DEVELOPMENT OF A LYOPHILIZED SYNBIOTIC PREPARATION CONTAINING *BIFIDOBACTERIUM* SPECIES AND INULIN

THESIS SUBMITTED TO THE
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IN PARTIAL FULFILMENT OF THE REQUIREMENT
FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY
IN
DAIRY MICROBIOLOGY

BY

LIGIMOL JAMES

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NATIONAL DAIRY RESEARCH INSTITUTE
(I. C. A. R.)
KARNAL - 132001 (HARYANA), INDIA

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Dedicated to

Almighty God

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This is to certify that the thesis entitled, **"DEVELOPMENT OF A LYOPHILIZED SYNBIOTIC PREPARATION CONTAINING *BIFIDOBACTERIUM* SPECIES AND INULIN"** submitted by **Ms. LIGIMOL JAMES** towards the partial fulfilment of the award of the degree of **DOCTOR OF PHILOSOPHY** in **DAIRY MICROBIOLOGY** of the **NATIONAL DAIRY RESEARCH INSTITUTE (DEEMED UNIVERSITY)**, Karnal (Haryana), India, is a bonafide research work carried out by her under my supervision, and no part of the thesis has been submitted for any other degree or diploma.

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ABSTRACT

The human large intestine is an intensely populated microbial ecosystem, where a delicate balance exists between the human intestinal microflora and its host. Upset of this system may lead towards the symptoms of acute gastroenteritis and chronic disorders. Probiotics, prebiotics and synbiotics are popular dietary strategies that have developed to target the gastrointestinal flora. Synbiotics, the combination of probiotics and prebiotics is a most novel idea to induce a favourable intestinal environment in hosts. Therefore, the present study was undertaken to select a *Bifidobacterium* culture based on probiotic attributes and to prepare a lyophilized synbiotic product incorporating the selected *Bifidobacterium* culture and inulin as well as to determine the effect of each factor (*Bifidobacterium*, inulin and their combination) on microecology of murine gut. Of the cultures tested *Bifidobacterium lactis* Bb-12 (Bb-12), *Bifidobacterium* species 420 (B-420) and Bifidobacteria (ABT-5) gave positive tests for the key enzyme of bifid shunt, fructose-6-phosphate phosphoketolase. Hence, these cultures were screened for probiotic attributes like cell surface hydrophobicity, antimicrobial activity, ability to utilize inulin and tolerance to acid, bile and lysozyme. Based on these attributes *Bifidobacterium* species 420 was selected for the development of the lyophilized synbiotic preparation incorporating inulin and using skim milk as base material. During refrigerated storage, viability of the probiotic bacteria in the product remained in the range of 10^8 to 10^9 cfu/g even after 60 days; whereas drastic reduction in cell number was observed on storage at room temperature. The feeding trial carried out in male albino mice showed significant differences in faecal, clostridial count, bifidobacterial count, coliform count and β-glucuronidase activity of probiotic, prebiotic and synbiotic groups compared to control group. Among the different dietary strategies tested, the synbiotic treatment was found to be most effective validating the improved benefits attributed to consumption of combination of probiotics and prebiotics rather than consuming either of them.

बाईफिडोबैक्टीरियम प्रजाति और इनुलिन समाविष्ट लायोफिलाइज़ड सेनबायोटिक का विकास

सारांश

मानव आन्त्र में गहन जीवसंख्या वाला सूक्ष्मजीव पारिस्थितकीय तन्त्र है जहां सूक्ष्मजीवों तथा पोषिता के बीच एक सामंजस्य होता है। यदि यह तन्त्र बिगड़ जाए तो एक्यूट गैसटरोइनटराइटिस, क्रोनिक डिसओडरस के लक्षण साफ दिखाई देते है। प्रोबायोटिक्स, प्रीबायोटिक्स और सेनबायोटिक्स चर्चित आहारिक योधन नीतियों को गैसटरोइनट्राइटिस रोगाणु को समाप्त करने के लिए बनाया गया है। सेनबायोटिक्स को प्रोबायोटिक्स एवम् प्रोबायोटिक्स के संयोजन से बनाया गया है जोकि एक नवीन विचार है जिससे पोषिता के लिए आन्त्र में अनुकूल परिस्थितियों का वातावरण प्रेरित किया जाता है। इसलिए वर्तमान अध्ययन में एक बाईफिडोबैक्टीरियम संवर्ध को प्रोबायोटिक विशिष्टताओं के आधार पर चुना गया और उसे इनुलिन के साथ समाविष्ट करके सेनबायोटिक उत्पाद विकसित किया गया। चूहे की आन्त्र का अणुजीवविज्ञान अध्ययन किया गया जिसमें प्रतिकारक का असर निर्धारित (बाईफिडोबैक्टीरियम, इनुलिन और उनका मिश्रण) किया गया। परीक्षण के दौरान केवल तीन संवर्धो ने मूल विकर, बाईफिड शंट, फ्क्टोस 6 – फोस्फेट फोस्फोकिटोलेस का सकरात्मक परीक्षण दिया जो है: *बाईफिडोबैक्टीरियम लैक्टिस* Bb-12, *बाईफिडोबैक्टीरियम* स्पैशिस 420, बाईफिडोबैक्टिरिया (ABT-5)। इसी तरह सभी संवर्धो का प्रोबायोटिक विशिष्टताओं जैसे कोशासतह हाइड्रोफोबिसिटी, विपरीत जीवाणु क्रियाशीलता, इनुलिन उपयोग क्षमता, ऐसिड, बाइल और लाइसोजाइम सहन करने की क्षमता का अध्ययन किया गया। इन सबके परीक्षण के बाद *बाईफिडोबैक्टीरियम* स्पैशिस 420 का लायोफिलाइजड सेनबायोटिक बनाने के लिए चयन किया गया जिसमें इनुलिन और मथित दूध को आधार सामग्री के रूप में प्रयोग किया गया। प्रशातित संग्रहण के दौरान भी प्रोबायोटिक बैक्टीरिया की गणना 60 दिन तक $10^8 - 10^9$ कोलोनी फोर्मिग यूनिट प्रति ग्राम रही जबकि कोष्ठ तापमान पर जीवाणु गणना में उग्र गिरावट अंकित की गई। एलबिनों चूहों पर सेनबायोटिक, प्रोबायोटिक और प्रीबायोटिक का अध्ययन किया गया जिसमें फीकल कलोस्ट्रीडिया, बाईफिडोबैक्टीरिया, कॉलीफार्म गणना, β ग़लूकोनिडेज़ क्रियाशीलता का नियंत्रण समूह से तुलनात्मक सार्थक अंतर पाया गया। परीक्षण की गई विभिन्न आहारिक योधननीतियों में प्रोबायोटिक और प्रीबायोटिक के मिश्रण सेनबायोटिक को अकेले प्रोबायोटिक या प्रीबायोटिक से अधिक फायदेमंद पाया गया।

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CHAPTER - 1

Introduction

1. INTRODUCTION

The human large intestine is an intensely populated microbial ecosystem. Several hundred species of bacteria are usually present with typical numbers of about 10^{11}-10^{12}/g, which live in a stable relationship with the host. Because of the microflora, the colon is one of the most metabolically active organs in the human body. The large-gut microflora is acquired at birth and initially facultatively anaerobic strains dominate. Thereafter, differences exist in the species composition that develops and this is largely controlled by the type of diet. The faecal flora of breast fed infants is dominated by bifidobacteria. In contrast, formula fed infants have a more complex microbiota with bifidobacteria, bacteroides, clostridia and streptococci all being prevalent (Ducluzeau, 1993). In adult humans the *Bacteriodaceae*, at 86% of the total flora are the most prevalent genera of bacteria. Bifidobacteria are the largest group in infants, but shift to the third largest group in adults. Normal healthy adults also possess other anaerobes including clostridia, veillonellae, coliforms, streptococci and facultative anaerobic lactobacilli (Mitsuoka, 1982).

A delicate balance exists between the human intestinal microflora and its host. Upset of this system may lead toward the symptoms of acute gastroenteritis and there is also the possibility of chronic disorders like inflammatory bowel disease, colonic cancer, etc. It is, therefore, important that gut microflora interactions be controlled and sustained in an optimal manner. Many environmental factors may affect the gut microbial ecology; these include diet, medication, stress, age and general living conditions. Knowledge of the gut microflora and its interactions lead to the development of dietary strategies that improve normal gastrointestinal microflora. Both pro- and pre-biotics are popular concepts that have been developed to target the gastrointestinal microflora.

Probiotics are "live microbial feed supplements that have beneficial effects on the host by improving its intestinal microbial balance" (Fuller, 1989, 1997). Although, yet to be proven conclusively it is believed that probiotic organisms compete with and suppress the growth of undesirable microorganisms in the colon and small intestine and thus help to stabilize the digestive system. Other benefits include prevention of intestinal infections, expression of antitumour activities and lactose utilization in the human gut (Mishra and Prasad, 2000; Sabikhi and Mathur, 2001). However, to exert these beneficial effects, probiotic organisms should survive in human gastrointestinal tract after ingestion, reach colon and should get established there. During their passage through gastrointestinal tract, probiotic organisms are confronted by many harsh physicochemical pressures, i.e., bile salts, pancreatic enzymes, etc. In the large intestine, the bacteria must compete effectively with a complex and metabolically active indigenous flora. Therefore, it is necessary to develop steps to overcome this problem in order to achieve beneficial health attributes from probiotics. The synbiotic approach offers an important solution for this problem (Ziemer and Gibson, 1998), where the probiotic is administered in conjunction with a specific prebiotic. A prebiotic is defined as a "non-digestible food ingredient that beneficially affects the host by selectively stimulating the growth and / or activity of one or a limited number of bacteria in the colon". Prebiotics are capable of modifying the composition of the colonic microflora in such a way that a few of the potentially health promoting bacteria become predominant in numbers. A synbiotic is defined as "a mixture of a probiotic and prebiotic that beneficially affects the host by improving the survival and implantation of live microbial dietary supplements in the gastrointestinal tract, by selectively stimulating the growth and / or by activating the metabolism of one or a limited number of health promoting bacteria" (Roberfroid, 1998). The combination could improve the survival of the probiotic organisms as its specific substrate is readily available for its fermentation and hence, result in advantages to the host that the live microorganisms and prebiotics offer.

It is now well established that there is a clear relation between diet and health. Although the primary role of diet is to provide enough nutrients to fulfil metabolic requirements, more recent discoveries support the hypothesis that, beyond nutrition in the conventional sense, diet may modulate various functions in the body. Hence, the concept of functional foods has developed as a food, or food ingredient, with positive effects on host health and / or well-being beyond its nutritive value (Huggett and Verschuren, 1996). In most recent years, the concept has become more directed towards food additives that may exert a positive benefit on the gut microbiota composition. This has largely concentrated on probiotics, but more recently interest in prebiotics has increased. Now-a-days probiotic foods are available in a number of forms. The most commonly used vector involves fermented milk products and over the counter freeze dried preparations. The majority of probiotic products already in market such as fermented milk and yoghurt are fresh products, and are generally consumed within days or weeks of manufacture. In contrast, dried preparations have longer shelf-life, and offer certain advantages over fermented products in terms of increased shelf-life, savings in the costs of transport and storage, and improvements in culture stability. Despite the fact that spray drying is more economical than freeze-drying, especially on large scale (Golker, 1993; Johnson and Etzel, 1993) many probiotics cannot tolerate the relatively high temperatures that are used during spray-drying. As a consequence, freeze drying is the most popular method for the production of dried probiotic preparations. The main challenge in the development of probiotic foods is the maintenance of stability and viability of organisms. Exploitation of the synergistic effect of combining a probiotic with a prebiotic might be advantageous to enhance the delivery of viable and metabolically active probiotics to the intestinal tract (Mattila-Sandholm *et al.*, 2002). Due to their perceived health benefits bifidobacteria are widely used in probiotic preparations and foods (Salminen *et al.*, 1998b). Several positive effects have been related to bifidobacteria. These include synthesis of vitamins, supplementation in digestion and absorption, inhibition of growth of exogenous organisms, and stimulation of the immune system (Mitsuoka,

1992). To maintain a high level of bifidobacteria in the gut different strategies can be applied. Number of bifidobacteria can be increased either by continuous ingestion of bifidobacteria containing preparations or foods, or food can be supplemented with substrates (bifidogenic factors or prebiotics) that beneficially promote the growth of endogenous bifidobacteria in the gut (Gomes and Malcata, 1999).

Keeping above facts in view, the present investigation has been undertaken to develop a lyophilized synbiotic preparation employing a combination of pro- and pre- biotic with the following objectives:

1. To screen and select a suitable strain of *Bifidobacterium* spp. based on its probiotic attributes.
2. To study the effect of different concentrations of prebiotic (inulin) on the growth of bifidobacterial strain and to study the ecological interaction of probiotic strain in the presence of inulin and gut associated harmful microflora.
3. To develop a lyophilized synbiotic preparation and to carry out its storage studies.
4. To study the efficacy of synbiotic preparation to improve the intestinal microbial ecology through *in-vivo* studies in mice.

Review of Literature

2. REVIEW OF LITERATURE

2.1 THE PROBIOTIC CONCEPT

2.1.1 History of the Term Probiotic

The concept of "probiotics", like many other evolutionary ideas appeared long before a neologism was proposed to describe it. At the beginning of the 20th century, Nobel Prize winning Russian Scientist, Elie Metchnikoff (1907), provided a thorough description of the concept based on the importance of the intestinal microflora on the general health status of the human body. The term probiotic, meaning "for life", is derived from the Greek language. Probably the term "probiotic' was introduced by Vergio (1954), when he compared in his manuscript "Anti-und Probiotika" the detrimental effects of antibiotics and other antimicrobial substances on the gut microbial population with factors ("Probiotika") favourable to the gut microflora. Later on in 1965, Lilly and Stillwell referred to probiotics as "microorganisms promoting the growth of other microorganisms". In 1971, Sperti applied this term to tissue extracts that stimulate microbial growth. Parker *et al.* (1974) proposed one early definition as organisms and substances that influence intestinal microbial balance. Several other recent definitions have also been proposed to describe probiotics, but a consensus has not been reached within the scientific community on a final definition. The most widely accepted definition in recent years is that of Fuller (1989) stating that a probiotic is a "live microbial feed supplement, which beneficially affects the host (animal) by improving its intestinal microbial balance". This version stressed the need for the supplement to be composed of viable microorganisms and introduced the aspect of a beneficial effect on the host, which was, according to his definition, an animal. In 1992, Havenaar and Huis int Veld broadened the definition of probiotics with respect to host and habitat of the microflora as

follows: "A viable mono- or mixed culture of microorganisms which applied to animal or man, beneficially affects the host by improving the properties of the indigenous microflora". However, for human nutrition, Salminen *et al.* (1998a) proposed the following definition: "a live microbial food ingredient that is beneficial to health". This emphasized the importance of definitive improvements in health. Presently, there is general agreement that a "probiotic" refers to viable microorganisms that promote or support a beneficial balance of autochthonous microbial population of the gastrointestinal tract (GIT; Holzapfel *et al.*, 2001).

2.1.2 Development of the Probiotic Concept - Historical Background

The large bowel harbours a nutritionally and physiologically diverse range of bacteria. This microflora offers the host protection against disease and promotes normal intestinal function (Salminen *et al.*, 1998c). The disruption of the gastrointestinal flora due to pathogens, dietary antigens or other harmful substances leads to dysfunction of the intestine and subsequently to a number of diseases. Certain bacterial genera, namely *Lactobacillus* and *Bifidobacterium,* which have a long and safe history in the manufacture of dairy products are, therefore, traditionally included in probiotic products to protect against such effects (Vaughan and Mollet, 1999).

The first significant introduction to the probiotic concept was by Metchnikoff at the beginning of the 1900s. He believed that the fermenting bacillus (now called *Lactobacillus*) contained in the fermented milk products consumed by Bulgarian peasants positively influenced the microflora of the colon and decreased toxic microbial activities (Metchnikoff, 1907). This was attributed to the health promoting values of the live microorganisms. He therefore abandoned his practice of surgical removal of the colon and began modification of the activity of the colonic microflora by the ingestion of soured milks.

Subsequent research looked to confirm that the consumption of lactic acid bacteria was having a beneficial effect on health. For example, preparations containing *Lactobacillus acidophilus* were used to alleviate

constipation (Rettger *et al.*, 1935), whilst concomitantly, in Japan Shirota selected beneficial strains of lactic acid bacteria which could survive passage through the intestine and subsequently used them to develop fermented milk drinks (Shortt, 1999). It was soon established that there were many species of lactic acid bacteria in the intestine and these have subsequently being incorporated into many probiotic preparations. Common probiotic preparations include *Lactobacillus, Bifidobacterium, Streptococcus*, and *Saccharomyces* (Goldin and Gorbach, 1992). *Lactobacillus* and *Bifidobacterium* species have achieved popularity in the manufacture of probiotic products because of their possession of generally regarded as safe (GRAS) status. Lactobacillus and Bifidobacteria are the most frequently used genera (Fooks and Gibson, 2002). The classical and most well used example of probiotic technology is the addition of lactobacilli and / or bifidobacteria to fermented milk products (Colombel *et al.*, 1987; Modler *et al.*, 1990a). In this case, it is proposed that the exogenous bacteria reach the large intestine in an intact and viable form and thus help to maintain the "balance" of the gut flora.

2.2 BIFIDOBACTERIA AS PROBIOTICS

The flora of the gastrointestinal tract is host and location specific and is a complex strictly anaerobic ecosystem. The intestinal flora comprises >500 different bacterial species. Overall, the microflora has positive effect on human health. It is generally assumed that *Bifidobacterium* species are the most important bacteria for this health promoting effect. This is based on the observation that bifidobacteria are normal inhabitants of the human intestinal tract throughout the life cycle, beginning just days after birth. Furthermore, breast-fed babies have higher numbers of *Bifidobacterium* than bottle-fed children, and they are less at risk for diarrheal disease / susceptible to infections than formula-fed infants (Heinig and Dewey, 1996). In contrast in elderly persons, the *Bifidobacterium* numbers decrease; whereas clostridial numbers significantly increase. Tissier (1906) was the first to promote the therapeutic use of bifidobacteria for relief from intestinal disorders. He

believed that the bifidobacteria displaced putrefactive bacteria, which are responsible for gastric upset, while re-establishing themselves as the dominant intestinal microorganisms. Their presence in the human intestine is almost universally accepted to be a contributing factor to a healthy well being (O'Sullivan and Kullen, 1998). The majority of the proposed physiological effects of bifidobacteria pertain to improvement of intestinal flora by preventing colonization of pathogens, amelioration of diarrhea or constipation, immune modulation, improvement of lactose-tolerance and digestibility of milk products. Yoghurts with bifidobacteria have been launched for decades in Europe and Japan, and their consumption is yet increasing (Mitsuoka, 2000). *Bifidobacterium bifidum*, *B. breve*, *B. longum* and *B. animalis* are commonly used for the production of fermented milks, in combination with other lactic acid bacteria.

2.2.1 Bifidobacteria – General Characteristics

Bifidobacteria were first isolated in 1899 by Tissier at the Pasteur Institute, Paris, France from the faeces of infants. These organisms are non-motile, Gram-positive curved rods often occurring as bifurcated Y-forms. They are non-sporulating organisms and catalase negative. Although considered anaerobic, bifidobacteria vary considerably in their tolerance to oxygen. Some are obligate anaerobes, while others tolerate oxygen in the presence of CO_2. The anaerobic requirements are strain-related; the less sensitive strains appear to possess weak catalase activity, which remove traces of H_2O_2 (Rasic and Kurmann, 1983). They ferment sugars with lactic and acetic acid as end products via an unusual glucose-metabolizing system that results in the production of acetic and lactic acid in molar ratios of 3:2 as primary metabolites (Scardovi and Trovatelli, 1965). Bifidobacteria do not produce CO_2, butyric or propionic acid (Kurmann, 1983). This genera of bacteria are also unique in that all the lactic acid produced is in the L(+) form, which is easily metabolized by infants. The optimum growth temperature for bifidobacteria is 37 to 43°C, but these bacteria will grow at extremes of 25 to

28°C and 43 to 45°C. Optimum pH is 6.5 to 7.0, and little or no growth occurs below the range of 4.5 to 5.0 or above 8.0 to 8.5 (Scardovi, 1986). Bifidobacteria also produce thiamine, riboflavin, and vitamins B and K (Modler *et al.*, 1990a).

Since 1957, research emphasis has been on the biochemical and taxonomical questions posed by the bifidobacteria and their relationship to their human hosts. Multiple biotypes of bifidobacteria were not widely recognized until 1957 (Rasic and Kurmann, 1983). Since their discovery, bifidobacteria have been assigned to the genera *Bacillus, Bacteroides, Bacterium, Tisseria, Nocardia, Lactobacillus, Actinomyces* and *Corrynebacterium* (Mitsuoka, 1984). In the eighth edition of "Bergey's Manual" the genus *Bifidobacterium* was defined as a member of the family Actinomycetaceae. The elucidation of the unusual means of hexose metabolism by the bifidobacteria revealed the enzyme, fructose 6-phosphate phosphoketolase, to be unique among lactose-utilizing bacteria. Assay for activity of this enzyme has become a key-differentiating test for the identification of bifidobacteria (Scardovi, 1981). In the latest edition of "Bergey's Manual" (Scardovi, 1986), twenty-four species have been defined. These species of *Bifidobacterium* found in humans are *B. bifidum*, *B. infantis*, *B. breve*, *B. longum*, *B. adolescentis*, *B. angulatum*, *B. catenulatum* and *B. pseudocatenulatum*. Other species occur in the intestinal tract of various animals and insects including honey bees (Scardovi, 1981). The most common species found in the infant gut are *B. infantis*, *B. breve* and *B. longum*, whilst *B. adolescentis* and *B. longum* are thought to predominate within the adult colon (Modler *et al.*, 1990a).

Newborn infants are firstly colonized with lactobacilli and bifidobacteria primarily via the birth and during delivery, a hypothesis supported by evidence that infants born via the genital tract are colonized more rapidly than those delivered through caesarean section (Mitsuoka *et al.*, 1974). The numerical differences in the lactobacilli and bifidobacterial populations between breast-fed and bottle-fed infants is significant. In healthy breast-fed infants,

lactobacilli may account for up to 10^7 per gram of faeces, with bifidobacterial levels at up to 10^{11} per gram, which are generally much higher than enterobacteria, and bacteroides within the first week of life (Mitsuoka, 1982; Yuhara *et al.*, 1983; Benno *et al.*, 1984). In contrast, in bottle-fed infants, lactobacilli numbers are only up to 10^6 per gram faeces, with the bifidobacterial population at around 10^{10} per gram, and this reduction in the number of lactobacilli and bifidobacteria is coupled with increase in numbers of enterobacteriaceae, streptococci, bacteroides, *E. coli* and Clostridia (Yuhara *et al.*, 1983).

Numbers of bifidobacteria fall significantly upon weaning, and this occurs concomitant with an increase in populations of bacteroides, clostridia, enterobacteria and eubacteria, streptococci and even lactobacilli, such that the colonic bacterial profile typically resembles the adult pattern. These populations usually remain more or less stable throughout adult population, until a decrease in bifidobacterial numbers is accelerated in the elderly (Mitsuoka, 1984). In order for the intestine to function optimally, however, the 'balance' of the bacterial flora must be maintained, and this appears to be increasingly difficult, as lifestyles have been changed. An increase in stress and modern day living, which makes a consequential demand on the immune system can disrupt homeostasis in the gut. Similarly, the direct effects of a change in dietary patterns and eating habits can affect overall functionality. Another contributory factor includes the consumption of pharmaceutical compounds, in particular antibiotics, which by design destroy bacteria, and therefore, can have a harmful effect on the balance of the gut microbiota. All of these combine to shift the balance of the gut microflora away from potentially beneficial or health promoting bacteria such as the lactobacilli, and bifidobacteria, towards an increase in harmful or pathogenic microorganisms, like the clostridia, sulphate-reducers and proteolytic bacteroides species.

Predominance of the latter may dispose towards a number of clinical disorders, including bowel cancer and inflammatory bowel diseases such as ulcerative colitis, whilst making the host more susceptible to infections by

transient enteropathogens such as *Salmonella, Campylobacter*, certain species of *Escherichia coli* and *Listeria*. It is of considerable benefit to the host, therefore, to maintain a good community structure, through increased levels of bacteria such as lactobacilli and bifidobacteria, preferably at the expense of harmful organism (Fooks and Gibson, 2002). One line of thinking as a means of combating undesirable changes in the gut microflora is to administer probiotics, prebiotics, etc. to re-establish the intestinal microbial balance, avoiding possible further infection, or subsequent sequlae, which can result from a disrupted gut flora.

2.3 SELECTION OF A PROBIOTIC STRAIN

The basis for selection of probiotic microorganisms include safety, functional aspects (survival, adherence, colonization, antimicrobial production, immune stimulation, antigenotoxic activity and prevention of pathogens) and technological details such as growth in milk and other food base, sensory properties, phage resistance and viability (Havenaar and Huis int Veld, 1992; Lee and Salminen, 1995; Salminen *et al.*, 1998b; Mishra and Prasad, 2000; Sabikhi and Mathur, 2001). Preferably, strains used should be of human origin. This is based on the observation that only human strains can be adhesive and colonise the human gastrointestinal tract; which is the first step in promoting colonization resistance (Huis int Veld *et al.*, 1994). It is proposed that species specificity does occur and for strains to be beneficial to a particular host they should be isolated from that species. One of the most important characteristics to establish regarding a probiotic strain is that it must be non-pathogenic and furthermore, should possess GRAS status. To survive and grow in the *in-vivo* conditions of the desired site of administration, the probiotic strain must be able to tolerate low pH and high concentrations of both conjugated and de-conjugated bile acids (Collins *et al.*, 1998). Probiotc bacteria perform best when they find adequate environmental conditions and when they are protected against various stresses they may encounter during their production at the industrial level or in the gastrointestinal (GI) system. Following table (Table 2.1) gives examples of criteria that aid the selection of efficacious probiotics.

Table 2.1 **Criteria for the selection of efficacious probiotics** (Gibson and Fuller, 2000).

Parameters	Requirement
Strain origin	Those isolated from the same species as the intended use ought to have an enhanced chance of survival.
Safety	Probiotics should be generally recognized as safe (GRAS) with minimal possibilities for the transfer of antiobiotic resistance
Survivability	Both in the product and after ingestion, strains that have improved, resistance to acid, bile secretions and able to attach to the gut epithelium would have better survival charactetristics.
Production characteristics	Able to be grown in bulk culture, without genetic variation
Processing	Robust enough to withstand the rigers associated with incorporation into oral delivery systems
Sensory properties	When added to foods, the quality should not be diminished
Microbiological properties	Required to survive the gastrointestinal ecosystem
Effects on the consumer	No adverse side effects such as bloating and effects on gut transit should occur

2.4 SUITABILITY OF BIFIDOBACTERIA AS PROBIOTICS : SELECTION CRITERIA

2.4.1 pH and Bile Salt Tolerance

One of the most important criteria for selection of probiotic organisms is their ability to survive in the acidic environment of the product and in the stomach, where the pH can reach as low as 1.5. Similarly, the organisms must be able to survive in the bile concentrations usually encountered in the intestine. Numerous studies had been conducted to evaluate different strains of bifidobacteria for their acid and bile salt tolerance, for their suitability to be used as probiotics. However, tolerance of bifidobacteria to acidic stomach conditions have been reported to be strain specific (Berrada *et al.*, 1991).

The survivability of *Bifidobacterium* from fermented milk during gastric transit was studied by Berrada *et al.* (1991). They incubated two strains of *Bifidobacterium* contained in fermented milks at 37°C, following acidification of the milk to pH 3.0. After only five min, one strain of *Bifidobacterium* had decreased from approximately 6×10^5 to 1×10^2 cfu/g. The other strain decreased slightly but still exceeded 10×10^7 cfu/g after incubation. This study demonstrated the strain specificity of acid tolerance among *Bifidobacterial* spp.

