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*Original Research Article*

# Analyzing Probiotic Attributes to Assess Comparatively Two Isolates of *Lactobacillus acidophilus* in Prebiotics, Honey and Inulin.

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**ABSTRACT:**

***Lactobacillus acidophilus* is most commonly used as a probiotic bacterium. Various factors affect their viability in gastrointestinal tract. Two isolates of *Lactobacillus acidophilus, Lactobacillus acidophilus* NCDC 13 and *Lactobacillus acidophilus* NCDC 291 were assessed comparatively for antimicrobial property against indicator organisms, bile and acid tolerance and lysozyme tolerance. *Lactobacillus acidophilus* NCDC 13 showed better resistance against indicator organisms, acid, bile and lysozyme. This study suggests the importance to identify the useful isolate in future synbiotic food development from the strain studied.**

**KEY WORDS: Lactobacillus, Probiotic attributes, Gastrointestinal.**

## INTRODUCTION:

The normal gastrointestinal tract is colonized by a variety of bacteria including, but not limited to, bacteria belonging the genera *Lactobacillus*, *Streptococcus*, *Bacteroides*, *Escherichia*, *Bifidobacterium*, *Clostridium* etc. Probiotic bacteria constitute a major part of the natural micro flora of human intestine and when present in sufficient numbers create a healthy equilibrium between beneficial and potentially harmful microflora in the gut (Beck *et al.,* 1961; Gilliland *et al*., 1977). To provide health benefits, the suggested concentration for probiotic bacteria is $10^6$ CFU/g of a product (Lankaputhra *et al.,* 1995). Lactobacilli have been isolated from all portions of the human gastrointestinal tract (Marshall *et al.,* 1985). They also have a long history of traditional use in many industrial and artisanal plant, meat, and dairy fermentations. Based on their putative or proven health-promoting effects, the lactobacilli are commonly marketed as probiotics (Shah, 2000; Tannock, 2004; Bernardeau *et al., Rev* 2006). Micro-organisms ingested with food begin their journey to the lower intestinal tract via the mouth and are exposed during their transit through the gastrointestinal tract to successive stress factors that influence their survival (Simon and Gorbach, 1987; Marteau *et al.,* 1993). The time from entrance to release from the stomach is about 90 min, but further digestive processes have longer residence times (Berrada *et al.,* 1991).

These bacteria must overcome biological barriers like lysozyme in the oral cavity, including low pH in the stomach and bile in the intestine (Lankaputhra and Shah, 1995). Strains need to be resistant to the stressful conditions of the stomach like pH as low as 1.5-3.0 (Lankaputhra and Shah, 1995) and bile in the upper intestine (Chou and Weimer, 1999; Çakır *et al.,* 2003). Bile secreted in the small intestine reduces the survival of bacteria by destroying their cell membranes, whose major components are lipids and fatty acids and these modifications may affect not only the cell permeability and viability, but also the interactions between the membranes and the environment (Gilliland *et al.,* 1984; Gilliland, 1987). Although the resistance mechanisms of these bacteria are still poorly understood, the inhibition of *Lactobacillus* growth by bile may be overcome in some cases by progressive adaptation to increasing concentrations of these compounds (Chung *et al*., 1999; Margolles *et al*., 2003).

The ability of lactic acid bacteria to inhibit the growth of various Gram-positive or Gram-negative bacteria is well known. This inhibition may be due to the production of organic acids such as lactic and acetic acid (Gilliland and Speck 1977), hydrogen peroxide, bacteriocins, bacteriocin-like substances and possibly biosurfactants (Velraeds *et al*., 1996), which are active against certain pathogens. Anti-microbial metabolites such as organic acids, short chain fatty acids, hydrogen peroxide, reuterin, diacetyl, bacteriocins and bacteriocin-like inhibitory substances are some of the metabolic products of these bacteria suggested to have potential antimicrobial effects (Holzapfel *et al.*, 1995; Ouwehand, 1998; Cleveland *et al*., 2001; Shah and Dave, 2002; Dieleveux *et al.,*1998) attributed phenyllactic acid to the inhibition of various pathogenic bacteria such as *Listeria monocytogenes, Staphylococcus aureus, Escherichia coli* and *Aeromonas hydrophila*.

