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In Vitro Evaluation of Prebiotic Attributes of Fermentable Fibres

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Abstract

Probiotics and prebiotics either alone or in combination are used to treat metabolic disorders. Many of the probiotics and prebiotics are already a part of our diet like *Lactobacillus, Bifidobacterium* and inulin, oligofructose, respectively. Many studies have got prominent results indicating that the probiotics and prebiotics have the potential to alter the gut microbiota composition. Recently, a new formulation called synbiotic, has been developed by the researchers. Selection of the suitable soluble fibre that have the ability to stimulate the growth of the particular probiotic strain is the key step in the formulation of synbiotic. *In vitro* studies are needed to evaluate the effect of different soluble fibre (prebiotic attributes) on the growth of probiotic strains. In the present study prebiotic attributes of soluble fibres viz. pectin and gum acacia were evaluated using *L. amylovorous* MTCC8129 and *L. bifermentum* MTCC3818. MRS media containing the pectin @1.5% had stimulated the growth of *L. amylovorous* MTCC8129, and can be used as potential prebiotic for the growth of *L. amylovorous* MTCC8129. It showed similar trends of growth pattern when compared to the positive control containing 2% dextrose. Other concentrations of media containing pectin and gum acacia didn't show any promising results on the growth of *L. amylovorous* MTCC8129 and *L. bifermentum* MTCC3818. In conclusion, pectin @1.5% can be used to enhance the number of beneficial bacteria in the gut of human. This leads into amelioration of metabolic disorders viz. obesity, diabetes, cardiovascular diseases etc.

1.0 Introduction

Human colon is home for more than trillions of micro-organisms which confers many beneficial effects on human health [1-3]. Number of genes in human microbiome outnumbers the number of genes in the human genome [2,3]. Microbiomes provide the human with added gene products that have an important role in maintaining human homeostasis [3]. The gut microbiota can be considered as a "microbial organ" which regulates many functions in our body without which the very existence of human become impossible [3]. Gut microbiota

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regulates many functions such as defence against pathogens at the gut level, immunity, development of intestinal microvilli, and the synthesis of several vitamins [4,5] and also these organisms have crucial role in conditions like obesity [6-8], diabetes [9-11], inflammatory bowel diseases [12], cardio vascular diseases and even cancer [13]. Even though human contains a large microbial diversity, only a small number of microbial phyla predominates in the intestine and this include *firmicutes* (~60%), *Bacteroidetes* (~15%), *Actinobacteria* (~15%), *Verrucomicrobia* (~2%), *Proteobacteria* (~1%), *Methanobacteriales* (~1%) [3]. However, the functions of the bacteria in each phylum varies and it is highly diverse. The main characteristic of intestinal microflora is that unlike human genome this can be modified, and this high plasticity of microbial flora can be used for manipulating the microflora thus improving the microbiota associated host disease or can be used to enhance the gut microbiota promoting health [3,14].

Functional food ingredients such as bifidogenic carbohydrates and sugar preparations are compounds which are mainly designed to promote beneficial effects on gut microbiota by positively influencing the balance of the gut microflora [15-17]. These compounds are popularly known as prebiotics and have the potential to act as an alternative to probiotics which are live microorganisms that are administered directly [15]. The main characteristic that should possessed by any compound to be considered as a prebiotic is that it should be able to pass through the upper intestinal section without being hydrolysed so that it can actively be used up by the gut microflora [16,18,19]. These prebiotic compounds can be obtained from various sources such as from different food and from different plant sources. There are two main classification of prebiotics i.e. soluble fibres and insoluble fibres. Soluble fibres are those compounds that are soluble in water and insoluble fibres are compounds that are insoluble in water. Insoluble fibres are also non-fermentable as it is resistant to the activity of intestinal microflora. Cellulose, hemicellulose, lignin and resistant starch are classified under non-soluble fibres. Soluble fibres include pectin, inulin, gums, β-glucan, fructo-oligosaccharides etc. and it has the capacity to form in to gels. Soluble fibres are fermentable as it can be used by the intestinal micro-organisms [20]. The consumption of dietary fibres promotes many beneficial effects such as inhibit pathogen growth, reduces gastro-intestinal disorders, reduces colonic cancer, stimulate immune system, reduce cardio vascular diseases, maintain mucosal integrity, reduce adipogenesis and increase lipolysis [21,22]. If non-dietary fibres are available in the intestine anaerobic bacteria could utilize it by fermentation, but the product thus formed by fermentation could be toxic and carcinogenic such as ammonia or phenolic compounds

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which will adversely affect the human health [23,24]. Thus, the importance of dietary-fibres for maintaining the balance of gut microflora and associated health benefits are evident here.

Viable strains of microorganisms can be administered orally to promote specific changes in gut-microbiota which are then integrated into the gut microflora for conferring health benefits [25]. These strains of bacteria which are administered orally in adequate amounts elicits beneficial effect on host health by improving the intestinal balance are called probiotics [26]. Probiotics are well known for their ability to promote health. Probiotics improve health by various means and it includes reducing LDL-cholesterol, decreases tissue inflammation, enhancing intestinal microflora balance, increases lipolysis and reduces lipogenesis, inhibiting the growth of harmful bacteria, promoting digestion, improving immune functions and by reducing obesity [26]. The most frequent strains used as probiotics belong to the genera *Bifidobacterium* and *Lactobacillus* [27]. The main characteristics that a probiotic should possess is to resist the physio-chemical conditions that prevail in the digestive tract and the ability to get integrated with the epithelial microflora and after integration it should not elicit any inflammation/immune response [26,28].

Recently many studies have conducted to prepare new formulation which contain both pro- and prebiotics. These combination of pro- and prebiotic are known as synbiotics [16,23, 28] and it may have two- fold positive effect on intestinal microflora. These preparations are done by growing probiotic bacterial strains in combination with sugars of prebiotic importance. These combinations will increase the rate of survival of probiotic strains in the harsh conditions that prevail in the intestine [29,30]. This research has been carried out to understand the dose dependent effect of pectin and gum acacia on growth of two different probiotic culture viz. *Lactobacillus amylovorous* MTCC8129 and *Lactobacillus bifermentum* MTCC3818 and to understand its potential as a synbiotic combination.

