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Comparing Adhesion Attributes of two Isolates of *Lactobacillus Acidophilus* for Assessment of Prebiotics, Honey and Inulin

Suman Saran[1]*, M.S. Bisht[1], K. Singh[2], U.V.S Teotia[3]**

[1]*Zoology Department, Pauri campus, Hemvati Nandan Bahuguna Garhwal University, Uttaranchal-246001, India
[2] Dairy Microbiology Division, National Dairy Research Institute, Karnal-132001, India.
[3] Teotia Education Consultancy Cell, Shastradhara Road, Dehradun-248001, India

***Abstract*-** Adhesion attributes of *Lactobacillus acidophilus* were studied for adhesion to hydrocarbons, aggregation abilities and autolysis to evaluate different prebiotics. Autoaggregation correlates with adhesion, which is a prerequisite for colonization and infection of the gastrointestinal tract by many pathogens and coaggregation has been related to the ability to interact closely with pathogens where autolysis decreases the adhesiveness. Hydrophobicity affects adhesion to intestinal surfaces. The strains *L. acidophilus* NCDC 13 and *L. acidophilus* NCDC 291 showed changes in aggregation abilities and adhesion properties in the presence of prebiotics, honey and inulin. The results for both strains indicate that the ability to autoaggregate and cell surface hydrophobicity, increased in the presence of inulin. While the ability to coaggregate increased in presence of honey and autolytic activity reduced highly in presence of inulin. This study suggest the importance to identify the useful prebiotic so as to enhance the effect of probiotic properties of lactobacillus strain, and also the relevance to future synbiotic food development from the strain studied.

Abbreviations Used: CFU, colony forming units; BHI, brain heart infusion; MRS, de man rogosa sharpe; PBS, phosphate buffered saline; PUM, phosphate urea magnesium sulphate.

***Index Terms*-** adhesion, autoaggregation, autolysis, coaggregation, prebiotics probiotics

I. INTRODUCTION

In the last 19 century microbiologists describe microflora in the gastrointestinal tract (GIT) of healthy individuals that different from those found in diseased individual. These beneficial microflora found in the GIT termed probiotic. Promising probiotic strains include members of the genera *Lactobacillus*, *Bifidobacterium* and *Enterococcus*. *L. acidophilus* is one of the most extensively studied probiotic. Lactic Acid Bacteria (LAB) are thought to be safe bacteria that have been ingested from foods without any problems for many years and are known as GRAS (Generally Recognized As Safe). Research on mass screening of probiotics for use in yoghurt has been performed from selecting points such as resistance to lysozyme in mouth, acidic conditions in stomach, and bile acids in intestine (Kurien and Singh, 2005). In addition to the selecting points, the adhesion of LAB to human intestine is thought to be one of the most important characteristics of probiotics preventing their immediate elimination by peristalsis and providing a competitive advantage in this ecosystem (Alander *et al*. 1997). Adherence of bacterial cells is related to cell surface characteristics (Bibiloni *et al*. 2001, Canzi *et al*. 2005 and Rahman *et al*. 2008). A number of reports have described the composition, structure, and forces of interaction related to bacterial adhesion (Del Re *et al* 2000; Tuomola *et al* 2000 & Collado *et al* 2005, Collado *et al* 2007a,b,c). The mechanism, by which *L. acidophilus* group adheres to the human gastrointestinal tract, has been partially elucidated (Saito *et al* 2004 and Buck *et al* 2005). Autoaggregation of probiotic strains appeared to be necessary for adhesion to intestinal epithelial cells, coaggregation abilities may form a barrier that prevents colonization by pathogenic microorganisms (Del Re *et al*. 2000, Schachtsiek *et al.* 2004 and Schellenberg *et al*. 2006) and autolysis reduces the number of probiotic bacteria (Kang *et al*. 1998 and Koch *et al*. 2007). Physicochemical characteristics of the cell surface such as hydrophobicity may affect autoaggregation and adhesion of bacteria to different surfaces (Del Re *et al*. 2000) but also conflicting results have been reported (Vinderola *et al*. 2004). When present in sufficient numbers, the lactobacilli are believed to be able to create a healthy equilibrium between beneficial and potentially harmful microflora in the gut (Tannock 1999 and S˘us˘kovic´ *et al*. 2001). Prebiotics are non-digestible food ingredients that stimulate the growth and /or activity of bacteria in the digestive system which are beneficial to the health of the body. Traditional dietary sources of prebiotics include soybeans, inulin sources (such as Jerusalem artichoke, jicama, and chicory root), raw oats, unrefined wheat, unrefined barley, honey, almonds and yacon. Prebiotics improve the number and/or activity of probiotic bacteria (Conway *et al*. 2001, Gibson *et al*. 2004, Salminen *et al*. 2004 & Macfarlane *et al.* 2008). Hence the aim of this study was to investigate various adhesion attributes; aggregation abilities, cell surface hydrophobicity property and autolytic activity of *L. acidophilus* NCDC 13 and *L. acidophilus* NCDC 291 to comparatively evaluate prebiotics honey and inulin.

