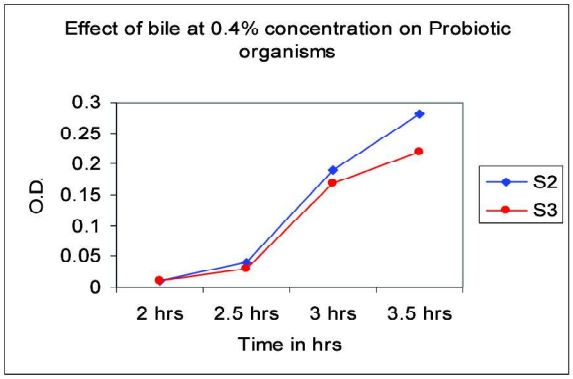

Figure.7 Effect of Bile 0.4% on Probiotic Organisms

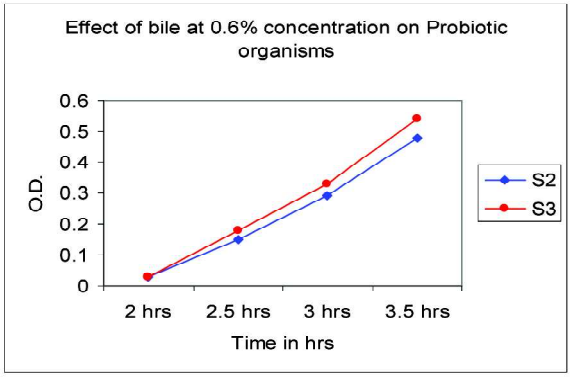

Figure.8 Effect of Bile 0.6% on Probiotic Organisms

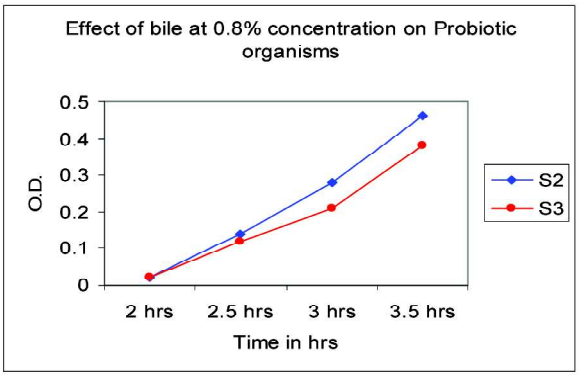

Figure. 9 Effect of Bile 0.8% on Probiotic Organisms

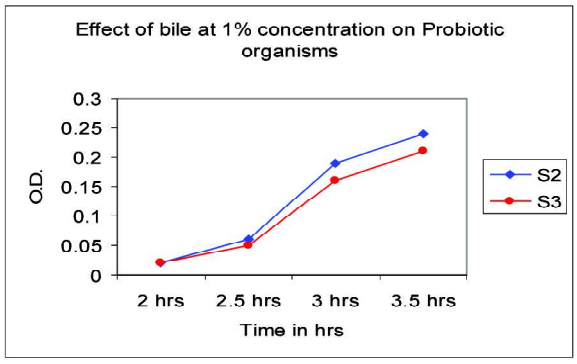

Figure. 10. Effect of Bile 1% on Probiotic Organisms

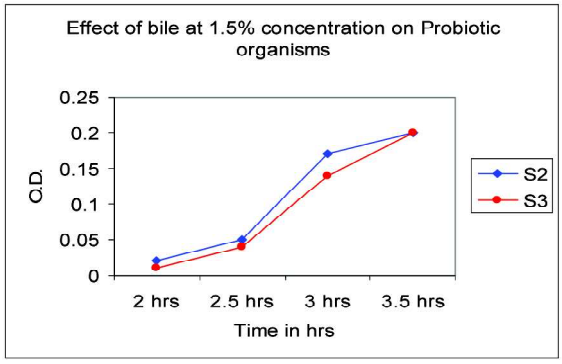

Figure. 11 Effect of Bile 1.5% on Probiotic Organisms

measured at 650nm at 30 minutes interval after two hours. Control broth was maintained at a pH 7.

Bile tolerance test (Khalil *et al.*, 2007)

Overnight cultures were inoculated into MRS broth containing 0.2 – 1.5% (w/v) of ox-gall, and incubated aerobically at 37ÚC for 4 h. The turbidity of the culture was determined at 650nm and at 30 minutes interval after two hours. Control was maintained in MRS broth without bile.

