

## Effect of Inulin on the Growth and Antimicrobial Activity of *Bifidobacterium animalis* Subsp. *Lactis* 420- an Assessment

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**Abstract:** Due to the wide variations among prebiotics in their ability to influence the growth and beneficial attributes of probiotics assessment of the compatibility between the probiotic and the prebiotic is an essential parameter while selecting potential synbiotic pairs. In this study effect of a prebiotic, inulin on the growth and antimicrobial activity of a probiotic *Bifidobacterium animalis* subsp. *lactis* 420 was assessed through *in vitro* and *in-vivo* studies. *In vitro* studies revealed the ability of inulin to support the growth and antimicrobial activity of the probiotic strain as a reduction in mean generation time of the probiotic and zone of clearance against test organisms was observed when grown on inulin as the sole carbon source. On administering either the inulin, *Bifidobacterium animalis* subsp. *lactis* 420 or their combination to adult albino mice, a significant increase in the fecal bifidobacterial counts and decrease in fecal coliform and clostridial counts compared to the control group were observed. Among the different treatments the combined administration of inulin and *Bifidobacterium animalis* subsp. *lactis* 420 has resulted in highest numerical increase in fecal bifidobacterial count and reduction in fecal coliform and clostridial counts. The synergistic effect between inulin and *Bifidobacterium animalis* subsp. *lactis* 420 was most prominent against coliforms as a significant reduction in their counts was observed on combined administration of the synbiotic rather than administering either of them alone. Our study exposes the possibilities of exploiting this combination of inulin and *Bifidobacterium animalis* subsp. *lactis* 420, as a potential synbiotic pair. However, human volunteer trials with placebo control and blind coded samples are to be conducted to reach a final conclusion as it is the ultimate way to demonstrate the beneficial effects on human health.

**Key words:** Probiotics • Prebiotics • Synbiotics • Bifidobacterium • Inulin

### INTRODUCTION

The resident bacterial microflora of human colon constitutes around 95% of the live cells of the human body and is identified as a vital contributor towards host nutrition, health and disease [1]. The concepts like probiotics and prebiotics has evolved considering the vital role the gut microflora play in the well being of the host. Dietary supplements like these are purported to elicit their beneficial effects through the favorable management of gut microbiota. Lactobacilli and bifidobacteria are the most widely recognized probiotics. Even though bifidobacteria is identified as the main health promoting group, due to the increased oxygen susceptibility and difficulty in maintenance it's incorporation in foods as probiotics is limited in comparison to the lactobacilli.

Administration of food-grade bifidogenic factors alone or in combination with bifidobacteria is being identified as an alternative for counteracting the problems pertaining to food applications of bifidobacteria. Among the different bifidogenic factors, inulin a fructooligosaccharide extracted from chicory roots is one of the most studied and well established prebiotic. A prebiotic is defined as “a selectively fermented ingredient that allows specific changes, both in the composition and/or activity in the gastrointestinal microflora, that confer benefits on host well-being and health” [2]. It is suggested that the survivability, colonization and the beneficial effects of feeding an probiotic can be enhanced and extended by simultaneous administration of a prebiotic that can be used by the probiotic [3]. Among the different beneficial properties attributed to probiotics, antagonistic activity

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against pathogens is considered as a major one and is reported to be influenced by the substrate. The strain specificity exhibited in prebiotic utilization [4] and the differential impact of prebiotics on probiotic attributes necessitates the assessment of the compatibility between the prebiotic and probiotic while formulating any synbiotic product. It is also observed that the development of synbiotic from properly selected probiotic, prebiotic combinations where the prebiotic supplements the specific probiotic strain significantly increases the effectiveness of the developed synbiotic product. Considering these aspects a study was conducted to assess the prebiotic effect of inulin on the growth and antibacterial activity of a commercially available probiotic strain *Bifidobacterium animalis subsp. lactis B420*.

