THE EFFECTS OF OLIGOSACCHARIDES AND PROBIOTIC BACTERIA ON THE INTESTINAL MICROFLORA AND VANCOMYCIN-RESISTANT ENTEROCOCCUS

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ABSTRACT

The objectives of this study were: 1) to investigate the effect of bifidobacteria and oligosaccharides on their role in suppressing the growth of pathogenic bacteria *in vitro*, 2) to investigate the growth, viability and activity of *Bifidobacterium* spp. in skim milk containing prebiotics, 3) to investigate the effect of storage temperature on freeze-dried bifidobacteria with inulin and commercial probiotic products, 4) to investigate the effect of humans consuming capsules containing freeze-dried bifidobacteria and inulin on several gastrointestinal indices, and 5) to investigate the effect of probiotic bacteria on the suppression of vancomycin-resistant enterococcus *in vitro*.

Inhibitory effect of supernatant obtained from *Bifidobacterium infantis* 1912, *B. longum* 1941, *B. longum* BB536 and *B. pseudolongum* 20099 was assessed against eleven different pathogens using inhibition assays and against five selected pathogenic organisms during growth. The supernatant obtained from all four bifidobacteria showed inhibition against all 11 pathogenic bacteria, while neutralized supernatant had no effect. Supernatants of *B. infantis* 1912 and *B. longum* 1941 had maximum inhibition against the 11 bacterial strains using inhibition assays and against five strains during growth in the presence of supernatants. Co-culturing *B. longum* 1941 with five selected pathogenic bacteria of intestinal origin showed high inhibition against these pathogens. All strains of bifidobacteria produced variable concentrations of acetic acid and lactic acid.

Evaluation of four prebiotics combined with five different *Bifidobacterium* spp. were assessed for refrigerated storage viability, doubling time, pH changes and acetic and lactic acid concentrations. Five *Bifidobacterium* species were cultured anaerobically at 37°C for 48 h in 12% (w/v) reconstituted skim milk containing 5% (w/v) Hi-maize™, lactulose, inulin or raftilose. Growth and activity of the cultures in the presence of Hi-maize™, lactulose, inulin and raftilose were determined. Viability of each *Bifidobacterium* strain was assessed before

and after 4 wks of refrigerated storage at 4°C. Mean doubling time (T_d) in the presence of prebiotics was also measured. Concentrations of acetic and lactic acids produced by bifidobacteria during fermentation were determined. The doubling time ranged from 158 to 457 min. The T_d time was lowest for *B. animalis* Bb12 with raftilose and inulin. Retention of viability of bifidobacteria was greatest with Hi-maize™. The pH of skim milk at the end of 4 wks storage averaged 4.34 (for *B. animalis* Bb12 with raftilose) to 4.07 (for *B. longum* 1941 with inulin). The greatest concentration of acetic acid was produced by *B. pseudolongum* 20099 with 5% (w/v) lactulose, while the highest concentration of lactic acid was produced by *B. infantis* 1912 with 5% (w/v) lactulose. The growth, activity and retention of viability are specifically dependent on *Bifidobacterium* strain studied. The addition of prebiotics to probiotic bacteria such as bifidobacteria will have a significant effect, where unabsorbed prebiotics can be selectively utilized. Hence, a combination of a suitable *Bifidobacterium* strain with a specific prebiotic would be a feasible approach in administering the beneficial bacteria *in vivo*.

Viability of bifidobacteria in freeze-dried probiotic products at various temperatures during 20 mo storage was assessed. *Bifidobacterium longum* 1941 and *B. longum* BB536 were freeze-dried and capsules manufactured. Five commercial probiotic capsule products were also tested. The capsules were stored at -18°C, 4°C or 20°C. *Bifidobacterium* cell counts were enumerated using MRS agar at 37°C for 72 h under anaerobic conditions at 0, 1, 2, 5 and 8 mo for commercial capsules and 0, 1, 2, 5, 8, 11, 14, 17 and 20 mo for experimental capsules. Storage of capsules containing probiotics at 20°C showed the greatest decline in the viability of bifidobacteria, while those stored at –18°C showed the least decrease.

Interest in consumption of probiotic and prebiotics (indigestible oligosaccharides) to improve human gastrointestinal health is increasing. Consumption of beneficial probiotic bacteria combined with oligosaccharides may provide enhanced gastrointestinal benefits and

improvements in internal health. The effectiveness of administering *Bifidobacterium longum* 1941 or *B. longum* BB536 and inulin was studied in healthy, adult volunteers over 2-wk to observe changes in gastrointestinal indices (bacterial counts in stool, stool defecation frequency and consistency, and in organic acids, β-glucuronidase and β-glucosidase enzyme concentration, pH and moisture). A randomised, double-blind and placebo-controlled parallel group comparison was carried out. Subjects (17 males and 13 females) were randomly assigned to receive 25 mg of freeze-dried bacterial preparation containing approximately $1x10^{10}$ cfu/g of either *B. longum* 1941 and 475 mg inulin (n = 10), *B. longum* BB536 and 475 mg inulin (n = 10) or a placebo containing 475 mg inulin (n = 10). Efficacy was based on comparison of initial values of gastrointestinal indices with final values. No significant difference between the baseline and the final reading among the three treatment groups was observed on bacterial counts, defecation frequency, stool consistency, pH, enzyme and organic acid concentrations or moisture percentage of stools. However, levels of butyric acid increased after subjects consumed probiotic capsules. No subjects reported worsening in gastrointestinal health after consumption of probiotic capsules. These results indicate that the administration of *B. longum* 1941 and *B. longum* BB536 did not significantly alter the intestinal environment, defecation frequency and faecal characteristics of healthy, human subjects. These results were possibly due to the short duration of the study and the participation of healthy, adult populations consuming probiotic bacteria and prebiotics.

Eleven strains of *Enterococcus faecium* and nine *Enterococcus faecalis* (VRE) were investigated for their inhibition against each other using a well diffusion inhibition test. Probiotic organisms including *Bifidobacterium longum* 1941, *B. longum* BB536, *B. infantis* 1912, *B. animalis* Bb12, *Lactococcus lactis* ssp. *lactis* and *Lactobacillus reuteri* 23272 were grown in de Mann, Rogosa and Sharpe (MRS) broth and centrifuged (2,714 x *g* for 15 min, 4°C). The supernatants were tested against the six most inhibitory-resistant VRE strains to

observe if probiotic bacteria suppress the growth of VRE. Probiotic strains *B. longum* 1941, *L. lactis* ssp. *lactis, L. reuteri* 23272 and a commercial freeze-dried probiotic combinations (VSL#3) were then grown for 24 h in MRS broth at 37°C with several strains of VRE and bacterial counts (log CFU/ml), optical density (OD_{610}) of culture and pH at regular intervals were measured. The effect of nisin, a bacteriocin, on inhibiting VRE strains was also studied.

Five *E. faecalis* and one *E. faecium* strains exhibited high inhibition towards other VRE strains. Cell-free supernatant of *B. longum* 1941, *L. lactis* ssp. *lactis* and *L. reuteri* 23272 exhibited high inhibition towards VRE strains with a zone of inhibition of up to 26 mm formed in the agar containing pure cultures of VRE. Commercial probiotic (VSL#3) containing 450 and 900 million cells reduced VRE by 2.84 to 2.95 log CFU/ml ($p<0.05$) over 24 h, respectively. The pH of VRE cultures (4.57) after 24 h was significantly higher ($p<0.05$) than those of probiotic cultures (pH 3.74). There was a significant reduction in VRE numbers when the culture was grown in MRS broth that decreased below pH 4.00. Nisin exhibited concentration dependent inhibition towards probiotic strains (27 mm diameter for 1 mg/ml nisin to 5.5 mm diameter for 1 µg/ml) while VRE strains displayed a zone of inhibition from 16 mm diameter for 1 mg/ml nisin to 1 mm diameter for 10 µg/ml. An adaptation by three VRE strains to increasing concentrations of nisin (1 to 20 µg/ml) over 24 h was observed.

In summary, addition of probiotic bacteria to VRE strains *in vitro* significantly reduced the bacterial count over a 24 h period. Increasing concentration of probiotic bacteria influenced the growth of VRE. The pH of the media had a significant effect on the survival of VRE. Decreasing pH of media resulted in lowering of VRE count. Furthermore, nisin had a greater effect on probiotic bacteria than VRE strains, which also exhibited resistance over time.

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LIST OF PUBLICATIONS

Refereed papers

1. Bruno, F.A. and Shah, N.P. 2002. Growth, viability and activity of *Bifidobacterium* spp. in skim milk containing prebiotics. *Journal of Food Science* 67(7):2740-2744.
2. Bruno, F.A. and Shah, N.P. 2002. Inhibition of pathogenic and putrefactive microorganisms by *Bifidobacterium* sp. *Milchwissenshaft* 57(11/12):617-621.
3. Bruno, F.A. and Shah, N.P. 2003. Viability of freeze-dried bifidobacteria at various temperatures over prolonged storage. *Journal of Food Science* 68(7):2336-2339.
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5. Bruno, F.A. and Shah, N.P. 2004. *In vitro* inhibition of vancomycin-resistant enterococcus (VRE) by probiotic bacteria, nisin and pH (in preparation).
6. Bruno, F.A. and Shah, N.P. 2004. Critical review of probiotics in humans (in preparation).

Conference abstracts

1. Bruno, F.A. and Shah, N.P. 2002. Selection of probiotic cultures and prebiotics for physiological benefits. A poster presented at the 9th World Congress on Clinical Nutrition. London, England (Poster No. AB001), 24-26th June, 2002.
2. Bruno, F.A., Shah, N.P. and Gibson, P.R. VRE. 2003. Inhibition of vancomycin-resistant enterococci (VRE) *in vitro* by probiotic bacteria. *Journal of Gastroenterology and Hepatology* 18: Supplement 2: B33.

LIST OF ABBREVIATIONS

ANOVA	Analysis of Variance
CFU	Colony Forming Units
CSIRO	Commonwealth Scientific and Industrial Research Organisation
DP	Degree of polymerisation
F6PPK	Fructose-6-phosphoketolase
FOS	Fructo-oligosaccharides
GIT	Gastrointestinal Tract
GRAS	Generally Regarded As Safe
HPLC	High Performance Liquid Chromatography
ICUs	Intensive care units
IMO	Isomalto-oligosaccharides
LAB	Lactic Acid Bacteria
LC	L-Cysteine Hydrochloride Monohydrate
MICs	Minimum inhibitory concentrations
MRS	De Mann, Rogosa and Sharpe
NDO	Nondigestible oligosaccharide
NNLP	Neomycin Sulphate, Nalidixic Acid, Lithium Chloride, Paromomycin Sulphate
rDNA	Ribosomal Deoxyribonucleic Acid
RSM	Reconstituted Skim Milk Powder
SD	Standard Deviation
SEM	Standard Error of the Mean
SOE	Soybean-oligosaccharides
TOS	Transgalacto-oligosaccharides
VRE	Vancomycin-resistant enterococci

LIST OF FIGURES

LIST OF TABLES

TABLE OF CONTENTS

INTRODUCTION

The human gastrointestinal tract includes a complex microbial ecosystem that contains over 400 different species of bacteria (Rao, 1999), of which bifidobacteria are generally considered beneficial and health promoting (Kimura *et al.*, 1997). The equilibrium that exists in the large intestine is dynamic and is affected by age, diet composition and other environmental factors. Although these factors are significant, the continued maintenance of the intestinal microflora which predominantly contains beneficial species such as probiotic bacteria can improve our well-being. The human gastrointestinal tract, especially the large intestine has only recently been recognised as an important reservoir of bacteria that carries out a process of fermentation consuming substrates and producing end products that significantly influence our health.

Over the last few decades there has been a significant increase in the consumption of various products containing probiotic bacteria by consumers. Probiotic bacteria have increasingly been the focus of intense research over the last few decades as their benefits have systematically been ascertained in regards to human gastrointestinal health. Probiotic bacteria are defined as 'mono- or mixed-cultures' of live microorganisms which, when applied to animal or man, beneficially affect the host by improving the properties of the indigenous microflora (Holzapfel *et al.*, 2001). Probiotics are bacterial preparations, most often lactic acid producing, that are administered orally or added to foods. Many different species of bacteria are used as probiotics, either as monocultures or as mixed cultures in commercial products with the most common being strains of *Bifidobacterium* and *Lactobacillus*.

Bifidobacteria, an important intestinal bacteria, were discovered at the turn of the 19th century by Tissier (1899), and since then research has been steadily increasing as the

continued benefits of these types of bacteria are established. Bifidobacteria are characterised as Gram-positive, obligate anaerobes, non spore-forming, nonmotile bacilli sometimes in the form of club-shaped or spatulate extremities. Acetic and lactic acids are produced primarily in the molar ratio of 3:2. Glucose is degraded, exclusively and characteristically, by the fructose-6-phosphate `shunt' metabolic pathway (Scardovi, 1986). Bifidobacteria have a number of beneficial health properties including inhibition of pathogenic bacteria, synthesis of B vitamins, lowering of blood ammonia levels, cholesterol absorption and inhibition of tumour formation. As a result, bifidobacteria have been incorporated into fermented dairy products and other foods.

Commercially, oligosaccharides are produced by enzymic processes either by hydrolysis of polysaccharides or synthesis from smaller sugars. Because of their prebiotic properties, oligosaccharides have received much recent attention as functional food ingredients. Prebiotics are defined as "food ingredients that selectively stimulate the growth and activity of specific species of bacteria in the gut, usually bifidobacteria and lactobacilli, with benefits to health."(Roberfroid, 1998a; Cummings *et al.*, 2001). The most common prebiotics are fructooligosaccharides and inulin. Prebiotics have been selectively manufactured to contain several or all of the following attributes; active at low dosages, varying viscosity, lack of side effects, varying sweetness, fine control of microflora modulation, persistence through the colon, good storage and processing stability and inhibition of pathogen adhesion. Established and possible effects of prebiotics include non-digestibility and low energy value, stool bulking effect and modulation of the gut flora, promotion of bifidobacteria and repression of clostridia.

Loss of viability of bifidobacteria is typically more pronounced in fermented milk than in unfermented milk due to acid injury to the organism (Dave and Shah, 1997a). Lankaputhra and others (1996) observed that viability of bifidobacteria strains such as *Bifidobacterium*

infantis in 12% (w/v) skim milk at pH 4.3 was decreased by 30% after 12 d of storage at 4°C. After 24 d at the same temperature the counts decreased by more than 82%. Medina and Jordano (1994) observed a 93% reduction in *Bifidobacterium* counts of fermented milk produced in Spain at 7°C. Thus, viability of bifidobacteria has received much attention (Dave and Shah, 1997a; Shah, 2000; Shin *et al.*, 2000b).

Recently, research has focused on the ability of probiotic bacteria to ferment oligosaccharides, which bypass metabolism and adsorption in the small intestine and may have a major influence on the selective growth of bifidobacteria (Gibson and Roberfroid, 1995; Fooks *et al.*, 1999; Shin *et al.*, 2000b). Oligosaccharides are mostly a group of short chain non-digestible polysaccharides that occur naturally in foods. They are typically defined as glycosides that contain between 3 to 10 sugar moieties and are characterized by the type and sequence of the monosaccharide moieties present (Crittenden, 1999). Several bifidobacteria strains added to fermented milk have reported higher viabilities when stored with Hi-maize™ at refrigerated temperatures, while mean doubling time was highest after inulin was added. The fermentation of oligosaccharides was optimal after inulin was added as compared to Hi-maize™, lactulose or raftilose. Measurements of pH were not significantly different after 4 wk of storage at 4°C between the four oligosaccharides tested (Shin *et al.*, 2000b).

The effects of prebiotics on colonic bifidobacteria have been investigated, however, there are limited reports on the effects of prebiotics on bifidobacteria in dairy foods during storage. The products containing probiotics and prebiotics are known as synbiotics. It is important to select appropriate prebiotics for improved retention of viability of bifidobacteria in dairy foods with an ultimate goal of delivering a large number of viable bifidobacteria in colon, and for stimulation of bifidobacterial growth in the colon. Viability of probiotics that are freeze-dried are significantly affected by temperature and duration of storage. Viability of

freeze-dried bifidobacteria stored at frozen temperatures (-18°C) was significantly less than probiotics stored at room temperature (20°C).

In the last 10 years, there has been an increasing interest in the consumption of probiotics and functional foods in western diets (O'Sullivan, 1996). Probiotic bacteria are able to suppress potentially pathogenic microorganisms in the gastrointestinal tract and enhance the population of beneficial microorganisms (Ibrahim and Bezkorovainy, 1993; Yaeshima *et al.*, 1997). The probiotic approach is based on the knowledge that the beneficial intestinal flora provides protection against various acute diseases (Fuller, 1991). Bifidobacteria have long been recognized as a major component of the faecal flora of humans and some animals (Chevalier *et al.*, 1990; Key and Marble, 1995; Yaeshima *et al.*, 1997; Amann *et al.*, 1998) and are associated with beneficial health effects as they suppress unfavourable bacteria such as *Escherichia coli* and *Clostridium perfringens* and stimulate host immune functions (Yaeshima *et al.*, 1997).

Bifidobacteria have recently been added to various dairy products such as yogurts and fermented milks, however, fermented dairy foods are not an ideal medium for the maintenance of bifidobacteria due to reduced viability of these organisms (Dave and Shah, 1997a; Medina and Jordano, 1994). Consequently, numerous attempts have been made to increase the level of bifidobacteria to $>10^8$ cells/ml, however, little work has been carried out on the effects of consuming capsules containing freeze-dried bifidobacteria with a prebiotic (inulin) on gastrointestinal health in healthy, adult populations.

The use of probiotic bacteria to control infectious nosocomial pathogens such as VRE have received minor attention since the problem was discovered nearly two decades ago. Vancomycin-resistant *Enterococcus faecium* and *E. faecalis* (VRE) were first described in Britain in 1986 and soon afterwards reported from other European countries and the United States (Bascomb and Manafi, 1998). The acquisition of resistance to vancomycin, used for

treating infections caused by Gram-positive cocci that are resistant to other drugs, has been on the increase since the late 1980s. Three types of resistance, VanA, VanB, and VanC, have been recognised. The fourth phenotype of resistance mediated by *E. faecalis* and *E. faecium* is due to VanD.

Since March 1996, multiple isolates of vancomycin-resistant *E. faecium* and *E. faecalis* have occurred throughout Australia. The VRE isolated in Australia to date show considerable diversity in their phenotypes, genotypes, and geographic locations. All four combinations of genotype and species have been found, with the commonest being *E. faecium* vanB (Bell *et al.*, 1998) While the clinical profiles of VRE-affected patients appear to be similar to those recorded in the United States and elsewhere, the predominance of *E. faecium* VanB rather than *E. faecium* VanA suggests an epidemiology different from that in Europe or the United States. The level of vancomycin use in Australia is relatively high and has been increasing over the last decade. There is significant regional variation in its use due to variation in prevalence of multidrug-resistant *Staphylococcus aureus* (Bell *et al.*, 1998).

Although a dozen *Enterococcus* species have been identified, only two are responsible for the majority of human infections. Until recently, *E. faecalis* had been the predominant enterococcal species, accounting for 80 to 90% of all clinical isolates, and *E. faecium* had accounted for 5 to 15%. Other *Enterococcus* species (*E. gallinarum, E. casseliflavus, E. durans, E. avium,* and *E. raffinosis*) were isolated much less frequently and account for less than 5% of clinical isolates (Cetinkaya *et al.*, 2000). Enterococci were originally classified as enteric gram-positive cocci and later included in the genus *Streptococcus*. In the 1980s, based on genetic differences, enterococci were removed from the genus *Streptococcus* and placed in their own genus, *Enterococcus*. Enterococci characteristically grow at both 10 and 45°C, at a pH 9.6, in 6.5% NaCl and survive at 60°C for 30 min. These organisms are facultative

anaerobes, with a G + C content of 37 to 45 mol% and have the ability to hydrolyse esculin in the presence of bile,

Enterococci are the second leading cause of nosocomial urinary tract infections and third leading cause of nosocomial wound and bloodstream infections (Bischoff *et al.*, 1999). Resistance to environmental conditions such as heat or desiccation allow prolonged survival, and poor compliance with hand-washing procedures by health care workers result in rapid spread of enterococci in hospitals. Moreover, strains of enterococci have acquired resistance to essentially all antimicrobial agents over the past three decades (Bischoff *et al.*, 1999). Enterococci have been recognised as an important cause of endocarditis for almost a century. In addition to this long-established role, enterococci began to be recognised as common causes of hospital-acquired infections in the 1970s. This was coincident with and probably related to the increasing use of third generation cephalosporins to which enterococci are naturally resistant. One of the major reasons why these organisms have survived in the hospital environment is their intrinsic resistance to several commonly used antibiotics and, perhaps more importantly, their ability to acquire resistance to all currently available antibiotics, either by mutation or by receipt of foreign genetic material through the transfer of plasmids and transposons (Cetinkaya *et al.*, 2000).

The requirement for a non-antibiotic product to reduce or eliminate VRE in hospitals is of significant importance. The bulk of the literature has referred to using several antibiotic combinations that have proven unsuccessful. This field of research may reduce the reliance of using antibiotics for this challenging infectious disease and reduce costs in medical care while beneficially helping the patient.

This study was divided into two parts. The first part of the study was aimed at examining the combined effects bifidobacteria and inulin on intestinal microflora *in vitro* and on human subjects to examine the influence of probiotics and prebiotics on pathogenic inhibition, viability during storage, growth and activity, organic acid production, enzyme production, human faecal analysis and other human gastrointestinal indices. The second part of the study investigated the effects of probiotic bacteria on inhibition of the growth of vancomycin-resistant enterococcus from human patients and commercial dairy sources.

The main aims of this project are to:

1. Determine the effects of probiotic bacteria and oligosaccharides on intestinal microflora, and
2. Determine the effects of probiotic bacteria on vancomycin-resistant enterococcus.

The specific objectives of the study were:

1. To investigate the effect of bifidobacteria and oligosaccharides on their role in suppressing the growth of pathogenic bacteria *in vitro*,
2. To investigate the growth, viability and activity of *Bifidobacterium* spp. combined with prebiotics,
3. To investigate the effect of storage temperature on freeze-dried bifidobacteria with inulin and commercial probiotic products over 2 years,
4. To investigate the effect of humans consuming capsules containing freeze-dried bifidobacteria and inulin on several gastrointestinal indices, and
5. To investigate the effect of probiotic bacteria on the suppression of vancomycin-resistant enterococcus *in vitro*.

1.0. LITERATURE REVIEW

1.1. Characteristics of probiotic bacteria

1.1.1. Historical background of probiotics

The first scientific observation of the normal microflora was probably made by Antony van Leeuwenhoek in the 17th century. He used his simple microscopes to examine scrapings collected from dental surfaces and observed microscopic cells of various shapes and sizes. Scientific interest in the activities of the normal microflora has been, however, largely a 20th century phenomenon although concepts and theories expressed early this century without a doubt had their basis in observations made in the last decades of the 19th century. Knowledge accumulated as a result of observations made by numerous researchers over many years has led to the recognition that the body is host to a large and varied collection of microbes. Major advances in the study of the normal microflora have been due, however, to technical and conceptual studies made by relatively few individuals (Tannock, 1995).

Early concepts of the normal intestinal microflora and its relationship with the host were largely influenced by two opposing views taken early in the history of bacteriology. Pasteur, arguing from an evolutionary viewpoint, suggested that the host and its microflora were interdependent and its removal would be disadvantageous to the host. Metchnikoff (1907) pioneered the concept of the probiotics at the beginning of the 19th century (Gibson and Fuller, 2000). Metchnikoff's initial observations and correlations between longevity and the size of caecum and colon of animals with their associated bacterial load led him to believe that on balance the intestinal microflora was detrimental to the host because of absorption of toxic bacterial metabolites. These two contrasting views at least indicate the need for speculation and hypothesis to be supported by experimentation since at the time next to

nothing was known of the quantitative and qualitative composition of the gut flora and even less of its relationship with the healthy host.

Early bacteriological studies frequently used highly selective media or aerobic incubation which led to the belief that the viable intestinal flora consisted of enterobacteria, staphylococci and easily isolated organisms. The frequent discrepancy between the microscopic and viable count, particularly in faeces, was attributed to the presence of large numbers of dead bacteria in the sample. However, this was obviously not true for all sites studied. For example, large populations of Gram-positive and, therefore, possibly viable morphologically distinct Y-shaped, bifid, organisms (now known to be *Bifidobacterium*) were regularly found in the faeces of breast-fed babies.

It was not until the 1950s that the use of better media and anaerobic incubation techniques led to a greatly improved recovery of bacteria unable to grow under the conditions used earlier. This led to the realization that the large intestine of animals and man was populated mainly with strictly anaerobic bacteria with aerobic organisms as a small minority. In-depth studies of the gut flora of human individuals involving detailed characterisation of large numbers of isolates have produced the finding that the colon of any individual at any time contains several hundred identifiable bacterial biotypes. Although the majority of them represent less than 1% of the total count, even these are still present at over 10^6 to 10^7 per gram of contents. The study of some of the numerically minor groups, particularly aerobes, should not be overlooked.

The consumption of fermented milks in many different forms has continued until the present day. The beneficial effects of yoghurt were put on a scientific basis at the beginning of the 19th century. Elie Metchnikoff, working at the Pasteur Institute in Paris, played a key role in the process (Fuller, 1991; Walker and Duffy, 1998). He had long regarded the microflora of the lower gut as having an adverse effect on the health of the human adult. So convinced was

he of this that he had advocated surgical removal of the colon. However, he was converted to a less invasive therapy by the finding that Bulgarian peasants, who ingested large amounts of soured milks, also live to a ripe old age. He was in no doubt that the two observations were related.

In recent years, the gastrointestinal microflora has featured strongly in scientific and medical research. As a result it has become obvious that the gut microflora is an essential component of human well-being. Not only is it involved in digestion of food; it is essential for the optimal resistance to disease. Infectious intestinal disease is frequently manifest as diarrhoea, sometimes producing severe dehydration. Thus in developing countries, enteric viruses, enteropathogenic *Escherichia coli* and *Vibrio cholerae* are still major causes of death in adults and young alike. Even in developed countries, where standards of hygiene are relatively high, salmonellosis and Shigella dysentery are still prevalent. A variety of bacterial and bacterial pathogens cause diarrhoea, with differing pathogenic mechanisms, only some of which are known and understood. Thus, the epidemiology, pathogenesis and prevention of intestinal diseases still present extensive fields for research and discovery. However, the development of probiotics has been largely empirical, failing to capitalise on the relevant research data. The bringing together of the basic information on gut microecology and the development of probiotic preparations is long overdue (Fuller, 1992).

1.1.2.1. Definition of probiotic bacteria

A method in which modulation of the gut microbiota composition has been attempted is through the use of live microbial dietary additions, as probiotics. Lilly and Stillwell (1965) introduced the term `probiotics' for growth promoting factors produced by microorganisms. `Probiotic' is derived from Greek and means 'pro life' and has had several meanings over the years (Gibson and Fuller, 2000). However, its use in this form did not persist and it was

subsequently used by Sperti (1971) to describe tissue extracts which stimulated the microbial growth. It was not until 1974 that Parker used it in the current context, `organisms and substances which contribute to intestinal microbial balance'. The term `substances' is imprecise and would include even antibiotics. However, this was subsequently refined by Fuller (1989) as: 'a live microbial feed supplement which beneficially affects the host animal by improving its intestinal balance'. This latter version is the most widely used definition and has gained widespread scientific acceptability. A probiotic would therefore incorporate living micro-organisms, seen as beneficial for gut health, into the diet. However, according to this definition probiotics are restricted to feed supplements, animals and the intestinal tract. This means that the term 'probiotic' cannot be used for live microorganisms administered in any other way other than in food or feed, for application in humans, or for locations other than the gut.

The Fuller's definition has been optimised by several researchers over the last few years. A recently proposed definition of probiotic state `a probiotic is a mono- or mixed-culture of live microorganisms which, applied to animal or man, affect beneficially the host by improving the properties of the indigenous microflora'. This proposed definition implies that the term `probiotic' is restricted to products which contain live microorganisms, e.g. as freeze-dried cells or in a fermented product; improve the health status of man or animals (which can include growth promotion of animals); and can have its effect in the mouth or gastrointestinal tract (e.g. applied in food or capsules), the upper respiratory tract (aerosol) or in the urogenital tract (local application). An improved definition proposed by Naidu *et al.* (1999) stated that 'Probiotic is a microbial dietary adjuvant that beneficially affects the host physiology by modulating mucosal and systematic immunity, as well as improving nutritional and microbial balance in the intestinal tract'. The beneficial properties of a probiotic should not only be used on theoretical aspects. Some scientific proof should be given that the

probiotic improves the properties of the indigenous microflora and/or beneficially affects the host.

Subsequent research has been directed towards the use of intestinal isolates of bacteria as probiotics. Over the years many species of microorganisms have been used. They mainly consist of lactic acid producing bacteria (lactobacilli, streptococci, enterococci, lactococci, bifidobacteria) but also *Bacillus* sp. and fungi such as *Saccharomyces* sp. and *Aspergillus* sp.

Despite the very widespread use of probiotics, the approach may have some difficulties. The bacteria used are usually anaerobic and do not relish extremes of temperature. To be effective, probiotic must be amenable to preparation in a viable form on a large scale. During use and under storage the probiotic should remain viable and stable, and be able to survive in the intestinal ecosystem, and the host animal should gain beneficially from harbouring the probiotic. It is therefore proposed that the exogenous bacteria reach the intestine in an intact and viable form, and establish therein and exert their advantageous properties. Therefore, the microbes must overcome a number of physical and chemical barriers in the gastrointestinal tract. These include gastric acidity and bile acid secretion. Moreover, on reaching the colon the probiotic(s) may be in some sort of stressed state that would probably compromise their chance of survival.

Table 1.1. Desired characteristics of a good probiotic (Salminen and von Wright, 1998).

• Exerts a beneficial effect on the consumer
• Non-pathogenic and non-toxic
• Contains a large number of viable cells
• Has the capacity to survive and metabolise in the gut
• Retains its viability during storage and use
• If incorporated into a food, should have good sensory qualities

1.1.2.2. Definition of normal microflora

The normal microflora is the term most commonly used when defining the microbial collection inhabiting the body. Other terms sometimes used are 'normal flora', 'commensals' and 'indigenous microbiota'. Of all of these the strictly correct term is 'indigenous microbiota' since it infers a collection of microscopic creatures that are native to the body. Many scientists would prefer the use of 'indigenous microbiota' since it is the more correct of those listed above. However 'normal microflora' has been used extensively in the medical literature for many decades, has international recognition, is therefore likely to remain in common usage.

1.1.3. Human gastrointestinal tract

The human gastrointestinal tract (GIT) is colonised by a vast and diverse community of microbes that are essential to its functions. A balanced, complex microflora is necessary for normal digestion and to maintain the homeostasis of intestinal ecosystem (Schell *et al.*, 2002). It is often stated that the human gastrointestinal (GI) tract serves two main purposes: main portal of entry for nutrients and acts a barrier to the external environment (Duggan *et al.*, 2002). Furthermore, the large bowel contained within the GI is also the most densely colonised region of the digestive tract of humans and it is proposed that there are at least 400 to 500 species of bacteria, as well as yeast, fungi, and protozoa. The GI includes a very complex population of aerobes, facultative anaerobes, and strictly anaerobic species with the nonsporing anaerobes predominating. It is generally recognised that the structure and function of the microbiota is regulated by environmental conditions such as nutrient availability, pH, redox potential, and microbial interactions. It can be generalised that the composition and function of the microbiota is environmentally controlled and varies between individuals. This generalisation is supported by the findings of van der Merwe and colleagues who report that

the aerobic and anaerobic intestinal flora differs between individuals. These workers note an exception for identical twins and suggest that genetic identity plays a role in the composition of the anaerobic flora of humans, which in turn can have implications for Crohn's disease patients (Gibson and Macfarlane, 1995b). However, bifidobacteria constitute a numerically major part of the faecal flora of healthy humans (McCartney *et al.*, 1996; Yaeshima *et al.*, 1997).

1.1.4. History of bifidobacteria

Bifidobacteria were first described in 1899, at the Institut Pasteur, by Tissier and named by him *Bacillus bifidus* (Ishibashi and Shimamura, 1993). They are Gram-positive, strictly anaerobic, fermentative rods, often Y-shaped or clubbed at the ends. Historically, bifidobacteria have been placed in different genera: *Bacillus, Bacteroides, Bacterium, Bifidobacteria, Tissierria, Nocardia, Lactobacillus, Actinomyces, and Corynebacterium.* Since Cruickshank (1925) and Weiss and Rettger (1934) reported serological, morphological, and cultural similarities between bifidobacteria and *L. acidophilus*, *L. bifidus* was the generally accepted designation in the USA. However, this was not so widely accepted in Europe. Considerable confusion in the taxonomy of bifidobacteria resulted in misunderstanding the ecological description of the intestinal flora. Bifidobacteria have been considered the most important organisms for infants, while *L. acidophilus* is the predominant beneficial bacterium for adults.

Tissier (1899) at the Pasteur Institute isolated a bacterium from the stools of infants with an unusual Y-shaped morphology. This was the first recorded observation of bifidobacteria, although researchers were unsure at the time of the group or genus to which these bacteria would belong to. In 1967, DeVries and Stouthamer demonstrated the presence of fructose-6-phosphate phosphoketolase (F6PPK) and the absence of aldolase and glucose-6-

phosphate-6-dehydrogenase in bifidobacteria. Based on these findings, DeVries and Stouthamer (1967) concluded that classification of bifidobacteria in the genus *Lactobacillus* was not justified.

1.1.4.1. Taxonomic history of bifidobacteria

Following the discovery of bifidobacteria in the beginning of last century, a number of other important discoveries were made after the advent of chemotaxonomy during the 1960s. It was shown that the G + C % in the DNA of bifidobacteria differed from that of *Lactobacillus, Corynebacterium* and *Propionibacterium*. In 1974, the 8th edition of Bergey's Manual of Determinative Bacteriology recognised *Bifidobacterium* as genus in its own right, consisting of 11 species. In 1986, 24 species were included in the genus *Bifidobacterium* (Scardovi, 1986). These 24 species were grouped according to their ecological origin and 15 strains were isolated from animals and the rest of the 9 species were found in the natural cavities and surface of the human body. The latest collection of species in the genus adds an additional 5 species, making a total of 29 species for the genus *Bifidobacterium* (Gibson *et al.*, 1994; Lankaputhra, 1997).

1.1.4.2. Characteristics of Bifidobacterium

The genus *Bifidobacterium,* a major bacterial group in the gastrointestinal tract (GIT), accounts for up to 25% of the total culturable bacteria in adults (Sghir *et al.*, 1998) and are generally considered to be health promoting and beneficial (Rao, 1999). Due to their wide probiotic activity, they are largely used in dairy and pharmaceutical products (Brigidi *et al.*, 2000). All members of the genus *Bifidobacterium* show a bacillar form of various shapes: short, regular, thin cells with pointed ends, coccoidal regular cells, long cells with slight bends or protuberances or with a large variety of branchings, pointed, slightly bifurcated club-

shaped or spatulated extremities. Some strains develop ramifications giving V, X, Y, or other shapes. However, their polymorphism depends mainly on culture medium and the growth conditions. While lower levels of N-glucosamine and amino acids produce more highly branched shapes, rich and favourable growth conditions produce longer and bacillus-form morphology. The levels of N-acetylglucosamine, which is involved in the synthesis of peptidoglycan, a component of the cell wall, affect the branching of bifidobacteria. They form single or in chains of many elements; in star-like aggregates or disposed in V or palisade arrangements. The colonies are smooth, convex, have entire edges, cream to white, glistening and of soft consistency. *Bifidobacteria* are Gram-positive, non acid-fast, non spore-forming and nonmotile (Scardovi, 1986).

They optimally grow in anaerobic conditions while some species can only tolerate O_2 only in the presence of CO_2. The optimum growth temperatures are 37-41°C; minimum growth temperatures 25-28°C and a maximum at 43-45°C. Optimum pH for initial growth is 6.5-7.0, with no growth occurring at pH 4.5-5.0 or 8.0-8.5. Below pH 4.1, most species die within less than a week even at 4°C and below pH 2.5 most species die within less than 3 h (Lankaputhra, 1997).

Resistance of bifidobacteria to antibiotics is an important parameter in assessing the possibility of maintaining bifidobacteria in the digestive tract without aggression, particularly during antibiotic treatment to the hosts. Knowledge of resistance to antibiotics also is important due to their applicability as selective agents in selective media for various species of bifidobacteria. Although sufficient research is not available regarding the antibiotic resistance of bifidobacteria, it has been claimed that bifidobacteria are resistant to antibiotics such as nalidixic acid, gentamycin, kanamycin, metronidazole, neomycin, polymixin B, and streptomycin. Sensitivity of these antibiotics vary from 10 to 500 μg/mL. Bifidobacteria could be strongly inhibited by ampicillin, bacitracin, chloramphenicol, clindamycin, erythromycin,

1.1.4.4. Role of lactic acid bacteria in human health

The intestinal flora is comprised of 100 trillion bacteria consisting of more than 100 species (Ogata *et al.*, 1997), and contains a variety of enzymes that perform extremely varied types of metabolism in the intestine. Thus, the intestinal flora can significantly influence the host's health, including nutrition, physiological function, drug efficacy, carcinogenesis, ageing as well as the host's immunological responses, resistance to infection, and responses to endotoxin and various other stresses. Within the intestine, bacteria are implicated in the conversion of various substances, resulting in the production of both beneficial and deleterious products to the host. In addition, bacterial toxins and cell components produced by some bacterial species modify the host's immune responses, enhancing or inhibiting immune function. The beneficial intestinal flora protects the gastrointestinal tract from proliferation or infection of harmful bacteria, while the deleterious bacterium manifests pathogenicity when the host's resistance is decreased (Mitsuoka, 1982).

Much recent research has focused on bifidobacteria to establish the importance of these bacteria in influencing certain normal functions of the intestinal tract and in exploring their role in human health and diseases. Bifidobacteria are used as dietary supplements or as starter cultures for yoghurt and other cultured milk products with the thought that such products may help the promotion of health. Some of the various effects of cultured dairy products are reported as follows:

- Suppression of putrefactive intestinal bacteria resulting in the prevention of constipation and geriatric diseases;
- treatment of antibiotic-associated diarrhoea; and
- stimulation of immune response.

1.1.4.5. Carbohydrate metabolism of lactic acid bacteria (LAB)

In most LAB, lactic acid is a major metabolic by-product produced as a result of carbohydrate metabolism. Some members of LAB group produce acetic and propionic acids in addition to lactic acid. The group of LAB can be homofermentative or heterofermentative based on their pattern of carbohydrate fermentation. LAB which produce lactic acid as a major by-product are referred to as homofermentative and those which produce CO_2, ethanol and acetic acid as major by-products in addition to lactic acid are referred to as heterofermentative. Homofermentative LAB follow Embden-Meyerhof-Parnas (EMP) pathway for glycolysis. LAB may either form D or L lactic acid or a racemic mixture of the two isomers. There are three major pathways associated with hexose metabolism in LAB.

Table 1.2. Major metabolic products of hexose metabolism through homo-, hetero- and bifidus-fermentative pathways (Lankaputhra, 1997).

Homo fermentative pathway	$C_6H_{12}O_6$----------→	$CH_3.CHOH.COOH$
Hetero fermentative pathway	$C_6H_{12}O_6$----------→	$CH_3.CHOH.COOH + CO_2$
Bifidus fermentative pathway	$C_6H_{12}O_6$----------→	$CH_3.CHOH.COOH + CH_3COOH$

Aldose plays a key role in EMP route of glycolytic homofermentation as compared with the phosphoketolase serving as a key enzyme in the other two pathways. The 6-phosphogluconate pathway which yields CO_2, lactate and ethanol is characteristic of some organisms exhibiting the heterolactic type fermentation. However, bifidobacteria utilise sugar via a different pathway known as `bifidus pathway'.

1.1.4.6. Genus Bifidobacterium metabolism utilising fructose-6-phosphate pathway

As suggested by the name, `bifidus pathway' is a major pathway of carbohydrate metabolism occurring in all bifidobacteria. This pathway is also known as `fructose-6-phosphate shunt'. In the genus *Bifidobacterium*, hexoses are metabolised exclusively and

specifically by the fructose-6-phosphate pathway (Amann *et al.*, 1998; Scardovi and Trovatelli, 1965). Many authors use the bifidus pathway as a unique marker for the genus *Bifidobacterium*. Studies by Grill *et al.* (1995b) characterised F6PPK from *Bifidobacterium* species.

The key enzyme for bifidus pathway is F6PPK, which hydrolyses hexose phosphate to erythrose-4-phosphate and acetyl phosphate. From tetrose and hexose phosphates through subsequent action of transaldolase and transketolase pentose phosphates are formed. The final fermentation products are formed by the action of transaldolase, transketolase, xylulose-5-phosphate phosphoketolase and enzymes belonging to EMP pathway (glyceraldehyde-3-phosphate dehydrogenase, pyruvate kinase and lactate dehydrogenase), which act on glyceraldehyde-3-phosphate. In bifidus pathway, fermentation of two moles of glucose leads to three moles of acetate and two moles of lactate. Phosphoraclastic cleavage of pyruate to formic and acetic acids and the reduction of acetate to ethanol can often alter the fermentation balance of end products to a great extent.

Although the enzyme F6PPK is found in bifidobacteria, aldolase and glucose-6-phosphate dehydrogenase are not present in these organisms. Absence of F6PPK in other Gram-positive anaerobic bacteria such as *Lactobacillus, Arthrobacter, Propionibacterium, Corynebacterium* and *Actinomycetaceae*, which could be morphologically confused with bifidobacteria, is important for identification of the members of the genus *Bifidobacterium*. Presented in Table 1.3 are fermentative characteristics that separate several *Bifidobacterium* spp.

Table 1.3. Fermentative characteristics distinguishing the species of the genus *Bifidobacterium* (Scardovi, 1986).

Bifidobacterium spp.	D-Ribose	L-Arabinose	Lactose	Cellobiose	Melezitose	Raffinose	Sorbitol	Starch	Gluconate
B. bifidum	-	-	+	-	-	-	-	-	-
B. longum	+	+	+	-	+	+	-	-	-
B. infantis	+	-	+	-	-	+	-	-	-

1.1.5. The adult intestinal microflora

The complex ecosystem of the adult intestinal microflora is estimated to harbour approximately 500 different bacterial species (Kimura *et al.*, 1997; Tannock, 1998; Isolauri, 2001a) with some of these species considered potentially harmful because of their abilities of toxin production, mucosal invasion, or activation of carcinogens and inflammatory responses (Isolauri, 2001b). These strains with health-promoting properties principally include bifidobacteria and lactobacilli. In infectious and inflammatory conditions the balance of the gut microecology is altered in such a way that the number of potentially pathogenic bacteria grows and the healthy interaction between the host and microbe is disturbed such that an immune response may be induced by resident bacteria. Probiotics are beneficial bacteria that exist in the healthy gut microflora (Bezkorovainy, 2001).

The intestinal microflora constitute a complex ecosystem. The most prevalent anaerobic bacteria are *Bacteroides, Clostridium, Bifidobacterium, Eubacterium, Peptococcus, Fusobacterium* and *Peptostreptococcus*. The bacteria of the human gastrointestinal tract are predominantly strict anaerobes and outnumber facultative bacteria by a factor of 10^2 to 10^4. The stomach of healthy individuals is populated by very low numbers of bacteria. The saliva from the oral cavity is a source of gastric bacteria, however, the highly acidic gastric juice destroys most of these organisms. Gram-positive facultative bacteria such as lactobacilli and

streptococci are most commonly isolated from gastric juice. The number of microorganisms isolated in the stomach is between 10^1 and 10^2/ml of gastric juice. The upper small intestine in healthy humans is also sparsely populated with organisms similar to those found in the stomach. However, the counts rise to 10^2 to 10^4 organism/ml of contents.

The ileum and caecum are heavily populated with microorganisms and the composition is much more complex. The most common organisms found in the lower small intestine are those that have been listed above as most prevalent in the gastrointestinal tract. The total bacterial counts vary between 10^4 and 10^8/ml of ileal contents. Within the colon the bacterial concentration rises dramatically and is between 10^{11} and 10^{12}/ml of faecal material. The composition of the colonic flora, as would be expected, comprises the bacteria listed above as most prevalent in the gastrointestinal tract.

1.2. Taxonomic diversity of probiotic bacteria

The gastrointestinal tract contains approximately 500 different bacterial species. Major bacterial species isolated from the human gastrointestinal tract fall generally into three distinct categories. These include 1) organisms almost always present in large number, and constituting the indigenous and resident flora, e.g. *Bacteroides, Bifidobacterium*; 2) organisms normally present in small or moderate numbers, and part of the resident flora, e.g. Enterobacteriaceae, *Streptococcus* and *Lactobacillus*; and 3) organisms present in small numbers, probably contaminants from other regions of the body e.g. *Staphylococcus, Haemophilus*, etc., or from the environment, e.g. *Bacillus, Corynebacterium*, which constitute transient flora.

More specifically, organisms of the human gastrointestinal tract include diverse bacterial genera or families, and are divided into the following three groups: 1) Lactic acid bacteria in a broad sense, including *Bifidobacterium, Lactobacillus, and Streptococcus* (including *Enterococcus)*; 2) Anaerobic group, including *Bacteroidaceae, Eubacterium, Peptococcaceae, Veillonella, Megasphera, Gemmiger, Clostridium* and *Treponema*; and 3) Aerobic group, including Enterobacteriaceae, *Staphylococcus, Bacillus, Corynebacterium, Pseudomonas* and yeasts.

During the past 20 years, significant advances have been made in bacterial taxonomy of indigenous intestinal bacteria. Newly developed research methods such as DNA-DNA homology, rRDNA-DNA homology or the guanine-plus-cytosine (G + C) content of DNA have contributed much to the advances in bacterial taxonomy, and numerous new taxa of intestinal anaerobes were described. Results of DNA homology are used to indicate relationships among strains, establish genospecies, and enable selection of those phenotypic tests that are the most useful for reliable identification of new isolates. Many different types

of bacteria, representing most bacterial groups, have been isolated from the intestine. Between 30-40 species constitute approximately 90% of the flora, but in order to be sure of identifying these species some hundred isolates from each sample should be examined. Such bacteria are generally identified on the bases of their morphology as determined by Gram stain, fermentation reactions and metabolic tests. While several schemes have been developed for the identification of indigenous anaerobic bacteria, it is still difficult to identify many of these organisms by conventional tests at species level. Differentiation of major intestinal bacterial groups according to Gram-staining, aerobic growth, spore production and major fermentation products are presented below in Table 1.4.

Table 1.4. Differentiation of major intestinal bacterial groups (Mitsuoka, 1992).

Bacterial group	Gram-staining	Aerobic growth	Spore production	Major fermentation products
Lactic acid bacteria group				
Lactobacillus	+	+	-	Lactic acid
Bifidobacterium	+	-	-	Acetic acid + lactic acid
Streptococcus	+	+	-	Lactic acid
Anaerobic group				
Bacteroidaceae	-	-	-	Various products
Anaerobic curved rods	-	-	-	Succinic acid, butyric acid
Eubacterium	+	-	-	Various products
Peptococcaceae	+	-	-	Various products
Veillonella	-	-	-	Acetic acid + propionic acid
Megasphaera	-	-	-	Caproic acid + butyric acid
Gemmiger	-	-	-	
Clostridium	+/-	-	+	Various products
Treponema	-	-	-	
Aerobic group				
Enterobacteriaceae	-	+	-	
Staphylococcus	+	+	-	
Bacillus	+	+	+	
Corynebacterium	+	+	-	
Pseudomonas	-	+	-	
Yeasts	+	+	-	

1.2.1. Lactic acid bacteria

The concept of the group name 'lactic acid bacteria' was created for bacteria causing fermentation and coagulation in milk, and defines as those which produce lactic acid from lactose. The family name Lactobacteriaceae was applied by Orla-Jensen (1919) to a physiological group of acid alone or to acetic and lactic acids, alcohol and carbon dioxide. Today, lactic acid bacteria are regarded as synonymous by and large with the family Lactobacillaceae (Breed *et al.*, 1957), which is now recognised as consisting of Gram-positive, non-sporing, carbohydrate-fermenting lactic acid producers, acid tolerant of non-aerobic habit and catalase negative; typically they are non-motile and do not reduce nitrate. They are sub-divided into four genera *Streptococcus, Leuconstoc, Pediococcus, and Lactobacillus*. For many years, bifidobacteria were included in the genus *Lactobacillus* as *Lactobacillus bifidus* (Mitsuoka and Kaneuchi, 1977). They are now excluded from this genus and the lactic acid bacteria (Sharpe and Fryer, 1972) and are classified in a separate genus *Bifidobacterium* as already suggested by Orla-Jensen (1924), which is included in the family Actinomycetaceae.

Recent taxonomic revisions suggest that LAB group could be comprised of genera Aerococcus, Carnobacterium, Enterococcus, Lactobacillus, Lactococcus, Leuconostoc, Pediococcus, Streptococcus, Tetragenococcus, and Vagococcus. Originally, bifidobacteria were included in the genus Lactobacillus and the organism was referred to as *Lactobacillus bifidus*. Although the classification of LAB into different genera is mainly based on the characteristics used by Orla-Jensen (1919) including morphology and mode of sugar fermentation, for some of the newly described genera of LAB, additional characteristics such as fatty acid composition and motility are used as the basis of classification.

Although most authors prefer to include the genus *Bifidobacterium* under the group of LAB, molecular percentage values of G + C contents of DNA show that all members of genus

Bifidobacterium contain >50 mol percent G + C in DNA. Other LAB contain <50 mol percent G + C in DNA. Based on the mol percent G + C contents, all lactic acid producers have been allocated into two branches called clostridium and actinomycetes (Klein *et al.*, 1998a). All members of bifidobacteria fall within the actinomycetes branch. Bifidobacteria show variable morphology characterised by branching and pleomorphism, Gram positive and prefer anaerobic conditions for growth. Phylogenically, bifidobacteria are also different from other lactic acid bacteria due to their higher molecular percentage of G + C contents in DNA and are placed in the Actinomycete branch as presented in Table 1.6. While other lactic acid bacteria are placed in the Clostridium branch. Presented in Table 1.5 and 1.6 compares the G + C contents in DNA of several genus of lactic acid bacteria and phylogenetic relationship of lactic acid bacteria according to G + C mol percent content in DNA, respectively.

Table 1.5. DNA base composition of different lactic bacteria (Salminen and von Wright, 1993).

Genus	G + C % range
Lactobacillus	34.7-50.8
Streptococcus	33-44
Leuconstoc	39-42
Bifidobacterium	57.2-64.5

Table 1.6. Phylogenetic relationship of lactic acid bacteria based on the mol percent of G + C content in DNA (Salminen and von Wright, 1993).

	Branch	
	Clostridium	**Actinomycete**
Mol % of G + C content in DNA	<50	>50
	Lactobacillus	*Bifidobacterium*
	Lactococcus	*Propionibacterium*
	Enterococcus	*Microbacterium*
	Leuconostoc	*Corynebacterium*
	Pediococcus	*Brevibacterium*
	Streptococcus	*Atophobium*
	Staphylococcus aureus	
	Bacillus subtilis	

1.2.2. Genus Bifidobacterium

1.2.2.1. Taxonomy of bifidobacteria

After bifidobacteria was first discovered in the beginning of the 19th century, a number of more important discoveries were made after the advent of chemotaxonomy during the 1960s. Sebald *et al.* (1965) showed that the percentage of G + C in the DNA of bifidobacteria differed from that of *Lactobacillus, Corynebacterium* and *Propionbacterium.*

In 1974, the 8th edition of Bergey's Manual of Determinative Bacteriology recognised *Bifidobacterium* as a genus in its own right consisting of 11 species. Scardovi (1986) included 24 species in the genus *Bifidobacterium*. These 24 species were grouped according to their ecological origin and 15 strains were isolated from animals and the rest of the 9 species were found in the natural cavities and surfaces of humans. According to the latest collection of species in the genus *Bifidobacterium*, an additional five species have been described making the total number of species as 29. Presented in Table 1.7. is a chronological order of the development of taxonomy of bifidobacteria over the last century.

Table 1.7. Chronological order of the development of taxonomy of bifidobacteria (Sgorbati *et al.* 1995).

Stage of development		Author/reference	Year
1	*Bacillus bifidus*	Tissier	1900
2	*Bacteroides bifidus*	Castellani	1919
3	*Bacteroides bifidus*	Chalmers	1923-34
4	*Lactobacillus bifidus*	Bergey's Manual eds	1920
5	*Bifidobacterium bifidum*	Holland	1924
6	*Bacterium bifidum*	Orla-Jensen	1927
7	*Tissier bifidia*	Lehmann and Neumann	1929
8	*Norcadia bifidia*	Pribram	1931
9	*Actynomyces bifidus*	Vuillemin	1934
10	*Actinobacterium bifidum*	Nanni	1937
11	*Lactobacillus acidophilus var. bifidus*	Weiss and Rettger	1938
12	*Lactobacillus parabifidus*	Weiss and Rettger	1938
13	*Bifidobacterium bifidum*	Weiss and Rettger	1938
14	*Lactobacillus bifidus*	Prevot	1939-57
15	*Cohnistreptothrix bifidus*	Bergey's Manual	1944

16	*Corynebacterium bifidum*	Negrovi and Fisher	1949
17	*Lactobacillus bifidus*	Olsen	1950
18	*Lactobacillus bifidus var. pennsylvanicus*	Norris *et al.*	1953
19	Description of human species	Dehnert	1963
20	New animal species	Reuter	1969
21	New animal species	Mitsuoka	1969
22	New animal species	Scardovi	1972
23	Creation of genus *Bifidobacterium* constituting 11 species	Holdeman and Moore	1974
24	Inclusion of 24 species to genus *Bifidobacterium*	Scardovi	1986
25	Inclusion of 29 species to genus *Bifidobacterium*	Sgorbati *et al.*	1995

1.2.2.2. Classification schema of bifidobacteria species

Prior to 1957, many workers recognised only one species in the genus *Bifidobacterium*. In 1957, Dehnert first recognised the existence of multiple biotypes of bifidobacteria and proposed a schema for the differentiation of five groups of bifidobacteria, based mainly on carbohydrate fermentation (Dehnert, 1957). Reuter (1963, 1964) has proposed, on the basis of carbohydrate fermentation and serological characteristics, seven species of *Bifidobacterium* in addition to the known *B. bifidum* four strains isolated from human infants and adults. He presented a schema for their identification and recognised the following species and biovars in the genus *Bifidobacterium*: *B. bifidum* var. a and b, *B. infantis, B. parvulorum* var. a and b, *B. breve* var. a and b, *B. lactentis*, *B. liberorum, B. adolescentis* var. a, b, c, and d, and *B. longum* var. a and b.

The next major classification schema was presented by Mitsuoka (1969); 483 strains of bifidobacteria, isolated from humans as well as a variety of animals, e.g., pig, chicken, calf, sheep, mouse, rat, and guinea pig, were studied and classified on the basis of physiological and biochemical characteristics, and the results compared with those obtained by Reuter (1963). In this study he clearly differentiated non-human strains from human by their carbohydrate fermentation patterns and ability to grow at 46.5°C, and proposed the creation of the two new species, *B. thermophilum* var. a, b, c and d, and *B. pseudolongum* a, b, c and d, and of a new variant, *B. longum* subsp. *animalis* a and b.

The latest collection of the *Bifidobacterium* genus includes 29 described species (Table 1.8). According to their ecological origin, 14 occur in humans, 12 in animals and 3 in honeybees (Shah and Lankaputhra, 2003b).

Table 1.8. Species of the genus *Bifidobacterium* and their mol percent G + C contents

No.	Species	G + C %	No.	Species	G + C %
1	*B. bifidum*	60.8	16	*B. boum*	60.0
2	*B. longum*	60.8	17	*B. magnum*	60.0
3	*B. infantis*	60.5	18	*B. pullorum*	67.5
4	*B. breve*	58.4	19	*B. gallinarum*	65.7
5	*B. adolescentis*	58.9	20	*B. suis*	62.0
6	*B. angulatum*	59.0	21	*B. minimum*	61.6
7	*B. catenulatum*	54.0	22	*B. subtile*	61.5
8	*B. pseudocatenulatum*	57.5	23	*B. coryneformes*	--
9	*B. dentium*	61.2	24	*B. asteroides*	59.0
10	*B. globosum*	63.8	25	*B. indicum*	60.0
11	*B. pseudolongum*	59.5	26	*B. gallicum*	61.0
12	*B. cuniculi*	64.1	27	*B. ruminatium*	57.0
13	*B. choerinum*	66.3	28	*B. mericicum*	59.0
14	*B. animalis*	60.0	29	*B. saeculare*	63.0
15	*B. thermophilum*	60.0			

Adapted from Sgorbati *et al.* (1995).

1.2.2.3. Differentiation of bifidobacteria from other LAB

At present, there is general agreement among taxonomists, that on the basis of morphological, cultural, and biochemical characteristics bifidobacteria should be classified in the genus *Bifidobacterium* as already suggested by Orla-Jensen (1924). Tentative identification of a bacterial strain as a member of the genus *Bifidobacterium* can be made on the basis of colony form and cell morphology, although *Lactobacillus, Propionibacterium, Actinomyces* and cell *Eubacterium* may closely resemble each other in their microscopical appearances. Reliable differentiation of bifidobacteria from these related genera, however, can only be achieved by biochemical tests as shown in the Table 1.9.

Table 1.9. Key for differentiation of *Bifidobacterium* from related genera (Mitsuoka, 1992).

Characteristics	*Bifidobacterium*	*Actinomyces*	*Propionibacterium*	*Eubacterium*	*Lactobacillus*
Major fermentation products	AL	S	PA	B,AF, none	L
Aerobic growth	-	+	V	-	V
Gas formation from glucose	-	-	+	V	V
Catalase production	-	V	$+^{-}$	-	-
Nitrate reduction	-	$+^{-}$	$+^{-}$	V	-
Indole production	-	-	V	-	-
Gelatin liquefaction	-	$-^{+}$	$+^{-}$	V	-
Acid from					
Rhamnose	-	V	$-^{+}$	$-^{+}$	V
Sorbose	-	V	V	$-^{+}$	$-^{+}$
Glycerol	-	-	$+^{-}$	$-^{+}$	$-^{+}$
Erythritol	-	-	$+^{-}$	$-^{+}$	$-^{+}$
Adonitol	-	-	V	$-^{+}$	$-^{+}$
Dulcitol	-	-	$-^{+}$	$-^{+}$	$-^{+}$

(+) = positive, (-) = negative, v = variable, ($+^{-}$) = major positive, ($-^{+}$) = major negative.
A = acetic acid, B = butyric acid, F = formic acid, L = lactic acid, P = propionic acid,
S = succinic acid.

The presence of the fermentation products, among which acetic acids generally predominates over lactic acid as the major final product, is considered one of the most important diagnostic tests for the genus *Bifidobacterium*. The most direct, reliable and most important, fruitful assignment of a bacterial strain to the genus *Bifidobacterium* is the demonstration in cellular extracts of the presence of fructose-6-phosphate phosphoketolase, the key enzyme of bifidobacteria hexose metabolism. Almost all biochemical tests, such as the demonstration of catalase, nitrate reduction, formation of indole, liquefaction of gelatin, gas formation from glucose, and response to rhamnose, sorbose, glycerol, erythritol, adonitol, and dulcitol, are negative for the genus *Bifidobacterium*.

1.2.2.4. Genetic adaptation of bifidobacteria to the gastrointestinal tract

Bioinformatic analysis of bifidobacteria strains have revealed several physiological traits that explain the adaptation of this bacteria to the colon. A large number of genes have been identified that specialise for catabolism of a variety of oligosaccharides, some possible released by rare or novel glycosyl hydrolases acting on nondigestible plant polymers or host-derived glycoproteins and glycoconjugates. Many genes for oligosaccharide metabolism appear to have arisen due to gene duplication or horizontal transfer. This ability to scavenge from a large variety of nutrients likely contributes to the competitiveness of bifidobacteria in the colon. Furthermore, identification of particular polypeptides showed homology to most major proteins needed for adhesion and persistence in the GIT (Schell *et al.*, 2002).

1.3. Human gastrointestinal function and ecology

The human gastrointestinal tract (GI) consists of the mouth, oral cavity, esophagus, stomach, small intestine and colon. The large intestine begins at the ileocecal junction, with anatomically distinct regions being the cecum, ascending colon, transverse colon, descending colon and sigmoid colon (Gibson and Roberfroid, 1995). Healthy animals and humans have a natural microflora consisting of prokaryotic and eukaryotic microorganisms on their external and internal body surfaces: skin, upper respiratory tract, lower urogenital tract, oral cavity and gastrointestinal tract. The composition of the natural microflora in these natural microbial habitats is very complex and strictly determined by the local environment conditions.

1.3.1. Microbiological aspects of the large intestine

The large intestine harbours the largest and most complex microbial ecosystem associated with the human body, consisting of several hundred different strains of anaerobic bacteria, with numbers exceeding 10^{11}/g of intestinal contents (Fooks *et al.*, 1999; McBain and Macfarlane, 1998). This is because of the resident microbiota which comprises 10^{12} bacterial cells for every gram of gut contents. As the large intestine usually contains about 200 g of contents, there is enormous biological activity. The fact that these activities can be modulated, or perhaps even controlled, through diet is of high relevance (Gibson *et al.*, 2000). This microbiota is involved in the catabolism of a vast range of dietary and endogenously secreted compounds. The products of these biotransformations are often of toxicological significance to the host. For example, the occurrence of colon cancer is greatly influenced by diet, while metabolism of dietary components by intestinal bacteria has been demonstrated to be an important factor in tumour initiation. The colonic microflora may be involved in the aetiology of large bowel cancer by chemical modification or activation of a wide variety of

chemical agents with carcinogenic or co-carcinogenic potential. Exposure of the intestinal microbiota to potential toxicants may occur due to their presence in the diet by biliary excretion of endogenously metabolised substances into the intestine, enzymic activation of procarcinogens by the gut microflora or by direct production of mutagenic substances by intestinal microorganisms (McBain and Macfarlane, 1998). In terms of functionality, the human colon is the body's most metabolically active organ.