Therefore before a probiotic can benefit human health it must fulfill several criteria such as the ability to tolerate acid and bile salts as well as to grow in the lower intestinal tract (Pereira & Gibson, 2002). Hence the aim of this study was to investigate the antimicrobial property against indicator organisms, bile and acid tolerance and lysozyme tolerance of *L. acidophilus* NCDC 13 and *L. acidophilus* NCDC 291 in prebiotics, honey and inulin, to assess these isolates comparatively.

**MATERIALS AND METHODS:**

**Bacterial strains and growth conditions**

*Lactobacillus acidophilus* NCDC 13, *Lactobacillus acidophilus* NCDC 291, *Escherichia* coli NCDC 135 (EC-135), *Enterococcus faecalis* NCDC 116 (EF-116), *Salmonella typhimurium* NCDC 125 (ST-125) and *Shigella flexneri* NCDC 103 (SF-103) were obtained from the National Collection of Dairy Cultures (NCDC), Dairy Microbiology Division, National Dairy Research Institute (NDRI), Karnal, India. Freeze dried lactic cultures were activated in chalk litmus milk at 37°C for 24 hr and the indicator organisms were activated and maintained in Brain Heart Infusion (BHI) medium (Himedia, Mumbai, India) and sub-cultured monthly. Before use, the lactic cultures were sub-cultured twice in de Man Rogosa Sharpe (MRS) broth (Himedia, Mumbai, India) and indicator organisms in BHI broth at 37°C for 24 hr. Four types of medium were used, which were G (glucose source) = MRS medium, H = (MRS – Dextrose) + Honey, I = (MRS – Dextrose) + inulin, M (minimal) = (MRS – Dextrose).

**A. Antimicrobial activity against indicator organisms**

Agar well method was followed for evaluation of the antimicrobial activity (Anand *et al*., 1984). Nutrient agar (20 ml) containing 0.1% Tween 80 was seeded with 200 µl of 24 hr culture of indicator organisms and poured into the plates and were allowed to solidify. Wells (8mm in diameter) were made and 50 µl cell free supernatants (CFS) of the 24 hr MRS broth cultures of lactobacilli were poured into the respective well and incubated at 37°C for 10-12 hr and the diameter (mm) of inhibition zone around the well was measured.

**B. Tolerance to low pH**

Ten ml of each of the pH solutions in distilled water (pH 1.5, 2.5 and 6.5; using 35.4% HCl, Reidel chemicals) were transferred into tubes and inoculated with both the culture suspensions of $10^8$ cfu/ml cell concentration (1ml) each. One ml from each tube was withdrawn at 1 and 3 hr of incubation at 37°C and viable cells were enumerated by plating in MRS agar (Clark *et al.,* 1993).

**C. Tolerance to high bile concentration**

Ten ml bile salt (s.d. Fine Chem Ltd.) solution (1 and 2%) was inoculated with 1 ml of both culture suspensions of $10^8$ cfu/ml cell concentration and 1ml from each tube was withdrawn at 3 and 12 hr of incubation at 37°C for viable cells enumeration (Gilliland and Walker, 1990).

**D. Lysozyme tolerance**

Ten ml MRS broth and distilled water with (100ppm) and without lysozyme (Himedia, Mumbai, India) were inoculated with 1 ml of both culture suspensions of $10^8$ cfu/ml cell concentration and incubated at 37°C for 24 hr. Samples were taken after 24 hr for enumeration of viable cells (Brennan *et al.,* 1986).