## MATERIALS AND METHODS

**Prebiotic and Probiotic:** Inulin (Raftiline HP, inulin >99.5%, average DP=23 monomers) was procured from Orafit, Belgium. The probiotic culture, *Bifidobacterium animalis* subsp. lactis B420 (B-420, Danisco, Germany) was maintained in MRS broth supplemented with 0.05% L-cysteine hydrochloride (mMRS) [5] with weekly subculturing. Purity of the culture was monitored periodically by plating on *Bifidobacterium* agar (HiMedia Laboratories Ltd., Mumbai). The culture was activated by 2 to 3 transfers in mMRS media with anaerobic incubation (using Anaero Hi Gas Pack, HiMedia Laboratories Ltd. Mumbai) at 37°C for 24 h.

**In vitro Assessment of Impact of Inulin on the Growth and Antibacterial Activity of *Bifidobacterium animalis* Subsp. Lactis B420:** 24 h old active culture of *Bifidobacterium animalis* subsp. lactis B420 ( $10^9$ cfu/ml) was inoculated into a semi-liquid (agar 0.1%, w/w) minimal media (meat peptone 1 percent (w/v), L-cysteine hydrochloride 0.04 percent (w/v), buffering salts and indispensable ions as in Garches medium [6] containing different levels of inulin (0, 0.5, 1, 3 and 5 percent (w/v) [7]. *Bifidobacterial* counts were determined at 0 h and after 24 h using *Bifidobacterium* agar and the growth stimulatory effect of inulin was assessed based on the percentage reduction in mean generation time in inulin containing media compared to that in control tube (0% inulin). Antibacterial activity of the bifidobacterial culture grown in mMRS media containing inulin as the sole carbon source was determined and compared with that grown in mMRS without inulin by agar well method [8].

Test organisms used were *E. coli* NCDC247, *S. typhimurium* NCDC113, *S. dysenteriae* NCDC107, *E. faecalis* NCDC116 and *S. aureus* NCDC109 (National Collection of Dairy Cultures, Karnal).

**In vivo Assessment of the Impact of Inulin on the Antibacterial Activity of *Bifidobacterium Animalis* Subsp. Lactis B420 Against Selected Fecal Organisms of Adult Albino Mice:** 6-7 weeks old adult male albino mice with an average body weight of 31g, obtained from Small Animal House, National Dairy Research Institute, Karnal, Haryana, India were used in this study. The basal diet given to mice was the one (crushed wheat-15%, crushed Bengal gram-57.6%, crushed groundnut cake - 4%, skim milk powder- 5%, casein-4%, salts mixture=4%, vitamins-0.2%, choline chloride mixture-0.2%) formulated institutionally and conventionally fed to the small animals maintained in the institute animal house. The animals were kept in plastic cages under conventional conditions and had free access to water during the entire experiment. The study was conducted with the approval of institutional ethics committee.

**Experimental Design of the Feeding Trial:** The mice were randomly assigned to four groups of seven each based on the type of feed given. During the feeding period, the control group was given the basal diet, the probiotic group basal diet supplemented with  $\sim 10^8$  live cells of *Bifidobacterium animalis* subsp. lactis B420/g feed, the prebiotic group, basal diet supplemented with inulin (5%, w/w feed) and the synbiotic group basal diet supplemented with both *Bifidobacterium animalis* subsp. lactis B420 ( $\sim 10^8$ cfu/g feed) and inulin (5% w/w feed). In the case of control and probiotic groups, inulin was replaced with the same amount of sucrose, a disaccharide which is completely digested and absorbed in the small bowel.

The study lasted for 30days and was divided into three consecutive periods: a 5 days adaptation period, a 15days supplementation period and a 10 days follow-up period to assess the sustainability of favorable changes, if any. The detailed experimental design of the study is given in Figure 1. For feeding trials, the culture (2 % v/v) was inoculated into mMRS and incubated at 37°C for 24h under anaerobic conditions. The cells were harvested by centrifugation at 12000 rpm for 10min at 4°C and mixed with the basal diet in order to get a count of  $10^8$  live cells of bifidobacteria/g feed. Throughout the experimental period food intake by all the groups were monitored daily. Fecal samples were collected at 5 days intervals by gently