Besides vertical transmission of microorganisms, the body surfaces mentioned are contaminated during and directly after birth with a variety of microbial strains from the immediate environment (horizontal transmission). A number of these microorganisms will colonise these body surfaces permanently or temporarily, while others disappear. The temporarily colonising strains act as pioneers, which initiate the successive domiciliation of other microorganisms. During time, under normal conditions, the microflora will mature to a balanced composition of many different microorganisms.

The occupation of special niches depends on the local environmental circumstances in these specific habitats. These local conditions are determined by multifactorial interactive processes between the host and the microorganisms. Therefore, the mature composition of the natural microflora is specific to the animal species and even specific to an individual. In general, the natural microflora is called the indigenous microflora of a given species (including autochthonous and allochthonous microorganisms), and the indigenous microflora of a given individual (mainly autochthonous microorganisms), respectively. However, the terminology around symbiosis of microorganisms on body surfaces is often confusing.

Species specificity of the microflora has been shown in several studies. Characterisation of *Lactobacillus* and *Bifidobacterium* from humans and different animal species shows various biotypes. The exchange of *Lactobacillus* from one species to another shows that these bacteria do not colonise mutually and that normalisation of the intestinal

microflora do not occur by inoculating germ-free animals with a complex microflora from other animal species. Colonisation in specific habitats within the intestinal tract is demonstrated by the fact that some strains are found in crypts while others are found on epithelial surfaces of the villi and that some microorganisms (filamentous bacteria) are found on specific sites (columnar epithelium) in the small intestine.

During one's lifetime further adaptation of the indigenous microflora occurs, due to the changing local circumstances on the condition that these changes take place gradually, such as due to ageing, and not abruptly (e.g. by a sudden change in food composition or drug use).

A schematic review of the predominant microflora of human body surfaces has been published by Tannock (1988). In mature microbial ecosystems, most microorganisms are obligately anaerobic, even on surfaces which are in direct contact with the air. The density of the microbial population varies from site to site, but can reach 10^8 bacteria per ml of saliva and 10^{10} per gram contents of the large intestine. The numbers of eukaryotic cells are very low in comparison to prokaryotic cells. The diversity of microbial species or strains is thrilling. Progress in isolation and identification techniques, such as the use of anaerobic chambers, cell-wall analysis and DNA-DNA hybridisation, offer an increasing possibility to discriminate between the different microbial strains, for example, on the gene level.

1.3.1.1. Importance of an indigenous microflora

A well-established and matured indigenous microflora on external and internal body surfaces of animals and man is very stable. The penetration and colonisation of non-indigenous microorganisms from the environment and/or from other animal species (xenochthonous microorganisms) onto these body surfaces is hindered.

The importance of an indigenous microflora in the gut as a natural resistance factor against potential pathogenic microorganisms was already recognised in the 19th century by Metchnikoff during his research on cholera. Many decades later the role of the indigenous microflora received renewed interest after findings in laboratory animals orally treated with antibiotics. The antibiotics caused intestinal disturbances owing to infectious agents. It was suggested that this effect was induced by suppressing the normal gut microflora. Later on, the protective effect of the normal intestinal microflora in chickens against *Salmonella infantis* infection was shown by Nurmi and Rantala (1973). Colonisation resistance of the gut microflora was further confirmed for *Salmonella* and for other pathogenic bacteria such as *Escherichia coli*, *Clostridium* and *Yersinia enterocolitica*. Although Impey *et al.* (1982) demonstrated a protective effect of 48 selected bacterial strains, the colonisation resistance is most effective when a complete species-species microflora has settled down. This has been demonstrated in 'normalisation' studies with germ-free animals inoculated with several dilutions of the total intestinal microflora from normal animals of the same species.

The colonisation resistance induced by an indigenous microflora is partly based on occupation of available niches (competitive inhibition of binding sites) and autogenic regulation factors (e.g. synthesis of fatty acids, hydrogen peroxide, bacteriocins). Another important factor might be the non-specific activation of the immune system. The gut, the mucosa, as well as the skin, have humoral and cellular immune systems which can influence the composition of the microflora (gut microflora). It has been shown that the activity of the immune system of germ-free animals is very low since less γ-globulin, smaller lymph nodes and fewer lymphocytes and phagocytes were found. Activation of macrophages was noticed following the introduction of indigenous microorganisms. In addition to this stimulating effect on non-specific resistance factors, the gut microflora has also an important complementary

function in the digestion of dietary components, such as plant polymers and the synthesis of vitamins (Wood, 1996).

1.3.1.2. Establishment of bifidobacteria in infants

The composition of human fecal flora changes with advancing age (Naidu *et al.*, 1999). Initially, the foetus exists in a sterile environment until birth (Mackie *et al.*, 1999). After birth it rapidly becomes colonised by bacteria, especially bifidobacteria (Wolin *et al.*, 1999) from the maternal vagina and other environmental sources (Mutai and Tanaka, 1987). In both breast-fed and bottle-fed infants, the large intestine is first colonised by Enterobacteria, Streptococci, including Enterococci, Clostridia on the 1st to 2nd day of life. On the 3rd day, bacteroides, bifidobacteria and clostridia have been isolated from over 40% of infants. Between days 4 and 7, bifidobacteria becomes more predominant accounting for 10^{10}-10^{11} organisms per gram of faeces of breast-fed infants, exceeding enterobacteria by 100-1000 times. Clostridia, bacteroides, streptococci and staphylococci decrease, whereas enterobacteria are the predominant organisms in the bottle-fed infants, exceeding bifidobacteria by about 10 times. Thus, nearly 100% of all bacteria cultured from the stools of breast-fed infants were bifidobacteria. At 1 month of age, bifidobacteria were the most prevalent organisms in both groups but the number of these organisms in the stool of bottle-fed infants was approximately one-tenth that of breast-fed infants.

Benno and Mitsuoka (1986) also made efforts to identify all isolates from the stools of healthy infants during the first week of life down to the species level. A total of 37 different species or biovars were obtained. No *Bifidobacterium* or *Eubacterium* sp. were recovered from any of the neonates on the first and second days of life. *Clostridium paraputrificum* and *Bacteroides fragilis* group were detected from the faeces on the first day of life. From the third to fourth day of age, however, *Bifidobacterium* sp. were recovered from the stools of

healthy neonates. The incidence of the *Bacteroides fragilis* group, *Clostridium tertium*, *C. paraputrificum* and *Klebsiella pneumoniae* also increased. *Escherichia coli* was the most common facultative species isolated from the stools of all healthy neonates. The next most common species, *Enterococcus faecalis*, *Streptococcus* and *Staphylococcus epidermis*, appeared on the first day after birth. At the end of the study period, *E. coli*, *E. faecalis*, *S. epidermis* and *Streptococcus* sp. had frequently been isolated from 90% of neonates.

1.3.1.3. Differences of the faecal flora between breast-fed and bottle-fed infants

Tissier's turn of the century microscope observations of the faeces of breast milk-fed infants are still valid, but the situation regarding formula-fed babies appears to have altered with improvements to formula feeds which now resemble, but still do not exactly match, the composition of human milk. Well documented modern studies show that bifidobacteria are just as common and likely to be numerically dominant in the faeces of formula-fed as in breast-fed infants. There is considerable infant-to-infant variation in the population size of particular bacterial genera during the first week of life in both infant groups which may have contributed to the somewhat variable interpretations of the status of the infant microflora reported in the literature. More consistent values are obtained in babies older than one week, however, and realistic comparisons between infant groups are possible.

Colonization of the gastrointestinal tract of newborn infants occurs within a few days of birth (Simon and Gorbach, 1984). The inoculum may be derived either from the mother's vaginal or faecal flora (in a conventional birth) or from the environment (in a caesarian delivery). Initially, facultative bacterial species such as *Escherichia coli* or streptococci, are transferred. These are relatively nutritionally undemanding bacteria. Subsequently, their activities create a highly reduced environment which allows the development of the strictly anaerobic bacteria that will later dominate the colon. Dependent on the type of feeding regime

given in early life, there appears to be variability in microflora development. The breast-fed infant has a preponderance of bifidobacteria, which easily outcompete other genera. In contrast, the formula-fed infant has a more complex flora which resembles the adult gut in that bacteroides, clostridia, bifidobacteria, lactobacilli, Gram positive cocci, coliforms and other groups are all represented in fairly equal proportions. The type of delivery, dietary constituents and gestational age influence the colonization pattern. The initial period of bacterial colonization in the colon takes place over approximately a two-week period. During this period the bacterial colonization is similar for formula- and breast-fed infants. *Escherichia coli* and *Streptococcus* are always the first organism to be detected, at concentrations between 10^8 and 10^{10} organisms per gram of faeces.

Several anaerobic organisms, namely *Bifidobacterium*, *Clostridium* and *Bacteroides*, often then take up residence in the gastrointestinal tract. In breast-fed infants a major decrease in the bacterial populations of *E. coli* and *Streptococcus* then occurs, as well as partial or complete disappearance of *Clostridium* and *Bacteroides* resulting in predominance of *Bifidobacterium*. In formula-fed infants these reductions or disappearances do not take place, resulting in a more complex flora. The relatively simple flora of the breast-fed baby remains until dietary supplementation occurs. Upon introduction of other foods to the diet of the breast-fed infant there is a return of *E. coli*, *Streptococcus* and *Clostridium* to the faeces. The differences between breast-fed and formula-fed infants disappear. There is then a transition period, which continues into the second year of life in which the intestinal flora evolves to resemble that of the adult.

The classical studies concerning the acquisition of the infant intestinal microflora are generally considered to be those of Tissier (1899). He divided the colonization of the intestinal tract of suckled infants into three phases. In the first phase, which consisted of the first few hours of life, the faeces were devoid of microbes. The second phase began between

the 10^{th} and 20^{th} hour of life with the detection of a heterogenous collection of microbial types in the faeces. After three days, by which time milk had passed through the length of the intestinal tract, the third colonization phase began. A Gram-positive bacillus became the numerically dominant microbe in the faeces at this stage judged by microscope examination of faecal smears. The other microbial types disappeared in a fairly consistent manner, and from the start of the third and fourth day of life until weaning the collection of microbes in the faeces remained the same.

The faecal flora of breast-fed infants is relatively simple. Bifidobacteria are the dominant organisms, accounting for about 99% (range 85% to more than 99%) of cultivable flora. Coliforms, enterococci and lactobacilli comprise about 1% (range 1-15%) of the faecal flora, while bacteroides, clostridia, and other organisms may be absent or insignificant. The stools have an acid pH (4·5-5·5). Since the work of Tissier (1899), it has been believed that bifidobacteria are found exclusively in faeces of breast-fed infants, whereas in bottle-fed infants *Lactobacillus acidophilus* is the most commonly found organism. Twenty-one genera and 103 species or biovars of microorganisms were isolated from the faeces of the breast-fed infants and 20 genera and 97 species or biovars from the bottle-fed infants. The organism that showed the highest number and the highest frequency of occurrence in both groups was *Bifidobacteium breve. Bifidobacterium infantis*, which was formerly the most prevalent *Bifidobacterium* species in baby faeces, was never isolated at this time.

1.3.1.4. The faecal flora of children and adults

The compositions of the bacterial flora in the large intestine and faeces of different age groups may differ. The most prevalent bacteria in the faeces of infants and adults are obligate anaerobes, while facultative anaerobes are generally expected to account for less than 10^3 of anaerobe numbers. During weaning, bifidobacteria decrease by 1 log, the species and biovars

alter from infant-type to adult-type, and a remarkable proliferation of bacteroides, eubacteria, peptostreptococcaceae, and clostridia occur. The faecal flora of children closely resembles that of adults, where the numbers of bacteroidaceae, eubacteria, peptococcaceae, and usually clostridia outnumbered bifidobacteria, which constitute 5 to 10% of the total flora. The counts of Enterobacteriaceae and Streptococci decrease to less than 10^8 per gram of faeces, but the counts are usually less than 10^7 per gram of faeces (Mitsuoka & Hayakawa, 1973).

In adults, the flora of the large intestine is more complex that that of children. The stools of adults have a low redox potential (E^h), a neutral or slightly alkaline pH (6·0-7·0 or above), a typical odour and colour, and they contain relatively large amounts of putrefactive products, such as ammonia, amines, phenols, and degraded bile acids. A comparison of faecal flora in healthy adults and elderly persons is shown in Table 1.10.

Table 1.10. Comparison of faecal flora of healthy adults and elderly persons (Wood, 1996).

Intestinal flora	Healthy adults (42)[a]		Aged (54)[a]	
Total counts	11.2 ± 0.2[b]		11.1 ± 0.2	
Bacteroidaceae	10.9 ± 0.2	(100)[c]	10.9 ± 0.3	(100)[c]
Eubacterium	10.4 ± 0.4	(100)	10.1 ± 0.7	(100)
Peptococcaceae	10.2 ± 0.3	(100)	10.0 ± 0.7	(100)
Bifidobacterium	10.0 ± 0.8	(100)	9.4 ± 0.8	(85.2)
Veillonella	7.4 ± 1.2	(78.6)	5.2 ± 2.0	(61.1)
Megasphaera	9.0 ± 0.5	(33.3)	8.5 ± 1.1	(16.7)
Curved rods	9.7 ± 0.5	(23.8)	9.3 ± 0.9	(29.6)
Clostridium perfringens	4.4 ± 1.2	(45.5)	6.6 ± 2.0	(83.3)
Clostridium-other	9.5 ± 0.5	(66.7)	9.6 ± 0.8	(100)
Lactobacillus	5.8 ± 2.1	(90.5)	7.5 ± 1.7	(98.1)
Enterobacteriaceae	7.8 ± 0.8	(100)	8.2 ± 1.3	(100)
Streptococcus	7.9 ± 1.4	(100)	7.4 ± 1.6	(100)
Staphylococcus	3.1 ± 0.7	(78.6)	3.8 ± 1.2	(44.4)
Corynebacterium	5.3 ± 2.2	(35.7)	4.7 ± 2.0	(7.4)
Yeasts	3.9 ± 1.6	(42.9)	4.7 ± 1.5	(72.2)

[a] Number of subjects examined.
[b] Mean ± SD of the bacterial counts $\log_{10}$ per gram wet faeces.
[c] Frequency of occurrence (%)

1.3.1.5. Faecal flora of elderly persons

In adults, little is known about the influence of the ageing process on faecal microflora (Andrieux *et al.*, 2002). Research conducted by Mitsouka & Hayakawa (1973) compared the faecal flora of healthy adults and elderly persons. In elderly persons bifidobacteria decrease or diminish, clostridia including *C. perfringens* significantly increase, while lactobacilli and Enterobacteriaceae also increased (Fujisawa *et al.*, 1990; Hopkins and Macfarlane, 2002). However, the counts of streptococci decrease as a person ages.

1.3.1.6. The bifidus factor in breast milk

The presence of certain glycoproteins and soluble oligosaccharides in human breast milk is thought to select for bifidobacteria. In fact, the original `bifidus factor' was isolated from human breast milk. Interestingly, these bacteria are protective with regard to large intestinal infections. As such, they are common targets for prebiotic intake and may also help to explain the low incidence of gut disorder seen in breast-fed infants. During the weaning stage, the microbiota becomes more developed and the ecosystem is thought to be reasonably stable at around two years of age. Microbial colonization of the neonate occurs within the first 24 hours following birth. Proliferation of microbial types in body sites appears to be initially unchecked, resulting in a heterogenous collection of microbes. Soon, however, regulatory mechanisms generated within habitats (autogenic factors) and by external forces (allogenic factors) permit the continuing presence of some microbial types in the body's ecosystems that occur in microbial populations inhabiting the young animal provide an example of a biological succession. Eventually, usually after weaning, the microbial composition of the microflora becomes more stable and the adult microflora (in ecological terms, the climax community) is attained.

A small amount of evidence is available that suggests that the normal microflora, at least in humans, is influenced by genetic determinants of the host. The oral and nasal microfloras, and perhaps that of the faeces, are more similar in comparisons of twins than in the case of singleton children. Although a climax faecal microflora characteristic of humans is recognisable, variation among individuals is greater than variation between samples collected from a single individual.

1.3.1.7. Activity of bacteria in the gastrointestinal tract

In terms of the microbiology of different digestive tract areas, there is variability both in terms of composition and activity. The lumen of the human stomach is essentially sterile due to a low gastric pH. However, micro-organisms are known to reside in the mucosal layer that overlies the gastric epithelium. In the small intestine, the transit time of gut contents tends to maintain bacterial numbers at below 10^6/ml contents. Intestinal secretions like pancreatic enzymes and physiochemical variables such as pH and E^h also contribute towards the type of microflora that develops. The upper small gut is dominated by facultatively anaerobic and aero-tolerant bacteria such as streptococci, staphylococci and lactobacilli, with bacterial numbers showing a progressive increase.

1.3.1.8. Gastrointestinal bacteria influenced by diet

Most work on the microbes of the large bowel of adults has concentrated on the analysis of fecal samples. The composition of the fecal microbiota has been the subject of many investigations and has been summarized in numerous reviews. Most studies focus on enumeration of the major groups of microbes with some studies characterised to the genus level. Few workers have been as thorough as Moore and Holdeman (1974) who identified the species level wherever possible.

Populations of faecal bacteria constitute a major proportion (approximately 50%) of faeces (Tomomatsu, 1994). Findings by Moore and Holdeman (1974) noted that despite considerably variation in diet and health, numbers of the major bacterial groups in all subjects were remarkably similar. Bacteroides were found in highest numbers in all fecal numbers. The most frequently isolated anaerobic cocci were peptostreptococci, ruminococci, viellonella, and anaerobic streptococci, with Peptostreptococcus being the most commonly isolated species. Of the anaerobic Gram-positive nonsporing rods, eubacteria were found in highest numbers.

1.3.2. Structure and function of the gastrointestinal tract (GIT)

The gastrointestinal tract is a tube extending from the lips to the anus and is divided into various well-defined anatomical regions. The digestive and absorptive functions are well known but, in addition to being an organ in the body, the intestine acts as a container for the most intimate portion of the chemical environment. Assimilation of food is not the only physiological function of the alimentary tract. It is also concerned with the excretion of chemical waste, the control of body metabolism and immune response. Furthermore, the gut harbours a complex ecosystem.

1.3.3. Bacterial fermentations in the large intestine

It is clear that a complex, resident gut flora is present in humans. While the transit of residual foodstuffs through the stomach and small intestine is probably too rapid for the microflora to exert a significant impact, this slows markedly in the colon. The average transit time is around 70 h, but can be higher. As such, colonic microorganisms have ample opportunity to degrade available substrates. These may be derived from either the diet or by endogenous secretions. Fermentations by gut bacteria consist of a series of energy yielding

reactions that do not use oxygen in the respiratory chains. The electron acceptors may be organic (e.g. some products of the fermentations) or inorganic (e.g. sulphate, nitrate). As carbohydrates form the principal precursors for fermentation, ATP is usually formed through substrate level phosphorylation by saccharolytic micro-organisms. The fermentation process in the large gut is influenced by a variety of physical, chemical, biological and environmental factors. Factors affecting fermentation in the large intestine are presented in Table 1.11.

Table 1.11. Factors affecting fermentation in the human large intestine (Gibson and Fuller, 2000).

- Amount of substrate available for fermentation
- Colonic transit time
- Physical form of the substrate
- Chemical composition of the substrate
- pH of gut contents
- Composition of the gut microbiota with respect to species diversity and relative numbers of different types of bacteria
- Ecological factors including competitive and cooperative interactions between bacteria
- Rates of depolymerisation of substrates
- Substrate specificities and catabolite regulatory mechanisms of individual gut species
- Fermentation strategies of individual substrate utilising bacteria
- Antibiotic therapy
- Availability of inorganic electron acceptors

Major substrates available for the colonic fermentation are starches that, for various reasons, are resistant to the actions of pancreatic amylases and can be degraded by bacterial enzymes as well as dietary fibres like pectins and xylans. Other carbohydrate sources available for fermentation in lower concentrations include oligosaccharides and a variety of sugars and non-absorbable sugar alcohols.

1.3.3.1. Metabolites produced in the large intestine

In terms of end products, a variety of different metabolites arise. Predominant among these are the short chain fatty acids (SCFA), acetate, propionate and butyrate. The majority are absorbed into the bloodstream and can be further metabolised systemically. Transport to, and further metabolism of SCFA in the liver, muscle or other peripheral tissues is thought to contribute about 7 to 8% of host daily energy requirements. Other products include sink metabolites, such as ethanol, pyruvate and lactate, which are mostly further converted to SCFA and therefore not allowed to accumulate to any significant level in the large bowel. The resulting end products due to carbohydrate fermentation with the major bacterial groups involved and metabolic fate are presented in Table 1.12.

Table 1.12. Predominant products of carbohydrate metabolism in the human colon (Gibson and Fuller, 2000).

End product	Bacterial group involved	Metabolic fate
Acetate	*Bacteroides, bifidobacteria, eubacteria, lactobacilli, clostridia, ruminococci, peptococci, Veillonella, peptostreptococci, propionibacteria, fusobacteria, butyrivibrio*	Metabolised in muscle, kidney, heart and brain
Propionate	*Bacteroides, propionibacteria, veillonella*	Cleared by the liver; possible gluceogenic precursor; suppresses cholesterol synthesis
Butyrate	*Clostridia, fusobacteria, butyrivibrio, eubacteria, peptostreptococci*	Metabolised by the colonic epithelium; regulator of cell growth and differentiation
Ethanol, succinate, lactate, pyruvate	*Bacteroides, bifidobacteria, Lactobacilli, eubacteria, peptostreptococci, clostridia, ruminococci, actinomycetes, enterococci, fusobacteria*	Absorbed; electron sink products further fermented to SCFA
Hydrogen	*Clostridia, ruminococci, fusobacteria*	Partially excreted in breath; metabolised by hydrogenotrophic bacteria

1.3.4. Cancer of the large intestine

The large intestine is the second most common site for carcinoma in man and faeces from individuals living in Western societies frequently contain mutagenic substances as indicated by the Ames test. There is no general agreement regarding the aetiology of bowel cancer, although factors such as diet, environment and genetics have been implicated. It has been speculated that tumours occur 100 times more often in the hindgut than in the small intestine, indicating that the colonic microbiota plays an important role in carcinogenesis. It has been suggested that a mechanism whereby intestinal bacteria may be involved in these processes is by the production of carcinogenic metabolites from non-toxic precursor molecules, and a variety of hydrolytic and reductive enzymes responsible for carcinogen production are produced by colonic micro-organisms (McBain and Macfarlane, 1998).

1.3.4.1. Conditions favourable for cancer

Colorectal cancer is the second largest cause of cancer deaths in western countries (Kulkarni *et al.*, 1994; Challa *et al.*, 1997; Singh *et al.*, 1997). Studies have suggested the involvement of intestinal microflora in the pathology of colon cancer. Epidemiological and experimental studies provide evidence that nutritional factors play a role in the aetiology of colon cancer (Kulkarni and Reddy, 1994). Lactulose, a disaccharide and keto hydrolytic product of β-galactosidase is not absorbed in the small intestine. These sugars are used as a substrate by bifidobacteria resulting in an increase in the number of bifidobacteria in faeces (Salminen *et al.*, 1993; Gibson *et al.*, 1994). These sugars enhance the selective proliferation and colonisation of bifidobacteria. Shifts in the colonic flora, i.e. an increase in anaerobes and a decrease in aerobes have been found in populations which are at increased risk of colon cancer. With respect to colon carcinogenesis, the fermentation of carbohydrate and dietary fibre by colonic bacteria to short chain fatty acids is of major interest. An acidic pH level in

the colonic lumen caused by increased production of short chain fatty acids inhibits the bacterial degradation of primary to secondary (deconjugated) bile acids, which have been shown to promote colon cancer in carcinogen-treated rats.

1.3.4.2. Bacterial prevention of cancer

Many reported studies have shown the beneficial effect of consuming specific lactic acid bacteria in the prevention of chronic conditions such as cardiovascular disease and cancer. These lactic cultures which are primarily used for fermentation of milk and other dairy products have shown to possess antimutagenic and anticarcinogenic properties and from epidemiological and experimental studies reduce certain types of cancer and inhibit tumour growth (Singh *et al.*, 1997). Japanese research by Kubota (1990) found that colon cancer incidence was lowest when the colonic population of bifidobacteria was highest and that of *Clostridium perfringens* was lowest.

Results of the study carried out by Challa *et al.* (1997) indicate that *Bifidobacterium* and lactulose exert an additive antitumorigenic effect in rat colon. While the mechanism of inhibition of colon carcinogenesis by dietary *B. longum* has not been clarified, it is likely that the effect of lactic bacteria can proceed through diverse mechanisms. These may include the alteration of physiological conditions in the colon affecting the metabolic activity of intestinal microflora, the action of bile acids, and to quantitative and/or qualitative alterations in the bile acid-degrading bacteria. The species of lactobacilli and *Bifidobacterium*, most often suggested as beneficial dietary supplements, have all been reported to exert anatagonistic actions toward several enteropathogenic organisms in the intestine such as *Escherichia coli* and *Clostridium perfringens*. *C. perfringens* and other enteropathogenic anaerobic bacteria contain high levels of 7α-dehydroxylase, which is an important enzyme in the formation of the secondary bile acids from the primary bile acids in the colon. These secondary bile acids have been shown to

play a role as tumour promoters in the colon. Evidence of correlations between the incidence of colon cancer and the number of bacteria per gram of faeces possessing 7α-dehydroxylase enzyme activity have been found in humans. It is then possible to assume that dietary lactic cultures modulate the metabolic activity of intestinal microflora and the activity of 7α-dehydroxylase thereby producing lower levels of secondary bile acids in the colon (Reddy *et al.*, 1993).

1.3.5. Natural microflora in gastrointestinal tract

Many different types of bacteria representing most bacterial groups have at some time been isolated from the intestine. Those isolated most frequently can be considered as members of the resident flora or as contaminants from the environment. The number of bacterial groups that may be detected is related to the methods used for their detection. Very few investigators have attempted a systematic investigation of the intestinal bacteria and so any list of the species present in the gut must be provisional.

Numerically, the most important genus of intestinal bacteria in animals and man is *Bacteroides*. This along with *Fusobacterium* which contains pathogenic species and *Leptotrichia* which is also found in the mouth comprise the family *Bacteroidaceae* which also contains members of the former genus *Sphaerophorus*. These are all Gram-negative, strictly anaerobic, non-sporing rods although some may show varying degrees of polymorphism. *B. fragilis* is ubiquitous in animals and man.

Amongst the Gram-positive, non-sporing rods several genera are numerically important in the gut. Obligately anaerobic types include *Propionibacterium* (mainly *P. acnes*), *Eubacterium* and *Bifidobacterium*, including *B. bifidum* and *B. infantis* from the faeces of breast-fed infants. Among the facultative anaerobes the genus *Lactobacillus* contains many species occurring in the gut of most warm-blooded animals. Although numerically important

throughout the alimentary tract their ecological significance has not been conclusively elucidated.

Several types of spore-forming rods and cocci are normal inhabitants of the gut. The genus *Clostridium* is probably the most ubiquitous. Others such as *C. perfringens, C. bifermentans* and *C. tetani* are found regularly, albeit in relatively low numbers but are of significance in humans.

Facultative and obligately anaerobic Gram-positive cocci are numerically important in the gut. The facultatively anaerobic streptococci are well represented by many species from Lancefield group D including *Streptococcus faecalis*, *S. bovis* and *S. equinus* and come from group K such as *S. salivarius* which is usually associated with the mouth. Gram-negative anaerobic cocci include the closely related genera *Veillonella* which utilises lactic acid and *Acidaminococcus* which can utilise amino acids as a sole energy source.

Although they are not numerically important, the Gram-negative facultatively anaerobic rods include a number of very important pathogens. Members of the related genera *Vibrio* and *Campylobacter* cause enteric disease in man and animals. The taxonomy of the *Enterobacteriaceae* is complex and while some genera such as *Proteus* and *Klebsiella* and many serotypes of *E. coli* and *Salmonella* are commensal in animals particular biotypes of the latter two genera are major animal pathogens, particularly affecting the young. Although *Shigella* causes dysentery in humans, it may be found existing as a commensal in the gut of other warm-blooded animals.

The significance of the presence of yeasts and moulds in the gut is uncertain. Some yeasts associate with the murine stomach wall, but in general they are thought to be transient contaminants. A total bacterial count of major groups of bacteria within each section of the gastrointestinal tract is presented in Table 1.13.

Table 1.13. Distribution of human gastrointestinal flora in different segments of the gastrointestinal tract (Simon and Gorbach, 1984).

	Stomach	Jejunum	Ileum	Faeces
Total bacterial count	$0\text{-}10^{3a}$	$0\text{-}10^{5}$	$10^{3}\text{-}10^{7}$	$10^{10}\text{-}10^{12}$
Aerobic or facultative				
Anaerobic bacteria				
Enterobacteria	$0\text{-}10^{2}$	$0\text{-}10^{3}$	$10^{2}\text{-}10^{6}$	$10^{4}\text{-}10^{10}$
Streptococci	$0\text{-}10^{3}$	$0\text{-}10^{4}$	$10^{2}\text{-}10^{6}$	$10^{5}\text{-}10^{10}$
Staphylococci	$0\text{-}10^{2}$	$0\text{-}10^{3}$	$10^{2}\text{-}10^{5}$	$10^{4}\text{-}10^{7}$
Lactobacilli	$0\text{-}10^{3}$	$0\text{-}10^{4}$	$10^{2}\text{-}10^{5}$	$10^{6}\text{-}10^{10}$
Fungi	$0\text{-}10^{2}$	$0\text{-}10^{2}$	$10^{2}\text{-}10^{3}$	$10^{2}\text{-}10^{6}$
Anaerobic bacteria				
Bacteroides	Rare	$0\text{-}10^{2}$	$10^{3}\text{-}10^{7}$	$10^{10}\text{-}10^{12}$
Bifidobacteria	Rare	$0\text{-}10^{3}$	$10^{3}\text{-}10^{5}$	$10^{8}\text{-}10^{12}$
Gram-positive cocci	Rare	$0\text{-}10^{3}$	$10^{2}\text{-}10^{5}$	$10^{8}\text{-}10^{11}$
Clostridia	Rare	Rare	$10^{2}\text{-}10^{4}$	$10^{6}\text{-}10^{11}$
Eubacteria	Rare	Rare	Rare	$10^{9}\text{-}10^{12}$

[a] Number per gram of intestinal contents.

Presented in Table 1.14 is a detailed description of bacterial species isolated from the healthy human intestine grouped by Gram stain, oxygen requirements and morphology.

Table 1.14. Some bacterial species isolated from the healthy human intestine (Drassar and Barrow, 1985).

Anaerobic bacteria Gram-negative anaerobic rods	**Gram-positive anaerobic rods**	**Gram-positive anaerobic cocci**	**Facultatively anaerobic bacteria**
Bacteroides assacharolyticu,	*Clostridium bejerinki*	*Peptococcus*	*Streptococcus faecalis*
Bacteroides capillosus	*Clostridium butyricum*	*asaccharolyticus*	*Streptococcus faecium*
Bacteroides coagulans	*Clostridium cadaveris*	*Peptococcus magnus*	*Streptococcus bovis*
Bacteroides distasonis	*Clostridium celatum*	*Peptococcus prevotii*	*Streptococcus agalactiae*
Bacteroides eggerthii	*Clostridium clostridiiforme*	*Ruminococcus albus*	*Streptococcus anginosus*
Bacteroides fragilis	*Clostridium difficile*	*Ruminococcus bromii*	*Streptococcus avium*
Bacteroides furcosus	*Clostridium inoculum*	*Ruminococcus*	*Streptococcus cremoris*
Bacteroides hypermegas	*Clostridium leptum*	*flavefaciens*	*Streptococcus equisimilius*
Bacteroides acteroides	*Clostridium malenominatum*	*Streptococcus*	*Streptococcus lactis*
Bacteroides multiacidus	*Clostridium nexile*	*constellatus*	*Streptococcus mitior*
Bacteroides ovalis	*Clostridium paraputrificum*	*Streptococcus*	*Streptococcus mutans*
Bacteroides ovatus	*Clostridium perfringens*	*intermedius*	*Streptococcus salivarius*
Bacteroides praeacutus	*Clostridium ramosum*	*Streptococcus*	*Streptococcus sanguis*
Bacteroides putredinis	*Clostridium tertium*	*morbillorium*	*Streptococcus epidermidis*
Bacteroides ruminocola	*Bifidobacterium adolescentis*	*Megasphera elsdenii*	*Lactobacillus casei*
Bacteroides s.s. brevis	*Bifidobacterium angulatum*		*Lactobacillus fermentum*
Bacteroides ruminocola s.s. ruminicola	*Bifidobacterium bifidum*	*Peptostreptococcus productus*	*Lactobacillus leichmannii*
	Bifidobacterium breve		*Lactobacillus minutes*
Bacteroides splanchinicus	*Bifidobacterium catenulatum*		*Lactobacillus plantarum*
Bacteroides thetaiotaomicron	*Bifidobacterium cornutum*	*Sarcina ventriculi*	*Lactobacillus rogosae*
Bacteroides uniformis	*Bifidobacterium dentium*		*Lactobacillus ruminis*
Bacteroides vulgatus	*Bifidobacterium infantis*	*Gemiger formicilis*	*Lactobacillus salivarius*
	Bifidobacterium longum		*Lactobacillus acidophilus*
	Bifidobacterium pseudolongum		*Lactobacillus brevis*
Fusobacterium mortiferum	*Eubacterium aerofacien*	*Acidaminococcus*	*Enterobacteriaceae*
Fusobacterium naviforme	*Eubacterium contortum*	*fermantans*	*Escherichia coli*
Fusobacterium necrogenes	*Eubacterium cylindroids*	*Veillonella parvula*	*Citrobactor freundii*
Fusobacterium nucleatum	*Eubacterium lentum*		*Klebsiella pneumoniae*
Fusobacterium plauti	*Eubacterium limosum*	*Coprococcus cutactus*	*Enterobacter cloacae*
Fusobacterium prausnitzii	*Eubacterium rectale*	*Coprococcus catus*	*Enterobacter aerogenes*
Fusobacterium russi	*Eubacterium ruminantium*	*Coprococcus comes*	*Proteus mirabilis*
Fusobacterium symbiosum	*Eubacterium tenue*		*Proteus morganii*
Fusobacterium varium	*Eubacterium tortuosum*		
	Eubacterium ventriosum		
Leptotrichia buccalis	*Propionbacterium acnes*		
Butyrivibrio fibriosolvens	*Propionbacterium granulosm*		
Sucinimonas amylolytia	*Propionbacterium jensenii*		
Desulfomonas pigra	*Lachnospira multiparus*		
Vibrio succinogenes			

1.3.6. Bacteria influenced by diet

1.3.6.1. Influence of diet on faecal bifidobacterial flora

It is often reported that the compositions of intestinal flora are influenced by diet. The quality of diet can immensely affect human health preventing and reducing susceptibility to particular diseases (Gibson, 1999; Kolida *et al.*, 2000). The physical and physiological characteristics of the gastrointestinal tract and its epithelial layer are greatly affected by the presence of a complex microflora whose density varies according to the section of the intestine colonized. The sensitivity of intestinal microorganisms to gastric acid and oxygen largely determines the sites of colonization. Since the oxidation-reduction potential (E^h) varies according to the microbial population level, the microflora itself controls certain aspects of its own environment. Some facultatively anaerobic groups of bacteria, such as the lactobacilli, streptococci and coliform bacteria, are ubiquitous and are distributed throughout most of the tract. Obligately anaerobic bacteria such as *Bacteroides* and *Bifidobacterium* are confined to parts of the gut where E^h values are very low. Such sites include the colon, caecum and the rumen or rumen-like anatomical modifications of the stomach in those animals possessing a fore-gut microbial fermentation.

Although diet is important in determining the qualitative and quantitative composition of the intestinal microflora, it is difficult to demonstrate experimentally. While the adult flora is characteristic of the host species that of the neonatal mammal is common to a wide range of species since the milk diet produces a common environment in the gut. Characteristic of the gut flora of neonates are low numbers of potentially pathogenic species such as *E. coli*. These low numbers are maintained by the inhibitory effects of specific antibody (mainly IgA) and several non-specific factors including the iron binding protein, lactoferrin.

Because of the complex intestinal flora, adult animals are normally extremely difficult to infect with enteric pathogens. Disturbance or removal of the flora (for example by antibiotics) thus increases susceptibility to colonisation by these organisms. An additional consequence of oral antibiotic administration is that commensal and pathogenic bacteria may become resistant to these drugs by mutation or by transferable drug resistance. Both these problems are of considerable significance to animal and public health. Because of this renewed attempts are being made to induce changes in the intestinal flora of animals and man, beneficial to host health, by feeding normal constituents of the gut flora or fermented milk products. The characteristics of particular genera commonly found in human faeces are presented in Table 1.15 including metabolic products and metabolic processes.

Table 1.15: Characteristics of bacterial genera commonly detected in human faeces (Tannock, 1995).

Genera	Characteristic
Bacteroides	Gram-negative, nonspore-forming bacilli. Obligate anaerobe. Metabolic products include combinations of acetic, succinic, lactic, formic or propionic acids. If *N*-butyric acid is produced, isobutyric and isovaleric acids are also present.
Bifidobacterium	Gram-positive, nonspore-forming, nonmotile bacilli sometimes club-shaped or spatulated extremities. Obligate anaerobe. Acetic and lactic acids are produced primarily in the molar ratio of 3:2. Glucose is degraded exclusively and characteristically by the fructose-6-phosphate 'shunt' metabolic pathway.
Clostridium	Gram-positive bacilli that form endoscopes. Obligate anaerobe.
Enterococcus	Gram-positive cocci. Facultative anaerobe. Lancefield group D. Can grow in 6.5% NaCl broth and in broth at pH 9.6.
Eubacterium	Gram-positive bacilli, nonspore-forming. Obligate anaerobe. Produces mixtures of organic acids including butyric, acetic and formic acids.
Fusobacterium	Gram-negative, nonspore-forming bacilli. Obligate anaerobe. *N*-butyric acid is produced, but isobutyric and isovaleric acids are not.
Peptostreptococcus	Gram-positive cocci. Obligate anaerobe. Can metabolise peptone and amino acids.
Ruminococcus	Gram-positive cocci. Obligate anaerobe. Amino acids and peptides are not fermented. Fermentation of carbohydrate produces acetic, succinic and lactic acids, ethanol, carbon dioxide and hydrogen.

The faecal flora of nine rural healthy Japanese and eight urban healthy Canadians were compared (Benno *et al.*, 1986). The two populations are typical Japanese and western diets, respectively. The numbers of eubacteria, bifidobacteria, lactobacilli and veilloneallae and the frequency of occurrence of bifidobacteria were higher in the Japanese group than in the Canadians. Higher numbers of bacteroides and *C. perfringens* were found in the Canadians. Presented in Table 1.16. is the faecal flora of vegetarian and non-vegetarian Seventh Day Adventists. comparing the faecal flora of volunteers consuming high- and low-beef diets.

Table 1.16. Faecal microbiota in various dietary groups including seventh-day Adventists who were strictly vegetarian, Japanese who consumed an oriental diet that included fish but no beef, and healthy subjects who consumed a Western diet with relatively large quantities of beef (Gibson and Macfarlane, 1995).

	Strict vegetarian (13)		Japanese (15)[c]		Western (62)		Total[d] (141)	
Microorganisms	%[a]	Mean[b]	%	Mean	%	Mean	%	Mean
Bacterioides	100	11.7	93	10.8	100	11.3	99	11.3
Fusobacterium	0	-	40	8.1	24	8.6	18	8.4
Anaerobic *Streptococci*	8	11.4	60	9.5	32	10.5	34	10.3
Peptococcus	8	11.2	47	9.4	37	10.1	33	10.0
Peptostreptococcus	23	11.1	80	10.2	35	10.2	45	10.1
Ruminococcus	54	10.2	60	10.3	45	10.0	45	10.2
Anaerobic cocci	85	10.3	100	10.7	98	10.6	94	10.7
Actinomyces	31	10.5	0	-	2	5.7	7.8	9.2
Arachnia-propionibacterium	38	10.0	0	-	2	5.5	9.2	8.9
Bifidobacterium	69	10.9	80	9.7	79	10.4	74	10.2
Eubacterium	92	11.0	93	10.6	95	10.6	94	10.7
Lactobacillus	85	11.1	73	9.0	73	9.3	78	9.6
Clostridium	92	9.4	100	9.7	100	10.2	100	9.8
Streptococcus	100	8.6	100	8.7	100	9.1	99	8.9
Gram-negative facultatives	100	8.2	100	9.2	98	8.9	98	8.7
Candida albicans	15	4.9	47	5.6	14	5.4	14.2	5.4
Other yeasts	23	5.6	53	5.8	31	5.2	36.2	5.6
Filamentous fungi	0	-	0	-	3	3.8	3.5	5.9
Bacillus sp.	69	4.2	80	6.2	82	5.0	82.3	5.2
Total[e]	100	12.6	100	11.8	100	12.2	100	12.2

[a]% Positive. [b]Mean count expressed as organisms log_{10}/g dry weight faeces. [c]Number of subjects per dietary group. [d]Total for all 141 subjects including polyp. Colonic cancer, and vegetarians who consume some meat. [e]Total of all microbes detected (including other genera and groups not listed above).

In summary, it seems that no general agreement exists in regard to whether or not the bifidobacterial flora of individuals on high-meat diets differ from those of individuals on low-meat diets. However, these results, which were obtained using a comprehensive method for cultivating intestinal flora, indicated that a Japanese-style diet is slightly better than a western-style diet from the viewpoint of bifidobacteria in the intestinal flora.

Presented in Table 1.17. contains the 25 most prevalent bacteria species that are present in the faeces of human subjects.

Table 1.17: The 25 most prevalent bacterial species in the faeces of human subjects consuming a Western diet (10^{9-10} bacteria per gram wet weight) (Gibson and Macfarlane, 1995).

Bacteroides vulgatus	*Ruminococcus albus*	*Bifidobacterium adolescentis* A
Bacteroides species, other	*Bacteroides distasonis*	*Bifidobacterium adolescentis* C
Bacteroides fragilis	*Peptostreptococcus intermedius*	*Bacteroides clostridiiformis* ssp. *clostridiiformis*
Bacteroides thetaiotaomicron	*Peptostreptococcus*	*Peptostreptococcus prevotii*
Peptostreptococcus micros	*Peptostreptococcus productus*	*Bifidobacterium infantis* ssp. *liberorum*
Bacillus species (all)	*Eubacterium lentum*	*Clostridium indolis*
Bifidobacterium adolescentis D	*Facultative streptococci, other*	*Enterococcus faecium*
Eubacterium aerofaciens	*Fusobacterium russii*	*Bifidobacterium longum*

1.4. Therapeutic properties of probiotic bacteria

Over the last 20 years, there has been a remarkable interest and awareness, by both consumers and food manufacturers as consumers are becoming more aware of maintaining their 'internal health' by taking preventative rather than curative approaches to today's health problems (O'Sullivan, 1996). The use of live microbes as dietary adjuncts or 'probiotics' is a subject of intense and growing interest. Probiotics have been defined as living organisms, which when included as part of the diet, confer favourable effects on the host (Fuller, 1991). Several health benefits have been claimed to be associated with consumption of fermented milk products containing viable cells of probiotic bacteria. The proposal that the ingestion of certain viable organisms may provide health benefits has recently resulted in numerous investigations into this area.

A comprehensive list of the potential health effects of fermented milks by Ouwehand *et al.* (2003) have revealed prevention and reduction of symptoms including: hypercholesterolaemia, diarrhoea, inflammatory bowel disease, constipation, allergies, ulcers and irritable bowel syndrome, as well as reduction of colorectal, cervical, breast and bladder cancers. Improvement of lactose assimilation, blood pressure, food digestibility and oral health were also linked to ingestion of fermented milks.

Consumption of probiotics is shown to reduce faecal putrefactive bacteria such as Clostridia and Coliforms and improve the intestinal microecology. These probiotic bacteria have been shown to possess antimutagenic and anticarcinogenic properties (Rafter, 1995; Lankaputhra and Shah, 1998a). Ingestion of *L. acidophilus* and bifidobacteria reduces the risk of certain types of cancer and inhibits tumour growth (Goldin and Gorbach, 1980; Challa *et al.*, 1997; Singh *et al.*, 1997). Ingestion of fermented milks containing *L. acidophilus* reduces both quantities of β-glucuronidase, azoreductase, and nitroreductase formed by intestinal

putrefactive bacteria presumably involved as tumour promoters and acknowledged procarcinogens. *L. acidophilus*, bifidobacteria and *L. casei* are the main probiotic bacteria used in commercial products. Bifidobacteria are major components of the human intestinal microbial flora (Simon and Gorbach, 1984; Ibrahim and Bezkorovainy, 1993).

1.4.1. Properties required for probiotics to be effective in nutritional and therapeutic settings

A probiotic can be used exogenously or endogenously to enhance nutritional status and/or the health of the host. In the case of exogenous use, microorganisms are most commonly used to ferment various foods and by this process can preserve and make nutrients bioavailable. In addition, microorganisms can metabolise sugars, such as lactose in yoghurt, making this food more acceptable for consumption by individuals suffering from lactose malabsorption. However, the most interesting properties that probiotics acting exogenously can have is the production of substances that may be antibiotics, anticarcinogens or have other pharmaceutical properties. The properties required for exogenously derived benefits from probiotics are the ability to grow in the food or the media in which the organism is placed, and the specific metabolic properties which result in the potential beneficial effects stated above. The selection of organisms that can be helpful therapeutically and nutritionally would be based on specific properties that are desired. This can be achieved by either classical biological selection techniques or genetic engineering. Probiotics that are ingested by the host and exert their favourable properties by virtue of residing in the gastrointestinal tract have to have certain properties in order to exert an effect.

1.4.1.1. Requirements for probiotics

It is of high importance that the probiotic strain can survive the location where it is presumed to be active. For a longer and perhaps higher activity, it is necessary that the strain can proliferate and colonise at this specific location. Probably only host-specific microbial strains are able to compete with the indigenous microflora and to colonise the niches. Besides, the probiotic strain must be tolerated by the immune system and not provoke the formation of antibodies against the probiotic strain. So, the host must be immuno-tolerant to the probiotic. On the other hand, the probiotic strain can act as an adjuvant and stimulate the immune system against pathogenic microorganisms. It goes without saying that a probiotic has to be harmless to the host: there must be no local or general pathogenic, allergic or mutagenic/carcinogenic reactions provoked by the microorganism itself, its fermentation products or its cell components after decrease of the bacteria.

For the maintenance of its favourable properties the strain must be genetically stable. For the production of probiotics it is important that the microorganisms multiply rapidly and densely on relatively cheap nutrients and that they remain viable during processing and storage. Besides the specific beneficial property, these general requirements must be considered in developing new probiotics, but also for determining the scientific value of a claimed probiotic. A number of these requirements can be screened during *in vitro* experiments. We advise the drawing up of a decision-tree for the minimal requirements which can be tested *in vitro*, such as culture conditions and viability of the probiotic strains during processing and storage; sensitivity to low pH values, gastric juice, bile, pancreas, intestinal juice and intestinal or respiratory mucus; adherence to isolated cells or cell cultures and interactions with other (pathogenic) microorganisms. If these *in vitro* experiments are successful, further research can be performed during *in vivo* experiments in animals or

humans. Requirements of probiotics that are important for their use in humans are presented in Table 1.18.

Table 1.18. Requirements of probiotics (Salminen and von Wright, 1998).

- Survival of the environmental conditions on the location where it must be active
- Proliferation and/or colonisation on the location where it is active
- No immune reaction against the probiotic strain
- No pathogenic, toxic, allergic, mutagenic or carcinogenic reaction by the probiotic strain itself, its fermentation products or its cell components after decrease of the bacteria
- Genetically stable, no plasmid transfer
- Easy and reproducible production
- Viable during processing and storage

1.4.2. Claimed beneficial properties of probiotics

So far, it was shown that the indigenous microflora is host-specific and location-specific, very complex in composition and that it has beneficial properties to the host. However, it is not precisely known which species of microorganisms play the principal part in these beneficial properties. For man it is suggested that specific microbial strains could play an important role in;

- formation or reconstruction of a well-balanced indigenous intestinal and/or respiratory microflora, for example, in newborn children during admission to an ICU;
- after gastrointestinal decontamination by antibiotics in connection with bone-marrow transplantation;
- improving the colonisation resistance of the indigenous microflora of the intestinal, respiratory and urogenital tracts;
- lowering the serum cholesterol level;
- inhibiting the mutagenicity of the intestinal contents and reducing the incidence of intestinal tumours;

- non-specific interactions with the immune system;
- metabolising lactose and hence reducing lactose malabsorption (Jiang *et al.* 1996);
- improving the absorption of calcium and hence inhibiting decalcification of the bones in elderly people;
- synthesis of vitamins and pre-digestion of proteins.

Probiotic foods are becoming increasingly popular. A number of health benefits have been claimed for *Bifidobacterium* sp. and therefore inclusion of these organisms in the diet is considered to be important in maintaining good health (Champagne *et al.*, 1996). Probiotics have anticarcinogenic properties, a specific probiotic effect, which are of three types: (1) elimination of procarcinogens; (2) modulation of procarcinogenic enzymes; and (3) tumour suppression (Wollowski *et al.*, 2001; Grill *et al.*, 1995a). Furthermore, consumption of these organisms is an ideal method to re-establish the balance in the intestinal flora after antibiotic treatment (Gibson *et al.*, 1994). There is a growing agreement relating to the beneficial aspects of specific dairy products such as fermented milk and yoghurt and of bacterial cultures that ferment the dairy products in human and animal nutrition. Experimental and epidemiological studies provide evidence that fermented milk and bacterial cultures that are routinely used to ferment the milk reduce the risk of certain types of cancer and inhibit the growth of certain tumours and tumour cells (Reddy and Rivenson, 1993).

Many health promoting affects have been attributed to certain *Bifidobacterium* sp. (Rolfe, 2000) These include reduction of ammonia levels, stimulation of the immune system, alleviation of lactose intolerance and prevention of gastrointestinal disorders (O'Sullivan, 1996). Several probiotic bacteria have been introduced in the market and the range of products in which probiotic bacteria are added is increasing. However, many of the prophylactic and therapeutic properties of these foods containing bifidobacteria are a matter

of speculation because there are inherent difficulties in obtaining definitive evidence for proposed effects of ingesting bifidobacteria.

1.4.2.1. Viability of probiotic organisms

Microorganisms introduced orally have to, at least, transiently survive in the stomach and small intestine. Although this appears to be a rather minimal requirement, many bacteria including the yoghurt-producing bacteria *L. delbrueckii* subsp. *bulgaricus* and *S. thermophilus* often do not survive to reach the lower small intestine. The reason for this appears to be low pH of the stomach. In fasting individuals, the pH of the stomach is between 1.0 and 2.0 and most microorganisms, including lactobacilli, can only survive from 30 seconds to several minutes under these conditions. Therefore, in order for a probiotic to be effective, even the selection of strains that can survive in acid at pH 3.0 for sometime would have to be introduced in a buffered system such as milk, yoghurt or other food.

1.4.2.2. Antimicrobial properties of probiotic bacteria

As indicated previously, the intestinal microflora is a complex ecosystem. Introducing new organisms into this highly competitive environment is difficult. Thus organisms that can produce a product or products that will inhabit the growth or kill existing organisms in the intestinal milieu have a distinct advantage. The growth media filtrates and sonicates from the bacterial cells of prospective probiotics should be tested for bactericidal and bacteriostatic activity in well-plates against a wide variety of pathogens. The ability of probiotics to establish in the gastrointestinal tract will be enhanced by their ability to eliminate competitors. Table 1.19 presents some examples of antimicrobial-producing organisms.

Table 1.19. Antimicrobial substances by probiotic bacteria (Fuller, 1992).

Probiotic	Compound
Lactobacillus GG	Wide spectrum antibiotic
L. acidophilus	*Acidolin, Acidophilin, Lactocidin*
L. delbrueckii ssp. *bulgaricus*	*Bulgarican*
L. plantarum	*Lactolin*
L. brevis	*Lactobacillin, Lactobrevin*
L. reuteri	*Reuterin*

1.4.2.3. Acid and bile 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 encountered in the intestine. Lankaputhra and Shah (1995) showed that, among several strains of *L. acidophilus* and *Bifidobacterium* sp. studied, only a few strains survived under the acidic conditions and bile concentrations normally encountered in fermented products and in the gastrointestinal tract, respectively. Therefore, it cannot be generalised that all probiotic strains are acid and bile tolerant. Clark *et al.* (1993) and Lankaputhra and Shah (1995) showed that *Bifidobacterium longum* survives better in acidic conditions and is able to tolerate a bile concentration as high as 4%. Acid and bile tolerance is strain dependent, and care should be taken to select strains based on these attributes.

1.4.2.4. Antagonism among bacteria

Bifidobacteria produce acetic and lactic acids in a molar ratio of 3:2. *L. acidophilus* and *L. casei* produce lactic acid as the main end product of fermentation. In addition to lactic and acetic acids, probiotic organisms produce other acids, such as hippuric and citric acid. Lactic acid bacteria also produce hydrogen peroxide, diacetyl and bacteriocin as antimicrobial substances. These inhibitory substances create antagonistic environments for foodborne pathogens and spoilage organisms. Yoghurt bacteria (*S. thermophilus* and *L. bulgaricus*) are

reported to produce bacteriocin against probiotic bacteria and vice versa (Dave and Shah, 1997b).

1.4.2.5. Anticarcinogenic properties of probiotic bacteria

In the last two decades, the number of people suffering from colon cancer has been gradually increasing, particularly in industrialised countries (Moore and Moore, 1995; Hidaka *et al.*, 1991). Studies by Goldin and Gorbach (1981, 1984a) have indicated that diet and antibiotics can lower the generation of carcinogens in the colon and reduce chemically induced tumours. These effects appear to be mediated through the intestinal microflora. Additional studies have shown that the introduction of *L. acidophilus* into the diet lowered the incidence of chemically induced colon tumours in rats (Goldin and Gorbach, 1980). A possible mechanism for these anticancer effects relies on inhibiting intestinal bacterial enzymes that convert procarcinogens to more proximal carcinogens. This technique can be expanded in the future by testing probiotics for their ability to inhibit the growth or organisms normally found in the flora that have high activities of enzymes such as β-glucuronidase (Reddy *et al.*, 1974), nitroreductase, azoreductase and β-glycosidase or the capability for nitrososation. The ability of probiotics to deactivate faecal mutagens can also be a marker used to introduce organisms that lower cancer risk.

1.4.2.6. Adherance of probiotic bacteria

It is not clear if adhesion to the intestinal epithelium is essential for the persistence of a probiotic in the human intestinal tract. However, adhesion seems to be a property that enhances long-term survival. The ability of microorganisms to adhere to epithelial cells is to a large extent species specific, although this may be relative. Screening of organisms for their ability to survive in the human gastrointestinal tract is not difficult. The selection of human

bacterial isolates will enhance the possibility of finding organisms that will survive. The isolates can then be tested by administering orally between 10^9 and 10^{11} viable organism in a single dose with an appropriate buffering agent and the bacterial counts of the specific organism are then measured in the faeces over a several week period. This technique is most successful if the natural flora does not contain the organism being tested or only in small numbers. The first question of transient survival can be established in 48 to 96 h. The evaluation of the ability of the organism to permanently establish in the gastrointestinal tract, by proliferation, can be established by continuous appearance in the faeces over several weeks to several months. The faecal counts should exceed 10^6/g of faeces. The application of this screen for selecting probiotics should be encouraged in the future. There are several tests for determining if a prospective probiotic can bind to intestinal epithelium. Radiolabelling the microorganisms with an amino acid and then counting for adhering radioactivity in either ileal cells recovered from ileostroma effluent or from buccal cells obtained by gently scraping the inside of the cheek are effective methods. Good adhesion properties should enhance the possibility of long-term survival of the organism in the intestinal tract by countering the peristaltic action of the intestine.

1.4.2.7. Immunological enhancement by probiotic bacteria

In recent years there have been several reports indicating that lactobacilli used in dairy products can enhance the immune response of the host. Organisms that have been identified as having this property are *Bifidobacterium longum*, *L. acidophilus*, *L. casei* subsp. *rhamnosum* and *L. helveticus* (Isolauri *et al.*, 2001b). In the future, prospective probiotics, in the appropriate settings (anticancer or infection resistance), should be tested for enhancement of the immunological response. The measurements that should be considered are lymphocyte

proliferation, interleukin 1, 2 and 6, tumour necrosis factor, prostaglandin E production and serum total protein, albumin, globulin and gamma interferon.

1.4.2.8. Cholesterol lowering by probiotic bacteria

Experiments by Gilliland *et al.* (1985) have shown that dietary elevation of plasma cholesterol levels in pigs can be prevented by introduction of a *L. acidophilus* strain that is bile resistant and assimilates cholesterol. These findings were supported by research conducted by Pereira and Gibson (2002a; b) who demonstrated that probiotic strains were able to assimilate cholesterol in the presence of bile into their cellular membranes. Results however, were influenced greatly by the bacterial growth stage and inoculum used as resting cells did not interact with cholesterol as also shown by studies conducted by Dambekodi and Gilliland (1998). St-Onge *et al.* (2000) extensively reviewed the existing studies from animal and human studies which detected moderate cholesterol-lowering was due to consumption of fermented products containing probiotic bacteria. Studies by Gopal *et al.* (1996) also showed cholesterol removal by *Bifidobacterium* spp. and *Lactobacillus acidophilus*.

1.4.2.9. Production of hormones and other agents

The possibility of genetically engineering strains of bacteria that can produce substances such as insulin, androgens, oestrogens, growth hormone or cholesterol-lowering compounds, just to mention a few is intriguing. The ability to produce *in situ* over a long period of time drugs or hormones that are constantly required by individuals suffering from various diseases (i.e. diabetes and hypercholesteremia) is of particular interest. These are problems to this approach, however; e.g. control of production and contamination of normal individuals with the organism. The first problem may be solved by establishing the maximum achievable production level of the organism in the gut and thereby setting an upper limit on

dose. The contamination problem may be more difficult to solve, although antibiotic sensitivity can be introduced into the strains, so that the organism could be rapidly eliminated if a normal individual is infected with a specifically designed probiotic. This idea may have too many regulatory problems associated with it; however, it is still something that may have potential use in human disease regulation.

1.4.2.10. Colonisation resistance

The indigenous microflora on body surfaces inhibit the colonisation of non-indigenous microorganisms. Nevertheless, in some cases (potential) pathogenic microorganisms are able to penetrate and/or colonise these body surfaces, due to a massive attack of the pathogens or to a (temporarily) reduced colonisation resistance. In different studies on humans and animals beneficial microorganisms are used to improve the colonisation resistance on body surfaces, such as gastrointestinal-, the urogenital-, and the respiratory-tract.

1.4.3. Application of probiotics

1.4.3.1. Importance of probiotic consumption in humans

The number of food and other dietary adjuncts products containing live *Bifidobacterium* and *Lactobacillus* bacteria have significantly increased over the last 20 years due in part to the beneficial effects these probiotic organisms are believed to provide (Laroia and Martin, 1990). Presented in Table 1.20 is a listing of bacterial species used as probiotic cultures in food products.

Table 1.20. Bacterial species primarily used as probiotic cultures (Krishnakumar and Gordon, 2001).

Species	Strains
Lactobacillus acidophilus	La2, La5 (also known as La1), Johnsonii (La1; also known as Lj1), NCFM, DDS-1, SBT-2062
Lactobacillus bulgaricus	Lb12
Lactobacillus lactis	La1
Lactobacillus plantarum	299v, Lp01
Lactobacillus rhamnosus	GG, GR-1, 271, LB21
Lactobacillus reuteri	SD2112 (also known as MM2)
Lactobacillus casei	Shirota, Immunitass, 744, 01
Lactobacillus fermentum	RC-14
Bifidobacterium longum	BB536, SBT-2928
Bifidobacterium breve	Yakult
Bifidobacterium bifidum	Bb-12
Bifidobacterium essensis	Danone, (Bio Activia)
Bifidobacterium lactis	Bb-02
Bifidobacterium infantis	Shirota, Immunitass, 744, 01

Although research is ongoing, the available evidence indicates that ingestion of probiotic bacteria may promote desirable changes in the gastrointestinal tract of humans (Kaplan and Hutkins, 2000). Table 1.21 describes several probiotics that are utilised in the food and agricultural industry.

Table 1.21. Organisms used as probiotics in the food and agricultural industry (Goldin and Gorbach, 1992).