**RESULTS AND DISCUSSION:**

**A. Antimicrobial activity against indicator organisms**

Among the probiotic properties, antimicrobial activity is one of the important criteria of selection of suitable strain of probiotic. Antimicrobial activity is an antagonistic activity against other bacteria. With the emergence of antibiotic resistant bacteria and natural ways of suppressing pathogens, the concept of probiotic has attracted much attention. Lactobacilli produce various antimicrobial substances, which causes the inhibition of pathogenic microorganism's growth and activities. Antimicrobial activities exhibited by different lactobacilli, used in the study, were determined by agar well diffusion in terms of zones of inhibition by culturing in MRS broth. The lactobacilli cultures showed comparable inhibitory activity (P>0.05) against all the indicator organisms (table 1). Greater inhibition was observed against *E. faecalis* NCDC 116 and *E. coli* NCDC 135 was inhibited least.

Table 1: Antimicrobial activity (mm, diameter of zone of inhibition including 8 mm well diameter) of lactobacilli strains against indicator organisms.

| **Indicator organisms** | **Lactobacilli cultures** | |
|---|---|---|
| | **LA-13** | **LA-291** |
| ***E. coli* NCDC 135** | 13.0 ± 0.34 | 13.2 ± 0.37 |
| ***S. flexneri* NCDC 103** | 13.8 ± 0.65 | 16.6 ± 1.07 |
| ***S. typhi* NCDC 125** | 16.2 ± 0.91 | 15.6 ± 0.25 |
| ***E. faecalis* NCDC 116** | 17.0 ± 0.01 | 16.7 ± 0.67 |

*L. acidophilus* NCDC 13 inhibited by enteropathogens *E. coli* and *S. typhi* when tested by agar well method (Reddy *et al*., 2006). Whereas Santos *et al*., (2003) reported the highest inhibition of *E. coli* and least inhibition of *S. typhi.* Tharmaraj and Shah, (2009), reported nearly equal inhibition of *E. coli* and *S. typhi* using spot on lawn assay. Jacobsen *et al*., (1999), reported almost same range of inhibition for *E. coli, S. flexneri* and *S. typhi.* While Fernandez, (2003) observed equal inhibition for *E. coli* and *E. faecalis* when tested by agar spot method. Mandal, (2006) reported same results for *E. coli* and *E. faecalis* when *L. acidophilus* NCDC 13 was checked for antimicrobial activity by agar well method. So our results are in conformation with Reddy *et al*., 2006 and Mandal, (2006) in case of *L. acidophilus* NCDC 13.

### B. Tolerance to Low pH

The pH of the different regions of GIT varies greatly. Stomach and the small intestinal region immediately following the stomach have the lower pH as low as 1.5 and colon region as high as 7.4. Probiotics should survive the high acidic conditions of stomach to transit the action site i.e., the colon, "the microbial fermenter". The pH of gastric juice in a fasting individual is approximately

1 to 2 and rises to 5 or more after food consumption (Betazzoni-Minelli *et al*., 2004). The tolerances of *L. acidophilus* strains to low pH were evaluated by exposing the cells to pH 1.5 upto 3 hr. At pH 6.5 and 2.5 there was no significant change (P>0.05) in viable cell numbers of both the strains during 3 hr of exposure (fig. 1 to 6). However drastic reduction in viable cell counts was observed at pH 1.5 (P< 0.05) after 1 and 3 hr (fig. 3 & 6).

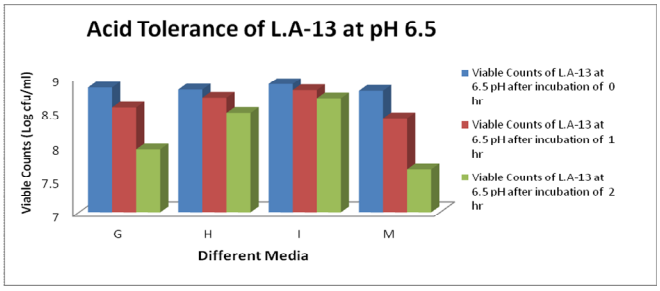


Figure1: Acid tolerance of *Lactobacillus acidophilus* NCDC 13 at pH 6.5 after incubation of 0, 1 and 2 hr in different media G, H, I and M.