2.0 Materials and Methods

2.1 Chemicals

All chemicals used in the present study were of AR grade. Beef extract powder, Proteose peptone, Yeast extract, Magnesium sulphate, Manganese sulphate, Sodium chloride, Tween 80 and Dipotassium hydrogen phosphate were purchased from Loba chemie Pvt Ltd, Mumbai, Maharashtra. Sodium acetate was purchased from Central Drug House (CDH) Pvt Ltd, New Delhi. Ammonium citrate were purchased from Molychem, Mumbai, Maharashtra.

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Lactobacillus MRS agar and Lactobacillus MRS broth were purchased from HiMedia Laboratories Pvt. Ltd., Mumbai

2.2 Soluble fibres

Pectin (100% pure) was purchased from Central Drug House (CDH) Pvt. Ltd., New Delhi and Gum Acacia was purchased from Loba Chemie Pvt. Ltd., Mumbai, Maharashtra.

2.3 Cultures

Two cultures viz. *Lactobacillus amylovorous* MTCC8129 and *Lactobacillus bifermentum* MTCC3818 were purchased from Institute of Microbial Technology (IMTECH) Chandigarh.

2.4 Maintenance and propagation of cultures

The probiotic cultures *L. amylovorous* MTCC8129 and *L. bifermentum* MTCC3818 purchased from IMTECH, Chandigarh were aseptically transferred (@1%) to freshly prepared sterile MRS broth, and was incubated for 24 hours at 37°C. Cultures were sub-cultured weekly, and kept at refrigeration temperature.

2.5 MRS Broth/Agar

MRS Broth was prepared by adding 55.15 gram, and MRS agar was prepared by adding 68.24 gram of powder in 1 L of distilled water and was microwaved to dissolve the powder thoroughly in distilled water. For sterilization purpose the broth/agar was autoclaved at 121°C for 15 mints at 15 psi pressure.

2.6 Basal medium

Carbohydrate free basal medium was prepared by adding all the ingredients of MRS broth except the dextrose. This media was used as the negative control. Positive control used was the MRS broth containing 2% dextrose. To evaluate the effects of soluble fibres (gum acacia/pectin) on the growth of Lactobacillus, gum acacia/pectin were added into basal media at different concentrations.

2.7 Preparation of media containing soluble fibres

Gum acacia/pectin added into basal media @ 0.5%, 1%, 1.5% and 2% (w/v). pH of the media was set to 6.5. Prepared media was then sterilised by autoclaving at 121°C for 15 min at 15 psi.

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2.8 Measurement of OD600 and pH

Twenty-four hours activated cultures viz. *L. amylovorous* MTCC8129 and *L. bifermentum* MTCC3818 were inoculated @1% into basal media, positive control and gum acacia/pectin containing basal media, respectively and incubated at 37 ^{o}C.

Media was thoroughly mixed and 1 ml of media was poured into the cuvette (path length = 1 cm) and OD was measured using spectrophotometer at 600 nm at different time intervals (0, 3, 6 and 24 hours). Basal media without any culture was used as blank.

At different time intervals (0, 3, 6 and 24 hours), decrease in pH of the media was measured using a pH metre. Decrease in pH indicated the proliferation of cultures in the media.

2.9 Measurement of viable count (log CFU/ml)

Viable counts of the bacterial cultures were determined using pour plate method. Briefly, 1.0 ml of culture was serially diluted in 0.85% saline, and plated on MRS agar plates. These plates were incubated for 24 hours at 37°C. Number of colonies were counted using the formula:

$$\text{CFU/ml} = \frac{\text{Number of colonies formed x Dilution Factor}}{\text{Volume of sample plated}}$$

3.0 RESULTS AND DISCUSSION

3.1 *In vitro* evaluation of prebiotic attributes of soluble fibres on *Lactobacillus amylovorous* MTCC8129

Gum acacia and pectin were added @ 0.5 %, 1%, 1.5% and 2% into 100 ml carbohydrate free basal media. Media without any carbohydrate (basal media) was used as negative control. MRS with 2% dextrose is used as the positive control.

3.1.1 Pectin as prebiotic fibre

Table 1: Effect of pectin on the growth (log CFU/ml) of *L. amylovorous* MTCC8129

CONCENTRATION (%)	**Time (h)**

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	0	**3**	**6**	**24**
0.5 % pectin	6.65 ± 0.09	6.92 ± 0.08	7.39 ± 0.51	7.63 ± 0.03
1 % pectin	7.14 ± 0.10	7.23 ± 0.04	7.85 ± 0.03	8.11 ± 0.01
1.5 % pectin	7.02 ± 0.07	8.3 ± 0.01	8.39 ± 0.08	8.55 ± 0.08
2 % pectin	7.14 ± 0.10	7.23 ± 0.12	7.53 ± 0.05	8.26 ± 0.02
Positive control	7.96 ± 0.02	8.08 ± 0.04	8.40 ± 0.02	8.73 ± 0.08
Negative control	6.48 ± 0.11	6.78 ± 0.06	7.00 ± 0.03	7.18 ± 0.06

Viable counts of *L. amylovorous* MTCC8129 grown on the MRS broth containing different concentrations of pectin were determined at different time intervals and are shown in Table 1. Viable counts in negative control were less as compared to the positive control. Positive control having 2% dextrose showed the highest viable count (8.73 ± 0.08) after 24 hour of incubation period. Viable counts in 1.5% pectin MRS plate were comparable to the positive control media and even showed higher values after 3 hour of incubation time. This implicates that 1.5 % pectin media is a good candidate for the growth of *L. amylovorous* MTCC8129 strain. However, 0.5% pectin media showed negligible growth and can be compared to the viable count showed by the negative control. Many studies have shown that the pectin is a good dietary fibre and have prebiotic potential. The trend in viable count showed by 1.5% pectin media strongly supports these statements.