Organism	Comment
Saccharomyces boulardii	Non-pathogenic yeast used for treatment of diarrhoea
Lactobacillus acidophilus	As a supplement in dairy products and used for fermentations; numerous health claims
L. plantarum	In dairy products, pickled vegetables and silage
Lactobacillus GG	In yoghurt and whey drink; numerous health claims
L. casei subsp. *rhamnosus*	In dairy products and silage
L. brevis	In dairy products and silage
L. delbrueckii subsp. *bulgaricus*	Production of yoghurt; health claims have been made
Streptococcus thermophilus	For the production of yoghurt
Bifidobacterium bifidum	Component of new dairy products and in preparation for new born; health claims
Bifidobacterium infantis	Similar to *B. bifidum*
Enterococcus faecium	Being introduced in certain health products; health claims
L. lactis ssp. *lactis* and *cremoris*	Used in production of buttermilk and most cheeses

1.5. Prebiotics

There is currently much interest in the concept of actively improving the host health by managing the colonic microflora. Traditionally, this has been attempted by using probiotics. An alternative approach is the consumption of food ingredients known as prebiotics (Rycroft *et al.*, 2001). Prebiotics, as currently conceived of, are all carbohydrates of relatively short chain length (Cummings *et al.*, 2001), additionally carbohydrates that have escaped digestion in the upper gastrointestinal tract form the predominant substrates for bacterial growth in the colon (Roberfroid *et al.*, 1998a). Present evidence concerning the two most studied prebiotics, fructooligosaccharides and inulin, is consistent with their resisting digestion by gastric juice and pancreatic enzymes *in vivo*. In the large intestine, prebiotics, in addition to their selective effects on bifidobacteria and lactobacilli, influence many aspects of bowel function through fermentation (Alles, 1998; Campbell *et al.*, 1997). Short-chain fatty acids are a major product of prebiotic breakdown, but as yet, no characteristic pattern of fermentation has been identified. Through stimulation of bacterial growth and fermentation, prebiotics affect bowel habit and are mildly laxative (Cummings *et al.*, 2001).

1.5.1. Definition of prebiotics

1.5.1.1. Definition and concept of prebiotics

The term `prebiotic' was first coined by Gibson and Roberfroid (1995). 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, that can improve the host health'. The function of prebiotics is to basically stimulate existing metabolisms in the colon (Coussement, 1996). Thus, the prebiotic approach advocates

administration of non-viable entities and therefore overcomes survival problems in the upper gastrointestinal tract. The prebiotic concept considers that many potentially health-promoting micro-organisms, such as bifidobacteria and lactobacilli, are already resident in the human colon. To be an effective prebiotic an ingredient must:

- Neither be hydrolysed nor absorbed in the upper part of the gastrointestinal tract;
- Have a selective fermentation such that the composition of the large intestinal microbiota is altered towards a healthier composition;
- Prebiotics may have many advantages over probiotics. This is firstly related to survivability problems.

These include:

- Maintenance of viability in the product (which, for obvious reasons, will usually be stored under conditions adverse to bacterial growth);
- Gastric acidity;
- Bile salts;
- Pancreatic enzymes and proteins;
- Competition for colonisation sites and nutrients with the resident gastrointestinal flora.

1.5.1.2. Definition of nondigestible oligosaccharides (NDO)

Oligosaccharides are carbohydrates with a low degree of polymersiation (DP) and therefore low molecular weight. They have been variously defined as including anything from 2 to 20 monosaccharide units. The main categories of nondigestible oligosaccharide presently available or in development as food ingredients include carbohydrates in which the monosaccharide unit is fructose, galactose, glucose, and/or xylose. NDOs are readily water soluble and exhibit some sweetness, but solubility decreases with longer chain length. Furthermore due to being undigested in the colon, they have caloric value but due to colonic

fermentation, they have an energy contribution to food of about 1.5 kcal/g, similar to soluble fibre (Roberfroid and Slavin, 2000). Presented in Table 1.22 is a selection of commonly available oligosaccharides, their properties and annual production.

Table 1.22. Commonly available oligosaccharides and annual production (Jelen & Lutz, 1998).

Source	Product	Estimated Annual Production (t)
Lactose (milk)	Lactulose	20,000
	Lactosucrose	1,600
	Lactitol	n/a
Chicory, Jerusalem artichoke	Fructo-oligosaccharides	12,000
Starch	Malto- and isomalto-oligosaccharides	21,000
Soybean	Raffinose, stachyose	2,000

1.5.2. Description of commonly used prebiotics

1.5.2.1. Oligosaccharides

Interest in fructooligosaccharides as a health-promoting food component is increasing. Oligosaccharides are a group of short chain nondigestible polysaccharides consisting of between approximately 2 and 20 saccharide units, may be linear or branched, and occur in a wide variety of foods (Shin *et al.*, 2000b). Fructo-oligosaccharides are widely distributed in plants such as onion, leek, asparagus, chicory, Jerusalem artichoke, garlic, wheat and oat as well as soybean asparagus and also made by the action of fructosyltransferase on sucrose. They are not hydrolysed by the human digestive enzymes, but are utilised by intestinal bacteria such as bifidobacteria (Kaplan and Hutkins, 2000; Hidaka *et al.*, 1986), the *Bacteroides fragilis* group, peptostreptococcaceae and klebsiellae.

Oligosaccharides can be commercially produced through the hydrolysis of polysaccharides (e.g. dietary fibres, starch) or through enzymatic transfer reactions from lower molecular weight sugars. An array of oligosaccharides have been tested using various

in vitro methods, animal models and human clinical trials for their prebiotics effect on probiotic bacteria. Significant increases of bifidobacteria populations in faeces after consumption of fructo-oligosacccharides after relatively short periods of time (Mitsouka *et al.*, 1987).

1.5.2.2. Inulin

Inulin is a blend of fructan chains found widely distributed in nature as plant storage carbohydrates (Wang and Gibson, 1993), and is present in more than 36,000 plant species. The majority of inulin commercially available today is extracted from chicory roots. Chemically, inulin is a polydisperse β-(2,1) fructan. The fructose units in the mixture of linear fructose polymers and oligomers are each linked by β-(2,1) bonds. A glucose molecule typically resides at the end of each fructose chain and is linked by an α-(1,2) bond, similar to sucrose. Chain lengths of these chicory fructans range from 2-60, with an average degree of polymerisation of 10 (Flickinger *et al.*, 2003). *In vitro* they selectively stimulate the growth of *Bifidobacterium* (Gibson *et al.*, 1995).

1.5.2.3. Isomalto-oligosaccharides

Isomalto-oligosaccharides exist in fermented foods such as miso, soy, sauce, sake and honey. The effect of isomalto-oligosaccharides on human faecal flora was also studied. Bifidobacteria and the *Bacteroides fragilis* group were able to utilise isomalto-oligosaccharides, but *Escherichia coli* and other bacteria were not. After the administration of 13.5g of isomalto-oligosaccharides per day for 2 weeks to healthy adults, bifidobacteria remarkably increased (Kohmoto *et al.*, 1988).

1.5.2.4. Lactulose

Lactulose is a synthetic disaccharide in the form Gal β1-4 fru. Lactulose has been used as a laxative as it is not hydrolysed or absorbed in the small intestine. However, at sub-laxative doses lactulose has received attention as a bifidogenic factor and has been administered as such (Tamura, 1983; Modler *et al.*, 1990; Modler, 1993; Kiyosawa, 1986). *In vitro*, lactulose increased lactobacilli and bifidobacteria and significantly decreased bacteroides in mixed continuous faecal culture. The feeding of lactulose to rats significantly increased bifidobacteria; however, only a limited number of bacterial groups were enumerated (Suzuki *et al.*, 1985).

In a human trial, bifidobacteria significantly increased while clostridia, bacteroides, streptococci and Enterobacteriaceae decreased on the feeding of 3 g/d lactulose to eight volunteers (five male, three female) for 14 days (Terada *et al.*, 1992). Small decreases in bacteroides and lactobacilli during the test period were also determined. In addition, decreases in the detrimental metabolites ammonia, indole, phenol, *p*-cresol and skatole, and enzymes β-glucuronidase, nitroreductase and azoreductase supported beneficial claims of lactulose.

Bifidobacterium longum has been shown to afford protection against colon tumourigenesis. Lactulose (4-O-β-D-galactopyranosyl-D-fructose), a keto analogue of lactose, serves as a substrate for preferential growth and increased development of *Bifidobacterium*). Lactulose is not metabolized by human or animal species and resists degradation by the lactases in the digestive tract.

1.5.2.5. Fructo-oligosaccharides (FOS)

Inulin and oligofructose have a specific chemical structure which our digestive enzymes cannot hydrolyse. Both substances are metabolised as dietary fibres in our body. Inulin and oligofructose also show beneficial dietary fibre effects such as a relief of

constipation, increased stool volume and an increased faecal acidity (Coussement, 1996). Inulin and oligofructose belong to a group of carbohydrates known as non-digestible oligosaccharides (NDO), which are commonly consumed in a standard Western diet (Gibson *et al.*, 1994).

Chemically speaking, inulin is a mixture of poly- and oligo-saccharides of which almost all have the chemical structure GF_n (G= glucose, F= fructose and n=number of fructose units linked to one another). The maximum amount of fructoses in inulin from chicory is about 60. The links between the molecules are of a very special type: the β(2-1) form, which makes these molecules indigestible for all higher animals.

Inulin-type fructans contain both GF_n (α D glucosyl-[β D fructosyl]$_{n-1}$-D fructoside) and FF_n (β D fructosyl-[β D fructosyl]$_{n-1}$-D fructoside) molecules, with the number of fructose units varying from two to more than 70 units. The structural relatives of inulin, fructo-oligosaccharides (FOS, a lower molecular weight version) are well documented oligosaccharides with regard to their effect on intestinal bifidobacteria and are considered important prebiotics substrates. Inulin naturally occurs in thousands of different plants with garlic, onion, asparagus, chicory, artichoke, wheat and leak being especially rich.

The two different types of fructo-oligosaccharides are common. Firstly, inulin extracted from chicory roots can be hydrolysed under controlled conditions by the enzyme inulinase to produce short-chain FOS represented as Glu-α1-2[β-D-Fru 1-2]^n where n = 2-9. Another FOS product known as 'neosugar' or 'meioligo' is a mixture of three oligosaccharides of different lengths, i.e. 1-ketose (Glu-Fru_2) and 1^F-β-fructosylnystose (Glu-Fru^4). The mixture is enzymatically synthesised from sucrose by the transfructosylation action of β-fructosidase from the fungus *Aspergillus niger*.

It is accepted that FOS are not degraded or absorbed in the upper human gastrointestinal tract. As such, they enter the colon intact where they are susceptible to

metabolism by the resident microbiota (Hidaka *et al.*, 1991). The β configuration of anomeric C_2 in fructose monomers, is thought to make FOS resistant to hydrolysis by human digestive enzymes which display a high degree of specificity for glycosidic linkages (Gibson *et al.*, 2000).

In pure culture, most species of bifidobacteria are adept at the utilisation of inulin-type fructans. Many other bacteria are also capable of metabolising these substrates including *Klebsiella pneumoniae, Staphylococcus aureus* and *S. epidermis*, *Enterococcus faecalis* and *E. faecium*, *Bacteroides vulgatus, B. thetaiotaomicron, B. ovatus* and *B. fragilis, Lactobacillus acidophilus* and *Clostridium* sp. In mixed batch and chemostat culture studies, it has been demonstrated that both inulin and its hydrolysate selectively stimulated the growth of bifidobacteria which, at the end of the incubation period, become numerically predominant.

Batch culture studies where faecal slurries were incubated with FOS, starch, polydextrose, fructose and pectin for 12 hours (Wang and Gibson, 1993) showed the greatest increase in bifidobacteria with the FOS, indicating the prebiotic nature of these substrates. Continuous culture systems inoculated with faecal slurries were later used to investigate the fermentation. In accordance with earlier studies, bifidobacteria and to a lesser extent lactobacilli preferred FOS to glucose. Three-stage chemostats (gut models) confirmed an enhanced proliferation of bifidobacteria by FOS in conditions resembling the proximal colon.

1.5.2.6. Galacto-oligosaccharides

Galacto-oligosaccharides are galactose-containing oligosaccharides of the form Glu α1-4[β Gal 1-6]$_n$ where n = 2 to 5, and are produced from lactose syrup using the transgalactosylase activity of the enzyme β-galactosidase. Studies by Taneka *et al.* (1983) showed good growth of all eight bifidobacteria strains tested, TOS was concluded to be a suitable bifidobacterial-promoting substrate.

1.5.2.7. Soybean oligosaccharides

The predominant oligosaccharides in soybeans are the trisaccharide raffinose and the tetrasaccharide stachyose which are able to reach the colon and are thought to stimulate bifidobacteria. In pure culture studies, soybean oligosaccharides were fermented to a far greater degree by bifidobacteria than any other organisms tested. Furthermore, the addition of a low concentration of soybean oligosaccharides to a two stage continuous culture of faecal bacteria resulted in a threefold increase in the proportion of bifidobacteria in the total bacterial count (Gibson *et al.*, 2001).

1.5.2.8. Lactosucrose

Lactosucrose is produced from a mixture of lactose and sucrose using the enzyme β-fructosidase and has been found to be bifidogenic in pure culture studies (Tamura, 1983). A later pure culture study compared lactosucrose with lactulose, FOS, soybean oligosaccharides, raffinose and glucose for its utilisation by various intestinal bacteria (Hara *et al.*, 1994). Six bifidobacteria and three lactobacilli strains grew to the same extent (comparable end pH) on lactosucrose and glucose, whereas all the other organisms tested preferred glucose.

1.5.3. Utilisation of prebiotics by consumers

Japan is at the forefront in the development and use of prebiotics due in principal to the influence of the food industry regulatory system. This allows some degree of health claim for a product. The permitted health claims are rather vague stating improvements in well-being and also carry warnings. Prebiotics have been incorporated into many functional foods and drinks in Japan. These products include; soft drinks, candies, biscuit, frozen yoghurt,

table-top sweetener, lactic acid bacteria drink, coffee drink and custard desserts. The oligosaccharides used in the application of these `foods for specified health use' (FOSHU) in Japan include; fructo-oligosaccharides, lactulose, lactosucrose, xylo-oligosaccharides, isomalto-oligosaccharides, soybean oligosaccharides and transgalacto-oligosaccharides.

The incorporation of prebiotic into foodstuffs in Europe is significantly less advanced than in Japan. However, the potential market of a dietary ingredient with a carbohydrate base is enormous. This includes yoghurts, cereals, confectionary, biscuits, cakes, sauces, powdered drinks, pasta, snack foods, processed vegetables, rice, cereal bars, breads, infant formula foods and fruit juices amongst others. Specific examples of prebiotic containing foods that already exist include *Frutex, Actilife, Symbalance, Aviva, LactoPro+, b2* and *Kinder-Flakes*. Although, these particular products are based on fructo-oligosaccharides, new biotechnological capabilities promise to expand the prebiotic market exponentially. In addition, the main European manufacturers of prebiotics (e.g. Sensus, Orafti and Solvay) all produce prebiotic supplements to be consumed on a daily basis.

1.5.4. Fermentation of inulin

Inulin-type fructans resist digestion in the upper part of the gastrointestinal tract. Furthermore, there is no evidence that they are absorbed to any significant extent within the gastrointestinal tract. Therefore, it has been proposed that inulin-type fructans be classified as 'colonic food,' i.e. a `food entering the colon and serving substrate for the endogenous bacteria, thus indirectly providing the host with energy and metabolic substrates' (Gibson and Roberfroid, 1995). Presented in Figure 1.1 are the chemical structures for three common prebiotic products.

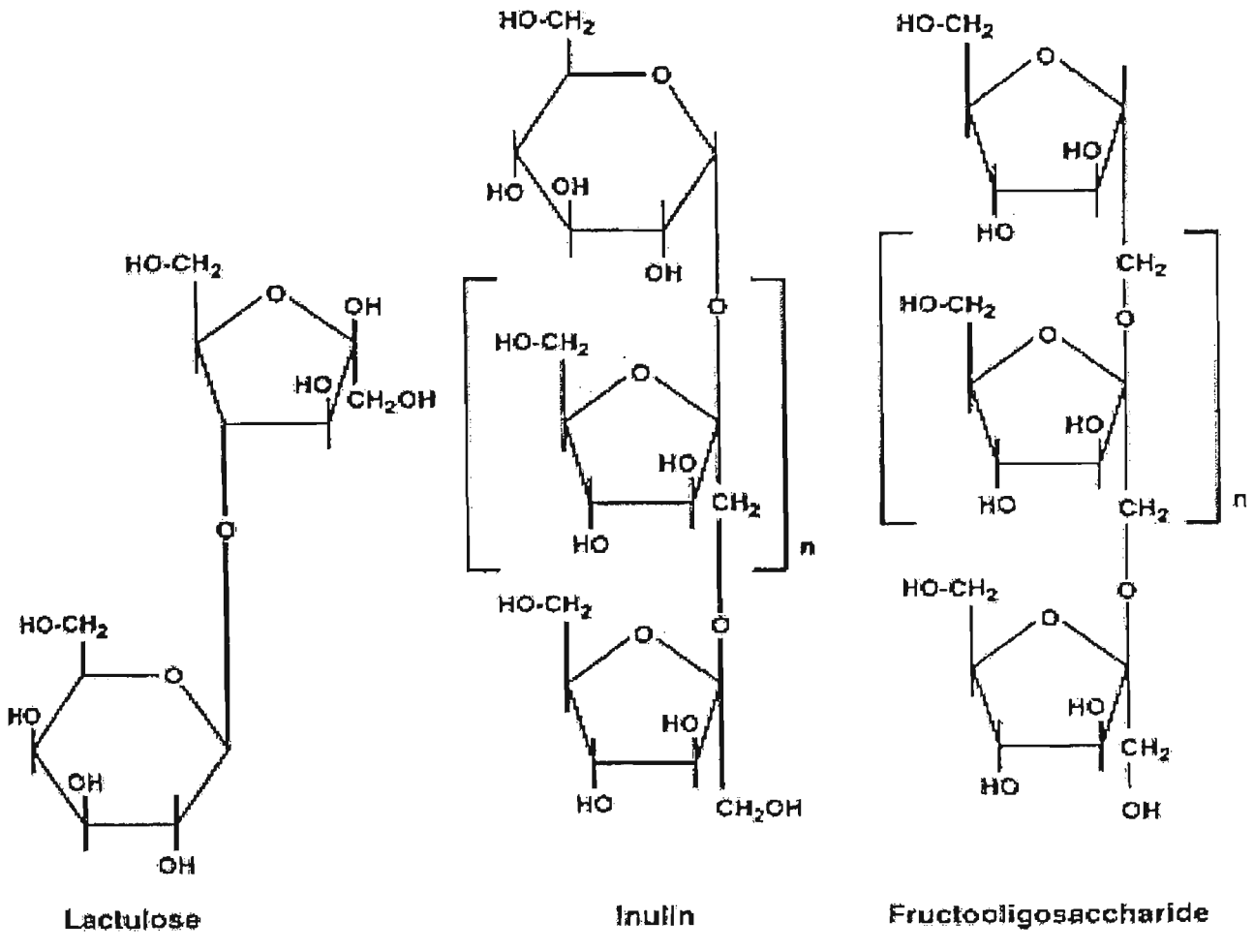

Figure 1.1. Chemical structures of three common prebiotic products (Gibson and Roberfroid, 1995a).

1.5.4.1. Fermentation in the large bowel

The large bowel is the most highly colonised section of the gastrointestinal tract containing approximately 10^{12} bacteria for every gram of gut contents. Colonic bacteria which are mainly anaerobes produce a wide variety of compounds during the process of fermentation. The fermentation of carbohydrates produce short-chain carboxylic acids (mainly acetate, propionate, and butyrate) and lactate, which allows the host to salvage part of the energy of nondigestible carbohydrates. Evidence that inulin-type fructans are fermented by bacteria colonising the large bowel is supported by a large number of *in vitro* and *in vivo* studies, which, in addition, confirm the production of lactic and short-chain carboxylic acids as end products of fermentation. In addition, it has been repeatedly demonstrated that in

human *in vivo* studies, this fermentation leads to the selective growth of bifidobacteria (Roberforid and Delzenne, 1998).

In vitro data demonstrate several facts: that inulin-type fructans are well fermented by human faecal slurries; that in pure cultures and among the bacteria known to be present in human feces, bifidobacteria among several other genus metabolise these carbohydrates; and that in mixed cultures, which replicate bacterial conditions similar to those in the large bowel, the growth of bifidobacteria is selectively stimulated to such an extent that these bacteria become largely predominant over the other populations (Roberfroid and Delzenne, 1998).

1.5.4.2. In vitro fermentation of inulin-type fructans

Evidence that inulin-type fructans are fermented by the colonic microbiota is the demonstration that these carbohydrates are metabolised when incubated with human fecal slurries in anaerobic batch cultures. During fermentation, production of various acids leads to changes in culture pH are convincing evidence as well as with pure cultures an easy method to identify which bacteria have the potential to perform such a metabolic process. Furthermore, by estimating the size of the drop in culture pH over a given period of incubation, it is possible to compare different substrates on a semiquantitative basis.

In summary, both *in vitro* and *in vivo* studies on the fermentation of inulin-type fructans demonstrate that they are metabolised by anaerobic bacteria that are normal constituents of the colonic microbiota. But even if in pure cultures miscellaneous bacteria species have the capacity to use inulin-type fructans as a fermentation substrate, in mixed cultures replicating the large bowel as well as *in vivo* in human volunteers, these fructans have convincingly been shown to selectively stimulate the growth of bifidobacteria.

1.5.5. Consumption of prebiotics by humans

The majority of reported trials carried out on the prebiotic effects of oligosaccharides are positive, however, the scientific control of these trials can be variable. Presented in Table 1.23 are a few examples of volunteer prebiotic human feeding trials carried out worldwide over the last two decades. Number of subjects, dosage consumed, substrate used, length of study and outcomes are presented.

Table 1.23. Examples of human studies with oligosaccharides (Gibson *et al.*, 2000).

Substrate	Subjects	Dose & duration	Results	Reference
FOS	10 adults	12.5 g/d for 12 d	Significant increase in bifidobacteria	Bouhnik *et al.* (1996)
Inulin	10 senile adults	20 g/d. for 8 d, then 40 g/d for 11 d	Significant increase in bifidobacteria	Kleesen *et al.* (1997)
Neosugar	23 senile adults	8 g/d for 14 d	Significant increase in bifidobacteria	Hidaka *et al.* (1986)
TOS	8 adults	10 g/d for 21 d	Significant increased bifidobacteria; significant increase in breath H_2	Bouhnik *et al.* (1997)
Raffinose	7 adults	15 g/d for 4 wk	Significant increase in bifidobacteria, significant decrease in bacteroides and clostridia	Benno *et al.* (1987)
Lactulose	12 adults	2 x 10 g/d for 4 wk	Bifidobacteria, streptococci and lactobacilli significantly increased.	Ballongue *et al.* (1997)
XOS	5 men	20 g/d for 3 wk	Significantly increased bifidobacteria, decrease in bacteroides	Okazaki *et al.* (1990)
SOE	6 adults	10 g/d for 3 wk	Significant increase in bifidobacteria and lactobacilli; large decrease in clostridia and peptostreptococci	Hayakawa *et al.* (1990)
Lacto-sucrose (55%)	13 senile adults	10.3 g/d for 3 wk	Significant increase in bifidobacteria	Kumemura *et al.* (1992)
IMO	9 men	10 g/d for 2 wk	Significantly increased bifidobacteria	Kohmoto *et al.* (1991)

1.6. Clinical evaluation methods for consumption of probiotic bacteria and prebiotics

1.6.1. Intestinal content sampling techniques

1.6.1.1. Testing for functionality: how to determine prebiotics effects

For prebiotics, a number of volunteer trials have already been carried out, with positive effects. However, it is imperative that the best technologies available are used in conjugation with well-controlled trials. The easiest way to determine how bacterial substrates are metabolised is the challenge the test material with pure cultures of selected microorganisms. The substrate would be added to a basal growth medium and bacterial growth determined during a time course incubation. Even so, for gut micro-organisms, the approach does involve something of a challenge in that anaerobic growth conditions must be induced along with standard microbiological aseptic techniques. The usual way to monitor the growth response is by measuring the optical density of the culture or plate count for viable colonies. The comparison would be with bacterial growth in the absence of test material.

This approach gives a reasonable comparative assessment of metabolism in monoculture, but does not induce any element of competition. The problem is that the gut microbiota has at least 500 described species, while latest estimates are that over 1000 different microbial types may exist. Thus, the approach cannot identify true selectivity and therefore the prebiotics activity of a particular substrate.

A more refined approach is to use mixed culture experiments with selected gut microbial species. This introduces some element of competition between the microorganisms, but again does not adequately resemble the complex interactions that occur in the human gut

microflora. However, the approach is very useful for predicting how the target micro-organisms for prebiotics intake may affect potentially pathogenic species.

1.6.1.2. Mixed cultural bacterial fermenters

A common approach towards the determination of gut microbial activities is to use batch culture fermenters inoculated with faecal bacteria. These would be kept anaerobic by the infusion of oxygen-free gas like nitrogen, have pH control and be continuously mixed. However, these are closed systems where the substrate is limited, so are only appropriate for time-course experiments. The recognised bacterial dynamics, of standard growth curves, would dictate that the substrate supply would diminish and cause 'stationary' culture response. In contrast, the human situation is that the colon is constantly replenished with microbial nutrients such that the ecosystem is frequently charge with growth substrates.

A more physiologically relevant approach is therefore continuous culture, whereby a constant input of nutrients may be supplied and other physiological parameters like varying dilution rate can also be imposed. Semi-continuous culture is one variable whereby 'pulse-type' experiments can be carried out where the medium is added and spent culture removed at specific intervals. The most conventional approach is the chemostat where a continuous supply of growth medium is fed to the culture. Usually, one substrate is given in growth-limiting quantities such that enrichment occurs whereby bacteria that can respond to the limited nutrient will eventually predominate in the system. This is an effective way to determine the metabolism of candidate prebiotics. The drawback is that the one-stage continuous culture chemostat is a homogenous system and varying physiochemical determinants cannot be imposed, while culture results obtained in these studies compose between 50 % and 80 % of the total microscopic count (Tannock, 2001).

1.6.1.3. In vitro gut models

In the proximal region, there is a very ready supply of substrate. Hence, bacteria grow quickly and the pH is acidic (due to the formation of acidic end products). In the left side bacteria grow more slowly, the nutrient supply is diminished and it is environmentally more pH neutral. As such, an efficient gut model system would minimise these different physiochemical parameters.

One model is the three-phase chemostat based on gut model simulation in different anatomical areas of the large gut, such as the right, transverse and left sides. This system has been validated against samples taken at autopsy and gives a very close approximation to fermentative bacterial events that occur *in situ*. Studies on the development of the microbial flora in the three vessels can be performed such that fermentation characteristics in the varying areas may be predicted. The system consists of three vessels, of increasing size, aligned in series such that a sequential feeding of growth medium occurs. The vessels are pH regulated to reflect *in vivo* differences. As such, vessel 1 has a high availability of substrate, bacteria grow quickly and is operated at an acidic pH, similar to events in the proximal colon. In contrast, the final vessel resembles the neutral pH, slow bacterial rate and low substrate availability which is characteristic of distal regions. After inoculation with faeces, an equilibration period is allowed such that the bacterial profiles respond to their imposed conditions. Such gut models have been applied to prebiotic research and gives very effective data. Future developments in chemical engineering, the use of intestinal cell tissues in such models and dialysis systems will help improve the situation whereby biofilm communities can also be predicted.

1.6.1.4. Animal models

Animals, often rats or mice, have been used to determine the prebiotic nature of a substrate. Conventional, gnotobiotic (germ-free) rats or those inoculated with one or a limited number of micro-organisms may be used to investigate prebiotic interactions, although this does not resemble the usual situation in the gut. Rats may be associated with a human faecal flora, known as HFA rats, and give a further representation of the situation in the human intestine, although the intestinal physiology is not the same. A major drawback with laboratory animal experiments is the differing (gut) anatomy as well as coprophagy. A more realistic model of the human gut would be to use primates; however, there are clear logistical and expense problems with this approach.

1.6.1.5. Human trials

The definitive assessment of prebiotic effect is to feed candidate substrates or food to human volunteers and assess microbiological changes in stools. As faeces are the only readily accessible area of gut contents, it is difficult to predict fermentation in more proximal gut contents. However, if a prebiotic can induce faecal changes in the microbiota, then it may be assumed that more saccharolytic environments (e.g. the right side of the large gut) have also been affected.

One major drawback towards microbial assessments on faeces is that conventional bacteriology dictates that freshly voided samples are processed. This is because gut bacteria react differently to storage, thus grossly affecting phenotypic (characterisation) traits. In this case, new discriminatory techniques that involve a molecular biological approach towards bacterial characterisation are of enormous value. Such technology is highly reliable and allows the processing of frozen specimens and therefore multiple centre trials.

Human trials may be carried out on volunteers who are on controlled diets, or are free living. The former is difficult to control, although the use of radio-opaque markers in the diet and their detection in stools is a useful measure of gut transit time and also compliance. Moreover, the trials should be carried out in a double-blind manner with good placebo control. In fact, some studies may be 'triple blind' in that not only the investigators and volunteers are unaware of the feeding form, but also those carrying out any statistical analyses. In this way, a candidate prebiotic could be fed and bacterial changes determined. Two types of challenges are possible. One is a crossover trial whereby volunteers would ingest both the test and placebo material (blind coded) for specific periods. Another form is the parallel study whereby volunteers would be randomly categorised into placebo or test material groups. Presented in Table 1.24 is a listing of strains of probiotic bacteria used in commercial production and their clinical evidence in human testing.

Table 1.24. Strains of probiotic bacteria in decreasing order of clinical evidence (Playne, 2002).

Strain	Conditions[1]
Lactobacillus rhamnous GG (Valio)	1, 2, 4, 5, 6, 7, 8, (12), 14, 15
Saccharomyces cerevisiae Boulardii (Biocodex)	2, 3, 4, 5, 7, 11
Lactobacillus paracasei Shirota (Yakult)	2, 5, 6, 9, (10), 11, (12), 15
Bifidobacterium lactis BB12 (Chr Hansen)	1, 2, 3, 4, 5, 6, 11, 15
Lactobacillus reuteri (Biogaia)	1, 5, (10), (12)
Lactobacillus johnsonii La1 (Nestle)	6, 11, 14, 15
Enterococcus faecium SF68 (Cernelle)	2, 5, 10, (12), 13
Lactobacillus acidophilus La5 (Chr Hansen)*	2, 4, 5, 6, 11
Bifidobacterium longum BB536 (Morinaga)	2, 5?, 11, (12), (15)
Bifidobacterium breve (Yakult)	(1), 5
Lactobacillus acidophilus NCFM (Rhodia USA)	1?, 5, 6, (12)
Lactobacillus plantarum 299v (ProViva, Sweden)	5, 13

[1] Condition: 1=rotaviral diarrhoea; 2=antibiotic-associated diarrhoea; 3=*Clostridium difficule* pseudomembranous colitis; 4=traveller's diarrhoea; 5=other acute bacterial diarrhoea; 6=lactose intolerance; 7=bacterial vaginitis; 8=atopic eczema and food allergy; 9=bladder cancer; 10=cholesterol; 11 chronic constipation; 12=bowel cancer; 13=irritable bowel syndrome; 14=*Helicobacter pylori*; 15=immune response modulation.
?=doubtful evidence; ( )=animal data and/or biomarkers only
* Data for this strain uncertain, as it was co-administered with *B. lactis* BB12 usually.

1.6.1.6. A molecular approach to bacterial characterisation

A critical aspect of prebiotic assessment, whether *in vitro* or *in vivo* models are used, is a reliable determination of the bacteriological aspects. The evaluation of the prebiotic potential of the oligosaccharides has, to date, been mostly performed using conventional microbiological techniques. Gut microbiology is usually carried out by plating faecal micro-organisms onto selective agars designed to recover the numerically predominant groups of bacteria. However, the agars used are only semi-selective, do not recover non-culturable bacteria (which may represent over 50% of the overall diversity) and allow operator subjectivity in terms of microbial characterisation, which is usually based on limited phenotypic procedures. As such, if this approach is to be used, then a vast array of selective agars should be involved with intensive characterisation of the colonies which develop. This may involve:

- Morphological description of colonies that develop (shape, margin, consistency, pigments and extracellular secretions;
- Microscopic characteristics (size of cells, nature of growth, shape, spores);
- Gram reaction (and possibly other staining techniques such as acid fast reaction);
- Biochemical traits (e.g. the use of analytical profile indices, analysis of fermentations end products which involves growing the colonies in a liquid growth medium and subsequent analysis);
- The assay of specific enzymes (e.g. fructosidase activities in bifidobacteria);
- Other biomarkers (such as specific cell wall antigens, cellular fatty acids, plasmid profiles, antibiotic sensitivities).

1.6.1.7. Genotypic characterisations

Many studies have reported the results of sampling small subunit ribosomal DNA (rDNA) from human faecal samples (Matsuki, 1999, 1998; Wilson and Blitchington, 1996). The genotypic identity of gut bacteria can be investigated by PCR-16S-rRNA gene restriction fragment length polymorphism (RFLP) and partial gene sequence analysis. 6S rRNA consists of a mosaic of universal, semi-conserved and non-conserved regions and enables the molecule to span great, as well as to measure close, genetic relationships. The `sequences' of the hypervariable regions of the molecule are characteristic of different organisms and to provide a rapid and reproducible means of determining genotype. Full 16S rRNA genes can be amplified from single colonies utilising primers to conserved regions proximal to the 5' to 3' termini of the gene. rDNA products are subjected to RFLP analysis (restriction with endonuclease and electrophorectic analysis), and each bacterial type is characterised by a simple, but highly specific, series of rDNA restriction patterns.

1.6.2. Conceptual approaches to bacterial research

Those bacteria that grow in the intestine are regarded as the indigenous flora and this can be regarded as having two major components, the normal flora and the autochthonous flora. The composition of the normal flora is variable and differs from population to population. The intestinal flora may be regarded as either the most intimate portion of the environment or as an organ of the body. Consideration of the flora as an organ, at least in a metabolic sense is considered when studies of ammonia metabolism, drugs and food additives by the bacteria in the intestine emphasize the practical importance of such metabolic considerations. It should be noted that if the flora is considered as an organ it is potentially the most metabolically adaptable and rapidly renewable portion of the body.

Through consideration of the evolutionary development of the gut flora and its metabolic activity provide important insights, one must remember that the bacteria growing in the intestines are an ecological unit. Study of the intestinal ecosystems may be performed at several levels. It has been stated that the ultimate aim of microbial ecology is to explain the ecosystem in terms of the biochemical properties of purified enzymes, explaining in this way the microbial interactions and the modifications to the environment. Five categories of experimental and conceptual complexity can usefully be distinguished:

The study of microorganisms in the natural environment are usually limited to enumeration of bacteria and measurement of the results of complex metabolic processes. One outcome of such studies may be the alteration or distinction of parts of the ecosystem.

The study of isolated strains of bacteria in the laboratory are directed towards examination of the ability of isolated strains to perform metabolic transformations known to occur in the intestine. Analysis of the chemical composition of isolated strains may be used as background data during attempts to estimate microbial biomass by chemical analysis of intestinal samples.

The intestinal ecosystem is complicated by the impacts of the physiology of the host. Thus, though natural homeostasis of the intestine acts to stabilise the environment, changes in host physiology, diet or immune status can cause short- or long-term environmental variations. Animal experiments may be regarded as attempts to elucidate the human intestinal environment; for example, physiological mechanisms only postulated to occur in man may be demonstrated in animals.

The study of cell suspensions enables the conditions under which ecologically significant reactions and transformations occur to be defined more closely. Suspensions may be prepared from samples of intestinal contents or isolated bacterial strains; faecal homogenates have been commonly investigated.

The study of purified enzymes in theory should provide a biochemical explanation for interactions between bacterial species and with their host. The genetic potential of particular bacterial strains may also be of crucial importance. It should be remarked that the bacteria chosen for study by biochemists and geneticists are seldom ecologically important.

1.6.2.1. In vivo testing

Following the characterisation or description phase in studying a microbial ecosystem in man, the ecological or pathogenic relationships of interests can be transferred to animals under controlled conditions or to *in vivo* model systems so that many sources of variation can be controlled or excluded. Extrapolation to models is always fraught with problems relating to the host and tissue specificity of microbial activity.

1.6.2.2. Obtaining samples of intestinal contents

Studies of the human intestine are complicated by the problems of obtaining samples of intestinal contents. Most of the intestinal tract is normally accessible for examination. Many studies of the intestinal flora are by necessity studies of the bacteria found in faeces. In some cases this is inevitable but it must be remembered that the faeces are probably a final descendant phase of the flora and accurately represents only the flora of the rectum. Samples must be processed as soon as possible after the removal, preferably immediately. As with human samples, if storage is to be carried out at all this should be done at -70°C and transport media should never be used in experimental work. Chemical analyses of the sample may be required, particularly for nutritional studies. Some of these, particularly volatile fatty acids, may require immediate processing while less volatile and stable compounds should resist storage at -80°C.

1.6.2.3. Estimates of microbial number

These often rely on culture techniques but microscopic examination of samples is a most useful means of evaluating the efficacy of other systems. The combination of microscopy with specific strains or fluorescent antibody enables particular organisms to be detected in mixed populations. Some organisms such as spirochetes can be recognised because of their unique morphology but most rods and cocci remain anonymous.

Two methods can be used to quantitate the microorganisms present in the sample: the total microscopic count and the viable count of the numerically most important groups of microorganisms, normally made from serial dilutions of the homogenised sample. For aerobes and facultative anaerobes, homogenisation presents little problem. For counting strict anaerobes a diluent such as Reinforced Clostridial Medium (RCM) should be chosen containing reducing substances such as cysteine or sodium thioglycollate and a small amount of agar in solution to reduce mixing caused by convection. Both methods for counting have inherent inaccuracies in addition to those arising from the dilution series. The total viable count made on a non-selective solid medium estimates the number of colony forming units (c.f.u) per gram or other unit. Since each colony can arise from one cell or a clump or chain of cells it is important to count microscopically the same way. Comparison of the results from using the two methods normally produces the finding that the total viable count is less than the microscopic count. In the past this was attributed to a large proportion of dead organisms in the sample. Although this may be true for highly inimical sites such as the adult stomach, in other sites, particularly those predominated by strict anaerobes, it is more likely to be due to an inability to culture the majority of the organisms present.

1.6.2.4. Culturing bacteria

Most aerobes and facultative anaerobes can be cultured relatively easily on commercially available highly selective media. Although these are inhibitory and even apparently reduce the overall count of the organisms for which they are selective they do at least allow the partial enumeration of easily recognised groups such as 'coliforms', staphylococci, streptococci, yeast and lactobacilli. Few media of this sort are available for strict anaerobes. Almost all the bacteria that grow in the intestine are non-sporulating anaerobes. Thus, *Escherichia coli*, usually thought of as a typical faecal organism, constitute less than 1% of the bacteria in faeces. Many of the bacteria are extremely sensitive to oxygen, especially on initial isolation, and thus the usual techniques for the study of anaerobes from clinical sources are not ideal.

The use of prereduced, anaerobically sterilised media or an anaerobic chamber is essential. Serial dilutions of specimens may be prepared in anaerobic dilution blanks, the manipulations being performed in an anaerobic chamber or, if the pre-reduced anaerobically sterilised medium is used, in tubes gassed with oxygen-free gas. Plates or roll tubes of pre-reduced media are inoculated from these dilution series. This enables an estimate to be made of the numbers of bacteria present by counting colonies; however, in faeces and with the contents of the large intestine the situation is complicated by the large number of bacterial species present. It is easy to obtain a total viable count but very laborious to obtain counts of the identifiable bacterial species.

1.6.3. Selection of prebiotics

One of the biggest constraints in the development of prebiotics is the limited knowledge of the structure-function relationships in these molecules. At the current time, there is little

information on the relative efficacy and selectivity of these molecules. A useful prebiotic would:

- Have low dosage forms;
- Be non-carcinogenic;
- Have a low calorific value;
- Have multifunctional properties;
- Easily incorporated into food vehicles;
- Exert good preservative and drying characteristics;
- Target the distal colon.

A summary of design parameters to improve the effectiveness of probiotics is presented in Table 1.25.

Table 1.25. Design parameters for enhanced activity prebiotics (Gibson *et al.*, 2000).

Desirable attribute in prebiotic	Properties of oligosaccharides
Active at low dosage	Highly selectively and efficiently metabolised by *Bifidobacterium* and/or *Lactobacillus* sp.
Lack of side effects	Highly selectively metabolised by `beneficial' bacteria but not by gas producers, putrefactive organisms, etc.
Persistence through the colon	High molecular weigh, correct choice of glycosyl residue, chemical modification
Varying viscosity	Available in different molecular weights and linkages
Good storage and processing stability	Possess 1-6 linkages and pyranosyl sugar rings
Fine control of microflora modulation	Selectively metabolised by restricted species
Varying sweetness	Different monosaccharide composition
Inhibits adhesion of pathogens	Possess receptor sequence

1.7. Vancomycin-resistant enterococci (VRE)

1.7.1. Characteristics of vancomycin-resistant enterococci

Enterococci, along with approximately 450 other taxa of aerobic and anaerobic bacteria, are part of the normal human intestinal flora (Mundy, 2000). The enterococci are ubiquitous and can be found as free-living in soil, on plants, in dairy and milk products, and as part of the normal flora of the gastrointestinal tracts of humans, canines, birds, cattle, pigs, horses, and other animals. They can cause infections at a wide variety of sites, including the urinary tract, bloodstream, endocardium, abdomen, and biliary tract, as well as burn wounds and indwelling devices. In the 1970s and 1980s, enterococci became firmly established as major nosocomial pathogens. They are intrinsically resistant to many antimicrobial agents (e.g. β-lactams, clindamycin), minimum inhibitory concentrations (MICs) of these drugs being higher than for most streptococci. They are also resistant to low levels of aminoglycosides and had acquired resistance to chloramphenicol and erythromycin as early as 1964. In 1973, there were reports of acquired resistance to high levels of aminoglycosides as well as to the synergistic combination of aminoglycosides as cell wall inhibitors. Enterococcal resistance due to the production of β-lactamase has also been reported. An inoculum of 10^7 CFU/ml, higher than that used by disk diffusion and broth microdilution methods, is required for the detection of enterococcal β-lactamase activities (Bascomb, 1998).

1.7.1.1. Historical background of VRE

Over the last two decades, enterococci have emerged as important pathogens. These organisms are the second leading cause of nosocomial urinary tract infections and the third

most commonly encountered nosocomial wound and bloodstream infection (Bhorade *et al.*, 1999). Although a dozen *Enterococcus* species have been identified, only two are responsible for the majority of human infections. *E. faecalis* had been the predominant enterococcal species, accounting for 80 to 90% of all clinical isolates, and *E. faecium* had accounted for 5-15% with other enterococcal species accounting for less than 5% of clinical isolates (Cetinkaya *et al.*, 2000). The identification of hospitalised patients infected with vancomycin-resistant enterococci (VRE) has become an important component part of infection control programs, endeavouring to minimise patient-to-patient transfer of these organisms (Hanson and Cartwright, 1999). The intrinsic antimicrobial resistance possessed by enterococci has limited the number of possible therapeutic agents (Chen *et al.*, 1998).

1.7.1.2. Taxonomic description of VRE

Enterococci were originally classified as enteric gram-positive cocci and later included in the genus *Streptococcus*. In the 1930s, with the establishment of the Lancefield serological typing system, enterococci were classified as group D streptococci and were differentiated from the nonenterococcal group D streptococci such as *Streptococcus bovis* by distinctive biochemical characteristics. Sherman (1937) further recommended that the term 'enterococcus' should be used specifically for streptococci that grow at both 10 and 45°C, at pH 9.6, and in 6.5% NaCl and survive at 60°C for 30 min. These organisms were also noted to hydrolyse esculin in the presence of bile, are facultative anaerobes, with a G + C content of 37 to 45 mol %. In the 1980s, based on genetic differences, enterococci were removed from the genus *Streptococcus* and placed in their own genus, *Enterococcus*. The previously used species designations such as *faecalis, faecium* and *durans* were retained but were preceded by the genus name *Enterococcus* in place of *Streptococcus*. Although a dozen *Enterococcus* species have been identified, only two are responsible for the majority of human infections.

Until recently, *E. faecalis* had been the predominant enterococcal species, accounting for 80 to 90% of all clinical isolates, and *E. faecium* had accounted for 5 to 15%. Other *Enterococcus* species (*E. gallinarum, E. casseliflavus, E. durans, E, avium,* and *E. raffinosis*) are isolated much less frequently and account for less than 5% of clinical isolates (Cetinkaya *et al.*, 2000).

1.7.2. Medical problems due to VRE

1.7.2.1. VRE resistance to antibiotics and associated medical issues

Enterococci are the second leading cause of nosocomial urinary tract infections and the third leading cause of nosocomial wound and bloodstream infections. Resistance to environmental conditions such as heat or desiccation allow prolonged survival, and poor compliance with hand-washing procedures by health care workers result in the rapid spread of enterococci in hospitals. Moreover, strains of enterococci have acquired resistance to essentially all antimicrobial agents over the past three decades (Bischoff *et al.*, 1999).

The requirement for a non-antibiotic product to reduce and eliminate VRE in hospitals is of significant importance. From the literature, there has been no research that has substantiated the use of probiotics to eliminating this problem although therapeutic and preventative options for the management of VRE have been administered (Kauffman, 2003). In the literature, there has been no discussion of using a probiotic such as bifidobacteria to reduce or eliminate the pathogen from the intestine. The bulk of the literature has referred to using several antibiotic combinations that have proven unsuccessful.

Enterococci have been recognised as an important cause of endocarditis for almost a century. In addition to this long-established role, enterococci began to be recognised as common causes of hospital-acquired infections in the 1970s. This was coincident with and

probably related to the increasing use of third-generation cephalosporins to which enterococci are naturally resistant. One of the major reasons why these organisms have survived in the hospital environment is their intrinsic resistance to several commonly used antibiotics and, perhaps more importantly, their ability to acquire resistance to all currently available antibiotics, either by mutation or by receipt of foreign genetic material through the transfer of plasmids and transposons (Cetinkaya *et al.*, 2000).

1.7.2.2. Hospital cases

Strains of vancomycin-resistant enterococcus (VRE) are prominent emerging nosocomial pathogens. Treatment of these organisms is difficult because they typically are resistant to multiple antibiotics, including penicillins and aminoglycosides. Enterococci are now the second most common organism isolated from sites of nosocomial infection, with a seeming increase in the fraction of isolates resistant to vancomycin. The Centres for Disease Control and Prevention reported an increase in isolation of VRE organisms as a fraction of all enterococci from infected sites from 0.3% in 1989 to 7.9% in 1993, a 20-fold increase in a 5-year period. In 1995, the incidence of VRE infection was estimated to be >10% of all enterococcal infections. The rate of increase for VRE isolates in ICUs is reported to be even higher, increasing approximately 34-fold from 1989 to 1993. Whereas VRE has not been described as more virulent than vancomycin-sensitive enterococcus, the rate of mortality attributable to VRE infections is significant and has been reported to be between 37 and 50 % (Bhorade, 1999).

Enterococci are the second leading cause of nosocomial urinary tract infections and the third leading cause of nosocomial wound and bloodstream infections. Resistance to environmental conditions such as heat or desiccation allow prolonged survival, and poor compliance with hand-washing procedures by health care workers result in the rapid spread of

enterococci in hospitals. Moreover, strains of enterococci have acquired resistance to essentially all antimicrobial agents over the past three decades. Infection or colonization with VRE results in a wide spectrum of possible deleterious effects for patients in health care settings. Studies have demonstrated that VRE can cause serious infections and attributable mortality of bacteremia caused by VRE has been estimated to be nearly 40%.

Enterococcal infections have emerged as one of the most common nosocomial infections in the past decade. They are an important cause of infection in organ transplant recipients and other seriously ill patients, and have become a common intestinal colonizer among hospitalised patients. Their management poses a tremendous problem as members of this species, particularly *Enterococcus faecium*, are resistant to a large number of antimicrobial agents (Baquero, 1997).

The resistance on enterococci to β-lactams (penicillin and ampicillin), through production of β-lactamase and alterations of PBPs, was overcome in the past by combined treatment with aminoglucosides and β-lactams which had a synergistic bactericidal effect. However, the development of high-level resistance to aminoglycosides over the past 15 years, which has been reported in as many as 25% of enterococcal isolates, has led to the failure of this treatment regimen.

Vancomycin, either alone or in combination regimens, replaced combined β-lactam/aminoglycoside therapy in this setting; however, the now widespread plasmid-mediated resistance to glycopeptides has significantly limited the use of this agent. As vancomycin-resistant *E. faecium* are also commonly resistant to ampicillin and to high-levels of aminoglycosides they have caused great concerns worldwide for the management of infections caused by these organisms.

Treatment options for patients infected with vancomycin-resistant enterococci are limited. Tetracyclines and fluoroquinolones, which show good activity *in vitro*, are the

standard agents; however, many isolates are resistant to the clinically achievable serum concentrations of these drugs. Furthermore, fluoroquinolones are not at present recommended for use in children and adolescents.

1.7.2.3. Patients suffering from VRE

Control of VRE may result in a reduced quality of life for patients, since isolation measures limit social exchange. In addition to possible increased length of hospitalisation, VRE carrier status makes the integration of patients more difficult, especially in rehabilitation or long-term health care settings. Overall, problems related to therapy and isolation for patients colonized or infected with VRE lead to increased health care costs. Preventative measures taken to avoid selection of VRE include the restricted use of antibiotics, especially vancomycin. Standard precautions should be followed for the handling of patients in the daily routine. Even if the patient is not known to be infected or colonised with a pathogen, these guidelines require the use of gloves for contact with blood, body fluids, secretions, excretions, and potentially contaminated items and hand washing after glove removal (Bischoff, 1999).

The identification of hospitalised patients infected with vancomycin-resistant enterococci (VRE) has become an important component of infection control programs aimed at minimising patient-to-patient transmission of these organisms (Mitchell, 2002). In addition to organisms possessing high-level, transferable vancomycin resistance (predominantly *Enterococcus faecalis* and *Enterococcus faecium*), those enterococci that intrinsically express low-level resistance to glycopeptide antibiotics, namely *Enterococcus casseliflavus* and *Enterococcus gallinarum*, will also grow on vancomycin-containing media. In contrast to vancomycin-resistant isolates of *E. faecalis* and *E. faecium*, isolates of intrinsically vancomycin-resistant enterococci (IVRE) have not been implicated in outbreaks of VRE infection control standpoint (Hanson, 1999).

Early studies dealing with the emergence of VRE in the United States revealed that most patients with VRE were in ICUs. Infection with VRE usually occurs in patients with significantly compromised host defences (Linden, 2002). However, VRE are now being seen with increasing frequency among patients with chronic renal failure (Trakarnwanich and Eima-Ong, 2000), or cancer (Zaas *et al.*, 2002), organ transplant recipients, patients who experience prolonged hospitalisation (Cetinkaya *et al.*, 2000) and long-term care facilities (Elizaga *et al.*, 2002). Patients that have urinary tract infections (Zhanel *et al.*, 2002), haematology patients (Timmers *et al.*, 2002; Tokars *et al.*, 2001) and patients that have wounds, bacteraemia, endocarditis or meningitis are also at constant risk of VRE infection (Kuriyama *et al.*, 2003).

1.7.2.4. Modes of transmission

Transmission of VRE by health care workers whose hands become transiently contaminated with the organism while caring for affecting patients is probably the most common mode of nosocomial transmission. This concept is supported by the recovery of VRE and other resistant enterococci from cultures of specimens from the hands of health care workers. As they remain viable for several days to weeks on dry surfaces (Wendt, 1998), it seems plausible that contaminated surfaces may act as a source from which personnel may contaminate their hands or clothing (Cetinkaya *et al*, 2000). Table 1.26 describes the characteristics of different phenotypes of glycopeptide-resistant enterococci.

Table 1.26. Characteristics of phenotypes of glycopeptide-resistant enterococci (Cetinkaya *et al.*, 2000).

Characteristic	Phenotype				
	VanA	VanB	VanC	VanD	VanE
Vancomycin MIC (μg/ml)	64->1000	4-1,024	2-32	128	16
Teicoplanin MIC (μg/ml)	16-512	≤ 0.5	≤ 0.5	4	0.5
Most frequent enterococcal species	*E. faecium*, *E. faecalis*	*E. faecium*, *E. faecalis*	*E. gallinarum*, *E. casseliflavus*, *E. flavescens*	*E. faecium*	*E. faecalis*
Genetic determinant	Acquired	Acquired	Intrinsic	Acquired	Acquired
Transferable	Yes	Yes	No	No	No

1.7.3. Colonisation of VRE in humans

Infection or colonization with VRE results in a wide spectrum of possible deleterious effects for patients in health care settings. Studies have demonstrated that VRE can cause serious infections and the attributed mortality of bacteremia caused by VRE has been estimated to be nearly 40% (Bischoff *et al.*, 1999). Control of VRE may result in a reduced quality of life for patients, since isolation measures limit social exchange. In addition to possible increased length of hospitalisation, VRE carrier status makes the integration of patients more difficult, especially in rehabilitation or long-term health care settings. Overall, problems related to therapy and isolation for patients colonized or infected with VRE lead to increased health care costs. Preventative measures taken to avoid selection of VRE include the restricted use of antibiotics, especially vancomycin. Standard precautions should be followed for the handling of patients in the daily routine. Even if the patient is not known to be infected or colonised with a pathogen, these guidelines require the use of gloves for contact with blood, body fluids, secretions, excretions, and potentially contaminated items and hand washing after glove removal (Bischoff *et al.*, 1999; Hosein *et al.*, 2002).

1.7.4. Global spread of VRE in humans

Vancomycin-resistant *Enterococcus faecium* and *E. faecalis* (VRE) were first described in Britain in 1988 and soon afterwards were reported from other European countries and the United States (Bascomb and Manafi, 1998). The acquisition of resistance to vancomycin, used for treating infections caused by gram-positive cocci that are resistant to other drugs, has been on the increase since the late 1980s. Three types of resistance, VanA, VanB, and VanC, have been recognised. Recently, a fourth type of resistance mediated by *E. faecalis* and *E. faecium* is due to VanD. Acquired resistance due to VanA has also been found in *E. avium, E. durans, E. hirae, E. mundtii,* and *E. raffinosus*. Resistance of *E. gallinarum, E. casseliflavus,* and *E. flavescens* can be intrinsic due to VanC1 and VanC2 or acquired due to VanA (Bascomb and Manafi, 1998).

Since March 1996, multiple isolates of vancomycin-resistant *E. faecium* and *E. faecalis* have occurred throughout Australia. The VRE isolated in Australia to date show considerable diversity in their phenotypes, genotypes, and geographic locations. All four combinations of genotype and species have been found, with the commonest being *E. faecium* vanB (Bell *et al.*, 1998). While the clinical profiles of VRE-affected patients appear to be similar to those recorded in the United States and elsewhere, the predominance of *E. faecium* vanB rather than *E. faecium* vanA suggests an epidemiology different from that in Europe or the United States. The level of vancomycin use in Australia is relatively high and has been increasing over the last decade. There is significant regional variation in its use due to variation in prevalence of multidrug-resistant *S. aureus* (Bell *et al.*, 1998). The general trend and usage of vancomycin in Australia has been similar with that used in Europe and the USA over the last two decades (Kirst, 1998).

1.7.5. Classification of VRE

Until recently, *E. faecalis* had been the predominant enterococcal species, accounting for 80 to 90% of all clinical isolates, and *E. faecium* had accounted for 5 to 15%. Other *Enterococcus* species (*E. gallinarum, E. casseliflavus, E. durans, E, avium,* and *E. raffinosis*) are isolated much less frequently and account for less than 5% of clinical isolates. Enterococci have been recognised as an important cause of endocarditis for almost a century. In addition to this long-established role, enterococci began to be recognised as common causes of hospital-acquired infections in the 1970s. This was coincident with and probably related to the increasing use of third-generation cephalosporins to which enterococci are naturally resistant. Enterococci are currently ascendant nosocomial pathogens, having become the second most common organisms recovered from nosocomial urinary tract and wound infections and the third most common cause of nosocomial bacteremia in the United States. One of the major reasons why these organisms have survived in the hospital environment is their intrinsic resistance to several commonly used antibiotics and, perhaps more important, their ability to acquire resistance to all currently available antibiotics, either by mutation or by receipt of foreign genetic material through the transfer of plasmids and transposons (Cetinkaya, 2000).

1.7.5.1. Identification of VRE by tests

Identification at the species level of enterococci isolated from clinical specimens is considered necessary, as is quantitative evaluation of their resistance to penicillin, ampicillin, vancomycin, and teicoplanin and high-level resistance to gentamicin and streptomycin. It is also necessary to distinguish the low-virulence motile enterococcal species with constitutive low-level resistance to vancomycin from the species that are more frequently isolated from clinical specimens, such as *Enterococcus faecalis* and *E. faecium*, which in some countries can often show high-level inducible and transmissible resistance to glycopeptides.

Commercially available kits often used by clinical laboratories as an alternative to the numerous physiological tests needed to identify enterococcal species; nevertheless, all commercial kits vary in their performance and persistently show many drawbacks, especially in cases of atypical strains and at best need supplementary manual tests, which somewhat impair their usefulness (Angeletti, 2001)

1.7.5.2. Strain identity

Enterococcus are characterised as cells arranged in pairs or chains, with no catalase activity. The genus *Enterococcus*, which included *E. faecalis* and *E. faecium*, was separated from *Streptococcus* on the basis of DNA hybridisation data by Schleifer and Kilpper-Balz (1984). Collins *et al.* (1984) added *E. avium, E. casseliflavus, E. durans, E. gallinarum*, and *E. malodoratus* to the list of *Enterococcus* species. With the exception of *E. malodoratus*, all the above species have been isolated from human sources. Since then, 12 new species have been described. *E. dispar*, *E. hirae, E. flavescens, E. mundtii,* and *E. raffinosus* have been isolated from humans and other sources. *E. pseudoavium*, and *E. saccharolyticus* have been isolated from cattle and other mammals. *E. columbae* and *E. cecorum* have been isolated from pigeons and chickens, respectively. *E. sulfureus* has isolated from plants. *E. solitarius*, which has been isolated from an ear exudate, from patients admitted to a public hospital, and from the rumens of domestic and wild ruminants, is now deemed closer to *Tetragenococcus halophilus*. Similarly, *E. seriolicida*, which has been isolated from yellow-tail fish and from water buffalo with subclinical mastitis, is now deemed closer to *Lactococcus garvieae*. It has now been recommended that these two species, which fail to react with the AccuProbe *Enterococcus* probe, should not be included in the genus *Enterococcus* at this time (Bascomb, 1998).

1.7.5.3. Descriptive identification

Enterococcus species are facultative anaerobes, with a G + C content of 37 to 45 mol %. *Enterococcus* species differ from members of the genus *Streptococcus* by being resilient organisms that can survive and multiply in harsh conditions (e.g., pH 9.6, at 10 and 45°C, and in the presence of 6.5 % NaCl and 40 % bile salts). Any isolate suspected of being an *Enterococcus* sp. is a gram-positive coccus, anaerobically facultative and catalase negative. It grows in 6.5 % NaCl, 40 % bile salts, and 0.1 % methylene blue milk and at pH 9.6. It grows at 10 and 45°C and resists 30 min at 60°C. All species produce LAP, and most produce PYR, but they do not contain cytochromes and are mostly negative in the catalase test. They are homofermentative; most strains produce the group D antigen, and some also produce the group Q antigen.

The AccuProbe Enterococcus DNA culture confirmation probe (Gen-Probe, San Diego, Calif.) has been recommended for positive identification of all enterococci because most species of enterococci react positively with the probe with the exceptions of the type strains of *E. cecorum, E. columbae,* and *E. saccharolyticus*. The taxonomy, virulence, clinical significance, and antibiotic susceptibility of the genus have been recently reviewed. The type strain of *E. hirae* has complex nutritional requirements and is used for the bioassay of vitamins and amino acids. Differentiation within the genus is based on acid production from a number of carbohydrates, motility, pigmentation, hydrolysis of arginine, ability to grow in the presence of tellurite, and production of PYR (Bascomb and Manafi, 1998).

1.7.5.4. Classification

Enterococci were originally classified as enteric gram-positive cocci and later included in the genus *Streptococcus*. In the 1930s, with the establishment of the Lancefield serological typing system, enterococci were classified as group D streptococci and were differentiated

from the nonenterococcal group D streptococci such as *Streptococcus bovis* by distinctive biochemical characteristics. Sherman further recommended that the term ''enterococcus" should be used specifically for streptococci that grow at both 10 and 45°C, at pH 9.6, and in 6.5% NaCl and survive at 60°C for 30 min. These organisms were also noted to hydrolyse esculin in the presence of bile. In the 1980s, based on genetic differences, enterococci were removed from the genus *Streptococcus* and placed in their own genus, *Enterococcus*. The previously use species designations such as *faecalis, faecium, durans,* and so forth were retained but were preceded by the genus name *Enterococcus* in place of *Streptococcus*. The genus named *Enterococcus* by Thiercelin and Jouhaud in 1903 was reviewed by Schleifer and Kilpar-Balz in 1984 with bacteria previously described as *S. faecalis* and *S. faecium*. Later, other streptococci having the characteristics of the enterococcus group were transferred to the genus *Enterococcus*. Although a dozen *Enterococcus* species have been identified, only two are responsible for the majority of human infections.

Table 1.27. Identification key for Enterococcus sp. (Manero & Blanch, 1999).

