

## ***Lactobacillus plantarum* shows more Probiotic Potential than *S. cremoris***

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Probiotics are live microorganisms which are regularly consumed as part of our daily diet and help to protect the host from various pathogens. The mechanisms by which the indigenous intestinal bacteria inhibit pathogens include competition for colonization sites and nutrients, production of toxic compounds, and stimulation of the immune system. *Lactobacillus plantarum* (C18) bacterial strain was isolated from yogurt and observed to be gram positive rod-shaped bacilli. After carrying out auto-aggregation and co-aggregation assay, it was seen that it displayed a strong auto-aggregating and co-aggregating phenotype. A known probiotic bacteria, *Streptococcus cremoris* was used as a control. Comparison studies proved that *L. plantarum* bacterial strain showed higher auto-aggregating and co-aggregating properties as compared to *S. cremoris*. Therefore, if *S. cremoris* is said to be one of the ideal candidates for probiotic use, this strain of bacteria could challenge the definition of ideal requirements for probiotic bacteria. Hypothesis could state that cells when cultured in broth secrete certain proteins which help in bacterial adhesion to the intestinal tract cells, and this was seen to be higher in supernatant diluted C18 bacterial culture than in saline diluted counterpart.

**Key words:** *Lactobacillus plantarum*, *Streptococcus cremoris*, Probiotic bacteria, Auto-aggregation, co-aggregation.

Probiotics are live microorganisms which are considered to be beneficial to the host organism. As defined by World Health Organisation (WHO) recently, probiotics were termed as 'live microorganisms which when administered in adequate amounts confer a health benefit on the host'<sup>1</sup>.

Most commonly used probiotic microbes are Lactic acid bacteria (LAB) and bifidobacteria, but certain yeasts and bacilli can also be helpful. Probiotics are regularly consumed as part of our daily diet with specially added active live cultures; such as in yogurt, soy yogurt, or even as dietary supplements.

Although there is good evidence for the effect of probiotic preparations in Gastrointestinal

(GI) health, little is known about the manner in which these changes occur. Several different mechanisms by which probiotics may protect the host from intestinal disorders have been suggested:

1. Production of anti-microbial substances<sup>1</sup>
2. Competition for adhesion receptors or sites<sup>2-3</sup>
3. Competition for nutrients.
4. Stimulation of immunity.
5. Degradation of toxin receptors.

The classical explanation of the effects of probiotics in the GI tract is through the direct or indirect modulation of the endogenous flora of the immune system. Evidence of cross-talk between lactobacilli and antigen-presenting cells of the intestine, such as M-cells, were observed in some of the electron microscopic studies. More recent microarray studies have shown that lactobacilli in contact with epithelial cells induce mRNA of several genes. The colonization of the intestine or the contact of probiotics with the

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intestinal wall might be achieved by different mechanisms, such as adherence, aggregation or high growth to avoid washing out, or continued consumption of probiotics.

According to Bengmark *et al*, probiotics seem to be effective in controlling overgrowth of potentially pathogenic microorganisms with a bacterial, viral, and fungal origin. In addition to the effects of probiotics in antagonizing noxious or unwanted microorganisms, eliminating toxins and stimulating the intestinal immune defense, some data suggest that probiotics also participate in the regulation of intestinal function. Thus, the mechanisms by which the indigenous intestinal bacteria inhibit pathogens include competition for colonization sites and nutrients, production of toxic compounds, and stimulation of the immune system.

Probiotics counteracted various enteric pathogens in experimental models such as *Salmonella typhimurium* in mice, *Shigella*, *Clostridium difficile*, *Campylobacter jejuni* and *Escherichia coli*. Studies currently aim to understand the cross-talk mechanisms between different bacteria and between bacteria and intestinal epithelial cells. The use of new technologies such as microarray provides a new view of the richness of these relationships.

In order to colonize the intestine and exert their beneficial effects, probiotic bacteria must be able to adhere to mucosal epithelial cells. Since colonization is species-specific, the microorganisms should be of human origin, as mentioned above, and survive in the intestine while ingested.

Adherence and colonization are crucial for the competitive exclusion of pathogens. Colonization also leads to immune modulation. Therefore, direct contact with intestinal epithelial cells is a prerequisite for some probiotic effects on the immune system. This helps to enhance leukocyte phagocytic activity against enterobacteria, an effect that was detected following administration of probiotic strains to healthy individuals.