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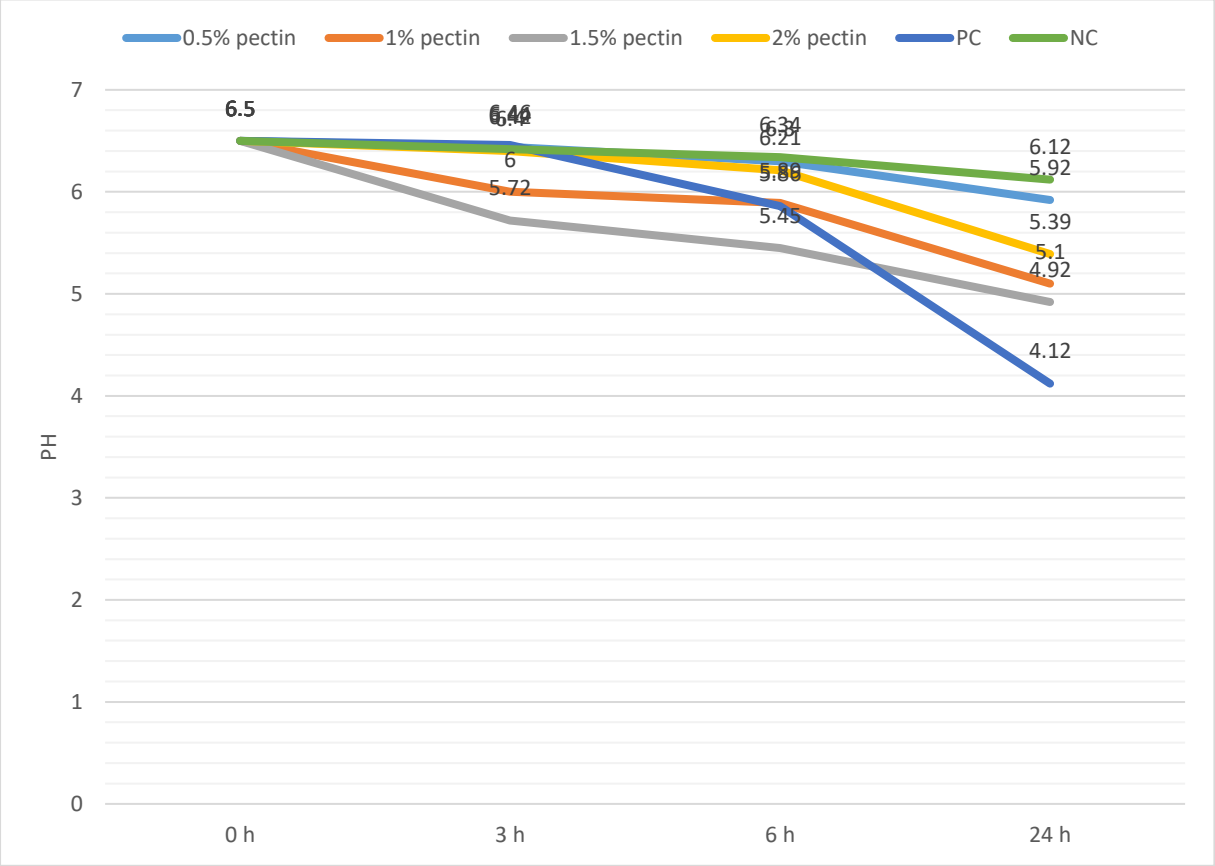

Fig 1. Evaluation of effect of pectin on the growth of *L. amylovorous* MTCC8129 by decrease in the pH of media at different time intervals

A decrease in the pH of media containing pectin at different time intervals due to growth of *L. amylovorous* MTCC8129 is shown in Fig. 1. Decrease in pH was observed to be maximum after 24 hour of incubation time in positive control plates. At 3 and 6 hours of incubation time, decrease in pH of media having 1.5% pectin was more than the positive control media. Negative control did not show any significant reduction in the pH, and is comparable to 0.5% pectin, both exhibited a similar trend in reduction in pH.

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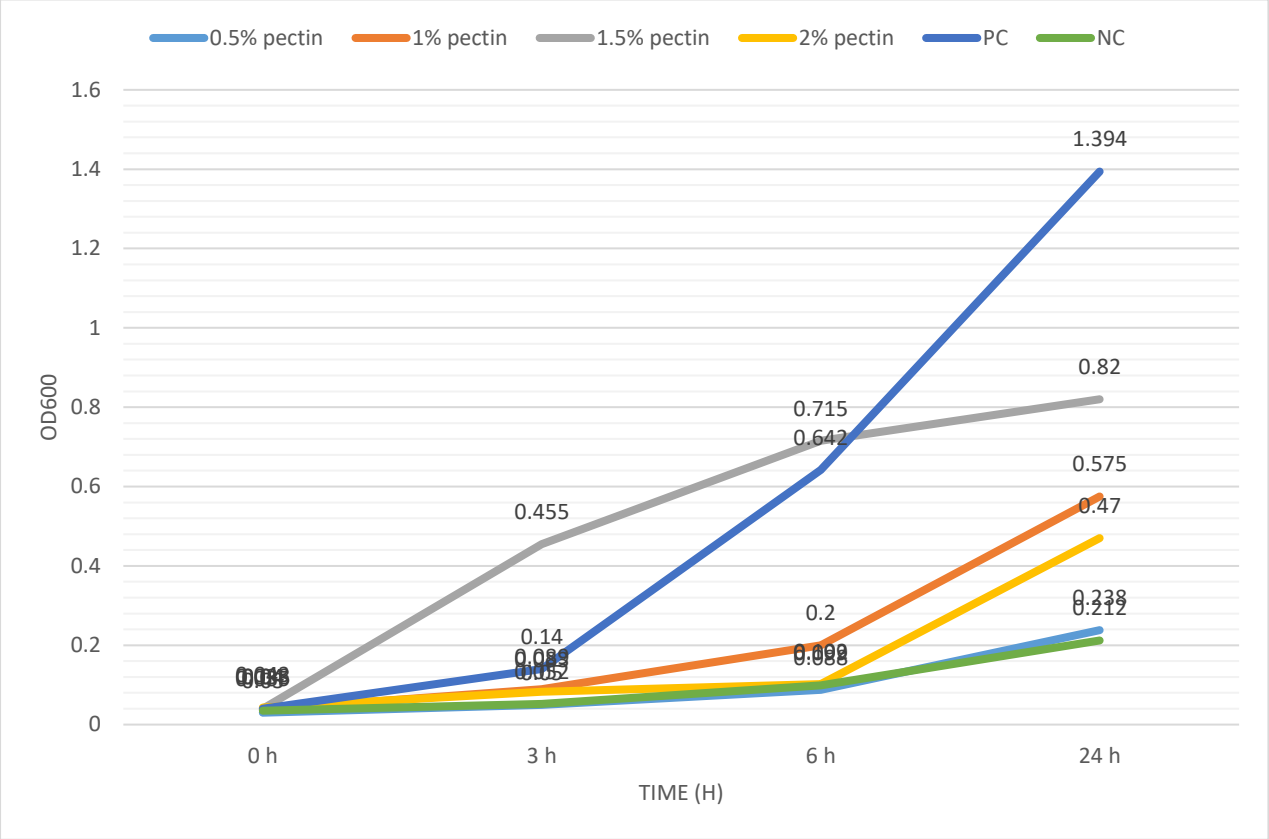