Pochart *et al.* (1992) investigated the ability of a strain of *Bifidobacterium* spp. (strain BB) to survive passage through the upper gastrointestinal tract when ingested in fermented milk (bifidus milk, BM) in six fasting healthy adults using *in vivo* ileal perfusion. They observed that the viability of BB bifidobacteria did not change significantly during 3 h of incubation at pH 3.0 and 1 h of incubation at pH 2.0. They also observed that during the 3 h after BM ingestion, the concentrations of bifidobacteria in the lumen of the upper gastrointestinal tract were higher than the bacterial concentrations usually found in the upper gastrointestinal tract of healthy people. Their results indicated that in healthy adults *Bifidobacterium* survives transit through the gastrointestinal tract when ingested in fermented milk.

Martin and Chou (1992) had screened eleven species / strains of bifidobacteria for their ability to survive in low pH (4.1 – 4.2) and high pH (5.5 – 5.6) plain yoghurts. They observed species and even strain specific variations in the acid tolerance of these organisms.

Clark *et al.* (1993) evaluated *in vitro* the tolerance of *B. infantis*, *B. adolescentis*, *B. longum* and *B. bifidum* to pH levels commonly exist in the human stomach (1.0, 2.0 and 3.0). This study indicated that certain species / strains of *Bifidobacterium* can be selected on the basis of their *in vitro* acid tolerance to be used successfully as dietary adjuncts in dairy foods. They suggested that the species of choice would be *B. infantis*, *B. adolescentis* and *B. longum*. They also suggested that *B. longum* may be the most logical species because of its intended viability *in vitro* even at pH 1.0.

In order to determine the suitability of the strains of *Bifidobacterium* spp. for use as dietary adjuncts in fermented dairy products, Lankaputhra and Shah (1995) evaluated the tolerance of nine strains of *Bifidobacterium* spp. to acidic conditions commonly exist in the human stomach (3.0, 2.5, 2.0 and 1.5), and bile concentrations (1 and 1.5%). Among the nine strains of *Bifidobacterum* spp., *B. longum* 1941 and *B. pseudolongum* 20099 survived best under acidic conditions. *B. longum* 20099 and *B. infantis* 1912 showed the best tolerance to bile.

Holcomb *et al.* (1991) tested resistance of *B. bifidum* to bile. They adjusted deMan Rogosa Sharp medium with Oxgall to bile concentrations of 0.0, 0.15, 0.3 and 0.45 percent. They reported no significant change in numbers of *B. bifidum* at these bile concentrations. The bile tolerance of *B. infantis*, *B. bifidum*, *B. breve* and *B. longum* to bile concentrations of 0.0, 0.6, 1.5, 3.0 and 6.0 g per litre of sodium glycolate in TPY medium was studied by Ibrahim and Bezkorovainy (1993b). They observed best survival rate by *B. infantis* followed by *B. bifidum* and *B. breve*. *B. longum* exhibited survival rates of approximately 10^7 cfu/ml after 12 h in 2 percent bile. Jiang *et al.* (1996) observed difference in bile sensitivity among different species. They noted that *B. longum* Bb was more tolerant to bile than *B. longum* ATCC 15078.

Clark and Martin (1994) evaluated *in vitro* the tolerance of four species of bifidobacteria to maximum (2.0%) and twice-maximum concentrations of bile reportedly produced in the human small intestine. They reported that among these organisms *B. longum* survived at bile concentrations as high as 4 percent.

The growth pattern of 15 *Bifidobacterium* strains in the modified MRS broth containing up to 2 percent oxgall bile was investigated by Chand Ram (1997). He observed substantial fall in the growth (O.D.), ranging from 0.006 to 0.106 with increase in bile salt concentration. He also studied the pH tolerance of these organisms at different pH levels (pH 1.0 to 10.0) and found

a pH of 7.0 as optimum for all the bifidobacterial cultures tested. As the pH was increased or decreased, growth of all the cultures showed gradual decrease corresponding to the changed pH.

Al-Saleh *et al.* (1998) tested 4 species of bifidobacteria for their ability to grow in MRSL broth medium with or without added bile. They observed that the growth of bifidobacteria in the presence of bile varied among the studied species. Although growth was reduced due to the presence of bile, all the tested species exhibited some degree of bile tolerance. *B. longum* and *B. angulatum* were more resistant to bile than *B. breve* and *B. bifidum*.

Prasad *et al.* (1998) studied the survivability of one putative strain of *B. lactis* (*B. lactis* HN019) at pH 3.0 for 0, 1, 2 and 3 h, and obtained a survival rate of more than 80 percent after 3 h exposure to pH 3.0. This organism not only survived up to one percent (w/v) bile concentrations, but also showed normal growth at bile concentrations of up to one percent (w/v). John (2000) screened *B. bifidum* NCDC 255 and *B. bifidum* NCDC 231 for their tolerance for simulated pH of the human stomach (pH 1.0, 2.0 and 3.0) and physiological bile concentrations (1.0 and 2.0%). *B. bifidum* NCDC 255 showed better pH tolerance, whereas *B. bifidum* NCDC 231 exhibited better bile tolerance.

Acid and bile tolerance is strain dependent, and care should be taken to select strains on the basis of these attributes. Reports suggest that *Bifidobacterium animalis* (which has recently been reclassified as *Bifidobacterium lactis*) an animal strain withstands harsh conditions compared to other strains (Shah, 2001). Alander *et al.* (2001) evaluated the bile and acid resistance properties of three commercially available bifidobacterial strains *B. lactis* Bb12, *Bifidobacterium* spp. 420 and *Bifidobacterium* spp. H in the basis of culture tests (loop-streakings) on TPY medium containing 1.5 percent (w/v) agar agar and different concentrations ranging from 0.1 to 0.5 percent (w/v) of bile salt mixture and on TPY agar of different pH values (3.5 – 5.0). All these cultures showed comparable bile salt and pH tolerance.

The pH tolerance of five *B. bifidum* cultures, *B. bifidum* NCDC 255, *B. bifidum* NIRD, *B. bifidum* VP and *B. bifidum* 1047 at different pH levels (1.0, 2.0 and 3.0) for 1, 2 and 3 h was determined by Kushal (2001). *B. bifidum* 255 showed highest pH tolerance with a log reduction of 2.3 and 1.7 at pH 1 and 2, respectively. On testing the bile tolerance of these cultures at 1 and 2 percent oxgall concentrations *B. bifidum* 255 exhibited better bile tolerance than other cultures.

2.4.2 Antibacterial Action

Bifidobacteria are known to inhibit the growth of various pathogens *in vitro*. This occurs due to several mechanisms and can be dubbed the *Bifidobacterium* barrier (Fig. 2.1).

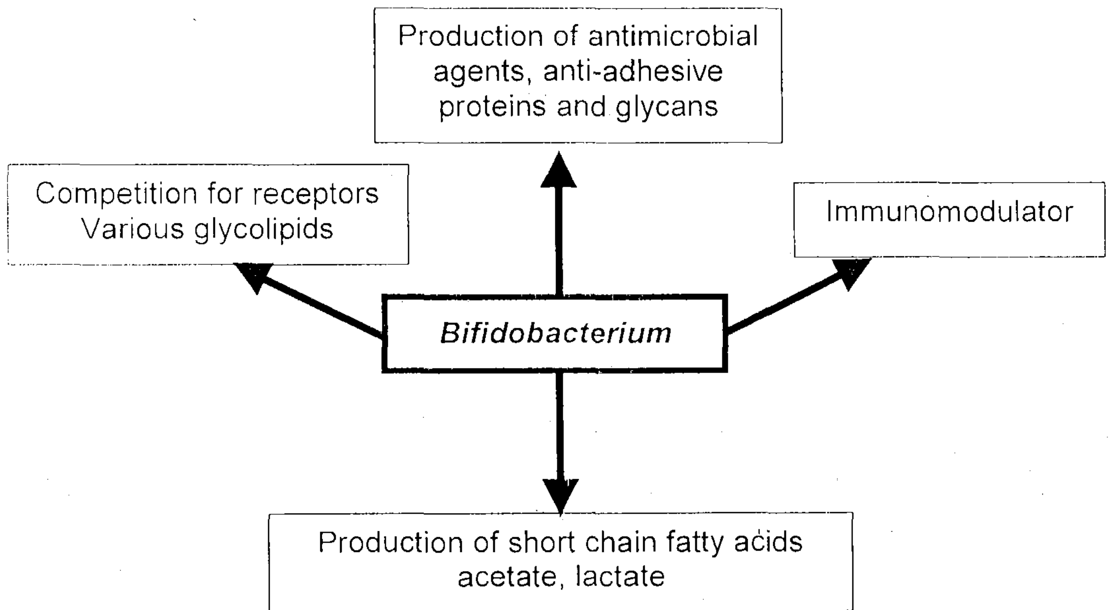

Fig. 2.1 ***Bifidobacterium* Barrier – Different mechanisms whereby Bifidobacteria may inhibit pathogen persistence in the gut (Rastall *et al.*, 2000).**

Bifidobacteria are known to exhibit inhibitory effects on many pathogenic organisms both *in vivo* and *in vitro*, including *Salmonella, Shigella, Clostridium*, *Bacillus cereus*, *Staphylococcus aureus*, *Candida albicans* and *Campylobacter jejuni* (Anand *et al.*, 1984; Tojo *et al.*, 1987; Tomoda *et al.*, 1988).

The infection-preventing effect of bifidobacteria is due to their antagonistic activity against undesirable microorganisms. Probiotic foods containing bifidobacteria have been found to be effective against *Clostridium difficile* (Corthier *et al.*, 1985) and childhood forms of diarrhoea (Hotta *et al.*, 1987; Saavedra *et al.*, 1994).

In another study about the infection preventing effect, Yamazaki *et al.* (1982) compared the mortality of *B. longum-gnotobioto* (mono-associated) mice and germ-free mice when *E. coli* was ingested orally. By ingesting *E. coli* in germ-free mice, the lethal activity or mortality became 64 percent. However, the mortality of *B. longum* mono-associated was zero. The same effect was observed by administering *E. coli* intravenously.

Nakaya (1984) and Okamura *et al.* (1986) studied the effect of *B. infantis* against Shigella infection in Hela cells. They observed reduction in the infection rate of *Shigella flexneri* on Hela cells in the presence of *B. infantis*, clearly indicating an inhibitory effect by *B. infantis* on Shigella.

A bifidus milk developed by Misra and Kuila (1992) using *B. bifidum* NDRI exhibited antibacterial activity against *Escherichia coli*, *Bacillus cereus*, *Shigella dysenteriae* and *Staphylococcus aureus* as determined by the agar diffusion technique.

Several strains of bifidobacteria were shown to adhere to Caco-2 cells and HT29-MTX cells, and while bound they prevented the binding of pathogenic strains of *E. coli*, *Salmonella typhimurium* and *Yersinia* strain (Bernet *et al.*, 1993). Ibrahim and Bezkorovainy (1993a) investigated the ability of 5 ATCC bifidobacterial species to produce antimicrobial substances by testing the effects of spent bifidobacterial broths on the growth of *Escherichia coli* in the thioglycollate medium. As the neutralization of the pH of these broths resulted in 57 to 70 percent reduction in their antimicrobial activity and such inhibition of *E. coli* growth could be duplicated by a 3:2 acetic and lactic acid mixture adjusted to neutral pH, the researchers concluded that no antibacterial substances other than acetic and lactic acids were produced by bifidobacterial strains used in this study.

Gibson and Wang (1994b) studied regulatory effects of bifidobacteria on the growth of other colonic bacteria. They observed that several strains of *Bifidobacterium* have broad spectrum antimicrobial secretions inhibiting pathogenic bacteria in general such as Salmonella, Listeria, Campylobacter as well as *Vibrio cholerae*. Bifidobacterial species have also been shown to secrete a proteinaceous factor that inhibits the binding of pathogenic strains of *E. coli* adding to their antimicrobial effects (Fujiwara *et al.*, 1997).

Chand Ram (1997) studied the antibacterial activity of various human bifidobacterial isolates against *Escherichia coli* NCDC 134, *Bacillus cereus* NCDC 66, *Salmonella typhi* NCDC 113, *Shigella dysenteriae* NCDC 107 and *Staphylococcus aureus* NCDC 110. He observed much variation in inhibitory spectrum of various *Bifidobacterium* strains against above mentioned pathogenic / spoilage causing microorganisms.

The antimicrobial activity of five *B. bifidum* cultures against *E. coli* NCDC 135, *Salmonella typhi* NCDC 113, *Staphylococcus aureus* NCDC 109, *Shigella dysenteriae* NCDC 107, *Bacillus cereus* NCDC 66 and *Micrococcus luteus* NCDC 131 were evaluated by Sabikhi (1999) and observed antibacterial activity by all tested *Bifidobacterium* cultures against all these organisms. But the Edam cheese containing a selected *Bifidobacterium* culture from these five cultures did not show any antagonistic activity.

John (2000) assessed the antimicrobial activity of the AB dahi containing *B. bifidum* NCDC 255 and *Lb. acidophilus* NCDC 13 against *Escherichia coli* NCDC 134, *Salmonella typhi* NCDC 113 and *Shigella dysenteriae* NCDC 107, and found to be inhibitory to the growth of all these organisms. Kushal (2001) studied the inhibitory activity of three *Bifidobacterium bifidum* cultures against *E. coli* and *S. typhimurium*, and found *B. bifidum* NCDC 255 as the one exhibiting maximum antibacterial activity.

Gopal *et al.* (2001) investigated the inhibitory effect of *Lactobacillus rhamnosus* DR20, *L. acidophilus* HNO17 and *Bifidobacterium lactis* DR10 against the intestinal cell monolayer colonization by a known enterotoxigenic strain of *Escherichia coli* (strain O157:H7). Pre-treatment of *E. coli* O157:H7

with 2.5-fold concentrated cell-free culture supernatants from the probiotics reduced the culturable *E. coli* numbers on TSB plates and also reduced the invasiveness and cell association characteristics of this toxic strain.

Kheadr *et al.* (2002) screened thirty-four isolates of human bifidobacteria for their ability to inhibit *L. monocytogenes* and out of this 6 strains showed antimicrobial activity against *L. monocytogenes*. An *in vitro* study of the inhibition of *Escherichia coli* O157:H7 by infant bifidobacterial strains was conducted by Gagnon *et al.* (2002). They showed that two infant isolates inhibited both enterohaemorrhagic *Escherichia coli* growth and adhesion to Caco-2 cells.

2.4.3 Adherence Properties

Adherence is one of the most important selection criteria for probiotic bacteria. The ability of probiotic bacteria to adhere to the intestine will improve their chances of winning the competition against "unfriendly bacteria" to occupy the intestinal "niches". Adhesion of *Bifidobacterium* strains to the colon surface may occur by association of bacteria with a secreted mucus gel or by adherence to underlying epithelium (Fontaine *et al.*, 1994). Generally, adhesion may be ascribed to the interplay of attractive and repulsive forces between the approaching surfaces (Ronner *et al.*, 1990; Geertsema-Doornbusch *et al.*, 1993; Greene and Klaenhammer, 1994).

Cell adhesion is a multistep process involving contact of the cell with the surface, and it is affected by the composition and structure of the cell membrane and interacting surfaces. The physical and chemical characteristics of the cell surface have been determined mainly by determining surface hydrophobicity and electrical mobility (Busscher *et al.*, 1993; Geertsema-Doornbusch *et al.*, 1993; Gilbert *et al.*, 1993; Crow *et al.*, 1995). The hydrophobicity of the surface and zeta potentials account for the attractive and repulsive forces, respectively, that takes place in auto-aggregation and adhesion of bacteria to different surfaces.

Bernet *et al.* (1993) studied the adhesion of human bifidobacterial strains to cultured human epithelial cells and reported inhibition of entero-pathogen host cell interactions in the presence of bifidobacteria. Most of the studies have reported adherence properties of bifidobacteria based on their adherence to Caco-2 and HT-29 cell lines. The validity of *in vitro* adhesion tests performed with cultured cell lines was determined by Crociani *et al.* (1995) by comparing with results obtained *in vivo* in a previous study. They demonstrated that the ability of the strain to adhere and colonize the intestinal cell wall *in vivo* or the cultured intestinal cells *in vitro* was similar.

On screening human bifidobacterial isolates for their hydrophobicity and adhesion index, Chand Ram (1997) observed wide variations among them. Highest percent hydrophobicity was observed in case of *B. bifidum* HI39 (79%) and *B. bifidum* HI48 (68%), which were followed by strain *B. bifidum* HI38 (52%). In a study by Lankaputhra and Shah (1998), only one out of 6 strains of *L. acidophilus* adhered properly; whereas 2 out of 9 strains of bifidobacteria showed good adherence properties. In general, *Bifidobacterium* spp. adhered better than *L. acidophilus. B. infantis* and *B. longum* showed the best adherence properties.

Adherence of 4 species of bifidobacteria to the epithelial cells of the small intestine of sheep was studied by Al-Saleh *et al.* (1998) and all the cultures tested exhibited adherence to sheep intestinal epithelial cells. The adherence of *Bifidobacterium* strains isolated from infant faeces and commercial fermented dairy products to enterocyte-like cells was studied by Perez *et al.* (1998) and correlated with the autoagglutination and hemagglutination properties of these organisms. They reported that the hydrophobicity appeared to be necessary for adhesion to enterocyte-like cells and autoagglutination. Adhesive strains were highly hydrophobic and the degree of adherence was slightly dependent on the surface potential.

Gopal *et al.* (2001) studied the *in vitro* adherence properties of *Lactobacillus rhamnosus* DR20, *Lactobacillus acidophilus* HN017 and *Bifidobacterium lactis* DR10 using the differentiated human intestinal cells

including HT-29, Caco-2 and HT-29-MTX. All these three strains showed strong adhesion with the human intestinal cell lines *in vitro*, which were comparable to the adhesion indices of two commercial probiotic strains *L. acidophilus* LA-1 and *L. rhamnosus* GG.

Twenty-four *Bifidobacterium* strains were examined for their ability to bind to immobilized human and bovine intestinal mucus glycoproteins. Among the tested bacteria, *B. adolescentis*, *B. angulatum*, *B. bifidum*, *B. breve*, *B. catenulatum*, *B. infantis*, *B. longum* and *B. pseudocatenulatum* adhered to human faecal mucus better than bovine faecal mucus, while the binding of *B. animalis* and *B. lactis* was not preferential. These results suggest that the mucosal adhesive properties of bifidobacteria may be a strain dependent feature and the mucosal binding of the human bifidobacteria may be more host specific (He *et al.*, 2001).

2.4.4 Immunomodulatory Effects

The immune system acts to protect the host from infectious agents that exist in the environment (bacteria, viruses, fungi, parasites) and from other noxious insults. The intestinal mucosa is the main interface between the immune system and the external environment. The gut-associated lymphoid tissues contain the largest pool of immunocompetent cells in the human body. The dialogue between host and bacteria at the mucosal interface seems to play a part in development of a competent immune system. The gut microflora is undoubtedly important in supporting a functional yet balanced immune system, the processes that lead to this balance can be emulated by transiently colonizing the gastrointestinal (GI) tract with appropriate strains of microbes – most common Gram-positive lactic acid bacteria (lactobacilli or bifidobacteria) that are delivered orally as probiotics. Modulation of host "immunity" is one of the most commonly purported benefits of the consumption of probiotics. Although several *in vitro* and *in vivo* studies on probiotic effects on immunity have been reported the specific mechanisms of the observed changes remain unclear (Erickson and Hubbard, 2000).

Presumably, to modulate immunity, probiotic organisms must reach immune cells that are endowed with recognition receptors or that are otherwise sensitive to probiotic-specific catabolites.

Recent studies of mucosal immunity suggest a key role for dendritic cells in the regulation of gut immune responses. Dendritic cells (DCs), the most potent and versatile of antigen presenting cells (APCs) have a central role in the immune activation of resting T cells and the initiation of primary immune response. DCs are distributed as immature cells in non-lymphoid organs and in the blood, where they perform a sentinel function for pathogens and foreign antigens. Immature dendritic cells are capable of decoding and integrating signals from invading microbes and transfer this information to naive T cells in the secondary lymphoid organs, undergoing a maturation process enroute. DC maturation is associated with enhanced production of inflammatory cytokines, with reduced endocytic and phagocytic capacity, and with acquisition of migratory functions that allow antigen loaded DCs to move from the marginal zones to the T cell areas or from non-lymphoid to lymphoid tissues (Granucci *et al.*, 2003). Their ability to prime T cells is very much dependent on their expression of cytokines and co-stimulatory molecules, which are differentially upregulated depending upon the stimuli inducing either Th1 or Th2 immune response. In the gut, resident DCs have been described in Peyer's patches, forming a dense layer of cells in the subepithelial dome, beneath the follicle epithelium and in the lamina propria, where they are distributed along the entire intestinal epithelium. Besides their sentinel function for incoming pathogens, DCs also actively participate in the microbial entry across mucosal surface by creeping up between epithelial cells and internalizing bacteria via their dendrites (Rescigno *et al.*, 2001).

Perhaps the most intriguing aspect of probiotic modulation of immune response is through its effects on cytokine production. Cytokines and their regulation of the immune system have been studied intensively in the last several years in cell lines and primary cells of both rodents and humans (Nicaise *et al.*, 1993; Marin *et al.*, 1998; Miettinen *et al.*, 1998; Tejada-Simon

et al., 1999). The effects of bacterial flora on cytokine production from mouse resident peritoneal macrophages were investigated. The production of interleukin (IL)-1, interleukin (IL)-6 and tumor necrosis factor-α (TNF-α) was determined in germ-free mice and mice implanted with either *Escherichia coli* or *B. bifidum*. Macrophages from the implanted mice produced significantly more IL-1 and IL-6 *in vitro* than macrophages from germ-free mice (Nicaise *et al.*, 1993). Miettinen *et al.* (1996) investigated the role of cytokines in interactions between lactic acid bacteria and the immune system by measuring the production of TNF-α, interleukin (IL)-6 and interleukin (IL)-10 from human peripheral blood mononuclear cells after stimulation with live or glutaraldehyde-fixed bacteria. They observed higher induction of TNF-α, IL-6 and in some cases, IL-10 in amounts even greater than those obtained with lipopolysaccharide as stimulant, suggesting that the lactic acid bacteria can stimulate non-specific immunity. The effects of four commercial strains of *Streptococcus thermophilus* found in yoghurt on cytokine production was evaluated with a macrophage cell line and a T-helper cell line and compared with active strains of *L. bulgaricus*, *Bifidobacterium adolescentis* and *B. bifidum* (Marin *et al.*, 1998). All cytokines studied, TNF-α, IL-6, interleukin (IL)-2 and interleukin (IL)-5 were affected by heat-killed *S. thermophilus* in a strain and dose dependent fashion. All bacteria induced significant increases of IL-6 production in the macrophage cell line with *S. thermophilus* 133 showing the greatest activity. The four *S. thermophilus* strains also strongly induced TNF-α production. IL-6 and to a lesser extent, TNF-α production were also increased when the macrophages were co-stimulated with LPS and cells of the three groups of lactic acid bacteria. After concurrent stimulation of a T cell line with phorbol 12-myristate-13-acetate, seven of the eight strains enhanced IL-12 and IL-5 production significantly. In another study, human peripheral blood mononuclear cells were stimulated with three non-pathogenic *Lactobacillus* strains and with one pathogenic *Streptococcus pyogenes* strain. All bacteria strongly induced interleukin (IL)-1β, IL-6 and TNF-α mRNA expression and protein production. *S. pyogenes* was the most potent inducer of secretion of

interleukin (IL)-12 and gamma interferon (IFN-γ), and the two of the *Lactobacillus* strains induced IL-12 and IFN-γ production. All strains induced IL-8 protein secretion (Miettinen *et al.*, 1998). More recent studies have assessed the effects of probiotics as cytokine gene transcription. For example, there was no effect of repeated oral exposure to viable or non-viable *L. acidophilus*, *L. bulgaricus*, *L. casei* or *S. thermophilus* on basal cytokine mRNA expression in Peyer's patches, spleen or lymph nodes of mice, after 14 d exposure (Tejada-Simon *et al.*, 1999).

The cytokine pattern produced from cord blood mononuclear cells relative to adult cells after stimulation with bacterial strains from normal flora was examined by Karlsson *et al.* (2002). The levels of IL-12 and TNF-α were similar in cord and adult cells. Gram-positive bacteria induced considerably higher levels of IL-12 and TNF-α than Gram-negative bacteria in both cord and adult cells. The results obtained from this study indicate that the innate immune response in newborns to commensal bacteria is strong and also suggest that different bacterial strains may have differential effects on the maturation of immune system of infants. Christensen *et al.* (2002) studied the effect of lactobacilli, on the expression of cytokines and maturation surface markers in murine dendritic cells. They showed that different species of lactobacilli exert very different activation pattern on the dendritic cells. Furthermore, *Lactobacillus reuteri* DSM 12246 inhibited the activities of the other species. Clearly, not all probiotics share the same immunomodulating properties and can even have opposite effects on some parameters. Moreover, in this model, the dose of probiotics also strongly influenced the nature of the immune response.

It has recently discovered that bacterial DNA contains immuno-stimulatory sequences (ISS-DNA), especially non-methylated CpG motifs, these sequences are potent activators of the innate immunity and exhibit anti-apoptotic properties in the mucosa. Rachmilewitz *et al.* (2002b) showed that the administration of ISS-DNA was beneficial for the colonic mucosa in mice with chemically induced colitis. They then showed that the beneficial effect of

the administration of the probiotic mixture VSL#3 was derived from its DNA, as VSL genomic unmethylated DNA was effective, whereas VSL methylated DNA and calf thymus DNA were ineffective (Rachmilewitz *et al.*, 2002a).

Hart *et al.* (2003) studied the ability of VSL#3 (combination of four species of lactobacilli, three species of bifidobacteria and one streptococcus species, effective in the treatment of inflammatory bowel diseases) components to regulate cytokine production by DC's. They observed that probiotic bacteria differentially modulate the cytokine production by DC's, in a manner that may shape the subsequent acquired immune response.

2.5 THE CONCEPT OF PREBIOTICS

Because of the survivability and colonization difficulties that abound with probiotics, the prebiotic approach was evolved as an alternative to take care of these constraints. Prebiotics exploit selective enzyme production by those gut microorganisms that may impart health benefits to the host. While some peptides, proteins and certain lipids are potential prebiotics, non-digestible carbohydrates, oligo- and poly- saccharides occur naturally and meet the criteria of prebiotics (Ziemer and Gibson, 1998). In order for a food ingredient to be classified as a prebiotic, it must:

i) be neither hydrolyzed nor absorbed in the upper part of the gastrointestinal tract,

ii) be a selective substrate for one or a limited number of beneficial bacteria commensal to the colon, which are stimulated to grow and / or are metabolically activated,

iii) consequently be able to alter the colonic flora in favour of a healthier composition, and

iv) induce luminal or systemic effects that are beneficial to host health (Gibson and Roberfroid, 1995; Mishra *et al.*, 2001).

The carbohydrate group that has received the most attention and research is the oligosaccharides. Crittenden and Playne (1996) described 12 food-grade oligosaccharides in commercial production; these include lactulose, galactooligosaccharides, fructo-oligosaccharides, isomalto-

oligosaccharides, malto-oligosaccharides, palatinose oligosaccharides, glucosyl sucrose and cyclodextrins, soybean oligosaccharides, lacto sucrose, gentio-oligosaccharides and xylo-oligosaccharides. The possible prebiotic effect of these oligosaccharides is that they may selectively stimulate growth of bifidobacteria (Ziemer and Gibson, 1998). Bifidobacteria have a number of health promoting properties such as improved intestinal environment, inhibition of pathogenic bacteria, immunomodulation, synthesis of B vitamins, improved calcium absorption, lowered blood ammonia and cholesterol concentrations and inhibition of tumour formation (Gibson *et al.*, 1996; Yaeshima, 1996). Many physiological and health claims have been made about prebiotics. Table 2.2 list these claims.