Auto-aggregation of probiotic strains appear to be necessary for adhesion to intestinal epithelial cells<sup>4-5</sup>. Thus it is very important for a particular strain of bacteria to aggregate together as it can then attach itself to the walls of the intestinal tract.

The aim of this study was to investigate auto-aggregation and co-aggregation properties of *Lactobacillus plantarum* which was isolated from yogurt and could possess the potential to be used as a probiotic organism. In numerous studies conducted around the world, it has been observed that an ideal candidate organism which could be of any probiotic use should possess certain properties. In this paper, the terms '*Lactobacillus plantarum*' and 'C18' are used interchangeably, as it was termed as 'C18' before its identification.

Firstly, auto-aggregation is the property of the bacterial strain to adhere to the walls of the intestinal tract of humans. There might be involvement of certain yet-unknown proteins which facilitate the adhesion of the bacterial cell with the cells of the intestinal tract. Thus, a potential probiotic bacterium should display a higher value of auto-aggregation. Here, we have compared the auto-aggregation of C18 bacterial strain with the auto-aggregation of a known bacterium *Streptococcus cremoris*.

Secondly, co-aggregation property of an organism enables the bacteria to create a barrier to prevent the adhesion of pathogenic microorganisms in the human intestinal tract. It is labelled to be one of the important features of the probiotic bacteria to prevent the colonization of pathogenic organisms. Thus, a potential probiotic bacterium should display a higher value of co-aggregation. Here, we used *S. cremoris* (control) and C18 to study the co-aggregation property of C18.

The main purpose of this study was to establish a relationship between auto-aggregation and co-aggregation of *Lactobacillus plantarum* (C18) to determine whether it could have a future as a potential probiotic bacterium.

## MATERIAL AND METHODS

*Streptococcus cremoris* strain was maintained on MRS medium at – 4p C. Before experimental use, *S. cremoris* was sub-cultured twice in MRS broth (composition stated below) for the enrichment of the culture. It was made sure that there were no precipitation after autoclaving as it would mean that there is a loss of nutrients.

The ingredients were dissolved in 1000 mL distilled water and the pH was adjusted at 6.2

to 6.6 and then medium was sterilized at 121°C for 15 minutes, under 15lb pressure.

#### Isolation of Lactobacilli from yogurt<sup>7-8</sup>

##### Collection of Samples

Yogurt samples were collected from a shop in Bhosari, immediately after their collection, they were stored aseptically in low temperature (-4p C) refrigerator to protect from contamination and deterioration.

##### Media

The medium used to culture the *Lactobacillus* strain was the same as the one used to culture *Streptococcus cremoris*. The composition of MRS broth is stated above.

##### Isolation of bacteria

*Lactobacillus* was isolated from yoghurts by using MRS medium. One gram of each sample was dissolved into 100 ml of MRS broth at pH 6.5.

After dissolving into MRS broth they were in homogeneously and were incubated at 37°C for 24 h in aerobic condition. The cultures were subjected to five subculture at 37°C under low pH (pH 4.5) and anaerobic condition in the presence of 10% CO<sub>2</sub> to remove unwanted bacteria. After serial dilution, the sample was further plated on MRS agar plates and a single colony of *Lactobacillus* was isolated by observing their colony morphology and some biochemical tests and the culture were maintained in MRS broth at pH 5.5.

##### Identification of Lactobacillus

The C18 isolated bacterial strain was identified as *Lactobacillus plantarum* by 16S rRNA gene sequencing.

##### Auto-aggregation and co-aggregation assays

Bacteria were grown for 18 h at 37p C with MRS liquid medium. The cells were harvested by centrifugation at 5000 g for 15 min, washed twice with phosphate buffered saline (PBS) and resuspended in their culture supernatant fluid to give viable counts of approximately 10<sup>8</sup> CFU/ml.

##### Auto-aggregation assay

Cell suspensions (4 ml) were mixed by vortex for 10 seconds and auto-aggregation was determined during 5 h of incubation at room temperature. 0.1 ml of the upper suspension was transferred to another tube with 3.9 ml of PBS and the absorbance (A) was measured at 600 nm at regular time intervals.

The auto-aggregation percentage is expressed as:

$$1 - (A_t/A_0) \times 100,$$

Where,

‘A<sub>t</sub>’ represents the absorbance at ‘t’ time and ‘A<sub>0</sub>’ the absorbance at t = 0.