Fig. 2 Evaluation of effect of pectin on the growth of *L. amylovorous* MTCC8129 by increase in the OD_{600} of media at different time intervals

Increase in the OD_{600} of media containing pectin due to growth of *L. amylovorous* MTCC8129 at different time intervals is shown in Fig. 2. After 24 hours of incubation time, OD_{600} was observed to be highest in the positive control media. At 3 and 6 hours of incubation time, OD_{600} was even higher in 1.5% pectin media than positive control media. Both the 0.5 % pectin media and negative control showed similar trends in increase in pH and is negligible.

3.1.2 Gum Acacia as prebiotic fibre

Table 2: Effect of Gum Acacia on the growth of (log CFU/ml) *L. amylovorous* MTCC8129

Concentration (%)	**Time (h)**			
	0	**3**	**6**	**24**
0.5% acacia	7.23 ± 0.12	7.42 ± 0.08	7.93 ± 0.04	8.12 ± 0.02
1% acacia	6.93 ± 0.10	7.54 ± 0.08	8.09 ± 0.04	8.11 ± 0.17

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1.5% acacia	7.02 ± 0.15	7.34 ± 0.08	7.65 ± 0.07	8.08 ± 0.02
2% acacia	7.33 ± 0.09	7.97 ± 0.04	7.67 ± 0.08	8.21 ± 0.02
Positive control	7.96 ± 0.02	8.08 ± 0.04	8.40 ± 0.02	8.73 ± 0.08
Negative control	6.48 ± 0.11	6.78 ± 0.06	7.00 ± 0.03	7.18 ± 0.06

Viable counts of *L. amylovorous* MTCC8129 grown on the MRS agar plates containing different concentrations of gum acacia were determined and are shown in Table 2. Positive control having 2% dextrose showed highest viable counts, followed by 2% acacia media, 1% acacia media, 0.5% acacia media and 1.5% acacia media. Viable counts in 2% acacia media were comparable to viable counts in positive control media. Gum acacia is considered as a bifidogenic carbohydrate, and it has showed increased proliferation of probiotic bacteria especially *Bifidobacteria*.

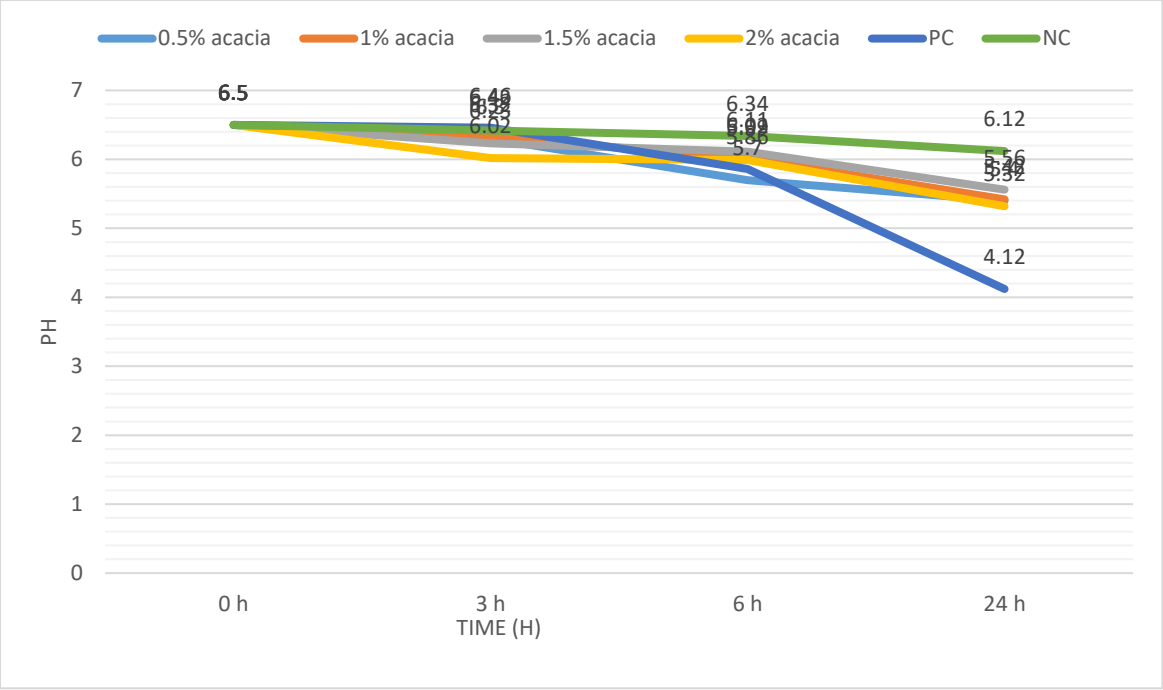

Fig 3. Evaluation of effect of pectin on the growth of *L. amylovorous* MTCC8129 by decrease in the pH of media at different time intervals