L-arabinose							
(-)	(+) Arginine dihydrolase (-)	(+) PYRase (-)	Alkaline phosphatase (-)		*E. saccharolyticus*		
			(+)		*E. cecorum*		
			Yellow pigment (-)	D-raffinose (-)	Sorbose (-)	*E. asini*	
					Sorbose (+)	*E. pseudoavium*	
				(+)	*E. malodoratus*		
			(+)		*E. sulfureus*		
		(+) Mannitol (-)	Methyl-α-D-glucopyranoside (-)	α-galactosidase (-)	*E. durans*		
					E. hirae		
			(+)	(+)	*E. dispar*		
			Ribose (-)		*E. solitarius*		
			(+)		*E. faecalis*		
(+)	(+) Yellow pigment (-)	(+) Sorbose (-)	Pyrase (-)		*E. columbae*		
			(+)	Methyl-α-D-glucopyranoside (-)	*E. faecium*		
				(+)	*E. gallinarum*		
			Sucrose (-)		*E. avium*		
			(+)		*E. raffinosus*		
		(+) Ribose (-)	*E. flavescens*				
			Methyl-α-D-glucopyranoside (-)		*E. mundtii*		
			(+)		*E. casseliflavus*		

1.7.5.5. Growth factors

The very frequent association of bacteriocin production and vancomycin resistance in different enterococcal clones isolated from human clinical samples suggests that the production of amensalistic substances may enhance extra-intestinal colonisation. In contrast, bacteriocin production was infrequently found among our vancomycin-resistant enterococcal strains from human faecal samples. That may suggest that these nonbacteriocinogenic vancomycin-resistant faecal isolates may remain at low density in the intestinal tract. This work indicates that a complete understanding of the epidemiology of vancomycin resistance in *Enterococcus* will probably require the simultaneous consideration of different factors involved in the dissemination and selection of particular strains in different environmental compartments (Del campo, 2001).

1.7.5.6. Media requirements

Enterococci need rich media for rapid growth. Brain-heart infusion medium has been recommended. The natural habitat of the intestinal enterococci is, however, largely anaerobic, and these facultative anaerobic bacteria have a preference for anaerobic conditions. These special requirements imply a need for specialised culture conditions (Butaye, 1998).

1.7.6. Acquired antibiotic resistance

1.7.6.1. Vancomycin-dependent enterococci

An interesting phenomenon that has developed in some strains of VanA-and VanB-type VRE is that of vancomycin dependence. These enterococci are not just resistant to vancomycin but now require it for growth. Vancomycin-dependent enterococci have been

recovered from apparently culture-negative clinical samples by plating them onto vancomycin-containing agar, such as that used for *Campylobacteria* or gonococci. A likely explanation for the phenomenon of vancomycin dependencies that these enterococci turn off their normal production of D-Ala-D-Ala and then grow only if a substitute dipeptide-like structure is made. With most VanA- and VanB-type enterococci, this occurs only in the presence of associated dehydrogenase (VanH) and ligase (VanA or VanB) that make D-Ala-D-Lac (Cetinkaya *et al.*, 2000).

1.7.6.2. Structure and properties of vancomycin

Vancomycin and other glycopeptide antibiotics often represent the last line of defence against Gram-positive bacteria that have developed resistance to antibiotics. Vancomycin acts by binding emerging cell wall mucopeptides terminating in the sequence D-Ala-D-Ala, resulting in weakening of the cell wall. This weak point creates a weak point resulting in making the cell susceptible to lysis when the osmotic pressure varies. Resistance to vancomycin results from the replacement of the D-Ala-D-Ala terminal petides by D-Ala-D-lactate, which binds less strongly to vancomycin because an amide NH group has been replaced by an oxygen group. Presented in Figure 1.2. is the structural formula of vancomycin.

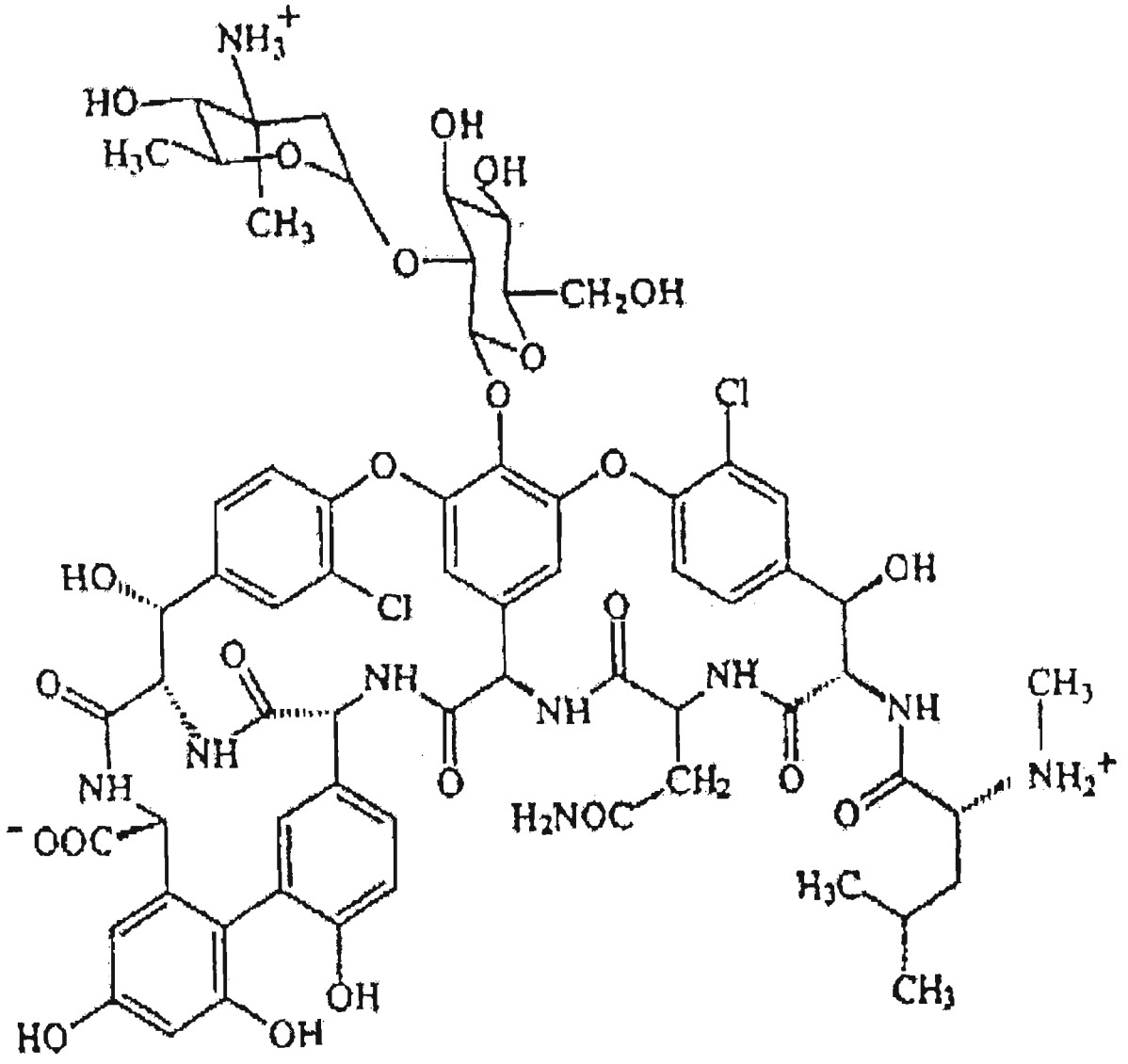

Figure 1.2. Structural formula of vancomycin (Schafer *et al.*, 1996)

1.7.6.3. Genetic acquired immunity

Glycopeptide antibiotics vancomycin and teicoplanin inhibit cell wall synthesis by forming complexes with the peptidyl-D-alanynl-D-alanine (D-Ala-D-Ala) termini of peptidoglycan precursors at the cell surface. Acquired resistance to these antibiotics is mostly due to two types of gene clusters, designated *vanA* and *vanB* that confer resistance by the same mechanism and encode related enzymes. In both cases, resistance is due to synthesis of

peptidoglycan precursors ending in the glycopeptides with reduced affinity (Arthur, 2001). The acquisition of resistance to vancomycin, used for treating infections caused by Gram-positive cocci that are resistant to other drugs, has been on the increase since the late 1980s. Three types of resistance, due to VanA, VanB, and VanC, have been recognised. Recently, a fourth type of resistance mediated by *E. faecalis* and *E. faecium* is due to VanA, VanB, and VanD. Acquired resistance due to VanA has also been found in *E. avium, E. durans, E. hirae, E. mundtii,* and *E. raffinosus*. Resistance of *E. gallinarum, E. casseliflavus,* and *E. flavescens* can be intrinsic due to VanC1 and VanC2 or acquired due to VanA (Bascomb, 1998). The intrinsic and acquired antimicrobial drug resistance in enterococci is present in Table 1.28.

Table 1.28. Intrinsic and acquired antimicrobial drug resistance in enterococci (Cetinkaya *et al.*, 2000).

Intrinsic resistance	Acquired resistance
β-Lactams (partially cephalosporins and penicillinase-resistant penicillins)	High concentrations of β-lactams, through alteration of PBPs or production of β-lactamase
Low concentrations of aminoglycosides	High concentrations of aminoglycosides
Clindamycin	Glycopeptides (vancomycin, teicoplanin)
Fluroquinolones	Tetracycline
Trimethoprim-sulfamethoxazole	Erthyromycin
	Fluoroquinolones
	Rifampin
	Chloramphenicol
	Fusidic acid
	Nitrofurantoin

Presented in Table 1.29 is a list of organisms and their corresponding antibacterial resistance. These organisms are all Gram-positive pathogens.

Table 1.29. Gram-positive pathogens with increasing resistance to antibacterial agents (Baquero, 1997).

Organism	Antibacterial resistance to
S. pneumoniae	β-lactams and macrolide
Viridans streptococci	β-lactams and aminoglycosides
Streptococus pyogenes	Macrolides and possibly penicillins
Groups B, C and G *streptococci*	Penicillins, macrolides and aminoglycosides
Enterococci	Glycopeptides (vancomycin, teicoplanin), penicillins and aminoglycosides
S. aureus	β-lactams, aminoglycosides, macrolides and lincosamides
Coagulase-negative *staphylococci*	β-lactams, aminoglycosides, macrolides and lincosamides and glycopeptides
Listeria sp. and *Corynebacterium* sp.	Multidrug resistance
Gram-positive *anaerobes* (*Peptostreptococcus* sp., *Clostridium* sp.)	Penicillins and macrolides

1.7.6.4. Identification of Enterococcus species

Microorganisms can be classified and identified on the basis of a variety of characteristics including biochemical, genetic, morphological, growth, tolerance and metabolic. Identification time of bacteria can vary from 2 h to several days, furthermore identification of clinical specimens are also variable.

Presented in Table 1.30 and Table 1.31 is a comprehensive consensus matrix test for the identification of the 19 different species of *Enterococci* according to biochemical tests or growth characteristics (Manero *et al.*, 1999)

Table 1.30. Consensus matrix test for identification of *Enterococci*[a] (Manero *et al.*, 1999).

Test or characteristic	*E. asini*	*E. avium*	*E. casseliflavus*	*E. durans*	*E. faecalis*	*E. faecium*	*E. gallinarum*	*E. hirae*	*E. malodoratus*	*E. mundtii*	*E. pseudoavium*	*E. solitarius*	*E. raffinosus*	*E. cecorum*	*E. dispar*	*E. saccharolyticus*	*E. sulfureus*	*E. columbae*	*E. flavescens*
Metabolism with:																			
N-Acetylglucosamine	+	+	+	+	+	+	+	+	+	+	+	+	+	+	N	N	+	+	+
Adonitol	-	+	-	-	-	-	-	-	+	-	+	-	D	-	-	-	-	-	-
Amygdalin	+	+	+	+	+	+	+	+	+	+	+	+	N	+	N	N	+	V	+
L-Arabinose	-	+	+	-	-	+	+	-	-	+	-	-	+	-	-	-	-	+	+
D-Arabitol	-	+	-	-	-	-	-	-	+	-	-	N	+	D	-	+	-	V	-
L-Arabitol	-	D	-	-	-	-	-	-	+	-	+	-	+	-	-	-	-	-	-
Arbutin	+	+	+	+	-	+	+	+	+	+	N	+	N	+	N	N	+	+	+
Cellobiose	+	+	+	+	+	+	+	+	+	+	+	+	N	+	N	N	+	+	+
Dextrin	N	(+)	+	N	+	V	+	N	N	N	N	N	N	N	N	N	N	N	N
Dulcitol	-	D	-	-	-	-	-	-	D	-	-	N	-	-	-	-	-	-	-
Erythritol	-	-	-	-	-	-	-	-	-	-	N	N	N	N	N	N	-	-	-
D-Fructose	+	+	+	+	+	+	+	+	+	+	N	+	N	+	N	N	+	+	+
D-Fucose	-	-	-	-	-	-	-	-	-	-	N	N	N	-	N	N	-	-	-
L-Fucose	-	-	-	-	-	-	-	-	-	-	N	N	N	-	N	N	-	-	-
Galactose	+	+	+	+	+	+	+	+	+	+	+	+	N	+	N	N	+	+	+
Gluconate	-	(+)	+	-	(+)	V	+	-	+	-	N	+	N	D	-	-	+	-	+
D-Glucose	+	+	+	+	+	+	+	+	+	+	N	N	N	+	N	N	+	+	N
Glycerol	-	D	V	-	+	D	D	D	D	D	-	+	+	-	+	-	-	-	-
Glycogen	-	-	-	-	-	-	D	-	-	(-)	-	-	-	-	-	D	-	-	-
Inulin	-	D	(+)	-	-	-	D	-	D	D	-	-	-	+	-	+	-	+	+
2-Ketogluconate	-	+	-	-	V	-	-	-	+	-	+	-	+	V	+	+	+	(-)	-
5-Ketogluconate	-	V	-	-	-	-	-	-	-	-	N	-	N	V	N	N	N	-	-
Lactose	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	N	+	(+)	+
D-lyxose	-	+	-	-	-	-	-	-	-	-	N	-	N	-	-	-	-	-	-
Maltose	+	+	+	+	+	+	+	+	+	+	+	+	N	+	N	N	+	+	+
Mannitol	-	+	+	-	+	+	+	-	+	+	+	+	+	D	-	+	-	+	+
D-Mannose	+	+	+	+	+	+	+	+	+	+	N	+	+	+	N	N	+	+	+
Melibiose	-	D	+	D	-	(+)	+	+	+	+	-	(-)	+	+	D	+	+	+	N
Melezitose	-	+	D	-	(+)	-	D	-	-	D	-	+	+	D	-	+	+	(-)	-
Methyl-α-D-glucopyranoside	(-)	+	+	-	(-)	-	+	-	D	-	+	+	+	D	+	+	+	+	+
Methyl xyloside	-	-	-	-	-	-	-	-	-	-	N	N	N	-	N	N	N	-	-
D-Raffinose	-	-	D	-	-	D	+	D	+	(+)	-	-	+	+	+	+	+	+	+
Rhamnose	+	+	(+)	-	V	-	-	-	+	+	-	-	+	-	N	N	-	(-)	+
Ribose	-	+	+	+	+	+	+	+	+	+	+	-	+	+	+	N	+	+	-
Salicin	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Sorbitol	-	+	D	-	+	-	D	-	D	D	+	D	+	D	-	+	-	+	-
Sorbose	-	+	-	-	-	-	-	-	+	-	+	N	+	-	-	-	N	-	-
Starch	(+)	-	V	D	D	D	D	(+)	N	D	-	-	V	+	-	N	N	+	-
Sucrose	-	(+)	+	D	+	+	+	+	+	+	D	+	+	+	+	N	+	+	+
D-Tagatose	-	+	-	-	+	-	+	(-)	D	-	-	+	+	-	N	N	-	V	-
Trehalose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	N	+	+	+
D-Turanose	-	D	V	-	-	-	+	(-)	V	-	N	+	N	-	N	N	N	(-)	-
L-Xylose	-	D	-	-	-	-	(-)	-	V	-	N	-	N	N	N	N	-	N	-
D-Xylose	+	D	+	-	V	D	+	-	V	+	N	-	N	-	-	-	-	+	+
Xylitol	-	+	-	-	-	-	-	-	+	-	N	-	N	-	-	-	-	(-)	-

[a] +, 90% or more of the strains of isolates are positives; (+) , 75 to 89% are positive; V, 26-74% are positive; (-), 11 to 25% are positive; -, 10 or less are positive; N, no data; D, discrepancies among reference studies.

Table 1.31. Consensus matrix of test for identification of *Enterococci* continued[a] (Manero *et al.*, 1999).

Test or characteristic	*E. asini*	*E. avium*	*E. casseliflavus*	*E. durans*	*E. faecalis*	*E. faecium*	*E. gallinarum*	*E. hirae*	*E. malodoratus*	*E. mundtii*	*E. pseudoavium*	*E. solitarius*	*E. raffinosus*	*E. cecorum*	*E. dispar*	*E. saccharolyticus*	*E. sulfureus*	*E. columbae*	*E. flavescens*
Growth at:																			
4°C	N	-	N	N	-	+	-	N	N	N	N	N	N	N	N	N	N	N	N
10°C	V	D	+	+	+	+	+	+	+	+	+	(+)	-	-	+	+	+	-	(-)
45°C	V	+	(+)	+	+	+	+	+	-	+	+	+	+	+	-	+	-	N	(+)
50°C	N	-	-	-	-	V	-	-	-	-	N	N	N	N	-	-	N	N	N
pH 9.6	N	+	+	+	+	+	+	+	N	+	+	N	+	N	N	N	N	N	N
Growth in:																			
6.5% NaCl	-	D	+	+	+	+	+	+	+	+	D	+	+	-	+	+	+	-	N
0.1% Methylene blue milk	N	-	N	+	+	+	V	N	N	N	N	N	N	V	N	N	N	N	N
0.04% Tellurite	N	-	D	-	+	-	D	-	-	D	-	-	-	N	N	N	N	N	N
0.01% Tetrazolium	N	N	N	-	+	-	+	N	N	N	N	N	N	N	N	N	N	N	N
Survival at 60°C for:																			
15 min	N	+	N	N	+	+	(+)	N	N	N	N	N	N	N	N	N	N	N	N
30 min	+	+	N	N	+	+		D	N	N	N	N	N	-	N	N	N	N	N
1 h	N	(-)	N	N	+	+	-	N	N	N	N	N	N	N	N	N	N	N	N
Gelatin liquefaction	N	-	-	-	D	-	-	-	-	-	N	N	N	-	N	N	N	N	N
H_2S production	N	D	-	-	-	-	-	N	+	N	N	N	N	N	N	N	N	N	N
α-hemolysis	N	+	+	V	V	D	D	D	-	-	D	-	-	D	N	N	N	N	-
β-hemolysis	N	-	-	V	V	D	D	-	-	-	-	-	-	D	N	N	N	N	-
Lancefield group D	+	+	+	(+)	+	V	+	V	+	+	-	+	N	-	-	-	-	-	+
Motility	-	-	+	-	-	-	+	-	-	-	-	-	-	-	-	-	-	N	+
Voges-Proskauer	N	D	D	+	+	+	D	+	D	+	D		D	D	N	-	N	+	+
Yellow pigment	-	-	+	-	-	-	-	-	-	+	-	-	-	-	-	-	+	-	+
Alkaline phosphatase	-	-	N	-	D	(-)	-	-	-	-	-	-	-	+	-	-	-	+	-
Arginine dihydrolase	-	-	D	+	+	+	+	+	-	+	-	+	-	-	+	-	-	-	+
Catalase	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Esculin hydrolysis	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
α-Galactosidase	-	-	+	-	-	D	+	+	+	(+)	D	+	D	D	+	+	+	+	+
β-Galactosidase	-	D	+	D	D	+	+	(+)	+	+	D	-	-	D	+	N	+	V	+
β-Glucuronidase	-	-	-	-	-	-	D	-	-	-	-	-	-	+	-	N	-	-	-
Hippurate hydrolysis	+	D	-	V	D	D	D	D		-	D	D	D	D	V	-	-	-	-
Leucine arylamidase	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Pyrrolidonyl aminopeptidase	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	-	+	-	+

[a] +, 90% or more of the strains of isolates are positives; (+) , 75 to 89% are positive; V, 26-74% are positive; (-), 11 to 25% are positive; -, 10 or less are positive; N, no data; D, discrepancies among reference studies.

2.0. INHIBITION OF PATHOGENIC AND PUTREFACTIVE MICROORGANISMS BY *BIFIDOBACTERIUM* SPECIES[1]

2.1. Introduction

An individual contains a gastrointestinal flora of 100 trillion viable bacteria, representing 100 or more different bacterial species (Simon and Gorbach, 1984; Mitsuoka, 2000). The major intestinal bacteria are classified into three main groups: 1) the lactic acid bacteria, including *Bifidobacterium*, *Lactobacillus* and *Streptococcus*: 2) the putrefactive bacteria, including *Clostridium perfringens*, *Escherichia coli* and *Staphylococcus*, and 3) other bacteria, including Eubacteria, *Ruminococcus* and *Candida* (Mitsuoka, 2000).

Bifidobacteria are the major constituent of the normal intestinal microflora of human infants and adults (Hughes and Hoover, 1991; Key and Marble, 1995; Ishibashi and Yamazaki, 2001). Initially named *Bacillus bifidus communis* or *Bacillus bifidus*, these anaerobic, lactic acid, Gram-positive, rodlike organisms possessed a characteristic Y or V-shaped end. Since then, the morphological, biochemical and physiological characteristics of these microorganisms have been ascertained and are currently classified as an independent genus, *Bifidobacterium* (Lim *et al.*, 1993), which now includes 29 species (Salminen *et al.*, 1993).

Bifidobacterium species are isolated from feces of human at any particular age and from birth are the first species to establish in the intestinal tract (Hoover, 1993). For infants, the most common species shortly after birth are *Bifidobacterium infantis, B. breve*, and *B. longum*. For breast-fed babies levels of 10^{10} to 10^{11}/g of bifidobacteria were commonly found in faeces. As infants mature, *B. infantis* and *B. breve* are replaced by *B. adolescentis* and *B.*

[1] A version of this chapter has been published: Bruno, F.A. and Shah, N.P. 2002. Inhibition of pathogenic and putrefactive microorganisms by *Bifidobacterium* sp. *Milchwissenshaft* 57 (11/12): 617-621

longum (Dubey and Mistry, 1996b; Mitsuoka, 2000). *Bifidobacterium* species produce acetic and lactic acids, which have been reported to suppress the proliferation of putrefactive bacteria such as *Escherichia* and *Clostridium* (Ibrahim and Bezkorovainy, 1993; Araya-Kojima *et al.*, 1995; Dubey and Mistry 1996b; Mitsuoka, 2000).

Probiotic foods containing bifidobacteria have been widely accepted in Japan, Europe, USA, and Australia (Arroyo *et al.*, 1994; 1995). Dairy foods containing bifidobacteria have potential benefits including inhibition of pathogens, increased immune response, and maintenance and restoration of normal intestinal flora (Dubey and Mistry, 1996a). *Bifidobacterium longum* is one of the most often used species industrially.

The antibacterial activity of lactic cultures is generally attributed to the presence of lactic acid (Anand *et al.*, 1985). Investigations conducted by Araya-Kojima *et al.* (1995, 1996), Chen *et al.* (1999), and Ibrahim and Bezkorovainy (1993) have all reported that *Bifidobacterium* strains inhibit the growth of pathogenic bacteria due to lowering of pH *in vitro* and *in vivo* as a consequence of acetic and lactic acid production.

Bifidobacterium longum has been reported to be acid and bile tolerant (Hughes and Hoover, 1991; Lankaputhra and Shah, 1995) and has displayed shown high levels of adherence to human colonic cells (Lankaputhra and Shah, 1998a). The viability of *B. longum* 1941 was not affected by the presence of acid and the organism maintained a high viable count after 6 wks of refrigerated storage at 4°C (Lankaputhra *et al.*, 1996). Therefore, some strains of *Bifidobacterium* are suitable for use as dietary adjuncts.

However, inhibition of organisms of intestinal origin by *B. longum* 1941 has not been reported. The objective of this study was to investigate based on earlier studies whether *B. longum* 1941 and three other strains of *Bifidobacterium* and their supernatants inhibit the growth of intestinal pathogenic and putrefactive microorganisms using inhibition assays and co-culture experiments in order to determine their suitability for use as a dietary adjunct.

2.2. Materials and Methods

2.2.1. Bacterial cultures and growth media

2.2.1.1. Probiotic and pathogenic cultures

Four bifidobacteria strains (*B. infantis* 1912, *B. longum* 1941, *B. longum* BB536 and *B. pseudolongum* 20099) were obtained from the Victoria University Culture Collection. The organisms were activated by growing three times consecutivelyuently in 12% reconstituted skim milk (RSM) containing 0.05% L-cysteine (LC) at 37°C for 24 h. The pathogens selected for this study included *Escherichia coli* LE392, *E. coli* TG2, *E. coli* Y10, *Clostridium chauvoei, C. perfringens, C. sporogenes, Candida albicans, Enterobacter aerogenes, Streptococcus agalactiae, S. mitis and S. pyogenes* were also obtained from the Victoria University Culture Collection. They were incubated in nutrient broth for 24 h at 37°C.

2.2.1.2. Growth media for probiotic and pathogenic bacteria

Twelve per cent (w/v) reconstituted skim milk (RSM) was prepared by dissolving 120 g of RSM to 1 L of distilled water at 45°C and dispensed into McCartney bottles followed by autoclaving at 115°C for 20 min. MRS broth (Oxoid Ltd., Hampshire, UK.) was prepared by dissolving 52 g of MRS powder in 1 L of distilled water followed by autoclaving at 121°C for 15 min. MRS agar (Oxoid) was prepared by dissolving 52 g of MRS powder and 10 g of bacteriological agar (Amyl Media Pty. Ltd., Dandenong, Australia) in 1 L of distilled water followed by autoclaving at 121°C for 15 min. Nutrient broth was prepared by dissolving 26 g of Nutrient powder (Amyl Media) in 1 L of distilled water followed by autoclaving at 121°C for 15 min. Eosine methylene blue (Oxoid) were prepared by dissolving 37.5 g in 1 L of distilled water, followed by autoclaving at 121°C for 15 min.

2.2.1.3. Preparation of NNLP and L-cysteine solutions

NNLP was prepared by dissolving 0.15 g/L of nalidixic acid, 1 g/L of neomycin sulphate, 50 g/L of lithium chloride, and 2 g/L of paromomycin sulphate (Sigma Chemical Co., Castle Hill, Sydney, Australia). The solution was filter-sterilised. To prepare MRS-NNLP agar, 100 mL of NNLP solution was added to 900 mL of autoclaved MRS agar just before pouring plates. L-cysteine hydrochloride (LC) is reported to lower the redox potential and improve the viability of bifidobacteria. To prepare 0.05% (w/v) L-cysteine·hydrochloride solution (LC), five grams of L-cysteine hydrochloride monohydrate (Sigma, St Louis, MO, USA.) were added to 100 mL of distilled water, stirred until dissolved and filter-sterilised. The solution was stored at 4°C.

2.2.2. Growth inhibition of pathogens with Bifidobacterium spp.

To obtain the supernatant for inhibition assays, each strain of bifidobacteria was incubated consecutivelyuently three times in MRS broth (Oxoid, UK.) containing 0.05% (v/v) LC at 37°C for 24 h using 1% inoculum, followed by centrifugation of 200 mL aliquots at 3838 x *g* for 15 min at 4°C. The cell free supernatant was divided into two portions. The pH of the first portion was recorded and left unadjusted, while the pH of the second portion was adjusted to 7.0 using 5 N NaOH. The pH was measured at 17-20°C using a pH meter (Model 8417; Hanna Instruments Pty. Ltd., Singapore) after calibrating with fresh pH 4.0 and 7.0 standard buffers. Both unadjusted and adjusted supernatants obtained from each strain of bifidobacteria were filtered using 0.45 μm Millipore filters (Millipore Corp., Bedford, MA, USA).

A thick lawn of each pathogenic microorganism was prepared by spreading 0.1 mL culture of each organism onto nutrient agar plates. Each plate was divided into 4 quadrants

and wells were formed in the agar using a sterile 6 mm borer. The bottom of each well was sealed with 0.9% (w/v) sterile agar, followed by addition of 50 µl of supernatant of each bifidobacteria. All inhibition assays were performed aerobically in triplicate for each pathogenic bacterium, for both adjusted and unadjusted supernatants using the method of Dave and Shah (1997b). The supernatant was left to diffuse into each well for 2 h, followed by aerobic incubation at 37°C for 48 h. The inhibition zones were then measured at 24 and 48 h.

2.2.3. Effect of growing pathogens with Bifidobacterium spp. supernatants

The cultures were activated by three consecutive transfers as previously discussed (2.2.1.1). The optical density (OD) at 620 nm was adjusted to 0.1. The supernatant was obtained by centrifuging as before and was divided into two portions. One portion was left unadjusted, while the other portion was adjusted to pH 7.0 using 5 N NaOH. One millilitre of the neutralised or unneutralized supernatant and 1 mL of *E. coli* LE392, *C. perfringens, E. aerogenes, C. albicans* and *S. agalactiae* were added to 13 mL of nutrient broth in McCartney bottles, followed by incubation for 24 h at 37°C. The controls contained 13 mL of nutrient broth, 1 mL of distilled water and 1 mL of each of the pathogenic microorganisms. Optical density readings at 620 nm were taken every 6 h for 48 h.

2.2.4. Effect of growing E. coli and E. aerogenes in the presence of four Bifidobacterium spp. supernatant

The effect of supernatant obtained from bifidobacteria on the growth of *E. coli* and *E. aerogenes* was assessed by adding separately 1 mL of either one of the four bifidobacteria supernatants to 1 mL of either *E. coli* or *E. aerogenes* in 8 mL of nutrient broth and incubated

at 37°C for 48 h. The control contained 1 mL of distilled water instead of the supernatant. Samples were taken at 0, 24 and 48 h to measure optical density, while pour-plates with EMB agar were used to enumerate both *E. coli* and *E. aerogenes*. The plates were incubated at 37°C for 48 h. Colony forming units were counted using a colony counter (Model Gallenkamp, Leicestershire, UK.)

2.2.5. Effect of co-culturing Bifidobacterium spp. and selected pathogens in MRS broth

The effect of culturing of *Bifidobacterium longum* 1941 with *E. coli* LE392, *C. albicans*, *S. agalactiae, E. aerogenes,* and *C. perfringens* was assessed by adding 1 mL of *B. longum* 1941 cells to 8 mL of MRS broth and 1 mL of each pathogenic bacteria cells. The co-culture was then incubated aerobically for 24 h. The control contained 1 mL of sterile distilled water instead of bifidobacteria cells. One millilitre aliquot was obtained at 6 h intervals for measurement of pH, optical density and enumeration for both groups of bacteria. Optical density was determined using a Novaspec II spectrophotometer (Pharmacia, Biotech, LKB, Biochrom, UK) at 620 nm. Enumeration of bifidobacteria was performed by pour plating using MRS-NNLP agar (Laroia and Martin, 1991; Dave and Shah, 1996). The plates were incubated anaerobically at 37°C for 72 h. Nutrient agar was used to enumerate pathogenic bacteria and the plates were incubated under aerobic conditions at 37°C for 48 h.

2.2.6. Fermentation activity by four Bifidobacterium spp.

The activity of each bifidobacteria strain was determined by measuring end products of fermentation (lactic and acetic acids) using high performance liquid chromatography (HPLC; Varian Australia Pty. Ltd., Mulgrave, Australia). Four *Bifidobacterium* strains (*B. infantis* 1912, *B. longum* 1941, *B. longum* BB536 and *B. pseudolongum* 20099) were

incubated at 37°C for 24 h consecutivelyuently three times in MRS broth containing 0.05% LC using 1% inoculum and HPLC analysis was performed using the method of Dubey and Mistry (1996a; 1996b). One-hundred microlitres of 15.8 M HNO_3 and 14.9 mL of 0.009 M H_2SO_4 were added to 1.5 mL of bifidobacteria cell extract and centrifuged at 4000 x *g* for 10 min using a benchtop centrifuge (Sorvall RT7, Newtown, CT, USA). The supernatant was filtered using 0.22 μm Millipore filters and 2 mL aliquots were stored at –20°C until analysed. The HPLC system consisted of a Varian 9012 solvent delivery system, Varian 9100 autosampler, Varian 9050 variable wavelength UV/Vis tunable absorbance detector and a 730 data module. An Aminex HPX-87H column (300 mm x 7.8 mm, Bio-Rad Laboratories, Richmond, CA, USA) and a guard column with disposable cartridges H^+ (Bio-Rad Laboratories) maintained at 65°C were used for organic acid analysis. The degassed mobile phase of 0.009 M H_2SO_4, filtered through a 0.45 μm membrane filter was used at a flow rate of 0.3 mL/min. The wavelength of detection was optimised at 220 nm. The standard solutions of lactic and acetic acids (Sigma St Louis, MO, USA) were prepared in mobile phase solution to establish elution times and standard curves. The retention times and standard coefficients for acetic and lactic acid were 16.2 and 25.7 min, 0.9991 and 0.9995, respectively.

2.3. Results and Discussion

2.3.1. Growth inhibition of pathogens with Bifidobacterium spp. as measured by inhibition assays

The growth inhibition of eleven pathogenic bacteria with four strains of bifidobacteria is shown in Table 2.1. In general, the zones of inhibition including the bore diameter were greater with the addition of unadjusted supernatants from *B. infantis* 1912 (10.25 ± 1.4 to 11.83 ± 0.5) and *B. longum* 1941 (10.00 ± 1.8 to 11.58 ± 0.5) as compared to that of *B. longum* BB536 (7.25 ± 0.6 to 8.50 ± 1.5) and *B. pseudolongum* 20099 (7.50 ± 0.5 to 9.00 ± 1.6). To examine whether the effect was due to acid, one portion of the supernatants was neutralised to pH 7.0. The neutralised supernatants of the four *Bifidobacterium* strains produced no detectable zones of inhibition of the eleven studied pathogenic bacteria (data not shown). The antimicrobial effects of *Bifidobacterium* spp. are attributed generally to the decrease of pH resulting from the production of lactic and acetic acids. These results are comparable to those of Ibrahim and Bezkorovainy (1993), Araya-Kojima *et al.* (1995; 1996) and Lankaputhra and Shah (1998b). Bifidobacteria produce acetic acid and lactic acid in appreciable amounts (Lankaputhra and Shah, 1998b) as a result of fermentation of glucose in the molar ratio of 3:2 (Scardovi and Trovatelli, 1965). The final pH of the unadjusted supernatant decreased from 6.34 at 0 h to pH 3.83 to 4.04 at 24 h depending on the strain (Table 2.1).

2.3.2. Effect of growing pathogens with Bifidobacterium spp. supernatant

The suppression of growth of five different groups of selected pathogenic bacteria with four bifidobacteria supernatants as measured by a decrease in optical density (OD) is

presented in Table 2.2. The values given in the table were a percentage difference from the control to standardise between pathogenic strains average values from three independent experiments. In general, the five pathogenic bacteria were greatly inhibited when incubated in the presence of bifidobacteria supernatant over 24 h as indicated by a decrease in OD. Addition of unadjusted supernatant of *B. infantis* 1912 and *B. longum* 1941 showed a noticeably stronger inhibition against pathogenic bacteria (42.73 to 65.59%) as compared to those of *B. longum* BB536 and *B. pseudolongum* 20099 (2.61 to 39.91%). The results were consistent with greater zones of inhibition with *B. infantis* 1912 and *B. longum* 1941. When the supernatant was neutralised to pH 7.0, the effect was substantially reduced (-8.16 to 9.33%). The pH of the unadjusted supernatants was 3.96 for *B. infantis* 1912, 3.83 for *B. longum* 1941, 4.04 for *B. longum* BB536 and 3.89 for *B. pseudolongum* 20099.

2.3.3. Effect of growing E. coli LE392 and E. aerogenes in the presence of Bifidobacterium spp. supernatant

The effect of growing selected pathogens (*E. coli* LE392 and *E. aerogenes*) with supernatant obtained from bifidobacteria as measured by optical density (OD) and bacterial counts (CFU) are shown in Table 2.3. In general, there was a decrease in OD and bacterial counts as compared to the control. The decrease in OD was greater at 24 h than at 48 h. A similar trend was observed with bacterial counts. The inhibition of *E. coli* and *E. aerogenes* was significant ($p<0.05$) when the supernatant was not neutralised. However, when the supernatant was neutralised to pH 7.0, the OD readings were similar to those of the control (Table 2.3).

2.3.4. Effect of co-culturing B. longum 1941 and five pathogenic bacteria

The effect of co-culturing *B. longum* 1941 and five pathogenic bacteria is shown in Table 2.4. In general, when the five pathogens were separately co-cultured with *B. longum* 1941, there was a significant decrease ($p<0.05$) in cell density as compared to the control except for *C. perfringens*. Similarly, the pH decreased by 0.97 to 2.24 log after *B. longum* 1941 cells were added with the pathogenic bacteria. *Clostridium perfringens* displayed the lowest decrease in colony forming units, and pH, and there was a slight increase in optical density. Studies conducted by Araya-Kojima *et al.* (1995; 1996) have also observed only minor changes in co-culture experiments when adding *B. longum* BB536 with *C. perfringens* as compared to other pathogenic bacteria. There was a substantial decrease in bacterial counts of all five of the pathogenic bacteria when *B. longum* 1941 was added. This decrease ranged from 74.3 to 13.2% with a decrease in pH of between 2.24 to 0.97. The combination of *E. aerogenes* and *B. longum* 1941 produced a final pH of 4.61, as compared to a pH of 6.85 for the control.

2.3.5. Activity of bifidobacteria

Bifidobacterium produce lactic and acetic acids as the products of sugar fermentation. This is an important and significant characteristic of bifidobacteria. The ideal *Bifidobacterium* fermentation pathway results in 3 moles of acetic acid and 2 moles of lactic acid per 2 moles of glucose in an ideal synthetic medium, therefore, generating a theoretical molar ratio (acetic: lactic) of 3:2 (Scardovi, 1965). Average results of three independent fermentations are presented in Table 2.5. These values demonstrate that *B. infantis* 1912 produced the closest ideal fermentation molar ratio of 1.65 of acetic to lactic acid (54.9 ± 24.79 mM: 33.2 ± 11.74 mM). The highest average concentration of acetic acid detected by the four *Bifidobacterium*

strains was 55.4 ± 10.21 mM was produced by *B. longum* BB536, while the lowest average concentration detected was 39.8 ± 3.57 mM produced by *B. longum* 1941. The highest average concentration of lactic acid (33.2 ± 11.74 mM) was produced by *B. infantis* 1912, while the lowest average concentration of lactic acid (17.1 ± 3.92 mM) was produced by *B. longum* BB536.

2.4. Tables

Table 2.1. Inhibition of 11 intestinal microorganisms with four strains of bifidobacteria supernatant

Pathogenic bacteria strains	Bifidobacteria supernatant Zone of inhibition (mm)[1, 2, 3, 4]			
	B. infantis 1912[4]	*B. longum* 1941[4]	*B. longum* BB536[4]	*B. pseudolongum* 20099[4]
E. coli LE392 (VUN104)	11.17 ± 0.4	11.58 ± 0.50	8.00 ± 0.9	8.00 ± 0.9
E. coli TG2 (VUN105)	10.75 ± 0.8	11.33 ± 0.8	8.17 ± 0.8	7.50 ± 0.5
E. coli Y10 (VUN109)	11.33 ± 1.2	11.42 ± 1.5	8.17 ± 1.3	7.75 ± 1.5
E. aerogenes (VUN025)	10.33 ± 0.5	10.75 ± 0.9	7.75 ± 1.0	8.67 ± 0.5
C. albicans (VUO002)	10.25 ± 1.4	10.92 ± 1.6	7.50 ± 0.6	7.67 ± 0.9
C. perfringens (VUP060)	10.58 ± 0.7	10.50 ± 1.0	8.33 ± 1.4	7.92 ± 0.5
C. sporogenes (VUP061)	10.50 ± 0.8	11.42 ± 0.7	7.92 ± 0.5	7.92 ± 0.4
C. chauvoei (VUP0062)	10.17 ± 1.1	10.00 ± 1.8	7.25 ± 0.6	7.67 ± 0.8
S. mitis (VUP052)	10.83 ± 0.5	10.92 ± 0.6	8.50 ± 1.5	7.83 ± 1.2
S. agalactiae (VUP053)	11.83 ± 0.5	11.33 ± 0.6	8.42 ± 1.2	9.00 ± 1.6
S. pyogenes (VUP058)	10.31 ± 0.9	10.83 ± 0.4	7.59 ± 1.1	7.65 ± 1.0

[1] Data are the means and standard deviations of two independent experiments (n = 6)

[2] Zone of inhibition includes a bore diameter of 6 mm

[3] Incubation conditions were: (O_2/48 h/37°C)

[4] pH of supernatant obtained from bifidobacteria was 3.96 for *B. infantis* 1912, 3.83 for *B. longum* 1941, 4.04 for *B. longum* BB536 and 3.89 for *B. pseudolongum* 20099

Table 2.2. Suppression of growth of pathogenic bacteria in the presence of supernatant obtained from bifidobacteria[1, 2]

	Supernatant obtained from bifidobacteria							
	B. infantis 1912[3]		*B. longum* 1941[3]		*B. longum* BB536[3]		*B. pseudolongum* 20099[3]	
Pathogenic bacteria	Adj[4]	Unadj[5]	Adj[4]	Unadj[5]	Adj[4]	Unadj[5]	Adj[4]	Unadj[5]
E. coli LE392 (VUN104)	-2.60%[6]	-61.17%	-8.16%	-51.94%	-0.73%	-4.67%	2.31%	-15.81%
C. perfringens (VUP060)	8.05%	-52.02%	7.82%	-42.73%	11.14%	-2.61%	9.33%	-14.77%
E. aerogenes (VUN025)	3.84%	-65.59%	-0.16%	-48.91%	7.07%	-3.77%	2.31%	-14.23%
C. albicans (VUO002)	1.43%	-62.67%	-7.92%	-45.01%	3.89%	-8.23%	4.81%	-15.11%
S. agalactiae (VUP053)	4.95%	-62.23%	-1.87%	-54.46%	4.56%	-26.00%	-2.70%	-39.91%

[1] Data are the means of three independent experiments (n = 6)

[2] Optical density measured at a wavelength of 620 nm

[3] Percentage difference in optical density between control and pathogenic bacteria with supernatant obtained from bifidobacteria

[4] Supernatant was adjusted to pH 7.0

[5] Unadjusted supernatant (pH of bifidobacteria supernatant 3.96 for *B. infantis* 1912, 3.83 for *B. longum* 1941, 4.04 for *B. longum* BB536 and 3.89 for *B. pseudolongum* 20099).

[6] Percentage difference = ((Co-culture OD/Control OD x 100)-100)

Table 2.3. Inhibition of *E. coli* and *E. aerogenes* with supernatant obtained from bifidobacteria as measured by OD and bacterial counts

			Bifidobacterium strains							
			B. infantis 1912		*B. longum* 1941		*B. longum* BB536		*B. pseudolongum* 20099	
Bacteria strain & code	Bacterial counts	Control	Unadj[5]	Adj[6]	Unadj[5]	Adj[6]	Unadj[5]	Adj[6]	Unadj[5]	Adj[6]
	OD (24 h)[2]	0.704	0.199	0.708	0.196	0.705	0.345	0.753	0.210	0.723
E. coli	CFU (24h)[3]	9.77	-59.3%	0.60%	-63.40%	-0.84%	-51.0%	5.88%	-52.2%	14.45%
LE392[3]	OD (48 h)[2]	0.999	0.525	0.895	0.539	0.903	0.710	0.967	0.642	0.914
(VUN104)	CFU (48h)[3]	9.78	-20.1%	-4.82%	-24.1%	-6.98%	-12.9%	2.00%	-18.0%	-3.99%
	OD (24 h)[2]	0.718	0.309	0.777	0.307	0.787	0.520	0.776	0.326	0.768
E. aerogenes[3]	CFU[2] (24 h)[3]	9.73	-47.5%	4.05%	-49.9%	19.15%	-42.1%	21.73%	-45.1%	4.05%
(VUN025)	OD (48 h)[2]	0.899	0.718	0.931	0.728	0.915	0.821	0.955	0.728	0.933
	CFU (48h)[3]	9.78	-15.8%	2.01%	-16.1%	-5.87%	-9.8%	1.67%	-23.8%	-3.69%

[1] Data are the means of three independent experiments (n = 6)

[2] Optical density measured at a wavelength of 620 nm

[3] CFU = Log_{10} Colony forming units per ml

[4] *E. coli* LE392 and *E. aerogenes* were incubated on EMB agar (O_2/37°C/48 h)

[5] Unadj = pH of supernatant not adjusted.

[6] Adj = pH of supernatant was adjusted to 7.0

Table 2.4. Effect of co-culturing *B. longum* 1941 with five pathogenic microorganisms after 24 h[1]

	Control			Co-culture[2]					
Bacteria strain & code	OD_{620}[3]	CFU[4]	pH[5]	OD_{620}	% OD difference	CFU	% CFU difference	pH	pH difference
E. coli (VUN104)	1.443	11.10	6.02	1.116	-22.6%	10.51	-74.3%	4.54	-1.48
C. albicans (VUO002)	1.290	11.06	6.48	1.105	-14.34%	10.59	-66.2%	4.53	-1.95
S. agalactiae (VUP053)	1.190	11.97	6.05	1.089	-8.57%	11.62	-55.4%	4.44	-1.61
E. aerogenes (VUN025)	1.483	11.08	6.85	1.079	-27.2%	10.68	-60.1%	4.61	-2.24
C. perfringens (VUP060)	1.022	11.67	5.64	1.086	6.26%	11.61	-13.2%	4.67	-0.97

[1] Data are the means of three independent experiments (n = 6)

[2] Co-culture of pathogenic bacteria and *B. longum* 1941

[3] Optical density of pathogenic bacteria (620 nm)

[4] Log_{10} Colony forming units incubated using nutrient agar (O_2/37°C/48 h); *B. longum* was incubated on MRS-NNLP agar (AnO_2/37°C/72 h)

OD for *B. longum* 1941 was 0.969, CFU/ml was 3.05×10^{11} and pH was 4.4.1 after 24 h incubation

[5] pH measurements of control and co-cultures

Table 2.5. Acetic and lactic acid production by four *Bifidobacterium* species

Bifidobacterium strain	Acetic acid (mM)[1]	Lactic acid (mM)[1]	Ratio (acetic: lactic)
B. infantis 1912	54.9 ± 24.79	33.2 ± 11.74	1.65 : 1.00
B. longum 1941	39.8 ± 3.57	19.4 ± 3.04	2.05 : 1.00
B. longum BB536	55.4 ± 10.21	17.1 ±3.92	3.24 : 1.00
B. pseudolongum 20099	43.1 ± 27.44	21.5 ± 7.57	2.00: 1.00

[1] Data are the means and standard deviations of three independent experiments (n = 6)

2.5 Conclusion

Overall, *Bifidobacterium infantis* 1912, *B. longum* 1941, *B. longum* BB536 and *B. pseudolongum* 20099 were able to successfully inhibit eleven selected pathogenic and putrefactive microorganisms as measured by inhibition assays and during growth with supernatants obtained from four strains of bifidobacteria. The inhibition of pathogenic and putrefactive bacteria was due to the lowering of the pH by the bifidobacteria strains. When the supernatant was adjusted to pH 7.0, the inhibition of growth and zones of inhibition were completely absent. The antimicrobial effects of *Bifidobacterium* spp against pathogenic and putrefactive microorganisms were generally attributed to the decrease of pH resulting from production of lactic and acetic acids as a result of fermentation of glucose. These selected *Bifidobacterium* strains will need to be studied further to substantiate additional beneficial antimicrobial properties *in vivo*.

3.0. GROWTH, VIABILITY AND ACTIVITY OF *BIFIDOBACTERIUM* SPP. IN SKIM MILK CONTAINING PREBIOTICS[1]

3.1. Introduction

The human gastrointestinal tract (GIT) comprises of a complex microbial ecosystem consisting of over 400 different species of bacteria (Simon and Gorbach, 1984; Parodi, 1999; Rao, 1999) of which the genus *Bifidobacterium* are generally considered to be health promoting and beneficial (Kimura *et al.,* 1997). The equilibrium of the ecosystem is dynamic and may be negatively altered by ageing, diet and other environmental factors (Collins and Gibson, 1999). It is believed that the maintenance of the intestinal bacterial population, which predominantly contains beneficial species and minimal putrefactive processes, is important for maintaining intestinal well being (Crittenden, 1999).

Recently, research has focused on the ability of probiotic bacteria to ferment oligosaccharides, which bypass metabolism and adsorption in the small intestine and may have a major influence on the selective growth of bifidobacteria (Gibson and Roberfroid, 1995c; Fooks *et al.,* 1999; Shin *et al.,* 2000b). Oligosaccharides are a group of short chain non-digestible polysaccharides that occur naturally in foods. They are typically defined as glycosides that contain between 3 to 10 sugar moieties and are characterized by the type and sequence of the monosaccharide moieties present (Crittenden, 1999).

Commercially, oligosaccharides are produced by enzymic processes either by hydrolysis of polysaccharides or synthesis from smaller sugars using transglycosylases. Certain oligosaccharides have the potential to increase bifidobacteria population in the colon without being utilized by other intestinal bacteria. Because of their prebiotic properties,

[1] A version of this chapter has been published: Bruno, F.A. and Shah, N.P. 2002. Growth, viability and activity of *Bifidobacterium* spp. in skim milk containing prebiotics. *Journal of Food Science* 67(7): 2740-2744.

oligosaccharides have received much recent attention as functional food ingredients. Some non-digestible carbohydrates have a number of functional effects on the gastrointestinal tract including reduced fat and cholesterol absorption, modulation of microbial fermentation, decreased pH and ammonia production, which therefore have a direct effect on reducing intestinal disturbances, cardiovascular disease and intestinal cancer (Ziemer and Gibson, 1998).

There has been a considerable interest in the use of prebiotics to enhance the survivability and colonization of probiotic bacteria that are added in food products (Ziemer and Gibson, 1998). Because of the difficulty in maintaining probiotic organisms in the gastrointestinal tract, significant research has been focused in discovering which prebiotic is most beneficial in increasing the levels of bifidobacteria in the gastrointestinal tract (Ziemer and Gibson, 1998). The most studied prebiotics are inulin and fructo-oliogosaccharides as they resist digestion by gastric acid and pancreatic enzymes *in vivo* (Cummings *et al.*, 2001).

Bifidobacteria are characterized as Gram-positive, obligate anaerobes, non-spore-forming, nonmotile bacilli sometimes in the form of club-shaped or spatulate extremities. Acetic and lactic acids are produced primarily in the molar ratio of 3:2. Glucose is degraded exclusively and characteristically by the fructose-6-phosphate 'shunt' metabolic pathway (Scardovi, 1986; Tannock, 1998; Bezkoravainy, 2001; Holzapfel *et al.*, 2001). Bifidobacteria have a number of beneficial health properties including inhibition of pathogenic bacteria, synthesis of B vitamins, lowering of blood ammonia levels, cholesterol absorption, and inhibition of tumor formation (Ziemer and Gibson, 1998; Roberfroid, 2001a, b). As a result, bifidobacteria are incorporated into fermented dairy foods and other foods (Crittenden, 1999). The ultimate intent of this approach is to provide the gastrointestinal tract of humans with elevated viable populations of bifidobacteria (Coussement, 1996). Although the effects of

oligosaccharides on colonic bifidobacteria have been investigated, there are few reports on the effects of oligosaccharides on bifidobacteria stored in dairy foods.

Loss of viability of bifidobacteria is typically more pronounced in fermented milk than in unfermented milk due to acid injury to the organism (Dave and Shah, 1997a). Lankaputhra *et al.* (1996) observed that viability of bifidobacteria strains such as *Bifidobacterium infantis* in 12% skim milk at pH 4.3 decreased by 30% after 12 d of storage at 4°C. After 24 d at the same temperature, the counts decreased by more than 82%. Medina and Jordano (1994) observed a 93% reduction in bifidobacterial counts of fermented milk produced in Spain at 7°C. Thus, viability of bifidobacteria has received much attention (Dave and Shah, 1997a; Shah, 2000; Shin *et al.,* 2000a). The effects of prebiotics on colonic bifidobacteria have been investigated, however, there are limited reports on the effects of prebiotics on bifidobacteria in dairy foods during storage.

The products containing probiotics and prebiotics are known as synbiotics (Ziemer and Gibson, 1998). It is important to select appropriate prebiotics for improved retention of viability of bifidobacteria in dairy foods with an ultimate goal of delivering a large number of viable bifidobacteria in colon, and for stimulation of bifidobacterial growth in the colon. Therefore, the objective of this study was to investigate the effects of commercially available Hi-maize™, inulin, raftilose and lactulose on growth, activity and viability, and pH reducing ability of five *Bifidobacterium* species.

3.2. Materials and Methods

3.2.1. Culture preparation

Five strains of *Bifidobacterium* (*B. infantis* 1912, *B. longum* 1941, *B. longum* BB536, *B. pseudolongum* 20099 and *B. animalis* Bb12) were obtained from Victoria University Culture Collection (Werribee Campus, Australia). *B. longum* 1941 is a commercial strain, *B. longum* BB536 was initially obtained from Morinaga Milk Industry Co. Ltd. (Tokyo, Japan), and *B. animalis* Bb12 is a commercial strain initially obtained from Chr. Hansen Pty. Ltd. (Bayswater, Australia). Each *Bifidobacterium* strain was cultured anaerobically in MRS broth (Oxoid Ltd, Basingstoke, Hampshire, UK.) containing 5% (w/v) lactose (MRSL) at 37°C for 48 h using Gas Paks (Anaerobic system BR-038B, Oxoid Ltd., Hampshire, UK.). The cells were centrifuged for 15 min at 1000 x *g* at 4°C and subsequently resuspended in pasteurized (63°C for 30 min) 12% w/v reconstituted skim milk (RSM).

3.2.2. Growth of bifidobacteria in the presence of prebiotics

Four types of commercially available prebiotics were used in this study including Hi-maize™ (Starch Australasia Ltd., Lane Cove, Sydney, Australia), lactulose (Sigma Chemical Co., St. Louis, MO, USA), raftilose and inulin (Orafti Pty. Ltd., Tienen, Belgium). Inulin and raftilose are extracted from chicory roots or Jerusalem artichoke and have a degree of polymerization (DP) ranging from 2-50. The method of Shin *et al.* (2000b) was used to measure growth of bifidobacteria with some modifications. Shin *et al.* (2000b) reported a maximum activity of bifidobacteria with 5% (w/v) oligosaccharides. Thus in the present investigation prebiotics were used at the rate of 5% (w/v). The prebiotics (5%) were added to 12% (w/v) RSM in 250 mL Schott bottles and pasteurized at 70°C for 15 min. Control samples did not contain any of the four prebiotics. Duplicate bottles of each treatment were

prepared. Pasteurised RSM was inoculated (5% v/v) separately with five strains of *Bifidobacterium* and incubated anaerobically at 37°C for 48 h. An aliquot from each sample was taken before and after incubation and diluted (1: 20, v/v) with 0.2% (w/v) EDTA (pH 12.0) and turbidity measured at 640 nm using a Novaspec II spectrophotometer (Pharmacia Biotech, LKB Biochrom, UK). Uninoculated pasteurized RSM diluted with 0.2% (w/v) EDTA (pH 12.0) was used as a blank.

Specific growth rate (μ) for each culture was calculated using the following equation: $\mu = (\ln X_2 - \ln X_1) / (t_2 - t_1)$, where X_2 and X_1 are the cell density at time t_2 and t_1. Mean doubling time (T_d) was calculated as: $T_d = \ln 2/\mu$ (Shin *et al.*, 2000b). Another sample was collected for a pH measurement of fermented skim milk.

3.2.3. Fermentation activity of Bifidobacterium spp.

The activity of each *Bifidobacterium* culture in the presence of different prebiotics was determined by measuring end-products of fermentation (lactic and acetic acids) using high performance liquid chromatography (HPLC, Varian Australia Pty. Ltd., Mulgrave, Australia). RSM containing 5% (w/v) Hi-maize™, lactulose, raftilose or inulin and fermented by *Bifidobacterium* (1912, 1941, BB536, 20099 and Bb12) as described previously (2.2.6) were prepared for HPLC analysis using the method described by Dubey and Mistry (1996). One hundred microlitres of 15.8 M HNO_3 and 14.9 mL of 0.009 M H_2SO_4 were added to 1.5 mL of sample and centrifuged at 4000 x *g* for 10 min using a benchtop centrifuge (Sorvall RT7, Newtown, CT, USA). The supernatant was filtered using 0.22 µm Millipore filters (Millipore Corp., Bedford, MA, USA) and 2 mL aliquots were stored in HPLC vials at –20°C until analyzed.

The HPLC system consisted of a Varian 9012 solvent delivery system, a Varian 9100 autosampler, a Varian 9050 variable wavelength UV/Vis tunable absorbance detector and a

730 data module. An Aminex HPX-87H column (300 mm x 7.8 mm, Bio-Rad Laboratories, Richmond, CA, USA) and a guard column with disposable cartridges H^+ (Bio-Rad Laboratories) maintained at 65°C were used for organic acid analysis. The degassed mobile phase of 0.009 M H_2SO_4, filtered through a 0.45μm membrane filter (Millipore, U.S.A.) was used at a flow rate of 0.3 mL/min. The wavelength of detection was optimized at 220 nm for the organic acids being quantified. The standard solutions of organic acids (lactic and acetic acids; Sigma, St Louis, MO, USA) were prepared in water to establish elution times and calibration curves. The retention times for lactic acid was 25.7 min and for acetic acid 16.2 min. The standard curve coefficients were 0.9991 and 0.9995 for lactic acid and acetic acid, respectively.

3.2.4. Determination of Bifidobacterium viability during refrigerated storage

Bifidobacterium strains; *B. infantis* 1912, *B. longum* 1941, *B. longum* BB536, *B. pseudolongum* 20099 and *B. animalis* Bb12 were cultured anaerobically at 37°C for 48 h with 5% (w/v) of each of the prebiotics. Samples containing no prebiotics were used as controls. All inoculated samples were stored at 4.0 ± 1°C for 4 wk. One millilitre of each fermented milk sample was diluted with 9 mL of sterile 0.1% (w/v) peptone water (Amyl Media Pty. Ltd., Dandenong, Australia) and mixed uniformly with a vortex mixer. Subsequent serial dilutions were prepared and viable numbers enumerated using pour plate technique to ensure consistent results among samples. Bifidobacteria were enumerated using MRS agar (Oxoid Ltd., Basingstoke, Hampshire, UK) containing 0.05% L-cysteine·hydrochloride (Sigma, St Louis, MO, USA). The inoculated plates were incubated anaerobically at 37°C for 72 h. The colonies were counted using a colony counter (Model Gallenkamp, Leicestershire, UK.). Viability of each *Bifidobacterium* spp with each particular added prebiotic was assessed immediately after a 1% (v/v) inoculation into the pasteurised milks and after 4 wk of

refrigerated storage. Percent viabilities of each culture in the presence of different prebiotics were calculated as follows:

Bifidobacterium viability % = Log_{10} ((CFU/ml at 4 wks of storage/initial CFU/ml) x 100))

3.2.5. Determination of pH

The pH of the fermented samples was measured in duplicate at 0 h, 48 h and 4 wk for each set of treatments of fermented milks containing *Bifidobacterium* spp and prebiotics. Determination of average pH of the fermented samples were performed at 17-20°C using a pH meter (Model 8417; Hanna Instruments Pty. Ltd., Singapore) after calibrating with fresh pH 4.0 and 7.0 standard buffers.

3.2.6. Statistical analysis

Each experiment was independently replicated three times in a completely randomized design. All analysis and enumerations were done in duplicate. Statistical analysis was conducted using Students t-tests where $p<0.05$ was considered statistically significant.

3.3. Results and Discussion

3.3.1. Mean doubling time of Bifidobacterium spp.

The mean doubling time in minutes of the five *Bifidobacterium* species grown in skim milk in the presence of 5% (w/v) each of Hi-maize™, lactulose, inulin and raftilose is presented in Table 3.1. Each of the four prebiotics added and five bifidobacteria strains displayed different mean doubling times after incubation for 48 h. Mean doubling time was used as a measure of the effectiveness of a specific carbon source in modulating growth rate. Growth promotion of *Bifidobacterium* strains by prebiotics was obtained dose dependently of either a control containing no prebiotics or a pasteurised skim RSM containing 5% (w/v) concentration of a particular prebiotic. Research conducted by Shin *et al.* (2000b) have shown increased activity of bifidobacteria with increase in oligosaccharides concentration, and maximum activity was observed at a concentration of 5%. Therefore, a 5% concentration of each prebiotic was used in this study.

In general, doubling time of *Bifidobacterium* species grown with prebiotics decreased as compared to the control, except with Hi-maize™. Mean doubling times were significantly lower ($p<0.1$) following the addition of 5% lactulose or inulin with strains *B. longum* 1941, *B. longum* BB536, *B. pseudolongum* 20099 and *B. animalis* Bb12 when compared to the control samples. The doubling time with raftilose and *B. animalis* Bb12 was also significantly lower ($p<0.1$) when compared with the control. The addition of Hi-maize™ and raftilose except for *B. animalis* Bb12 had no significant effect. The doubling time for the strain *B. infantis* 1912 was not altered significantly by addition of any of the four prebiotics. Among the prebiotics tested in this study, lactulose, raftilose and inulin were equally effective in enhancing growth rate of *B. animalis* Bb12 in skim milk. A concentration of 5% (w/v) raftilose and inulin resulted in the shortest mean doubling time of 158 min among the four prebiotics tested for *B.*

animalis Bb12. The highest doubling time for the five *Bifidobacterium* strains tested was 457 ± 20 min also by *B. animalis* Bb12 with the prebiotic Hi-maize™.