II. MATERIALS AND METHODS

A. Bacterial strains and growth conditions

Lactobacillus acidophilus NCDC 13, *Lactobacillus acidophilus* NCDC 291 and *Escherichia* coli NCDC 135 (EC-

135) were obtained from the National Collection of Dairy Cultures (NCDC), Dairy Microbiology Division, National Dairy Research Institute (NDRI), Karnal, India. Freeze dried lactic culture was activated in chalk litmus milk at 37°C for 24 hr and *E. coli* was activated in Brain Heart Infusion (BHI) medium (Himedia, Mumbai, India) and sub-cultured monthly. Before use, the lactic culture was sub-cultured twice in de Man Rogosa Sharpe (MRS) broth (Himedia, Mumbai, India) and *E. coli* in BHI broth at 37°C for 24 hr. Four types of medium were used to evaluate the prebiotics, which were G (glucose source) = MRS medium, H = (MRS – Dextrose) + Honey, I = (MRS – Dextrose) + inulin, M (minimal) = (MRS – Dextrose).

B. Autoaggregation assays

Auto aggregation assays were performed according to Del Re *et al*., (2000) with certain modifications. Fresh lactic cultures were inoculated in all the four media (G, M, H, I) for 16-18 hr. Cells were harvested by centrifuging at 10,000 rpm for 15 min at 4°C. Cells were washed twice in Phosphate Buffered Saline (PBS) having viable counts of 10^8 CFU ml^{-1} .

Cell suspensions (10 ml) were mixed by vortexing for 10 sec in acid washed tubes and left undisturbed. Upper layer was removed after 3 and 5 hr and absorbance (A) was measured at 610 nm. The autoaggregation percentage is calculated by the formula:

$$Autoaggregation\ \% = 1 - (A_t / A_0) \times 100$$

Where A_t represents the absorbance at time t = 3 or 5 hr and A_0 the absorbance at t = 0.

C. Coaggregation assays

Coaggregation assays was also performed according to Del Re et al., (2000) with certain modifications. Fresh cultures of *L. acidophilus* NCDC 13 and *L. acidophilus* NCDC 291 were inoculated in all the four media (G, M, H, I) and *E. coli* in BHI broth for 16-18 hr. Cells were harvested by centrifuging at 10,000 rpm for 15 at 4°C. Cells were washed twice with PBS having viable counts of 10^8 CFU ml^{-1}. Equal volume (5+5 ml) of each cell suspensions were mixed together by vortexing for 10 sec. Control tubes were set up at the same time, containing 10 ml of each bacterial suspension on its own. The absorbance (A) at 610 nm of the single and mixed suspensions were measured after 3 and 5 hr of incubation at room temperature. Samples were taken in the same way as in the auto aggregation assay. The percentage of coaggregation was calculated using the equation (Handley *et al*. 1987):

$$Coaggregation\ \% = \frac{[(A_X + Ay)/2 - A(x+y)]}{A_X + Ay/2} \times 100$$

Where x and y represent each of the two strains in the control tubes, and (x + y) the mixture.

D. Autolytic assay

The method for preparing the cell suspensions for autolysis was the same as that for auto aggregation assays. The samples for checking absorbance was taken after mixing the cell suspensions after 3 and 5 hr. The percentage of autolytic activity was calculated by the following formula:

$$Autolytic\ activity\ \% = 1 - (A_t / A_0) \times 100$$

Where A_t represents the absorbance after mixing at time t = 3 or 5 hr and A_0 the absorbance after mixing at t = 0.