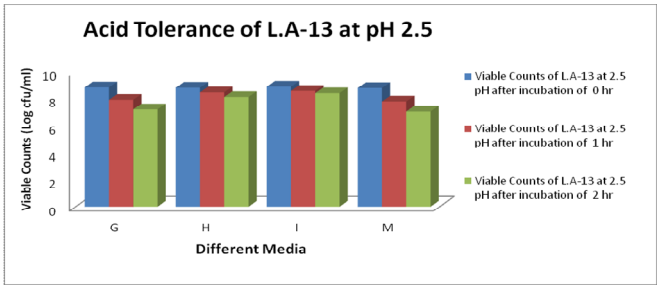


Figure 2: Acid tolerance of *Lactobacillus acidophilus* NCDC 13 at pH 2.5 after incubation of 0, 1 and 2 hr in different media G, H, I and M.

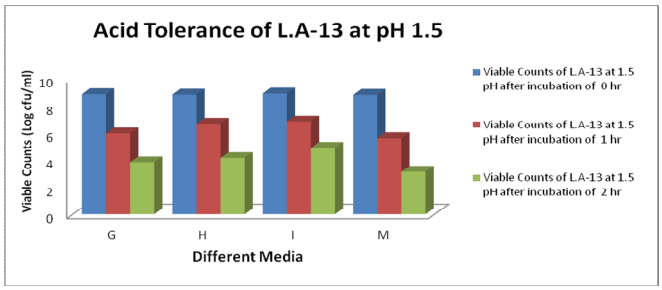


Figure 3: Acid tolerance of *Lactobacillus acidophilus* NCDC 13 at pH 1.5 after incubation of 0, 1 and 2 hr in different media G, H, I and M.

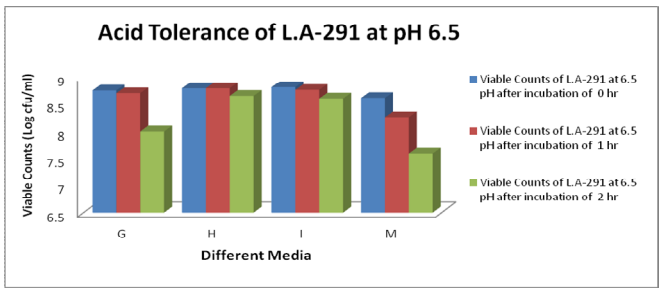


Figure 4: Acid tolerance of *Lactobacillus acidophilus* NCDC 291 at pH 6.5 after incubation of 0, 1 and 2 hr in different media G, H, I and M.

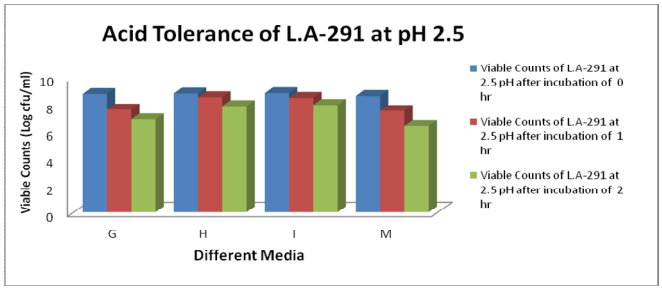


Figure 5: Acid tolerance of *Lactobacillus acidophilus* NCDC 291 at pH 2.5 after incubation of 0, 1 and 2 hr in different media G, H, I and M.