Table 2.2 **Claimed gastrointestinal effects of prebiotics (Cummings and Macfarlane, 2002).**

Through fermentation in the large bowel :
• Production of short-chain fatty acids and lactate • Gas, mainly CO_2 and H_2 • Increase in biomass • Increased faecal energy and nitrogen • Mild laxative properties
On the microflora :
• Selective increase in bifidobacteria and lactobacilli in planktonic and biofilm communities • Reduction in clostridia • Increase in colonization resistance to pathogens • Potential benefit in preventing pathogen invasion
Small intestine :
• Osmotic effect of low molecular weight prebiotics (DP3,4) which occasionally cause diarrhoea • Improved calcium, magnesium and iron absorption • Interaction with mucus to change binding sites for bacteria, lectins, etc.
Mouth :
• Protection against caries
Other effects :
• Bile acid metabolism – no consistent changes reported • Variable effects on microbial enzymes with potential to affect carcinogenesis • Stimulation of apoptosis

2.5.1 Fructo-Oligosaccharides

A number of studies have investigated the effects of fructo-oligosaccharides (FOS) on human gut bacteria. Chemically, fructo-oligosaccharides are short and medium length chains of β-D fructans in which fructosyl units are bound by a β-2-1 osidic linkage. Depending on the chain length, as defined by the number of osyl units called the degree of polymerization (DP), fructo-oligosaccharides are named oligofructose (DP<9, average DP=4.8) or inulin (DP up to 60, average DP=12). The inulin extracted from chicory roots contains some fructo-oligosaccharides in addition to polysaccharides (Gibson and Roberfroid, 1995). The Belgian company ORAFTI market their inulin products under the trade name Raftiline. Raftiline contains fructose chains ranging in size from DP 3 to larger than DP 50 (Crittenden and Playne, 1996). Inulin is prepared by hot water extraction of chicory roots, and oligo-fructose is obtained by serial enzymatic hydrolysis of inulin under strictly controlled conditions (Gibson and Roberfroid, 1995). In general, feeding of FOS increases bifidobacteria and lactobacilli, increases short chain fatty acids (SCFA) concentrations and decreases clostridia, fusobacteria and bacteroides and pH (Gibson *et al.*, 1995, 1996; Fuller and Gibson, 1997). The intake doses of FOS, which have elicited a bifidogenic effect in human studies, ranged from 4 to 15 g d^{-1} (Gibson *et al.*, 1996; Roberfroid, 1996). It is always preferable to use possible lowest dose that gives a demonstrable effect. Buddington *et al.* (1996) have demonstrated that fructose oligosaccharides are prebiotic at 4 g d^{-1}.

2.5.2 Lactulose

Lactulose is commonly found in small quantity in thermally processed dairy products (condensed milk, evaporated milk, UHT products, etc.). The quantities produced are insignificant (Andrews, 1984) in comparison to the amounts which can be utilized by bifidobacteria; however, commercial processes are now in place for the production of lactulose from lactose. Evaporated milk contains 0.4 to 0.9% of lactulose. Evaporated milk had been used for infant feeding and, therefore, lactulose had been administered to

infants without recognition. The glucose moiety of lactose can easily be isomerized to fructose to yield the disaccharide referred to as lactulose (4-O-β-D-galactopyranosyl-D-fructose). Commercially, it is produced by heating, using borate as a catalyst (Mizota *et al.*, 1987).

The significance of lactulose as bifidogenic factors have been recognized as early as 1957 (Harju, 1991). Lactulose plays the role of an energy source to stimulate the growth of bifidobacteria, a beneficial intestinal microbe, selectively. This compound also has many other medical applications: treatment of systemic encephalopathy (Bircher *et al.*, 1966); use as a laxative (Brocklehurst *et al.*, 1983) and in the prevention of endotoxemia (Pain and Bailey, 1986). Lactulose is not metabolized by humans or animals due to the absence of enzymatic activity to split the galactose-fructose bond.

2.5.3 Lactitol

Lactitol (4-O-β-D-galactopyranosyl-D-glucitol) is a sugar alcohol which can be prepared by a variety of techniques such as electrolytic reduction, reduction with sodium bicarbonate or hydrogenation with nickel catalyst (Harju, 1988). Petuely (1966) proposed the use of lactitol as a bifidogenic factor. It is similar to lactulose in that it stimulates the growth of bifidobacteria. It is hydrolyzed at a very slow rate in the small intestine (Harju, 1988). Most of it is delivered to the large intestine, where it is utilized by intestinal flora to produce short chain fatty acids (Booy, 1987). These acids are in turn absorbed and serve as sources of energy for the host. Lactitol appears to have some positive benefits in terms of reducing serum cholesterol (Sugimoto, 1976) and altering the metabolism of cholic acids in the intestine (Booy, 1987). Other uses of lactitol include the treatment of hepatic encephalopathy and constipation. Lactitol has a low level of cariogenicity (Grenby, 1989) and can be used in dietetic sweetners.

2.5.4 Xylo-Oligosaccharides

All land plants contain a group of polysaccharides known as D-xylans which form a polymer-homologous series such as xylobiose, xylotriose, up to xyloheptaose. Xylobiose (4-O-β-D-xylopyranosyl-D-xylopyranose) has been

isolated from a variety of sources (corn cobs, aspen, wheat straw) using concentrated acid hydrolysis and endoxylanases or xylans. Limited data are available on the properties of xylobiose or its application as a bifidogenic factor; however, work published by Okazaki *et al.* (1990) found the xylo-oligosaccharides (XOS), principally xylobiose to be effective as bifidobacterial factors when administered at 1 or 2 g daily.

2.5.5 Transgalactosylated Oligosaccharides

These oligosaccharides do not occur naturally, but a number of transgalactosylated oligosaccharides (TOS) have been produced by synthesis. Minami *et al.* (1983) prepared three TOS: Isogalactobiose, Galsucrose, and Lactosucrose. TOS compounds are available commercially from two Japanese suppliers: Ensuiko Ltd. produces lactosucrose, while Yakult offers a commercial preparation containing approximately 51% (w/w) tri-saccharides, 35% tetra-saccharides and 13% penta- and hexa-saccharides (Wijsman *et al.*, 1989).

2.6 INULIN AS A PREBIOTIC

Any food that reaches the colon such as non-digestible carbohydrates, some peptides and proteins as well as certain lipids is a prebiotic candidate (Gibson, 1999). Non-digestible carbohydrates, in particular fructose oligosaccharides are authentic prebiotics. Inulin escape digestion in the upper gastrointestinal tract and reach the large intestine virtually intact. This attribute contribute them as being ideal for fermentation in the colon by the saccharolytic resident microbiota. The effects of inulin on the human gut microbiota has been extensively studied both *in vivo* and *in vitro*, and the majority of studies report selective fermentation by the beneficial flora, namely, bifidobacteria and to a lesser extent lactobacilli (Kolida *et al.*, 2002).

2.6.1 *In-vitro* Trials

Bifidobacteria possess hydrolytic enzymes known as inulases (2,1-β-D-fructan-fructanohydrolase), which unlike human and animal digestive enzyme, cleave fructo-oligosaccharides.

Yazawa and Tamura (1982) have observed that ruminal bifidobacteria (*B. infantis*) utilized short chained FOS well, but were unable to metabolize longer-chained polymers (DP>25). Both, Hidaka *et al.* (1986) and Mitsuoka *et al.* (1987) observed that *B. bifidum* did not grow in media supplemented with short-chained FOS (neosugars). *B. longum* grew poorly while both *B. adolescentis* and *B. infantis* readily metabolized this carbohydrate. Generally, *E. coli* and the *Clostridium* species do not grow well in neosugars, nor does *Lactobacillus casei* (Hidaka *et al.*, 1986; Mitsuoka *et al.*, 1987).

Both human and animal strains of bifidobacteria can readily metabolize short-chained polymers (DP 3-6), however, animal strains metabolize inulin much more effectively than bifidobacteria of human origin (McKellar and Modler, 1989).

In-vitro studies have demonstrated that *B. longum* has difficulty in metabolizing long-chained polymers such as inulin: *B. adolescentis* metabolizes inulin slightly better than *B. longum*, but generally *B. thermophilum* has the best growth and inulase activity with 2 to 4% inulin as the carbohydrate source (McKellar and Modler, 1989).

Gibson and Wang (1994a) observed higher specific growth rates of eight species of bifidobacteria in media containing oligo-fructose as the sole carbon and energy source in comparison to glucose. Members of *Clostridium* and *Bacteroides* preferred glucose to FOS, suggesting that bifidobacteria would have a selective growth / survival advantage in the colon, if FOS is available. This has been confirmed in *in-vitro* studies using mixed human faecal cultures and pure strains in both batch and continuous culture.

Hopkins *et al.* (1998) documented the ability of seven *Bifidobacterium* isolates to utilize a selection of fifteen different carbohydrate sources in 48 h batch culture experiments. In a continuous culture study, Sghir *et al.* (1998) demonstrated through molecular techniques that inulin and oligofructose were selectively fermented not only by bifidobacteria but also by lactobacilli. Oligofructose and galacto-oligosaccharides preferentially supported growth of the test bacteria.

The prebiotic properties of inulin and oligofructose are now well documented, but few groups have looked at the role of these carbohydrates in determining the composition of the mucosa associated flora. When growing on surfaces, intestinal bacteria form biofilms (Macfarlane *et al.*, 1999) and behave differently from those existing in planktonic or free form. Using a novel design (Horan and Cummings, 1999) glass fermentation chambers have been adapted to suspend several mucin covered glass slides within a culture vessel. These are run at constant temperatures and pH and used to assess the effect of inulin and oligofructose on the planktonic and biofilm populations of bacteria. Faecal inocula from six healthy volunteers were used. After 12 h, the effect of the prebiotic mixture was to increase counts of bifidobacteria in the planktonic phase by 0.45 log_{10} cfu/ml ($P=0.06$) and the biofilm count by 0.77 log_{10} cfu/slide ($P<0.001$). Count of clostridia fell and lactobacilli showed a small increase.

Karppinen *et al.* (2000) compared the *in vitro* fermentability of inulin by faecal bacteria to that of rye, wheat and oat bran. Inulin was the most rapidly fermented of the test substrates giving most butyrate production and the largest decrease in pH but also the highest and fastest acid production.

Kaplan and Hutkins (2000) screened a selection of twenty-eight lactic acid bacteria and bifidobacteria for their ability to ferment inulin and oligofructose on MRS agar. Twelve of sixteen *Lactobacillus* strains and seven of eight *Bifidobacterium* strains tested were able to ferment the substrates.

2.6.2 *In-vivo* Trials

Gibson *et al.* (1995) studied the selective stimulation of bifidobacteria by inulin and oligofructose in a 45-day study of eight healthy male human subjects. Both oligofructose and inulin caused significant increases in faecal bifidobacteria. Bacteroides, clostridia and fusobacteria all decreased during oligofructose supplementation and Gram-positive cocci were reduced during inulin supplementation. Total bacterial levels remained unaffected, while little change was observed in faecal SCFA and breath CH_4.

The effect of dietary supplementation of inulin and lactose on faecal flora, microbial activity and bowel habit in thirty-five elderly constipated patients was studied by Kleessen *et al.* (1997). A significant increase was observed in bifidobacterial levels in the inulin group, while a decrease in enterococci numbers and enterobacteria occurred. Lactose had no effect on bifidobacteria, while it increased enterococci counts and decreased lactobacilli levels. A better laxative effect was reported with inulin.

The effect of inulin on faecal bifidobacteria in eight healthy free living humans was investigated by Kruse *et al.* (1999). Subjects consumed a typical western diet (45% energy as fat, 40% energy as carbohydrate), followed by a reduced fat diet (30% energy as fat) using inulin as fat replacement (maximum inulin consumed 34 g per day). Controls consumed identical diets but without inulin supplementation. The effect on faecal flora was monitored using fluorescent probes targeting diagnostic regions of the 16S rRNA molecule. A significant increase in bifidobacterial populations was observed, while short chain fatty acids (SCFA) blood lipids and gas production remain unaffected.

In two studies, the effect of inulin on dextran sulphate sodium (DSS) induced colitis rats was reported (Videla *et al.*, 1998; Videla, 1999). It was established that dietary inulin promoted growth of lactobacilli in the rat colon, reduced the severity of DSS induced colitis and reduced the luminal pH in a wide area extending from left to right colon.

Reddy (1999) evaluated inulin (raftiline) and oligofructose (raftilose) for their potential inhibitory properties against the development of colonic aberrant crypt foci (ACF) in rats. The results of this study indicated that the dietary administration of oligofructose and inulin inhibits the development of ACE in the colon, suggesting the potential colon tumor inhibitory properties of chicory fructans.

Bouhnik *et al.* (1999) assessed the tolerance and threshold dose of oligofructose (from sucrose) that significantly increased faecal bifidobacteria counts in a 7-day study of forty healthy human volunteers. They reported that the optimal dose for increased bifidobacteria without significant side effects, such as flatulence, was 10 g per day.

Den Hond *et al.* (2000) investigated the effect of high performance inulin on constipation in six healthy humans with a low stool frequency in a double-blind placebo control crossover study. A significant increase in stool frequency and faecal bulk was observed with inulin administration.

Langlands *et al.* (2000) carried out a human feeding study to investigate the effect of inulin and oligofructose on mucosal microflora. Fifteen healthy subjects were selected from a colonoscopy waiting list and supplemented their usual diet with 15 g/day of an inulin + oligofructose mixture for 2 weeks before their colonoscopy. The effect on the mucosal flora was to increase significantly both bifidobacterial and lactobacilli counts on the epithelium, while counts of bacteroids, clostridia and enterobacteria were unchanged.

2.7 SYNBIOTICS

The concept of prebiotics has become very popular since its introduction in 1995 (Gibson and Roberfroid, 1995). The concept of synbiotic is in its infancy, and is yet to be applied to the full extent in developing new foods. When combining both a probiotic and a prebiotic in a single food product, the expected benefits are an improved survival during the passage of probiotic bacteria through the upper intestinal tract. A more efficient implantation in the colonic microbiota together with a stimulating effect of the prebiotic on the growth and / or the activities of both the exogenous (probiotic) and endogenous bacteria (e.g., bifidobacteria) (Roberfroid, 1998). Modler *et al.* (1990b) revealed that approximately 90% survival of all bacterial species was achieved over a 70 day study period, when *B. longum*, *B. brevi* and *B. infantis* were mixed with bifidogenic factors and incorporated in ice-cream.

Gibson *et al.* (1995) reported a statistically significant effect on the composition of faecal flora on feeding a group of volunteers a daily supplement of a synbiotic composed of 125 ml *Lactobacillus* fermented milk containing 2.75 g oligofructose continuously for 7 weeks. They reported an increase in the number of bifidobacteria cfus leading to an equivalent and parallel increase in total anaerobes cfu. The coliforms cfus were not modified but, surprisingly, the number of lactobacilli cfu was decreased. Even though

this study does not really demonstrate the efficacy of the synbiotic approach, the results are interesting because they demonstrate the bifidogenic / prebiotic effect of quite a low daily dose (2.75 g) of chicory fructan (oligofructose) continuously fed over a period of 7 weeks. Martin (1996) studied the effects of bifidogenic growth factors on survival of *B. longum, B. infantis* and *B. adolescentis* in various dairy products. They supplemented 10% solids skim milk containing *B. longum* or *B. infantis* with 0.5% FOS, 0.5% lactulose and 0.5% units/ml of oxyrase. Their data indicated that the level of FOS and lactulose used (0.5%) does not significantly affect numbers of *B. longum* or *B. infantis* in skim milk during incubation at 37°C for 48 h. Dubey and Mistry (1996) studied the effect of bifidogenic factors on growth characteristics of Bifidobacteria in infant formulae. They incorporated a bifidogenic factor, lactulose or fructo-oligosaccharides (0.5%) into infant formulae, which were then inoculated (2.5%) with *Bifidobacterium bifidum*, *Bifidobacterium breve*, *Bifidobacterium infantis* or *Bifidobacterium longum* or their mixture. Lactulose did not influence maximal counts or generation times in either formula for any species except *B. infantis*, which had lower counts. Maximal counts and production and change in pH and biochemical metabolites at maximal bacterial counts were not influenced by the presence of FOS in the infant formulae.

Without specifically referring to it as a `synbiotic approach', Bouhnik *et al.* (1996) have assessed in healthy humans, the effects of prolonged ingestion of *Bifidobacterium* spp. fermented milk with or without inulin (equivalent to 18 g/day) on faecal bifidobacteria. They concluded that the *Bifidobacterium* spp. fermented milk substantially increased the number of bifidobacteria colony-forming units (cfu), but that the concurrent administration of inulin did not enhance the effect. This observation is not surprising in view of the fact that the number of bifidobacterial cfu in the faeces of probiotic-fed volunteers was already so much increased (from 10^7 to 10^9) that they could hardly be additionally increased by the prebiotic. Indeed, it has been argued by Roberfroid *et al.* (1997) that the stimulation of growth of bifidobacteria by prebiotics very much depends on their initial level. Moreover, an in-depth analysis of the data reported by these authors reveals that 2 weeks after

stopping the consumption of the dairy products, the volunteers who received the synbiotic type product still had a significantly higher number of bifidobacterial cfu than those receiving the probiotic alone. This could indicate either a better implantation of the probiotic bifidobacteria in the colonic microbiota or a prebiotic-type effect on endogenous bifidobacterium.

One of the principal benefits of synbiotics is believed to be increased persistence of the probiotic in the gastrointestinal tract. A synbiotic preparation of *Lactobacillus acidophilus* (probiotic strain 74-2) and FOS has been studied in an *in vitro* model of the human gut (Gmeiner *et al.*, 2000). The model used was the SHIME reactor and the synbiotic resulted in higher levels of lactobacilli (an increase of 0.89 log) in the vessel corresponding to the ascending colon. An increase in bifidobacteria was seen in the vessels corresponding to the ascending colon (1.27 log) transverse (0.9 log) and descending (0.47 log) colon, presumably due to the prebiotic component of the synbiotic. Increases were also seen in levels of propionate and butyrate, and in β-galactosidase. A decrease was seen in β-glucuronidase levels.

Synbiotics based around resistant starch have been traditionally developed (Crittenden *et al.*, 2001). A screen of 40 bifidobacteria revealed that a particular *B. lactis* strain had a good growth rate on the resistant starch, survived a simulated passage through the stomach and small intestine, and was suitable for the manufacture of yoghurt. Resistant starch is a promising medium for synbiotic development as it is prebiotic (Silvi *et al.*, 1999) and results in high levels of bifidobacteria in the large intestine (Topping and Clifton, 2001).

The ability of a synbiotic preparation [*Bifidobacterium breve* Yakult with galactooligosaccharide (GOS)] to protect against Salmonella infection in mice has been investigated (Asahara *et al.*, 2001). Mice were treated with streptomycin to compromise the gut flora by selective removal to undetectable levels of bifidobacteria, lactobacilli and enterobacteria. Feeding with *B. breve* at 10^8 cfu/mouse/day or the synbiotic preparation, which additionally contained GOS at a concentration of 2 to 50 mg/mouse/day, resulted in recolonization of the gastrointestinal tract with *B. breve*. Mice fed with

probiotic and synbiotic displayed reduced faecal excretion of *Salmonella enterica* serovar *typhimurium* after pathogen challenge. In addition, the synbiotic blocked extra-intestinal translocation of the pathogen; whereas GOS alone did not.

Alander *et al.* (2001) screened three commercially available bifidobacterial strains *B. lactis* Bb-12, *Bifidobacterium* spp. 420 and *Bifidobacterium* spp. H for their bile and acidity resistance properties, and ability to utilize carbohydrates and prebiotics in order to select a bifidobacterial strain for incorporation into the test product. In spite of comparable resistance and utilization patterns of all three strains, Bb-12 was chosen, mainly due to two criteria, i.e., its extensive background of documentation regarding the probiotic properties and its higher viable count stability in the test product. They also investigated the persistence of the selected probiotic *B. lactis* Bb-12, on ingestion, as a synbiotic preparation with GOS using randomly amplified polymorphic DNA genotyping. A total of 30 healthy volunteers consumed either prebiotic, probiotic or synbiotic in a yoghurt medium for two weeks. Volunteers consuming each supplement showed increases in lactic acid bacteria and all of the volunteers taking the probiotic (or synbiotic) had Bb-12 present in the faecal flora. No increase in survival of the Bb-12 due to GOS was seen; however, as pointed out by the authors, *B. lactis* Bb-12 has very good survival properties to begin with. The development of synbiotics might be more important for strains with poorer survival properties. Kanamori *et al.* (2002) designed a new protocol for synbiotic therapy to demonstrate the beneficial effects of this therapy. The synbiotic contained three agents, *Bifidobacterium breve*, *Lactobacillus casei* and galactooligosaccharides. More than $1x10^9$ of probiotic bacteria were contained in each 1.0 g pack and they administered 3.0 g per day of each agent to the patient. They treated a critically ill 9-month old girl with laryngotracheo-esophageal cleft. Abundant amounts of the administered bacteria were detected in the faeces suggesting that these bacteria affected intestinal function *in situ*. Bowel movements resumed soon after the commencement of synbiotic therapy and considerable amounts of short chain fatty acids were detected in the faeces. Growth of the

patient was satisfactory under this treatment, enabling the researchers to recommend synbiotic therapy for critically ill patients in intensive care units as an important immunonutritional therapy.

Bielecka *et al.* (2002) studied *in vitro* the influence of fructan type oligosaccharides (as prebiotics) on growth and acidifying activity of *Bifidobacterium* strains (probiotics). The selected synbiotic pairs of bifidobacteria strains and oligosaccharides enhancing their activity were studied *in vivo* to determine the effect of probiotics, prebiotics and synbiotics on microecology of murine gut. *In vitro* studies showed that the majority of *Bifidobacterium* species utilized FOS and low polymerized inulins, but only 18 out of 30 strains tested (mostly of *B. longum* and *B. animalis* species) were stimulated. Incorporation of oligofructose into the diet stimulated proliferation of faecal bifidobacteria by 1.6 log cfu/g in comparison to the control. *B. longum* KN29.1 and KNA1 selected *in vitro*, slightly increased the faecal bifidobacteria live number by about 0.6 log cfu/g under *in vitro* conditions; whereas *B. animalis* KSp4 was not effective. But administration of bifidobacteria together with the prebiotic (as synbiotics) improved the bifidogenic effect by 1.4 log cfu/g faeces. Supplementation of diet had almost no effect on the other determined groups of gut microflora [coliforms, total count of mesophilic bacteria, spores of anaerobic bacteria (saccharolytic, proteolytic), and spores of aerobic bacteria].

Number of synbiotic products containing Bifidobacteria and Lactulose are already available in Japanese markets. Some of these products are Hounyu Milk Powder for adults [Lactulose (g/100g) – 8.3 and Bifidobacteria - $>3x10^7$], Sawayaka sour milk [Lactulose (g/100 g) – 4 and Bifidobacteria - $>1x10^8$], etc. (Mizota *et al.*, 1987). Some synbiotic dairy products have been marketed in Europe also, e.g., Symbalance, mixture of *Lactobacillus reuterii*, *L. acidophilus* and *L. casei* along with RAFTILINE, an inulin and John apres Jour a UHT skimmed milk with ACTILIGHT, etc. (Young, 1998), probiotic plus oligofructose (yoghurt), two *Lactobacillus* strains plus inulin, Actimel (cholesterol control yoghurt), *L. acidophilus* plus oligofructose, Fysiq (dairy drink), *L. acidophilus* plus inulin (Kolida *et al.*, 2002).

CHAPTER - 3

Materials and Methods

3. MATERIALS AND METHODS

3.1 BACTERIAL CULTURES

The bacterial cultures used in this study were obtained from different sources as given below :

Cultures	Source
Bifidobacterium :	
Bifidobacteria	From ABT-5 (Defined Mixed Strain Culture, Chr. Hansen, Denmark)
Bifidobacterium lactis Bb-12	Chr. Hansen, Denmark (obtained from Heritage Food India Ltd., Tirupati)
Bifidobacterium species 420	Danisco, Germany [obtained from Dohler (India) Flavourade Ltd., Pune]
Bifidobacterium bifidum NCDC255, *Bifidobacterium bifidum* NCDC229A,	National Collection of Dairy Cultures, DM Division, NDRI, Karnal
Bifidobacterium adolescentis	Human isolate (obtained from Dairy Microbiology Division, Gujarat Agricultural University, Anand)
Bifidobacterium bifidum VP, *Bifidobacterium bifidum* A3, *Bifidobacterium bifidum* 1047, *Bifidobacterium bifidum* NCDC255 (GM Strain)	Infant Food Laboratory, DT Division, NDRI, Karnal
Lactobacillus :	
Lactobacillus rhamnosus GG	Valio Ltd., Helsinki, Finland
Indicator organisms :	
Escherichia coli NCDC247, *Salmonella typhimurium* NCDC113, *Staphylococcus aureus* NCDC109, *Shigella dysenteriae* NCDC107, *Enterococcus faecalis* NCDC116	National Collection of Dairy Cultures, DM Division, NDRI, Karnal

3.2 MAINTENANCE AND PROPAGATION OF CULTURES

The *Bifidobacterium* cultures were maintained in modified MRS broth (MRS + 0.05% L-cysteine hydrochloride, mMRS) at 2 percent inoculation level and were sub-cultured at weekly intervals. The cultures were activated with 2 to 3 transfers in mMRS media and grown anaerobically (using Anaero Hi Gas Pack, HiMedia Laboratories Ltd. Mumbai) at 37°C for 24 h.

Indicator organisms were maintained in nutrient agar slants and sub-culturing was done after every 30 days. Before using for experiments, the cultures were activated by 2-3 transfers in nutrient broth and incubation was done at 37°C for 24 h.

3.2.1 Composition of DeMan Rogosa Sharpe (MRS) Broth / Agar (deMan *et al.*, 1960)

Composition per litre :

Peptone	:	10.0 g
Beef extract	:	8.0 g
Yeast extract	:	4.0 g
Dextrose	:	20.0 g
Sorbitan mono-oleate	:	1.0 ml
Dipotassium hydrogen phosphate	:	2.0 g
Magnesium sulphate heptahydrate	:	0.2 g
Manganese sulphate tetrahydrate	:	0.05 g
Triammonium citrate	:	2.0 g
Sodium acetate	:	5.0 g

Distilled / deionized water to make volume to 1000 ml. pH 6.2 ± 0.2 at 25°C.

The ingredients were suspended in water and boiled to dissolve. Addition of agar at the rate of 15 g/L resulted in a solid culture medium. The medium was autoclaved at 121°C and 15 psi for 15 min.

3.2.1.1 mMRS broth / agar (Arroyo *et al.*, 1994)

A 5 percent solution of L-cysteine hydrochloride monohydrate was filter sterilized and stored at 5 to 7°C. It was then added to molten, cooled MRS broth / agar (50°C) so that the final concentration of cysteine hydrochloride in the medium is 0.05 percent. This media was used for cultivating pure cultures of bifidobacteria.

3.2.2 Composition of Nutrient Broth / Agar (Atlas, 1993)

Peptone	:	5.0 g
NaCl	:	5.0 g
Yeast extract	:	2.0 g
Beef extract	:	1.0 g

Distilled / deionized water to make the volume to 1000 ml, pH 7.4 ± 0.2.

The components were dissolved in water by gentle heating. Addition of agar at the rate of 15 g/L resulted in a solid culture medium. Distributed into tubes or flasks, and autoclaved at 121°C and 15 psi for 15 min. This medium was used for the cultivation and maintenance of indicator organisms.

3.3 PURITY AND CONFIRMATION OF CULTURES

3.3.1 Microscopic Examination

Gram stained smears of all cultures were examined microscopically in order to observe their morphology and to check the purity.

3.3.2 Catalase Test

The catalase test was performed as per slide method. Using an inoculating needle, culture from a well-isolated colony was placed onto a clean glass slide. A drop of 3 percent hydrogen peroxide solution was added to this culture and closely observed for the evolution of bubbles.