##### Co-aggregation assay

Co-aggregation properties of the probiotic bacteria form a barrier that prevents the colonization of pathogenic micro-organisms. The method for preparing the cell suspensions for co-aggregation was the same as that for auto-aggregation assay. Equal volumes (2 ml) of each cell suspension were mixed together in pairs by vortex for 10 seconds. Control tubes were set up at the same time, containing 4 ml of each bacterial suspension on its own.

The absorbance (A) at 600 nm of the suspensions was measured after mixing and after 5 h of incubation at room temperature. Samples were taken in the same way as in the auto-aggregation assay.

The percentage of co-aggregation was calculated using the equation of Handley et al. (1987)<sup>6</sup>.

$$\frac{(Ax + Ay) / 2 - A(x + y)}{Ax + Ay / 2} \times 100$$

Where,

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

##### Gram staining

Gram staining was done to confirm that the strain was not contaminated. A smear was made and was air dried and heat fixed. Crystal violet dye was added to the smear and kept at room temperature for 30-40 seconds. It was then washed twice with water and then grams iodine was added and left for another 1 - 1.5 minutes at room temperature. The smear was washed twice with water. Alcohol washes were given to the smear till violet color faded away. It was again washed twice with water and safranin was added for 5-6 minutes at room temperature. Lastly, it was washed twice with water before air drying and observing under oil immersion lens at the magnification 40x.

## RESULTS

It has been known for some time now, that probiotic bacteria have considerably higher

percentage of auto-aggregation and higher percentage of co-aggregation. Higher auto-aggregation enables them to attach to the walls of intestinal tract with ease whereas higher co-aggregation helps them to create a barrier to prevent the colonization of pathogenic organisms in the intestinal tract. The culture of bacteria was dissolved in its own MRS broth supernatant as there would be some extracellular components concerned with auto-aggregation which might interfere with the absorbance readings.

#### Auto-aggregation

The absorbance of *S. cremoris* (in Saline v/s Supernatant) goes on decreasing as time progresses (Fig. 1). This is expected as the protein present in the extracellular solution seems to go on degrading as time progresses. These proteins are said to be responsible in aiding the adhesion of the probiotic bacteria to the walls of intestinal tract. Thus, the ideal probiotic organism should be capable of fast auto-aggregation.

This experiment was performed again to make sure that the results observed in Fig. 1 were reproducible. Different time intervals were used for this set. Similar results were seen as those

observed in Fig. 1 (Data not shown). As seen before, supernatant diluted bacterial cultures show much higher and quicker increase in the auto-aggregation as compared to saline diluted bacterium.

The absorbance observed when the same protocol was applied when C18 strain of bacteria was diluted in supernatant and saline solution. This bacterial strain was isolated from yogurt and gram staining was performed. It was observed that for this bacterial strain as well, there is higher absorbance seen at the earlier stages of supernatant diluted bacterial culture as compared to saline diluted cultures (Fig. 2).

Significant increase in the absorbance was observed in terms of supernatant diluted C18 bacterial culture as compared to the saline diluted C18 culture (Fig. 2). As the supernatant forms of both the C18 bacteria and the control *S. cremoris* are giving expected results, we decided to compare the percent auto-aggregation of those two bacterial cultures in supernatant solution (Fig. 3).

Higher percentage of auto-aggregation was observed when C18 strain of bacteria was diluted in supernatant solution as compared to the

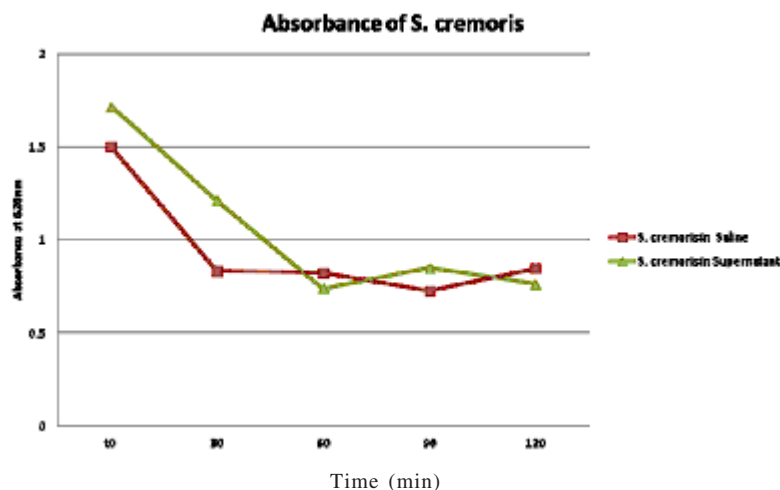


Fig. 1. Absorbance at 620 nm when *S. cremoris* diluted in Saline and Supernatant performed at time intervals of 30 mins each