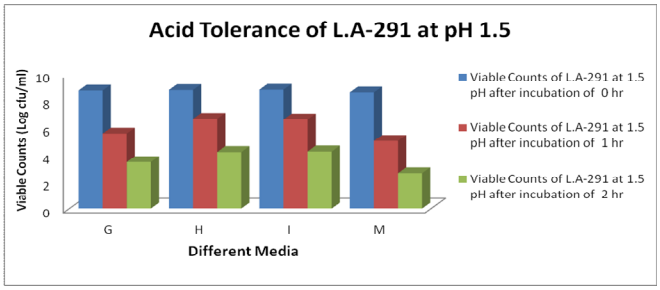


Figure 6: Acid tolerance of *Lactobacillus acidophilus* NCDC 291 at pH 1.5 after incubation of 0, 1 and 2 hr in different media G, H, I and M.

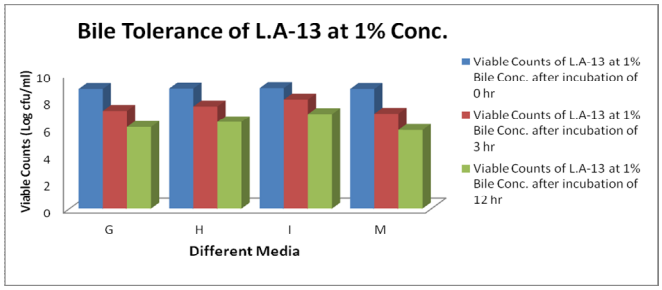


Figure 7: Bile tolerance of *Lactobacillus acidophilus* NCDC 13 at 1% bile concentration after incubation of 0, 3 and 24 hr in different media G, H, I and M.

Jin *et al.,* (1998) reported complete loss of viability after exposure of 1 hr at pH below 2. Millette *et al*., (2008) also reported high sensitiveness at pH 1.5 after exposure of 3 hr. Shukla *et al.,* (2010) reported results similar to Liong and Shah, (2005) by stating a small decrease in viable counts at pH 2 after exposure of 3 hr. Jacobsen *et al*., (1999) documented that 28 strains of *Lactobacillus* were sensitive to pH 2.5. Similarly, complete loss of viability of *Lactobacillus* at pH 2.5 after 3 hr of exposure (Charteris *et al*., 1998) was observed. While Fernandez *et al.,* (2002) and Muhammad *et al.,* (2009) reported less effect on viability at pH 2.5 after exposure of 3 hr. So our results are in confirmation with Millette *et al*., (2008) at pH 1.5 and with Fernandez *et al.,* (2002) and Muhammad *et al.,* (2009) at pH 2.5.

### C. Tolerance to High Bile Salt Concentrations

The 80% of stomach contents (after consumption of fermented product) had passed through to the intestine after 90 min (Charteris *et al.,* 1998). The concentration of bile varies from 0.5 to 2 % during the first hour of digestion; the levels may decrease during the subsequent period. Most foods pass through the small intestine by 12 hr (Clark and Martin, 1994). Hence tolerances of *L. acidophilus* strains were evaluated by exposing the cells to 1 and 2 % bile salt solution upto 12 hr at 37C°. The viable counts of both the strains got decreased (P>0.05) more in 2% bile concentration after 3 and 12 hr of exposure (fig. 7 to 10).

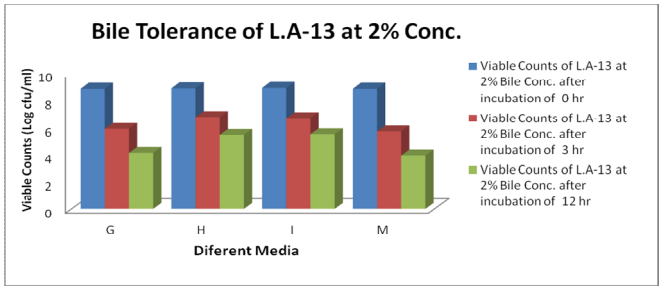


Figure 8: Bile tolerance of *Lactobacillus acidophilus* NCDC 13 at 2% bile concentration after incubation of 0, 3 and 24 hr in different media G, H, I and M.