E. Cell surface hydrophobicity

Cell surface hydrophobicity was measured according to the method of Rosenberg *et al*. (1980) with some modifications (Crow and Gopal, 1995 and Bellon-Fontaine *et al*. 1996). Fresh lactic cultures were inoculated in all the four media (G, M, H, I)for 16-18 hr. Cells were harvested by centrifuging at 10,000 rpm for 15 min at 4°C. Cells were washed twice with Phosphate Urea Magnesium sulphate (PUM) buffer having viable counts of 10^8 CFU ml^{-1}. The absorbance of the cell suspension (10 ml) was measured at 600 nm (A_0). Distributed 4.2 ml of cell suspension in acid washed tubes and added 0.8 ml of solvent n-hexadecane and incubated for 15 min at 37°C. The two phase system was mixed well by vortexing for 2 min. The aqueous phase was removed after 1 hr of incubation at room temperature and its absorbance at 600 nm (A_t) was measured. The percentage of bacterial adhesion to solvent was calculated as:

$$Cell\ surface\ hydrophobicity\ \% = 1 - (A_t / A_0) \times 100$$

Where A_t represents the absorbance at time t = 1 hr and A_0 the absorbance at t = 0.

III. RESULTS

A. Autoaggregation assay

The sedimentation rate of *L. acidophilus* NCDC 13 and *L. acidophilus* NCDC 291 was measured over a period of 3 and 5 hr in four different media (G, H, I, M). Results showed that autoaggregation of both the *lactobacilli* strains is raised upto 35.23-38.19% after 3 hr incubation and 40-43.24% after incubation of 5 hr in the case of inulin than honey (Fig.1 & 2). However the rise is more in case *L. acidophilus* NCDC 13.

B. Coaggregation assay

The coaggregation abilities of probiotic strains might enable it to form a barrier that prevents colonization by pathogenic bacteria. Coaggregation of *L. acidophilus* NCDC 13 and *L. acidophilus* NCDC 291 with enteropathogen *E. coli* was measured over a period of 3 and 5 hr in four different media (G, H, I, M). Results are expressed as percentage reduction after 3 and 5 hr in the absorbance of a mixed suspension compared with the individual suspension. *L. acidophilus* NCDC 291 showed more percentage reduction in absorbance (upto 18.97%) in honey (Fig. 3) while *L. acidophilus* NCDC 13 showed more reduction in percentage in absorbance (upto 19.95%) in inulin (Fig. 4).

C. *Autolytic assay*

Autolysis is the spontaneous disintegration of the bacterial cell as a result of age or unfavourable physiological conditions, which activate autolysins, the enzymes found in the cell that are capable of hydrolyzing the cell wall peptidoglycan. The autolytic process proceeds by endogenous autolysin that hydrolyzes the covalent bonds of peptidoglycan, the main cell wall component in LAB. Autolytic activity of *L. acidophilus* NCDC 13 and *L. acidophilus* NCDC 291 was measured over a period of 3 and 5 hr

in four different media (G, H, I, M). The extent of autolysis is highest in MRS and reduced in all the other media. This shows that there is reduction in autolysis in the presence of prebiotics (Fig. 5 & 6), and the effect is reduced more in the presence of inulin (upto 5.6-6.09%).

D. Cell surface hydrophobicity

The measurement of cell surface hydrophobicity can be considered as an indicator of the ability of cells to adhere to the intestinal epithelial cells. The adherence of probiotics to the intestinal epithelial tissues is an important prerequisite, which depends on the hydrophobicity of the bacterial cell surface (Jacobsen *et al*., 1999, Tuomola *et al*., 2001). It is the interaction of the bacterial cell with the organic compounds. Adhesion to hydrocarbon like n-hexadecane is considered as a biochemical marker for adherence to the epithelial cell in gut. Hence the cell surface hydrophobicity of *L. acidophilus* NCDC 13 and *L. acidophilus* NCDC 291 was measured with organic solvent n-hexadecane after 1 hr. The interaction got drastically increased in the presence of inulin (upto 47.22-48.12%) as compared to honey (Fig. 7).

IV. DISCUSSION

Cell adhesion is a multistep process involving contact of the bacterial cell membrane and interacting surfaces. The ability of probiotic bacteria to form cellular aggregates is considered a desirable characteristic, as they can potentially inhibit adherence of pathogenic bacteria to intestinal mucosa either by forming a barrier via self-aggregation or coaggregation with commensal organisms on the intestinal mucosa or by direct coaggregation with the pathogens to facilitate clearance (Bujnakova *et al*., 2002, Schachsteik *et al.*, 2004). In addition, studies have suggested aggregation as an important mechanism for genetic exchange, adhesion, and colonization in the host environments, as well as Immunomodulation of colonic mucosa (Cesena *et al*., 2001, Voltan et al., 2007).