***In vitro* cell adhesion assay (Khalil *et al.*, 2007)**

Treatment of bacteria prior to adhesion

The isolates were propagated in MRS broth overnight at 37°C. Bacteria were harvested by centrifugation (10,000 X g for 10 minutes) at 4°C, and washed twice with phosphate buffer saline (PBS; 10mM, KH_2PO_4, 150mM NaCl, pH 7.2). The optical density was measured at 600nm to asses the growth of bacteria.

Preparation of intestinal mucosa (Ouwehand *et al.*, 1999)

Faecal samples containing mucosal cells were suspended in ice-cold PBS containing 0.5g/l NaN_3 to prevent bacterial growth. The suspension was shaken for 1 h at 4ÚC and centrifuged at 15,000x g for 30 minutes. From the clear supernatant, mucous was precipitated with 60% ice-cold ethanol, and dissolved in pure water and then resuspended in HEPES HANKS (HH) buffer (10mg/ml with pH 7.4).

***In vitro* adhesion assay (Vesterlund *et al.*, 2005)**

Mucous stock suspension was prepared by dissolving 10 mg/ml in HEPES HANKS (HH) buffer. Microtitre wells were coated with 150µl of intestinal mucous. 100 µl of test cultures with different concentration from 10^3-10^7 were added into Microtitre plate. Plate was incubated at 37ÚC for 1 hour, so that the bacteria could adhere to the mucous. Microtitre plate was washed thrice with 250µl of phosphate buffer saline (PBS). Then the plate was dried at 60ÚC for 20 minutes in hot air oven and stained with 100µl of crystal violet solution for 45

minutes. Wells were subsequently washed five times with phosphate buffer saline (PBS) to remove excess stain. 100µl of 20mM citrate buffer was added to the wells to remove excess stains from bacteria. After 45 minutes incubation at room temperature the absorbance was measured at 640nm.

Hemolytic activity

Isolates S2 and S3 were evaluated for hemolysis on Blood agar plates supplemented with 5% sheep blood which were incubated at 37°C for 24 h (Lombardi *et al.,* 2004).

Antimicrobial activity against human pathogens

The cell free filtrate of the isolates *Bifidobacterium bifidum* (S3) and *Weissella confusa* (S2)) were tested for their antimicrobial activity against enteric pathogens *Escherichia coli, Bacillus cereus, Staphylococcus aureus, Salmonella enteritidis* and *Shigella dysentriae* by using well diffusion assay method on Muller Hinton agar previously inoculated with 0.1 ml of 24 hrs. Broth culture of pathogenic bacteria, wells were made and filled with different concentrations (50 ìl, 75 ìl, 100 ìl) of cell free filtrate. Petri dishes were incubated at 37°C for 24 hrs. The antimicrobial activity was determined by measuring the clear zone developed around the wells.

RESULTS

Acid tolerance

The growth pattern of the test organisms under different pH are presented in (Figure-1-5). Among the two organisms, *Bifidobacterium bifidum* showed a better growth at pH 3.5 than *Weissella confusa* with increase in the incubation time.

Bile tolerance test

Increase in the concentration of bile did not affect the multiplication of viable cells up to 0.8% beyond which the multiplication was slow in the case of both strains (Figure 6-11).

***In vitro* cell adhesion assay**

When the test cultures at different concentration from 10^3-10^7 were added to the microtitre plate, there was a progressive increase in the OD value in case of both S2 and S3 cultures. This indicated that adhesion of S2 and S3 increased with increase in the concentration of organism from 10^3-10^7 (Table 1). The tubes containing the load 10^6 and 10^7 cells showed very high absorbance (0.46 and 0.96 respectively) in case of both S2 and S3, indicating a very high amount of cell adhesion to intestinal mucosa (Table 1).

Hemolytic activity

The strains of *Bifidobacterium bifidum (S3) a*nd *Weissella confusa (S2)* did not show hemolysis over blood agar plate.

Antimicrobial activity against human pathogens

The zone of inhibition formed against various enteric pathogens is presented in table 2. *Weissella confusa* showed better antagonistic activity against pathogens such as *E.coli, Staphylococcus aureus, Bacillus cereus, Salmonella enteritidis and Shigella dysteriae* when compared to *Bifidobacterium bifidum*. On other hand *Bifidobacterium bifidum* showed better antagonistic activity against *E. coli*.