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A decrease in the pH of media containing gum acacia at different time intervals due to growth of *L. amylovorous* MTCC8129 is shown in Fig. 3. Decrease in pH was observed to be maximum after 24 hour of incubation time in positive control plates. Compared to the positive control, at 3 and 6 hours, decrease in pH of media having different concentration of acacia media was more or less same when compared to positive control plates. After 6 hours of incubation, positive control showed a drastic decrease in pH reaching a value of 4.12 followed by the 2% acacia, 0.5% acacia, 1% acacia and 1.5% acacia respectively.

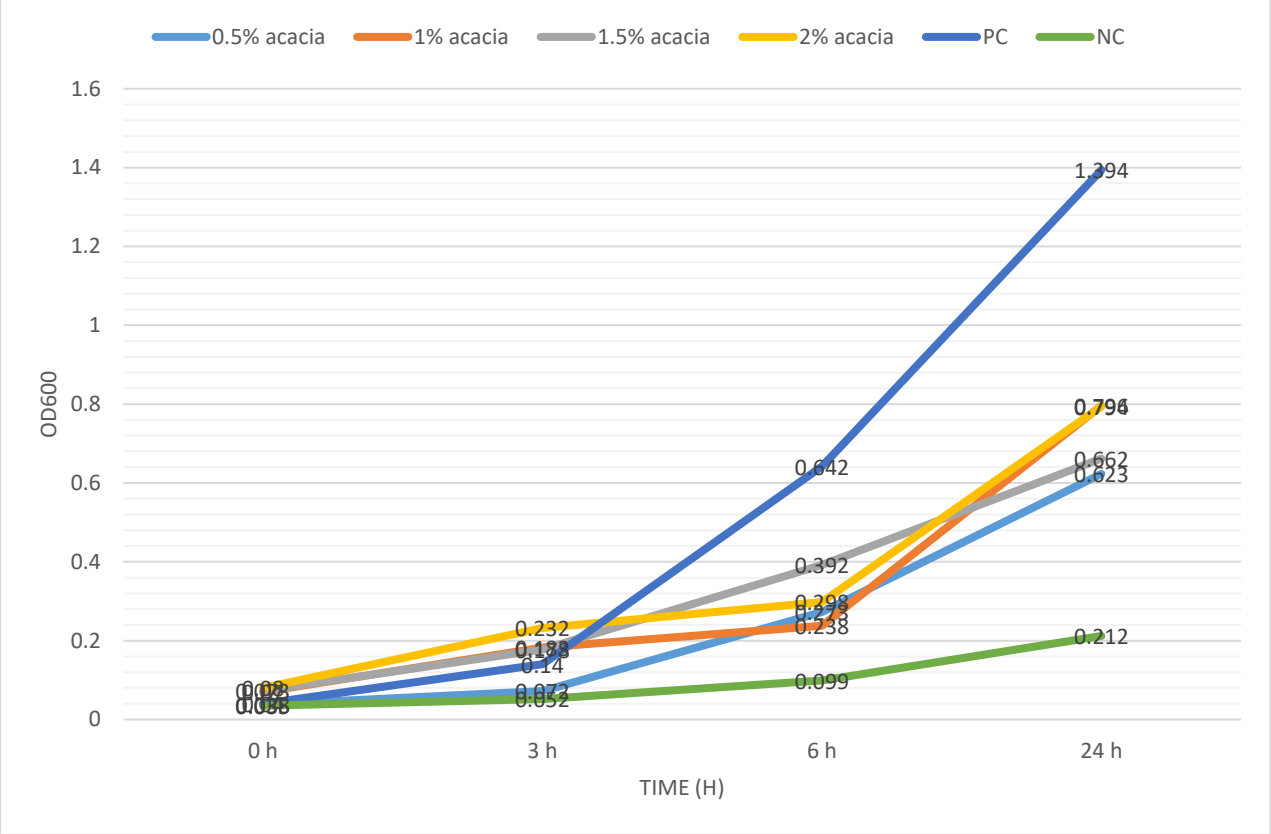

Fig 4. Evaluation of effect of gum acacia on the growth of *L. amylovorous* MTCC8129 by increase in the OD_{600} of media at different time intervals

Increase in the OD_{600} of media containing gum acacia due to growth of *L. amylovorous* MTCC8129 at different time intervals is shown in Fig. 4. After 24 hours of incubation time, OD_{600} was observed to be highest in the positive control media. Increase in OD_{600} of media having 0.5%, 1% and 2% of gum acacia at different time intervals was lower than positive control but higher than negative control media.

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3.2 *In vitro* evaluation of prebiotic attributes of soluble fibres on *Lactobacillus bifermentum* MTCC3818

Effect of pectin and gum acacia on the growth of *L. bifermentum* MTCC3818 was evaluated. Gum acacia and pectin were added @ 0.5 %, 1%, 1.5% and 2% into 100 ml carbohydrate free basal media. Media without any carbohydrate (basal media) was used as negative control. MRS with 2% dextrose is used as the positive control. Each media was inoculated @1% (v/v) with 24 h activated culture and incubated at 37°C. Aliquots were aseptically withdrawn at 0, 3, 6 and 24 h of incubation period, and tested for decrease in pH and increase in OD600. Viable counts (log CFU/ml) also determined at different time intervals.