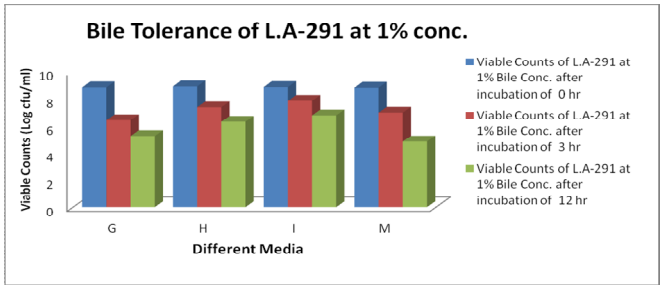


Figure 9: Bile tolerance of *Lactobacillus acidophilus* NCDC 291 at 1% bile concentration after incubation of 0, 3 and 24 hr in different media G, H, I and M.

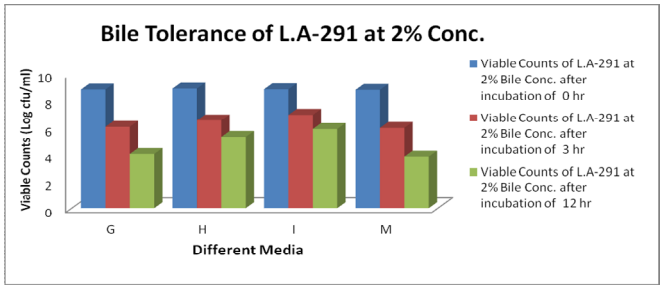

Figure 10: Bile tolerance of *Lactobacillus acidophilus* NCDC 291 at 2% bile concentration after incubation of 0, 3 and 24 hr in different media G, H, I and M.

Bile resistance is necessary for an organism that is expected to grow in an intestine (Gilliland *et al.,* 1984) and hence an important characteristic to be considered in the selection of a culture as dietary adjunct (Walker and Gilliland, 1993). Millette *et al.,* (2008) reported loss of activity at bile conc. below 0.5%. While the viable counts were between 6-8 Log cfu/ml at 2% bile salt conc. and decrease drastically below this conc. as reported by Succi and Coppola, (2005), Hamon *et al.,* (2011) checked bile tolerance in nine strains of *Lactobacillus* and reported lesser decrease in viable counts at 0.5-3.6% conc. of bile salts. Desai and Shah, (2007) checked the viable count of twenty two different *Lactobacillus* strains in the bile concentrations of 1% and 1.5%. Eight strains showed lesser decrease in viable counts while 14 strains showed large decrease in viable counts at 1% conc. While at 1.5% only seven strains were able to show small amount of growth while the other strains even failed to grow. So our results confirmed the results of Millette *et al.,* (2008) at 2% bile salt conc. and of Desai and Shah, (2007) at 1% conc.

### D. Lysozyme Tolerance

The lysozyme content in saliva varies from 10 to 200 µl and in the gastric juice from 43 to 106 µl. Therefore their ability to survive at these lysozyme concentrations can be an additional parameter for selecting probiotics. Resistances to the lysozyme by *L. acidophilus* strains were evaluated in MRS at 37°C for 24 hr. No significant difference (P>0.05) of viable counts was observed in MRS broth with and without lysozyme. Lysozyme is capable of lysing bacteria, but doesn't significantly impair activities of lactic acid bacteria. The results indicated that the culture was resistant to lysozyme (Fig. 11 & 12) and even the viable counts got increased in presence of lysozyme.