Inulin was found to be least effective in stimulating the growth of *Bifidobacterium* spp. Bf-1 and Bf-2 in the study of Shin *et al.* (2000b). However, in the present investigation inulin was effective in stimulation of growth of bifidobacteria. This could be due to differences in strains that were used in the study of Shin *et al.* (2000b). Our results are consistent with previous reports by Gibson and Wang (1994a) on the ability of inulin to stimulate the proliferation of bifidobacteria relative to other intestinal microflora. Research conducted by Roberfroid *et al.* (1998b) have also reported improved *in vitro* fermentation of inulin by human fecal bacteria when molecules had degree of polymerisation greater than 10.

Autoclaved milk (heated at 121°C for 15 min) contains some lactulose resulting from isomerization of lactose; however, the level of lactulose in milk heated at 70°C for 15 min is expected to be very low. The amount of lactulose formed in all RSM samples that were used to grow bifidobacteria in this study would be similar, hence the stimulation of growth or reduction in doubling time would be due be the presence of added prebiotics. All *Bifidobacterium* spp with added Hi-maize™ consistently exhibited the highest doubling time among the four prebiotics tested and the values were distinctly higher than the control samples.

3.3.2. Acetic and lactic acid production

The lactic and acetic acid concentrations produced after fermentation by each of the five *Bifidobacterium* spp with added prebiotics in skim milk is presented in Table 3.2. The effects of selected prebiotics in the metabolic production of acetic acid and lactic acid were varied among the *Bifidobacterium* spp studied. The highest concentration of acetic acid produced was by *B. pseudolongum* 20099 with lactulose (69.3 ± 9.3 mM), while the lowest

was produced by *B. animalis* Bb12 with inulin (31.9 ± 21.99 mM). The highest concentration of lactic acid was produced by *B. infantis* 1912 with lactulose (24.8 ± 10.56 mM), and the lowest by *B. longum* BB536 with lactulose (11.8 ± 5.59 mM). The samples that contained inulin showed an average molar ratio of 1.9:1 as compared to the theoretical ratio of 3 moles of acetic acid to 2 moles of lactic acid. Acetic acid concentration produced by the five strains of bifidobacteria in combination with the four prebiotics was only significant ($p<0.05$) by *B. pseudolongum* 20099 with lactulose when compared to the controls that contained no prebiotics. Lactic acid concentration was significantly lower ($p<0.05$) when compared to the controls by *B. infantis* 1912 in Hi-maize™ and raftilose.

We observed a higher average molar ratio of acetic to lactic production when Hi-maize™ was added (3.3:1), however, the addition of inulin with the five individual strains of bifidobacteria produced the closest to optimum fermentation average ratio of acetic to lactic acid of 1.9:1, which is close to the theoretical molar ratio of 3 acetic acids to 2 lactic acids. The control samples containing bifidobacteria in 12% RSM but no added prebiotic produced an average ratio of 2.1:1. Overall, the highest average fermentation ratio of acetic to lactic acid using a particular prebiotic was 3.3 (Hi-maize™) and the lowest was 1.9 (Inulin).

Bifidobacteria produce lactic and acetic acids as metabolic products of sugar fermentation. This is an important characteristic of bifidobacteria. The *Bifidobacterium* fermentation pathway results in 3 moles of acetic acid and 2 moles of lactic acid per 2 moles of glucose in an ideal synthetic medium, therefore generating a theoretical molar ratio (acetic: lactic) of 3:2 (Scordavi and Trovatelli, 1965). Although lactic acid production is desirable in fermented dairy foods, a high concentration of acetic acid can result in a distinct vinegar flavour in products. Thus, a high acetic to lactic acid ratio is typically undesirable in fermented dairy products.

Roberfroid *et al.* (1998) reported *in vitro* fermentation of inulin by human fecal bacteria. Molecules with DP > 10 were fermented on the average half as quickly as molecules with DP < 10. Hopkins *et al.* (1998) reported that galactooligosaccharides (GOS) and fructo-oligosaccharides (FOS) having lower DP were best in supporting growth of bifidobacteria. In contrast, carbohydrates with high DP were poor substrates for bifidobacteria. There has not been considerable research conducted on the mechanism of carbohydrate uptake by bifidobacteria; however, it appears likely that the substrate transport systems may be more efficient for dimeric and oligomeric carbohydrate sources.

3.3.3. Viability of Bifidobacterium spp. at refrigerated storage temperatures

The viability of the five *Bifidobacterium* strains grown in RSM containing prebiotics after 48 h incubation and after 4 wks of refrigerated storage at 4°C is presented in Table 3.3. As growth of bifidobacteria was affected by the type of prebiotic added, the initial viable cell counts varied prior to refrigerated storage. Averaged percent viabilities were therefore calculated and reported. In general, the viability of all five strains of bifidobacteria stored at 4°C was reduced the greatest by 76.35% (*B. animalis* Bb12 with raftilose) to the lowest of 24.66% (*B. animalis* with Hi-maize™). However, the retention of viability during the 4 wks of storage was significantly higher ($p<0.05$) when they were grown in the presence of prebiotics as compared to the control without any prebiotic added. The highest retention in viability was observed when cultures were grown in the presence of Hi-maize™ at a concentration of 5% (w/v). The highest viability of 75.3% was retained by strains *B. longum* BB536 and *B. animalis* Bb12 combined with Hi-maize™. The average viability of the five *Bifidobacterium* strains containing Hi-maize™ was 58%. The prebiotic inulin was the least effective overall in retaining the viability ($p<0.05$) with an average viability of only 36.93%. The control samples containing bifidobacteria but containing no prebiotics had a significantly

lower average retention of viability of 27.15%, with a range from 24.02 to 31.73% ($p<0.05$). In our study, 75 to 24% of the viability was retained after 28 d of storage at 4°C when prebiotics were added. These results are similar to those of Shin *et al.* (2000a).

The loss in viability of bifidobacteria occurs in fermented milks due to several factors including production of lactic and acetic acid and presence of oxygen as discussed elsewhere (Shah, 2000). Research conducted by Lankaputhra *et al.* (1996) had also observed a decrease in viability of *B. infantis* of 30% in 12% (w/v) RSM at pH 4.3. It is interesting to note while Hi-maize™ was least effective in stimulation of growth of bifidobacteria, this carbohydrate however was also the most effective in maintaining viability during refrigerated storage. The solution of 5% Hi-maize™ with skim milk was distinctly more viscous than that of other prebiotics used and may have provided enhanced protection of bifidobacteria during refrigerated storage at 4°C. Overall, pasteurised skim milk containing a concentration of 5% (w/v) prebiotics and bifidobacteria had a viability of between 36.93 to 58 % over 4 wks at 4°C.

3.3.4. Measurement of pH of RSM

The average pH values of RSM after addition of prebiotics, after 48 h of incubation with each strain of bifidobacteria in the presence of each prebiotics and after 4 wks of storage is presented in Table 3.4. The initial pH of RSM after addition of prebiotics ranged from log 6.24 ± 0.1 to 6.26 ± 0.1. The average pH values after 48 h incubation dropped 1.5 to 2.0 log and varied from log 4.33 to 4.57. The average pH of all fermented samples decreased slightly during the 4 wks of storage, possibly due to post-fermentation production of acetic acid and lactic acid by bifidobacteria strains. These results were similar to those samples with or without prebiotics added.

Bifidobacteria grow well under anaerobic conditions, especially in the presence of 0.05% of L-cysteine (Dave and Shah, 1997a). If optimum growth conditions were provided, most strains of bifidobacteria would decrease the pH of sterile RSM to 4.5 in 24 h (Dave and Shah, 1997a). Overall, the pH of samples with prebiotics after 4 wks of storage ranged from pH 4.07 ± 0.4 (*B. longum* 1941 with inulin) to pH 4.34 ± 0.3 (*B. animalis* Bb12 with raftilose). These pH values were not significant when compared to the control. Individual *Bifidobacterium* strains, which were significant ($p<0.05$) when combined with prebiotics were *B. infantis* 1912 with raftilose (pH 4.28) and lactulose (pH 4.13), *B. animalis* Bb12 with inulin (pH 4.13) and raftilose (pH 4.34) and *B. longum* 1941 with inulin (pH 4.07) and raftilose (pH 4.29). The pH of the Hi-maize™ samples was not significantly different ($p<0.05$) as compared to the control samples containing no prebiotics.

3.4. Tables

Table 3.1. Doubling time of five *Bifidobacterium* spp. in skim milk containing prebiotics[1]

	Mean doubling time (min)[2]				
	Prebiotic[3]				
Bifidobacterium spp.	Control	Hi-maize™	Lactulose	Raftilose	Inulin
B. infantis 1912	323 ± 19	378 ± 14	214 ± 8	271 ± 8	221 ± 11
B. longum 1941	348 ± 22	392 ± 15	205 ± 9[a]	252 ± 7	203 ± 9[a]
B. longum BB536	351 ± 22	426 ± 20	192 ± 8[a]	251 ± 7	181 ± 6[a]
B. pseudolongum 20099	337 ± 22	449 ± 20	175 ± 6[a]	246 ± 7	174 ± 6[a]
B. animalis Bb12	330 ± 22	457 ± 20	171 ± 6[a]	158 ± 8[a]	158 ± 3[a]

[1] Means ± standard deviations of 3 replicates for all treatments.

[2] Mean doubling time (T_d)= ln2 / μ (specific growth rate); $\mu = \ln X_2 - \ln X_1 / t_2 - t_1$

[3] Prebiotic was added at 5% (w/v) to 12% (w/v) RSM

[a] Significantly different ($p<0.1$) from the control.

Table 3.2. Acetic and lactic acid production by five *Bifidobacterium* spp. grown in skim milk containing 5% (w/v) prebiotics[1]

Prebiotic\species		Control	Hi-maize™	Lactulose	Raftilose	Inulin
B. infantis 1912	Acetic acid (mM)[2]	54.9 ± 24.79	53.7 ± 2.86	41.7 ± 12.45	40.8 ± 16.31	36.6 ± 3.73
	Lactic acid (mM)[2]	33.2 ± 11.74	12.6 ± 6.99[a]	24.8 ± 10.56	19.2 ± 3.89[a]	22.5 ± 7.7
	Ratio[3]	1.65	4.26	1.68	2.13	1.63
B. longum 1941	Acetic acid (mM)	39.8 ± 3.57	42.3 ± 21.33	42.3 ± 18.85	43.5 ± 11.59	38.0 ± 13.09
	Lactic acid (mM)	19.4 ± 3.04	19.3 ± 2.03	21.7 ± 3.98	22.3 ± 4.17	23.1 ± 4.57
	Ratio	2.05	2.19	1.95	1.95	1.65
B. longum BB536	Acetic acid (mM)	55.4 ± 10.21	50.4 ± 9.40	53.6 ± 16.25	51.2 ± 9.83	57.7 ± 7.19
	Lactic acid (mM)	17.1 ± 3.92	12.3 ± 5.29	11.8 ± 5.59	14.2 ± 3.31	15.1 ± 4.56
	Ratio	3.24	4.10	4.54	3.61	3.82
B. pseudolongum 20099	Acetic acid (mM)	43.1 ± 27.44	45.4 ± 18.45	69.3 ± 9.3[a]	45.7 ± 18.27	42.5 ± 29.97
	Lactic acid (mM)	21.5 ± 7.57	14.0 ± 8.73	20.1 ± 8.6	17.0 ± 5.49	24.0 ± 10.38
	Ratio	2.00	3.24	3.45	2.69	1.77
B. animalis Bb12	Acetic acid (mM)	38.5 ± 16.86	53.4 ± 14.38	34.8 ± 26.99	43.7 ± 14.92	31.9 ± 21.99
	Lactic acid (mM)	19.3 ± 3.47	15.7 ± 8.17	20.1 ± 4.02	17.4 ± 7.1	24.1 ± 4.75
	Ratio	1.99	3.40	1.73	2.51	1.32
Average	Acetic acid (mM)	46.34 ± 8.22	49.04 ± 5.03	48.34 ± 13.52	44.98 ± 3.89	41.34 ± 9.9
	Lactic acid (mM)	22.1 ± 6.4	14.78 ± 2.86	19.7 ± 4.82	18.02 ± 2.99	21.76 ± 3.78
	Ratio	2.1	3.3	2.5	2.5	1.9

[1] Measurements are means ± standard deviations of 3 replicates for all treatments.

[2] mM = millimole per ml of fermented milk

[3] Acetic acid: lactic acid ratio.

[a] Significantly different ($p<0.05$) from the control.

Table 3.3. Viability of five *Bifidobacterium* spp. grown in skim milk containing prebiotics after 4 wks of refrigerated storage at 4°C[1, 2]

Bifidobacterium spp.	Sampling time	Prebiotic				
		Control	Hi-maize™	Lactulose	Raftilose	Inulin
B. infantis 1912	0 week	8.47	8.30	8.20	8.36	8.41
	4 week	7.88	7.89	7.75	7.11	7.89
	Viability %	29.66 ± 0.1	44.29 ± 0.2[a]	37.85 ± 0.3[a]	56.22 ± 0.1[a]	49.99 ± 0.1[a]
B. longum 1941	0 week	8.25	8.37	8.21	8.13	8.25
	4 week	7.71	8.09	7.67	7.74	7.71
	Viability %	26.30 ± 0.1	51.17 ± 0.3[a]	27.49 ± 0.1[a]	29.7 ± 0.2[a]	34.61 ± 0.2[a]
B. longum BB536	0 week	8.16	8.05	8.07	8.61	8.16
	4 week	7.52	7.88	7.68	8.29	7.52
	Viability %	24.04 ± 0.1	75.32 ± 0.1[a]	55.43 ± 0.3[a]	44.86 ± 0.1[a]	30.04 ± 0.1[a]
B. pseudolongum	0 week	8.64	8.61	8.44	8.11	8.64
20099	4 week	7.92	8.00	7.91	7.82	7.92
	Viability %	24.02 ± 0.1	43.88 ± 0.3[a]	36.01 ± 0.1[a]	45.37 ± 0.1[a]	33.53 ± 0.1[a]
B. animalis Bb12	0 week	8.19	8.05	8.46	8.19	8.19
	4 week	7.70	7.93	7.03	7.59	7.76
	Viability %	31.73 ± 0.1	75.34 ± 0.1[a]	58.59 ± 0.3[a]	23.65 ± 0.1[a]	36.47 ± 0.2[a]
Average	Viability %	27.15 ± 0.1	58.00 ± 0.2[a]	43.07 ± 0.1[a]	39.96 ± 0.1[a]	36.93 ± 0.1[a]

[1] Measurements are Log_{10} means ± standard deviations of 3 replicates for all treatments.

[2] *Bifidobacterium* viability %= Log_{10} ((CFU/ml after 4 wks of storage / initial CFU/ml) x 100)).

[a] Mean values that are significantly different from the control ($p<0.05$).

Table 3.4. The pH of fermented samples after 4 wks of refrigeration at 4°C[1]

Bifidobacterium spp.	Reading interval	Prebiotic				
		Control	Hi-maize™	Lactulose	Raftilose	Inulin
B. infantis 1912	0 h	6.26 ± 0.1	6.23 ± 0.1	6.24 ± 0.1	6.25 ± 0.1	6.22 ± 0.1
	48 h	4.41 ± 0.1	4.40 ± 0.1	4.39 ± 0.1	4.42 ± 0.1	4.43 ± 0.1
	4 wk	4.24 ± 0.2	4.22 ± 0.2	4.13[a] ± 0.3	4.28[a] ± 0.2	4.15 ± 0.2
B. longum 1941	0 h	6.26 ± 0.1	6.24 ± 0.1	6.23 ± 0.1	6.25 ± 0.1	6.23 ± 0.1
	48 h	4.42 ± 0.1	4.40 ± 0.1	4.46 ± 0.1	4.54 ± 0.2	4.39 ± 0.1
	4 wk	4.26 ± 0.2	4.23 ± 0.2	4.16 ± 0.3	4.29[a] ± 0.3	4.07[a] ± 0.4
B. longum	0 h	6.25 ± 0.1	6.22 ± 0.1	6.21 ± 0.1	6.26 ± 0.1	6.20 ± 0.1
BB536	48 h	4.38 ± 0.1	4.33 ± 0.2	4.38 ± 0.1	4.38 ± 0.1	4.47 ± 0.2
	4 wk	4.28 ± 0.3	4.18 ± 0.3	4.20 ± 0.4	4.23 ± 0.5	4.24 ± 0.4
B. pseudolongum	0 h	6.26 ± 0.1	6.25 ± 0.1	6.20 ± 0.1	6.21 ± 0.1	6.23 ± 0.1
20099	48 h	4.49 ± 0.2	4.57 ± 0.2	4.44 ± 0.2	4.52 ± 0.2	4.48 ± 0.2
	4 wk	4.24 ± 0.3	4.31 ± 0.3	4.19 ± 0.3	4.20 ± 0.3	4.20 ± 0.4
B. animalis Bb12	0 h	6.24 ± 0.1	6.24 ± 0.1	6.25 ± 0.1	6.22 ± 0.1	6.24 ± 0.1
	48 h	4.37 ± 0.1	4.57 ± 0.2	4.37 ± 0.2	4.54 ± 0.2	4.41 ± 0.2
	4 wk	4.27 ± 0.1	4.32 ± 0.3	4.23 ± 0.1	4.34[a] ± 0.3	4.13[a] ± 0.1

[1] Measurements are means ± standard deviations of 3 replicates for all treatments.

[a] Mean values that are significantly different from the control ($p<0.05$).

3.5. Conclusion

The growth, fermentation activity and retention of viability during refrigerated storage of five strains of *Bifidobacterium* in skim milk were dependent on the prebiotic present as well as the strain of *Bifidobacterium* studied. Doubling time was significantly decreased with the addition of prebiotics as compared to the control except with Hi-maize™. However, the addition of 5% (w/v) prebiotics improved retention of viability of bifidobacteria, in particular with Hi-maize™. The final pH of fermented milks with prebiotics after 4 wk was similar to those of the controls. The metabolism of prebiotics would be more evident *in vivo* where prebiotics reach the colon unabsorbed and are selectively utilized by numerous indigenous *Bifidobacterium* species. Hence a combination of a *Bifidobacterium* strain with a specific prebiotic would be a feasible approach in administering the beneficial bacteria *in vivo*.

4.0. VIABILITY OF FREEZE-DRIED BIFIDOBACTERIA AT VARIOUS TEMPERATURES DURING PROLONGED STORAGE[1]

4.1. Introduction

The human body is inhabited permanently and transiently by a myriad of bacteria that have a significant influence on health and longevity (Collins and Gibson, 1999; Macfarlane and Cummings, 1999). Moreover, the human gastrointestinal tract contains an ecosystem consisting of billions of living microorganisms that typically coexist in a symbiotic relationship. The human intestinal flora contains over 400 distinct species of microorganisms inhabiting the various regions of the human digestive tract (Simon and Gorbach, 1984; Parodi, 1999; Rao, 1999) cumulating in a population greater than the number of tissue cells (Dunne *et al.*, 2001). The maintenance of this internal microflora contributes to the overall function of the intestinal tract, suppressing harmful microorganisms, providing the body with enzymes, nutrients and chemicals to the body.

Bifidobacteria are an important genus that inhabits the normal colonic microflora (Hopkins *et al.*, 1998). *Bifidobacterium* plays a significant role in human health from birth to old age (Fuller and Gibson, 1997). The intestinal tract of a breast-fed infant comprises of approximately 90% bifidobacteria which produce beneficial byproducts for protecting the infant from harmful pathogenic bacteria (Hoover, 1993; Saavedra, 2001). However, the bifidobacterial population within the human intestinal tract decrease progressively with ageing, which in turn, gradually favours the growth of harmful viral, fungal and putrefactive, disease causing bacteria (Hopkins *et al.*, 1998; Gomes and Malcata, 1999).

[1] A version of this chapter has been published: Bruno, F.A. and Shah, N.P. 2003. Viability of freeze-dried bifidobacteria at various temperatures over prolonged storage. *Journal of Food Science* 68(7):2336-2339.

The consumption of probiotic products has risen considerably, especially over the last two decades due significantly to the considerable amount of credible, scientific evidence in human studies that regular probiotic consumption maintains a healthy gastrointestinal tract (Macfarlane and Cummings, 1999; Salminen, 1999). These numerous benefits include a reduction in carcinogen production (Kailasapathy and Chin, 2000), regression of tumours (Tannock, 1999), inhibition of intestinal pathogenic bacteria (Bruno and Shah, 2002a), production of B vitamins (Hawkins, 1993), decreased duration of diarrhoea (De Ross and Katan, 2000; Playne and Salminen, 2001; D'Souza *et al.*, 2002), reduction in blood pressure and serum cholesterol concentration (Hiatt, 1984; Klaenhammer and Kullen, 1999; De Ross and Katan, 2000). There is sufficient evidence that these microorganisms improve colonic flora, however, other claims require further studies.

Probiotic cultures in food should be 1) well defined and correctly taxonomically named, 2) available in a viable state in sufficient quantities for the entire period of shelf life, 3) able to reach the intestine in a viable state and in sufficient numbers, 4) be able to provide beneficial effects to the consumer and, 5) genetically stable (Reuter *et al.*, 2002). However, the viable microbial count of many probiotic products has been questionable as analysis of products has confirmed poor survival of probiotic bacteria in yoghurts and fermented milks (Shah, 2000). In order to provide health benefits a minimum concentration of 10^6/ml cells must be consumed (Dave and Shah, 1997a; Kailasapathy and Chin, 2000).

The addition of selected prebiotics increases the number of bifidobacteria in the human large intestine (Gibson *et al.*, 1995a). Prebiotics are defined as "non-digestible carbohydrates that beneficially affect the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon, and thus improve the hosts health" (Gibson and Fuller, 2000). Inulin is a mixture of poly- and oligo-saccharides of fructose units linked via β(2-1) occur naturally in several plant foods including onion, chicory and garlic

(Gibson *et al.*, 1994c) and is indigestible to all higher animals. Inulin has been extensively studied and it is reported to increase bifidobacteria numbers both *in vitro* (Rycroft *et al.*, 2001; Bruno *et al.*, 2002b,) and *in vivo* (Gibson *et al.*, 1995).

Viability of bifidobacteria in fermented products declines over time due to the acidity of the product, storage temperature, storage time and depletion of nutrients (Dave and Shah, 1997a). Loss of viability of probiotic bacteria occurs in fermented products and these products have limited shelf life (Dave and Shah, 1996). As a result, freeze-dried probiotic bacteria are marketed as capsules or packaged in foil sachets. There is limited information available on viability of bifidobacteria in probiotic products at various temperatures. A correct storage temperature of probiotic preparations is essential to maintain viable populations of freeze-dried probiotic bacteria. This study examined the survival of experimental freeze-dried bifidobacteria and commercial probiotic preparations at different storage temperatures (-18°C, 4°C, and 20°C) for a prolonged period.

4.2. Materials and Methods

4.2.1. Bacteria

Bifidobacterium longum 1941 and *Bifidobacterium longum* BB536 were obtained from the Victoria University Culture Collection (Werribee, Australia). *B. longum* 1941 was originally obtained from the Commonwealth Scientific and Industrial Research Organisation (CSIRO, Highett, Australia). Freeze-dried *B. longum* BB536 was originally obtained from Morinaga Milk Industry Co. Ltd (Tokyo, Japan) through the courtesy of Pacific Medical Pty. Ltd. (Richmond, Australia). *B. longum* BB536 has been shown to possess many health benefits including; alleviation of constipation, prevention of diarrhea, reduction in cancer risk, development of immunopotentiation, enhancement of bone strength, improves intestinal environment and suppress infection (Ogata and Matsukuma, 1995).

The organisms were stored at -80°C in (12% w/v) sterile (121°C/15 min) reconstituted skim milk (RSM) and glycerol (20% v/v). Both strains were identified as bifidobacteria using the F6PPK test (Orban and Patterson, 2000), Gram staining (Scardovi, 1986) and carbohydrate fermentation test using a rapid ID 32A kit (Biomerieux Pty. Ltd., Lyon, France).

4.2.2. Growth media

The organisms were grown in sterile de Mann, Rogosa, Sharpe (MRS) broth (Oxoid Ltd., West Heidelberg, Australia). Filter-sterilized L-cysteine·HCl (LC) solution (5% w/v) was also added to the agar prior to inoculation, to lower the oxidation-reduction potential of the medium and enhance the growth of bifidobacteria.

4.2.3. Freeze-drying of Bifidobacterium spp

Pure cultures of *B. longum* 1941 and *B. longum* BB536 were transferred from frozen stock culture vials stored at -80°C into MRS broth containing 0.05% LC and incubated anaerobically at 37°C for 18-20 h. Three successive transfers were carried out. The cultures were then centrifuged at 4,500 x *g* at 4°C for 15 min. Twenty millilitres of sterile 0.1 M sodium phosphate buffer (pH 6.8) were added to the cell pellet and re-centrifuged (2,714 x *g* at 4°C, 15 min). The cell pellet was suspended in sodium phosphate (20 ml) containing Unipectine™ RS150 (Savannah Biosystems, Balwyn East, Australia) (2% w/v) as a cryoprotectant. The cell suspension was then aseptically poured into large petri dishes, sealed with paraffin film and aluminium foil and frozen overnight at -18°C. Freeze-drying for 48 h immediately followed this. The freeze-dryer (model FD-300, Airvac engineering Pty. Ltd., Dandenong, Australia) was programmed to operate for 10 min of initial freezing after reducing internal pressure to –100 Torr, 44 h primary freezing and finally 4 h of secondary freezing. The temperature inside the freeze-dryer was maintained at -88°C. The two batches of freeze-dried *Bifidobacterium* cells were then aseptically transferred from the petri-dishes and stored into separate sterile, sealed, plastic bags and stored at -18°C. An initial bacterial count was performed using MRS agar with 0.05% LC, the plates were then stacked into anaerobic jars with Gas-paks and incubated at 37°C for 3 d.

4.2.4. Capsule manufacture of experimental probiotic preparations

Two sealed plastic bags containing a mixture of freeze-dried bifidobacteria and inulin (Orafti Pty. Ltd., Tienen, Belgium) were transported frozen to Cottee Pharmaceuticals Pty. Ltd. (Frenchs Forest, Sydney, Australia) where the capsules were manufactured. Manufacturing of the probiotic capsules involved aseptically adding freeze-dried powder

concentration of LC (Dave and Shah, 1996). The commercial probiotic preparations were incubated using anaerobic jars with two Gas-paks at 37°C for 72 h.

4.2.6. Commercial probiotic capsules

Five different commercial brands of capsules containing bifidobacteria and other probiotic species were purchased from a local health food shop stored in a commercial refrigerator (4°C). The five commercial brands consisted of four Australian and one New Zealand manufacturer. All five brands of probiotic capsules contained *Bifidobacterium* species, which included *B. longum, B. infantis*, *B. bifidum* and *B. lactis*. Each of these products contained information on the number of bifidobacteria (CFU/ml) contained in each capsule at the time of manufacture (Table 4.3)

4.2.7. Statistics

Three capsules were enumerated in duplicate and data was presented in mean $\log_{10}$ CFU ± standard error (n = 6). Statistical analysis was performed using analysis of variance (ANOVA) using the software package Decision Tools Suite software StatPro™ for Microsoft® Excel (NY, USA). ANOVA was used to test for differences over time and between different storage temperatures for each product where $p<0.05$ was considered statistically significant.

4.3. Results and Discussion

4.3.1. Viable counts of B. longum 1941 over 20 months

The average bifidobacterial counts of three randomly selected capsules over 20 mo containing *Bifidobacterium longum* 1941 stored at -18°C, 4°C and 20°C are presented in Table 4.1. The counts of *B. longum* 1941 decreased significantly ($p<0.05$) from 10.18 ± 0.02 log to 8.70 ± 0.02 log cfu/ml over the twenty mo of storage at -18°C. The same strain stored at 4°C exhibited a significant decrease ($p<0.05$) in viability and a greater drop in bifidobacterial count from 10.18 ± 0.02 log cfu/ml to 4.69 ± 0.07 log cfu/ml over 8 mo of storage. The bifidobacterial counts of the capsules stored at 20°C decreased significantly ($p<0.05$) from 10.18 ± 0.02 with no bifidobacteria cells detected after 5 mo.

4.3.2. Viable counts of B. longum BB536 over 20 months

The viable counts of three randomly selected capsules of *Bifidobacterium longum* BB536 stored at -18°C, 4°C and 20°C over 20 mo are presented in Table 4.2. The counts of *B. longum* BB536 exhibited a similar pattern as *B. longum* 1941, however, the decrease in the rate of viability was slower in both the capsules stored at -20°C and 4°C. The bifidobacterial count decreased significantly ($p<0.05$) from 10.61 ± 0.02 log to 9.52 log ± 0.03 cfu/ml at -18°C over 20 mo. Counts of bifidobacteria stored at 4°C dropped significantly ($p <0.05$) from 10.61 log ± 0.02 to 6.02 ± 0.04 log cfu/ml over 20 mo of storage. Viability was significantly decreased ($p<0.05$) when the capsules were stored at 20°C and the organisms were not detected after 5 mo of storage. The loss of viability of the two freeze-dried *Bifidobacterium* species after 5 mo of storage at room temperature is possibly due to the increased metabolic and cellular activity at 20°C that may led to exhaustion of nutrients stored within the cell. The

microorganisms that were stored at frozen temperatures contained the maximum bacterial counts due to their reduced metabolic and cellular activity, therefore any loss of viability was due to cells that were affected storage at frozen conditions. Freeze-dried probiotic microorganisms that were stored at refrigerated conditions had lower viability than those stored at frozen temperatures due to slight metabolic or cellular activity at refrigerated temperatures.

4.3.3. Description of commercial probiotic products

A description of commercial probiotic preparations is given in Table 4.3. The criteria for choosing these products were that the probiotic bacteria were freeze-dried, contained within a capsule at least one species of *Bifidobacterium*, displayed information in regards to the bacterial composition including bacterial counts per capsule, a product of Australia or New Zealand, and displayed an expiry date of more than 12 mo from the date of purchase. All of these products were stored at refrigerated temperatures (≤4°C) when purchased. Three of the five products also contained some form of prebiotics. The concentration of *Bifidobacterium* in each product ranged from log 5.52 to 9.30 cfu/ml.

4.3.4. Viable counts of 5 commercial products over 8 months

The viable counts of the five commercial products A, B, C, D and E containing bifidobacteria stored at -18°C (freezer), 4°C (refrigerator) and 20°C (room temperature) over 8 mo are presented in Table 4.4. The initial bifidobacterial counts of the five commercial products ranged from 9.97 ± 0.03 log (Product E) to 6.41 ± 0.05 log cfu/ml (Product C). After 8 mo of storage all products exhibited a significant decrease ($p<0.05$) in bacterial viability over time in all conditions but there was a significant decrease in viability for capsules stored at room temperature as compared to those capsules stored at frozen conditions.

The bifidobacterial cell counts of commercial products were enumerated by using a selective medium (MRS-NNLP agar) and anaerobic incubation at 37°C for 3 d. The cell counts of Product A stored at -18°C and 4°C over 8 mo declined significantly by 0.50 log ($p<0.05$) and 0.30 log ($p<0.05$) respectively, while the capsules stored at 20°C declined by 5.92 log ($p<0.05$). Cell counts of Product B capsules contained maltodextrin derived from rice, stored at -18°C and 4°C declined significantly by 0.41 log ($p<0.05$) and 0.59 log ($p<0.05$) respectively, while the capsules stored at 20°C declined significantly by 6.01 log ($p<0.05$). Cell counts of Product C capsules stored at -18°C and 4°C declined significantly by 0.39 log ($p<0.05$) and 0.77 log ($p<0.05$) respectively, while the capsules stored at 20°C declined by 3.31 log ($p<0.05$). Cell counts of Product D capsules which contained glucose, stored at -18°C and 4°C declined significantly by 0.37 log ($p<0.05$) and 0.30 log ($p<0.05$) respectively, while the counts of the capsules stored at 20°C declined significantly by 7.33 log ($p<0.05$). Finally, cell counts of Product E, which contained 200 mg of oligofructose stored at -18°C and 4°C declined significantly by 0.26 log ($p<0.05$) and 0.42 log ($p<0.05$) respectively, while the capsules stored at 20°C declined significantly by 3.15 log ($p<0.05$).

The largest decrease in bifidobacterial counts stored at -18°C over 8 mo was 0.50 log ($p<0.05$) in Product A. The largest decrease in bifidobacterial counts for Product B stored at 4°C over 8 mo was 0.77 log ($p<0.05$). The largest reduction in bifidobacterial counts stored at 20°C was 7.33 log ($p<0.05$) in Product D. Product E exhibited the lowest decline in the bifidobacterial counts over 8 mo stored of 0.26 log ($p<0.05$) at -18°C, 0.22 log ($p<0.05$) at 4°C and of 3.15 log ($p<0.05$) at 20°C. Independent testing of *B. longum* BB536 capsules has also shown a decline of 40% in the survival of *Bifidobacterium* at 25°C compared to 18% decline at 5°C over 12 mo in storage. The greater decrease in viable populations at room temperature is due to the metabolic activity of the organism.

Overall, commercial probiotic capsules had a slower rate of decrease in bifidobacterial counts at all storage temperatures as compared to laboratory freeze-dried bifidobacterial preparations. The manufacturing conditions of the commercial products are not known. However, it is assumed that the manufacturing conditions were better than our experimental probiotic preparations. This was reflected in the improved survival of the bacteria in commercial probiotic preparations. In general, it is important to maintain probiotic preparations at appropriate temperatures (preferably in the freezer at -18°C) to ensure enhanced survival over a prolonged storage period.

4.4. Tables

Table 4.1. Viable microbial count (log_{10} CFU/ml) of capsules containing freeze-dried *B. longum* 1941 cells stored for 20 months at -18°C, 4°C and 20°C

	Log_{10} CFU/ml[1, 2]		
Sampling period (months)	Frozen (-20°C)	Refrigerated (4°C)	Shelf (20°C)
0[3]	10.18 ± 0.02 [a, A]	10.18 ± 0.02 [a, A]	10.18 ± 0.02 [a, A]
1	9.77 ± 0.02 [a, B]	9.68 ± 0.02 [a, A]	7.85 ± 0.06 [b, B]
2	9.37 ± 0.01 [a, C]	8.78 ± 0.01 [b, B]	6.42 ± 0.18 [c, C]
5	8.76 ± 0.08 [a, DE]	8.42 ± 0.51 [a, BC]	ND[4]
8	8.66 ± 0.01 [a, DEFG]	8.21 ± 0.01 [b, BCD]	ND
11	8.57 ± 0.04 [a, DEFGH]	7.99 ± 0.13 [b, BCDEF]	ND
14	8.67 ± 0.06 [a, DEF]	8.20 ± 0.04 [b, BCDE]	ND
17	8.53 ± 0.08 [a, DEFGHI]	5.09 ± 0.21 [b, G]	ND
20	8.70 ± 0.02 [a, DE]	4.69 ± 0.07 [b, GH]	ND

[1] Mean ± standard error (n = 6).

[a, b, c,] Means in the same row with different lowercase superscript are significantly different ($p<0.05$);

[A, B, C, D, E, F, G, H, I] Means in the same column with different uppercase superscript are significantly different ($p<0.05$).

[2] One-way ANOVA of means in the same row and column ($\alpha = 0.05$).

[3] 0 = initial counts immediately after manufacture of capsules.

[4] ND = Not detectable.

Table 4.2. Viable microbial count (log_{10} CFU/ml) of capsules containing freeze-dried *B. longum* BB536 cells stored for 20 months at -18°C, 4°C and 20°C

	Log_{10} CFU/ml[1, 2]		
Sampling period (months)	Frozen (-20°C)	Refrigerated (4°C)	Shelf (20°C)
0[3]	10.61 ± 0.02 [a, A]	10.61 ± 0.03 [a, A]	10.61 ± 0.03 [a, A]
1	10.24 ± 0.01 [a, B]	9.97 ± 0.22 [a, B]	9.83 ± 0.40 [a, A]
2	9.80 ± 0.02 [a, C]	9.52 ± 0.01 [b, BC]	7.08 ± 0.01 [c, B]
5	9.30 ± 0.01 [a, EFGHI]	8.49 ± 0.04 [b, D]	ND[4]
8	9.56 ± 0.03 [a, CD]	8.77 ± 0.01 [b, DE]	ND
11	9.37 ± 0.03 [a, DEFGH]	8.10 ± 0.06 [b, EF]	ND
14	9.52 ± 0.11 [a, DEF]	7.95 ± 0.01 [b, FG]	ND
17	9.45 ± 0.01 [a, DEFG]	6.44 ± 0.04 [b, H]	ND
20	9.52 ± 0.03 [a, DE]	6.02 ± 0.04 [b, HI]	ND

[1] Mean ± standard error (n = 6).

[a, b, c,] Means in the same row with different lowercase superscript are significantly different ($p < 0.05$);

[A, B, C, D, E, F, G, H, I] Means in the same column with different uppercase superscript are significantly different ($p<0.05$).

[2] One-way ANOVA of means in the same row and column ($\alpha = 0.05$).

[3] 0 = initial counts immediately after manufacture of capsules.

[4] ND = Not detectable.

Table 4.3. Commercial probiotic products[1]

Product[2]	Prebiotic added	Probiotic bacteria[3]	Log_{10} (CFU/ml)[3]	Expiry date
A	None	*Lactobacillus acidophilus*	9.00	09/03
		Bifidobacterium longum	9.00	
		L. delbrueckii ssp. *bulgaricus*	8.00	
B	Maltodextrin	*L. acidophilus* (NCFM)	10.00	11/03
		B. infantis (BBI)	10.00	
C	None	*L. acidophilus*	9.48	04/04
		L. plantarum	9.22	
		L. casei	8.52	
		L. brevis	8.52	
		L. delbrueckii ssp. *bulgaricus*	5.52	
		B. bifidum	5.52	
		L. rhamnosus	5.52	
		L. kefir	5.52	
D	Glucose	*L. acidophilus*	8.70	05/04
		B. lactis	8.70	
E	Oligofructose (200mg)	*L. acidophilus*	9.90	05/04
		B. longum	9.30	
		L. delbrueckii ssp. *bulgaricus*	8.30	

[1] Probiotic products purchased on 13/09/02

[2] Product name not given for confidentiality reasons

[3] Strain name not given for confidentiality reasons.

[4] Bacterial cell count claimed by manufacturer in log_{10} colony forming units (CFU/ml)

Table 4.4. Viable bifidobacterial count (log_{10} CFU/ml) of product A, B, C, D and E between 0 to 8 months at –18°C, 4°C and 20°C[1]

Product & sampling period (months)	Log_{10} CFU/ml[1] Storage temperature		
	Frozen -18°C	Refrigerated 4°C	Shelf 20°C
Product A			
0[2]	9.95 ± 0.03 $^{a, A}$	9.95 ± 0.03 $^{a, A}$	9.95 ± 0.03 $^{a, A}$
1	9.88 ± 0.02 $^{a, A}$	9.91 ± 0.01 $^{a, A}$	9.50 ± 0.01 $^{b, B}$
2	9.64 ± 0.04 $^{b, BC}$	9.80 ± 0.01 $^{a, B}$	9.33 ± 0.01 $^{c, C}$
5	9.58 ± 0.02 $^{b, C}$	9.71 ± 0.01 $^{a, CD}$	7.26 ± 0.04 $^{c, D}$
8	9.45 ± 0.01 $^{b, D}$	9.65 ± 0.01 $^{a, D}$	4.03 ± 0.02 $^{c, E}$
Product B			
0[2]	9.84 ± 0.01 $^{a, A}$	9.84 ± 0.01 $^{a, A}$	9.84 ± 0.01 $^{a, A}$
1	9.79 ± 0.01 $^{a, A}$	9.74 ± 0.02 $^{b, B}$	9.06 ± 0.01 $^{c, B}$
2	9.60 ± 0.01 $^{a, BC}$	9.48 ± 0.02 $^{b, C}$	9.03 ± 0.02 $^{c, BC}$
5	9.57 ± 0.01 $^{a, C}$	9.25 ± 0.03 $^{b, D}$	6.76 ± 0.05 $^{c, D}$
8	9.43 ± 0.01 $^{a, D}$	9.25 ± 0.01 $^{b, DE}$	3.83 ± 0.02 $^{c, E}$
Product C			
0[2]	6.41 ± 0.05 $^{a, A}$	6.41 ± 0.05 $^{a, A}$	6.41 ± 0.05 $^{a, A}$
1	6.39 ± 0.07 $^{a, A}$	6.31 ± 0.02 $^{a, A}$	5.72 ± 0.02 $^{b, B}$
2	6.36 ± 0.03 $^{a, A}$	5.80 ± 0.01 $^{b, B}$	5.70 ± 0.02 $^{c, BCD}$
5	6.23 ± 0.03 $^{a, AB}$	5.66 ± 0.02 $^{b, CD}$	5.67 ± 0.01 $^{b, BCD}$
8	6.02 ± 0.01 $^{a, B}$	5.64 ± 0.02 $^{b, D}$	3.10 ± 0.01 $^{c, E}$
Product D			
0[2]	9.05 ± 0.05 $^{a, A}$	9.05 ± 0.05 $^{a, A}$	9.05 ± 0.05 $^{a. A}$
1	8.83 ± 0.03 $^{b, BCD}$	8.92 ± 0.01 $^{a, BCD}$	8.39 ± 0.01 $^{c, BC}$
2	8.87 ± 0.01 $^{a, BCD}$	8.90 ± 0.01 $^{a, BCD}$	8.30 ± 0.01 $^{b, BC}$
5	8.76 ± 0.02 $^{a, BCD}$	8.85 ± 0.01 $^{a, BCDE}$	3.82 ± 0.04 $^{b, D}$
8	8.68 ± 0.02 $^{a, DE}$	8.75 ± 0.02 $^{a, DE}$	1.72 ± 0.02 $^{b, E}$
Product E			
0[2]	9.97 ± 0.03 $^{a, A}$	9.97 ± 0.03 $^{a, A}$	9.97 ± 0.03 $^{a, A}$
1	9.75 ± 0.02 $^{a, BCDE}$	9.72 ± 0.01 $^{a, BCD}$	9.41 ± 0.01 $^{b, BC}$
2	9.77 ± 0.01 $^{a, BCDE}$	9.66 ± 0.02 $^{b, BCD}$	9.32 ± 0.01 $^{c, BC}$
5	9.79 ± 0.01 $^{a, BCDE}$	9.66 ± 0.02 $^{b, BCD}$	8.34 ± 0.02 $^{c, D}$
8	9.71 ± 0.02 $^{a, BCDE}$	9.55 ± 0.02 $^{b, E}$	6.82 ± 0.02 $^{c, E}$

[1] Mean ± standard error (n = 6).

$^{a, b, c,}$ Means in the same row with different lowercase superscript are significantly different ($p<0.05$);

$^{A, B, C, D, E,}$ Means in the same column with different uppercase superscript are significantly different ($p<0.05$).

[2] 0 month = initial counts immediately after purchase.

[3] One-way ANOVA of means in the same row ($p = 0.05$).

4.5. Conclusion

This study has demonstrated that a temperature maintained at –18°C in a freezer was ideal for the long-term storage of probiotic capsules to maximise viability of freeze-dried bifidobacteria. The study demonstrated that *Bifidobacterium* counts were noticeably reduced at refrigerated temperatures (4°C), whereas storage temperature of 20°C reduced the viable counts significantly over the same period. Although commercial and experimental probiotic preparations showed a general trend of decline in bacterial counts over time when placed at similar storage temperatures, commercial probiotic products showed a reduced rate of decrease in viability as compared to experimental probiotic preparations.

5.0. EFFECTS OF FEEDING *BIFIDOBACTERIUM LONGUM* AND INULIN ON SOME GASTROINTESTINAL INDICES IN HUMAN VOLUNTEERS[1]

5.1. Introduction

In the last 10 years, there has been an increasing interest in the consumption of probiotics and functional foods in Western diets (O'Sullivan, 1996). Probiotics are defined as "living organisms, which when included as part of the diet, confer favourable effects on the host when given in sufficient numbers" (Fuller, 1991). Probiotic bacteria are able to suppress potentially pathogenic microorganisms in the gastrointestinal tract and enhance the population of beneficial microorganisms (Ibrahim and Bezkorovainy, 1993; Yaeshima *et al.*, 1997). The probiotic approach is based on the knowledge that the beneficial intestinal flora provides protection against various acute diseases (Fuller, 1991).

It is now recognised that the human large intestine isn't exclusively an organ involved in the storage and excretion of undigested dietary material but an extremely complex microbial ecosystem of which prokaryotic cells account for approximately 90% of the total cells (Kolida *et al.*, 2000). The bacterial inhabitants of the human gastrointestinal tract constitutes both anaerobic and aerobic microorganisms, of which the large intestine contains several hundred distinct strains of anaerobic bacteria often exceeding 10^{11}/g cfu per g of its content (Gibson and Wang, 1994b; Gibson *et al.*, 1994; McBain and Macfarlane, 1998).

The genus *Bifidobacterium* have long been recognized as a major component of the faecal flora of humans and some animals (Chevalier *et al.*, 1990; Key and Marble, 1995;

[1] A version of this chapter has been published: Bruno, F.A. and Shah, N.P. 2004. Effects of feeding *Bifidobacterium longum* and inulin on some gastrointestinal indices in human volunteers. *Journal of Bioscience and Microflora* 23(1): 1-10

Yaeshima *et al.*, 1997; Amann *et al.*, 1998) and are associated with beneficial health effects as they reduce unfavourable bacteria such as *Escherichia coli* and *Clostridium perfringens* and stimulate host immune functions (Yaeshima *et al.*, 1997).

Morphological, biochemical and physiological characteristics of bifidobacteria have been established. *Bifidobacterium* are classified as an independent genus consisting of 29 species (Sgorbati *et al.*, 1995; Lankaputhra, 1997). Several species have shown protection against colon tumourigenesis and antimutagenic and anti-carcinogenic properties (Reddy and Rivenson, 1993; Singh *et al.*, 1997).

Prebiotics are defined as "non-digestible carbohydrates that beneficially affect the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon, and thus improve the host health" (Gibson and Roberfroid, 1995b; Ziemer and Gibson, 1998; Roberfroid, 2001b). Prebiotics, such as oligosaccharides, are used as a substrate by bifidobacteria resulting in their increased numbers in the large intestine and faeces (Salminen *et al.*, 1993; Gibson and Wang, 1994a). Inulin occurs naturally in several plant foods including onion, garlic, asparagus, banana, chicory, artichoke and leek (Gibson *et al.*, 1994; Gibson *et al.*, 2000). Chemically, inulin is a mixture of poly- and oligo-saccharides of fructose units linked via β(2-1). Thus, inulin is typically indigestible to all higher animals (Coussement, 1996) due to the lack of degradation in the digestive tract. Inulin selectively enhances the proliferation of bifidobacteria, which in turn decrease pH and lowers the oxidation-reduction potential in the intestine (Gibson and Wang, 1994b). According to previous research carried out by Shin *et al.* (2000b) and Bruno and Shah (2002b), the addition of 5% inulin improves the mean doubling time (T_d) of *B. longum* strains in milk as compared to other prebiotics. This finding is supported by Gibson and Wang (1994a), which have also supported the concept of the ability of inulin to stimulate the proliferation of bifidobacteria relative to other intestinal microflora.

Bifidobacterium longum 1941 is of human origin and was initially obtained from Commonwealth Scientific & Industrial Research Organisation (CSIRO, Highett, Australia). *B longum* BB536 was isolated from a breast-fed Japanese infant faeces, and is commercially found in many dairy products (Ogata *et al.*, 1997). These two strains have been thoroughly studied and both are ideal probiotic bacteria to be used in dairy applications due to their acid and bile resistance (Lankaputhra and Shah, 1995), high adherence capabilities (Lankaputhra and Shah, 1998a), and favourable organic acid production *in vitro* especially acetic and lactic acids (Pochart *et al.*, 1992).

A fundamental problem with commercial preparations is stability of the probiotic during storage. Probiotic bacteria such as bifidobacteria have recently been added to various dairy products such as yogurts and fermented milks; however, dairy foods are not an ideal medium for the maintenance of bifidobacteria due to reduced viability of these organisms (Medina and Jordano, 1994; Dave and Shah, 1997a). Consequently, numerous attempts have been made to increase the level of bifidobacteria to $>10^8$ cells/ml. Although numerous studies *in vitro* and *in vivo* have shown that normal intestinal flora is an extremely effective barrier against opportunistic and pathogenic microorganism (Fuller, 1991), little work has been carried out on the effects of consuming capsules containing freeze-dried bifidobacteria with a prebiotic (inulin) on gastrointestinal health in healthy, adult populations.

In this study, we have evaluated the effectiveness of these two strains (*B. longum* 1941 and *B. longum* BB536) combined with inulin. The total daily dose of freeze-dried probiotic bacteria and inulin was restricted to 1 g per subject. Both bifidobacteria strains were administered to 30 healthy human volunteers to test their effects on their faecal microflora, faecal characteristics and defecation frequency. The objective of this study was to investigate whether gastrointestinal indices are influenced by the daily consumption of capsules containing freeze-dried *B. longum* and inulin in healthy human subjects over a 2 wk period.

5.2. Materials and Methods

5.2.1. Subjects

The study was approved by the Victoria University Human Ethics Committee (HRETH 2001/03). Participants were recruited from the vicinity of the Werribee campus of Victoria University by advertisement in local newspapers and flyers. Subjects who were chosen were healthy, 18-65 years of age, not currently taking antibiotics, and were prepared to consume capsules containing bifidobacteria and inulin over a 2 wk period. Subjects that had recently consumed probiotic bacteria, had gastrointestinal problems, used strong analgesics or laxatives repeatedly were excluded from the study. The subjects were able to discontinue the study under the following conditions: voluntary withdrawal and unacceptable treatment response. All subjects signed an informed consent form after receiving written and oral information of the aim, duration of study and potential risks of the trial. A copy of the consent form is available in the Appendix (A1). Analysis of BMI, sex and age was analysed between each treatment group. BMI was calculated for each subject by weight (kg) divided by height $(m)^2$.

5.2.2. Bacterial strains

Bifidobacterium longum 1941 was obtained from the Victoria University Culture Collection (Werribee Campus). *B. longum* BB536 was obtained from Pacific Medical Pty. Ltd. (Richmond, Australia). Both strains were identified as *B. longum* using the F6PPK test (Orban and Patterson, 2000), Gram-staining (Scardovi, 1986) and carbohydrate fermentation test using a rapid ID 32 A kit (BioMerieux, Lyon, France). *B. longum* 1941 was chosen based on previous studies including acid resistance, adherence properties, β-glucuronidase and β-

glucosidase enzyme activity, antimutagenic properties and inhibitory activity against pathogens (Lankaputhra, 1997).

5.2.3. Freeze-drying of bacteria for capsule manufacture.

B. longum strain 1941 and BB536 were grown in MRS broth separately at 37°C for 18 h. Sterile L-cysteine hydrochloride monohydrate (Sigma., St Louis, Mo., USA) was added to the broth at a final concentration of 0.05% (w/v) to lower the redox potential and to improve their survival (Dave and Shah, 1997a). The cultures were transferred successively three times in MRS broth before use. The culture was then centrifuged at 2714 x *g* at 4°C for 15 min, the pellet washed twice with sterile water, resuspended by vortexing in 50 mL of 0.1 M phosphate buffer (pH 6.8), and re-centrifuged at 2714 x *g* at 4°C for 15 min using a benchtop centrifuge (Sorvall RT7, Newtown, Conn., USA). The supernatant was discarded and 50 mL of sterile 0.1 M phosphate buffer (pH 6.8) containing 2.0% (w/v) of food grade cryoprotectant Unipectine^{TM} RS 150 (Savannah Bio Systems, Balwyn East, Australia) was added, vortexed until dissolved, the contents poured into large petri-dishes and frozen overnight at –18°C. Freeze-drying was carried out using a Dynavac FD300 freeze-drier (Airvac Engineering Pty. Ltd., Rowville, Australia) at –88°C for 44 h of primary freezing and 4 h of secondary freezing under vacuum (-100 Torr). After freeze-drying, the cultures were carefully removed and transferred to sterile containers, subsequently crushed to a fine powder using a spatula under sterile conditions. The packaged freeze-dried powder was then stored at –18°C in sealed plastic bags under vacuum until enumeration was carried out. Viability of *B. longum* 1941 and BB536 was monitored before the study commenced and at monthly intervals for 8 mo after freeze-drying at frozen (-18°C), refrigerated (4°C) and shelf temperature (20°C).

Twenty-five mgs of freeze-dried bifidobacteria cells were mixed with 475 mg of inulin (Orafti Pty. Ltd., Tienen, Belgium) to obtain a final concentration per capsule of approximately $\geq 1 \times 10^{10}$ cfu/g. Placebo capsules contained only 475 mg of inulin. Inulin is a fructo-oligosaccharide produced by the extraction from chicory root (Gibson *et al.*, 1994c). The mixture of freeze-dried bacteria and inulin was transported frozen (-18°C) to Cottee Pharmaceuticals Pty. Ltd. (Frenchs Forest, Sydney, Australia), where the capsules were filled under aseptic conditions. The weight of the manufactured capsules were 606.9 ± 4.7 mg (containing *B. longum* 1941), 610.7 ± 8.0 mg (containing *B. longum* BB536) and 586.9 ± 12.4 mg (placebo containing only inulin). A total of 1250 capsules were manufactured for the study.

5.2.4. Study design

The study was designed to be a randomised, double-blind and placebo-controlled. Each subject was listed in a sequentially manner in order of agreeing to the conditions of the experiment and then randomly given a 3-digit coded sealed container by a third-party, which contained 30 capsules of one of three treatments which were coded BAA, AAB or ABA. The co-ordinator was unaware of the person's identity and what capsules they were consuming.

Treatment group BAA (n = 10) consumed capsules containing 25 mg of *B. longum* 1941 and 475 mg of inulin, treatment group AAB (n = 10) consumed capsules containing 25 mg of *B. longum* BB536 and 475 mg of inulin, while treatment ABA (n = 10) was the placebo capsules containing only 475 mg inulin. Each subject was asked to consume two capsules 30 min before a meal with a glass of water, every morning for 14 d. The subjects kept their capsules refrigerated at 4°C during the 14 d trial to maintain viability of the bifidobacteria. The subjects were also asked to complete a 14 d table relating to defecation frequency and

any intestinal discomfort i.e. constipation or diarrhoea by assigning a rating of 1 (soft) to 5 (hard). Changes that were observed from the baseline (day 1) were recorded.

5.2.5. Randomisation procedure

Randomisation of the 3-digit numbers was performed using a computer-generated list of random numbers. The three-digit number was allocated to the particular subject for the whole period of the study. The treatment groups were uncoded after completion of the study.

5.2.6. Faecal sampling and analysis

Whole freshly voided faeces were collected from each subject at day 1 before consumption of capsules at day 1 (baseline) and at day 15 (final). Each of the faecal samples was kept under anaerobic conditions using anaerobic waterless sachet (AN010C, Oxoid Pty. Ltd., Hampshire, UK.) in a sterile plastic bag. Analysis of the faecal microflora, pH, and moisture contents were performed within 3 h after excretion. The bacteriological analysis of the faecal microflora was performed according to Mitsuoka *et al.* (1987). Five g of faeces were placed in a sterile homogeniser bag with 50 mL of 0.1 M sodium phosphate buffer (pH 7.0) containing 100 µl of L-cysteine·HCl (5% w/v). The contents were homogenized in a stomacher on low speed for 2 min, followed by serial dilutions in half-strength Wilken-Chalgren broth (CM643, Oxoid Pty. Ltd., Hampshire, UK).

5.2.7. Stool moisture content

The moisture content in stool samples was determined by weighing approximately 1 g of faeces in triplicate from each subject before and after feeding of capsules, in an aluminium

dish, drying the sample at 105°C for 2 h. Each stool sample and dish were weighed until dried to a constant weight. The percentage moisture in the original sample was then calculated for each subject.

5.2.8. Colour measurement

The colour of the faecal samples for each subject was recorded at day 1 and day 15 using a hand-held chromameter (Model CR-300; Minolta Camera Co. Ltd., Osaka, Japan) used specifically in colour analyses for measuring reflective colours of surfaces. The colour of each stool was randomly measured in triplicate. The instrument provides a printout of colour values of the L^*, a^* and b^* colour scale values.

5.2.9. Stool pH

The pH of stool sample from each subject was measured in triplicate at a temperature of 17-20°C before and after feeding of capsules using a pH meter (Model 8417; Hanna Instruments Pty. Ltd., Singapore) after calibrating with fresh pH 4.0 and 7.0 standard buffers.

5.2.10. Organic acid analysis

Faecal samples for the analysis of organic acids were stored frozen (-20°C) after obtaining from subjects on day 1 and day 15 of the study. Organic acids were measured by HPLC according to the method of Shin *et al.* (2000b). An Aminex HPX-87H column (300 mm x 8.7 mm, Bio-Rad Laboratories., Richmond, CA., USA) and a guard column with disposable cartridges H^+ (Bio-Rad Laboratories) was maintained at 65°C using an UV/vis detector set at 220 nm for organic acids. The HPX-87H column specifically separates organic

acids using primarily ion exclusion and reversed phase mechanisms. The mobile phase used was 0.009 M H_2SO_4. Stool samples were prepared by thawing duplicate samples at room temperature by digesting with 0.1 ml of 15.8 M HNO_3 and 14.9 ml of 0.009 M H_2SO_4 for 30 min, vortexing for 30 sec, and centrifuged at 14,000 x *g* for 15 min using a benchtop centrifuge (Model 5415C; Eppendorf Pty. Ltd., Engelsdorf, Germany). Samples were then filtered using a 0.22 μm Millipore filter into a HPLC vial and then injected in duplicate. Results were then calculated in mgs of organic acid per g of faecal sample. Single and combined organic acid standards were performed including pyruvic acid, lactic acid, formic acid, acetic acid, isobutyric acid and n-butyric acid in order to identify organic acid retention times.

5.2.11. Enzyme analysis

The enzymes β-glucosidase and β-glucuronidase were quantified in subject's stool samples before and after feeding of experimental capsules. One g of stool sample from each subject was homogenised using a homogeniser (Model Braun; Melsungen AG., Germany) with 1 g of 0.2 mm sterile sand for 2 x 1 min intervals and then centrifuged at 14,000 *x g* for 15 min. Five hundred millilitres of crude sample was added to each test tube together with 1000 μl of 0.005 M ρ-nitrophenyl β-D-glucopyranoside or ρ-nitrophenyl β-D-glucuronide. The reaction was carried out for 30 min at 37°C and was stopped by the addition of 1 ml of cold 1 M Na_2CO_3. All measurements were carried out in duplicate and the absorbance was measured using a spectrophotometer (Pharmacia, Novaspec II, Uppsala, Sweden) at a wavelength of 420 nm (Goldin and Gorbach, 1976). The enzyme concentration was measured in duplicate as μg of enzyme activity per g of faeces per 30 min at 37°C.

5.2.12. Stool consistency

Consistency of stools was evaluated subjectively by each subject based on a simple 5-point scale over the 14 d of capsule consumption. The scale was based on 1 as being extremely soft to 5 being hard, with intervals of 0.5. A mean consistency score was calculated for each subject and averaged for the treatment group. Also, subjects were provided with a 14 d tabulated form to enter their observations and to make any comments relating to their intestinal health over the feeding period including: bloated feelings, occurrence of flatulence, diarrhoea or constipation. A copy of the stool consistency subject form is available in the Appendix (A2).

5.2.13. Defecation frequency

The subjects recorded their defecation frequency on a 14 d, 24 h chart by marking a cross through the appropriate hour of every bowel motion completed. A mean defecation score was calculated for each subject and averaged for the treatment group. A copy of the subject defecation frequency table is available in the Appendix (A3).

5.2.14. Enumeration and sampling of faecal bacterial isolates

Faecal samples were collected from the subjects at the start (day 1) and the end of the feeding trial (day 15) using sterile resealable plastic bags containing a waterless anaerobic sachet and were taken immediately to the laboratory. Five g of faeces was removed from the middle of the sample and mixed in 50 ml of phosphate buffer (pH 7.0) with 0.05 % L-cysteine and homogenised for 2 min at low speed using a stomacher (Model; Stomacher 400 lab blender; Seward Medical Pty. Ltd., UK.). One millilitre of the homogenate was then aseptically transferred into half-strength Wilken-Chalgren anaerobe broth (CM643; Oxoid)

and serial dilutions (10^{-1} to 10^{-9}) were made. One-millilitre aliquots of each dilution were plated in duplicate using selective agar and appropriate incubation conditions (temperature, duration, anaerobic or aerobic conditions). Blood agar base (CM55; Amyl Media Pty Ltd., Dandenong, Australia) with 40 ml/L defibrillated horse blood, nutrient agar (CM3; Amyl Media), MRS agar (CM359; Oxoid) and 10 ml/ L of NNLP solution (nalidixic acid, neomycin sulfate, lithium chloride and paromomycin sulfate), Chromocult *E. coli* coliform medium (CM956; Oxoid) and reinforced clostridial agar (CM151; Amyl Media) with Polymyxin-B-sulfate (Sigma Aldrich Pty. Ltd., Castle Hill, Australia) were used to selectively isolate anaerobes, aerobes, bifidobacteria, *E. coli* and clostridia strains, respectively (Laroia and Martin, 1991). Further confirmation of bacterial isolates was based on Gram-staining. Plates containing 25 to 250 colonies were counted as CFU per g (wet weight) of faeces. In a preliminary experiment, serial dilutions using three homogenates were prepared from a single faecal sample and bacterial counts were within 0.5-log range (data not shown).