3.3.3 Fructose-6-Phosphate Phosphoketolase (F6PPK) Detection

3.3.3.1 Reagents for fructose-6-phosphate phosphoketolase (F6PPK) test

a) Phosphate buffer (0.05 M, pH 6.5)

Thirty-one ml of a 71.6 g/L solution of disodium hydrogen orthophosphate was added to 67.5 ml from a 31.21 g/L solution of sodium dihydrogen phosphate in distilled water, to prepare 0.2 M buffer. The solution was then diluted 4 times with distilled water to prepare 0.05 M buffer.

b) Fructose-6-phosphate

D-fructose-6-phosphate (Disodium salt, Sigma Chemicals, USA) (80 mg/ml) in distilled water.

c) Hydroxylamine solution

Hydroxylamine hydrochloride 28 percent was neutralized with the addition of equal volume of 1.4 percent sodium hydroxide. The mixture had the pH of 6.4 and freshly prepared solution was used.

d) Sodium flouride – iodoacetic acid solution

Sixty mg of sodium fluoride and 100 mg of iodoacetic acid in 10 ml of distilled water.

e) Other reagents

Fifteen percent solution of trichloroacetic acid, four N hydrochloric acid solution and five percent w/v of ferric chloride.$6H_2O$ solution in 0.1 N HCl were prepared.

3.3.3.2 Preparation of cell mass

Bifidobacteria were grown anaerobically in mMRS broth at 37°C for 24 h and cells were harvested by centrifugation (Kubota, Japan) at 12000 rpm for 10 min at 4°C. Harvested cells were washed twice with 0.05 M phosphate

buffer, pH 6.5 and resuspended in 1 to 2 ml phosphate buffer, and the cells were disrupted by sonication (MSE, England) in an ice bath at high power for 5 min (with intermittent breaks to avoid over heating of the culture).

3.3.3.3 Enzyme (F6PPK) detection

To one ml of the cell lysate taken in an acid-rinsed test tube, 0.25 ml each of sodium fluoride – iodoacetic acid solution and fructose-6-phosphate solutions were added. Distilled water was added in place of fructose-6-phosphate solution so as to prepare the control. After incubation at 37°C for 30 min, the reaction was stopped with 1.5 ml of hydroxylamine hydrochloride reagent. After 10 min at room temperature, 1.0 ml each of 15 percent trichloroacetic acid and 4 N HCl were added. The mixture was left at room temperature for 5 min and 1.0 ml of colour developing reagent ferric chloride ($FeCl_3.6H_2O$ – 5%) was added. The formation of acetyl phosphate from fructose-6-phosphate was detected by the reddish violet colour formed by the ferric chelate of its hydroxyamate. The contents of the control tube appear bright yellow (Scardovi, 1986).

3.4 TESTING OF PROBIOTIC ATTRIBUTES

Only those cultures which gave positive results for F6PPK test were used for further experiments.

3.4.1 *In vitro* Tolerance to Simulated pH of Human Stomach

3.4.1.1 Preparation of solutions to simulate pH of human stomach

Solutions of 35.4 percent HCl (Reidel Chemicals) in distilled water were adjusted to pH levels of 1.5, 2.0 and 2.5. Sterile distilled water (pH 6.5) served as the control. The prepared solutions were transferred to test tubes in 10 ml volumes, sterilized and stored at room temperature until needed.

3.4.1.2 Enumeration of bifidobacteria in pH solution

Each of the four pH solutions (pH 1.5, 2.0, 2.5 and 6.5) were inoculated with 24 h old active culture of *Bifidobacterium* at 2 percent level. Contents of the tubes were mixed well and one milliliter of culture from each tube was

taken immediately (0 h) and after 1, 2 and 3 h of anaerobic incubation at 37°C. Serial dilutions were prepared in 0.1 percent sterile peptone solution and appropriate dilutions were transferred to sterilized screw capped tubes. Bifidobacterium agar (HiMedia Laboratories Ltd., Mumbai) at approximately 45°C was then poured to screw capped tubes containing appropriate dilution and mixed well. The tubes were incubated anaerobically at 37°C and colonies developed were counted after 48 h (Clark *et al.*, 1993).

3.4.2 *In vitro* Tolerance to Simulated Bile Concentrations of the Human Small Intestine

Ten ml mMRS broth containing different concentrations (0, 2, 2.5 and 3%) of bile salt (s.d. Fine Chem. Ltd.) were inoculated with active *Bifidobacterium* cultures at 2 percent level. One milliliter of culture from each tube was taken immediately (0 h) and after 3 and 12 h of anaerobic incubation at 37°C. Serial dilutions were prepared in 0.1 percent sterile peptone solution and appropriate dilutions were transferred to sterilized screw capped tubes. Bifidobacterium agar was used for enumeration and colonies developed were counted after 48 h of anaerobic incubation at 37°C (Gilliland and Walker, 1990).

3.4.3 Lysozyme Tolerance

Ten ml mMRS broth with (100 ppm) and without lysozyme (Brennan *et al.*, 1986) was inoculated with active cultures of bifidobacteria at 2 percent level, and incubated anaerobically at 37°C for 24 h. Samples were taken at 0 and 24 h, appropriate dilutions were prepared in 0.1 percent sterile peptone solution and transferred to screw capped tubes for enumerating the initial and final number of viable cells. The colonies developed were counted after 48 h of anaerobic incubation at 37°C.

3.4.4 Surface Hydrophobicity

Adhesion to n-Hexadecane was carried out to assess the cell surface hydrophobicity of bifidobacterial cultures.

3.4.4.1 *Adhesion to n-Hexadecane*

The method of Rosenberg *et al.* (1980) with slight modification was adopted to measure the cell surface hydrophobicity.

a) **Buffer**

Phosphate urea magnesium sulphate (PUM) buffer

Ingredients	**g/L**
$K_2HPO_4.3H_2O$	22.2
KH_2PO_4	7.26
Urea	1.8
$MgSO_4$	0.2
pH	7.1

b) **Protocol**

Bifidobacterium cells grown at 37°C for 18 h anaerobically, were harvested by centrifuging at 12000 rpm for 10 min at 4°C. The pellet obtained was resuspended in PUM buffer and washed twice by centrifuging as mentioned above. The washed pellet was resuspended in PUM buffer and the absorbance was adjusted to 0.8 to 0.9 at 610 nm using Spectronic 21D Spectrophotometer (Milton Roy). The bacterial suspension (with absorbance 0.8 to 0.9) was taken (4.8 ml) in clean acid washed test tubes. To this, 0.8 ml of n-Hexadecane was added and incubated at 37°C for 10 min. After this, the two phases were mixed using a vortex mixer for 2 min at full speed. The mixed solution was kept at 37°C for 1 h for phase separation. The lower aqueous phase was separated with the help of glass pipette and the light absorbance of the aqueous phase was determined at 610 nm. The fraction of adherent cells was taken as percent decrease in absorbance of the aqueous phase after mixing and phase separation as compared to that of original suspension. The cell surface hydrophobicity was calculated as follows :

$$\text{Cell surface hydrophobicity (\%)} = \frac{\text{Initial O.D.} - \text{Final O.D.}}{\text{Initial O.D.}} \times 100$$

3.4.5 Antimicrobial Activity against Enteric Organisms

Agar well method was followed for measuring the antimicrobial activity (Anand *et al.*, 1984). Nutrient agar containing 0.1 percent Tween-80 was seeded with the test culture (*E. coli* NCDC247, *S. typhimurium* NCDC113, *S. dysenteriae* NCDC107, *E. faecalis* NCDC116, *S. aureus* NCDC109) at the rate of one percent and poured into the plates. These were then allowed to solidify. The plates were marked into three different zones to represent three bifidobacterial cultures and a well of 0.7 cm was made in each of the marked zones. One hundred µl of the 24 h old culture of bifidobacteria grown in mMRS was poured into the respective well. The plates were kept in the refrigerator for 4 to 5 h for allowing the diffusion of the material in the well to the agar. Plates were incubated at 37°C for 48 h and the diameter of the zone of inhibition formed was measured.

3.5 EFFECT OF INULIN (PREBIOTIC) ON THE GROWTH OF BIFIDOBACTERIAL CULTURES

The ability of bifidobacteria to ferment inulin (Orafti, Belgium, obtained from SA Chemicals, Mumbai) was examined in semi-liquid (agar 0.1%, w/w) minimal media (Bielecka *et al.*, 2002) which contained 0, 0.5, 1, 3 and 5 percent (w/v) of inulin, meat peptone 1 percent (w/v), L-cysteine hydrochloride 0.04 percent (w/v), buffering salts and indispensable ions as in Garches medium (Rasic, 1990). The final pH was 6.4. The minimal media containing different concentrations of inulin was inoculated with 24 h old active cultures of bifidobacteria at 2 percent levels and incubated anaerobically at 37°C for 24 h. Samples for determining the bifidobacterial counts were taken at 0 h and after 24 h of anaerobic incubation. Bifidobacterium agar was used for enumeration and the colonies developed were counted after 48 h of anaerobic incubation at 37°C. The growth stimulatory effect of inulin was assessed on the basis of percentage reduction in mean generation time on inulin supplementation, compared to control (0% inulin).

3.6 ANTIBACTERIAL ACTIVITY OF THE SELECTED BIFIDOBACTERIAL CULTURE AGAINST ENTERIC ORGANISMS IN THE PRESENCE OF INULIN

The antimicrobial activity of the selected bifidobacterial culture grown in mMRS containing inulin was determined and compared with that grown in mMRS containing dextrose by agar well method (Anand *et al.*, 1984). For this, nutrient agar containing 0.1 percent Tween-80 was seeded with the test culture (*E. coli* NCDC247, *S. typhimurium* NCDC113, *S. dysenteriae* NCDC107, *E. faecalis* NCDC116 and *S. aureus* NCDC109) at the rate of one percent and poured into the plates. These were then allowed to solidify. Wells of 0.7 cm diameter were drawn and one hundred µl of the 24 h old culture of bifidobacteria grown in mMRS containing either dextrose or inulin as the sole carbon source was added into them. The plates were kept in the refrigerator for 4 to 5 h for allowing the diffusion of the material in the well to the agar. Plates were incubated at 37°C for 48 h and the diameter of the zone of inhibition formed was measured.

3.7 DEVELOPMENT OF A FREEZE-DRIED (LYOPHILIZED) SYNBIOTIC PREPARATION CONTAINING *BIFIDOBACTERIUM* AND INULIN

The freeze-dried synbiotic preparation was developed on the basis of the method followed by Collins and Hall (1984). For this, the selected culture was inoculated into mMRS broth @ 2 percent and incubated at 37°C for 48 h under anaerobic conditions. After this, cells were harvested by centrifugation at 12000 rpm for 10 min at 4°C, washed once in sterilized distilled water and resuspended @ $1 \times 10^9 - 2 \times 10^9$ cfu/ml in a sterilized mix containing 5 percent non-fat dry milk (NFDM), 20 percent inulin, 8 percent sucrose and 1.5 percent gelatin (gelatin was separately sterilized and mixed with the solution containing other components under aseptic conditions). The mix thus obtained was poured into sterile petri plates and freeze dried (Alpha 1-4 Freeze dryer; Martin Christ, Germany).

In order to carry out storage studies the synbiotic product was aseptically transferred into sterile moisture proof 150 ml polypropylene bottles and stored at room and refrigeration temperatures. The viability of probiotic organisms were determined on 0, 7, 14, 21, 30 and 60 day of storage. Bifidobacterium agar was used as the growth media and colonies were enumerated in screw capped tubes.

3.8 *IN VIVO* STUDIES IN MICE

To check whether the use of a combination of probiotic and prebiotic can provide better results in terms of improvement of the survival of the added probiotic bacteria than the use of either of them, feeding trials were carried out as given below :

3.8.1 Experimental Animals

The trial was conducted on adult male albino mice (mean initial body weight – 31 g) obtained from Small Animal House, NDRI, Karnal. The animals were divided into four groups, each containing seven animals. The animals were kept in plastic cages under conventional conditions and fed with feed and water *ad libitum.*

3.8.2 Model of Study

Mice were randomly assigned to four groups, seven mice in each group. All groups were fed on basal diets for 5 days before and 10 days after the treatment period. During treatment period, the control group was given the basal diet, the probiotic group was additionally receiving ~10^8 live cells of bifidobacteria/g feed, the prebiotic group was given basal diet supplemented with inulin (5%, w/w feed) and the synbiotic group received both bifidobacteria (~10^8 cfu/g feed) and inulin (5% w/w feed). In case of control and probiotic groups, inulin was replaced with same amount of sucrose. Faecal samples were collected during pre-feeding (0, 5 day), feeding (10, 15, 20 day), post-feeding (25, 30 day) periods and analyzed for total plate count, bifidobacterial count, clostridial count, direct microscopic count and β-glucuronidase activity (Bielecka *et al.*, 2002).

3.8.3 Determination of Bacteria in Faeces

All bacterial enumerations were done within 1.5 h of sampling. Faecal samples were collected fresh by gently squeezing the rectal area of the mice. Anaerobic conditions were maintained as far as possible during collection and analysis of faecal samples. Fresh faeces 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 used for enumerating the bacterial count.

Total plate count was determined on nutrient agar with 1 percent glucose, 1 percent yeast extract and 1 percent peptone (Bielecka *et al.*, 2002). The plates were incubated at 37°C for 48 h. Violet red bile agar (VRBA, HiMedia Laboratories Ltd., Mumbai) was used for the enumeration of coliforms (John, 2000). Plates were incubated at 37°C for 48 h. Differential reinforced clostridial agar (HiMedia Laboratires Ltd., Mumbai) was used for the determination of clostridial count. Bifidobacterial count was enumerated on *Bifidobacterium* iodoacetate medium-25 (BIM-25; Campbell *et al.*, 1997). Clostridial and bifidobacterial counts were enumerated in screw capped tubes and the colonies developed were counted after 72 h of anaerobic incubation at 37°C.

3.8.3.1 Preparation of Bifidobacterium iodoacetate medium-25 (BIM-25)

Composition of reinforced clostridial agar :

(Composition per litre)

Ingredient		Amount
Agar	:	13.5 g
Beef extract	:	10.0 g
Pancreatic digest of casein	:	10.0 g
NaCl	:	5.0 g
Glucose	:	5.0 g
Yeast extract	:	3.0 g
Sodium acetate	:	3.0 g
Soluble starch	:	1.0 g
L cysteine.HCl.H_2O	:	0.5 g

pH 6.8 ± 0.2 at 25°C.

The components were added to distilled water and the volume was made up to 1000 ml., Mixed thoroughly and dissolved by gentle heating. Distributed in tubes or flasks and autoclaved for 15 min at 15 psi and 121°C.

After bringing down the temperature of sterilized reinforced clostridial agar to 45-50°C, 1 percent (w/v) filter sterilized solutions of nalidixic acid, polymyxin B sulfate, iodoacetic acid and 2,3,5-triphenyl tetrazolium chloride and kanamycin sulfate were added to attain concentrations of 20, 8.5, 25, 25 and 50 ppm, respectively. The final pH of BIM-25 was 6.8 ± 0.2 (Munoa and Pares, 1988). On BIM-25 agar *Bifidobacterium* spp. forms convex colonies with a white edge and purple center.

3.8.4 Direct Microscopic Count (DMC)

Appropriate dilutions of faecal samples (0.01 ml) were spread over a known area (1 sq.cm) on a glass slide (Breed's slide). The smear was stained and examined under microscope. The number of cells in a certain number of microscopic fields were counted and the average number per field was calculated. The average number of cells per field was multiplied by the microscopic factor and the corresponding dilution to get the final DMC count/g faeces.

3.8.5 Determination of β-Glucuronidase Activity

The release of p-nitrophenol from p-nitrophenyl-β-D-glucuronide was used as the indicator of faecal β-glucuronidase activity. The method followed was that of Marteau *et al.* (1990). Fresh faecal samples were suspended in 10 ml buffer (0.1 mol Na_2HPO_4/L, 0.15 mol NaCl/L, pH 7.4) and sonicated in an ice bath. This solution was then filtered through Whatman No.1 filter paper and the filtrate was used for β-glucuronidase and protein determination (Section 3.8.5.1). Two ml of the appropriately diluted sample was then combined with 1 ml of a solution (0.13 mol/L) of the substrate p-nitrophenyl β-D-glucuronide prepared in the above mentioned buffer. The reaction, which proceeded at 37°C, was stopped after 15 min by adding 1 ml of cold 1 M

Na_2CO_3/L solution. After centrifugation for 10 min at 4000 Xg and 4°C, the absorbance of the supernatant was read at 405 nm. The blank was prepared in the same way as the sample except that it contained 1 ml of buffer instead of the substrate solution. The amount of p-nitrophenol released was determined by comparing with a standard p-nitrophenol curve and expressed as specific activity (μmol/mg protein per 30 min).

3.8.5.1 Protein determination

Protein concentration of faecal sample was determined by slightly modified Lowry's method (1951) as below :

3.8.5.1.1 Reagents :

1) **Lowry's Reagent**

Reagent A : Copper sulphate solution

One gram of anhydrous copper sulphate crystals were dissolved in 100 ml of double distilled water.

Reagent B : Sodium-potassium tartrate solution

Two grams of sodium-potassium tartrate was dissolved in 100 ml of distilled water.

Reagent C : NaOH (0.2 M) solution

Sodium hydroxide (0.8 g) was dissolved in 100 ml of distilled water.

Reagent D : Sodium carbonate solution

Four grams of sodium carbonate was dissolved in 100 ml of distilled water.

Reagent E : Lowry's reagent (Copper alkali solution)

For preparing this 49 ml of reagent D was added to 49 ml reagent C. To this, was added 1 ml of reagent A followed by 1 ml of reagent B. This is copper alkali solution, which must be prepared fresh when needed.

2) **Follin's Coicalteau Reagent (FC Reagent)**

Follin's coicalteau reagent was diluted 1:1 with double distilled water just before use.

3) **Standard Protein Solution**

Bovine serum albumin (BSA) solution with a concentration of 1 mg/ml in double distilled water.

3.8.5.1.2 Estimation of protein concentration of faecal samples :

Protein concentration of the faecal samples, which were analyzed for β-glucuronidase activity was determined as follows:

To 0.2 ml of appropriately diluted faecal samples, 0.3 ml of distilled water was added. To this, 2.5 ml of Lowry's reagent was added and incubated for 10 min at room temperature. This was followed by the addition of 0.25 ml FC reagent and kept at room temperature, for 30 min, away from direct light. The intensity of colour developed was measured for their absorbance value at 660 nm, with a blank containing distilled water instead of faecal samples. From the corresponding absorbance on standard curve of bovine serum albumin (BSA), the relative protein concentration of samples was calculated.

WORK DONE IN GERMANY UNDER DAAD FELLOWSHIP

3.9 EFFECT OF PROBIOTICS ON HUMAN MONOCYTE DERIVED IMMATURE DENDRITIC CELLS (DCs)

3.9.1 Bacterial Cultures

Two probiotic cultures, *Bifidobacterium* species 420 (B-420; Danisco, Germany) and *Lactobacillus rhamnosus* GG (LGG, Valio Ltd., Finland) were used in this study. Lactobacillus culture was grown aerobically in deMan, Rogosa, Sharpe broth (MRS broth, Merck) and bifidobacterial culture in MRS broth supplemented with 0.05 percent L-cysteine hydrochloride monohydrate (Merck), anaerobically (using Anaerogen, oxoid products) at 37°C for 24 h. Bacteria were collected by centrifugation at 12000 rpm for 10 min at 4°C,

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washed two times in phosphate buffered saline (PBS; Gibco, pH 7.4), resuspended in the cell culture medium and stored at –80°C until use. Viability of cultures was ensured by plating them on their respective growth media.

3.9.2 Generation of Immature DCs *In Vitro*

Peripheral blood mononuclear cells (PBMCs) were isolated by density-gradient centrifugation (Ficoll-Paque PLUS, Amersham Biosciences AB; Uppasala, Sweden), from sodium-heparinized blood obtained from healthy adult volunteers. Monocytes were purified by positive selection of $CD14^+$ cells, using magnetic beads (Miltenyi Biotec, Germany), according to the manufacturer's instruction. Immature DCs were generated by culturing of monocytes, in VLE RPMI 1640 medium (Biochrom AG), supplemented with human blood serum (1%) in the presence of Granulocyte-macrophage colony stimulating factor (GM-CSF; 800 IU/ml; Leucomax®, Essex-Pharma, München, Germany) and interleukin (IL)-4 (800 IU/ml; Promocell), in 6-well plates (TPP), at 37°C in 5 percent CO_2. On 6th day, non-adherent immature DCs were harvested and used for stimulation experiments (Romani *et al.*, 1996).

3.9.3 Induction of Surface Markers and Cytokine Release

Immature dendritic cells developed as mentioned above were washed out of cytokine containing media on the 6th day of incubation, counted and placed in 6 well plates at the rate of 6×10^5 cells per well in a total volume of 1.5 ml culture medium supplemented with 500 IU/ml GM-CSF. Medium containing bacteria were added to this culture to get a DC : Bacteria ratio of 1:5 and 1:50. A cytokine cocktail consisting of tumor necrosis factor-α (TNF-α; 250 IU/ml, Promo Kine, Germany), interleukin (IL)-1β (20 ng/ml, Promocell), and prostaglandin E_2 (PGE_2; 1 micro mol/ml, Sigma) was used for developing mature dendritic cells to serve as the positive control. Immature dendritic cells cultivated in the same way without any stimulants served as the unstimulated (negative) control. After a stimulation period of 48 h, dendritic cells were harvested for flow cytometric analysis. Culture supernatants were collected and stored at –20°C until cytokine analysis.

3.9.3.1 Immunocytostaining and flow cytometry

The DCs were suspended in FACS buffer (PBS + 1% FCS), incubated at 6 to 12°C with combinations of fluorescence conjugated antibodies (Becton Dickinson) for 30 min, washed with FACS buffer, and resuspended in 300 µl of FACS buffer. The cells were analyzed for the surface expression of HLA-DR, CD80, CD83, CD86 and CD25, with a FACS caliber flow cytometer (Becton Dickinson) using CELLQUEST (Becton Dickinson) software. The data are expressed as percent of cells expressing the particular marker.

3.9.3.2 Cytokine quantification in culture supernatants

Interleukin (IL)-8, Interleukin (IL)-1β, interleukin (IL)-6, interleukin (IL)-10, tumor necrosis factor-α (TNFα), and interleukin (IL)-12p70 in the culture supernatants were analyzed using commercially available cytometric bead array (Human inflammation kit, Becton Dickinson) according to manufacturer's instructions (Chen *et al.*, 1999).

3.10 STATISTICAL ANALYSIS

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 and Discussion

4. RESULTS AND DISCUSSION

4.1 CONFIRMATION OF CULTURE IDENTITY AND PURITY

Bifidobacteria are non-motile, Gram-positive rods often occurring as bifurcated Y-forms. They are non-sporulating organisms and catalase negative (Rasic and Kurmann, 1983). The most direct, reliable and most important fruitful assignment of a bacterial strain to the genus *Bifidobacterium* is the one based upon demonstration in cellular extracts of fructose-6-phosphate phosphoketolase (F6PPK), the key enzyme of bifidobacterial hexose metabolism (Scardovi, 1986). The purity of bifidobacterial cultures was confirmed through Gram's reaction and catalase reaction. The F6PPK test was done for ensuring the authenticity of cultures as members of *Bifidobacterium* genus (Table 4.1).

4.1.1 Microscopic Examination : Morphology and Gram's Reaction

All the *Bifidobacterium* cultures were Gram-positive rods or cocci. The cultures *B. lactis* Bb-12, *Bifidobacterium* species 420 and Bifidobacteria (ABT-5) occurred in typical Y shape and as rods with bulbed edges (Plate 4.1). This is in agreement with the description of characteristics of *Bifidobacterium* genus in Bergey's Manual of systematic bacteriology. Genus *Bifidobacterium* occur as rods of varied shapes, usually somewhat curved and clubbed and are often branched. Arranged singly, in pairs, in V arrangements, sometimes in chains, in palisades of parallel cells or in rosettes. Occasionally, exhibit swollen coccoid forms (Scardovi, 1986).

4.1.2 Catalase Test

Catalase is an enzyme produced by many microorganisms as well as other organisms, including man (present in saliva). Since it is present in many microorganisms, its absence is rare enough to make it a significant diagnostic characteristic. The enzyme if present catalyses the breakdown of hydrogen peroxide to produce oxygen and water, and does so vigorously enough to

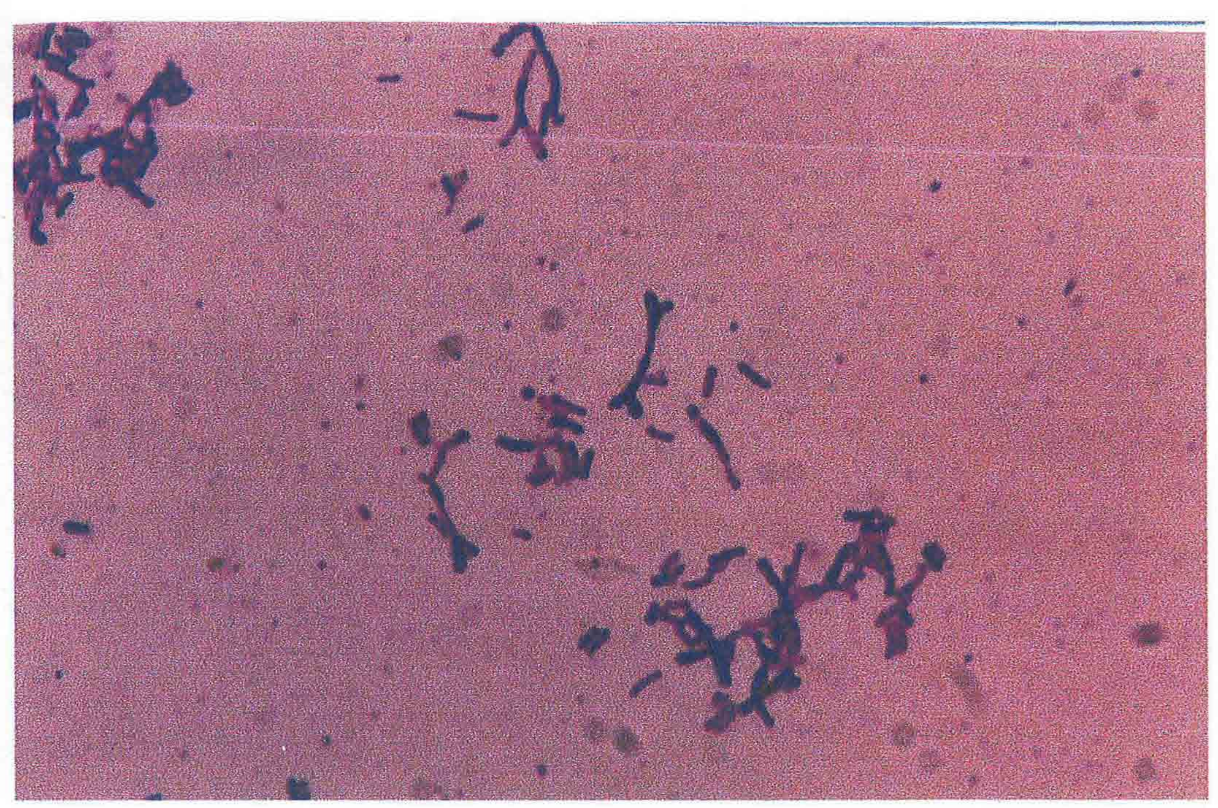


Plate 4.1 Typical Y shaped morphology of *Bifidobacterium* culture

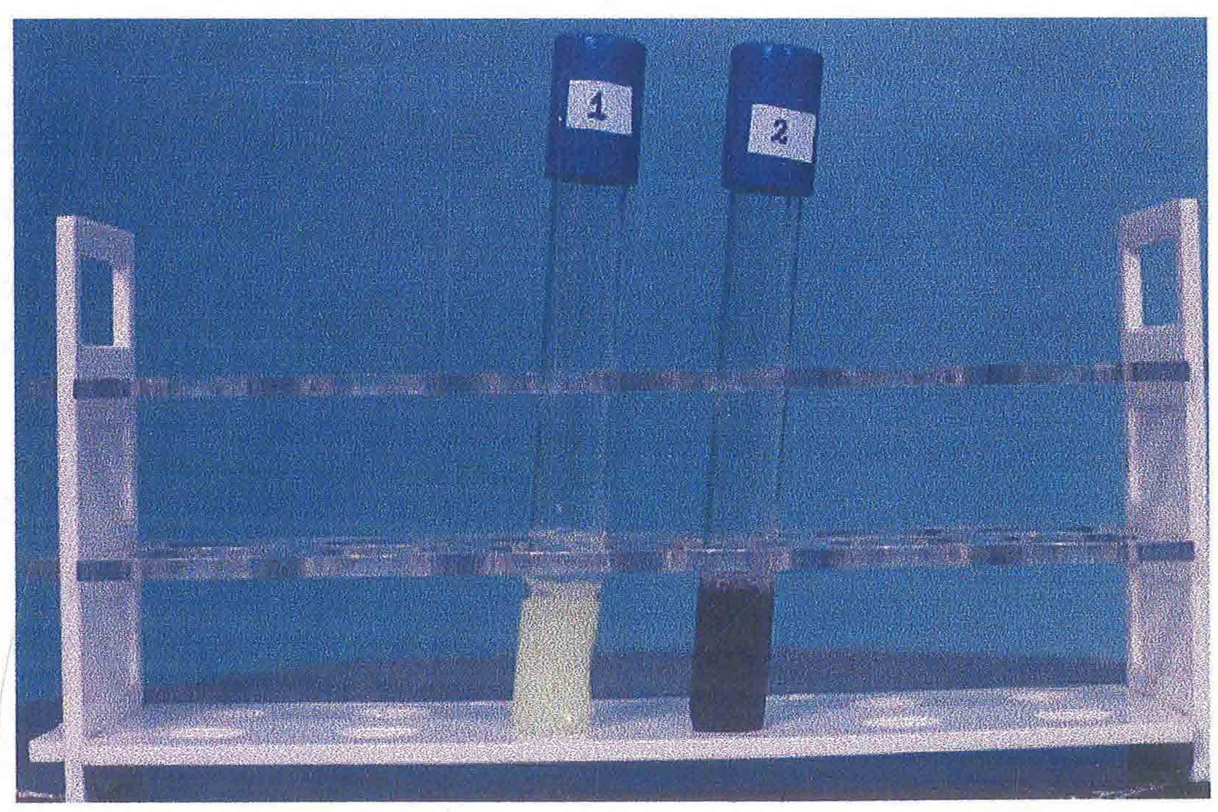


Plate 4.2 Fructose 6 phosphate Phosphoketolase Test
(1 - Negative and 2 - Positive)

Table 4.1 Culture purity and confirmation tests.