3.2.1 Pectin as prebiotic fibre

Table 3: Effect of pectin on the growth (log CFU/ml) of *L. bifermentum* MTCC3818

Concentration (%)	**Time (h)**			
	0	**3**	**6**	**24**
0.5% pectin	6.61 ± 0.08	6.90 ± 0.06	6.90 ± 0.16	7.92 ± 0.05
1% pectin	7.32 ± 0.08	7.48 ± 0.09	7.75 ± 0.08	8.24 ± 0.01
1.5% pectin	7.15 ± 0.08	7.30 ± 0.11	7.35 ± 0.14	8.39 ± 0.02
2% pectin	6.98 ± 0.11	7.17 ± 0.09	7.35 ± 0.14	8.31 ± 0.01
Positive control	7.98 ± 0.03	8.14 ± 0.03	8.52 ± 0.02	8.80 ± 0.04
Negative control	6.73 ± 0.21	7.01 ± 0.08	7.13 ± 0.03	7.28 ± 0.11

Viable counts of *L. bifermentum* MTCC3818 grown on the MRS broth containing different concentrations of pectin were determined at different time intervals and are shown in Table 1. Highest viable counts were observed in positive control after 24 hours of incubation. After 24

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hours of incubation, viable counts in media containing pectin @ 1%, 1.5% and 2% reached near to viable counts in positive control.

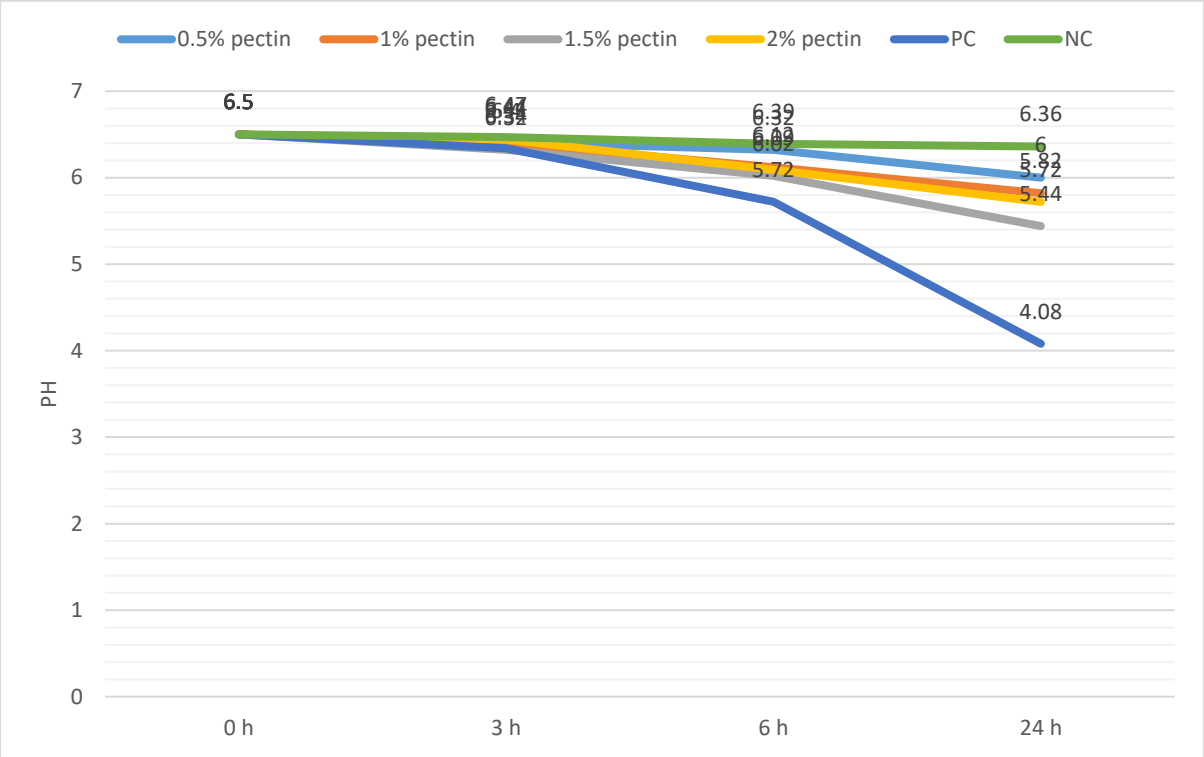

Fig. 5 Evaluation of effect of pectin on the growth of *L. bifermentum MTCC3818* by decrease in the pH of media at different time intervals

Change in the pH of media with or without different concentration of pectin @0.5%, 1%, 1.5% and 2% due to growth of *L. bifermentum* MTCC3818 was determined and shown in Fig. 5. Positive control showed the highest drop in pH after 24 hour of incubation time. At 6 and 24 hours of incubation, drop in the pH of media with pectin was not significant when comparing to the positive control. Decrease in the pH showed by the negative control and 0.5% pectin were almost similar.