It was reported by Kos *et al*., (2003) that the cell surface proteins (S-layer proteins) influenced autoaggregation property and adhesiveness of *L. acidophilus* M92. Tomas *et al*., (2007) analyzed that autoaggregation increases with the concentration of glucose in the growth medium. Collado and Salminen (2007) reported that dadih lactic acid bacteria strains presented higher autoaggregation abilities than the pathogens after incubation of 24 hr. Goh and Klaenhammer, (2010) analyzed that the aggregation promoting factors increases self-aggregation with incubation. So our results confirmed the results of Tomas *et al*., (2007), Collado and Salminen (2007) and Goh and Klaenhammer, (2010). As the autoaggregation increases with incubation time and also got improved with the glucose concentration.

Ehrmann *et al.,* (2002) studied the co-aggregation properties of nine *Lactobacillus* strains with three different indicator strains, *E. coli*, *S. enteritidis* and *S. typhimurium*. All strains showed maximum coaggregation with *S. enteritidis*. Kos *et al*., (2003) reported maximum coaggregation ability of *L. acidophilus* M92 with *Enterococcus faecium L3* rather than *E. coli; Salmonella serotype Typhimurium and Lactobacillus plantarum* L4. Schachtsiek, *et al*., (2004) analyzed that *Lactobacillus* coryniformis coaggregated with *Escherichia coli* K88, *Campylobacter coli* and *Campylobacter jejuni* but not with other human pathogens. Collado and Salminen, (2007) analyzed the dadih lactic acid bacteria strains and pathogens *B. vulgatus*, *C. histolyticum* and *difficile*, *St. aureus*, *Enterobacter sakazakii*, and *E. coli* for coaggregation abilities. The results of coaggregation were dependent on dadih strain, pathogen strain and time. Ekmekci *et al*., (2009) studied the coaggregation ability of 19 vaginal *Lactobacilli*. Coaggregation ability of all *lactobacilli* with *Escherichia coli* ATCC 11229 was positive under both aerobic (71%) and anaerobic conditions (62%). So our results are in confirmation with Schachtsiek, *et al*., (2004) and Ekmekci *et al*., (2009) and are similar to that they have reported about coaggregation ability of *Lactobacillus* strains with *Escherichia coli*.

Riepe *et al*., (1997) analyzed that the two highly autolytic *Lactococcus lactis* subsp. *cremoris* strains (CO and 2250) showed maximum lysis when grown in M17 broth containing a limiting concentration of glucose (0.4 to 0.5%) as the carbohydrate source. Lysis was reduced when strains were grown on lactose or galactose. Whereas Kang *et al.,* (1997) showed that rate and extent of autolysis of *Lb. bulgaricus* and *Lb. casei* was dependent upon temperature, pH, NaCl concentration, growth phase and strain. Masuda *et al*., (2005) evaluated 7 strains of *L. gasseri* and 5 strains of *L. acidophilus* for the autolytic activity. *L. gasseri* strains showed more prominent results in dispersed solutions than *L. acidophilus* strains. So our results in confirmation with Riepe *et al*., (1997) when inuln and honey were used as carbohydrate source which have limiting concentration of glucose.

Kushal (2001) reported a higher cell surface hydrophobicity of *L. acidophilus* NCDC 13 in presence of inulin. Pascual *et al.,* (2008) reported the rise in cell surface hydrophobicity percentage was upto 36.12 when *Lactobacillus* strains were grown in MRS broth for 3 hr. Kos *et al*., (2003) recorded maximum cell surface hydrophobicity in chloroform when the strains *L. acidophilus* M92, *Lactobacillus plantarum* L4 and *Enterococcus faecium* L3 were tested against xylene, chloroform and ethyl acetate. So our results are in confirmation with Kushal (2001) and Pascual *et al.,* (2008).