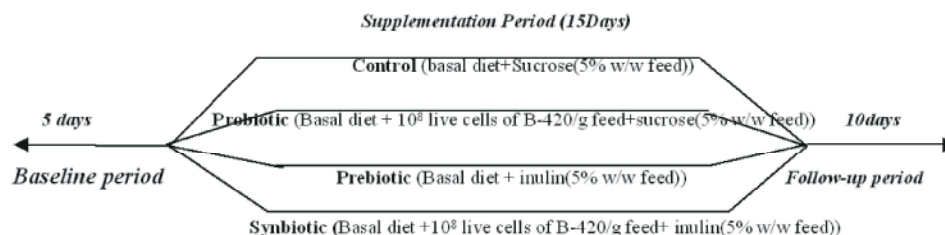


Fig. 1: Experimental Design

squeezing the rectal area of the mice. Analysis of fecal sample was done within 1.5 h of sampling. For this fresh feces were weighed and immediately diluted with peptone saline solution (0.1% peptone and 0.9% NaCl) containing L-cysteine hydrochloride (0.5 g/L). After dispersion, serial dilutions were made in the same diluent and were used for determining the bacterial count. Bifidobacterial count was enumerated on *Bifidobacterium* iodoacetate medium-25 (BIM-25) [9]. Coliforms and clostridia were enumerated on Violet red bile agar and Differential reinforced clostridial agar (HiMedia Laboratories Ltd., Mumbai) respectively.

Data was statistically analyzed using ANOVA according to the General Linear Models procedure of Systat Version 6.0.1 (1996, SPSS Inc.). When significant (1 and 5% levels) differences were observed, individual values were compared by Fisher's Least Significant difference.

## RESULTS

**In Vitro Assessment of Impact of Inulin on the Growth and Antibacterial Activity of *Bifidobacterium Animalis* Subsp. Lactis B420:** The growth and antibacterial activity of *Bifidobacterium animalis* subsp. lactis B420 on inulin was assessed in terms of reduction in mean generation time and formation of zone of clearance respectively (Table 1). The ability of *Bifidobacterium animalis* subsp. lactis B420 to utilize inulin was evident from the reduction observed in its mean generation time when grown in media containing inulin as the sole carbon source compared to that in a media devoid of any carbon source. On comparing the impact of level of inulin supplementation on growth stimulation it was observed that supplementation at 1, 3 and 5% resulted in a significant reduction in mean generation time compared that on 0.5 percent supplementation. No significant differences were observed between inulin concentrations of 1, 3 and 5% ( $P < 0.05$ ) in their growth stimulatory effect. Inulin could support the antimicrobial activity exhibited by *Bifidobacterium animalis* subsp. lactis B420 as zone

of clearance was observed against all the tested organisms except *E. faecalis*. The antibacterial activity exhibited by *Bifidobacterium animalis* subsp. lactis B420 was a broad one as it was antagonistic against both gram positive and gram negative bacteria. However the antibacterial activity exhibited by the organism grown in inulin added media was significantly lower than that grown in dextrose containing media ( $P < 0.05$ ).

**In-vivo Studies– Impact on Fecal Bifidobacterial, Coliform and Clostridial Counts:** As the potential of inulin to support the growth and antagonistic activity of *Bifidobacterium animalis* subsp. lactis B420 was established through the *in vitro* studies, we have subjected this combination to an *in vivo* assessment for its impact on fecal bifidobacterial, coliform and clostridial counts of albino mice. A significant increase in the fecal bifidobacterial counts and decrease in fecal coliform and clostridial counts of treatment groups compared to the control group was observed on feeding the dietary supplements ( $P < 0.05$ ; Table 2). However there was no significant difference in between the treatments in their effect on fecal counts except on the coliform count ( $P < 0.05$ ). Among the treatments, the combined administration of inulin and the probiotic was found to be most effective against coliforms, as a significant reduction in the fecal coliform counts of this group was observed compared to other two groups ( $P < 0.05$ ). On assessing the numerical increase in fecal bifidobacterial count of treatment groups from that during the pre-feeding period, the highest log increase was noted in the synbiotic group (1.46) followed by the probiotic group (0.86 log). Similar was the result in the case of reduction in fecal clostridium and coliform counts also, with the synbiotic treatment showing a higher reduction in log values (1.38 and 1.57) than the other two treatments (probiotic-1.11, 0.62, prebiotic-0.49, 0.83). On withdrawal of the supplementation, the fecal bifidobacterial and clostridial counts of all the groups returned to the baseline values ( $P < 0.05$ ), whereas the coliform counts remained at a low level even during the follow-up period.