5.2.15. Statistical analysis

The baseline readings of all subjects were compared with the final reading including faecal microflora, pH, moisture, organic acid concentration, β-glucuronidase and β-glucosidase activity, colour and gastrointestinal symptoms. Each treatment group was compared and statistical significance was assessed by using ANOVA, where $p<0.05$ was the determining level for all analysis of data.

5.3. Results and Discussion

5.3.1. Anthropotometric characteristics

A comparison of anthropotometric characteristics of the three treatment groups that participated in the study were analysed. Presented in Figure 5.1 is a comparison of the treatment group by age. There was a greater range of subject age for the treatment groups consuming capsules containing freeze-dried *B. longum* BB536 with inulin and capsules containing only inulin (placebo group) as compared to the treatment group consuming capsules containing freeze-dried *B. longum* 1941 with inulin. However, there was no significant difference ($p>0.05$) between the three treatment groups. The Body Mass Index (BMI) of the subjects in each treatment group was also analysed and presented in Figure 5.2. The BMI was similar in all three treatment groups ($p>0.05$). The thirty subjects who participated in the study were comprised of students and local residents of different ethnicity and dietary patterns. The average distribution of sex, age, body weight, and height were not significantly different ($p>0.05$) in all three treatment groups.

5.3.2. Viability of bifidobacteria in the capsules

The levels of bifidobacteria in every capsule were carefully monitored and corrected in order to maintain balance of capsule weight given to each subject. During production, a sample of 20% of all capsules produced were weighed and plotted on a control chart. The total production run of 1250 capsules was certified by the company's technical officer at Cottee Health Pty. Ltd. (Frenchs Forest, NSW, Australia). Enumeration of capsulated *B. longum* 1941 and *B. longum* BB536 was carried out over 8 mo, both before and after feeding of subjects at different storage temperatures, which included freezer (-18°C), refrigerator

(4°C) and shelf (20°C) and presented in Figure 5.3. Viability of bifidobacteria was highest when the capsules were stored in the freezer at -18°C and lowest when stored at room temperature (20°C), where there were none detectable after 5 mo of storage. Consequently, all manufactured capsules were stored frozen at -18°C in sealed containers for the feeding trial to maintain the highest viability over an extended period of time.

5.3.3 Bacterial counts of faecal samples

The bacterial levels of all 30 subjects, before and after consumption of capsules, were enumerated using selective media with added supplements, appropriate anaerobic or aerobic conditions and incubation temperature. These methods were optimised before the trial proved to be successful in differentiating and enumerating the five different groups of bacteria present in the stool samples. The bacterial counts are presented in Figure 5.4 as average measurements of bacterial levels according to the three treatment groups.

The total anaerobic bacteria increased for the placebo (0.35 log) and *B. longum* BB536 (0.07 log) treatment groups but decreased slightly for the treatment group consuming *B. longum* 1941 (-0.15 log) after 14 d of consuming these capsules. The highest anaerobic average count was 9.86 ± 0.20 log after 14 d of consuming placebo capsules. The total aerobic bacterial count increased for the placebo group (0.19 log) and *B. longum* 1941 group (0.44 log) but decreased for the group consuming *B. longum* BB536 (-0.46 log). There was no significant difference between the three treatment groups when comparing anaerobic counts.

The average levels of aerobic bacteria observed from the three treatment groups ranged between log 6.43 ± 0.25 to 7.25 ± 0.17. After 14 days of consuming the capsules the highest aerobic average count was 7.25 ± 0.17 log after 14 d of consumption of placebo capsules, while the lowest was 6.47 ± 0.19 after 14 d of consumption of *B. longum* BB536.

Bifidobacteria colonies were counted based on presumptive colony morphology as well as Gram-staining and F6PPK testing. The average bifidobacteria plate counts increased slightly for the group consuming *B. longum* 1941 (0.43 log) and decreased for the placebo group (-0.07 log) and the *B. longum* BB536 group (-0.22 log) after 14 d of consuming the experimental capsules. The highest bifidobacteria average count was 8.91 ± 0.17 log after 14 d was the treatment group consuming *B. longum* 1941 capsules.

The counts of *E. coli* decreased for the placebo group (-0.22 log) and *B. longum* BB536 group (-0.55 log), while the group consuming *B. longum* 1941 observed a slight elevation in numbers (0.05 log) after 14 d of consuming these capsules. The highest *E. coli* average count was 6.72 ± 0.25 log observed in the placebo group after 14 d of consumption. Finally, the levels of clostridia increased in the placebo (0.54 log), *B. longum* 1941 group (0.18 log) and *B. longum* BB536 group (0.40 log).

For all three treatment groups in the study, there was a non-significant increase in the level of clostridia after 14 d of consuming the experimental capsules. The treatment group with the highest clostridia average count was 8.99 ± 0.34 log after 14 d of consuming *B. longum* BB536.

Overall, there was no significant difference ($p>0.05$) between initial (day 1) and final (day 15) average bacterial counts from subject's stool samples of either anaerobic, aerobic, bifidobacteria, *E. coli* and clostridia from the three treatment groups.

5.3.4. Faecal colour profile (L, a* and b*)*

The colour of the faecal samples for all subjects is presented in Figure 5.5. The purpose was to detect any abnormal changes in colour (lightness/darkness) upon consumption of probiotic capsules. Colour changes in stool samples are normally in combination with diet

change or relative well being of the individual due to significant alterations of bacterial levels. Overall, the stool colour for treatment *B. longum* 1941 showed a slight lightening of colour (L^* 43.3 to 43.9), while *B. longum* BB536 (L^* 46.8 to 44.1) and the placebo (L^* 45.9 to 44.1) showed a slight darkening of colour after 14 d of capsule consumption. In general, no significant differences ($p>0.05$) were observed in the L^*, a^* and b^* colour scales for all subjects within or between each treatment group.

5.3.5. Defecation frequency and consistency of subjects

Defecation frequency and consistency were recorded individually using an individually tailored 24 h, 14 d tabulated chart and presented in Figure 5.6. Each subject was asked to circle the hour at which they passed a bowel motion during a 24 h period over 14 d. On average, subjects had between 1 to 2 bowel motions per day, with several subjects recording 3 to 5 occurrences per day. Defecation frequency of subjects was slightly higher but not significant with subjects consuming *B. longum* BB536 (1.164 ± 0.22) and placebo groups (1.143 ± 0.23) as compared to treatment group *B. longum* 1941 (1.057 ± 0.15).

Average faecal consistency as recorded by the thirty subjects is presented in Figure 5.7. The average results between the three treatment groups were not significantly different ($p>0.05$) over the 14 d period. The placebo group recorded the highest value of 3.182 ± 0.2, treatment group *B. longum* 1941 with inulin recorded a value of 3.036 ± 0.17, while treatment group *B. longum* BB536 with inulin recorded the lowest value of 2.964 ± 0.10.

5.3.6. Faecal moisture content

Faecal moisture is an important criterion in the evaluation of the effectiveness of probiotic capsules in the gastrointestinal tract. Figure 5.8 compares the faecal moisture

content of subjects for each treatment group over a 14 d period. The average faecal moisture remained relatively constant over the 14 d period between the three treatment groups. The faecal moisture of the placebo group increased slightly from 70.43% ± 3.10 to 70.96% ± 2.85 (p>0.05), while the treatment groups consuming *B. longum* 1941 and BB536 faecal moisture decreased from 72.67% ± 2.78 to 71.77% ± 2.50 and 75.28% ± 1.52 to 74.20% ± 2.10, respectively. The results indicate that there was no significant difference between the placebo and probiotic treatment groups. These results were similar to those of Ogata *et al.* (1997), who recorded similar faecal moisture of values of approximately 70%. Analysis of the results from the three treatment groups show that the consumption of these probiotic strains have no significant impact on the level of moisture within the expelled faeces and therefore do not result in noticeable gastrointestinal discomfort as compared to normal.

5.3.7. Faecal sample pH

The pH of all subject's baseline and final faecal samples are presented in Figure 5.9. Overall, there was an increasing trend of pH of all three treatments after 14 d of consuming. However, there was no statistically significant alteration in faecal pH over the 14 d of each treatment group. The placebo group increased slightly from pH 6.10 ± 0.16 to 6.15 ± 0.20. Treatment group *B. longum* 1941 and BB536 both increased from 5.97 ± 0.21 to 6.07 ± 0.15 and 6.23 ± 0.21 to 6.24 ± 0.20, respectively. Most pH measurements of faecal samples were recorded within a range of 5.0 to 7.0. The averaged values do show that treatment with *B. longum* 1941 and *B. longum* BB536 produced higher average pH than the placebo however, the range of pH in the probiotic treatment groups produced several lower pH results than the placebo, the overall difference in pH between the three treatments was not significant. While the pH results of treatment group *B. longum* 1941 with inulin appears to be shifted more

acidic, the median and average values are similar. However, the treatment group *B. longum* BB536 with inulin has a wider spread of values, the average and median pH values are slightly higher than the placebo. In general, the results presented were also observed by Ogata *et al.* (1997) who showed lower faecal pH was detected after probiotic consumption.

5.3.8. Faecal organic acid profile

Organic acid profile of all three treatment groups is presented in Figure 5.10. Overall, we detected small variations in concentrations of organic acids in stool samples of the subjects. Levels of pyruvic and isobutyric acid remained relatively consistent over the 14 d for all treatment groups. Consumption of *B. longum* 1941 and inulin resulted in increases in lactic acid, formic acid and butyric acid with decreases in acetic acid. Subjects consuming *B. longum* BB536 and inulin showed increases in lactic acid, formic acid, acetic acid and butyric acid with decreases in pyruvic acid and isobutyric acid. Subjects consuming inulin only had increases in pyruvic acid, lactic acid, formic acid and acetic acid, while decreases in isobutyric acid and butyric acid were observed. From the results, we observe that the consumption of *B. longum* 1941 and BB536 with inulin resulted in significant increases in butyric acid, while the level decreased when subjects consumed inulin only. This is an important observation. While the major products of fermentation produced by bifidobacteria are lactic and acetic acids, increased levels of butyric acid are of great significance as butyric acid has been shown to produce antimutagenic activity (Lankaputhra and Shah, 1998b). Butyric acid has been claimed to prevent tumour formation in rats by altering transcription rate of a few genes and inhibits or suppress DNA synthesis *in vitro* or *in vivo* (Lankaputhra and Shah, 1998b). While all treatment groups showed an increase in lactic acid concentration,

the level of acetic acid increased for both *B. longum* BB536 and placebo but not when consuming *B. longum* 1941.

5.3.9. Faecal enzyme concentration

Levels of β-glucosidase and β-glucuronidase were measured before and after feeding of capsules are presented in Figure 5.11. Measurements of enzyme are presented as μg of enzyme per g of faecal sample. Levels of β-glucosidase activity increased for treatments *B. longum* 1941 (40.86 ± 9.33 to 43.54 ± 9.10) but decreased for *B. longum* BB536 (35.17 ± 9.07 to 27.24 ± 4.75). The level of β-glucosidase activity for the placebo group slightly increased after consumption of capsules (34.76 ± 6.89 to 34.91 ± 7.84). There was no significant difference ($p>0.05$) between the three treatment groups of β-glucosidase activity within the faecal samples.

The concentration of β-glucuronidase enzyme in the faecal samples decreased for all of the three treatment groups. The placebo treatment group decreased from 42.59 ± 9.40 to 38.20 ± 5.56), *B. longum* 1941 treatment group decreased from 41.79 ± 8.57 to 38.72 ± 6.76, while *B. longum* BB536 treatment group decreased from 41.36 ± 6.79 to 39.27 ± 4.48. In general there was no significant difference between treatment groups for concentration of β-glucuronidase enzyme.

5.3.10. Wellbeing assessment

The assessment of well-being was documented objectively by the subjects on a daily basis and overall assessment at the end of the study is presented in Figure 5.12. Subjects were asked to report any feelings of gastrointestinal well-being at the completion of the study on a feedback form. Gastrointestinal health as observed by three treatment groups containing 30

subjects reported no adverse condition as a result of the consumption of the capsules over the 14 d. The proportion of subjects that consumed probiotic capsules reported a feeling of slight to marked improvement more than subjects consuming the placebo capsules. Feeling of unchanged gastrointestinal condition was reported by 50% of the subjects.

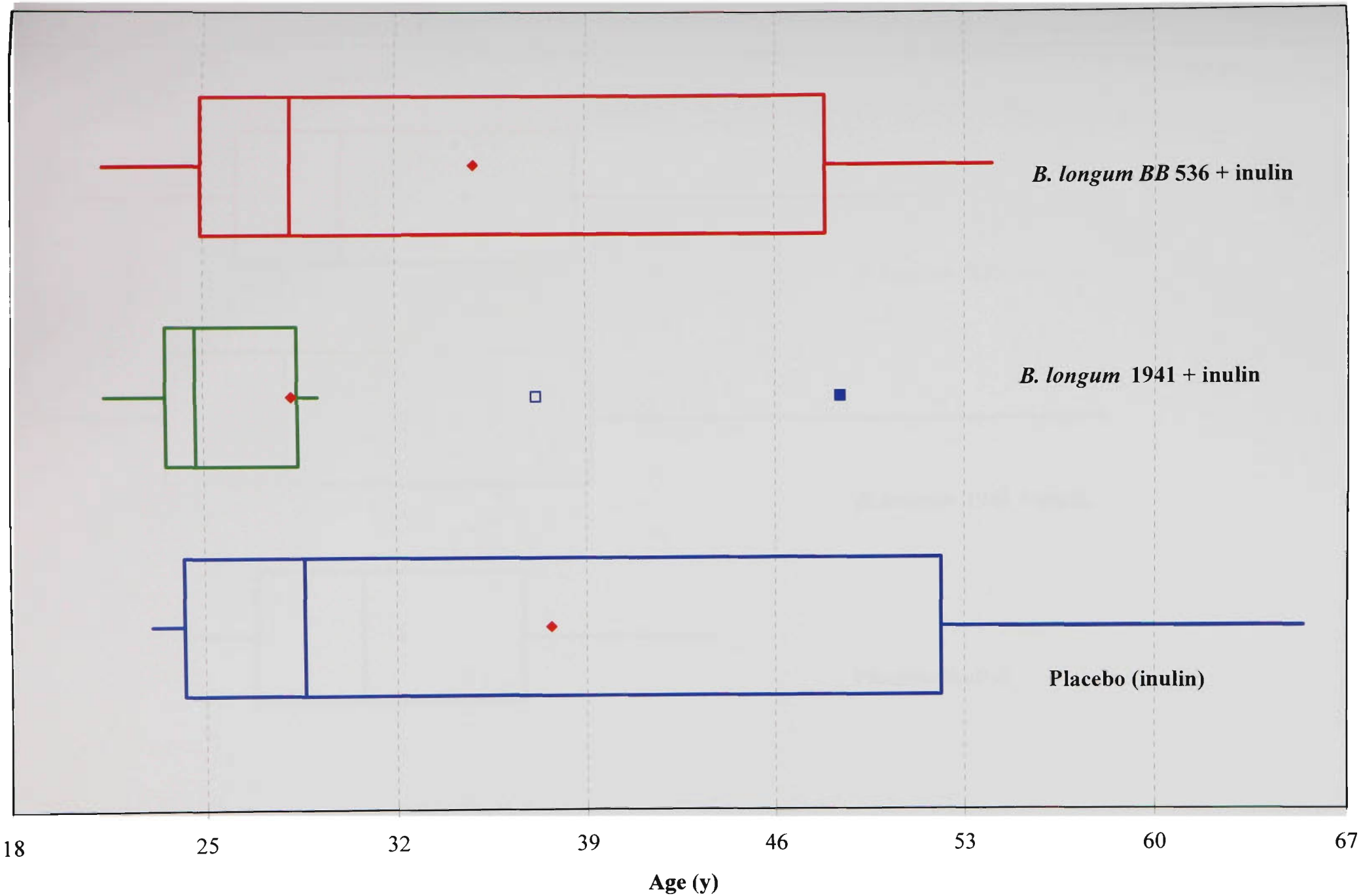

Figure 5.1. Age of subjects in each treatment group

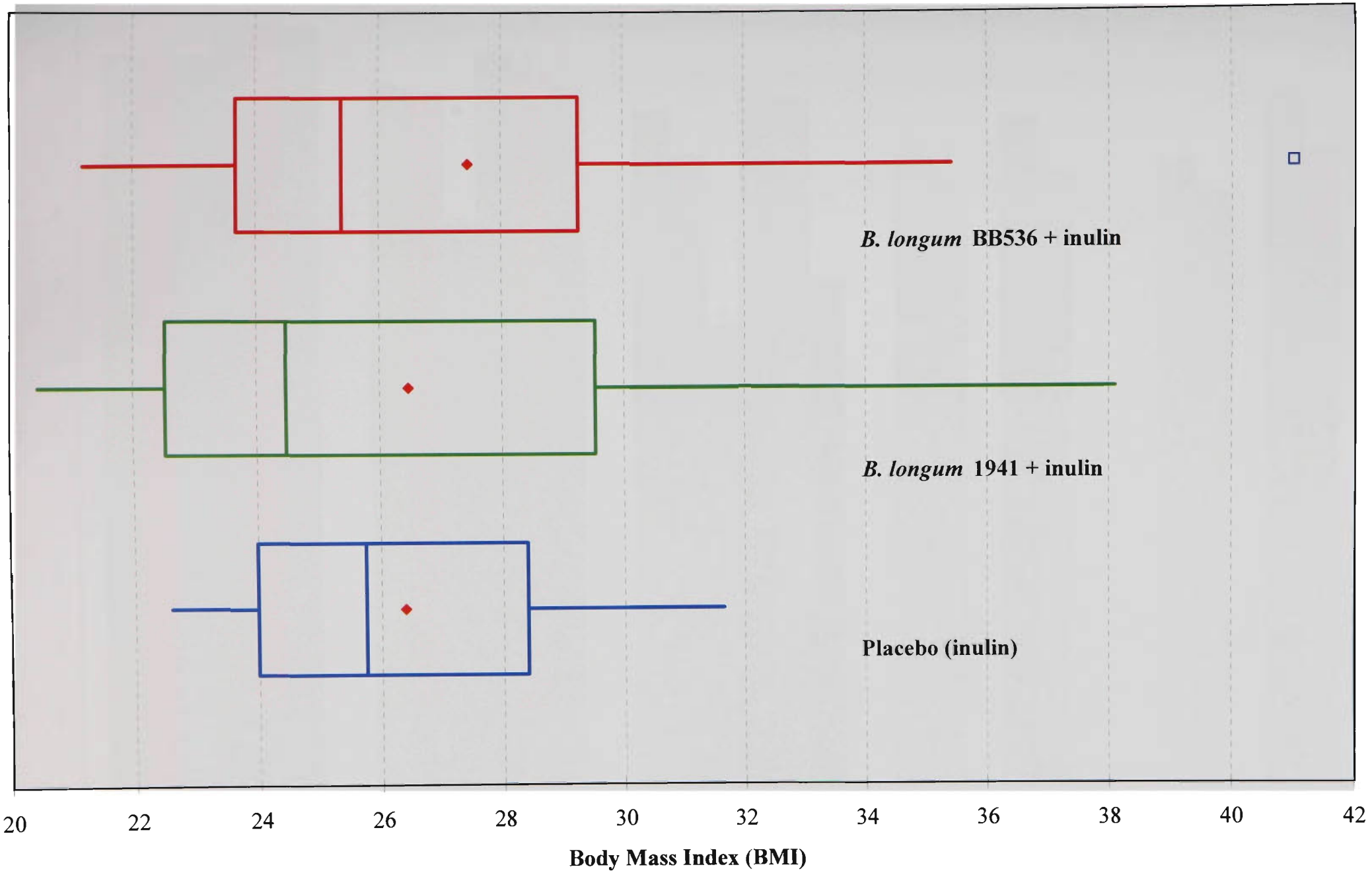

Figure 5.2. Body Mass Index of subjects

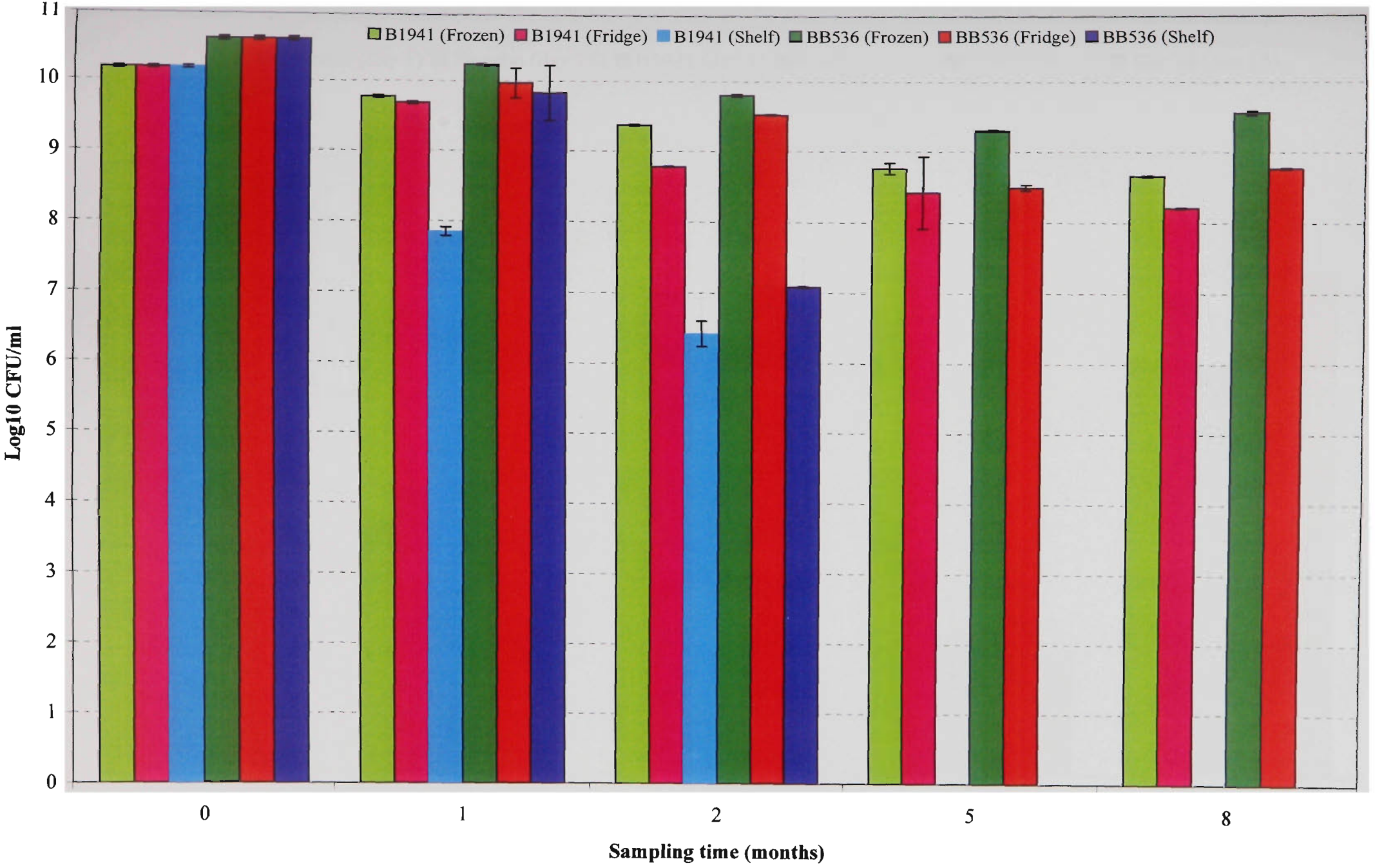

Figure 5.3. Viability counts of *Bifidobacterium longum* 1941 and BB536 over 8 months of storage at frozen, refrigerated and shelf temperatures

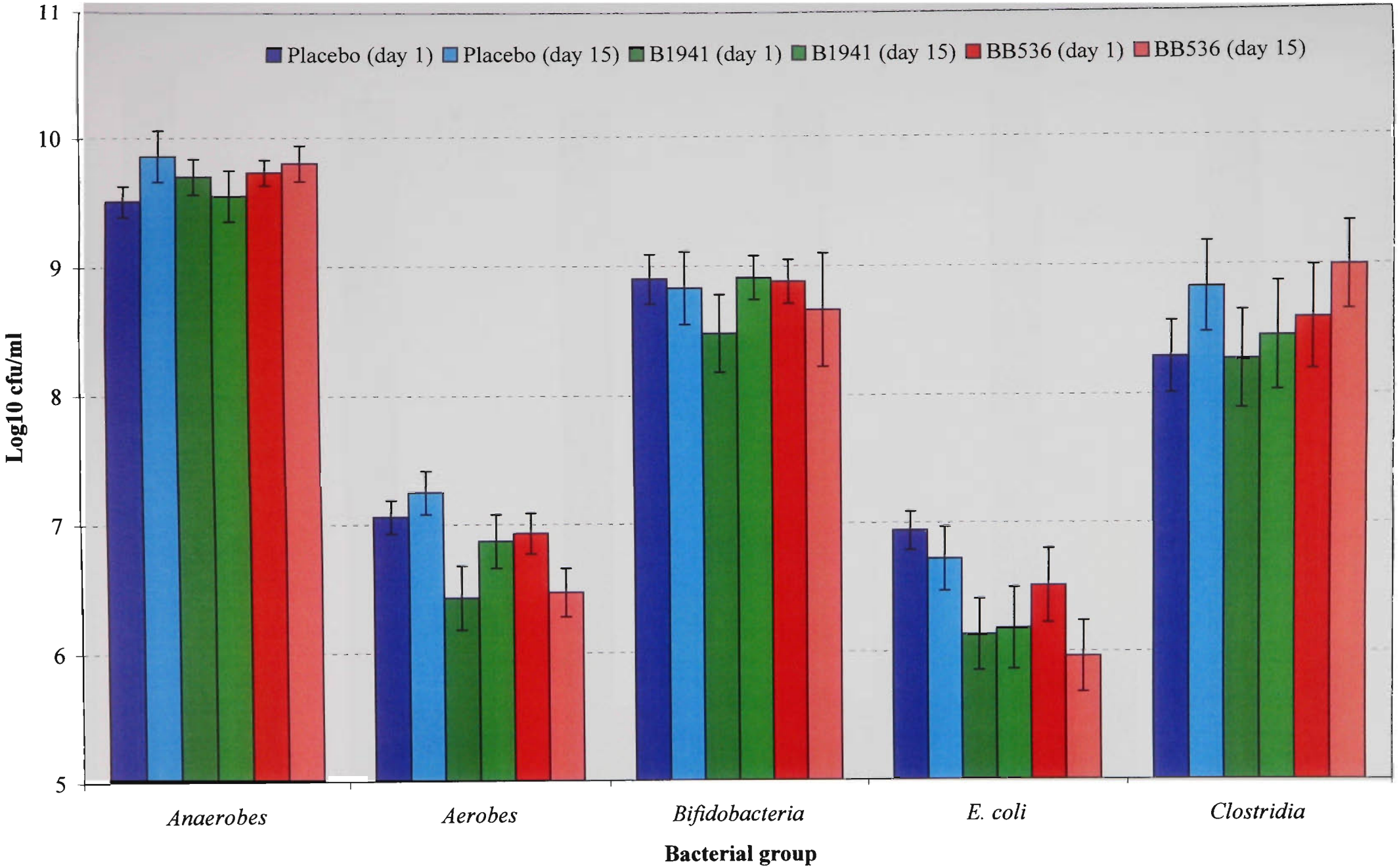

Figure 5.4. Bacteriological counts of the three treatment groups before and after consumption of capsules (Mean ± SE, n = 10)

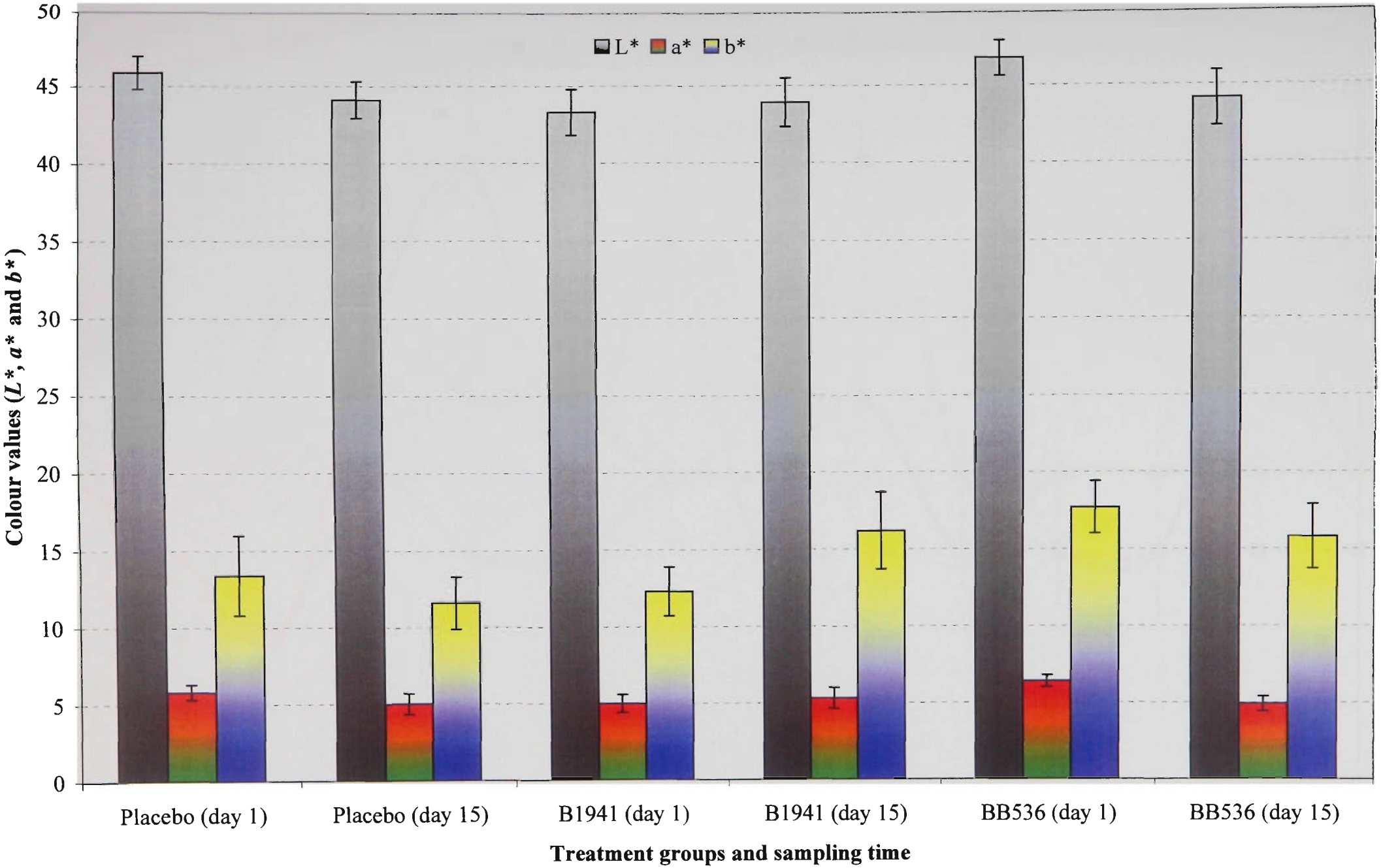

Figure 5.5. Colour of stools before and after consumption of capsules for each treatment group

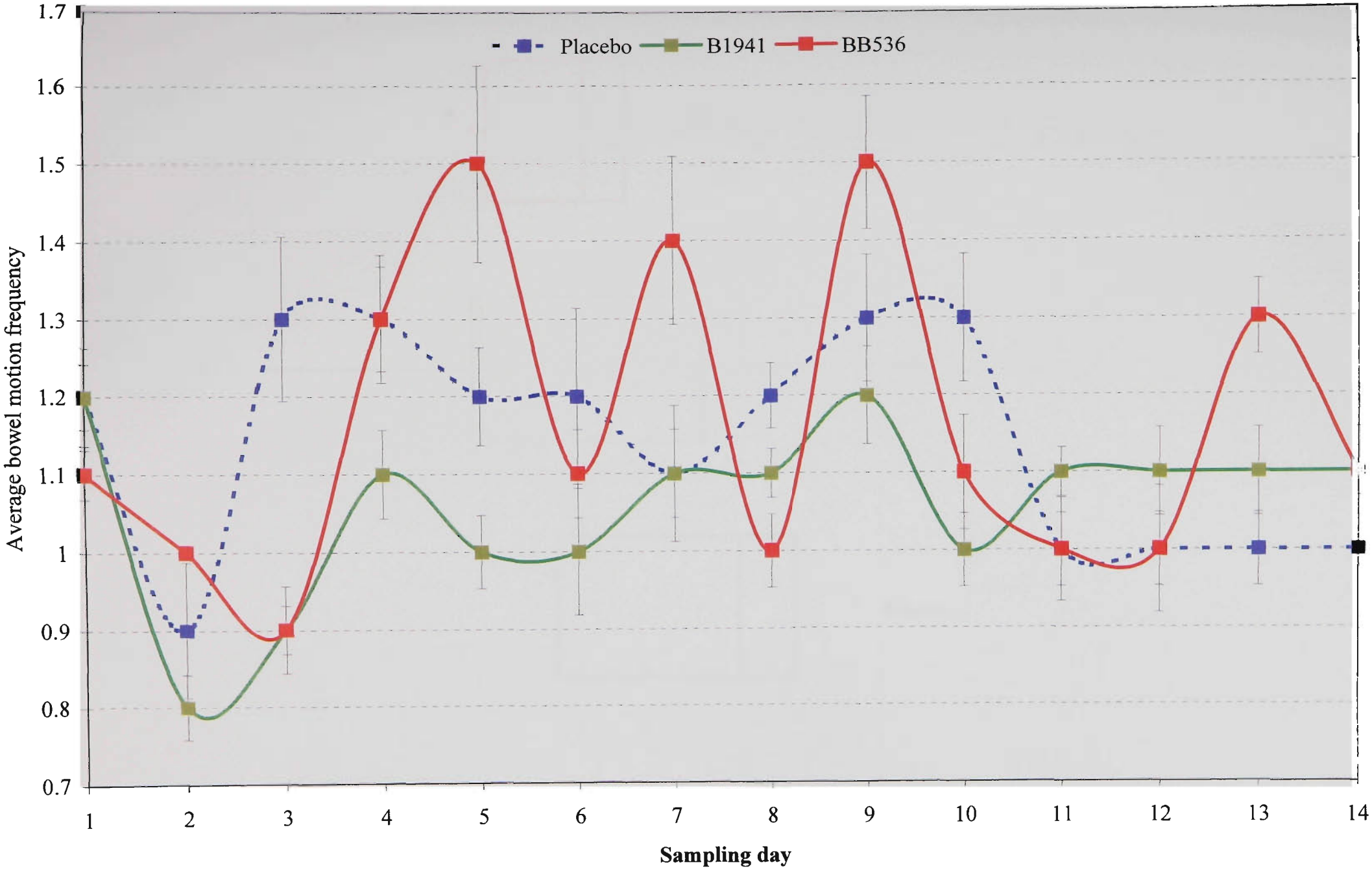

Figure 5.6. Average daily defecation frequency by treatment group (Mean ± SE, n = 10)

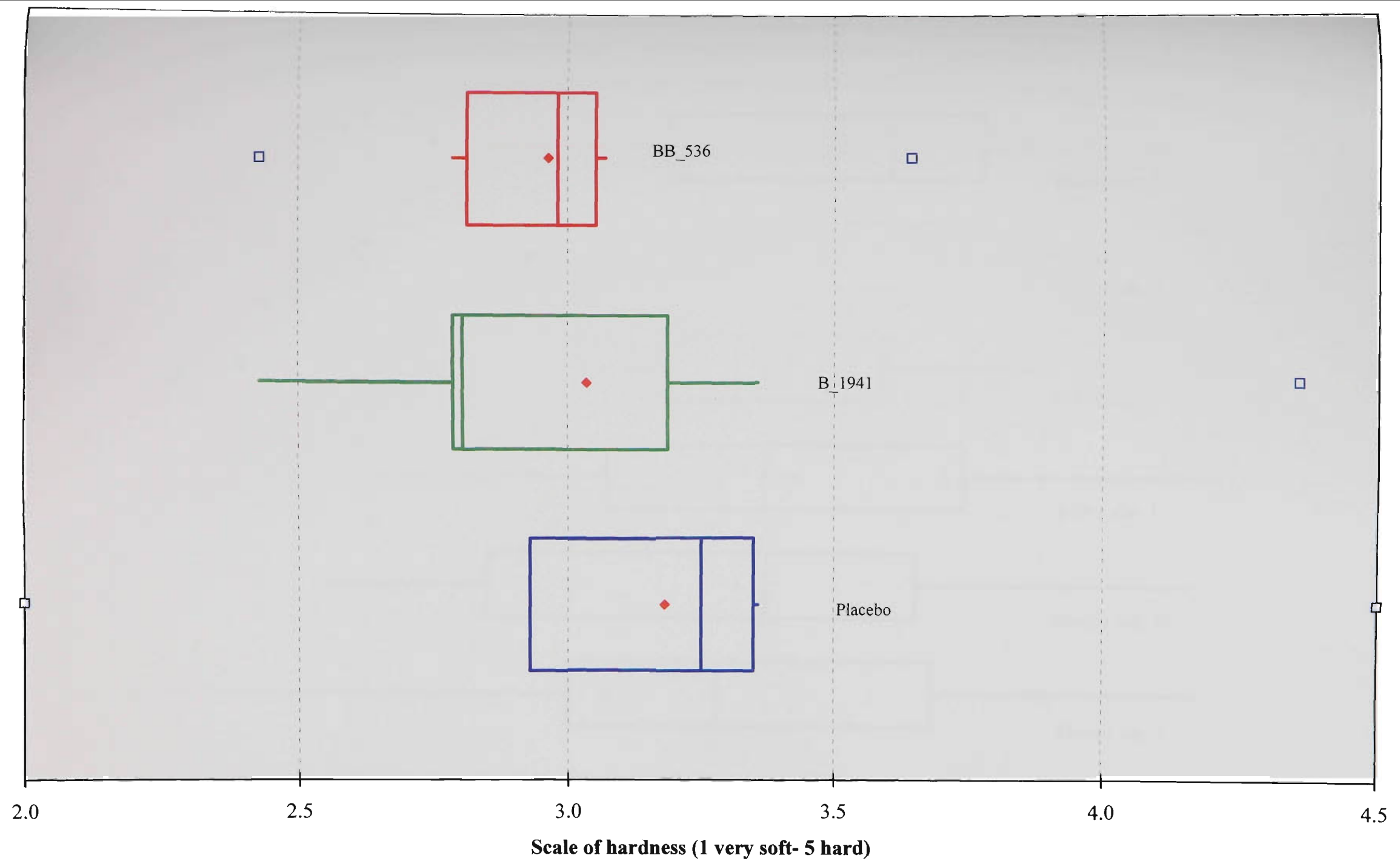

Figure 5.7. Consistency of stool samples by treatment group (n=10)

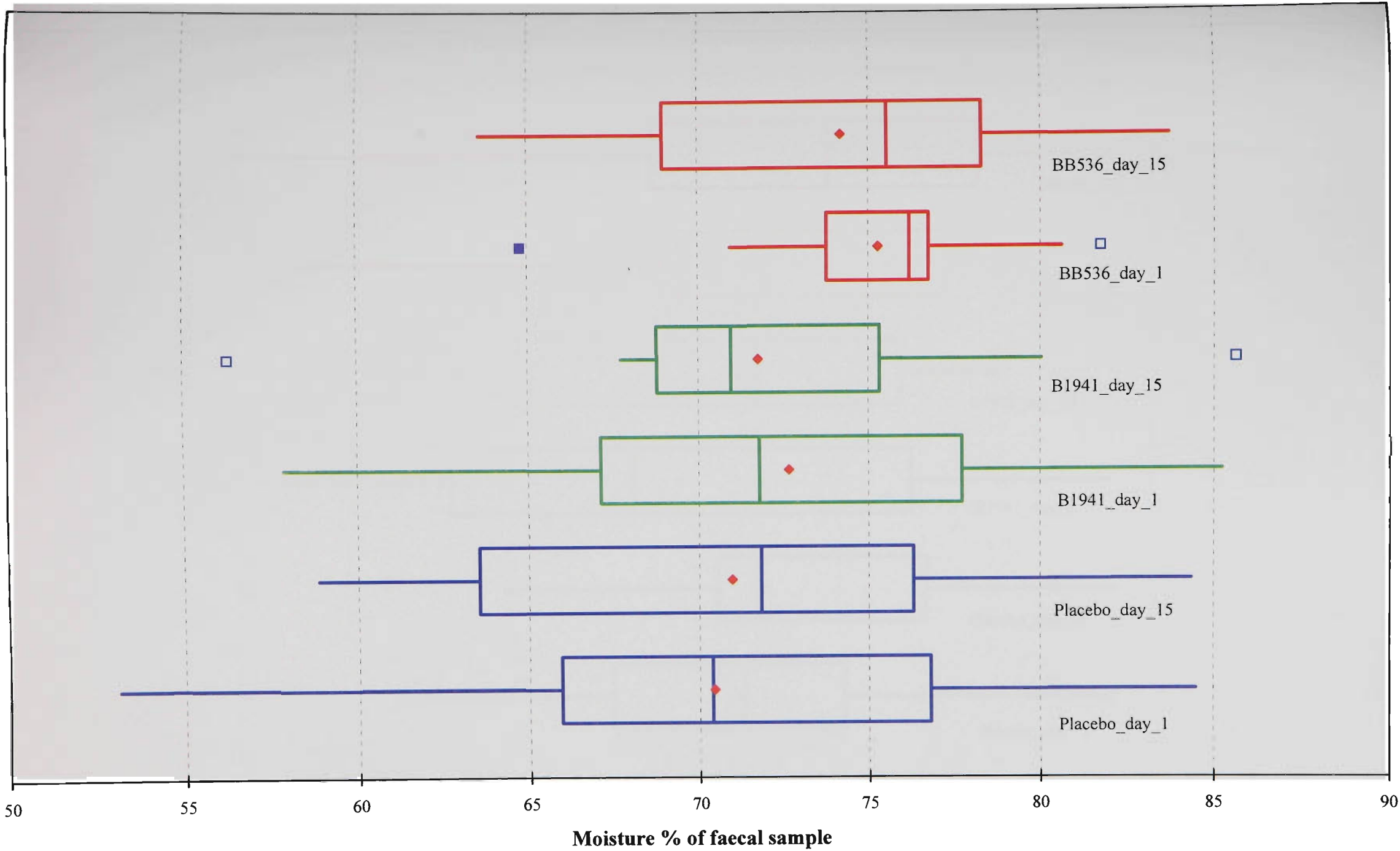

Figure 5.8. Moisture % of faecal samples of each treatment group before and after consumption of capsules (n=10)

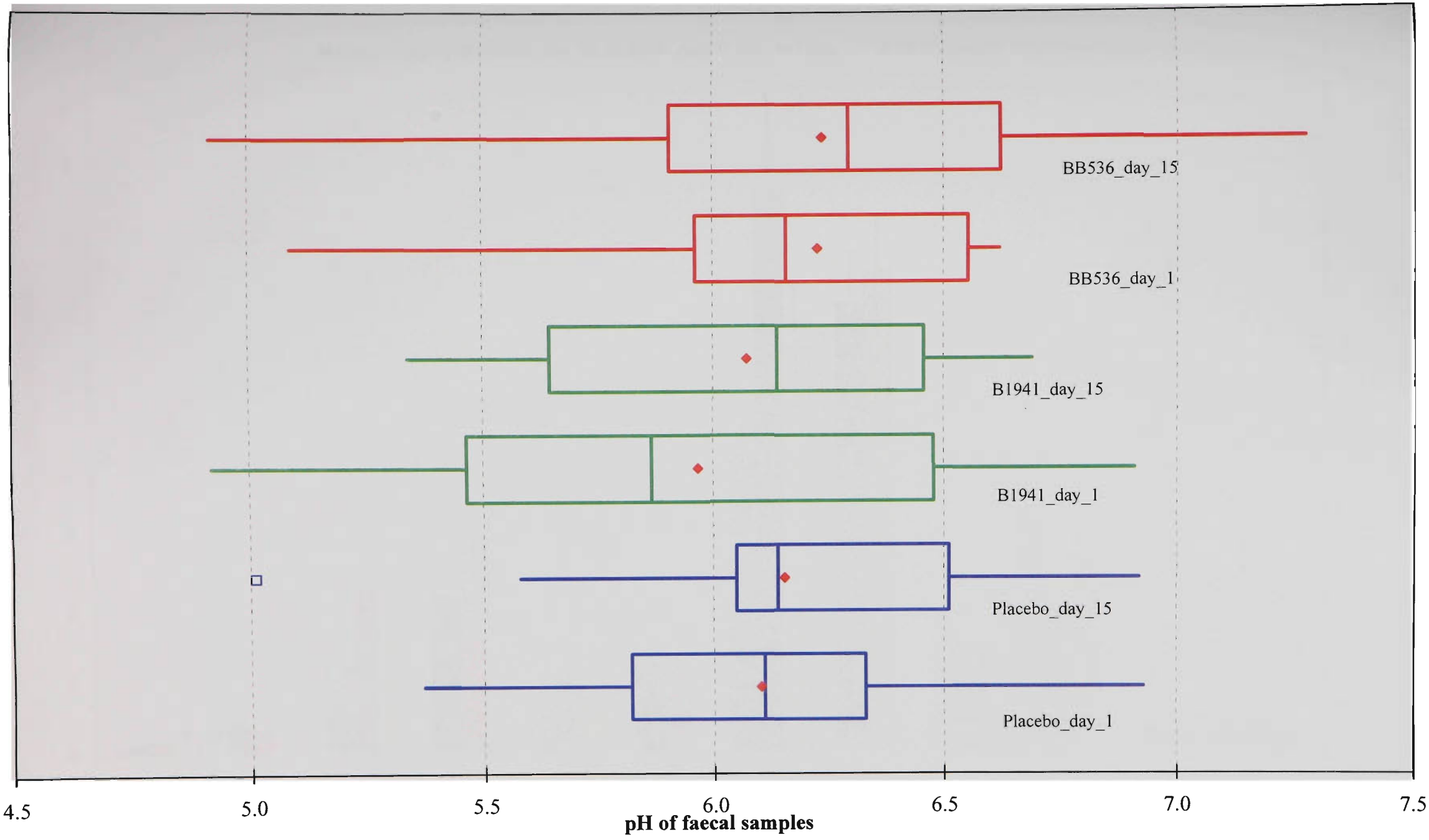

Figure 5.9. Measurement of faecal pH of each treatment group (n=10)

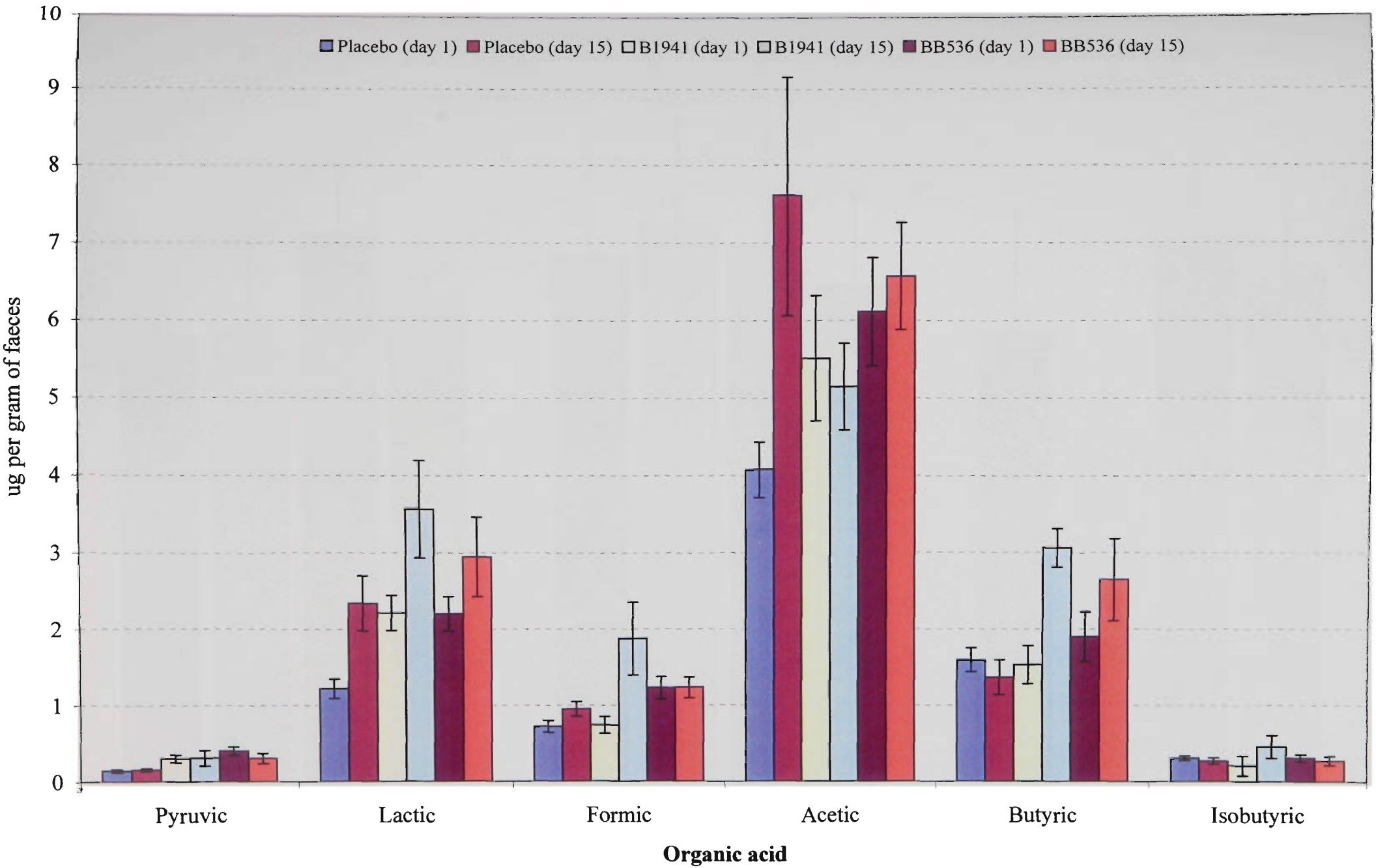

Figure 5.10. Comparison of organic acids present in faecal samples of each treatment group before and after feeding (Mean ± SE, n = 10)

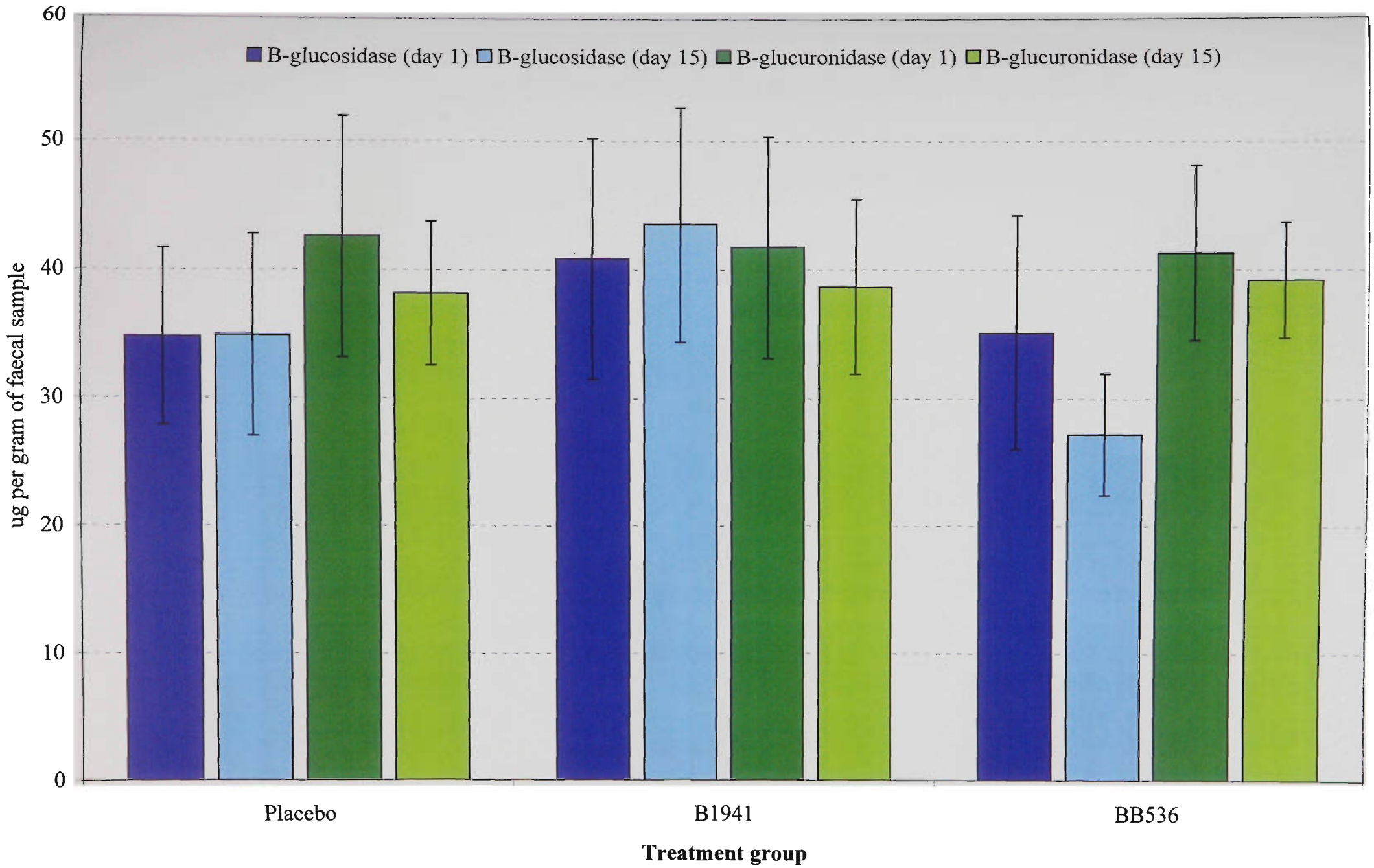

Figure 5.11. Comparison between treatment groups measuring enzyme levels in faecal samples (Mean ± SE, n = 10)

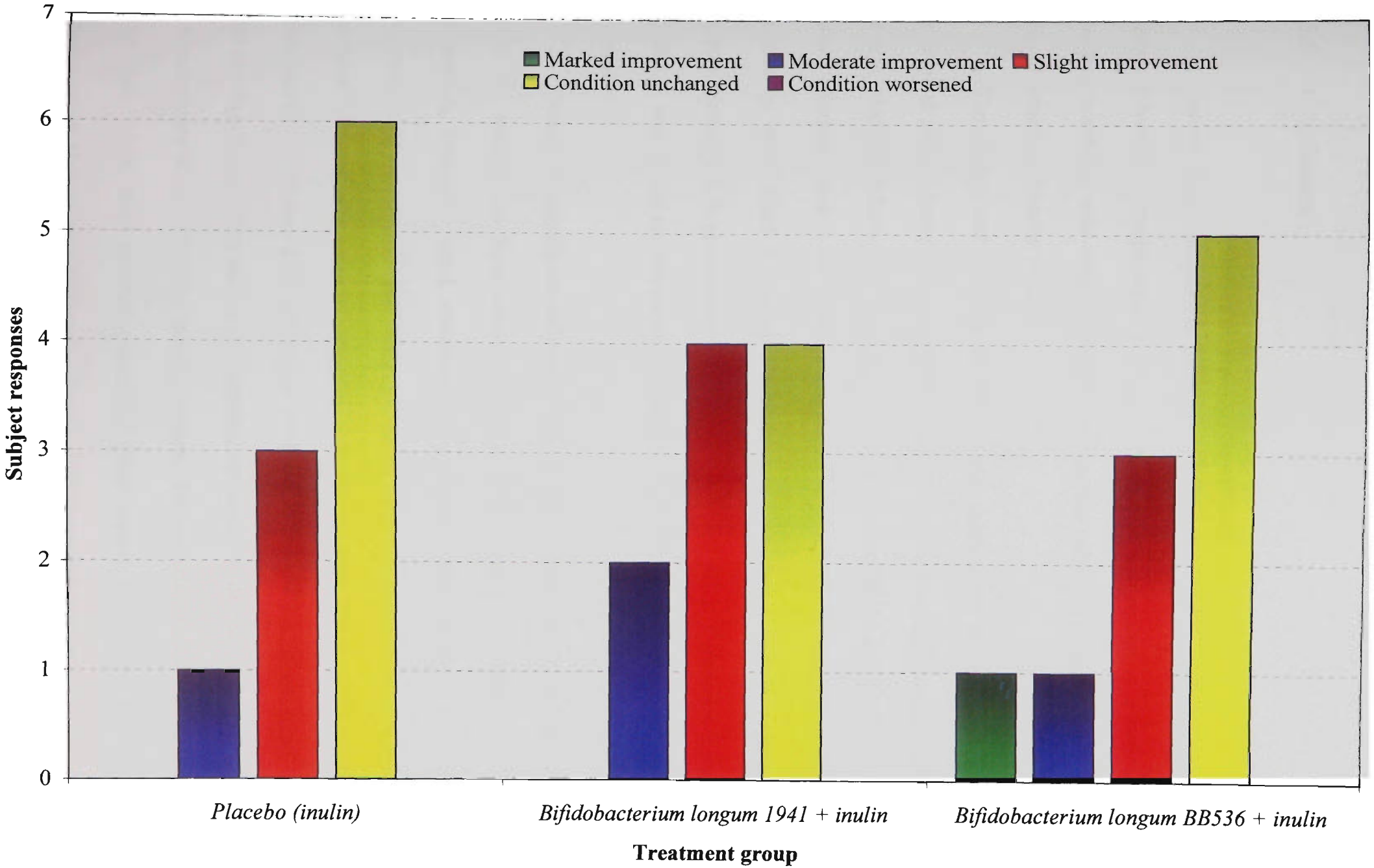

Figure 5.12. Response of subjects by treatment group after consumption of capsules for 14 d (n = 30)

5.5. Conclusion

Interest in consumption of probiotic and prebiotics (indigestible oligosaccharides) to improve human gastrointestinal health is increasing. Consumption of beneficial probiotic bacteria combined with oligosaccharides may provide enhanced gastrointestinal benefits and improvements in internal health.

The design and rationale of this *in vivo* randomised, double-blind and placebo-controlled parallel group comparison study was to evaluate the effectiveness of administering freeze-dried *Bifidobacterium longum* 1941 or *B. longum* BB536 and inulin to 30 healthy, adult volunteers over 2-wk. Results based on changes in gastrointestinal indices (bacterial counts in stool, stool defecation frequency and consistency, and in organic acids, β-glucuronidase and β-glucosidase enzyme concentration, pH and moisture were detected). Efficacy was based on comparison of initial values of gastrointestinal indices with final values.

Overall, no significant difference between the baseline (day 1) and the final reading (day 15) among the three treatment groups was observed on faecal bacterial counts, defecation frequency, stool consistency, faecal pH, faecal enzyme and organic acid concentrations or moisture percentage of stools. However, levels of butyric acid in faecal samples increased after subjects consumed probiotic capsules. These results indicate that the administration of freeze-dried *B. longum* 1941 and *B. longum* BB536 combined with inulin did not significantly alter the human intestinal environment, defecation frequency and faecal characteristics of thirty healthy, human subjects. The bacterial counts, defecation frequency and stool colour, stool moisture percentage, stool organic acid profile and faecal enzyme concentration showed no significant difference from the placebo group.

The feeding of *B. longum* 1941 and *B. longum* BB536 with inulin appears to be safe for consumption as the subjects did not experience any cases of illness or being unwell during the feeding trial period. Furthermore, the subjects did not experience any gastrointestinal discomfort and there was slight to marked improvement in well-being of most subjects participating in the study.

The absence of significant differences on several gastrointestinal indices may be due to several factors including; the relative short duration of the study (2 wks), the participation of only 30 adults consuming probiotic bacteria and prebiotics and insufficient quantity of prebiotic given. As science advances in this field, we should be increasingly able to explain why sometimes probiotics/prebiotics work *in vivo*, and why sometimes they do not.

6.0. *IN VITRO* INHIBITION OF VANCOMYCIN-RESISTANT ENTEROCOCCUS (VRE) BY PROBIOTIC BACTERIA

6.1. Introduction

During the past decade there has been a major interest in the emergence and spread of enterococci resistant to vancomycin (VRE). Shortly after the first isolates of VRE were reported by investigators in the United Kingdom and France in 1986, similar strains of VRE were detected in hospitals located in the eastern half of the United States in 1987. Consequently, VRE has spread with unanticipated rapidity and now encountered by hospitals in most states in the USA and other countries (Boyce, 1997; Johnson *et al.*, 2000; Kawalec *et al.*, 2000). In the last decade, enterococci became the second most frequently reported cause of surgical wound infections and nosocomial urinary tract infections and the third most frequently reported cause of bacteremia (Klein *et al.*, 1998b; Markopulos *et al.*, 1998; Silverman *et al.*, 1998; Kirschner *et al.*, 2001).