Culture Name	Microscopy	Catalase	F6PPK
Bifidobacteria (ABT-5)	Gram +ve branched rods	-ve	+ve
B. lactis Bb-12	Gram +ve branched rods	-ve	+ve
Bifidobacterium species 420	Gram +ve branched rods	-ve	+ve
B. bifidum NCDC255	Gram +ve rods	-ve	-ve
B. adolescentis	Gram +ve rods	-ve	-ve
B. bifidum NCDC229A	Gram +ve rods	-ve	-ve
B. bifidum VP	Gram +ve cocci	-ve	-ve
B. bifidum 1047	Gram +ve cocci	-ve	-ve
B. bifidum A3	Gram +ve cocci	-ve	-ve
B. bifidum NCDC255 (GM strain)	Gram +ve cocci	-ve	-ve

pathogens, lysozyme resistance, etc. Therefore, the three *Bifidobacterium* cultures were subjected to following tests in order to select the organism for further studies.

4.2.1 *In vitro* Tolerance to Simulated pH of the Human Stomach

Different regions of the gastrointestinal tract have varying acid levels. Stomach and the regions immediately following stomach have the highest acidity and the pH of these areas may fall to as low as 1.5. Therefore, it is a must that a probiotic should survive under these acidic conditions to reach their site of action, i.e., the colon to elicit beneficial effects expected from them.

Figure 4.1 illustrates the acid tolerance pattern of Bifidobacteria (ABT-5). It is evident from this depiction that Bifidobacteria (ABT-5) was resistant to pH 2.5, showing somewhat similar trend in that of control (pH 6.5). At pH 2.0 also, this organism could survive well, showing only a slight decrease in its viability, i.e., this organism did not show any significant difference in its viability at pH 2.0, 2.5 and 6.5 ($P<0.05$). However, at pH 1.5, the number of viable cells reduced by about 1.6 log cycles (from 8.70 to 7.03) after 1 h of exposure and decreased further by 3 log cycles in the next hours, and then to essentially nil after 3 h. The pH 1.5 had a significant inhibitory effect on this organism compared to other pH levels ($P<0.05$).

As expected *B. lactis* Bb-12 (Bb-12), the most thoroughly studied probiotic *Bifidobacterium* strain currently on the market, exhibited very high tolerance at pH 2.0 and 2.5 without any reduction in their cell numbers even after 3 h of incubation at these pH levels. At pH 1.5 also Bb-12 showed comparatively better resistance showing no reduction in cell numbers in the first hour, and 4 log reduction (from 8.18 to 4.30) after 2 h. But Bb-12 also could not withstand this low pH further falling down to zero after 3 h of incubation at pH 1.5 (Fig. 4.2). However, the different pH levels used in this study did not show any significant difference in their effect towards the viability of this organism ($P<0.05$)

Fig. 4.1 Survival of Bifidobacteria (ABT-5) during three hours in hydrochloric acid solutions (pH 1.5, 2.0, 2.5 and 6.5).

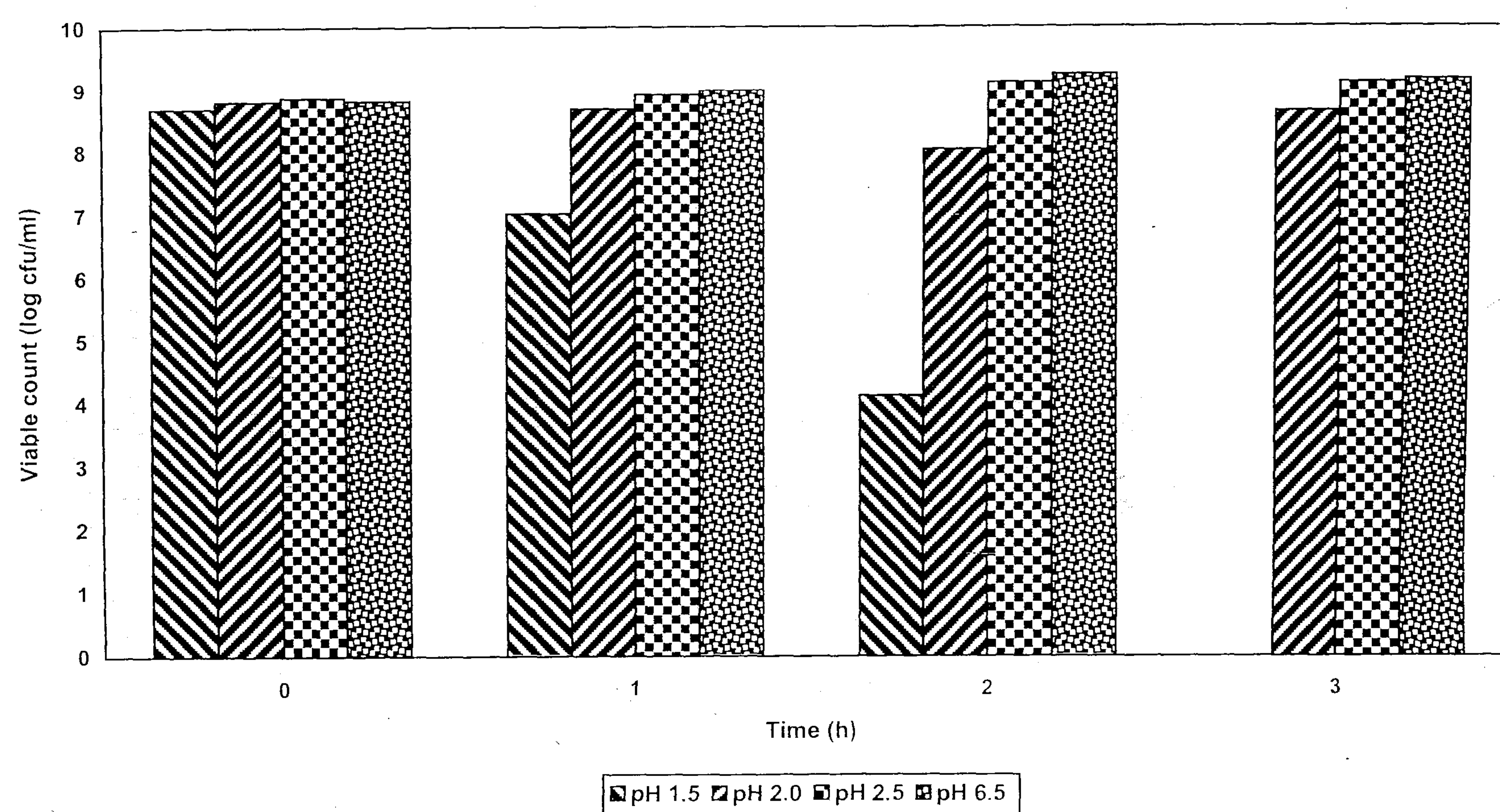
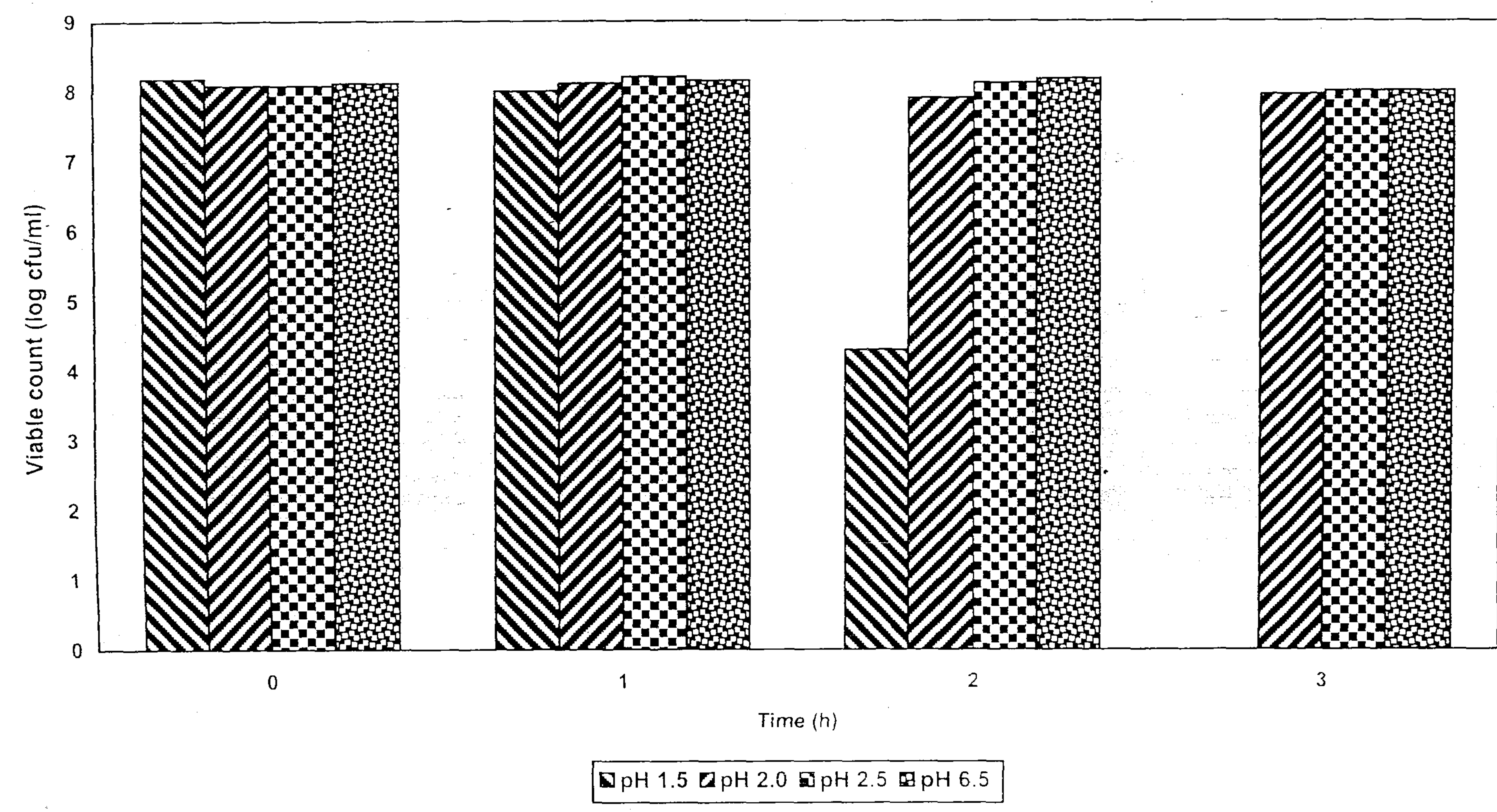


Fig. 4.2 Survival of B. *lactis* Bb-12 during three hours in hydrochloric acid solutions (pH 1.5, 2.0, 2.5 and 6.5).

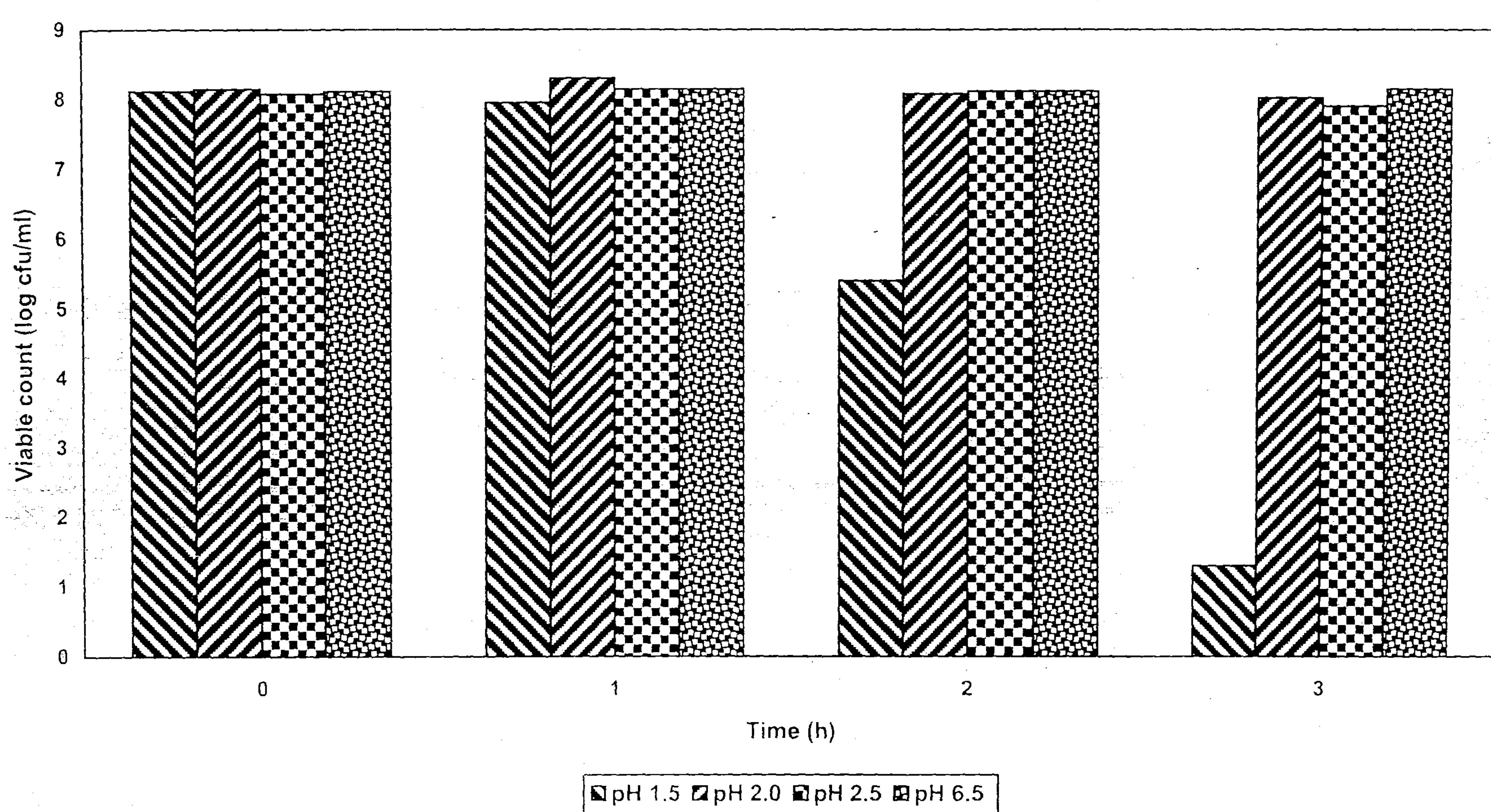


For *Bifidobacterium* species 420 (B-420), results were much the same as for *B. lactis* Bb-12 (Fig.4.3). No decrease in viable numbers occurred at pH 2.0, 2.5 or in the control (pH 6.5) during three hours of incubation. At pH 1.5, the viable number did not show any reduction during the first hour, dropped by 2.7 log cycles (from 8.1 to 5.4) and 6.8 log cycles (from 8.1 to 1.30) after two and three hours at this pH. However, no significant difference was observed in the viability of B-420 at different pH levels used in this study ($P<0.05$).

In the distilled water control (pH 6.5), there was essentially no change in numbers of any of the tested culture during 3 h of incubation. Obviously, all the tested cultures could survive well at pH 2.0 and 2.5, but at pH 1.5, drastic reduction in the viable numbers was observed. No significant difference was observed between the cultures in terms of their pH tolerance ($P<0.05$). The viability of the cultures at pH 1.5 was significantly lower than their viability at other pH levels used in this study. However, no significant difference was observed in the viability of cultures at pH 2.0, 2.5 and 6.5.

Several other studies (Berrada *et al.*, 1991; Martin and Chou, 1992; Clark *et al.*, 1993; Lankaputhra and Shah, 1995) have reported a variation in pH tolerance by different members of the *Bifidobacterium* species. However, we could not observe any significant difference between the cultures used in the study with respect to pH tolerance. This is quite reasonable as these are commercially available cultures, which might have gone through stringent selection procedures before their introduction in the market. In our study, pH 1.5 was observed as the most lethal among all the pH levels used. The results are in accordance with several studies, which reported a substantial reduction in the viability of cells at pH 2.0 or below (Pochart *et al.*, 1992; Clark *et al.*, 1993; Lankaputhra and Shah, 1995). The comparable pH tolerance of *Bifidobacterium* species 420 and *B. lactis* Bb-12 observed by us is in agreement with that of Alander *et al.* (2001). Results obtained in the present study are in accordance with the report of Shah (2001) suggesting better survivability of *B. animalis* (reclassified as *Bifidobacterium lactis*) an animal strain, compared to other strains under harsh conditions.

Fig. 4.3 Survival of *Bifidobacterium* species 420 during three hours in hydrochloric acid solutions (pH 1.5, 2.0, 2.5 and 6.5).



4.2.2 *In vitro* Tolerance to Simulated Bile Concentration of the Human Small Intestine

Gastrointestinal systems have varying concentration of bile which play an important function in upper GIT for digestion of food. The bile acids cause emulsification of large fat particles into simple particles, which can be acted upon by lipases and aid in transportation and absorption of digested fat end products to and through intestinal mucosal membrane. The rate of secretion of bile and the concentration of bile in different regions of the intestine can range between 0.5 to 2.0 percent during the first hour of digestion, the levels may decrease during the second hour. A period of twelve hours has been reported as sufficient time for most foods to pass through the small intestine (Clark and Martin, 1994). Keeping in view these observations, the bifidobacterial cultures used in the present study were examined for their tolerance to 2.0, 2.5 and 3.0 percent bile salts up to 12 h of exposure at 37°C.

Figure 4.4 shows the survival of the Bifidobacteria (ABT-5) in different bile salts concentration of 2.0, 2.5 and 3.0 percent. At bile salts concentration of 2.0, 2.5 and 3.0 percent, viability of Bifidobacteria (ABT-5) decreased drastically (about 3.5 log reduction compared to the control) by the time, it took for determining the initial numbers. After the initial drastic reduction in the numbers, the reduction was not that much prominent on further exposure. It might be due to the fact that the organism, after surviving an initial exposure might have got adapted to high bile salts concentration. Compared to the control a significant reduction in the viability of Bifidobacteria (ABT-5) was observed on exposure to bile ($P<0.05$). However, there was not any significant difference in its viability at different bile salts concentration.

B. lactis Bb-12 survived very well even at the highest bile concentration tested, i.e., 3.0 percent (Fig. 4.5). There was a slight reduction in the viability of Bb-12 after 3 h of exposure to bile, and a further reduction on 12 h of incubation. This much reduction appears to be quite reasonable at this high bile concentration. There was not any significant difference in the count of Bb-12 at different bile concentrations, indicating that this organism could survive very well in bile in the small intestine, where the presence of bile is an important constraint a probiotic has to get through.

Fig. 4.4 Survival of Bifidobacteria (ABT-5) at various bile concentrations (2.0, 2.5 and 3.0%) during 12 h incubation at 37°C.

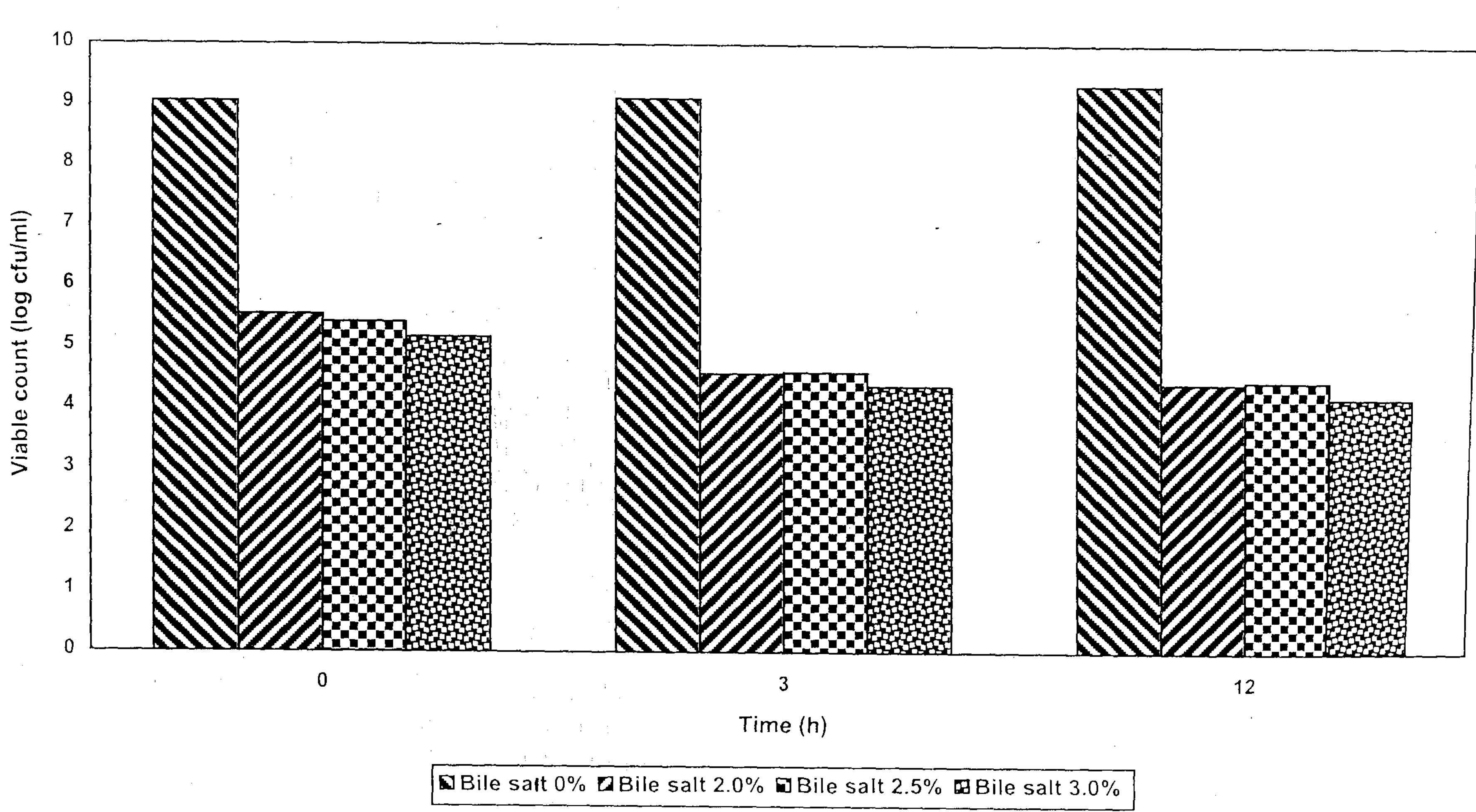
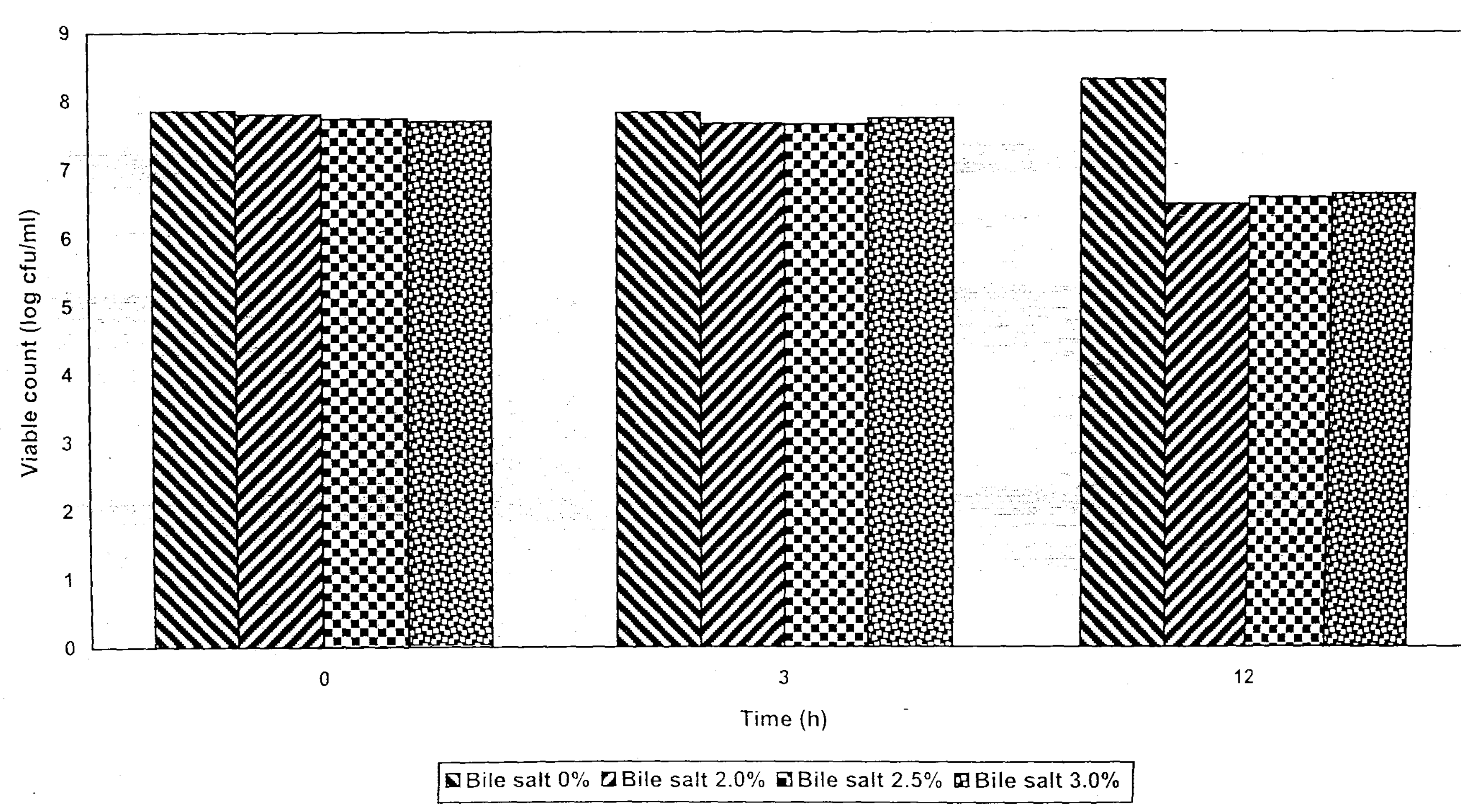


Fig. 4.5 Survival of *B. lactis* Bb-12 at various bile concentrations (2.0, 2.5 and 3.0%) during 12 h incubation at 37°C.