Table 1: Effect of inulin on the growth and antibacterial activity of *Bifidobacterium animalis* subsp. lactis B420

					Antimicrobial activity (zone of inhibition, diameter, mm, inclusive of well diameter 7 mm).		
Growth stimulatory effect (mean generation time, hours)					Growth media		
Inulin concentration					Test organisms	mMRS containing Dextrose	mMRS containing inulin
0%	0.5%	1.0%	3.0%	5.0%	<i>Salmonella typhimurium</i>	18 <sup>a</sup>	12 <sup>b</sup>
58.8(0%)*	20.50 <sup>b</sup>	19.80 <sup>c</sup>	11.40 <sup>c</sup>	12.12 <sup>c</sup>	<i>Escherichia coli</i>	21 <sup>a</sup>	11 <sup>b</sup>
	(65.11%)	(66.32%)	(80.61%)	(79.39%)	<i>Staphylococcus aureus</i>	20 <sup>a</sup>	13 <sup>b</sup>
					<i>Shigella dysenteriae</i>	19 <sup>a</sup>	13 <sup>b</sup>

The values shown are the average of values obtained in three independent experiments. \*Values in parenthesis are percent reduction in mean generation time compared to control (0% inulin), ab - values bearing different superscripts differ significantly (P<0.05).

Table 2: Effect feeding of probiotic (B-420), prebiotic (inulin) and synbiotic (B-420 + inulin) on fecal bifidobacterial, coliform and clostridial counts (log cfu/g wet weight) of albino mice

Group (Days of fecal sample collection)	Parameter tested	Control group	Probiotic group	Prebiotic group	Synbiotic group
Baseline Period <sup>p</sup> (0, 5 day)	Bifidobacterial count	8.32 ±0.03 <sup>a</sup>	8.50±0.19 <sup>a</sup>	8.72±0.03 <sup>a</sup>	8.39 ± 0.07 <sup>a</sup>
Supplementation Period <sup>q</sup> (10,15,20 day)		8.40 ±0.16 <sup>b</sup>	9.36±0.25 <sup>a</sup>	9.44±0.11 <sup>a</sup>	9.85 ± 0.09 <sup>a</sup>
Follow-up period <sup>p</sup> (25, 30 day)		8.26 ±0.07 <sup>a</sup>	9.16±0.19 <sup>a</sup>	8.45±0.33 <sup>a</sup>	8.89 ± 0.04 <sup>a</sup>
Baseline period <sup>p</sup> (0, 5 day)	Clostridial count	4.87 ±0.04 <sup>a</sup>	4.67 ±0.01 <sup>a</sup>	4.566 ±0.17 <sup>a</sup>	4.86 ± 0.09 <sup>a</sup>
Supplementation period <sup>q</sup> (10,15,20 day)		4.68 ±0.15 <sup>b</sup>	3.56 ±0.21 <sup>a</sup>	4.07±0.04 <sup>a</sup>	3.48 ± 0.31 <sup>a</sup>
Follow-up period <sup>p</sup> (25, 30 day)		4.68 ±0.09 <sup>a</sup>	4.29 ±0.23 <sup>a</sup>	4.43 ±0.16 <sup>a</sup>	4.27 ± 0.25 <sup>a</sup>
Baseline Period <sup>p</sup> (0, 5 day)	Coliform count	5.51 ±0.15 <sup>a</sup>	5.43 ±0.16 <sup>a</sup>	5.56 ±0.23 <sup>a</sup>	5.82 ± 0.13 <sup>a</sup>
Supplementation period <sup>q</sup> (10,15,20 day)		5.23 ±0.04 <sup>c</sup>	4.81 ±0.12 <sup>b</sup>	4.73 ±0.11 <sup>b</sup>	4.25 ± 0.19 <sup>a</sup>
Follow-up period <sup>q</sup> (25, 30 day)		5.58 ±0.09 <sup>c</sup>	4.74 ±0.03 <sup>b</sup>	4.49 ±0.10 <sup>a</sup>	4.69 ± 0.09 <sup>b</sup>

Values are mean ± SE of counts obtained during the corresponding feeding period.

ab - Means with different superscripts within rows differ significantly (P<0.05).

pq - Sampling points with different superscripts differ significantly (P<0.01).