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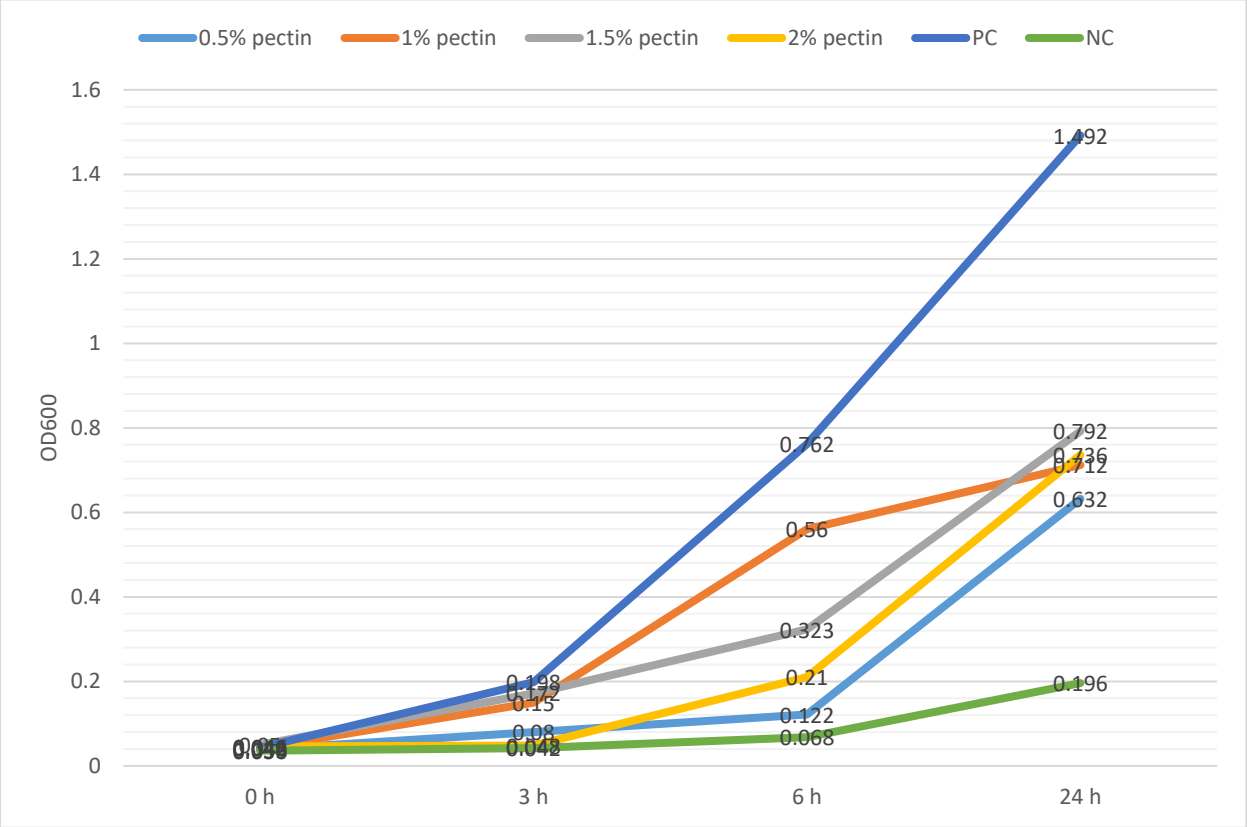

Fig 6: Evaluation of effect of pectin on the growth of *L. bifermentum MTCC3818* by increase in the OD_{600} of media at different time intervals

Change in OD_{600} of media with or without different concentrations of pectin due to the growth of *L. bifermentum* MTCC3818 was determined at different time intervals and presented in Fig. 6. OD_{600} was highest in case of positive control after 24 hours of incubation time. Increase in OD_{600} of media having different concentrations of pectin media was not significant when compared to the positive control. However, at 24 hours, 1.5% pectin media showed highest OD_{600}, followed by 2% pectin, 1% pectin, and 0.5% pectin, respectively.

3.2.2 Gum Acacia as prebiotic fibre

Table 4: Effect of Gum Acacia on the growth of (log CFU/ml) *L. bifermentum* MTCC3818

Concentration (%)	**Time (h)**			
	0 h	**3 h**	**6 h**	**24 h**
0.5% acacia	7.28 ± 0.06	7.41 ± 0.07	7.56 ± 0.04	8.02 ± 0.03

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1% acacia	7.25 ± 0.09	7.54 ± 0.03	7.71 ± 0.06	8.06 ± 0.02
1.5% acacia	7.11 ± 0.11	7.38 ± 0.08	7.52 ± 0.09	8.2 ± 0.03
2% acacia	7.23 ± 0.09	7.47 ± 0.05	7.52 ± 0.09	8.01 ± 0.06
Positive control	7.98 ± 0.03	8.14 ± 0.03	8.52 ± 0.02	8.80 ± 0.04
Negative control	6.73 ± 0.21	7.01 ± 0.08	7.13 ± 0.03	7.28 ± 0.11

Viable counts of *L. amylovorous* MTCC8129 grown on the MRS agar plates containing different concentrations of gum acacia were determined and are shown in Table 4. Highest viable counts were observed in case of positive control after 24 hours of incubation period. Positive control was followed by the 1.5% acacia, 1 % acacia and 2% acacia, respectively. Compared to the positive control, different concentrations of acacia media showed less increase in viability count. However, 1.5% acacia showed a viability count of 8.2 ± 0.03 CFU/ml compared to the 8.80 ±0.04 CFU/ml of the positive control after 24 hours of incubation.

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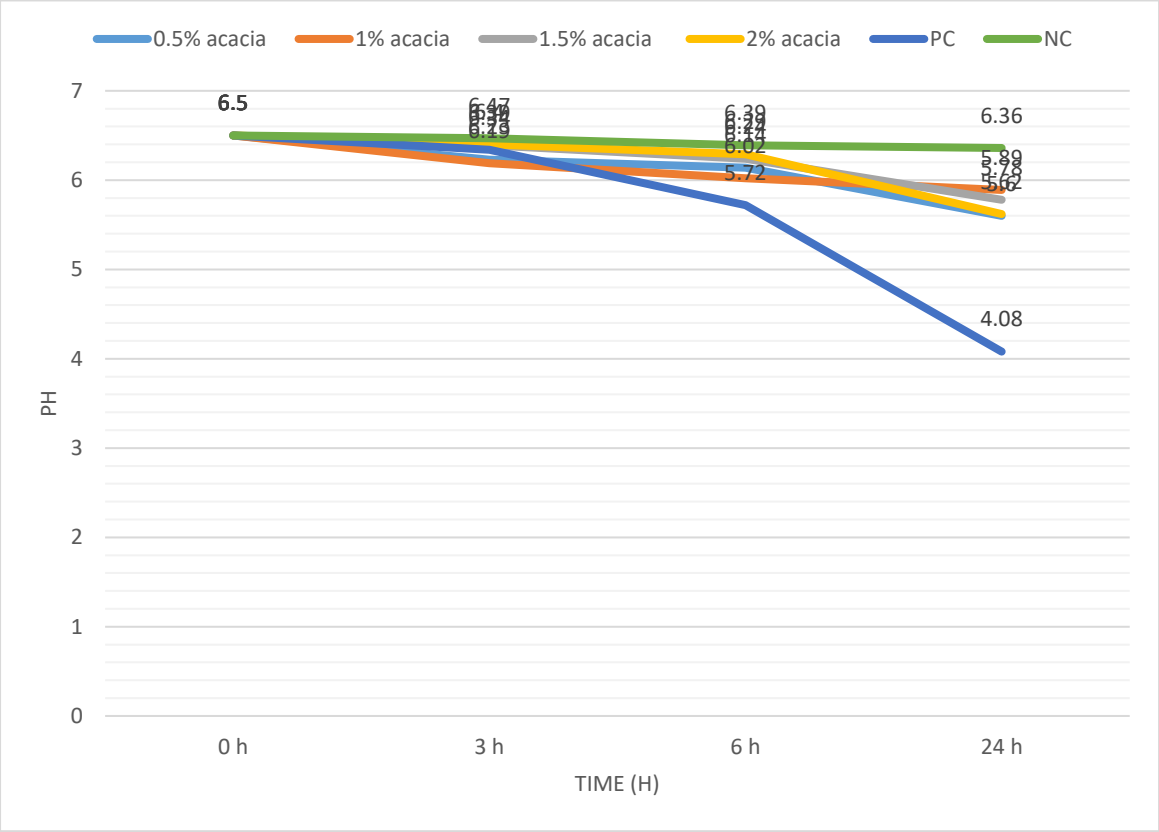