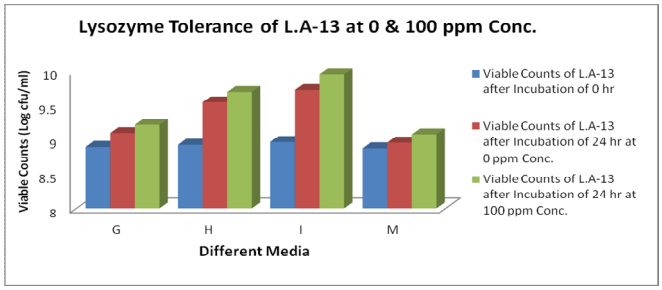


Figure 11: Lysozyme tolerance of *Lactobacillus acidophilus* NCDC 13 at 0 & 100 PPM lysozyme concentration after incubation of 0 and 24 hr in different media G, H, I and M.

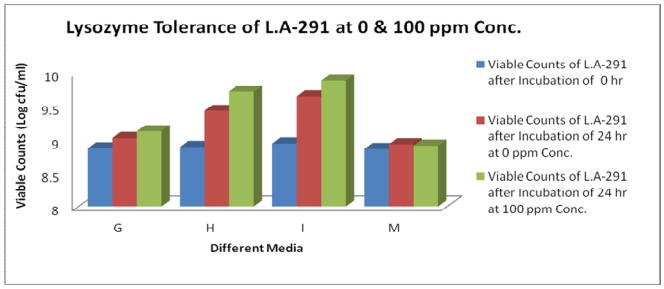


Figure 12: Lysozyme Tolerance of *Lactobacillus acidophilus* NCDC 291 at 0 & 100 PPM Lysozyme Concentration after Incubation of 0 and 24 hr in Different Media G, H, I and M.

Lysozyme is capable of lysing bacteria, but doesn't significantly impair activities of lactic acid bacteria (Lodi *et al*., 1983). Some strains of *L. acidophilus* may sensitive to 100 mg/ml lysozyme, but some showed considerable resistance (Brennan *et al*., 1986). Neviani and Veaux (1991) reported the acquisition of lysozyme resistance in *L. helveticus* cells grown in milk, MRS agar containing lysozyme, due to cell adaptation. The results indicated that the culture was resistant to lysozyme and even the viable counts got increased (upto 9.88-9.95 log cfu/ml) in presence of lysozyme (Fig. 11 & 12).

## CONCLUSION

The two strains of *Lactobacillus acidophilus* (*Lactobacillus acidophilus* NCDC 13 and *Lactobacillus*

*acidophilus* NCDC 291) were comparatively analyzed for the probiotic attributes in two prebiotics viz., honey and inulin. Various probiotic attributes checked were antimicrobial activity against indicator organisms (*E. coli, E. faecalis, S. typhimurium* and *S. flexneri)*, tolerance to low pH (1.5, 2.5 and 6.5), bile toxicity (1 and 2% conc.) and lysozyme concentrations (0 and 100 ppm conc). The strain *Lactobacillus acidophilus* NCDC 13 showed more inhibitory actions than *Lactobacillus acidophilus* NCDC 291 against indicator organisms (*E. coli, E. faecalis, S. typhimurium* and *S. flexneri*) as determined by agar well assay method. Both the strains could withstand the pH upto 2.5 but at pH 1.5 the survival of *Lactobacillus acidophilus* NCDC 13 was more than *Lactobacillus acidophilus* NCDC 291 in spite of the significant decrease in viable counts on exposure upto 3 hr. Both the strains showed more tolerance to pH in inulin than honey. Both the strains showed more decrease in viable counts at 1% and 2% bile salt concentration in honey than inulin after 12 hr of exposure but the decrease was more in case of *Lactobacillus acidophilus* NCDC 291. The lactic culture strains were resistant to lysozyme and both showed even an increase in viable counts after exposure of 1 hr to 0 and 100ppm conc. of lysozyme. In conclusion it can be said that *Lactobacillus acidophilus* NCDC 13 showed better probiotic attributes than *Lactobacillus acidophilus* NCDC 291in prebiotic inulin than honey.

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