V. CONCLUSION

Two prebiotics viz., honey and inulin were evaluated for the probiotic and functional attributes of the two strains of *Lactobacillus acidophilus* (*Lactobacillus acidophilus* NCDC 13 and *Lactobacillus acidophilus* NCDC 291) to assess them comparatively. Various adhesional attributes checked were the autoaggregation, coaggregation with *E. coli*, autolysis and cell surface hydrophobicity. *Lactobacillus acidophilus* NCDC 13 showed remarkable autoaggregation efficiency in inulin than *Lactobacillus acidophilus* NCDC 291. While the strain *Lactobacillus acidophilus* NCDC 291 showed more coaggregation in presence of honey than *Lactobacillus acidophilus* NCDC 13. Autolytic activity gets remarkably decreased in presence of prebiotics and the decrease is more in inulin than honey. The property of cell surface hydrophobicity of both the strains was much increased in inulin than honey. In conclusion it can be said that both the strains *Lactobacillus acidophilus* NCDC 13 and *Lactobacillus acidophilus* NCDC 291showed better attributes in prebiotics inulin than honey.

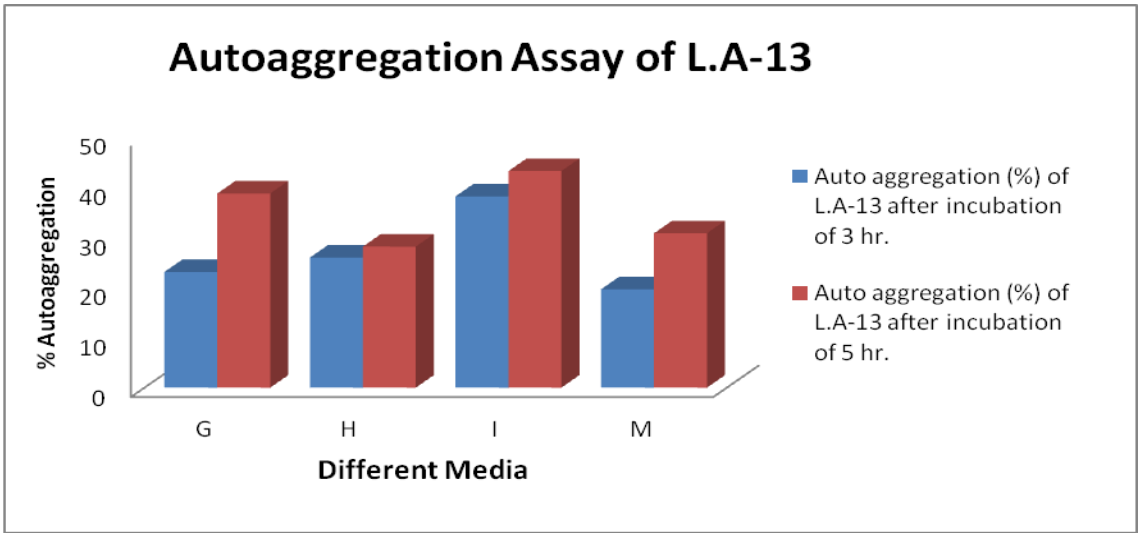

Figure 1: Autoaggregation assay of *Lactobacillus acidophilus* NCDC 13 after incubation of 3 and 5 hr in different media G, H, I and M.