Fig 7: Evaluation of effect of gum acacia on the growth of *L. bifermentum* MTCC3818 by decrease in the pH of media at different time intervals

Change in the pH of media with or without different concentrations of gum acacia due to the growth of *L. bifermentum* MTCC3818 was determined, and represented in the Fig. 7. Because of the growth *L. bifermentum* MTCC3818, a decrease in pH was obtained. Highest drop in pH was shown by the positive control. Drop in pH of media having different concentrations of gum acacia was not significant when compared to the positive control after 24 hour of incubation period. At 3 hours, pH differences of different media were negligible. After 3 hours, positive control showed a huge drop in pH because of the fast proliferation of bacteria in the media. The bacteria ferment the gum acacia for their growth and produce various acidic compounds like short chain fatty acid (SCFA) and it is one of the reasons for the decrease in pH in the media

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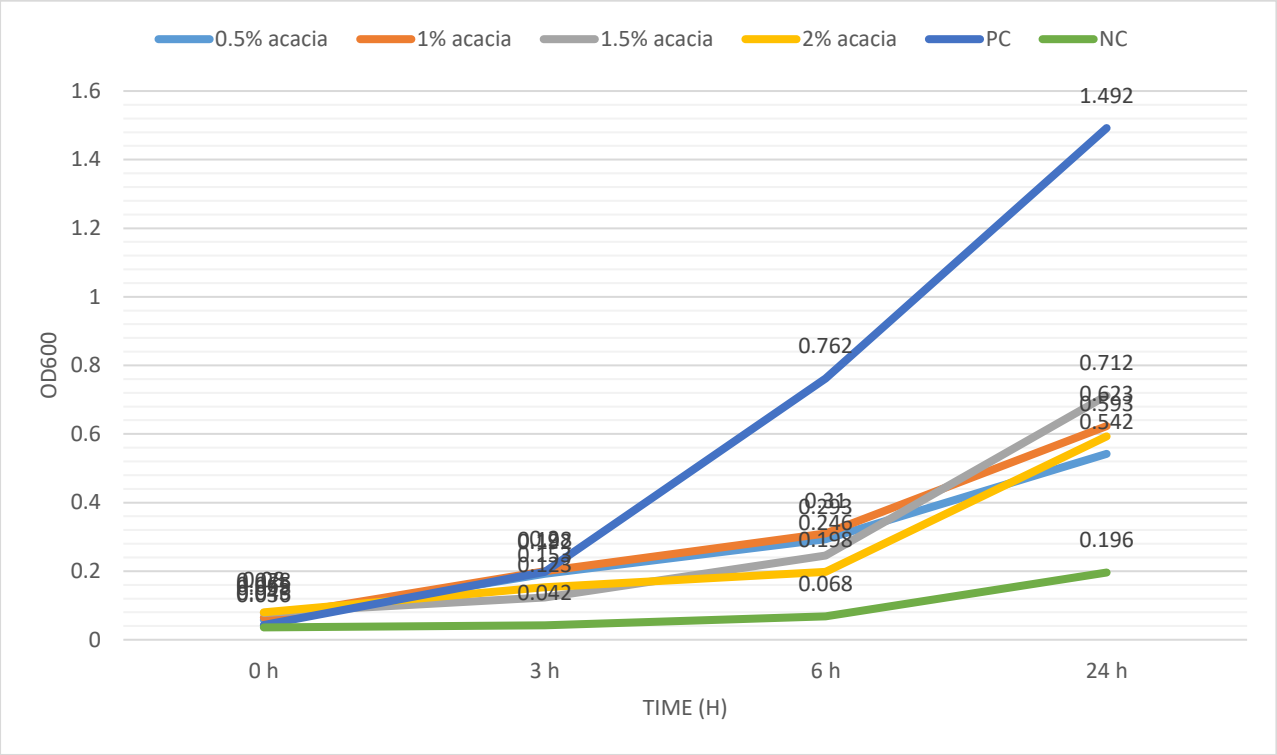

Fig 8: Evaluation of effect of gum acacia on the growth of *L. bifermentum* MTCC3818 by increase in the OD_{600} of media at different time intervals

Change in the OD_{600} of media with or without gum acacia due to the growth of *L. bifermentum* was determined and presented in Fig. 8. Compared to the positive control, the increase in OD_{600} of media having different concentrations of gum acacia was not significant, but acacia media promote the growth of *L. bifermentum* MTCC3818 to some extent. The maximum increase in OD_{600} was shown by the positive control. 1.5% acacia showed an increase in OD600 by a value of 0.712 followed by 1% acacia, 2% acacia and 1.5% acacia, respectively. The negative control showed a negligible increase in OD_{600}.

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