control (*S. cremoris*) auto-aggregation (Fig. 3). The auto-aggregation of C18 strain was not only quicker than *S. cremoris*, but also longer lasting.

Results obtained from auto-aggregation assays (described in Methods) showed that C18 strain displays a strong auto-aggregation property

as compared to that of *S. cremoris*. It was also seen that the auto-aggregation was more when the bacterium was cultured in broth (dissolved in supernatant) than when grown in agar. Also, the auto-aggregation was lost when the strain was washed and suspended in PBS (Data not shown).

### Co-aggregation

Higher co-aggregation helps them to create a barrier to prevent the colonization of pathogenic organisms in the intestinal tract. Percentage of Co-aggregation was calculated

between *S. cremoris* and C18 when diluted in both supernatant (Fig. 4). This set of experiment was performed in triplicates and the average value of each reading is represented in the graph below.

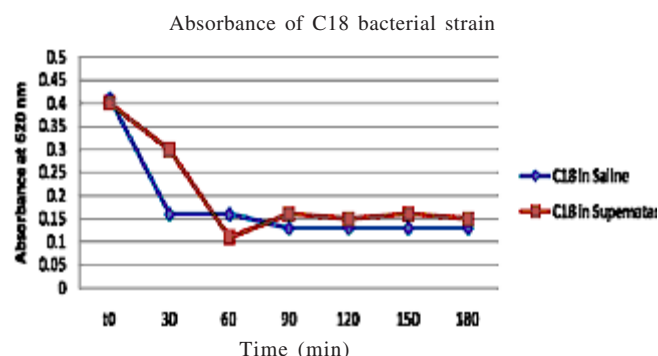


Fig. 2. Reduction is seen in the absorbance at 620 nm as time progresses

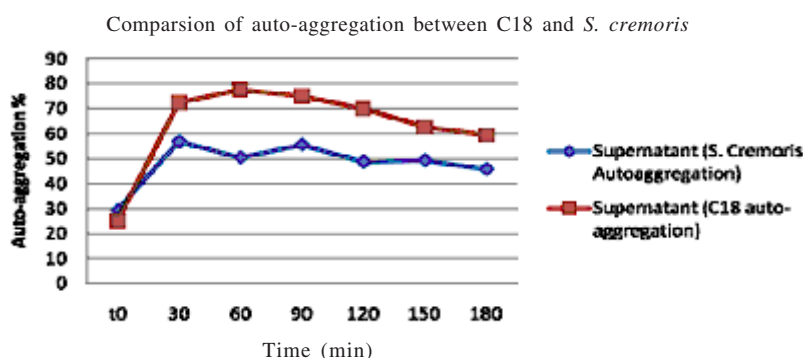


Fig. 3. Comparison between auto-aggregation of C18 bacterial culture and *S. cremoris*

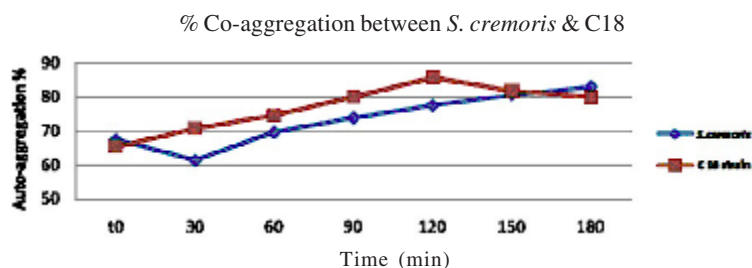


Fig. 4. Percentage of Co-aggregation between *S. cremoris* and C18

### DISCUSSION

Based on the papers published before on the auto-aggregation and co-aggregation properties of probiotic bacteria, we have managed to support the hypothesis stating that probiotic bacteria show higher auto-aggregation and higher co-aggregation as quickly as possible (30-60 mins range) <sup>(8)</sup>. As the probiotic bacteria needs higher

auto-aggregation properties to successfully get attached to the walls of the intestinal tract and higher co-aggregation to successfully defend off pathogenic organisms from the human gut, we can say that *S. cremoris*, the organism used in this experiment is a good candidate for probiotic use.