DISCUSSION

Acid tolerance test

The viability of probiotic cells after consumption remains obscure as the bacteria are also subjected to unfavourable physiological conditions of the gastrointestinal (GI) tract such as acidic environment and bile secretions (Holzapfel *et al.,* 1998). The threshold point to determine acid resistance was set at pH value of 3.0 and incubation period of 3 hours (Prasad *et al.,* 1998; Haddadin *et al.,* 2004). This is in accordance with findings of Liong and Shah (2005) who stated that resistance at pH 3 is set as standards for acid tolerance of probiotic culture. According to Fernandez *et al*. (2003), Good probiotic sources should withstand at least pH 3.0. In the present study it was found that the increased duration of exposure to acid did not affect the viability of the cells of both the strains (Fig1-5). The ability of the isolates S2 and S3 to resist pH 2-3.5 even after 2 h revealed that these organisms tolerated the intestinal pH range of 2-3.5. Thus they satisfy the acid tolerance ability, and hence they could be used as potential probiotic strains.

Bile tolerance test

As bile stress takes place after pH stress in the stomach, Leyer and Johnson (1993) and Lin *et al,* (2006) postulated that sub-lethally injured microorganisms may have a different and unpredictable resistance to new stress factors. The enhanced survival capabilities of probiotics to bile appeared to be due to the acclimatization of the bacteria to the low pH environment, and thus minimise the relative toxicity to glycoconjugates in the intestine (Begley *et al*., 2005; Martoni *e*t al., 2007). The protective effect of food matrix also may prevent the bacteria from bile exposure and hence, giving rises to the increased bile resistance of the strains (Begley *et al.,* 2005). The bile concentration selected for the present study was in the range of 0.2-1.5%, which was more or less equivalent to the physiological concentration in the duodenum (0.4%) or the human bile juice. The viability of the strains of *Bifidobacterium bifidum* and *Weissella confusa* was seemed to improve when exposed to high levels of ox-gall (0.4%). Increase in the concentration of bile did not affect the multiplication of viable cells up to 0.8% beyond which the multiplication was slow in case of both *Weissella confusa* (S2) and (S3) *Bifidobacterium bifidum* (Fig.2). Thus

the strains *Bifidobacterium* and *Weissella confusa* showed greater acid tolerance and higher levels of bile salt.

***In vitro* cell Adhesion assay**

Adhesion to intestinal mucosal is an important property of probiotic organisms (Beachey, 1981; Chen *et al*., 1993; Schiffrin *et al*., 1997), Adherence to the intestinal epithelium and mucus is associated with stimulation of the immune system and adhesion to the intestinal mucosa is also crucial for transient colonization. Intestinal mucus has a dual role of protecting the mucosa from certain microorganisms while providing an initial binding site, nutrient source, and matrix on which probiotic bacteria can proliferate. Finlay and Falkow (1997 showed an important prerequisite for probiotics is to control the balance of the intestinal microûora. Tuomola *et al*. (1999) used human ileostomy glycoproteins as a model for the small-intestinal mucus to investigate adhesion of probiotics, and *in vitro* adhesion to mucous glycoproteins extracted from faeces has been shown to correlate with the adhesion to ileostomy glycoproteins Thus, *in vitro* evaluation of the adhesion to human intestinal mucus provides a suitable model for estimating the ability of probiotics to adhere to intestinal surfaces. From the study it is clear that both the organism showed good adhesion to intestinal mucosa.

Antimicrobial activity against human pathogens

The enteric pathogens *E.coli*, *Staphylococcus aureus, Bacillus cereus, Salmonella enteritidis and Shigella dysentriae were highly susceptible* to the isolates S2 and S3 used in the test. The diameters of the inhibition zones varied and ranged between 6 to15 mm. This revealed that S2 and S3 inhibited all the pathogenic bacteria used in the study. Schillinger and Lucke (1989) reported that inhibition was scored positive if the width of the clear zone around the colonies of the producer strain was 0.5 mm or larger. Thus *b*oth the test strains S2 and S3 showed good inhibitory effect on the test pathogens (Table 2).

CONCLUSION

Invitro probiotic tests conducted on *Bifidobacterium bifidum* and *Weissella confusa* showed that they are the potential Probiotic strains adopted to adhere to intestinal mucous, tolerate the gastro intestinal tract acid and bile conditions and antagonistic to enteric pathogens.

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