## DISCUSSION

Contradictory to the widely accepted bifidogenic nature of inulin, the inability of some pure cultures of bifidobacterium strains to utilize inulin is also being reported and is attributed to their failure to produce extracellular enzymes that hydrolyze long-chain fructans [1]. The probiotic used in our study, *Bifidobacterium animalis* subsp. lactis B420, was able to utilize inulin suggesting its ability to synthesize extracellular enzymes that hydrolyze long-chain fructans. This finding is of relevance as many bifidobacteria are reported to possess only inducible cell-associated  $\beta$ -fructofuranosidases [10]. The ability of *Bifidobacterium animalis* subsp. lactis B420 to produce extracellular enzymes offers an added advantage in the intestinal milieu, as these enzymes could support the mutual metabolic and nutritional dependencies among numerous Bifidobacterial species present there.

Nowadays inulin is incorporated in food products as a fat replacer, dietary fiber or for enhancing their therapeutic potential [11]. In this study it was observed that inulin supplementation at the rate of 1% is was sufficient to elicit the same growth stimulatory effect as that of 5%. This is of significance from an economical

point of view as it allows the incorporation of the prebiotic in a lower level to. We also observed a reduction in mean generation time of *Bifidobacterium animalis* subsp. lactis B420 upto an inulin concentration of 3% and though not statistically significant an increase upon further raising the inulin concentration to 5%. The observation is of significance while developing fermented milks incorporating this prebiotic as the level of addition could have an impact upon the growth kinetics of the starter culture used.

Pathogen inhibition and the production of benign and beneficial metabolites are identified as the mechanisms through which the beneficial bacteria reduce the risk of disease. One of the reason for the wide use of Bifidobacteria as a probiotic is its ability to exert powerful antipathogenic effect by excreting natural antibiotics [12]. As inulin was found to support the growth of *Bifidobacterium animalis* subsp. lactis B420 we have evaluated its effect on the antimicrobial activity of this probiotic. It was observed that the probiotic grown on inulin could inhibit the growth of all indicator organisms except *E. faecalis*. The resistance exhibited by *E. faecalis* is understandable as Enterococci are found to exhibit natural resistance to numerous antibiotics [13]. The antibacterial activity exhibited by microorganisms is

attributed to the fermentation products of the substrate available to them. So the significantly high antibacterial activity exhibited by *Bifidobacterium animalis* subsp. lactis B420 grown on dextrose could be attributed to the easy fermentation of dextrose, a simple sugar resulting in faster production of antimicrobial agents like organic acids than from the hydrolysis of a complex sugar like inulin. This is in agreement with the observation of [14] who reported differential modulation of antimicrobial activity of Lactobacilli by carbohydrate growth substrates.

The prebiotic, inulin, due to its ability to resist enzymatic digestion in the upper gastrointestinal tract and due to the presence of higher proportion of long chain fructans is found to survive transit through proximal colon and reach the distal colon intact [15]. As a result inulin might potentially have more effects on distal colonic fermentation and bacterial populations than a shorter chain fructan-type prebiotic like FOS or oligofructose, which are metabolically more active in the proximal colon [16]. The significance of the antimicrobial activity exhibited by *Bifidobacterium animalis* subsp. lactis B420 grown on inulin lies in the competitive advantage it would get over other microorganisms in a mixed culture environment such as the human gut due to its ability to utilize a substrate, which reaches the colon intact and is not easily utilized by many other organisms. So it could be suggested that a synbiotic combination of *Bifidobacterium animalis* subsp. lactis B420 and inulin could be more effective against distal colonic infections.