Even after repeating the experiments twice in order to confirm the results obtained the first time were reproducible, we have observed that

there are certain extracellular components are present in the supernatant of the broth where the bacterium was cultured and it plays an important role in auto-aggregation. The results obtained seemed to be perfectly matching the essential requirements of ideal probiotic bacteria, thus helping us re-confirm that *S. cremoris* is rightly an ideal candidate for probiotic use.

Although there was no difference seen in the absorbance when the bacterial culture was diluted in saline instead of its own broth's supernatant, it helps us to assume that the certain extracellular components which are helping in adherence of the bacteria to the walls of intestinal tract were missing. This could also mean that certain proteins are secreted out of the bacterial cell after their production which helps in attaching of the two surfaces.

There was higher amount of this component present in the supernatant, thus leading to higher absorbance values. It was also understood that the absorbance values of the supernatant (or even saline) diluted bacterial cultures will decrease as time progresses as there would be degradation of protein(s) at room temperature as it is advisable to keep the samples at 4p C.

When comparison was made between the percentages of auto-aggregation observed in supernatant diluted form of *L.plantarum* and *S. cremoris*, using this bacterium as control, we observed that the *L.plantarum* strain shows better auto-aggregation properties as compared to the control. Although further experiments are required, we can assume that *Lactobacillus plantarum* could be an ideal candidate for probiotic use.

Gram staining was performed to confirm the presence of gram-positive bacteria isolated from yogurt which was used for the experiments.

## CONCLUSION

*S. cremoris* shows high auto-aggregation and high co-aggregation property when grown in MRS culture broth. It could be used as control for probiotic bacteria as it shows elevated levels of aggregation during the initial stages of the

experiment (30-60 mins). *Lactobacillus plantarum* showed not only a higher percentage of auto-aggregation than that of *S. Cremoris*, but also a quick response. The co-aggregation seen in *L.plantarum* was higher than that observed in *S.cremoris*. Although further experiments like adhesion assays and testing the bacterial strain on CaCo - 2 cell lines are required to confirm that this bacterial strain would be ideal for probiotic use. Encapsulation could also be performed to figure out the commercial delivery of this bacterial strain.

## REFERENCES

1. Rolfe, R.D. The role of probiotic cultures in the control of gastrointestinal health. *Journal of Nutrition*, 2002; **130**(2): 396S-402S.
2. Wood, B.J.B (ed) Lactic Acid Bacteria in Health and Disease, vol. 1. Cambridge: Elsevier Science Publishers Ltd 1992; pp. 171-192.
3. Fooks, L.J. & Gibson, G.R. Probiotics as modulators of the gut flora. *British Journal of Nutrition*, 2002; **88**: S39-S49.
4. Boris, S., Suarez, J.E. and Barbes, C. Characterization of the aggregation promoting factor from *Lactobacillus gasseri*, a vaginal isolate. *Journal of Applied Microbiology*, 1997; **83**: 413-420.
5. Reid, G., McGroarty, J.A., Angotti, R. and Cook, R.L. Lactobacillus inhibitor production against *Escherichia coli* and coaggregation ability with uropathogens. *Canadian Journal of Microbiology*, 1988; **34**: 344-351.
6. Hoque, M.Z., Akter, F., Hossain, K.M., Rahman, M.S.M., Billah, M.M., and Islam, K.M.D. Isolation, Identification and Analysis of Probiotic Properties of *Lactobacillus Spp.* From Selective Regional Yoghurts. *World Journal of Dairy & Food Sciences*, 2010; **5**(1): 39-46.
7. De Man, J.C., M. Rogosa and M.E. Sharpe, 1960. A medium for the cultivation of lactobacilli. *Journal of Applied Bacteriology*, 1960; **23**(1): 130-135.
8. Kos, B., Suskovic, J., Vukovic, S., Simpraga, M., Frece, J. and Matosic, S. Adhesion and aggregation ability of probiotic strain *Lactobacillus acidophilus* M92. *Journal of Applied Microbiology*, 2002; **94**: 981-987.