In Europe, a diversity of VRE types has also been isolated from sewage, animal waste, meat and meat products, and feces of healthy persons, suggesting a heterogenous pool of VRE outside hospitals, whereas in the United States VRE has not been isolated from animal sources and it seems to be more or less absent outside hospitals (Stobberingh *et al.*, 1999). The percentage of vancomycin-resistance in *Enterococcus faecium* in blood cultures in the UK increased from 6.3% in 1993 to 24% in 1998. While in the United States, the percentage of vancomycin-resistant enterococcal isolates in intensive care units (ICUs) increased from 0.3% in 1989 to 25.2% in 1999 (Harbath *et al.*, 2002) with 47% of all *E. faecium* are now resistant to vancomycin (Moellering *et al.*, 1999).

Enterococci, along with approximately 450 other taxa of anaerobic and aerobic bacteria are normal inhabitants of gastrointestinal tract of humans (Ke *et al.*, 1999; Mundy *et al.*, 2000; Harbath *et al.*, 2002). However, enterococci are ubiquitous and can be found free-living in soil, on plants, or in dairy products. The genus *Enterococcus* comprises Gram-positive cocci that are catalase negative, usually facultative, anaerobic bacteria that grow in 6.5% NaCl, 40% bile salts, and 0.1% methylene blue milk at pH 9.6. They grow between 10 and 45°C and can resist 30 min at 60°C (Manero and Blanch, 1999). Enterococci are important opportunistic nosocomial pathogens and have intrinsic and acquired resistance to numerous antibiotics. Intrinsic resistance is present in all or most strains of enterococci, and the genes appear to reside on the chromosome. Intrinsic resistance includes resistance to semisynthetic, penicillinase-resistant penicillins, cephalosporins, and low levels of aminoglycosides and clindamycin. Acquired resistance on the other hand results from a mutation in cellular DNA or acquisition of new DNA. Examples of which include resistance to chloramphenicol, erythromycin, tetracycline, high levels of aminoglycosides and clindamycin, penicillinase, fluoroquinolones, and vancomycin (Knudtson and Hartman, 1993).

The increasing incidence of multidrug-resistant enterococcal infections, including vancomycin-resistant strains, has caused difficulties in treating some cases of infection with routinely used antimicrobials. The prospect of a 'postantibiotic' era of untreatable bacterial infections might arise as currently rates of about 14 to 25% vancomycin-resistance in enterococcal isolates in ICUs are detected (Huebner *et al.*, 2000). Although 85-90% of clinical isolates of enterococci are *Enterococcus faecalis*, most vancomycin-resistant enterococci are *Enterococcus faecium* (Johnson *et al.*, 2000) with the incidence of more unusual strains such as *E. durans*, *E. hirae*, *E. gallinarum* and *E. casseliflavus* has increased significantly (Kirschner *et al.*, 2001). Five vancomycin-resistant enterococci phenotypes,

VanA-VanE have been distinguished on the basis of the level and inducibility of resistance to vancomycin (Kobayashi *et al.*, 2000).

The prospect of other alternatives to antibiotics to reduce the incidence of VRE in patients has rarely been discussed in the literature. To date, efforts to decolonise the gastrointestinal tract of VRE using oral antimicrobial agents has been unsuccessful. Nisin, is an antimicrobial peptide belonging to the family of lantibiotics, a group of small peptides (< 4 kDa) containing (β-methyl) lanthionine residues (Breukink *et al.*, 1997). The use of nisin, an antimicrobial substance produced by *Lactobacillus lactis* has been used successfully against other Gram-positive organisms (Severina *et al.*, 1998). However, nisin is also known to be inhibitory to bifidobacteria (Rada and Dlabal, 1998; Kot, 2001).

Presumably, the best strategy for preventing VRE infection is to prevent VRE gastrointestinal colonisation (Montecalvo, 2003). A non-chemical, non-classical approach to reversing the resistance problem would be the revival of the susceptible strains. By encouraging their regrowth and repopulation in areas where they have been severely reduced, they can replace resistant strains. One action is to re-introduce a susceptible, competitive flora. This approach has been typified by the use of probiotics (Levy *et al.*, 2002). Reducing the number of VRE-colonised patients may be more important than ever, given the recent emergence of vancomycin-resistant *S. aureus* in which the vancomycin resistance determinant was the *vanA* gene, present in VRE. The use of probiotics to alter the colonic microbiota in reducing VRE *in vivo* has not been explored. Therefore, this chapter will investigate the therapeutic potential of probiotic bacteria in reducing or eliminating VRE isolated from patients *in vitro*.

6.2. Materials and Methods

6.2.1. Bacterial strains, media and pH instruments

6.2.1.1. Bacterial identification and storage of probiotic strains

Five strains of *Bifidobacterium* (*B. infantis* 1912, *B. longum* 1941, *B. longum* BB536, *B. pseudolongum* 20099 and *B. animalis* Bb12) were obtained from the Victoria University Culture Collection (Werribee, Australia). *B. infantis* 1912, *B. longum* 1941 and *B. pseudolongum* 20099 were originally obtained from the Commonwealth Scientific and Industrial Research Organisation (CSIRO, Highett, Australia). Freeze-dried *B. longum* BB536 was originally obtained from Morinaga Milk Industry Co. Ltd. (Tokyo, Japan) through the courtesy of Pacific Medical Pty. Ltd. (Richmond, Australia). Freeze-dried *B. animalis* Bb-12 is a commercial strain was initially obtained from Chr. Hansen Pty. Ltd. (Bayswater, Australia). The organisms were stored at -80°C in (12% v/v) sterile (121°C/15 min) reconstituted skim milk (RSM) and glycerol (40% v/v). All strains were identified as bifidobacteria based on the fructose-6-phosphate phosphoketolase (F6PPK) test (Orban and Patterson, 2000), Gram-staining (Scardovi, 1986) and carbohydrate fermentation test using a rapid ID 32A kit (Biomerieux, Lyon, France). One strain of *Lactococcus lactis* ssp. *lactis* and *Lactobacillus reuteri* 23272 were obtained from Melbourne University (Gilbert Chandler campus, Werribee, Australia) and Australian Starter Culture Centre (Werribee, Australia), respectively.

6.2.1.2. Bacterial identification and storage of enterococci strains

Eleven strains of *E. faecium* and nine strains of *E. faecalis* (VRE) were obtained from Box Hill Hospital (Box Hill, Australia), Victorian College of Pharmacy (Carlton, Australia) and Food Science Australia (Werribee, Australia). All enterococci were previously identified

as *E. faecalis* and *E. faecium* by biochemical tests according to Manero and Blanch (1999). Vancomycin-resistance was further confirmed by vancomycin-resistant disc diffusion tests (4-64 μg/ml). The twenty VRE strains for the study were labelled A to T.

6.2.1.3. Growth and storage media

Probiotic bacteria were grown in sterile DeMann, Rogosa, Sharpe (MRS) broth (Oxoid Ltd., West Heidelberg, Australia)(Dave and Shah, 1996). Filter-sterilised L-cysteine·hydrochloride monohydrate (LC) solution (0.05% w/v final concentration) was also added to the agar or broth prior to inoculation to the lower the oxidation-reduction potential of the medium to enhance the growth of bifidobacteria. Enterococci (*E. faecalis* and *E. faecium*) were grown in BHI broth (Oxoid Ltd., West Heidelberg, Australia). Probiotic and enterococci strains were stored at −80°C in 1.8 ml vials containing 20% (v/v) glycerol solution.

6.2.1.4. Determination of pH

The pH of the media was measured using a pH meter (Model 8417, Hanna Instruments Pty. Ltd., Singapore) after calibrating with fresh pH 4.0 and 7.0 standard buffers. Adjustment of pH of media was performed using freshly prepared 5 N HCl or 5 N NaOH solutions.

6.2.2. Vancomycin resistance of 20 VRE strains

Vancomycin resistance was performed twice in triplicate of each VRE strain used in this study. Twenty VRE strains (A to T) were seeded at an initial concentration of 1% (v/v) in BHI agar (0.9% w/v) and left to set for 2 h until the agar solidified. Four individual wells were subsequently cut out of the agar using a 6 mm borer. Vancomycin was dissolved in phosphate buffer (pH 6.8) and stored in vials with increasing concentrations from 4-128

μg/ml. An aliquot of 50 μl of increasing concentration of vancomycin was added in triplicate to each well and VRE agar plate. Each plate also contained a control using distilled water. Plates were left for another 2 h to allow the vancomycin solution to diffuse from the wells into the agar and then were incubated for 24 h at 37°C. Zones of inhibition (measured in mms) around each well were later recorded as an average (n=3).

6.2.3. Effect of pH on VRE bacterial counts and optical density

6.2.3.1. Measurement of pH of 20 VRE strains after 24 h

Twenty VRE strains were grown separately in 100 ml of BHI broth at 37°C for 24 h. Duplicate samples were taken at 0 h and 24 h and incubated aerobically in BHI agar at 37°C for 24 h. Bacterial counts of VRE were then enumerated. Measurement of pH was carried out on duplicate samples at 0 and 24 h.

6.2.3.2. Effect of pH of media on VRE bacterial counts, optical density and pH over 24 h

A 1% (v/v) inoculum of VRE strain A (*E. faecium* 01B149594) was added to bottles containing 100 ml of BHI broth which had pH adjusted at 2.0, 3.0, 4.0, 5.0, 6.0, 7.0 and a control (unadjusted pH). Measurement of pH was recorded in duplicate using a pH meter (Hanna Instruments Pty. Ltd., Singapore) every 2 h over 24 h for each bottle containing pH adjusted BHI broth containing VRE strain A. Adjustment of pH was performed every 2 h using 5 N NaOH. Measurements of bacterial counts of VRE were conducted over 24 h every 2 h by taking a 1 ml sample from each bottle and incubating them in BHI agar at 37°C for 24 h. Optical density was also measured from each bottle every 2 h. One ml samples in duplicate were obtained from each treatment bottle and using a spectrophotometer (Novaspec Pty Ltd., Uppsala, Sweden) set at 620 nm, an optical density reading was recorded for each sample.

6.2.4. Organic acid production of six VRE strains at different pH over 24 h

Organic acids present in media after inoculation with six enterococci strains with various pH, were measured by HPLC according to the method of Shin *et al.* (2000b). An Aminex HPX-87H column (300 mm x 8.7 mm, Bio-Rad Laboratories, Richmond, CA., USA) and a guard column with disposable cartridges H^+ (Bio-Rad Laboratories) was maintained at 65°C using an UV/vis detector set at 220 nm for organic acids. The column specifically separates organic acids using primarily ion exclusion and reversed phase mechanisms. The mobile phase was 0.009 M H_2SO_4. Culture samples were prepared by digesting with 15.8 M HNO_3 and 0.009 M H_2SO_4 for 30 min, vortexed, and centrifuged at 14,000 x *g* for 15 min using a benchtop centrifuge (Model 5415C; Eppendorf Pty. Ltd., Engelsdorf, Germany). Samples were then filtered using a 0.45 µm millipore filter into a HPLC vial and then injected in duplicate. Results were then calculated in milligrams of organic acid per ml of sample. Single and combined organic acid standards were performed including lactic acid, formic acid, acetic acid, isobutyric acid and n-butyric acid in order to identify organic acid retention times.

6.2.5. Inhibition of VRE with VRE and probiotic supernatant

6.2.5.1. VRE supernatant inhibition of VRE strains in well diffusion tests

Level of inhibition between 20 VRE strains was analysed by comparing the effect of VRE supernatant diffused into agar seeded with 20 pure VRE cultures. BHI broth with 0.9% bacteriological agar was autoclaved (121°C for 15 mins) and added to 1 ml of 20 individual VRE strains. The agar was left to solidify for 1 h at room temperature. Four wells were cut into the agar using a 6 mm borer in each agar plate. Twenty VRE strains were previously grown in BHI broth at 37°C for 24 h consecutively for 3 d. A benchtop centrifuge (Sorvall,

USA) was used to separate the supernatant (3838 x *g*, 4°C for 15 min) from the cells contained within the culture. A 0.45 μm Millipore filter was used to sterilise the supernatant. The supernatant was divided into pH unadjusted or adjusted to 6 for each experiment using 5 N NaOH. An aliquot of 50 μl of supernatant from each VRE strain was added to each well. The supernatant was left to diffuse for 2 h. The plates were incubated at 37°C for 24 h. Zones of inhibition were recorded. The experiment was performed in duplicate for each VRE strain.

6.2.5.2. Well diffusion tests using probiotics

To obtain supernatant for inhibition assays, each probiotic strain was grown consecutively 3 times in MRS broth containing 0.05% LC at 37°C for 24 h using 1% inoculum, followed by centrifugation of 200 ml aliquots at 3838 x *g* for 15 min at 4°C. The cell free supernatant was divided into two portions. The pH of the first portion was recorded and left unadjusted, while the pH of the second portion was adjusted to 6.0 using 5 N NaOH. Both unadjusted and adjusted supernatants obtained from each probiotic strain were filtered using 0.45 μm Millipore filters (Millipore Corp., Bedford, MA, USA.).

An aliquot of 0.1 ml of culture of each of the 20 VRE strains was added with approximately 20 ml of BHI agar to separate plates. Each plate was divided into four quadrants and wells were cut into the agar using a 6 mm borer. The bottom of each well was sealed with 0.9% (w/v) sterile agar, followed by the addition of 50 μl of supernatant of each probiotic strain. All inhibition assays were performed in triplicate for each VRE strain, for both adjusted and unadjusted supernatants using the method of Dave and Shah (1997b). Supernatant was diffused for 2 h into the agar, followed by incubation at 37°C for 24 h. The inhibition zones (mms) were then measured.

6.2.5.3. Disc diffusion tests

Twenty VRE strains were grown consecutively three times in BHI broth at 37°C for 24 h. An aliquot of 0.1 ml of each VRE strain was transferred to a plate where BHI agar was pour plated. The agar was left to solidify for 2 h. Seven probiotic strains, *B. infantis* 1912, *B. longum* 1941, *B. longum* BB536, *B. pseudolongum* 20099, *B. animalis* Bb12, *L. lactis*, and *L. reuteri* 23272 were grown separately at 37°C for 24 h, three times in 50 ml of MRS broth. The cultures were centrifuged at 3838 x *g* at 4°C for 15 min and filter sterilised using a 0.22 μm Millipore filter. Sterile 6 mm discs were dipped into each probiotic supernatant for 30 sec and then placed in triplicate onto a solidified agar plate containing a single VRE strain. The discs were left to diffuse for 2 h and then the plates were incubated at 37°C for 24 h. The zone of inhibition around the discs was measured for inhibition of VRE strains from each probiotic supernatant.

6.2.6. Laboratory probiotic strains used to inhibit VRE

6.2.6.1. Inhibition of VRE using bifidobacteria

Bifidobacterium longum 1941 was grown consecutively three times in 100 ml of MRS broth at 37°C for 24 h using 1% (v/v) inoculum. VRE strain C was grown consecutively three times in 100 ml of BHI broth (Oxoid, UK.) at 37°C for 24 h using 1% inoculum. *B. longum* 1941 and VRE strain C were both added (1% v/v) to 200 ml of MRS broth. Controls of *B. longum* and VRE were also used. The co-culture and single strain cultures (controls) were grown at 37°C for 24 h in duplicate and 1 ml samples taken every 3 h. *B. longum* 1941 was selectively grown anaerobically on MRS agar (37°C for 72 h) while VRE was selectively grown aerobically on MacConkey agar (37°C for 24 h). Colony forming units were counted

after the respective period of incubation was completed. Measurement of pH was also taken in duplicate every 3 h from co-culture and control media.

6.2.6.2. Inhibition of VRE using two strains of probiotic bacteria

L. lactis and *L. reuteri* 23272 were grown consecutively three times in 100 ml of MRS broth at 37°C for 24 h using 1% (v/v) inoculum. VRE strain A was grown consecutively three times in 100 ml of BHI broth at 37°C for 24 h using 1% (v/v) inoculum. Each organism was added (1% v/v) to 100 ml of MRS broth, which was incubated at 37°C for 24 h. Combinations of organisms included VRE (A) as a control, VRE (A) with *L. lactis*, VRE (A) with *L. reuteri* and VRE (A) with *L. lactis* and *L. reuteri*. A one ml sample was taken in duplicate every 6 h for 24 h. VRE (A) was selectively grown aerobically on MacConkey agar (37°C for 24 h) while *L. reuteri* and *L. lactis* were grown anaerobically in MRS agar (37°C for 72 h). Colony forming units were recorded for each strain used.

6.2.7. Commercial probiotic applications used to inhibit VRE

6.2.7.1. VSL#3 used at different concentrations to inhibit VRE over 24 h

A commercial freeze-dried probiotic preparation VSL#3™ (VSL Pharmaceuticals, Inc., Gaithersburg MD, USA.) was supplied by the Department of Gastroenterology at Box Hill Hospital (Box Hill, Australia). VSL#3™ contains a mixture of 8 distinct probiotic strains, supplied in a 5 g sachet containing a reported 450 billion live lactic acid bacteria, which were stored at a refrigerated temperature (4°C).

To measure the effectiveness of VSL#3™ on a VRE strain, 1 or 2 packets of VSL#3 was added to 100 ml of MRS broth (Oxoid, Australia) containing 1% (v/v) inoculum of VRE strain A. A control containing no VSL#3™ was also used. The media were incubated for 24 h

at 37°C. One ml of sample was taken at 0 and 24 h and incubated aerobically in MacConkey agar at 37°C for 24 h for VRE strains, while MRS agar containing 0.05% L-cysteine was used to selectively enumerate probiotic strains grown anaerobically for 3 d at 37°C. All experiments were performed in duplicate.

In the second experiment, one bottle containing 100 ml of MRS broth was inoculated with 1% inoculum of VRE, and the second bottle also inoculated with VRE but also contained 1 packet of VSL#3™. The bottles were mixed thoroughly and incubated at 37°C over 24 h. Duplicate one ml samples were taken every 3 h for 24 h and plated in BHI agar at 37°C for 24 h. Colony forming units (CFU) were recorded. The experiment was performed in duplicate.

In the third experiment, VSL#3™ was added in a dose-dependent fashion of 0.01, 0.1, 1.0 and 2.0 g to 100 ml of BHI broth (Oxoid, Ltd., West Heidelberg, Australia) containing 1% inoculum of VRE strain A. A control containing no VSL#3™ was also used. The media were incubated at 37°C. Duplicate one ml samples were taken at 0, 3, 6, 9, 12 and 24 h to measure CFU/ml in BHI agar incubated at 37°C for 24 h. PH readings were also taken simultaneously. The experiment was performed in duplicate.

6.2.8. VRE & probiotic inhibition by nisin

6.2.8.1. Effect of nisin on VRE strains optical density

The effect of nisin on 3 strains of VRE was conducted. Nisin at a final concentration of 0, 1, 5, 10, 15 and 20 μg/ml was added to 50 ml of BHI broth, containing 1% (v/v) inoculum of VRE strains A (*E. faecium* 01B149594), C (*E. faecium* 01B074188) and E (*E. faecium* e1971g) and were incubated at 37°C over 24 h. Duplicate samples were taken at 2 h intervals for 24 h. Optical density was measured using a spectrophotomer (Novaspec, Uppsala, Sweden) set at 620 nm. The experiment was performed in duplicate.

6.2.8.2. Effect of nisin inhibition on VRE and probiotic strains

The effect of nisin on five strains of VRE (A, B, C, D and E) and 6 strains of probiotic bacteria (*B1941, BB536, BB12, B1912, L. reuteri* and *L. lactis*) was performed. An inoculum of 1% (v/v) of VRE was added to BHI broth containing 0.9% (w/v) bacteriological agar (Amyl Media Pty Ltd., Dandenong, Australia). An inoculum of 1% (v/v) of probiotic strains was added to MRS broth containing 0.9% (w/v) bacteriological agar. Plates were divided into quadrants and a well in the middle of the agar of each sector was cut using a 6 mm borer. The bottom of the well was sealed with 0.9% (w/v) of sterile agar. Dissolved nisin at a concentration of 0, 1, 5, 10, 50, 100, 250, 500 and 1000 μg/ml was added to wells in duplicate. The agar plates were left to diffuse for 2 h and then incubated at 37°C for 24 h. The zone of inhibition from each well was measured in mms using a metric ruler for both VRE and probiotic strains.

6.2.8.3. Inhibition of VRE strains using serially diluted probiotic strains

Two probiotic strains (*L. reuteri* 23272 and *L. lactis*) were grown consecutively three times in MRS broth at 37°C for 24 h using 1% inoculum. Three probiotic strains were then serially diluted using 0.1% peptone water (10^{-1} to 10^{-9}). One ml from each dilution was plated in triplicate using MRS agar. The organisms were grown at 37°C for 72 h. Individual 5 ml overlay containing five separate VRE strains (1% v/v) in sterile BHI broth containing 0.9% (w/v) bacteriological agar was poured over the probiotic plates. The plates were left to harden for 2 h and incubated at 37°C for 24 h. The zones of inhibition were recorded for each probiotic strain for each dilution. In a separate experiment, VRE strains (A, B, C, D and E) were spread plated on BHI agar and incubated for 24 h. Molten BHI agar was then inoculated with the same VRE strains which were overlayed separately in duplicate onto each plate. The

plates were then incubated separately at 30, 37 and 45°C for a further 48 h. Inhibition zones were recorded as a clearing of the overlay inoculated agar.

6.2.8.4. Overlay matrix design inhibition tests

One percent inoculum of five VRE strains (A, B, C, D and E) and two probiotic strains (*L. lactis* and *L. reuteri* 23272) were grown at 37°C for 24 h in MacConkey agar (Oxoid Ltd., West Heidelberg, Australia) and MRS agar, respectively. Autoclaved (121°C for 15 mins) MacConkey broth and MRS broth with 0.9% bacteriological agar were inoculated with pure VRE strains and probiotic strains (0.1% v/v), mixed, and approximately 5 to 7 ml was overlayed onto a total of 49 VRE and probiotic plates. The plates were incubated for a further 24 h at 37°C. Zones of inhibition (mms) around individual colonies were recorded.

6.2.8.5. Spot on lawn test

Five VRE strains were grown in BHI broth at 37°C for 24 h and then 1% v/v was pour-plated using BHI broth containing 0.9% (w/v) bacteriological agar. Three probiotic strains (*L. reuteri* 23272, *L. lactis* and *B. longum* 1941) were grown consecutively three times in 100 ml of MRS broth at 37°C for 24 h using 1% (v/v) inoculum. The culture was then centrifuged (3838 x *g*, 15 min at 4°C). The pH of the supernatant was then adjusted to 6 using 5 N NaOH and serially diluted (10^{-1} to 10^{-9}) using 0.1% peptone water. One drop (20 µl) of each dilution of probiotic supernatant was placed onto the VRE inoculated agar plates. The drop was left to diffuse for 2 h and then incubated at 37°C for 24 h. The zone of inhibition (mms) by the supernatant of each probiotic was recorded.

6.2.9. Effect of concentrated supernatant on VRE

6.2.9.1. Inhibition of VRE using concentrated probiotic supernatant

Probiotic strains *L. reuteri* 23272 and *L. lactis* was grown consecutively three times in MRS broth (Oxoid) at 37°C for 24 h using 1% inoculum. The culture was centrifuged (3838 x *g*, 4°C for 15 min) and filtered using a 0.45 µm Millipore filter. The supernatant was then concentrated using two Vivacell 70 concentrators which contained a 10 kDa cutoff (Sartorius Australia Pty. Ltd., East Oakleigh, Australia) in a centrifuge (Model J2-HS, Beckmann Pty Ltd., USA) set at 1,000 x *g* (4°C for 2 h). The Vivacell 70 concentrator was centrifuged four times until a total of 200 ml of supernatant was concentrated. The probiotic supernatant of both strains was then separated into two 50 ml portions with one portion pH adjusted to 6 using 5 N NaOH. VRE strain A was grown in BHI broth at 37°C for 24 h over three consecutive days. The VRE culture was serially diluted (10^{-1} to 10^{-6}) and was inoculated into autoclaved (121°C for 15 min) BHI broth containing 0.9% bacteriological agar. Two wells were cut into each agar plate using a 6 mm borer. The bottom of the well was sealed with sterile 0.9% bacteriological agar. An aliquot of 50 µl of each pH adjusted and unadjusted concentrated permeate was added to each well. The wells were left to diffuse for 2 h and then incubated at 37°C for 24 h. Zones of inhibition were recorded.

6.2.9.2. Effect of probiotic permeate and retentate supernatant on VRE

The previous procedure was followed, except that five VRE strains (A, B, C, D and E) were used and were not serially diluted. While both the retentate and permeated were filtered (0.45 µm Millipore) and autoclaved (121°C for 15 min) from the Vivacell 70 concentrator using a 10 kDA cutoff. Permeate and retentate controls were not concentrated.

6.3 Results and Discussion

6.3.1. Vancomycin resistance by 20 VRE strains

Initially, twenty strains of VRE *E. faecalis* and *E. faecium* were tested for their resistance to vancomycin using inhibition well diffusion tests to measure minimum inhibitory concentration (MIC) between 0 to 128 μg/ml. Experiments were performed in triplicate on two occasions with all 20 *E. faecalis* or *E. faecium* strains confirmed to have prior vancomycin resistance of between 8 to 64 μg/ml as shown in Figure 6.1. Strains F, G, H and Q had the lowest vancomycin MIC of 8 ug/ml, while strains I, M, N and O had the highest vancomycin MIC of 64 ug/ml. Vancomycin resistance was previously known to be contained within these enterococcal strains as they were isolated using Enterococcosel agar with 6ug/ml vancomycin from samples tested at Box Hill Hospital and Food Science Australia. The level of variability of vancomycin resistance between the 20 strains was dependent on their vancomycin phenotype i.e. VanA, B, C, D or E, strain identity and isolated environment.

6.3.2. Assessment of final pH of 20 VRE strains after 24 h incubation

An assessment of final pH was taken from 20 VRE strains of *E. faecalis* and *E. faecium* that were grown for 24 h at 37°C in Brain Heart Infusion broth (Oxoid, UK.). The pH average of duplicate results ranged between a high of 5.83 ± 0.01 (VRE D) to a low of 5.62 ± 0.01 (VRE S) as shown in Figure 6.2. Final pH for the 20 VRE strains indicate that the enterococci assessed optimally survive at a final pH that is approximately 1 log scale higher than other probiotic genus such as *Bifidobacterium* or *Lactobacillus*. However, we can consider that while VRE produce a final pH within a small band between the various strains,

different survival mechanisms other than pH adjustment are utilised to survive and colonise *in vivo*.

6.3.3. Effect of media pH on VRE final pH, bacterial counts and optical density over 24 h

6.3.3.1. Effect of initial pH on VRE final pH

The adjustment of pH was further evaluated with 24 h growth experiments adjusting the initial pH of brain heart infusion (BHI) broth to 2.0, 3.0, 4.0, 5.0, 6.0 and 7.0 and adding 1% (v/v) initial inoculum of VRE strains I, M, N, O, R and S. As shown in Figure 6.3, the initial BHI broth pH of 2.0, 3.0 and 4.0 produced only slight increases in pH, while initial BHI broth pH of 5.0, 6.0 and 7.0 showed a significant decrease in final pH, especially strains that were initially exposed to a pH of 6.0 or 7.0. Although, differences between strains were non-significant at any initial pH exposure tested between 2.0 and 7.0, we can hypothesise that the higher the initial pH, the greater the production of organic acids due to increased metabolic activity of the VRE. Furthermore, more energy could be expedited into replication and gastrointestinal colonisation rather than using large amounts of energy at a very low pH (<3.0) where conditions are too adverse for survival and growth. Low pH conditions as demonstrated *in vivo* have a detrimental effect on the survival of VRE and may be a significant factor in reducing gastrointestinal VRE populations *in vitro*.

6.3.3.2. Effect of maintenance of pH over 24 h on bacterial counts.

Maintenance of BHI pH at 2.0, 3.0, 4.0, 5.0, 6.0 and 7.0 resulted in variable growth patterns as presented in Figure 6.4. VRE (A) grown in pH 2.0 and 3.0 resulted in zero survival after 4 and 8 h, respectively. Growth at pH 4.0 produced a stable survival of VRE with no significant increase in bacterial numbers. However, maintenance of pH at 5.0, 6.0 and 7.0

produced a final bacterial count similar and non-significant to the control broth which was not pH adjusted. From these results, low pH has a dramatic effect on growth and survival patterns of VRE.

6.3.3.3. Effect of maintenance of pH over 24 h on optical density.

Maintenance of BHI media at a pH at 2.0, 3.0, 4.0, 5.0, 6.0 and 7.0 resulted in a similar pattern of optical density (OD) presented in Figure 6.5 as that of bacterial counts in Figure 6.4. BHI broth adjusted to pH 2.0, 3.0 and 4.0 displayed a flat OD reading over the 24 h incubation period. Results obtained from VRE grown at pH 5.0 showed a significantly slower increase in OD than VRE grown at pH 6.0 and 7.0, which were similar to that of the control.

6.3.4. Organic acid production of six VRE strains at different pH over 24 h

HPLC was used to measure organic acid production of 6 VRE strains (I, M, N, O, R and S) at pH 2.0, 3.0, 4.0, 5.0, 6.0 and 7.0 over 24 h. Lactic acid, formic acid, acetic acid, isobutyric acid and butyric acid were specifically isolated using standards of each acid to determine retention time. Injection of samples was performed in duplicate (n = 4). A summary of organic acid production by each VRE strain is shown in Figure 6.6 (VRE I), Figure 6.7 (VRE M), Figure 6.8 (VRE N), Figure 6.9 (VRE O), Figure 6.10 (VRE R) and Figure 6.11 (VRE S). Overall, organic acid production by each strain showed that lactic and acetic acids were produced in the highest quantities after 24 h incubation. More specifically, the profile of the 6 strains can be separated into low organic acid producer VRE strains (I, R and S) and high organic acid producer VRE strains (M, N and O). VRE (I) produced higher levels of lactic and acetic acid concentrations at pH 2.0 to 4.0 than pH 5.0 to 7.0. VRE (R) displayed higher concentrations of lactic acid and acetic acid than VRE (I) with others acids similar at

each pH concentration and time interval. Analysis of VRE (S) shows a selective production of lactic acids at a higher pH range of 5.0 to 7.0 than pH 2.0 to 4.0. Analysis of organic acids by VRE (M) demonstrated slightly higher production of lactic acid towards the lower end of the pH range (2.0 to 4.0). Analysis of VRE (N) showed a pronounced skew towards higher production of lactic acids at the higher pH range between 4.0 to 7.0. However, examination of VRE (O) results displayed higher values of lactic acid at all pH levels after 24 h incubation and no significant trend amongst the five organic acids analysed.

6.3.5. Effect of 20 VRE supernatant on 20 VRE strains using well diffusion inhibitory tests

The analysis of 20 VRE strains inhibition was analysed using the supernatant from the same VRE strains to show whether they displayed inhibitory properties against similar species. As represented in Figure 6.12, inhibition produced by the 20 VRE strains is highly variable with strains I, M, N, O, R and S generally producing the highest inhibition towards the VRE strains. Zones of inhibition produced by strain M have a maximum of 21 mms while VRE strains F and G produced no detectable zones of inhibition. Eleven VRE strains of the 20 tested produced zones of inhibition greater than 10 mms against one or more VRE strains.

When comparing the target VRE strains, we can observe those strains that are highly resistant to inhibition. Furthermore, some VRE strains are both resistant to inhibition and do not cause inhibition to other VRE strains. However, strains that produce high inhibition also are highly resistant to inhibition from other similar strains. This is observed in Figure 6.13 where strains I, M, R and S are not significantly affected by other VRE supernatants, however produce high levels of inhibition against other VRE strains.

6.3.6. Comparison of inhibition against 20 VRE strains using pH adjusted and unadjusted supernatants of probiotic supernatant

The level of inhibition produced by adjusted and unadjusted probiotic supernatant against 20 strains of VRE is presented in Figure 6.14. In general, the level of inhibition is greater when the supernatant is pH unadjusted as compared to the supernatant that is adjusted to pH 6.0 when added using well diffusion inhibition tests. Levels of inhibition against VRE strains were higher for probiotic strains *L. lactis*, *L. reuteri* and *B. longum* 1941 as compared to *B. animalis* Bb12 and *B. longum* BB536. Highest average zone of inhibition of 25 mm was recorded by *B. longum* 1941 and *L. lactis* using unadjusted supernatant while *B. longum* BB536 and *B. animalis* recorded several strains with no zones of inhibition detected. Overall, probiotic strains *L. lactis* and *L. reuteri* provided the most consistent inhibition against the 20 VRE strains.

6.3.7. Comparison of VRE inhibition using discs soaked in probiotic unadjusted supernatant

Figure 6.15 compares the zone of inhibition of 20 VRE strains that were inhibited by the unadjusted supernatant of 7 different probiotic strains (*L. reuteri, L. lactis, B. longum* 1941, *B. longum* BB536, *B. pseudolongum* 20099 and *B. animalis* Bb12) soaked on blank 6 mm paper discs. In general, the supernatant of *L. reuteri* and *L. lactis* produced higher zones of inhibition than *B. longum* 1941 for the 20 VRE strains tested. Zones of inhibition ranged from 6.5 mm to 13 mm after 48 h of incubation at 37°C on BHI agar with 1% VRE inoculum of each strain.

6.3.8. *Growth curves using probiotic cultures*

6.3.8.1. Inhibition of VRE using bifidobacteria

Inhibition of VRE (A) was significantly inhibited by the addition of *B. longum* 1941 when incubated together and bacterial counts were enumerated over a 24 h period. Figure 6.16 displays bacterial counts and the pH of the culture of both the control and co-culture of *B. longum* 1941 and VRE (A). We can observe a steady increase of the control VRE until the 12 h mid-point and then a slight decrease of CFU/ml. However the media containing VRE and *B. longum* 1941 displayed a significantly reduced bacterial count of VRE over 24 h as compared to the control. In addition to bacterial counts, pH data was also displayed on the same figure, we can observe that the co-culture maintained a significantly lower pH than the control VRE (A). Overall, the low pH had a significant impact on the viability of VRE in co-culture over 24 h of approximately 0.5 log CFU/ml.

6.3.8.2. Inhibition of VRE using other probiotic bacteria

The growth of VRE with *L. lactis* and *L. reuteri* over 24 h is presented in Figure 6.17. As shown in the figure, *L. lactis* and *L. reuteri* produced a lower viability for VRE as compared to the control. Although, when both probiotic strains were added together, the final result was slightly higher than that of a single probiotic strain, however, the viability was still lower than that of the control. These results demonstrate that addition of probiotics to VRE *in vivo* have a significant effect on VRE bacterial counts.

6.3.9. Inhibition of VRE strains using adjusted probiotic supernatant

The zone of inhibition produced by three supernatants of probiotic bacteria against 5 strains of VRE were analysed. The results for inhibition by supernatant of probiotic bacteria

which was adjusted for pH, hydrogen peroxide production, catalase and trypsin are presented in Figure 6.18. When comparing the five probiotic strains supernatant inhibition of the five VRE strains was affected mostly by unadjusted supernatant especially by *B. longum* 1941, while pH adjustment of supernatant significantly altered the level of inhibition of VRE strains. Addition of H_2O_2, catalase and trypsin to the supernatant slightly reduced the level of inhibition, if inhibition was present when adjusted supernatant was added. Overall, we can observe variable inhibition of the five VRE strains as a result of probiotic supernatant addition which was pH unadjusted or pH adjusted with NaOH and H_2O_2, trypsin and catalase.

6.3.10. VSL used at different concentrations to inhibit VRE over 24 h

6.3.10.1. Effect of adding VSL#3™ over 24 h to VRE

The inhibition effectiveness of VSL#3™ was assessed by measuring the zone of inhibition against VRE (A) when different concentrations of VSL#3™ were added over a 24 h incubation period and was presented in Figure 6.19. The level of bacterial inhibition was 0.91 to 1.01 log reduction of VRE from initial bacterial counts using 1 and 2 packets of VSL#™, respectively. The control showed an increase of approximately 2 log as compared to the initial bacterial counts without any VSL#3™ addition.

6.3.10.2. Effect of adding different concentrations of VSL#3™ over 24 h to VRE bacterial counts

The bacteria counts of VRE taken at 3 h intervals for 24 h to measure the inhibitory effect of different concentrations of VSL#3™ over 24 h are presented in Figure 6.20. Comparing each time interval, we can observe that the decrease in VRE was greater when the

initial concentration of VSL#3™ was greater. There was a significant decrease in VRE when 0.1, 1.0 and 2.0 g of VSL#3™ was added resulting in the lowest bacterial count after 24 h of 4.3 log after 2 g of VSL#3™ was initially added. The inhibition of VSL#3™ as a concentrated probiotic inhibitor was evident at each time interval, especially at the final 24 h measurement.

6.3.10.3. Effect of adding different concentrations of VSL#3™ over 24 h to culture pH

The addition of VSL#3™ also had a significant effect on the pH of the VRE culture that was dependent on the concentration of initial added VSL#3™. Results presented in Figure 6.21 illustrate a significant drop in pH after only 3 h for 1 and 2 g of VSL#3 while 0.1 g VSL#3™ displayed a linear decrease. A concentration of 0.01 g VSL#3™ showed a pattern of pH drop similar to VRE but significantly drops after the 12 h point. Therefore, from Figures 6.20 and 6.21 we can conjecture that the drop in bacterial counts is correlated with the significant drop in pH of the growth media.

6.3.11. Effect of nisin on VRE strains optical density

6.3.11.1. Effect of nisin inhibition on single strains of VRE optical density

The optical density of VRE strains (A, C and E) after increasing concentrations of nisin were added (0, 1, 5, 10, 15 and 20 ug/ml) into BHI broth incubated at 37°C was measured every 2 h for 24 h. Results presented in Figure 6.22, 6.23 and 6.24 show a similar pattern impeded growth when organisms containing higher concentrations of nisin were added. Although the pattern for reaching stationary phase for the VRE strains was similar. VRE (C) was significantly more sensitive to nisin than VRE (A) or VRE (E). These results suggest that the VRE organisms that were resistant to nisin were selected for, while those

cells that were sensitive to nisin underwent lysis. From these results, we can observe the growth characteristics of VRE after exposure to a bacteriocin produced by *Lactococcus* subsp. *lactis*. Unfortunately, the inhibition of VRE due to nisin is brief and unknown cellular mechanisms by VRE adapt relatively quickly to the harsh environment.

6.3.11.2. Effect of nisin inhibition on VRE and probiotic strain optical density

The inhibitory effect of nisin was authenticated further with well diffusion inhibition tests on 6 probiotic and 5 VRE strains. Concentrations of nisin ranged from 1 to 1000 ug/ml. From the results presented in Figure 6.25, we can observe the inhibitory effect of nisin on probiotic and VRE strains. The higher the concentration of nisin, the greater the average zone of inhibition observed around the wells. Probiotic strains were more sensitive to nisin than VRE strains by all nisin concentrations used. *Bifidobacterium longum* 1941 was the most sensitive and produced a inhibitory zone at 5 ug/ml, while most other probiotics and VRE strains were in general sensitive down to 50 ug/ml of nisin.

6.3.12. Further inhibition tests

6.3.12.1. Sandwich overlay of VRE and probiotic strains

The results from the overlay test were mixed with no evidence of inhibition as a result of probiotic bacteria towards the VRE strains, however inhibition was recorded against probiotic and other VRE strains by VRE. As shown in Table 6.1, we can observe inhibition of *L. reuteri* by all VRE strains while *L. lactis* was inhibited by VRE (A), (B) and (C). VRE (A) inhibited VRE (B) and (D), VRE (B) inhibited VRE (A), (C) and (D), VRE (C) inhibited VRE (D), while VRE (D) and (E) was unable to inhibit other VRE strains.

Comparing the VRE inhibition of other VRE overlayed strains incubated at different temperatures, we were unable to detect any zone of inhibition after 24 and 48 h of incubation of the overlayed strains. Results presented in Table 6.2 show no observable zones of inhibition after duplicate experiments using five strains of VRE.

6.3.12.2. Spot on lawn test

The further testing of supernatant concentration of VRE inhibition was researched by utilising different concentrations of three probiotic strains (*L. reuteri, L. lactis* subsp *lactis* and *B. longum* 1941) against five strains of VRE (A, B, C, D and E). The supernatant was firstly pH adjusted to 6.0 and the concentration of stationary phase supernatant (18 h growth) was then serially diluted from 10^{-0} to 10^{-9}. One drop (50 ul) was placed in decreasing concentration in a matrix design on 9 points of seeded BHI agar containing 1% v/v VRE (A, B, C, D or E) overnight grown culture. Of the inhibition plates that were tested, no observable zones of inhibition were recorded as presented in Table 6.3. From these results, we can assume that the combination of pH adjustment to 6.0 and the serial dilution of supernatant were ineffective to produce a recordable zone of inhibition against VRE.

6.3.13. Concentration of probiotic supernatant

6.3.13.1. Effect of concentrated probiotic supernatant on VRE

Concentration of probiotic supernatant was performed in order to enhance the inhibitory properties of the supernatant against VRE. The inhibitory properties of the supernatant were further defined by separating the permeate (<10 KDa) and retentate (>10 KDa) using a Vivacell™ with a 10 KDa filter. Concentrated retentate which was adjusted (pH 6.0) and unadjusted (pH 4.5) was used in well diffusion tests against VRE (A, B, C, D and E).

The probiotic supernatant was concentrated approximately five times from 300 ml to 60 ml total volume. The retentate of both *L. reuteri* and *L. lactis* was ineffective in producing observable zones of inhibition against the 5 strains of VRE as shown in Table 6.4.

6.3.13.2. Effect of probiotic permeate and retentate supernatant on VRE

The problem of diffusibility of the thick retentate through the agar was tested. Retentate was serially diluted from 10^{-2} to 10^{-6} and was tested against VRE (A) using well diffusion inhibition tests. Observations recorded and presented in Table 6.5. From these inhibition tests showed negative results for any zone of inhibition from both supernatant retentate that was unadjusted and pH adjusted to 6.0.

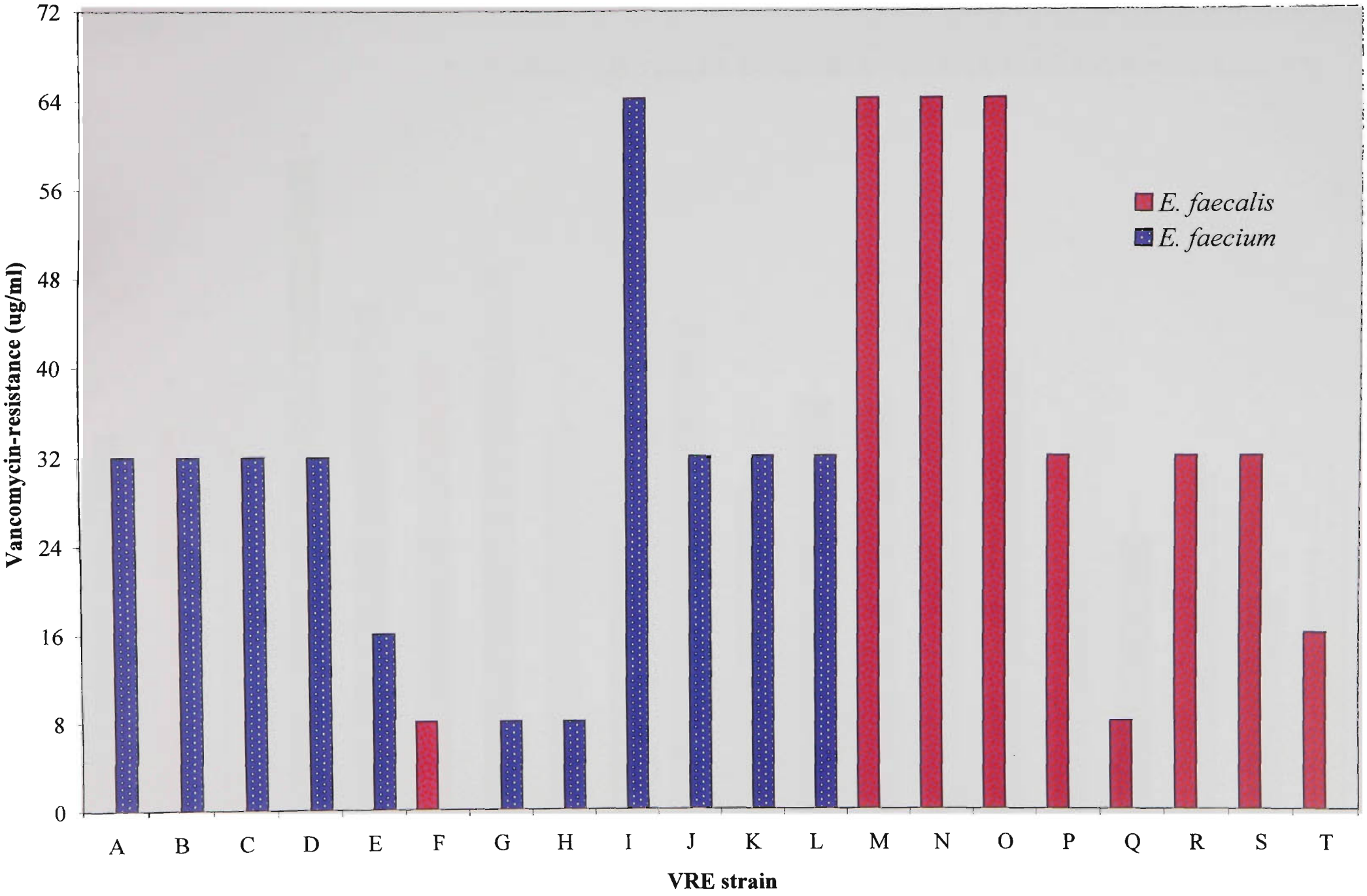

Figure 6.1. Vancomycin-resistance (MIC) of 20 VRE strains (Mean values, n = 3)

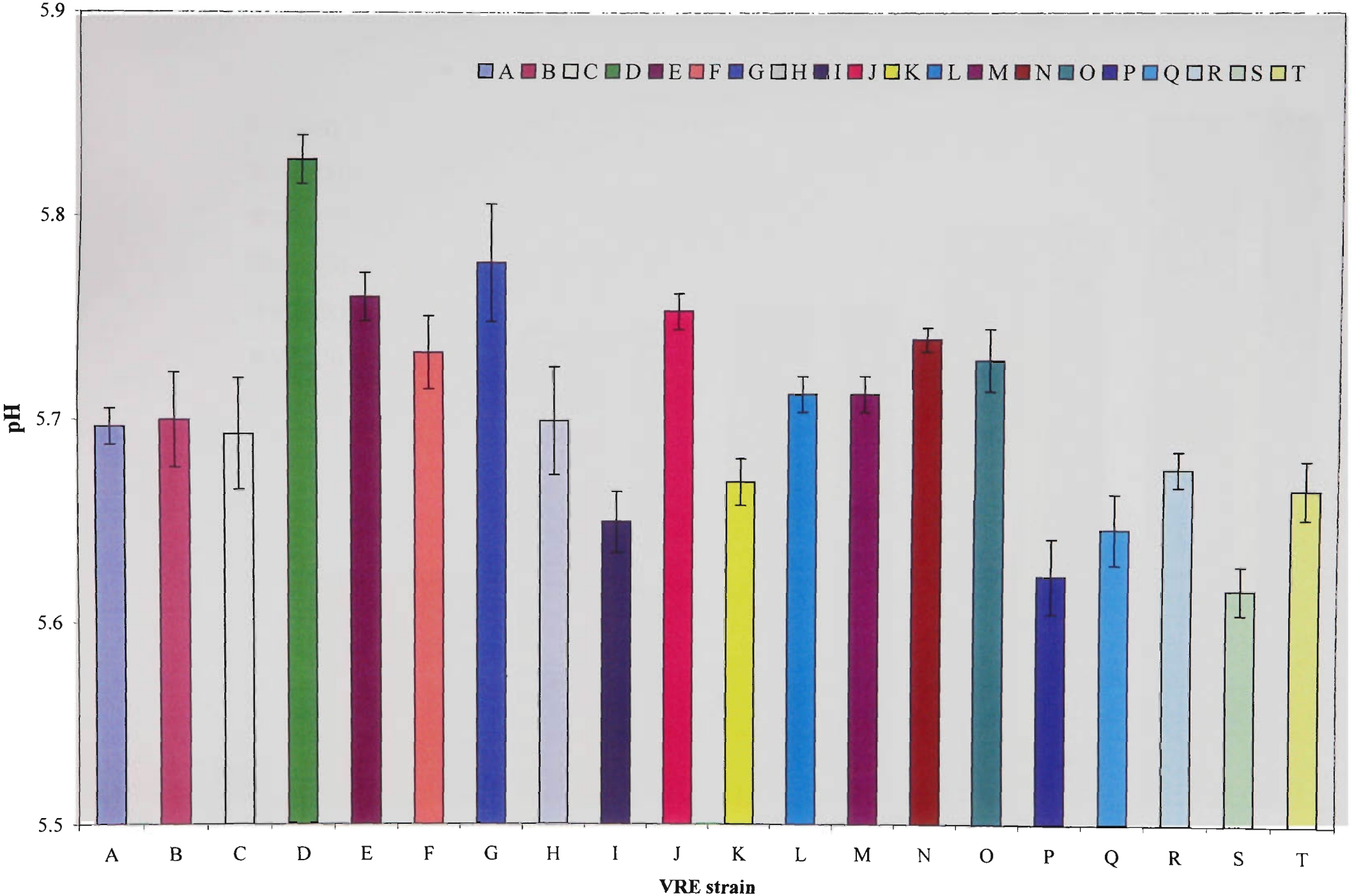

Figure 6.2. PH of 20 VRE strains after 24 h incubation (Mean ± SE, n = 4)

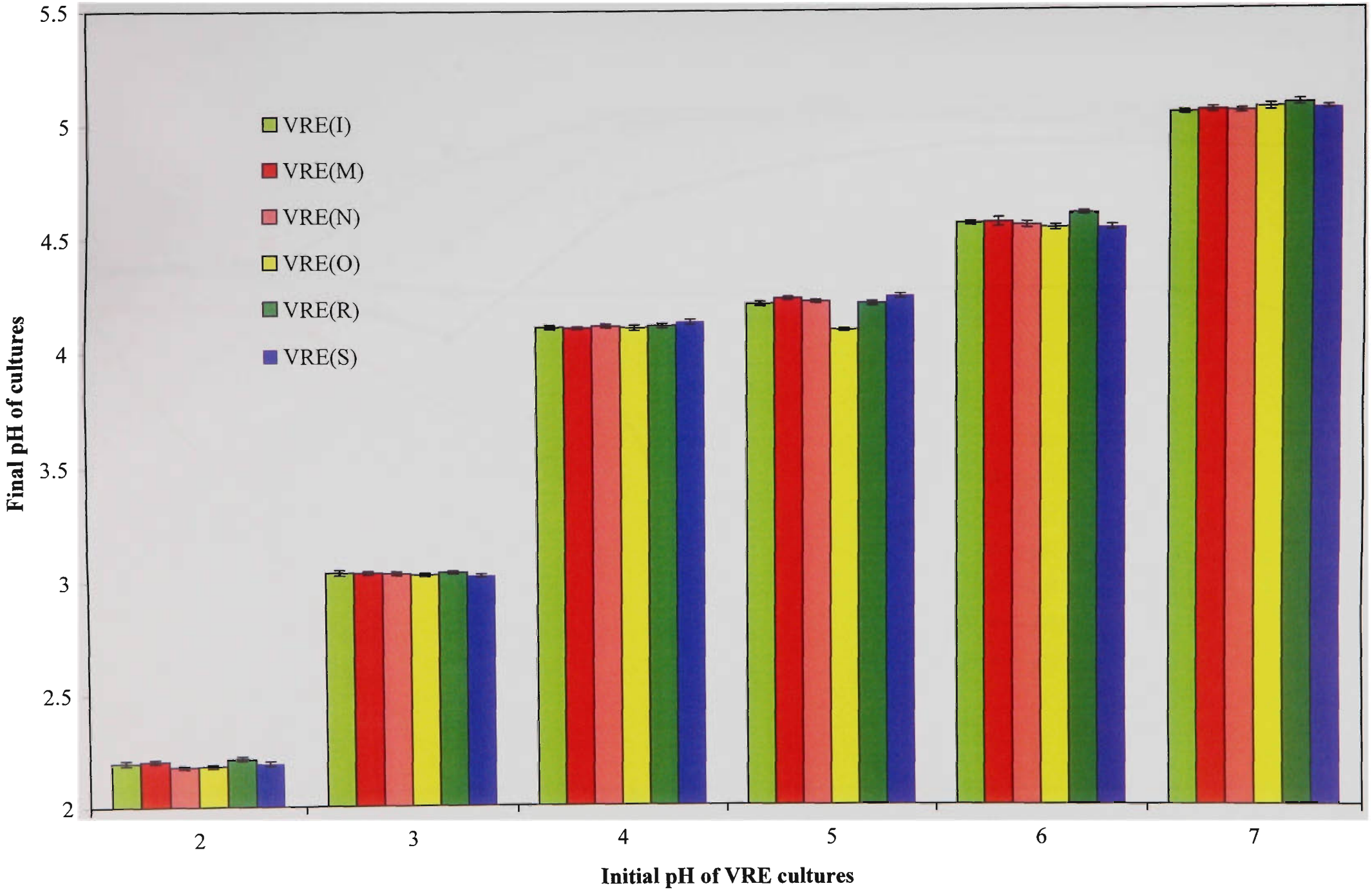

Figure 6.3. Final pH of 6 VRE strains after initial pH adjustment (Mean ± SE, n = 6)

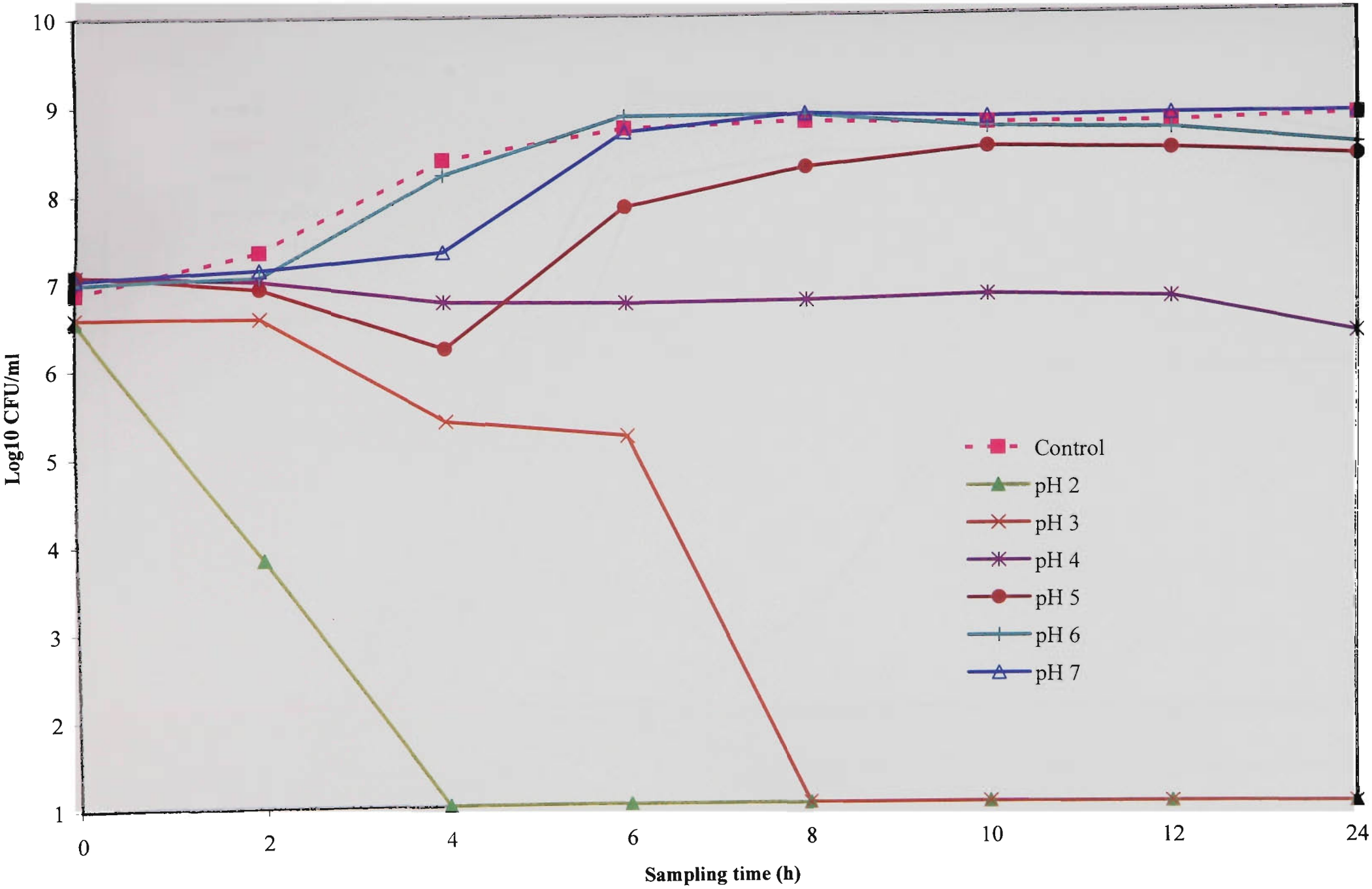

Figure 6.4. Bacterial counts of VRE after maintenance of pH over 24 h (Mean values, n = 3)