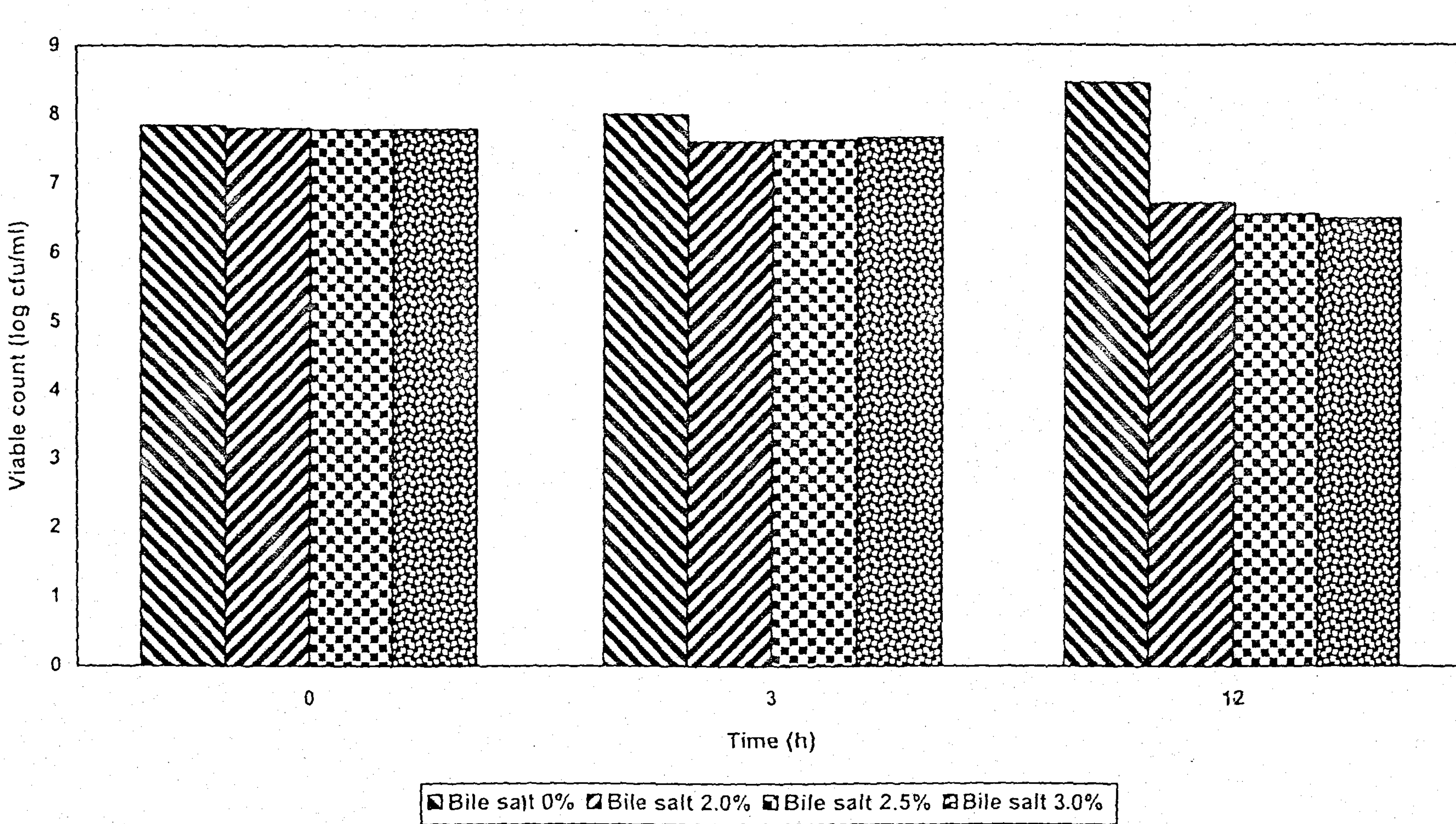
In the case of *Bifidobacterium* species 420, somewhat similar trend of that of *Bifidobacterium lactis* Bb-12 was observed. B-420 also exhibited high survivability at high bile concentration even after 12 h of exposure (Fig. 4.6), i.e., only 1 log reduction (from 7.70 to 6.63). This organism also did not show any significant difference in their viability on exposure to bile, i.e., no significant difference in its viability in control as well as at 3.0 percent bile ($P<0.05$).

Other groups of workers have reported widely varying results about the bile tolerance of *Bifidobacterium* species. Ibrahim and Bezkorovainy (1993b) found *B. infantis* to be most tolerant, followed by *B. bifidum*, *B. breve* and *B. longum*. Clark and Martin (1994) observed better tolerance by *B. longum* (at 2 and 4% bile concentrations for 12 h) compared to *B. infantis*, *B. adolescentis* and *B. bifidum*. Similar to these studies, we also observed variation among *Bifidobacterium* cultures in their bile tolerance. Significant difference in the bile tolerance was observed between Bifidobacteria (ABT-5) and the other two cultures Bb-12 and B-420; whereas no significant difference was observed between Bb-12 and B-420 in terms of bile tolerance. This is in accordance with the results of Alander *et al.* (2001) who observed comparable bile tolerance by *B. lactis* Bb-12 and *Bifidobacterium* species 420. Bifidobacteria (ABT-5) though tolerant to pH, possessed poor tolerance to bile. In contrast, *B. lactis* Bb-12 and *Bifidobacterium* species 420 exhibited good tolerance to low pH and bile salts as well, and can be expected to survive harsh conditions of the upper digestive tract in appreciably large numbers.

4.2.3 Lysozyme Tolerance

Lysozyme is a protein that leads to the osmotic lysis of bacteria by cleaving the glycosidic bonds of the bacterial cell wall polymer of N-acetyl glucosamine and N-acetylmuramic acid. It is a part of various normal body secretions, such as tears and saliva, and body fluids presumably functioning as a major line of defense against infection by bacteria. The lysozyme content in saliva varies from 10 to 200 µg/ml and in the gastric juice from 43 to 106 µg/ml. Therefore, their ability to survive at these lysozyme concentrations can

Fig. 4.6 Survival of *Bifidobacterium* species 420 at various bile concentrations (2.0, 2.5 and 3.0%) during 12 h incubation at 37°C.



be an additional parameter for selecting probiotic microorganisms, thereby ensuring the better survivability of the selected organism under normal physiological situations.

All the *Bifidobacterium* cultures used in this study, could tolerate and even grow in the presence of lysozyme (100 ppm; Table 4.2). No significant difference was observed in their growth in mMRS broth with or without lysozyme (P<0.05). Similar results were reported by other researchers also. Loh and Maznah (1999) observed a growth promoting effect of lysozyme (1 mg/ml) on *Bifidobacterium infantis*. Tolerance of *Bifidobacterium* cultures isolated from infant faeces to a lysozyme concentration of 0.5 mg/ml was reported by Kheadr *et al.* (2002). Kushal (2001) observed high tolerance of *Bifidobacterium* cultures, *B. bifidum* NCDC-255, *B. bifidum* NIRD and *B. bifidum* 1047 to lysozyme concentration of 100 ppm.

4.2.4 Cell Surface Hydrophobicity

Attachment of a strain to the intestinal mucosa can be one of the significant selection criteria for probiotic microorganisms. Traditionally, one reason for this is that adhesion is generally considered to be a complex mechanism and a pre-requisite for bacterial colonization. The hydrophobicity of the cell surface and zeta potentials account for the attractive and repulsive forces, respectively, that takes place in auto-aggregation and adhesion of bacteria to different surfaces. Therefore, the measurement of cell surface hydrophobicity can be considered as an indicator of the ability of cells to adhere to epithelial cells.

Bifidobacterium cultures were evaluated for their cell surface hydrophobicity on the basis of their ability to adhere to the hydrocarbon, n-hexadecane. Wide variations were observed in their cell surface hydrophobicity ranging from 11.85 to 94.51 percent (Table 4.3). *Bifidobacterium* cultures Bb-12 and B-420 exhibited significantly high cell surface hydrophobicity than Bifidobacteria (ABT-5; P<0.05). As the hydrophobicity appears to be necessary for adhesion to epithelial cells (Perez

Table 4.2 Resistance of *Bifidobacterium* cultures to lysozyme.

Culture	Viable Counts (Log_{10} cfu/ml)			
	mMRS Broth		mMRS Broth + Lysozyme	
	0 h	24 h	0 h	24 h
Bifidobacteria (ABT-5)	7.38	9.05	7.36	9.33
B. lactis Bb-12	7.30	9.10	7.37	9.14
Bifidobacterium species 420	7.26	8.81	7.24	8.60

Table 4.3 Percent hydrophobicity of *Bifidobacterium* cultures to n-hexadecane.

Culture Name	Percent Hydrophobicity
Bifidobacteria (ABT-5)[a]	11.85
B. lactis Bb-12[b]	88.77
Bifidobacterium species 420[b]	94.51

ab – *Bifidobacterium* cultures bearing different superscripts differ significantly ($P<0.05$)

et al., 1998), the cultures exhibiting high cell surface hydrophobicity can be better performers in terms of their ability to adhere to intestinal epithelial cells, a feature contributing towards better possibilities of colonization of this organism thereby enhancing their beneficial effects such as competitive exclusion of pathogenic organisms. The results obtained in the present study are in agreement with that of Chand Ram (1997) who observed wide variations among human bifidobacterial isolates in their cell surface hydrophobicity and adhesion index. Gopal *et al.* (2001) observed exhibition of good adhesion properties by *Bifidobacterium lactis* HT-29, which was comparable to that of two commercial probiotic cultures. They also reported the inhibitory effect of this strain against the intestinal cell monolayer colonization by an enterotoxigenic strain of *Escherichia coli*.

4.2.5 Inhibition of Enteric Organisms

With the emergence of antibiotic resistant bacteria and natural ways of suppressing pathogens, the concept of probiotics has attracted much attention. Therefore, their antagonistic activity against pathogens is an important criterion for the selection of potential probiotics. Bifidobacteria are natural components of human intestinal microbial flora, and these fermentative organisms produce acetic and lactic acids, which tend to lower the intestinal pH and this inhibit the proliferation of pathogenic microorganisms. The antimicrobial activity exhibited by different *Bifidobacterium* cultures used in this study against common enteric organisms was determined by well diffusion method in terms of zone of inhibition (Table 4.4). All the tested *Bifidobacterium* cultures were found to be effective against *Shigella dysenteriae* NCDC107, *Staphylococcus aureus* NCDC109, *Salmonella typhimurium* NCDC113 and *Escherichia coli* NCDC247. None of the cultures used in this study showed inhibitory activity against *Enterococcus faecalis* NCDC116. No significant differences were observed in the antibacterial activity exhibited by the *Bifidobacterium* cultures used in the present work ($P<0.05$). Moreover, the inhibitory effect was more or less similar against all the indicator organisms.

Table 4.4 Antimicrobial activity of *Bifidobacterium* cultures against enteric organisms (inclusive of well diameter 7 mm).

Culture Name	**Zone of Inhibition (diameter, mm)**			
	E. coli **NCDC247**	***S. typhimurium*** **NCDC113**	***S. dysenteriae*** **NCDC107**	***S. aureus*** **NCDC109**
Bifidobacteria (ABT-5)	14.75	17.75	15.00	18.50
B. lactis Bb-12	22.00	16.50	18.50	16.50
Bifidobacterium species 420	21.50	18.25	18.50	18.00

Earlier studies have also reported antimicrobial activity of bifidobacteria against *Salmonella*, *Shigella*, *Staphylococcus aureus*, *Escherichia coli*, etc. (Anand *et al.*, 1984; Tojo *et al.*, 1987; Tomoda *et al.*, 1988; Ibrahim and Bezkorovainy, 1993a). Chand Ram (1997) studied the antibacterial activity of various human bifidobacterial isolates against *E. coli* NCDC134, *B. cereus* NCDC66, *S. typhi* NCDC113, *S. dysenteriae* NCDC107 and *S. aureus* NCDC110, and observed variation in inhibitory spectrum of various *Bifidobacterium* strains against these microorganisms. Similar results were obtained by Kushal (2001). Results obtained in the present study are in agreement with that of Sabikhi (1999) who observed antimicrobial activity by all tested *Bifidobacterium* cultures against *E. coli* NCDC135, *S. typhi* NCDC113, *S. aureus* NCDC109, *S. dysenteriae* NCDC107 and *B. cereus* NCDC66. Antibacterial action of bifidobacteria can be ascribed to different attributes, such as production of acids, bacteriocins, etc. (Ibrahim and Bezkorovainy, 1993a; Gibson and Wang, 1994b; Fujiwara *et al.*, 1997). As the *Bifidobacterium* cultures tested in the present investigation exhibited similar inhibitory effect against all the indicator organisms, this property could not be considered as a criteria for the selection of *Bifidobacterium* culture for the preparation of synbiotic powder.

4.3 EFFECT OF INULIN (PREBIOTIC) ON THE GROWTH OF BIFIDOBACTERIA

On comparison with lactobacilli, bifidobacteria are much more susceptible to heat and oxygen, and this limits its application in foods, as probiotics. Therefore, the incorporation of bifidogenic factors in foods can be an alternative approach to increase the number of beneficial organisms of the human large intestine. Inulin is one of the most studied and well-established prebiotics. The effects of inulin on the human gut flora has been extensively studied *in vivo* and *in vitro*, and the majority of studies reported selective fermentation by the beneficial flora, namely, bifidobacteria and to a lesser extent lactobacilli.

The ability of the *Bifidobacterium* cultures Bifidobacteria (ABT-5), *B. lactis* Bb-12, and *Bifidobacterium* species 420 to utilize, inulin was determined by monitoring their growth in a semi-liquid minimal media (Table 4.5).

Since the mean generation time of Bifidobacteria (ABT-5), *B. lactis* Bb-12 and *Bifidobacterium* species 420 was decreased on inulin supplementation, it can be inferred that all of them could utilize inulin. However, wide variations were observed between cultures in their ability to utilize inulin. The growth stimulatory effect of inulin was most prominent in the case of *Bifidobacterium* species 420 as a marked reduction in its mean generation time was observed compared to other two cultures. Inulin supplementation had a significantly lower impact on the growth of Bifidobacteria (ABT-5) compared to Bb-12 and B-420 ($P<0.05$). Among different concentrations of inulin used, supplementation at the rate of 0.5 percent showed a significantly lower growth stimulatory effect compared to other concentrations. However, no significant difference was observed among other inulin concentrations (1, 3 and 5%) in terms of growth stimulatory effect ($P<0.05$).

The ability of bifidobacterial cultures to ferment inulin had been reported by other researchers also. Dubey and Mistry (1996) could not observe any difference in the maximal counts and generation times of *Bifidobacterium breve*, *Bifidobacterium infantis* and *B. longum* on growing them in infant formulae supplemented with fructooligosaccharides (0.5%). Kaplan and Hutkins (2000) observed that seven out of eight *Bifidobacterium* were able to ferment inulin and oligofructose on MRS agar. Bielecka *et al.* (2002) studied the influence of fructan type oligosaccharides (as prebiotics) on growth of *Bifidobacterium* strains. *In vitro* studies showed that the majority of *Bifidobacterium* species utilized fructo-oligosaccharides and low polymerized inulins, but only 18 out of 30 strains tested (mostly of *B. longum* and *B. animalis* species) were stimulated. To sum-up, all the bifidobacterial cultures used in the present study could use inulin as the sole carbon source, suggesting better colonization chances of these organism on consuming them together with inulin (as a synbiotic), as this prebiotic will serve as a ready source of energy for these organisms in the colon.

Table 4.5 **Utilization of different concentrations of inulin (mean generation time, hours).**

Culture Name	**Inulin Concentration**				
	0%	**0.5%**	**1.0%**	**3.0%**	**5.0%**
Bifidobacteria (ABT-5)[a]	12.99	10.90 (16.00%)	6.90 (46.88%)	7.10 (45.34%)	7.02 (45.96%)
B. lactis Bb-12[b]	46.30	26.10 (43.63%)	19.40 (58.10%)	18.18 (60.73%)	19.20 (58.53%)
Bifidobacterium species 420[c]	58.80	20.50 (65.11%)	19.80 (66.32%)	11.40 (80.61%)	12.12 (79.39%)

abc – *Bifidobacterium* cultures bearing different superscripts differ significantly ($P<0.05$).

Values in parenthesis are percent reduction in mean generation time compared to control (0% inulin).

4.4 SELECTION OF BIFIDOBACTERIAL CULTURE FOR PREPARING THE SYNBIOTIC PRODUCT

The results obtained for different probiotic attributes of *Bifidobacterium* cultures are summarized in Table 4.6. No significant difference was observed between cultures in their pH tolerance as well as antimicrobial activity ($P<0.05$). However, in case of bile tolerance and cell surface hydrophobicity, the Bifidobacteria isolated from ABT-5 turned out to be a poor performer compared to the other two cultures, *B. lactis* Bb-12 (Bb-12) and *Bifidobacterium* species 420 (B-420). Although, the growth rate of the culture B-420 was significantly lower than other two cultures, there was not any significant difference in its growth pattern in media with and without lysozyme ($P<0.05$). Thus, it can be concluded that all the three cultures behaved in a similar pattern in the presence as well as in the absence of lysozyme in their growth medium. While comparing the inulin utilizing ability of *Bifidobacterium* cultures, it was observed that all of them could utilize inulin. However, in terms of percentage reduction in mean generation time on inulin supplementation, the growth stimulatory effect of inulin was most prominent in the case of B-420.

Table 4.6 Selection of *Bifidobacterium* culture for incorporation into the test product.

Selection Criteria	Bifidobacteria (ABT-5)	*B. lactis* Bb-12	*Bifidobacterium* species 420
pH tolerance	$++^{a}$	$++^{a}$	$++^{a}$
Bile salt tolerance	$+^{b}$	$++^{a}$	$++^{a}$
Lysozyme tolerance	$++^{a}$	$++^{a}$	$+^{b}$
Surface hydrophobicity	$+^{b}$	$++^{a}$	$++^{a}$
Antimicrobial activity	$++^{a}$	$++^{a}$	$++^{a}$
Growth stimulatory effect of inulin	$+^{c}$	$++^{b}$	$+++^{a}$

abc – symbols in the same row with different superscripts differ significantly ($P<0.05$; +++ > ++ > +).

On overall comparison of results obtained in different tests conducted for evaluating the probiotic attributes of *Bifidobacterium* cultures, it can be

concluded that the performance of Bb-12 and B-420 was better than that of Bifidobacteria (ABT-5). However, no significant difference was observed between Bb-12 and B-420 in any of the tested parameters, except in the case of growth stimulatory effect of inulin. Based on all these results, the culture *Bifidobacterium* species 420 was selected for the formulation of product as it showed comparatively better performance than Bb-12 in terms of cell surface hydrophobicity, pH tolerance and inulin utilization.

4.5 ANTIBACTERIAL ACTIVITY OF THE SELECTED BIFIDOBACTERIAL CULTURE IN THE PRESENCE OF INULIN

In order to test whether the incorporation of inulin into the growth media has any enhancing effect on the antibacterial activity of the selected culture, a comparison was made between the antibacterial activity exhibited by the culture grown in mMRS containing either dextrose or inulin as the sole carbon source. *Bifidobacterium* species 420 grown either in inulin containing media or dextrose containing media exhibited inhibitory effect against all the tested enteric organisms except *E. faecalis*. The antibacterial activity exhibited by the organism grown in inulin added media was significantly lower than that of those grown in dextrose containing media ($P<0.05$; Table 4.7). This difference may be due to the comparatively faster utilization of the monosaccharide (dextrose) by the organism than that of an oligosaccharide (inulin).

Table 4.7 Antimicrobial activity of the selected *Bifidobacterium* culture (B-420) against enteric organisms in the presence of inulin (zone of inhibition, diameter, mm, inclusive of well diameter 7 mm).

Enteric Organisms	Growth Media	
	mMRS Containing Dextrose[a]	mMRS Containing Inulin[b]
Salmonella typhimurium	18	12
Escherichia coli	21	11
Staphylococcus aureus	20	13
Shigella dysenteriae	19	13

ab – Growth media bearing different superscripts differ significantly ($P<0.05$).

Oyarzabal and Conner (1995) evaluated the ability of *B. bifidum*, *Lactobacilli* and *Salmonella* spp. to grow in media containing FOS (FOS-50 or FOS-pure formulation) through *in vitro* experiments. They observed clear inhibition of growth of all salmonella serotypes grown in media containing the pure formulation of FOS as the only carbohydrate source. Bomba *et al.* (2002) investigated the influence of administration of *L. paracasei* and maltodextrin KMS X-70, on *E. coli* adhesion in the gastrointestinal tract of gnotobiotic piglets. They observed a stimulatory effect of maltodextrin KMS X-70 on the inhibitory effect of *L. paracasei* on the adhesion of *E. coli* to the jejunal mucosa of gnotobiotic piglets. As in this case, we also observed inhibition of growth of *E. coli*, *Salmonella typhimurium*, *S. aureus* and *S. dysenteriae* by culture supernatants obtained by growing B-420 culture in the medium containing inulin as the sole carbon source. This can be attributed to the bifidogenic nature of inulin, which thereby contributes towards the inhibition of undesirable organisms by the enhanced growth as well as the metabolic activity of bifidobacteria in the colon.

4.6 DEVELOPMENT OF A FREEZE-DRIED (LYOPHILIZED) SYNBIOTIC PREPARATION CONTAINING *BIFIDOBACTERIUM* AND INULIN

Fermented dairy products are well recognized as suitable carrier medium for probiotics and prebiotics. Now-a-days, they also have been incorporated into drinks as well as marketed as supplements in the form of tablets, capsules and freeze-dried preparations. Considering the limited shelf-life of fermented dairy products necessitating sophisticated storage facilities the possibilities of developing probiotic foods with increased shelf-life are well considered. The present work was an attempt towards this direction. A freeze-dried synbiotic powder was prepared as per the flow diagram (Fig. 4.7).

4.6.1 Storage Studies

The success of probiotic food development relies on the maintenance of suitable level of viable cells during the products shelf-life. In order to elicit beneficial effects, probiotic cultures must remain viable and active in the product during storage until consumption (Gilliland, 1989; Hull *et al.*, 1992).

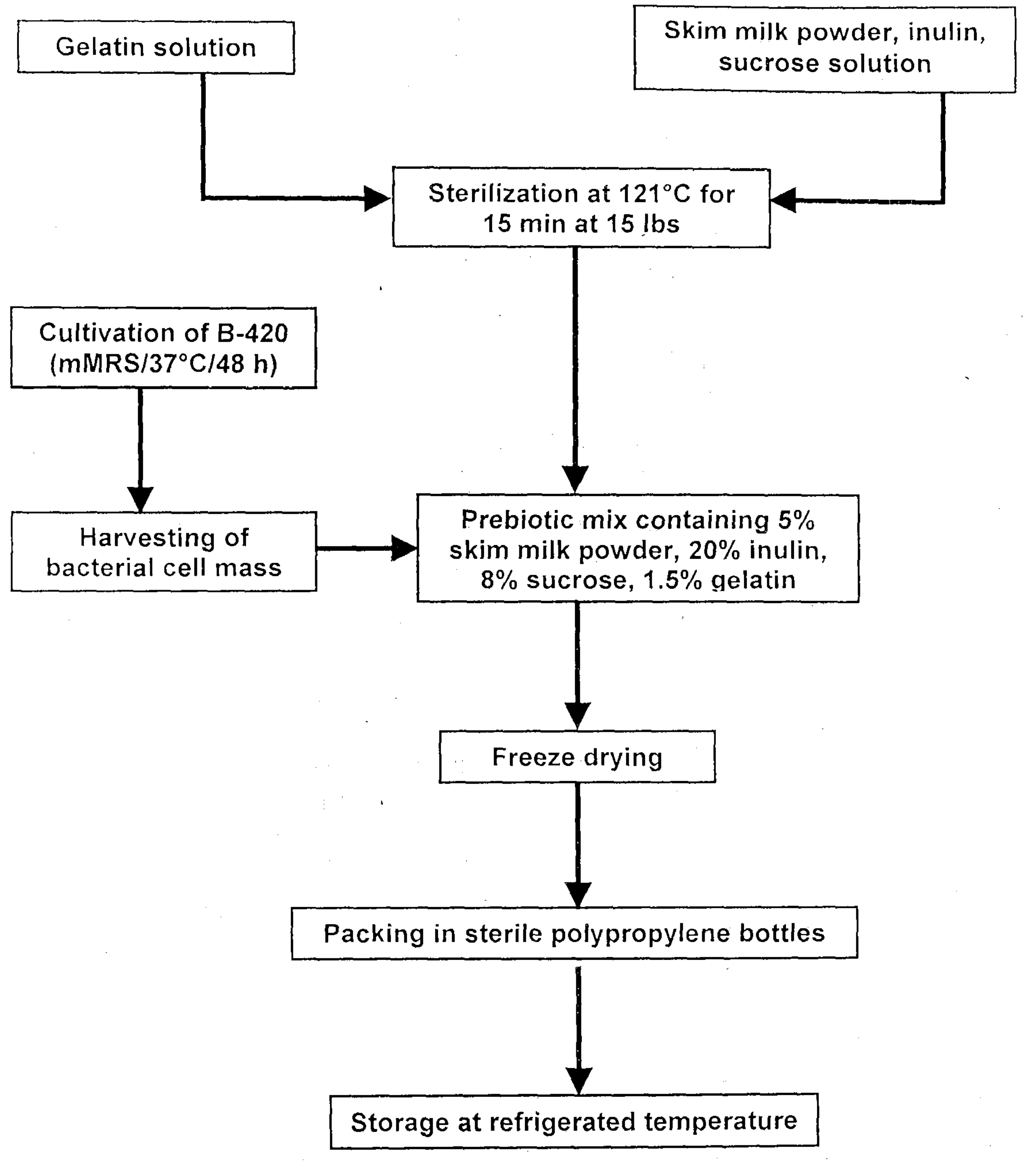


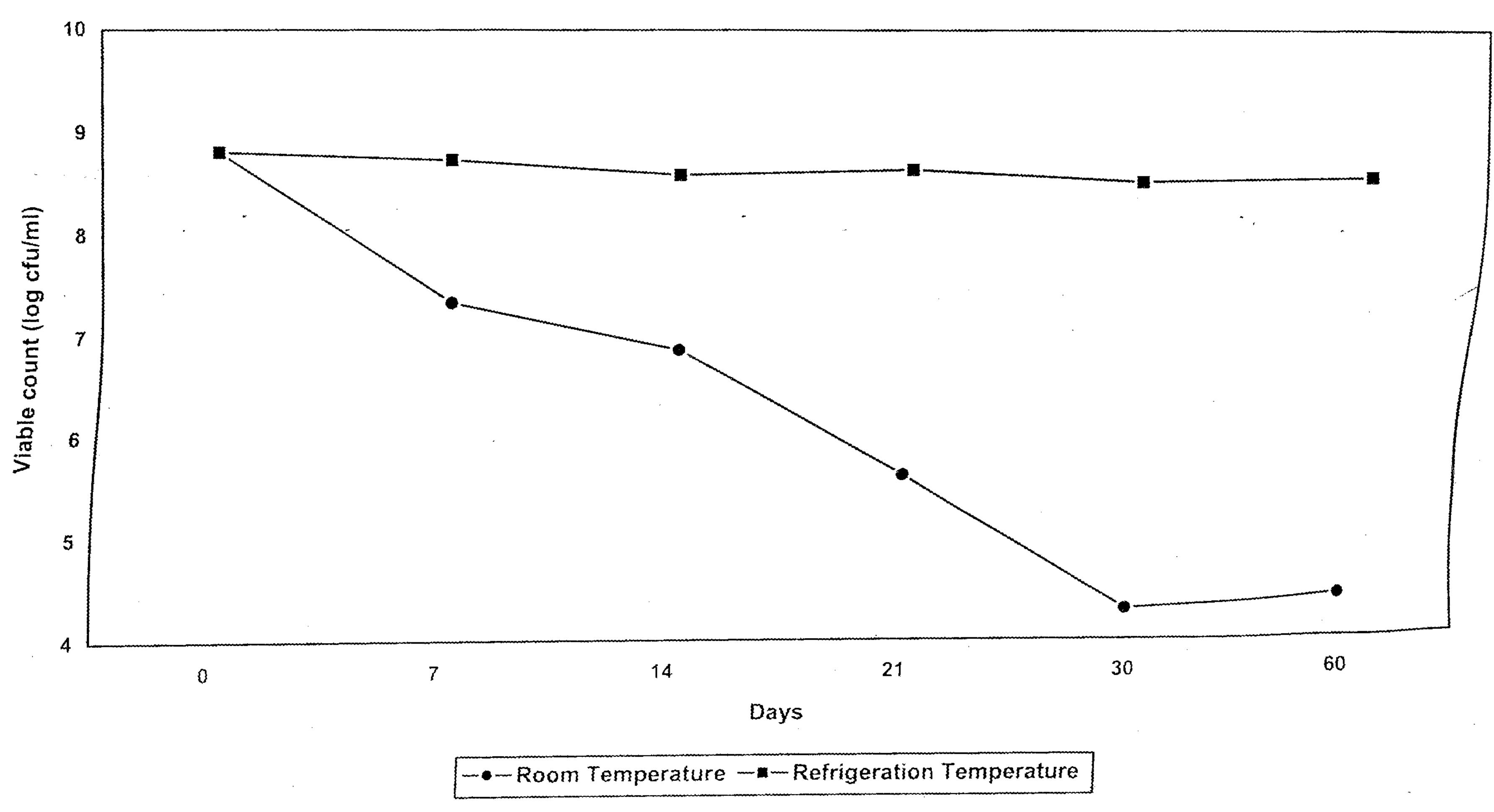
Fig. 4.7 Flow chart for development of lyophilized synbiotic preparation.