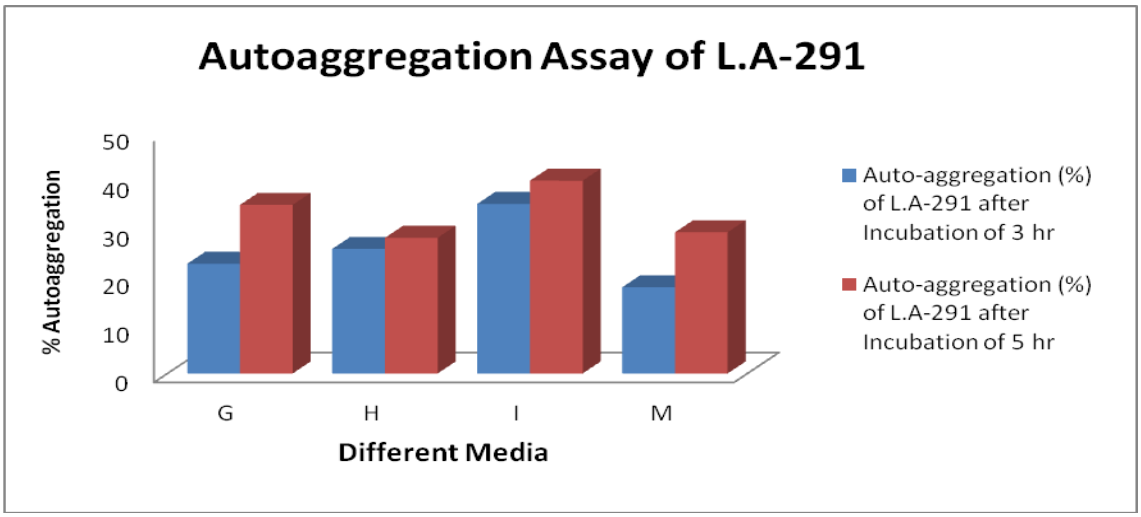

Figure 2: Autoaggregation assay of *Lactobacillus acidophilus* NCDC 291 after incubation of 3 and 5 hr in different media G, H, I and M.

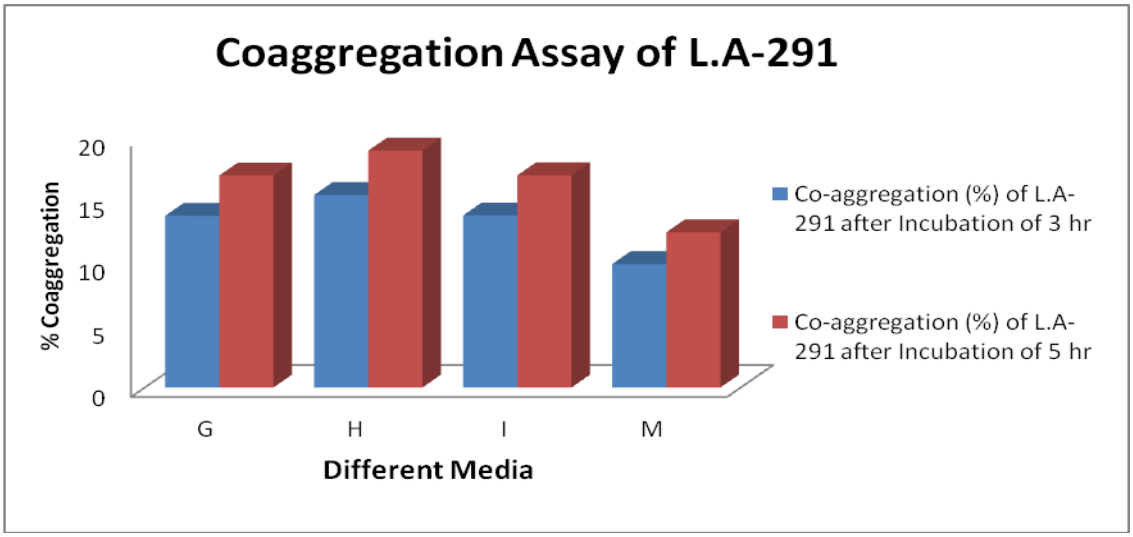

Figure 3: Coaggregation assay of *Lactobacillus acidophilus* NCDC 291 after incubation of 3 and 5 hr in different media G, H, I and M.