On conducting the *in-vivo* assessment of impact of inulin on the modulation of indigenous microbiota of albino mice by a proven probiotic *Bifidobacterium animalis* subsp. lactis B420, it was observed that the co-supplementation resulted in a higher increase in fecal bifidobacterial count and reduction in fecal clostridium and coliform counts from the baseline values. The findings of our study are consistent with many other studies which reported added advantages on combined administration of probiotics and prebiotics compared to feeding any of them alone [17, 18]. A numerical increase of 0.5 to 1.0 log<sub>10</sub> in bifidobacterial counts is considered sufficient to cause a major shift in the gut microbiota towards a "healthier" composition [19]. In this context all the three treatments resulted in more than 0.5 log increase in bifidobacterial count, thereby establishing their potential to beneficially manipulate the GI microbiota. The highest increase obtained on administration of the probiotic along with inulin, suggests an added advantage

on combined administration of probiotic and prebiotic rather than using either of them alone. The understanding that the proliferation of certain undesirable bacteria could be controlled by increasing bifidobacterial counts is based on the observation that consequent to the decline in bifidobacterial count during old age an increase in microorganisms like clostridia and members of Enterobacteriaceae family are noted [20]. In our study also concurrent to the increase in bifidobacterial count obtained in all the treatments groups, a significant reduction in fecal clostridial and coliform counts were also observed. This is of significance as Clostridia are toxin producers and have been implicated in many intestinal ailments such as nosocomial diarrhea, antibiotic-associated diarrhea, necrotizing enterocolitis, and gastrointestinal (GI) infections [21]. An inverse relationship between bifidobacterial and clostridial numbers has been reported in other bifidobacterium feeding studies also [22, 23]. In the present study, the synergistic association between *Bifidobacterium animalis* subsp. lactis B420 and inulin was most evident against coliforms as significant reduction in their counts was observed on administering the synbiotic rather than administering either of them alone. Among, the two main types of fermentations that occur in the gut, the saccharolytic fermentations are considered favorable to the host than the proteolytic fermentations because of the types of metabolic end products formed [19]. So it is obvious that the significant reduction in the number of a fecal putrefactive bacteria such as the coliforms [24] observed in this study on feeding the synbiotic is surely going to benefit the host by reducing the presence of toxic metabolites of proteolytic fermentation, some of which are even carcinogenic [25]. The inhibitory effect of synbiotic supplementation on members of coliform group observed in this study is consistent with other studies also [26, 27].

On assessing the persistence of the impact of the tested dietary interventions on fecal counts it was observed that on bifidobacterial and clostridial counts it was a transient one as the counts reverted to baseline values on withdrawing the supplementation. It suggests that the administered probiotic could only survive the transit through the gastrointestinal tract, but was unable to colonize and establish in the colon. It may be that the period of supplementation was not sufficient to allow them to get colonized. The transient nature of increase in bifidobacterial count observed in our study is consistent with other studies which reported the disappearance of

*Bifidobacterium animalis* subsp. lactis Bb-12, another well established probiotic belonging to the same subspecies as in our study, following cessation of feeding[28, 23]. However persistency was observed in the antagonistic effect against coliforms as the reduction in all treatment groups was significant even during the follow-up period. It could be that the probiotic, prebiotic and the synbiotic treatments somehow elicited a more prolonged antagonistic effect on fecal coliform counts than that towards other microbial populations evaluated in this study.

In conclusion, the *in vitro* and *in vivo* trials revealed the ability of inulin to support the growth and antimicrobial activity of a well established probiotic, *Bifidobacterium animalis* subsp. lactis B420. *Bifidobacterium animalis* is the most common species used in foods, as it has the highest tolerance to oxygen and acids [23]. These inherent features of *Bifidobacterium animalis* subsp. lactis B420, along with its ability to utilize a highly polymerized prebiotic inulin warrants better functional possibilities while developing synbiotic products incorporating them. The potential of a synbiotic pair of inulin and *Bifidobacterium animalis* subsp. lactis B420 to modulate the gut microbiota towards a healthier one was also established in this study and is worth looking at in larger studies in human volunteers.

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