Storage of the synbiotic powder at room temperature resulted in marked reduction in the count of *Bifidobacterium* species 420, the count decreased by 3.5 logs (from 8.8 to 4.42 log cfu/g) after 60 days of storage (Fig. 4.8). No significant difference was observed in the count of *Bifidobacterium* species 420 in the synbiotic powder stored under refrigerated conditions. The count remained in the range of 8 to 9 log cfu/g even after 60 days of refrigerated storage. Consumption of bifidobacteria at a level between 5 to 7 log cfu/ml is considered necessary to derive the probiotic benefit. Based on the results obtained from the storage study, it is quite clear that our product could serve as a good carrier of probiotic organism even after 60 days of storage at refrigeration temperature. Our results are comparable with that of Collins and Hall (1984), who reported no decrease in viability (6 x 10^8/ml) of lyophilized *Bifidobacterium infantis* during storage for 4 months at 4°C in a desiccator. Maintaining the viability, stability and functionality of probiotics during processing, formulation and storage is essential for delivering the health benefits of these ingredients to consumers. Therefore, attempts are being made to develop functionally enhanced prebiotics and synbiotic combinations (Saarela *et al.*, 2002). This includes the technology to encapsulate probiotics within starch granules that are then coated with amylose (Myllarinen *et al.*, 2000). The benefits of using resistant starch extend beyond traditional prebiotics, since resistant starch can be used to ensure the viability of probiotic populations from the food to the large intestine. Incorporation of a probiotic along with prebiotic may enable relatively selective utilization of the substrate by the probiotic, providing a selective competitive advantage for the added probiotic within the intestinal tract (Mattila-Sandholm, 2002). This is applicable in our product also as this consists of both, a probiotic organism (B-420) and prebiotic (inulin). Synbiotic powders such as Hounyu Milk Powder for adults [Lactulose (g/100 g) – 8.3 and Bifidobacteria - >3 x 10^7] are already available in Japanese markets (Mizota *et al.*, 1987).

4.7 FEEDING TRIALS

The use of diet to fortify certain gut flora components is a popular current aspect of functional food sciences. In this context, probiotics,

Fig. 4.8 Viability of the probiotic organism (B-420) during storage of synbiotic powder at room and refrigeration temperatures.



prebiotics and synbiotics all have a significant role. It has been demonstrated that each of these dietary interventions can have an effect on the gut flora 'balance'. Of the several hundred species of bacteria that colonize large intestine, bifidobacteria are generally considered to be health promoting and beneficial. Probiotics, prebiotics and synbiotics are reported to stimulate the growth of beneficial bacteria like lactobacilli and bifidobacteria at the expense of undesirable organisms like bacteroides, clostridia or coliforms. Therefore, a feeding trial was carried out to determine the effect of feeding probiotic (*Bifidobacterium* species 420), prebiotic (inulin) or their combination (synbiotics) on bifidobacterial, clostridial, coliform, total aerobic count, direct microscopic count and β-glucuronidase (a procarcinogenic faecal enzyme) activity of the faeces of adult albino mice.

4.7.1 Effect on Faecal Microflora

Compared to control group significant increase in the faecal bifidobacterial counts of probiotic, prebiotic and synbiotic groups were observed during the feeding period ($P<0.05$; Table 4.8). Probiotic, prebiotic and synbiotic treatments were comparable with each other in terms of their effect on faecal bifidobacterial count. However, a slight increase in faecal bifidobacterial counts of synbiotic group (9.85 log cfu/g) was observed compared to probiotic (9.36 log cfu/g) and prebiotic (9.44 log cfu/g) groups. During pre-feeding as well as post-feeding periods, no significant differences were observed between faecal bifidobacterial counts of control and experimental groups ($P<0.05$). Therefore, the increase in faecal bifidobacterial counts of experimental groups during the feeding period can be considered as an effect of administration of probiotics, prebiotics or synbiotics. However, the increase in faecal bifidobacterial count appeared to be a transient one with the counts returning to the initial level on withdrawal of feed supplements.

As in the case of faecal bifidobacterial count, no significant differences were observed between the faecal clostridial counts of control and experimental groups during the pre-feeding as well as post-feeding periods ($P<0.05$; Table 4.9). During the feeding period, the faecal clostridial counts of

Table 4.8 Effect of feeding of probiotic (B-420), prebotic (inulin) and synbiotic (B-420 + inulin) on faecal bifidobacterial count (log cfu/g wet weight) of albino mice.

Sampling Point	Control Group	Probiotic Group	Prebiotic Group	Synbiotic Group
Pre-feeding period^{p} (0, 5 day)	8.32 ± 0.03^{a}	8.50 ± 0.19^{a}	8.72 ± 0.03^{a}	8.39 ± 0.07^{a}
Feeding period^{q} (10,15,20 day)	8.40 ± 0.16^{b}	9.36 ± 0.25^{a}	9.44 ± 0.11^{a}	9.85 ± 0.09^{a}
Post-feeding period^{p} (25, 30 day)	8.26 ± 0.07^{a}	9.16 ± 0.19^{a}	8.45 ± 0.33^{a}	8.89 ± 0.04^{a}

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 ($P<0.01$).

Table 4.9 Effect of feeding of probiotic (B-420), prebotic (inulin) and synbiotic (B-420 + inulin) on faecal clostridial count (log cfu/g wet weight) of albino mice.

Sampling Point	Control Group	Probiotic Group	Prebiotic Group	Synbiotic Group
Pre-feeding period[p] (0, 5 day)	4.87 ± 0.04^{a}	4.67 ± 0.01^{a}	4.56 ± 0.17^{a}	4.86 ± 0.09^{a}
Feeding period[q] (10,15,20 day)	4.68 ± 0.15^{b}	3.56 ± 0.21^{a}	4.07 ± 0.04^{a}	3.48 ± 0.31^{a}
Post-feeding period[p] (25, 30 day)	4.68 ± 0.09^{a}	4.29 ± 0.23^{a}	4.43 ± 0.16^{a}	4.27 ± 0.25^{a}

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 ($P<0.01$).

probiotic, prebiotic and synbiotic treatments (3.56, 4.07 and 3.48 log cfu/g, respectively) were significantly lower than that of control group (4.68 log cfu/g; P<0.05). Though statistically non-significant, the faecal clostridium counts of synbiotic and probiotic groups were lower than that of prebiotic group. However, as in the case of increase in bifidobacterial count, this effect was also transient with the faecal clostridial count shifting towards the pre-feeding period levels on stoppage of feed supplementation.

During feeding period, significant reduction in faecal coliform counts of probiotic, prebiotic and synbiotic groups (4.81, 4.73 and 4.25 log cfu/g, respectively) was observed as compared to control group (5.23 log cfu/g; P<0.05; Table 4.10). Among treatments, the synbiotic treatment was found to be most effective in terms of antagonistic activity against coliforms. Both probiotic and prebiotic treatments were observed to be equally effective. While comparing among sampling points, the faecal coliform counts of pre-feeding period was significantly higher than that of feeding as well as post-feeding period (P<0.01). As no significant differences were observed between faecal coliform counts of feeding and post-feeding periods, it can be inferred that the inhibitory effect of different treatments towards coliforms was persistent even after the withdrawal of feed supplements. During the post-feeding period, the faecal coliform counts of experimental groups were significantly lower than that of control group (P<0.05).

The total aerobic count of all the groups remained in the range of 9 to 9.8 log cfu/g throughout the feeding trial period (Table 4.11). There was no significant difference between the total aerobic count of control group and experimental groups, at any stage of the study.

As in the case of faecal bifidobacterial and clostridial counts, no significant differences were observed between direct microscopic counts (DMC) of control and experimental groups during pre-feeding as well as post-feeding periods (P<0.05; Table 4.12). However, during the feeding period, the faecal direct microscopic counts of synbiotic and probiotic groups (10.55 and 10.63 log DMC/g, respectively) were significantly higher than that of prebiotic

Table 4.10 Effect of feeding of probiotic (B-420), prebotic (inulin) and synbiotic (B-420 + inulin) on faecal coliform count (log cfu/g wet weight) of albino mice.

Sampling Point	Control Group	Probiotic Group	Prebiotic Group	Synbiotic Group
Pre-feeding period^{p} (0, 5 day)	5.51 ± 0.15^{a}	5.43 ± 0.16^{a}	5.56 ± 0.23^{a}	5.82 ± 0.13^{a}
Feeding period^{q} (10,15,20 day)	5.23 ± 0.04^{c}	4.81 ± 0.12^{b}	4.73 ± 0.11^{b}	4.25 ± 0.19^{a}
Post-feeding period^{q} (25, 30 day)	5.58 ± 0.09^{c}	4.74 ± 0.03^{b}	4.49 ± 0.10^{a}	4.69 ± 0.09^{b}

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

abc – Means with different superscripts within rows differ significantly ($P<0.05$).

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

Table 4.11 Effect of feeding of probiotic (B-420), prebotic (inulin) and synbiotic (B-420 + inulin) on faecal total aerobic count (log cfu/g wet weight) of albino mice.

Sampling Point	Control Group	Probiotic Group	Prebiotic Group	Synbiotic Group
Pre-feeding period (0, 5 day)	9.29 ± 0.13	9.49 ± 0.32	9.17 ± 0.06	9.41 ± 0.18
Feeding period (10,15,20 day)	9.35 ± 0.09	9.52 ± 0.11	9.38 ± 0.08	9.48 ± 0.11
Post-feeding period (25, 30 day)	9.56 ± 0.19	9.61 ± 0.12	9.47 ± 0.26	9.77 ± 0.21

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

Table 4.12 Effect of feeding of probiotic (B-420), prebotic (inulin) and synbiotic (B-420 + inulin) on faecal direct microscopic count (log DMC/g wet weight) of albino mice.

Sampling Point	Control Group	Probiotic Group	Prebiotic Group	Synbiotic Group
Pre-feeding period^{p} (0, 5 day)	9.69 ± 0.06^{a}	9.39 ± 0.28^{a}	9.41 ± 0.14^{a}	9.25 ± 0.20^{a}
Feeding period^{q} (10,15,20 day)	10.27 ± 0.15^{c}	10.63 ± 0.16^{a}	10.44 ± 0.17^{b}	10.55 ± 0.20^{a}
Post-feeding period^{r} (25, 30 day)	11.32 ± 0.14^{a}	11.55 ± 0.03^{a}	11.32 ± 0.05^{a}	11.39 ± 0.25^{a}

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

abc – Means with different superscripts within rows differ significantly ($P<0.05$).

pqr – Sampling points with different superscripts differ significantly ($P<0.01$).

group (10.44 log DMC/g; P<0.05). The higher direct microscopic counts can be attributed to the exogenous supply of *Bifidobacterium* species 420. Although the DMC of prebiotic group was lower than that of other two treatment groups, it was significantly higher than that of control group (P<0.05). Significant differences in faecal direct microscopic counts of groups were observed among pre-feeding, feeding and post-feeding periods.

Based on the results obtained from the feeding trial, it can be inferred that the probiotic, prebiotic and synbiotic treatments were effective in modulating the intestinal flora of mice towards a more beneficial one with increased faecal bifidobacterial counts and reduced faecal clostridial and coliform counts. Synergistic effect between *Bifidobacterium* species 420 and inulin was most prominent in terms of reduction in faecal coliform counts, however, though statistically non-significant the synbiotic treatment was observed to be more effective than either the probiotic or prebiotic treatment in terms of increase in faecal bifidobacterial count. Therefore, the administration of probiotics in combination with prebiotics can be exploited for getting enhanced beneficial effects from them. Gmeiner *et al.* (2000) studied a synbiotic preparation of *Lactobacillus acidophilus* and FOS in an *in vitro* model of the human gut and observed an increase in bifidobacteria in the vessels corresponding to the ascending colon (1.27 log) transverse (0.9 log) and descending (0.47 log) colon, presumably due to the prebiotic component of the synbiotic.

To study the effect of GOS-containing syrup (60% GOS) alone or together with the probiotic strain *Bifidobacterium lactis* Bb-12 on selected components of the faecal flora and the effect of GOS supplementation on colonization of *B. lactis* Bb-12, Alander *et al.* (2001) performed a feeding trial on 30 healthy volunteers. They observed a slight increase in mean numbers of bifidobacteria in all study groups during the feeding period. No differences in the prevalence or numbers of isolates with *B. lactis* Bb-12 genotype could be observed between groups, suggesting that GOS-containing syrup did not

enhance the survival or persistence of *B. lactis* Bb-12 in the gut. In the GOS-containing syrup group, a slight decrease in the mean number of *Clostridium perfringens* ($P<0.05$) was observed. GOS-containing syrup consumption together with Bb-12 showed no effect on *C. perfringens* counts. No statistically significant changes were detected in other bacterial numbers (coliforms, lactic acid bacteria). Bielecka *et al.* (2002) studied the effect of synbiotic pairs of bifidobacteria and oligosaccharides enhancing their growth *in vivo* to determine the effect of probiotics, prebiotics and synbiotics on the microecology of murine gut. Daily, $>10^9$ live cells of bifidobacteria strains and / or 5 percent (w/w) of oligofructose in the diet were orally administered to Wistar rats. They observed that the incorporation of oligofructose into the diet stimulated the proliferation of faecal bifidobacteria in comparison to the control and noticed variations in the survivability of administered bifidobacteria under *in vivo* conditions. Administration of bifidobacteria together with the prebiotic (as synbiotics) improved the bifidogenic effect. Supplementation of diet had almost no effect on the other determined groups of gut microflora [Total count of mesophilic bacteria, coliforms, spores of anaerobic bacteria (saccharolytic, proteolytic), spores of aerobic bacteria].

Bomba *et al.* (2002) examined the effect of administration of *L. paracasei* and a mixture of *L. paracasei* and FOS on faecal bifidobacterial counts of weanling pigs under field conditions. Significantly higher counts of *Lactobacillus* spp. ($P<0.01$), *Bifidobacterium* spp. ($P<0.05$), total anaerobes ($P<0.05$) and total aerobes were found in faeces of experimental animals receiving the mixture of *L. paracasei* and FOS compared with the controls. Moreover, significantly higher numbers of anaerobes ($P<0.05$), total aerobes ($P<0.05$), *Bifidobacterium* ($P<0.05$), and *Lactobacillus* ($P<0.05$) counts were found compared to the *L. paracasei* group. The results of this study point to a synergistic effect of the *L. paracasei* and FOS combination on faecal microflora of weaned pigs. Similar to the results obtained in this study, we also observed a synergistic effect of *Bifidobacterium* species 420 and inulin on the bifidobacterial and coliform count of adult albino mice.

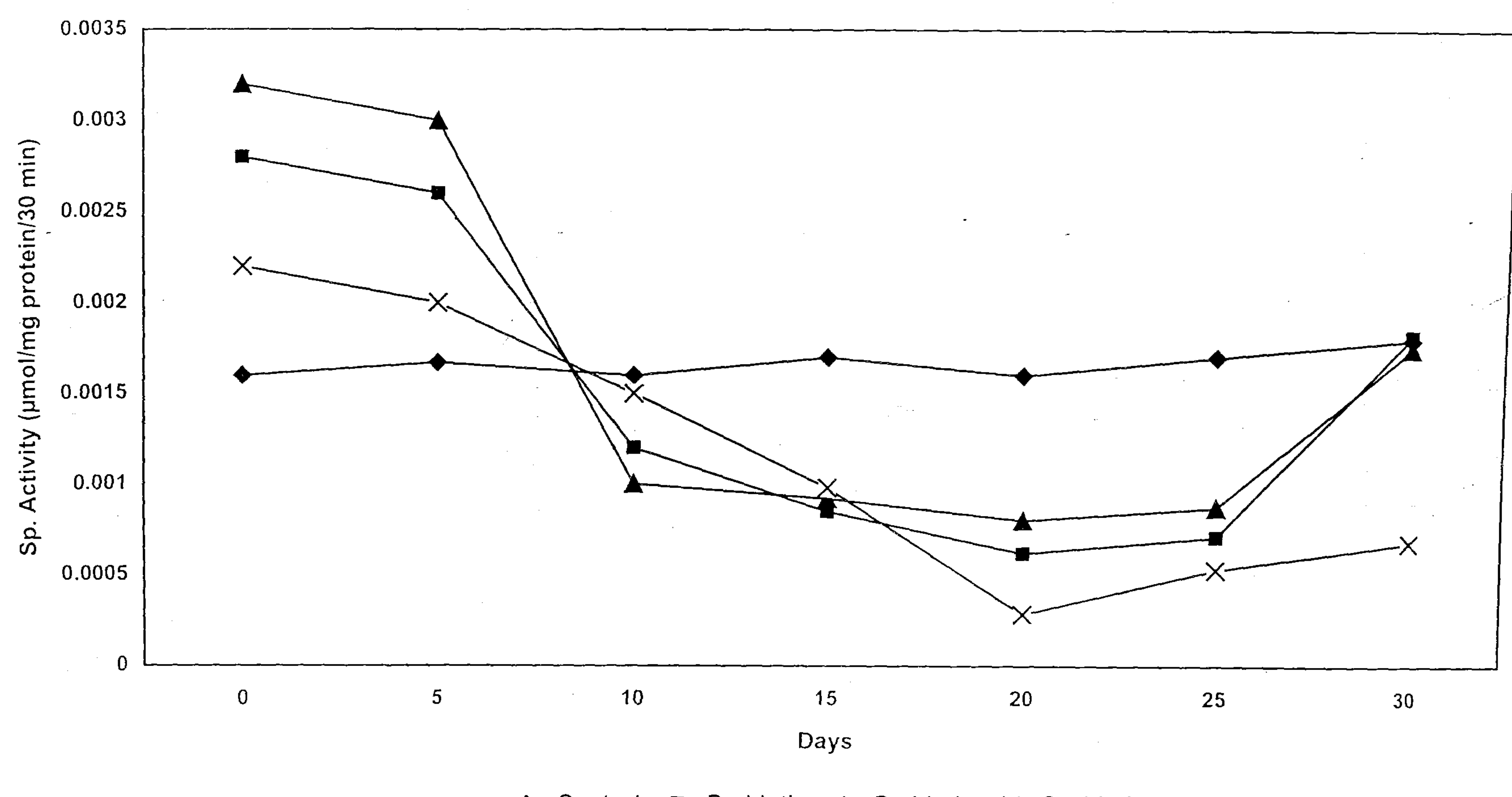
4.7.2 Effect on Faecal β-Glucuronidase Activity

The intestinal microflora comprise a complex ecosystem of a large variety of bacteria. These complex microflora exhibits many physiological functions and is also very likely to be involved in pathological states such as pseudomembraneous colitis and colonic carcinogenesis. Therefore, the alteration of the intestinal microflora through the consumption of probiotics and prebiotics can be an alternative way for tackling their negative effects. The determination of metabolic properties of the flora, such as bacterial enzyme activities or gas production, provides an alternative method for assessing dietary influence and, moreover, is more directly relevant to the flora's effect on humans than are the classical bacteriological counts. Faecal enzymes like β-glucuronidase, azoreductase and nitroreductase have the capacity to convert pro-carcinogens to carcinogens in colon. The activities of these enzymes have been negatively correlated to the number of lactic acid bacteria in the intestine. Intestinal microflora can metabolize indirect-acting mutagens by means of specific microbial enzymes that reduce nitro and azo groups or hydrolyze glucuronides (Sreekumar and Hosono, 2000).

In the present study, we have evaluated the effect of feeding probiotics, prebiotics or synbiotics on faecal β-glucuronidase activity of mice. A significant reduction in the faecal β-glucuronidase activity was observed after 10 days of feed supplementation and this effect was persistent for further 5 days even after the withdrawal of feed supplements (Fig. 4.9). However, no significant difference was observed among probiotic, prebiotic and synbiotic treatments in terms of their ability to reduce the faecal β-glucuronidase activity ($P<0.05$). In control group, the β-glucuronidase activity remained almost in the same level throughout the study.

Goldin *et al.* (1980) and Goldin and Gorbach (1984) extensively studied the effects of *Lactobacillus acidophilus* in rats and men, and showed that oral administration of some strains decreased the concentrations of three faecal enzymes (β-glucuronidase, azereductase and nitroreductase) that have the capacity to convert pro-carcinogens to carcinogens in the colon.

Fig. 4.9 Effect of feeding of probiotic (B-420), prebiotic (inulin) and synbiotic (B-420 + inulin) on faecal ß-glucuronidase activity.



Marteau *et al.* (1990) did not observe any change in the faecal β-galactosidase, β-glucuronidase and azoreductase activities of nine healthy volunteers on consumption of a fermented dairy product containing *Lactobacillus acidophilus* and *Bifidobacterium bifidum*. Sabikhi (1999) reported decrease in faecal β-D glucuronidase activity of rats when *Bifidobacteirum bifidum* was fed along with Edam cheese. The immediate effect of *Lactobacillus acidophilus* on the intestinal flora and faecal enzymes of rats was studied by Sreekumar and Hosono (2000). They observed significant reduction in faecal β-glucuronidase on administration of *L. acidophilus* SBT2074. Gmeiner *et al.* (2000) studied a synbiotic preparation of *Lactobacillus acidophilus* (probiotic strain 74-2) and FOS in an *in vitro* model of the human gut and observed a decrease in β-glucuronidase levels.

WORK DONE IN GERMANY UNDER DAAD FELLOWSHIP

4.8 EFFECT OF PROBIOTICS ON HUMAN MONOCYTE DERIVED IMMATURE DENDRITIC CELLS

4.8.1 Surface Markers

Dendritic cell (DC) maturation is characterized by the upregulation of major histocompatibility complex (MHC) II molecules, costimulatory molecules CD40, CD80 and CD86, maturation surface markers CD25 and CD83 as well as the secretion of several cytokines and chemokines (Cella *et al.*, 1997; Banchereau and Steinman, 1998; Luster, 2002). CD83 is considered as the hallmark molecule of fully matured DCs (Zhou and Tedder, 1996). In the present study, immature dendritic cells were co-cultured with live cultures of two probiotic cultures: *Bifidobacterium* species 420 (B-420) and *Lactobacillus rhamnosus* GG (LGG) and the expression of different DC surface markers, CD80, CD86, CD83, CD25 and HLADR were assessed after 48 h of incubation (which was found to be optimum in preliminary kinetic analysis) by flow cytometry.

Exposure to both the probiotic cultures, B-420 and LGG resulted in an increase in the number of cells expressing CD83, the typical maturation surface marker. The CD25 expressing cells were also significantly higher in

the probiotic treated groups ($P<0.05$; Fig. 4.10). The stimulation by the probiotic organisms also caused an increase in the number of cells expressing the costimulatory molecules CD80 and CD86. However, in the case of HLA-DR expressing cells, the bifidobacterium probiotic culture B-420 exhibited comparable effect to that of a DC maturation cocktail, but LGG did not elicit any significant effect ($P<0.05$). In comparison to unstimulated group CD80 expressing cells were significantly higher in probiotic-stimulated groups. Among the two probiotics, stimulation with B-420 induced enhanced expression of costimulatory molecules CD80 and CD86. Although both the probiotic cultures were able to induce maturation in dendritic cells, significant differences were observed in their ability to induce expression of CD80, CD86, CD25 and HLA-DR ($P<0.05$). Similar results were reported by Christensen *et al.* (2002) who observed differential production of maturation surface markers MHC-II and B7.2 (CD86) in murine dendritic cells by lactobacilli. They also observed an inhibitory effect of one of the tested lactobacilli culture (*L. reuteri*) on the induction of CD86 (B7.2) by *L. casei*. Considering the comparatively higher ability of B-420 to modulate the surface markers, this culture can be considered as a more potent immunostimulator than LGG. However, in order to reach a final conclusion, further trials are to be carried out incorporating more blood donors in the study.

As both the probiotic cultures were capable of inducing phenotypic maturation of dendritic cells, we have evaluated the effect of increase in bacterial concentration on the expression of surface markers. The results obtained for B-420 are depicted in Figure 4.11. A significant increase in the number of cells expressing the maturation markers CD83 and CD25 was observed on increasing the DC:bacteria ratio from 1:5 to 1:50. But in the case of co-stimulatory molecules CD80 and CD86, stimulation with increased number of cells resulted in significant reduction in the number of cells expressing these markers. No significant differences were observed in the expression of antigen presenting molecule HLA-DR, with increase in bacterial population.

Fig. 4.10 Effect of probiotics on surface marker expression of dendritic cells.