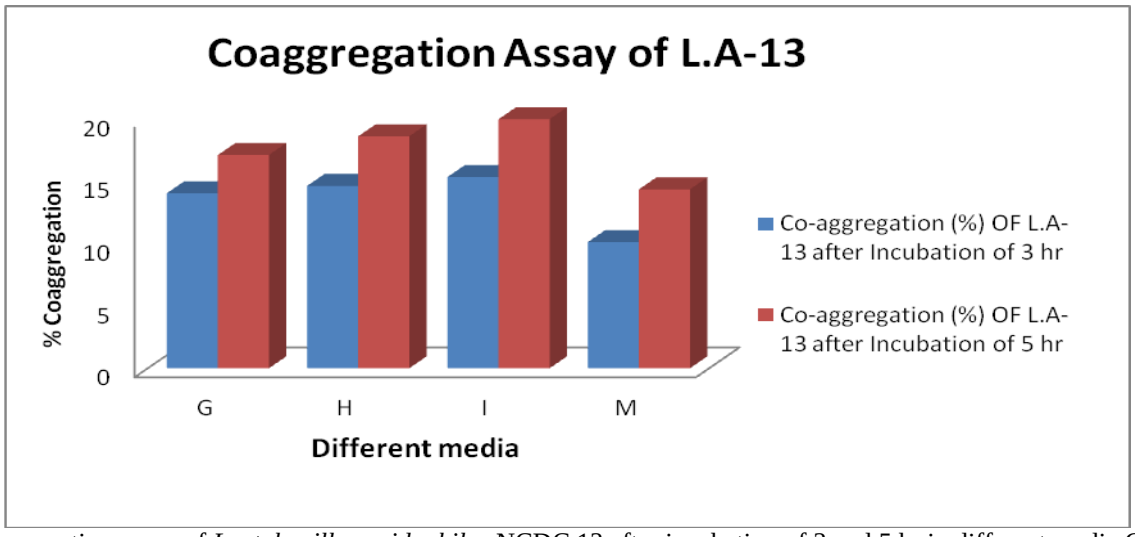

Figure 4: Coaggregation assay of *Lactobacillus acidophilus* NCDC 13 after incubation of 3 and 5 hr in different media G, H, I and M.

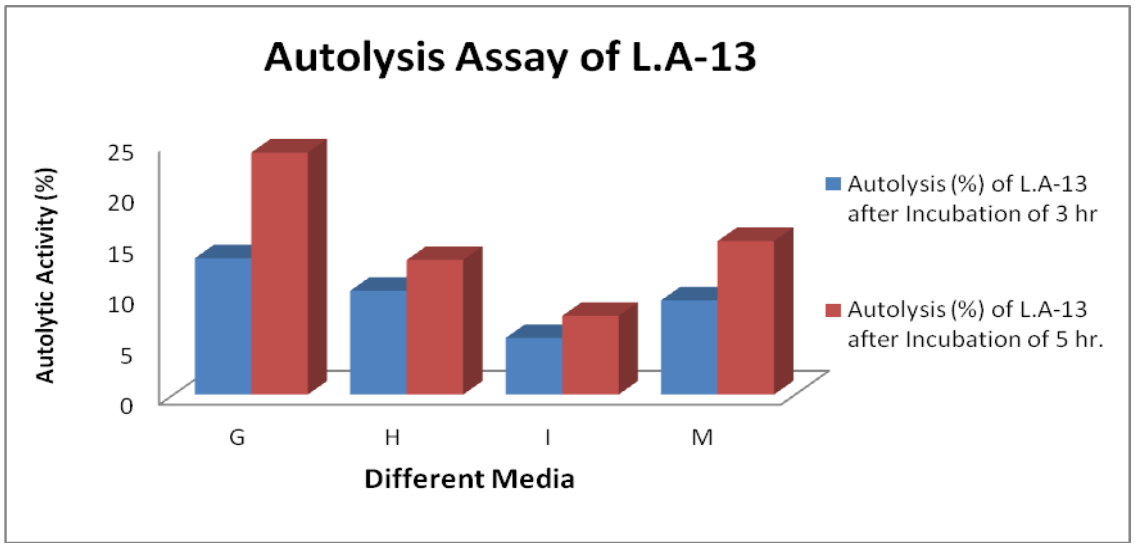

Figure 5: Autolysis assay of *Lactobacillus acidophilus* NCDC 13 after incubation of 3 and 5 hr in different media G, H, I and M.