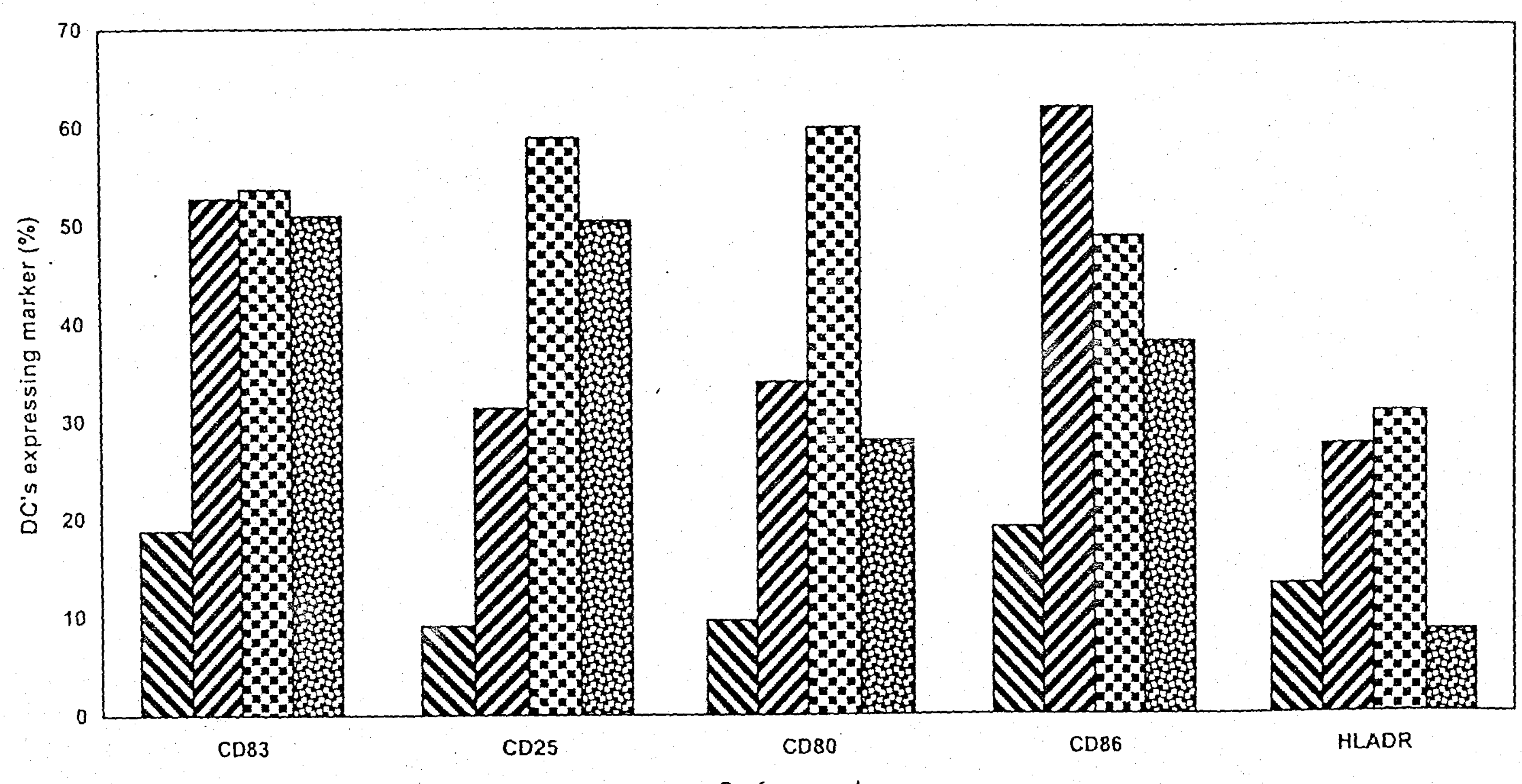
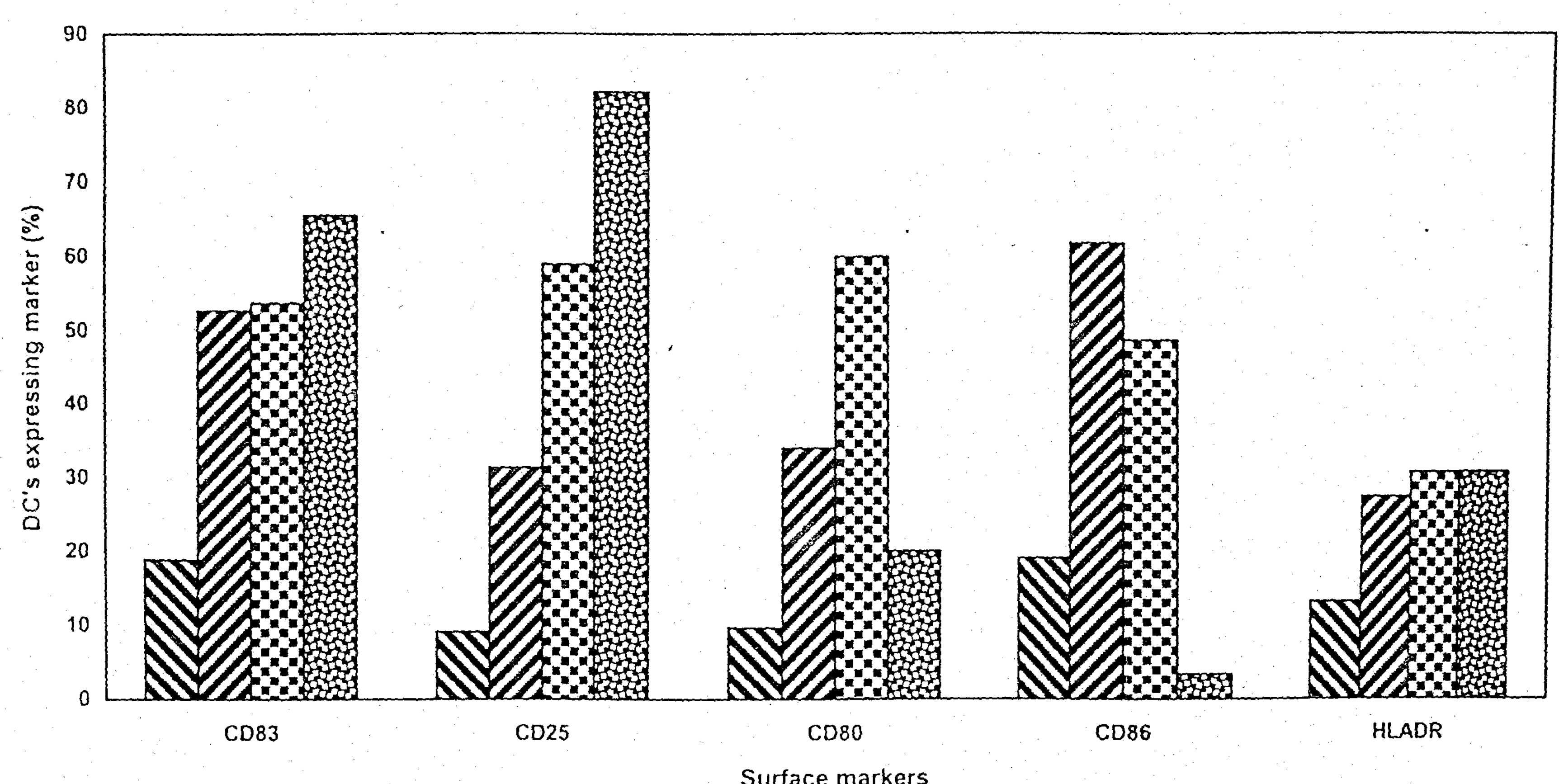


Fig. 4.11 Effect of *Bifidobacterium* species 420 concentration on expression of surface markers by dendritic cells.



As observed in the case of B-420, an increase in the DC:LGG ratio from 1:5 to 1:50 resulted in significant reduction in the number of cells expressing CD80 and CD86, and increase in the number of cells expressing CD25 ($P<0.05$; Fig. 4.12). No significant differences were observed in HLA-DR and CD83 expressing cells on increasing the LGG concentration. In contrast to the results reported by Christensen *et al.* (2002) who observed an increase in the CD86 expression on increasing the bacterial concentration, a decrease in the expression of this co-stimulatory molecule was observed in our study. The use of viable cultures rather than irradiated culture in the present study might be a contributing factor in this regard.

4.8.2 Cytokine Secretion

To assess the effect of different probiotics on cytokine secretion by DCs, live cultures of *Bifidobacterium* species 420 (B-420) and *Lactobacillus rhamnosus* GG (LGG) were added at DC : Bacteria ratio of 1:5 to immature DC cultures. After 48 h of incubation, the cytokines interleukin (IL)-12, interleukin (IL)-6, interleukin (IL)-10, interleukin (IL)-1β, interleukin (IL)-8 and tumor necrosis factor-α (TNF-α) were measured in culture supernatants. Culture supernatants of DCs matured with a cytokine cocktail served as the positive control; whereas the culture supernatant of DCs cultured in the growth medium without any stimulant served as the negative control. The effect of probiotic stimulation on the secretion of IL-12p70, IL-6, IL-10, IL-1β and TNF-α is showed in Table 4.13. The IL-8 content in all culture supernatants was extremely high that it was beyond the detection limit even after 100 times dilution of the culture supernatant.

The cytokines TNF-α and IL-6 were produced in much higher levels than other tested cytokines. In contrast to other cytokines, the IL-1β production was higher by DCs stimulated by LGG than those stimulated with B-420. LGG stimulated DCs secreted more IL-10 than IL-12; whereas in the case of B-420 stimulation, there was not much difference between IL-12 and IL-10 levels. This observation might be of clinical significance as IL-12 and IL-10 produced by antigen presenting cells (APCs) exert largely opposite

Fig. 4.12 Effect of LGG concentration on expression of surface markers by dendritic cells.

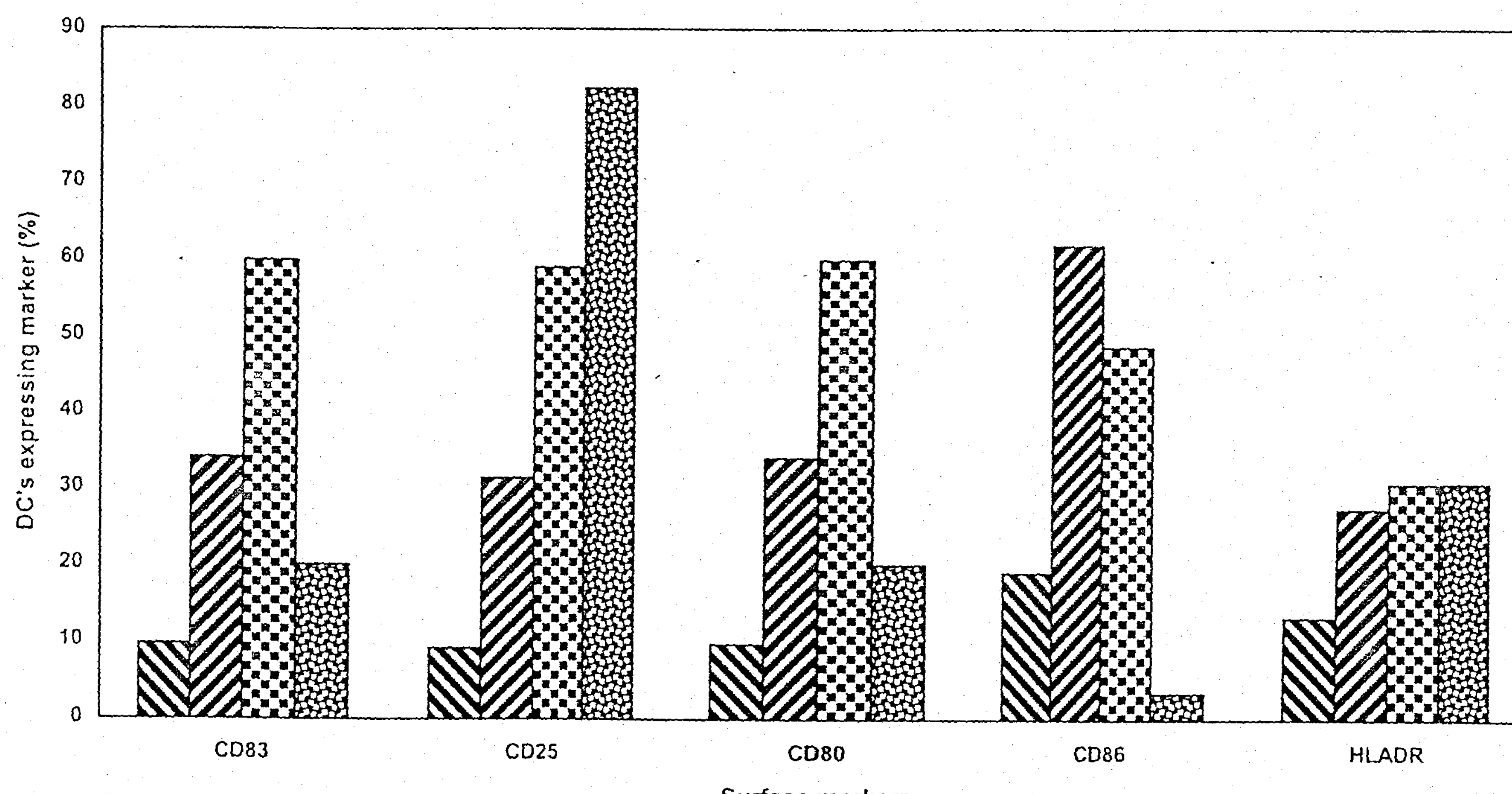


Table 4.13 Cytokine production by dendritic cells upon exposure to probiotics (B-420 and LGG).

Exposure to	**Cytokine (pg/ml)**				
	IL-12p70	**IL-10**	**TNT-α**	**IL-6**	**IL-1β**
None	1.5	0	1.4	0	0
Cytokine cocktail	0	0	26.5	44.7	8.9
B-420 (DC:B-420 – 1:5)	1021.1	855.8	131905	132560	3223
LGG (DC:LGG – 1:5)	83.2	229.5	44165	20838	4250.5

immunoregulatory effects. IL-12 is a critical Th1 skewing cytokine that elicits IFN-γ production by T cells and by NK cells (Heufler *et al.*, 1996). IL-10 on the contrary is an anti-inflammatory cytokine that suppresses IL-12 production and subsequently IFN-γ production, thus favouring a Th2 or Th3 response. The comparatively higher production of IL-10 than IL-12 by LGG renders this organism as a suitable candidate for the treatment of patients with inflammatory bowel diseases. Moreover, DCs stimulated with LGG produced lower levels of TNF-α than B-420 stimulated DCs. The cytokines IL-12 and TNF-α are both implicated in the enteropathy of inflammatory bowel diseases, as they are increased in the mucosa of patients suffering from diseases like Crohn's and for whom administration of TNF-α neutralizing antibodies has been a successful treatment (Shanahan, 2000). Therefore, probiotic bacteria producing comparatively lower levels of IL-12 and TNF-α, and higher levels of the anti-inflammatory IL-10 might be a potential fine-targeted treatment, thus providing an alternative therapeutic approach to counter balance the pro-inflammatory cytokine milieu.

Compared to LGG, stimulation by B-420 resulted in much higher production of IL-12, TNF-α, IL-10 and IL-6 by DCs. This may be due to the inhibitory effects arising from comparatively higher acid production as well as faster growth of LGG than B-420. These possibilities are to be clarified by carrying out further experiments in this direction. Pro-inflammatory cytokines such as tumor necrosis factor alpha (TNF-α) and IL-6 are among the first cytokines produced in response to bacteria and have a role in the early induction of the immune response and in the clearance of pathogens. IL-6 promotes terminal differentiation of B cells into plasma cells and has furthermore been found to be able to polarize naïve CD4 + T cells to effecter Th2 cells. The high levels of TNF-α and IL-6 secretion by DCs on probiotic stimulation might be a contributory factor towards the antipathogenic and immuno-stimulatory activity exhibited by probiotic organisms. IL-1β is a cytokine produced by antigen presenting cells, which specifically stimulates T

cells to proliferate and differentiate. Only in the case of IL-1β secretion LGG, was found to be more effective than B-420. Whether this difference from the induction pattern of other cytokines has any significance is to be studied.

Among the two probiotic cultures tested, the culture B-420 induced significantly higher production of most cytokines than the culture LGG. This is in agreement with the surface marker induction also as B-420 was observed to be more effective than LGG in inducing maturation markers also. The differential modulation of cytokine production as well as surface marker expression might be critical in shaping the subsequent adaptive immune response. Dendritic cells play a critical role in shaping the emerging immune response. Their activation by microbial antigens induces the production of cytokines such as IL-12, IL-18 or IL-10, which may promote either type 1 T helper (Th1) cells or type 2 helper (Th2) cells (Reise-Sousa *et al.*, 1997; Moser and Murphy, 2000). Th1 cells secrete interferon (IFN)-γ, which is effective against intracellular pathogens, because it stimulates microbicidal activities in macrophages; whereas Th2 cells produce IL-4 and IL-5, which drive B cells to release IgG1 and IgE antibodies. IgE is involved in effecter mechanisms protecting against worm infections. An important aspect of the capacity of dendritic cells to tailor the emerging T cell response is their potential to produce different cytokines in response to different stimuli.

The results obtained from the study is similar to that of Christensen *et al.* (2002). They studied the effect of various lethally irradiated *Lactobacillus* spp. on the cytokine (IL-6, IL-10, IL-12 and TNF-α) secretion pattern of bone marrow derived murine DC. They observed substantial differences among strains in the capacity to induce IL-12 and TNF-α production in the DC. Similar but less pronounced differences were observed among lactobacilli in the induction of IL-6 and IL-10. Hart *et al.* (2003) reported inhibition of ongoing IL-12 production and further enhancement of IL-10 production of CD11c + DC by the combination of probiotics in VSL#3 (four species of lactobacilli, three species of bifidobacteria and one streptococcus species). They also observed inhibition of ongoing IL-12 production by VSL#3 components. IL-10 production

was enhanced by *B. infantis* and *B. breve*. The response of the CD11c – subpopulation was similar to that of CD11c + DC to the bacterial stimuli, with the exception of *S. salivarius* and *L. bulgaricus*, which differentially influenced IL-10 and IL-12 production, respectively.

B-420 and LGG exhibited differences in their ability to induce DC maturation. B-420 was more effective than LGG in terms of upregulation of surface markers as well as cytokine secretion. This study does not lead to any conclusion by itself, and is just a preliminary attempt towards elucidating the immuno-stimulatory mechanisms of probiotics. The mature dendritic cells generated through probiotic stimulation should be assessed for their ability to stimulate T cells or to alter T cell phenotype, in order to see whether the differential activation of DCs by probiotics has any real impact on subsequent immune response.

Summary and Conclusion

5. SUMMARY AND CONCLUSION

The human gastrointestinal tract is a kinetic microecosystem that enables normal physiological functions of the host organism unless harmful and potentially pathogenic bacteria dominate it. In order for the intestine to function optimally, the 'balance' of the bacteria must be maintained, and this appears to be increasingly difficult as lifestyles change. Various factors may shift the balance of the gut microflora away from potentially beneficial or health promoting bacteria such as lactobacilli and bifidobacteria, and towards a predominance of potentially harmful or pathogenic microorganisms, like clostridia, sulphate reducers and certain *Bacteroides* species. Predominance of these latter populations may pre-dispose to a number of clinical disorders. Therefore, maintenance of a good community structure through increased predominance of bacteria such as lactobacilli and bifidobacteria is of considerable benefit to the host. Maintaining a proper equilibrium of the microflora may be ensured by systematic supplementation of the diet with probiotics, prebiotics or synbiotics. Hence, the present investigation was undertaken to select a *Bifidobacterium* culture based on probiotic attributes and to prepare a lyophilized synbiotic product incorporating the selected *Bifidobacterium* culture and inulin as well as to determine the effect of each factor (*Bifidobacterium*, inulin and their combination) on microecology of murine gut. The major results obtained during course of this investigation are summarized as follows :

5.1 Out of several cultures tested for the purity and presence of fructose-6-phosphate phosphoketolase only *Bifidobacterium lactis* Bb-12 (Bb-12), *Bifidobacterium* species 420 (B-420) and Bifidobacteria (ABT-5) tested positive for this enzyme, so only these three cultures were selected for evaluation of different probiotic attributes (Section 5.2 to 5.7).

5.2 These three cultures were screened *in vitro*, for their tolerance to simulated pH of the human stomach and physiological bile concentrations. *Bifidobacterium* cultures @ 10^8 to 10^9 cfu/ml were exposed to acidified distilled water (pH 1.5, 2.0 and 2.5). The number of surviving cells was determined immediately on exposure (0 h) and after 1, 2 and 3 h of holding at 37°C. No significant difference was observed among the three cultures in terms of pH tolerance ($P<0.05$). The pH 1.5 was observed to be more inhibitory than other pH levels, however, no significant difference was observed in the viability of cultures at pH 2.0, 2.5 and 6.5 ($P<0.05$).

5.3 In order to study bile tolerance, the cultures were exposed to modified MRS broth containing bile salt at different concentrations (2, 2.5 and 3.0%). Viability of cultures was observed at 0, 3 and 12 h. *Bifidobacterium lactis* Bb-12 and *Bifidobacterium* species 420 exhibited better bile tolerance than Bifidobacteria (ABT-5). However, no statistically significant difference was observed between *B. lactis* Bb-12 and *Bifidobacterium* species 420 in terms of bile tolerance.

5.4 As lysozyme, a protein capable of causing bacterial cell wall damage is a part of various normal body secretions such as saliva and gastric juice, the cultures were screened for their tolerance towards lysozyme (100 ppm). All the cultures tested could tolerate and even grow in the presence of lysozyme. No significant difference was observed in the growth of these cultures in modified MRS broth with or without lysozyme ($P<0.05$).

5.5 One of the important criteria for a potentially probiotic strain is believed to be its ability to adhere to mucosal surfaces of the human gastrointestinal tract. As the cell surface hydrophobicity is an indicator of the ability of cells to adhere to epithelial cells, the cultures were screened for their cell surface hydrophobicity on the basis of their adherence to the hydrocarbon, n-hexadecane. Wide variations were observed in their cell surface hydrophobicity varying from 11.85 to

94.51 percent. *Bifidobacterium lactis* Bb-12 and *Bifidobacterium* species 420 exhibited significantly high cell surface hydrophobicity than Bifidobacteria (ABT-5).

5.6 Antagonistic effects and improved resistance to pathogens are the most promising beneficial effects of probiotics. Keeping in view this factor, *Bifidobacteirum* cultures were tested for their antimicrobial activity against *Shigella dysenteriae*, *Staphylococcus aureus*, *Salmonella typhimurium*, *Escherichia coli* and *Enterococcus faecalis*. None of the cultures showed inhibitory activity against *E. faecalis*; whereas all were effective against all other enteric organisms. No statistically significant differences were observed in the antibacterial activity exhibited by the *Bifidobacterium* cultures.

5.7 Some indigestible but fermentable dietary carbohydrates may selectively stimulate certain bacterial groups resident in the colon such as bifidobacteria, lactobacilli and eubacteria, considered beneficial for the human host, and are collectively called prebiotics. Inulin and oligofructose are the most commonly used prebiotics. The ability of *Bifidobacterium* cultures to utilize inulin was determined by monitoring their growth in a semi-liquid minimal media. All the cultures were able to utilize inulin. However, on the basis of percentage reduction in mean generation time on inulin supplementation, the growth stimulatory effect of inulin on *Bifidobacterium* species 420 was significantly higher than that on other cultures ($P<0.05$).

5.8 On the basis of probiotic attributes, it was concluded that the overall performance of *B. lactis* Bb-12 and *Bifidobacterium* species 420 was better than Bifidobacteria (ABT-5). However, no statistically significant difference was observed between B-420 and Bb-12 in any of the tested parameters, except in the case of growth stimulatory effect of inulin. Yet, on the basis of its better performance in terms of pH tolerance, inulin utilization and cell surface hydrophobicity, the culture *Bifidobacterium* species 420 was selected for the formulation of synbiotic powder and further investigation.

5.9 In order to evaluate whether the incorporation of a prebiotic (inulin) in the growth medium of the selected culture *Bifidobacterium* species 420 has any enhancing effect on the antimicrobial activity of this culture, the antagonistic activity of B-420 grown in dextrose containing media was compared with that grown in inulin containing media. However, no enhancing effect was observed on incorporating inulin in the growth medium, rather the antibacterial activity exhibited by B-420 grown in inulin containing media was lower than that of B-420 grown in dextrose added media.

5.10 Good viability and activity of probiotics are considered as pre-requisites for optimal functionality. Therefore, the development of products in which a suitable level of viable probiotic cells are retained for a longer period is one of the key research and development area for probiotic foods. As prebiotics selectively stimulate the proliferation and / or activity of populations of desirable bacteria *in situ*, consumption of prebiotics along with probiotics might result in a competitive advantage for the probiotic. In the present study, a lyophilized synbiotic preparation was prepared incorporating *Bifidobacterium* species 420 and inulin using skim milk as base material. The product contained very high numbers (~10^9 log cfu/g) of viable *Bifidobacterium* cells, satisfying the criteria of consisting viable populations of 10^5 to 10^7 cfu/ml to elicit the beneficial effect.

5.11 The success of probiotic food development depends on the maintenance of viability of probiotic culture during storage. Therefore, viable counts of *Bifidobacterium* species 420 in the synbiotic powder was monitored at room and refrigeration temperatures for a period of 60 days. The product contained high numbers (10^8 to 10^9 cfu/g) of viable cells even after 60 days of storage under refrigerated conditions; whereas in the case of room temperature storage marked reduction (from 10^8 to 10^4 cfu/g) in the number of viable cells was observed.

5.12 The ultimate test for probiotic, prebiotic or synbiotic is the exhibition of their functionality under *in vivo* situations. Animals usually rats or mice have been used to determine the effect of substrate on the faecal microflora. A 30 days feeding trial was conducted using adult albino mice for the comparative evaluation of effectiveness of probiotic (*Bifidobacterium* species 420), prebiotic (inulin) and synbiotics on faecal, bifidobacterial, clostridial, coliform, total aerobic and direct microscopic counts and β-glucuronidase activity. The probiotic, prebiotic and synbiotic treatments were effective in terms of increase in faecal bifidobacterial count and reduction of faecal coliform, clostridial counts, and β-glucuronidase activity. No significant differences were observed in faecal total aerobic count of groups at any stage of the feeding trial. During the feeding period, the faecal direct microscopic counts of probiotics, prebiotics and synbiotics were significantly higher than that of control group. Among the different dietary strategies studied, the synbiotic treatment was most effective in reducing the faecal coliform counts and also exhibited a comparatively better stimulatory effect on faecal bifidobacterial count. Therefore, it can be inferred that the administration of *Bifidobacterium* species 420 along with inulin can elicit better results than administering either of them.

5.13 It is widely accepted that probiotics play an important role for the health of the host and possess immunomodulatory capacity. However, mechanisms by which probiotics modulate the immune system is yet to be elucidated. In an attempt towards clarifying, the mechanisms behind the immunoregulatory effect, the ability of *Bifidobacterium* species 420 to modulate the expression of cytokines and maturation surface markers in human monocyte derived dendritic cells were studied and compared with that of a well established probiotic culture, *Lactobacillus rhamnosus* GG. *Bifidobacterium* species 420 was observed to be more effective than *Lactobacillus rhamnosus* GG in terms of upregulation of surface marker expression and cytokine secretion by human dendritic cells.

CONCLUSION

The use of diet to fortify certain gut flora components is a popular current aspect of functional food sciences. In this context, probiotics, prebiotics and synbiotics have a significant role. A number of steps are essential in the development of efficacious probiotic, prebiotic or synbiotic functional foods. An important area of research is the development of probiotic foods incorporating bacterial cultures with proven probiotic attributes and in which the viability of added organism is maintained throughout its shelf-life. Additionally, the efficacy of probiotic foods can be enhanced by exploiting synergistic interaction between functional ingredients, as is potentially the case with synbiotics. Once a product is developed, it has to be subjected to *in vivo* trials in order to ensure its functionality. All these aspects were taken into consideration in the present study, in which a lyophilized synbiotic preparation was developed incorporating *Bifidobacterium* species 420, selected on the basis of its probiotic attributes and a prebiotic, inulin. The product contained high and constant levels of the probiotic culture even after 60 days of refrigerated storage. *In vivo* feeding trials in mice have showed better functionality of synbiotics compared to probiotics and prebiotics, 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.

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