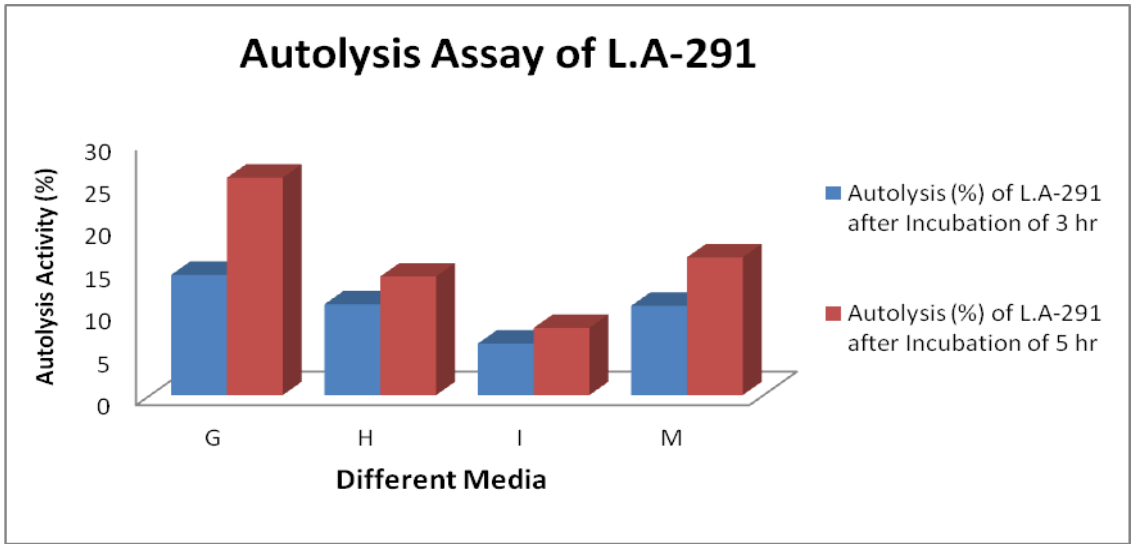

Figure 6: Autolysis assay of *Lactobacillus acidophilus* NCDC 291 after incubation of 3 and 5 hr in different media G, H, I and M.

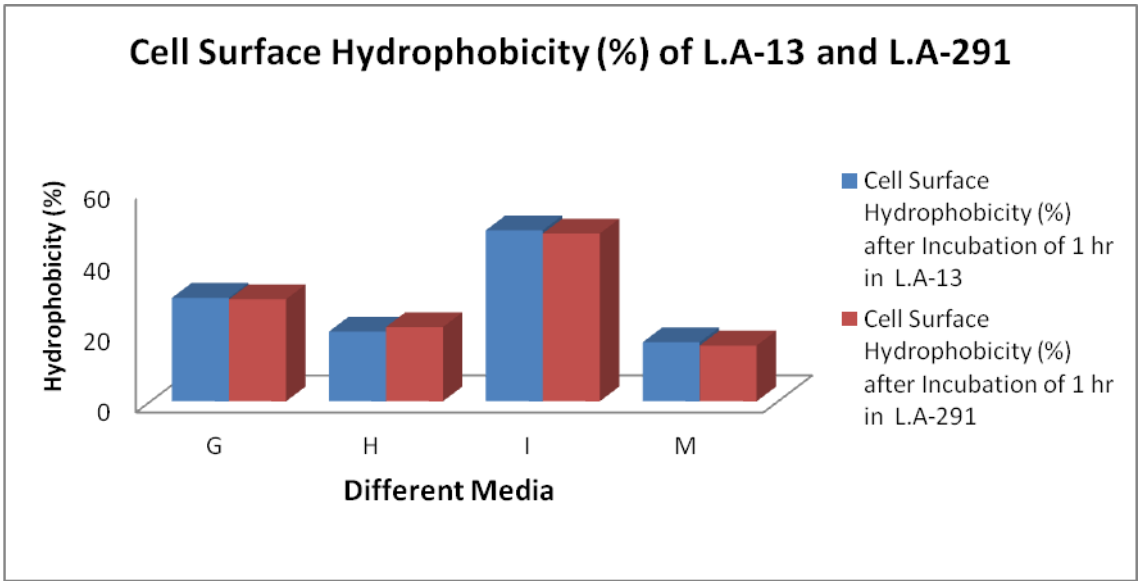

Figure 7: Cell surface hydrophobicity assay of *Lactobacillus acidophilus* NCDC 13 and *Lactobacillus acidophilus* NCDC 291 after incubation of 1 hr in different media G, H, I and M.

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AUTHORS

First Author – Suman Saran, Ph.D Scholar, Zoology Department, Pauri Campus, Hemvati Nandan Bahuguna Garhwal University, Uttaranchal-246001, India

Second Author – Dr. Kishan Singh, Dairy Microbiology Division, National Dairy Research Institute, Karnal-132001, India, Email: Ksinghndri@gmail.com

Third Author – Dr. M.S Bisht, Zoology Department, Pauri campus, Hemvati Nandan Bahuguna Garhwal University, Uttaranchal-246001, India

Forth Author - Dr. Uday Veer Singh Teotia. Teotia Education Consultancy Cell, Shastradhara Road, Dehradun-248001, India

Corresponding Author - Dr. Kishan Singh, Dairy Microbiology Division, National Dairy Research Institute, Karnal-132001, India, Email id: Ksinghndri@gmail.com