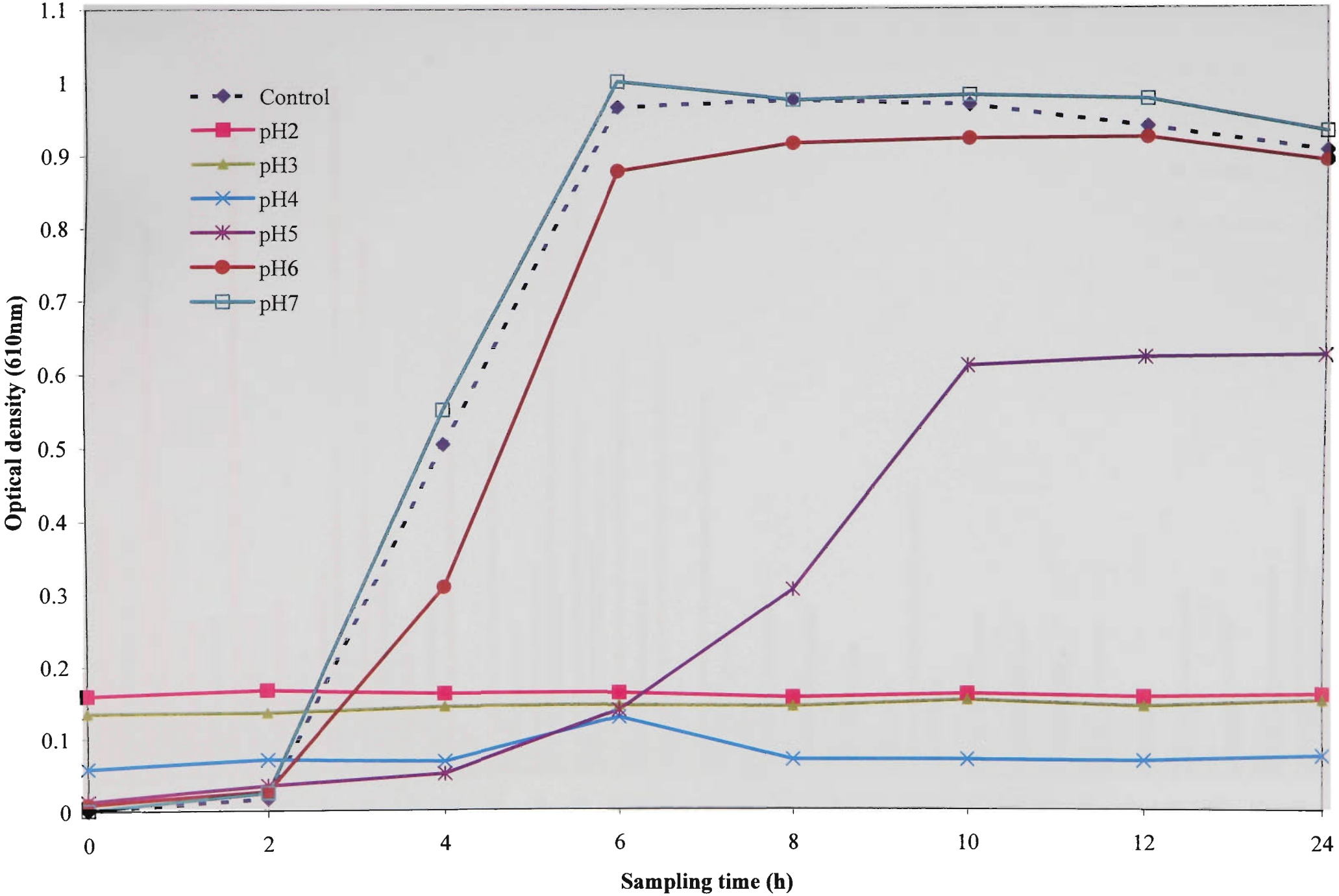

Figure 6.5. Optical density of VRE while maintaining pH levels over 24 h

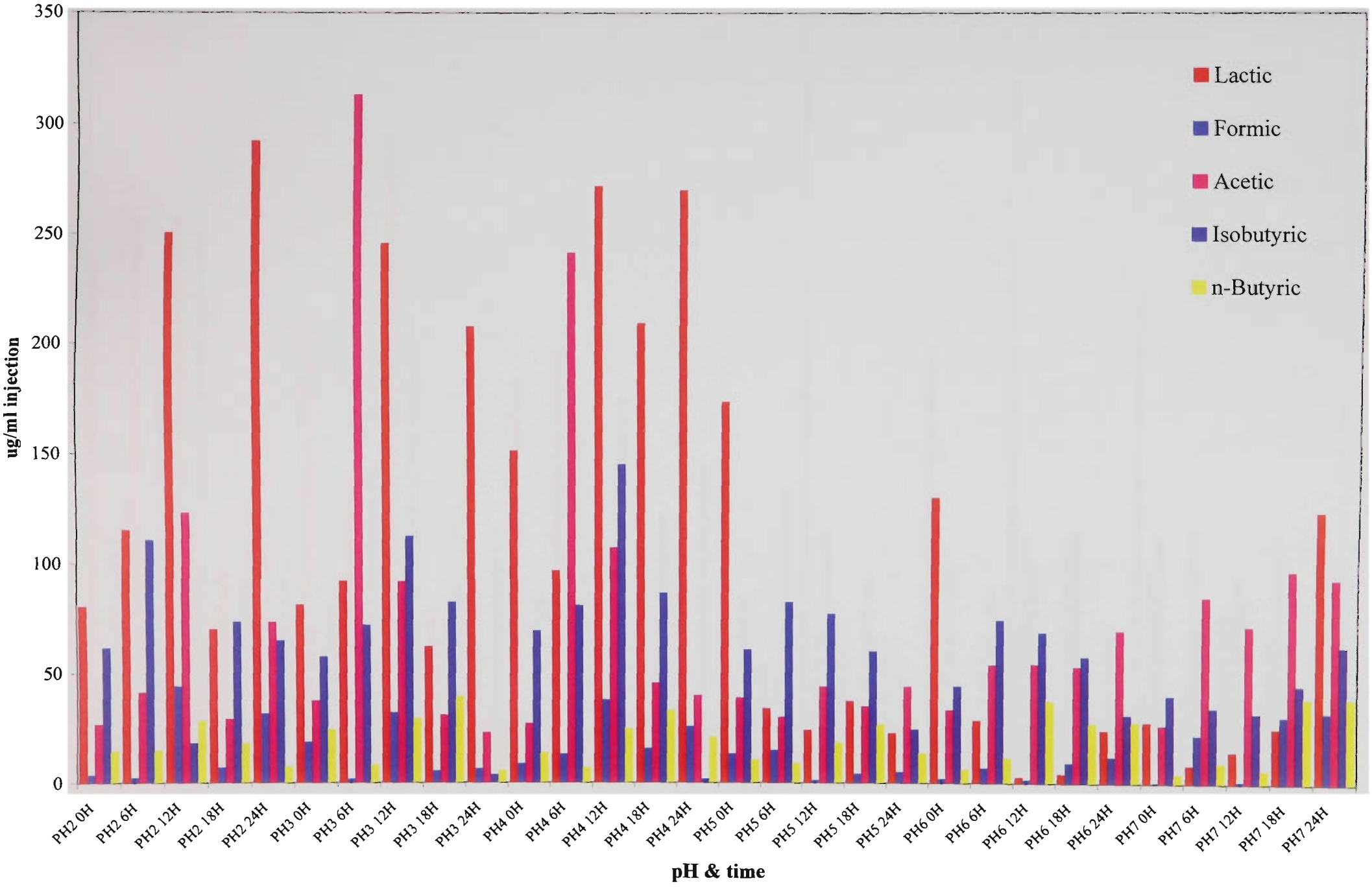

Figure 6.6. Production of organic acids by VRE (I) at different pH over 24 h

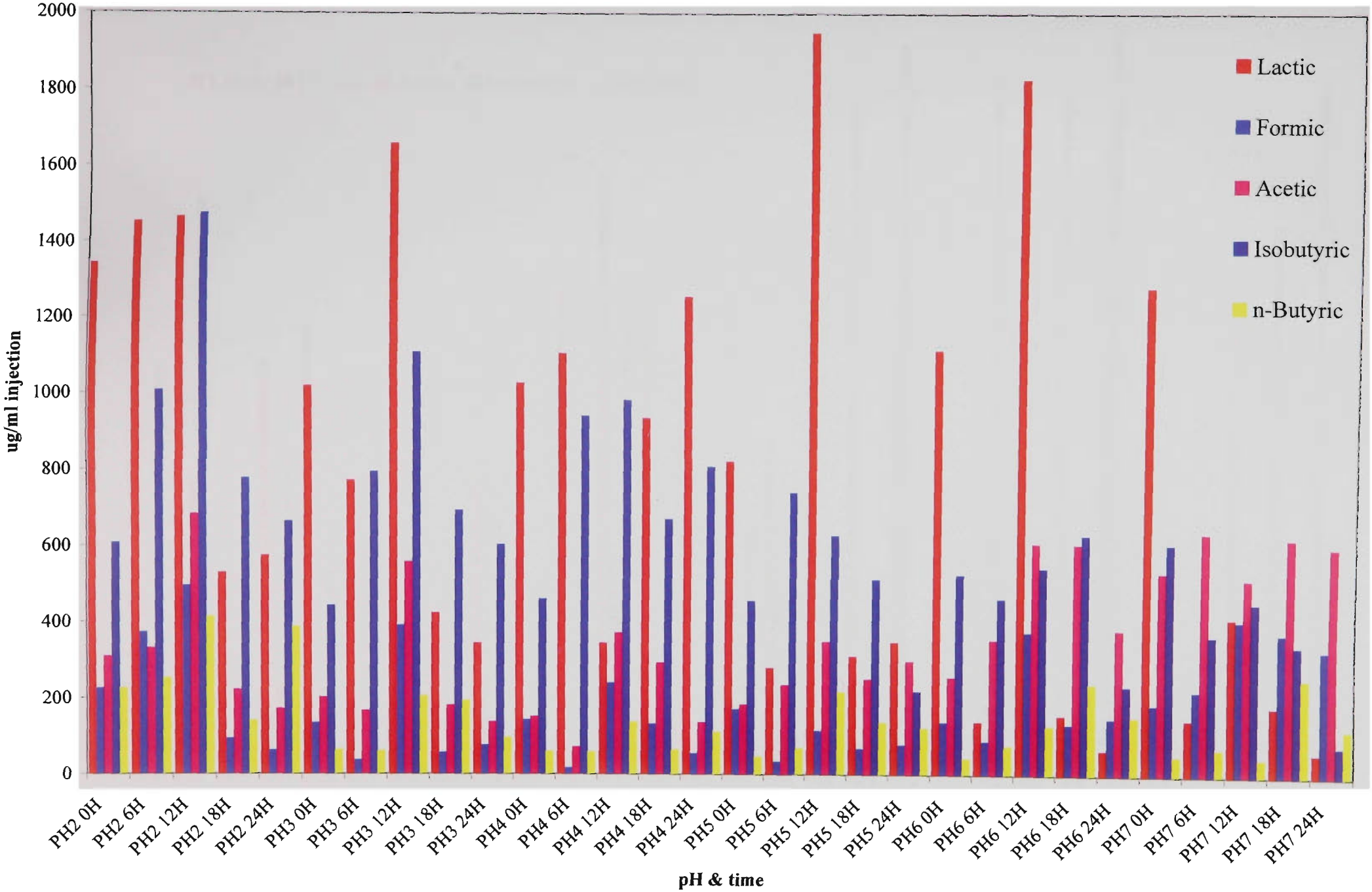

Figure 6.7. Production of organic acids by VRE (M) at different pH over 24 h

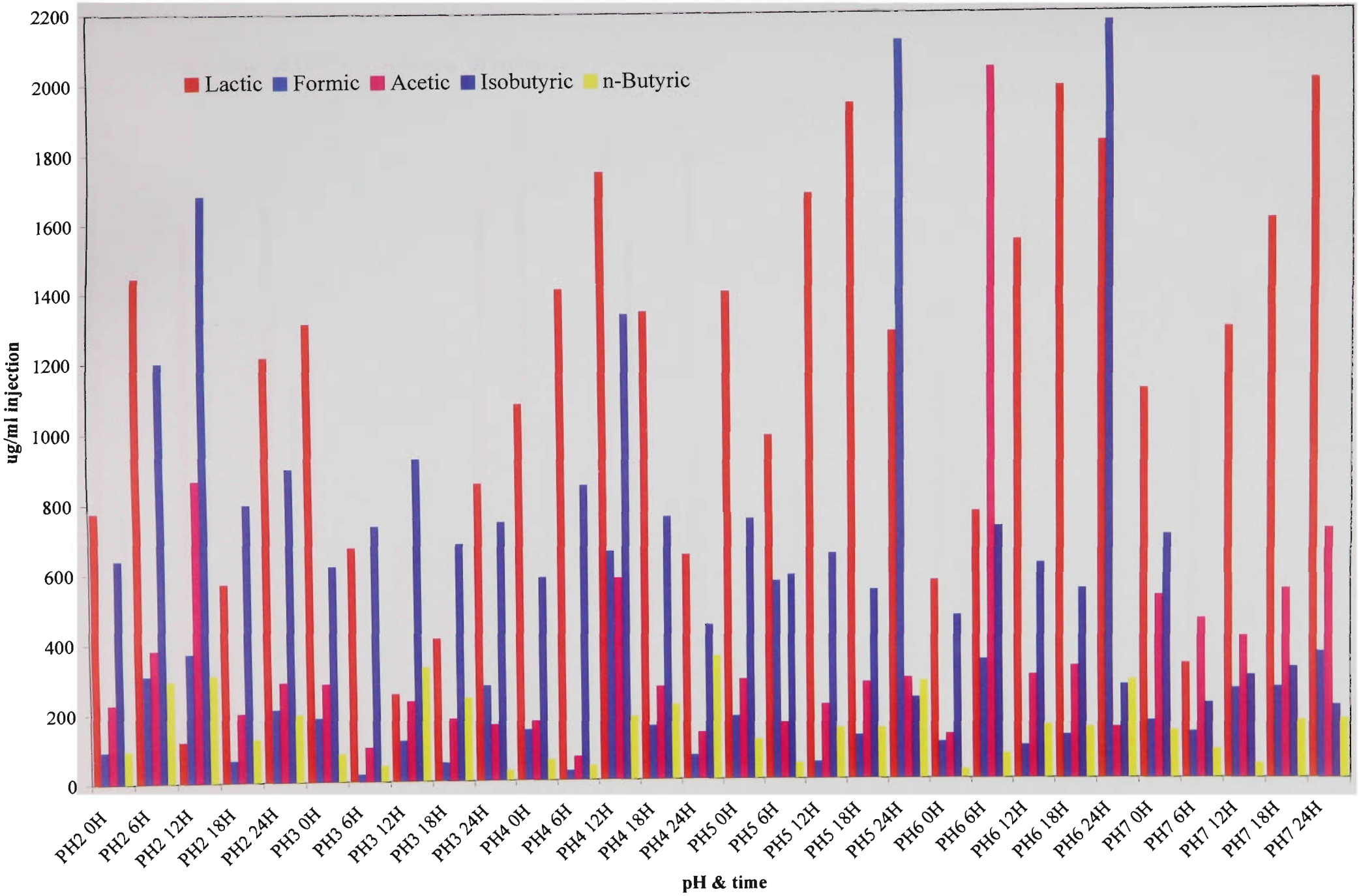

Figure 6.8. Production of organic acids by VRE (N) at different pH over 24 h

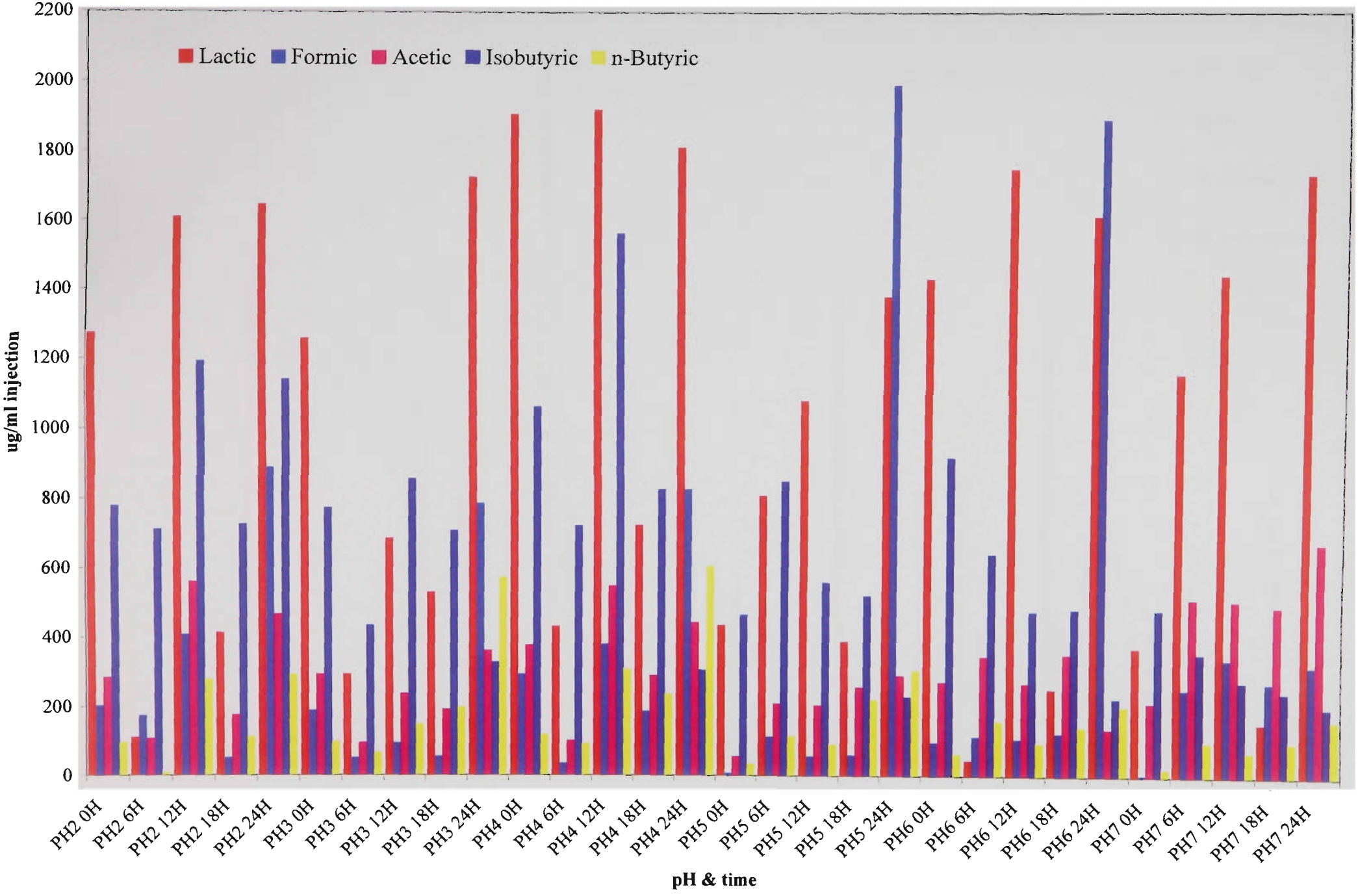

Figure 6.9. Production of organic acids by VRE (O) at different pH over 24 h

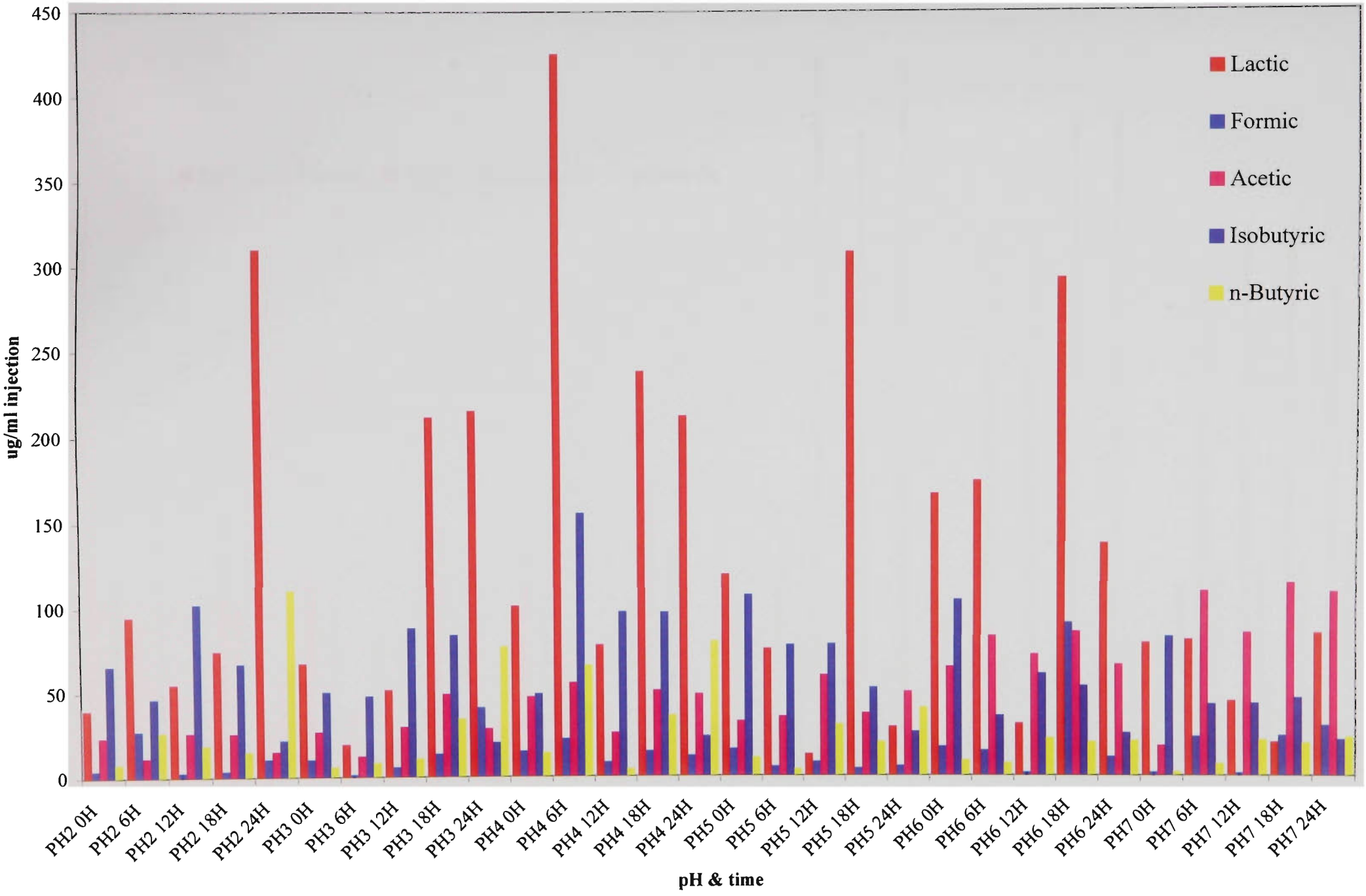

Figure 6.10. Production of organic acids by VRE (R) at different pH over 24 h

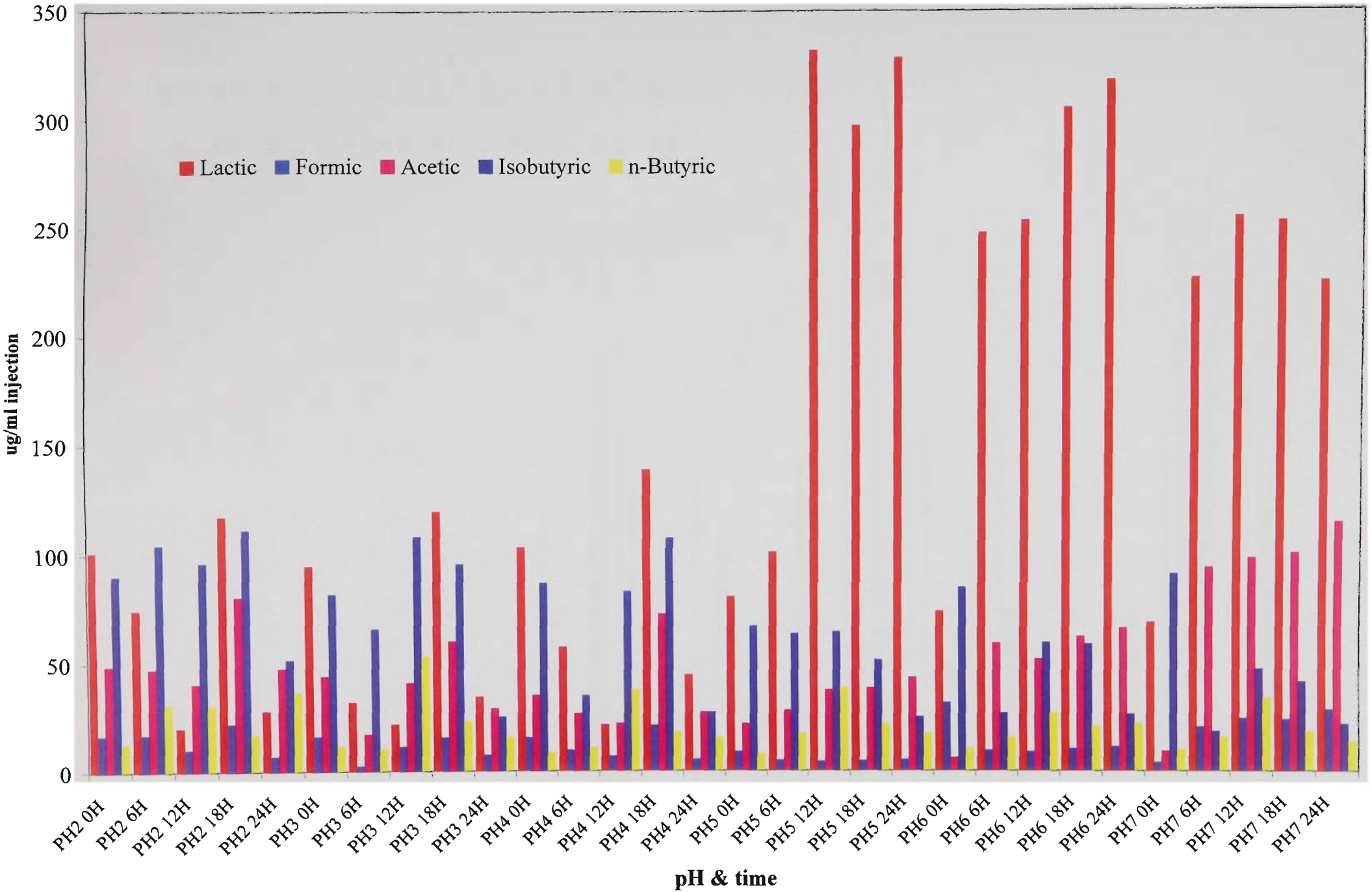

Figure 6.11. Production of organic acids by VRE (S) at different pH over 24 h

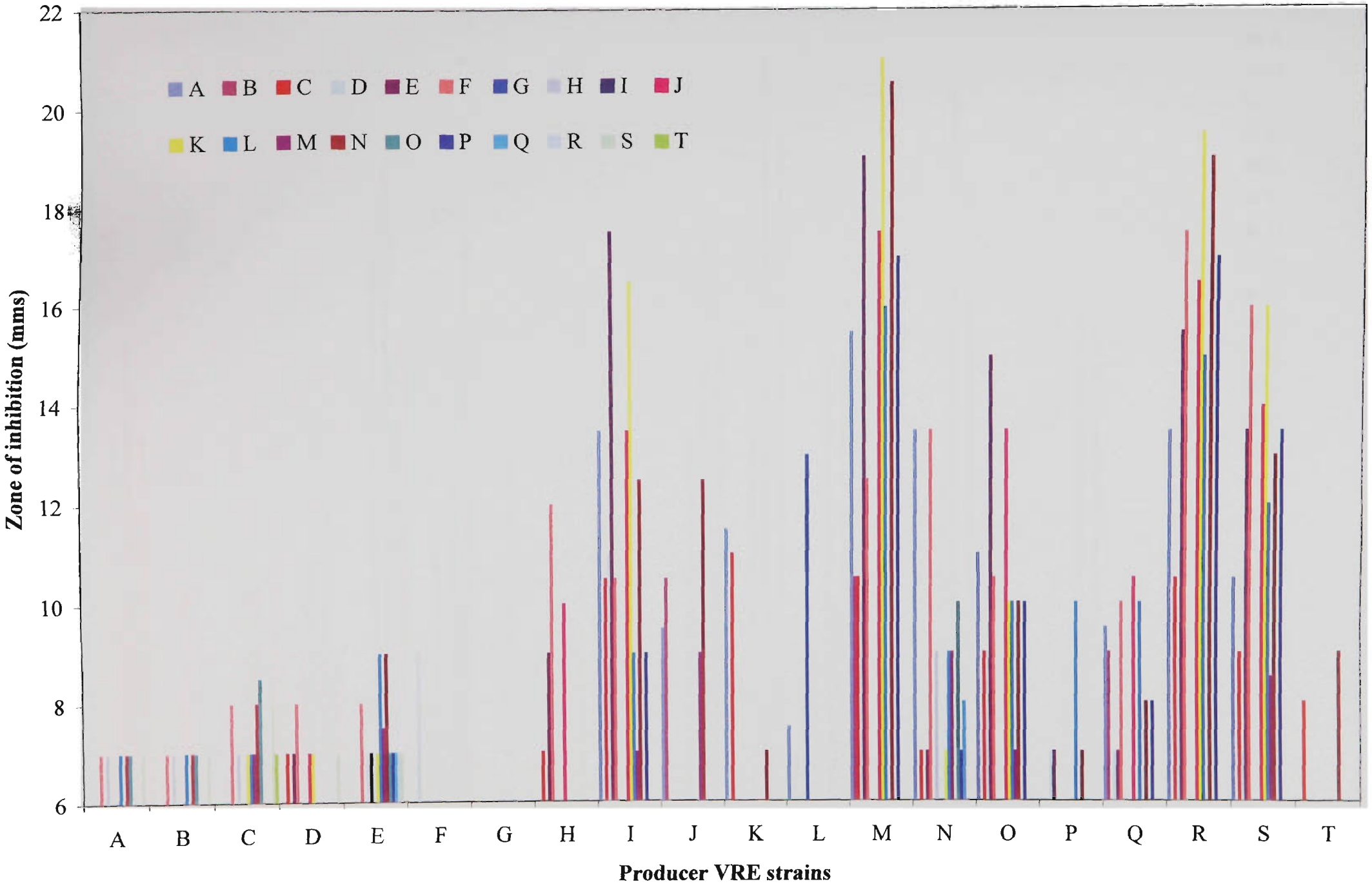

Figure 6.12. Level of inhibition by VRE producer strains versus target VRE strains (Mean values, n = 4)

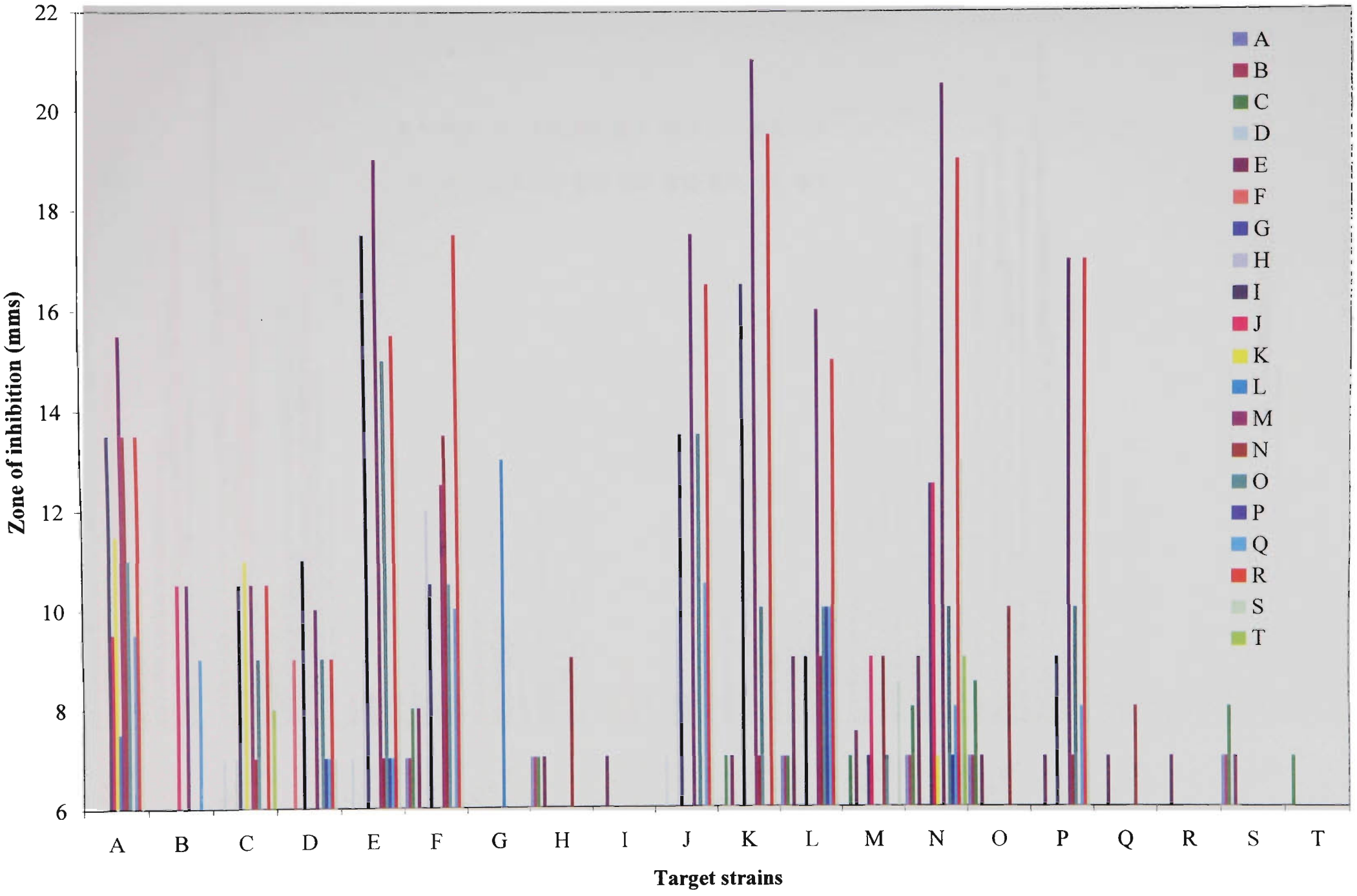

Figure 6.13. Comparison of inhibition of target VRE strains by producer VRE strains (Mean values, n = 4)

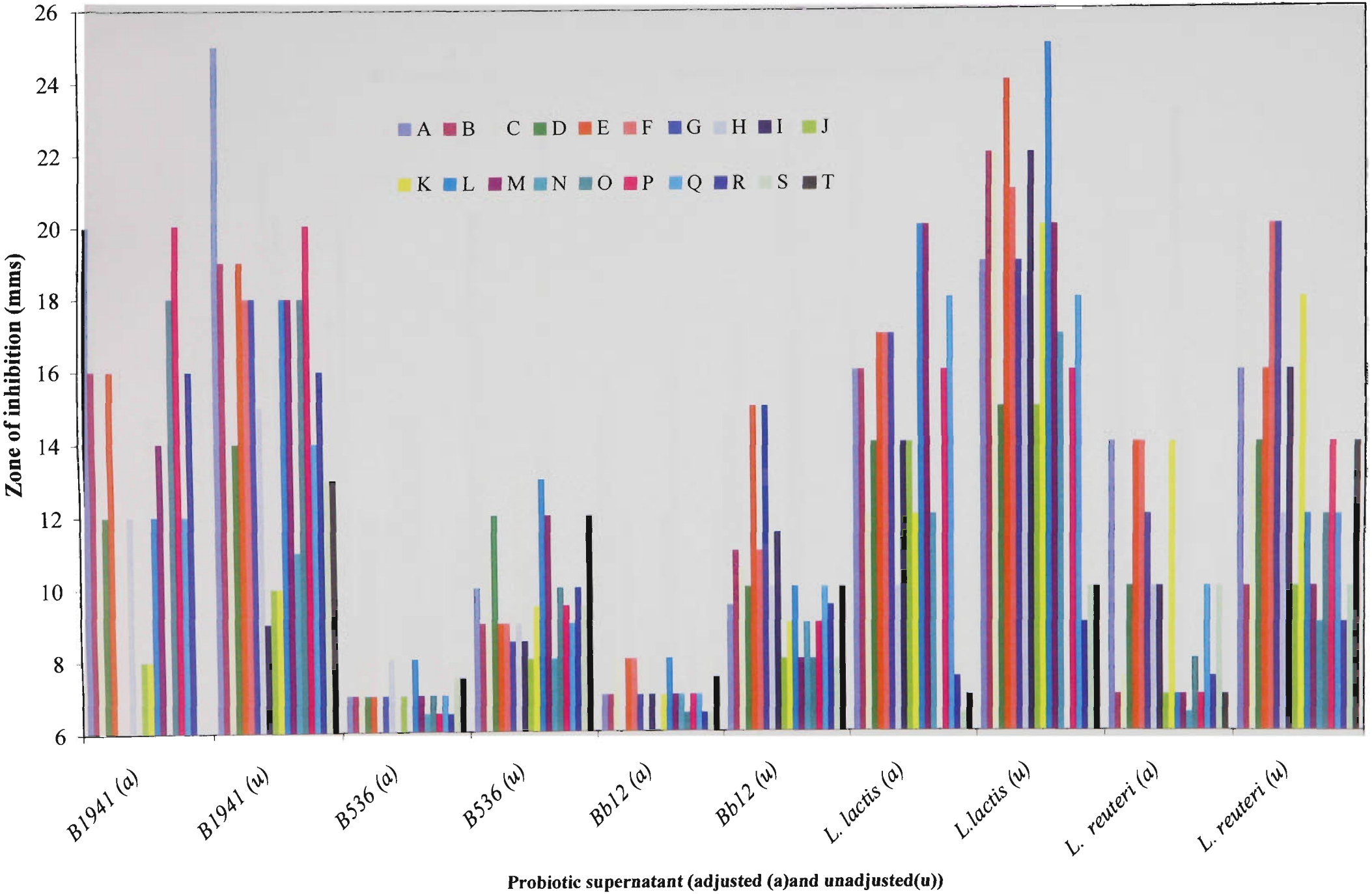

Figure 6.14. Comparison of inhibition against 20 VRE strains using adjusted and unadjusted supernatant of several probiotic bacteria (Mean values, n = 4)

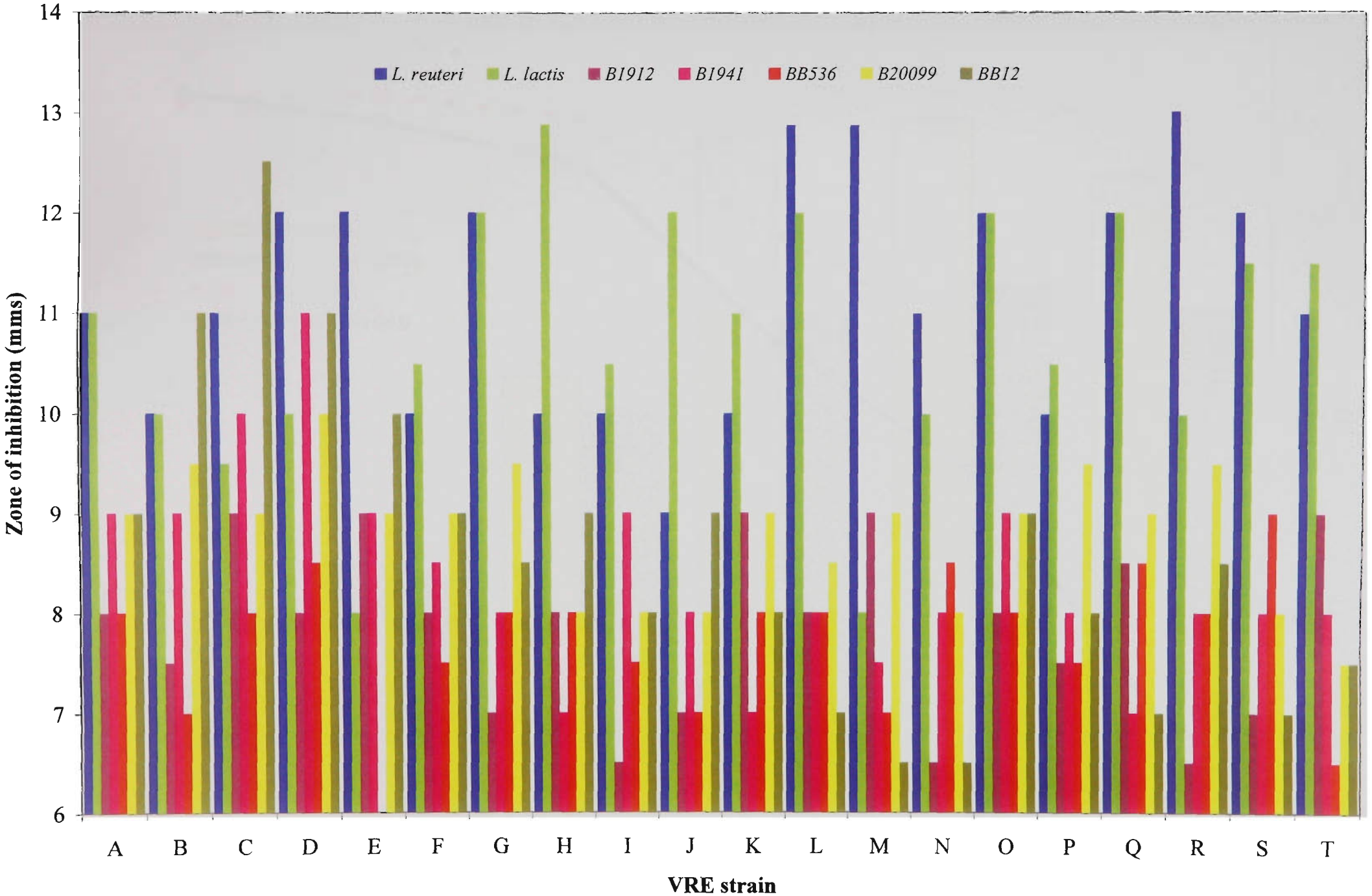

Figure 6.15. Comparison of VRE inhibition using discs soaked in probiotic unadjusted supernatant (Mean values, n = 4)

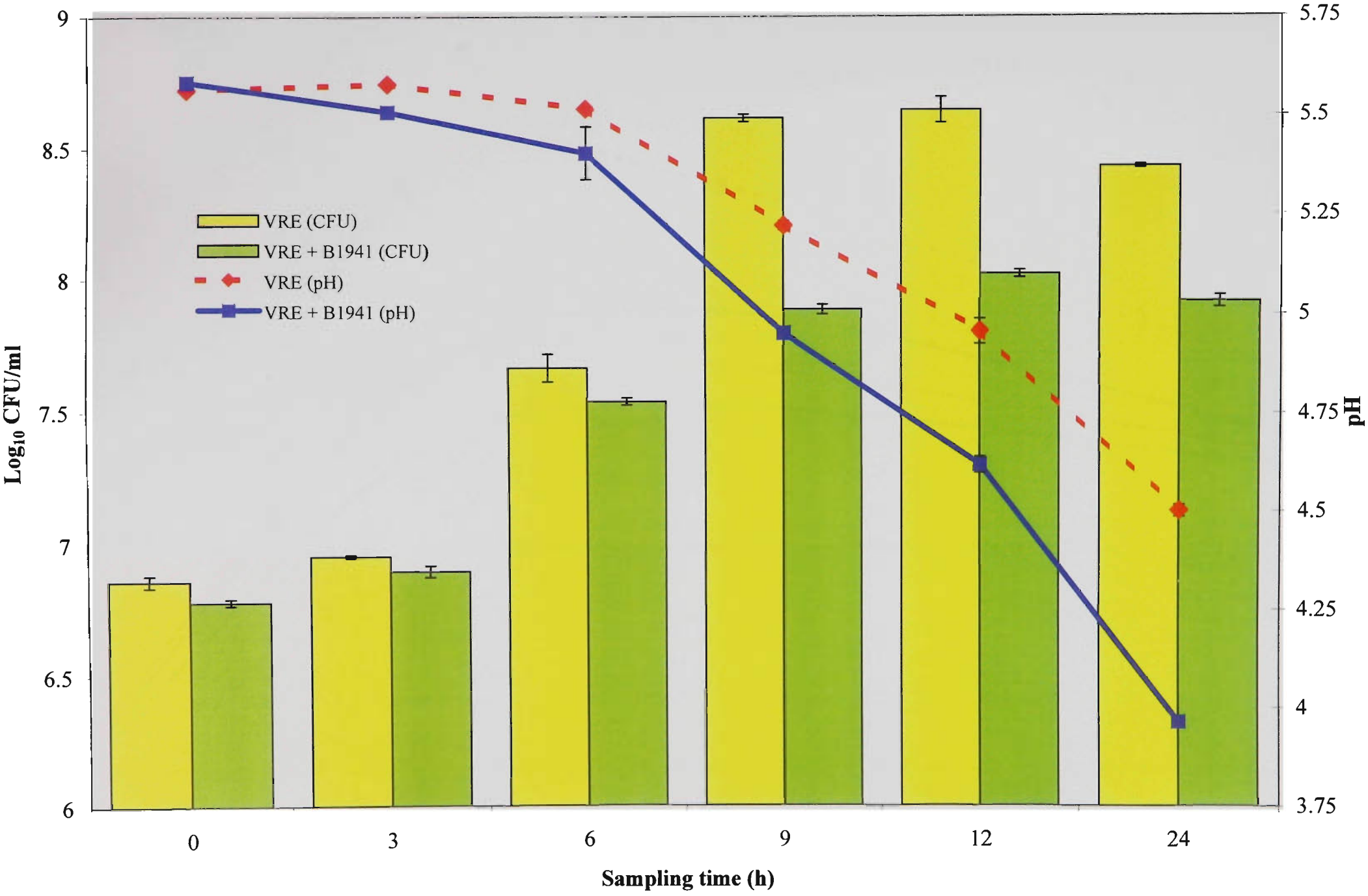

Figure 6.16. Measurement of bacterial counts and pH of VRE with B. longum 1941 over 24 h (Mean ± SE, n = 4)

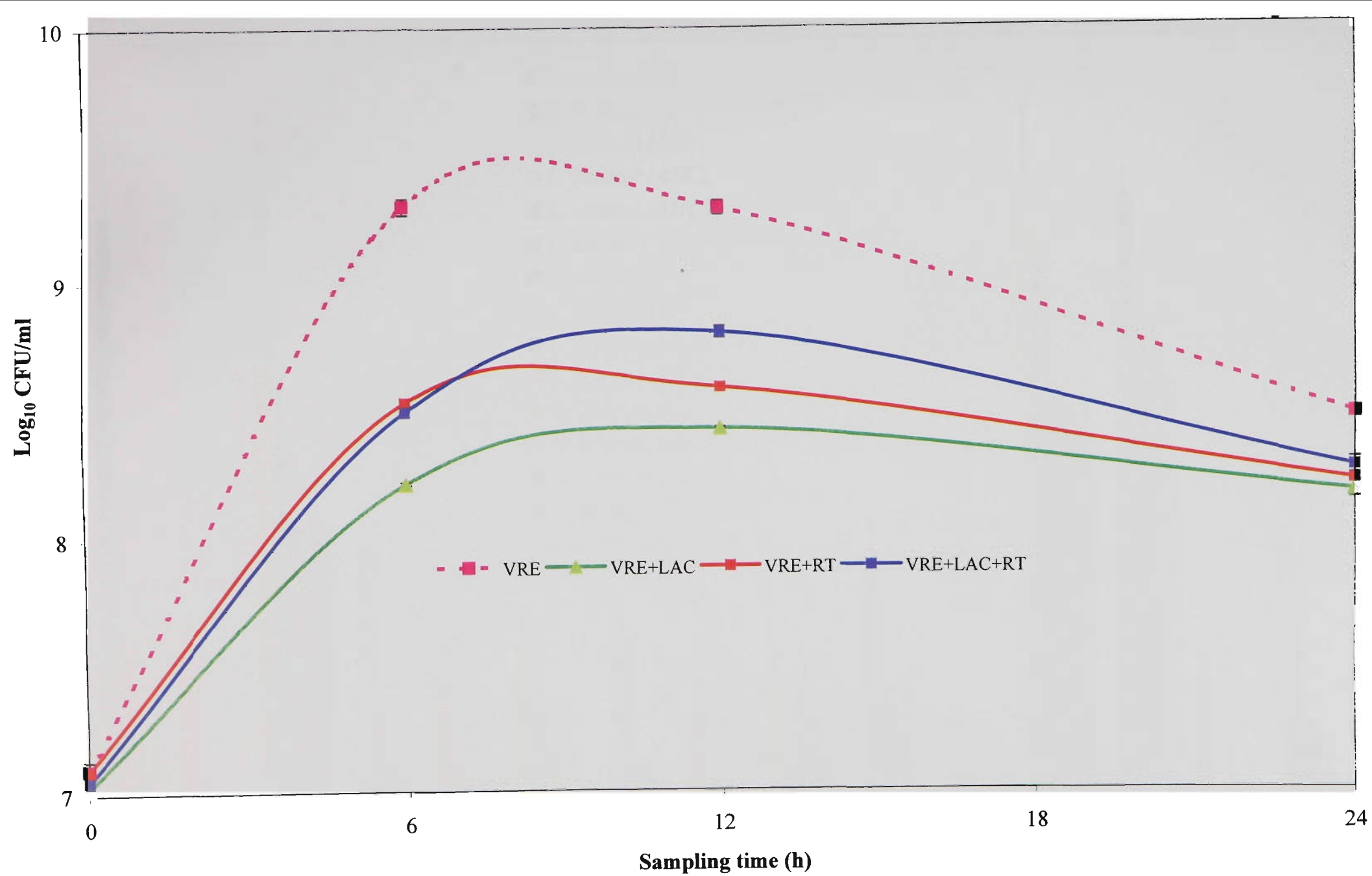

Figure 6.17. Growth curves of VRE with *L. reuteri* and *L. lactis* (Mean values, n = 4)

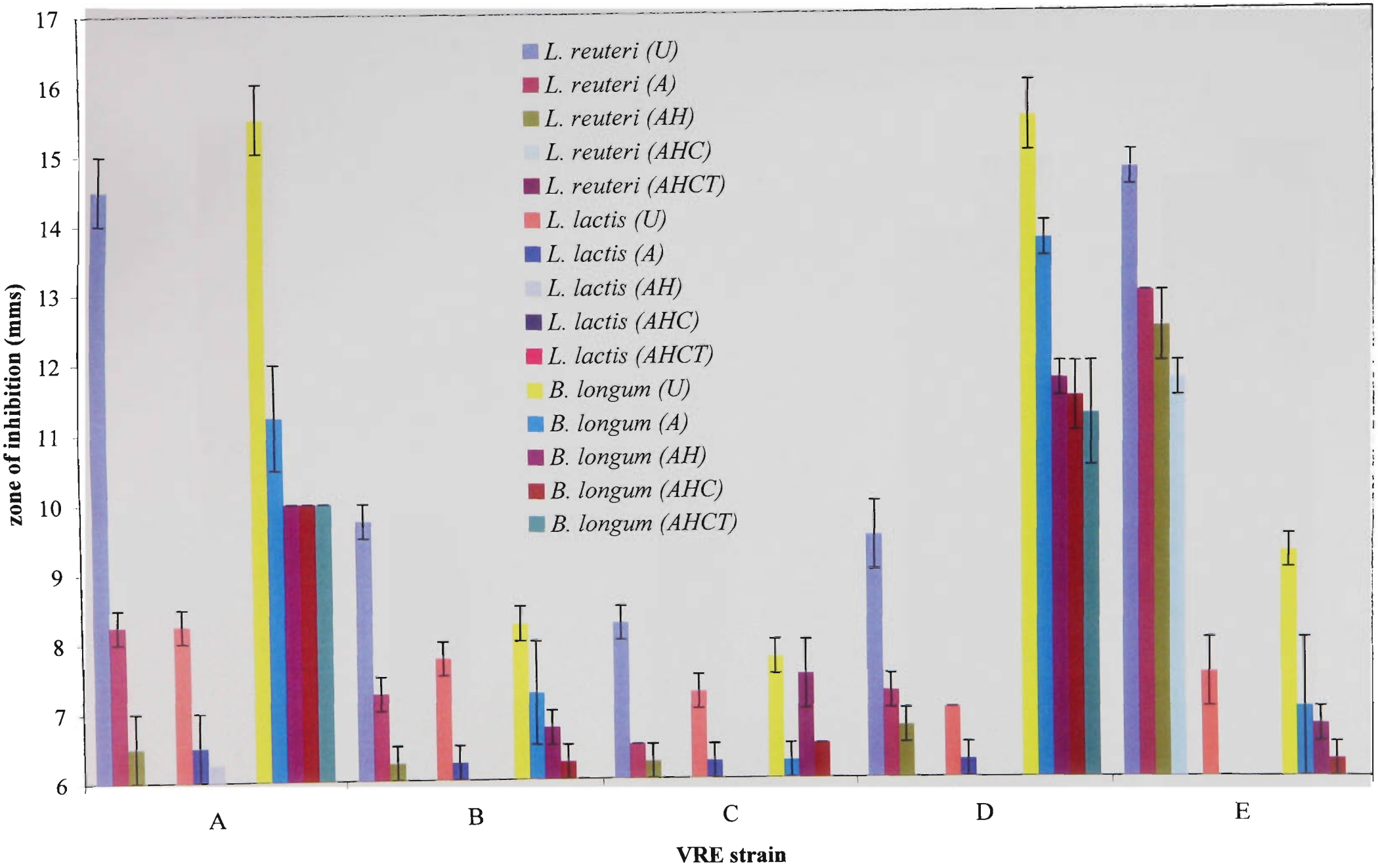

Figure 6.18. Inhibition of 5 VRE strains using probiotic supernatant that has been unadjusted (U), pH adjusted (A), H_2O_2 (H), catalase (C) and trypsin (T) (Mean ± SE, n = 4)

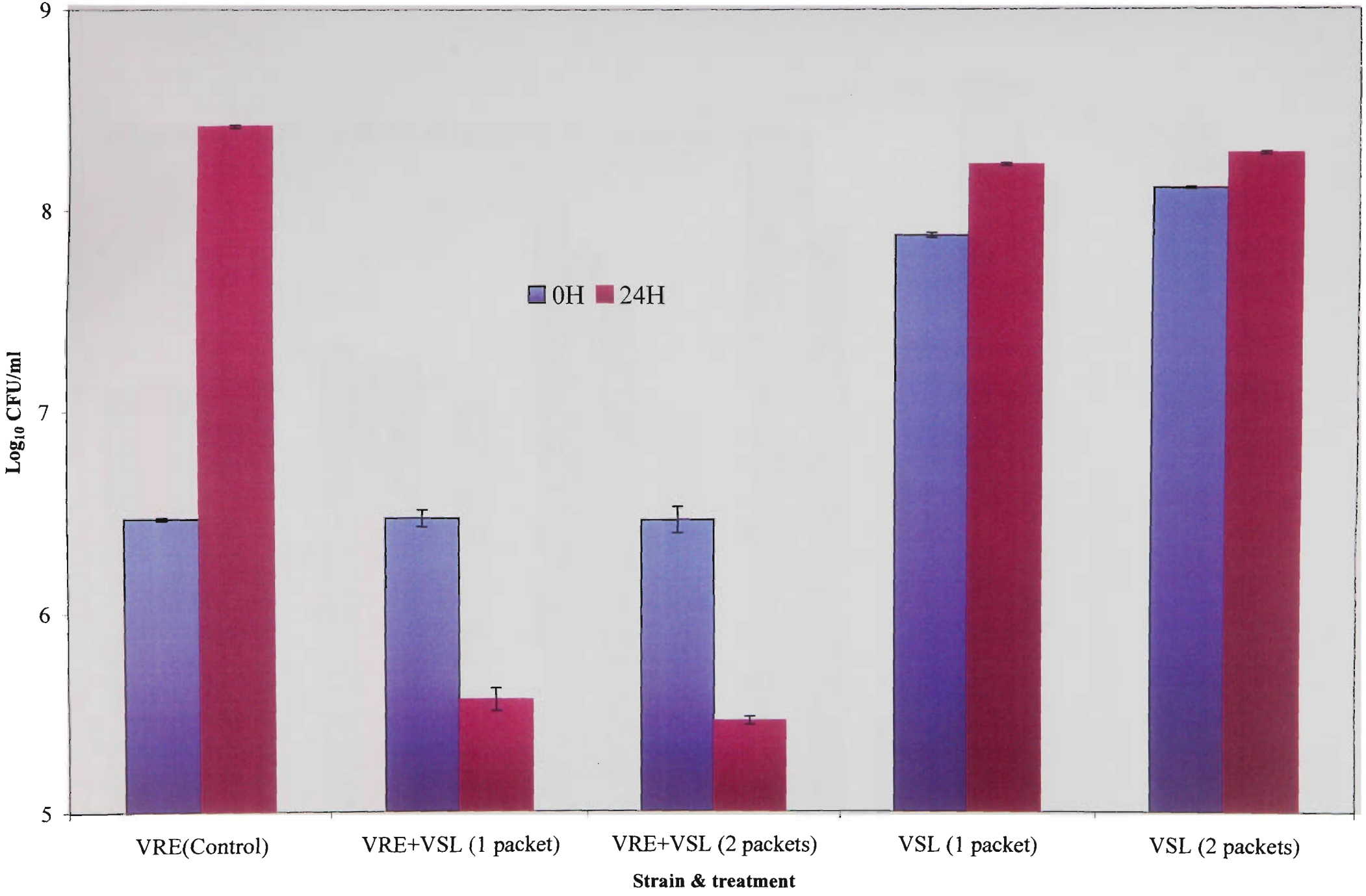

Figure 6.19. Inhibition of VRE (A) by VSL#3^{TM} at 0 and 24 h (Mean ± SE, n = 4)

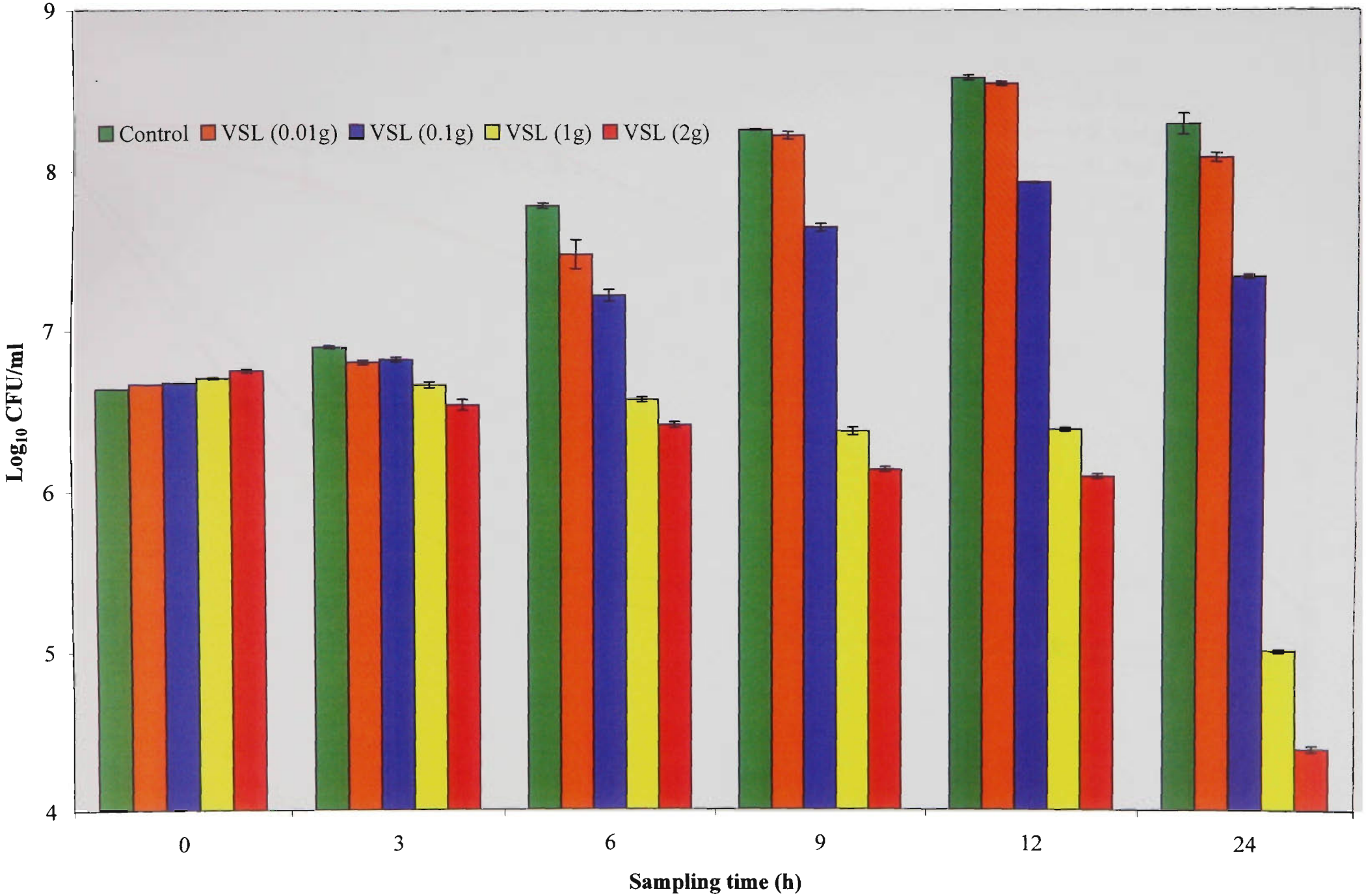

Figure 6.20. Inhibition of VRE (A) using increasing amounts of VSL#3 incubated for 24 h (Mean ± SE, n = 4)

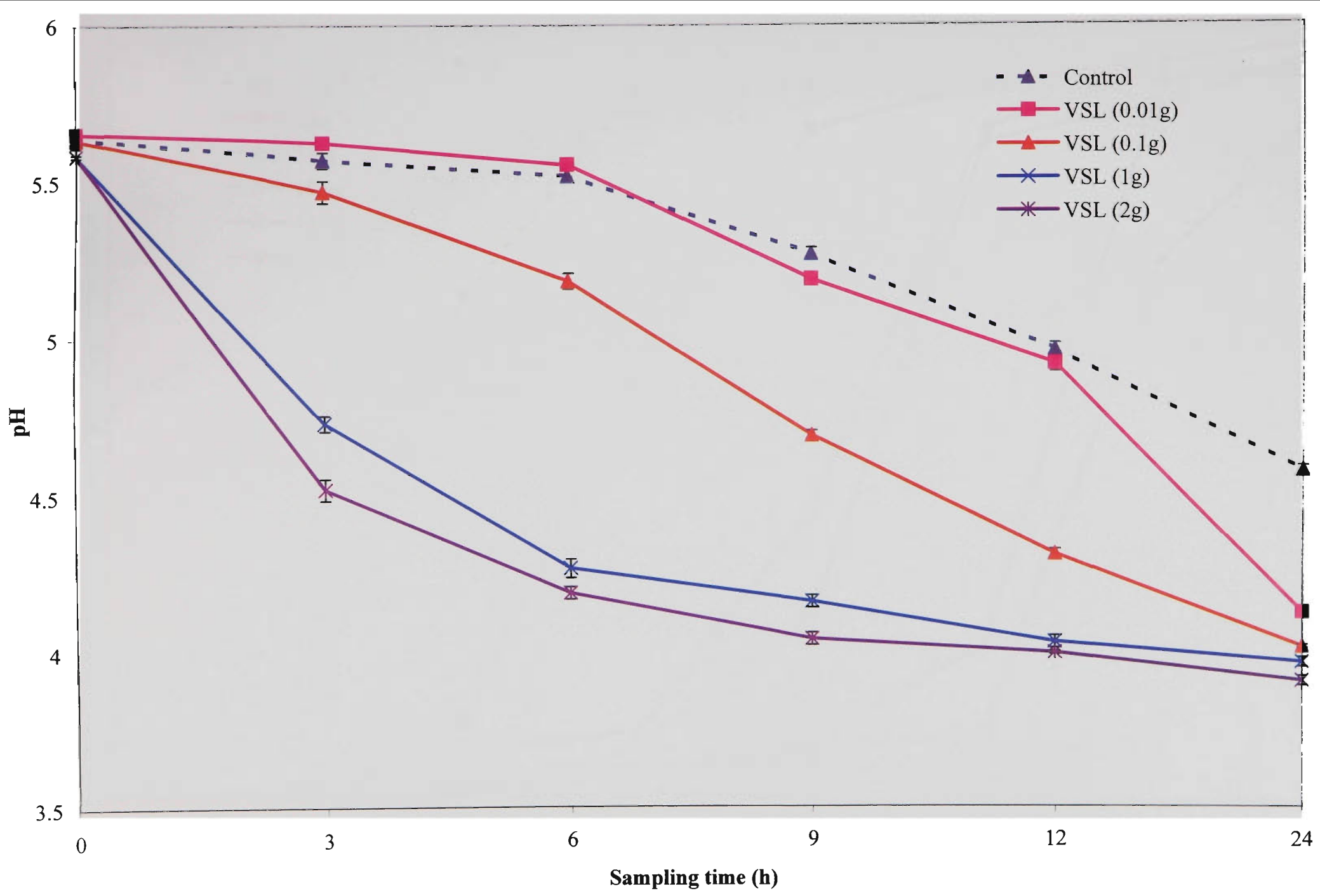

Figure 6.21. PH of media when increasing amounts of VSL added over 24 h (Mean ± SE, n = 4)

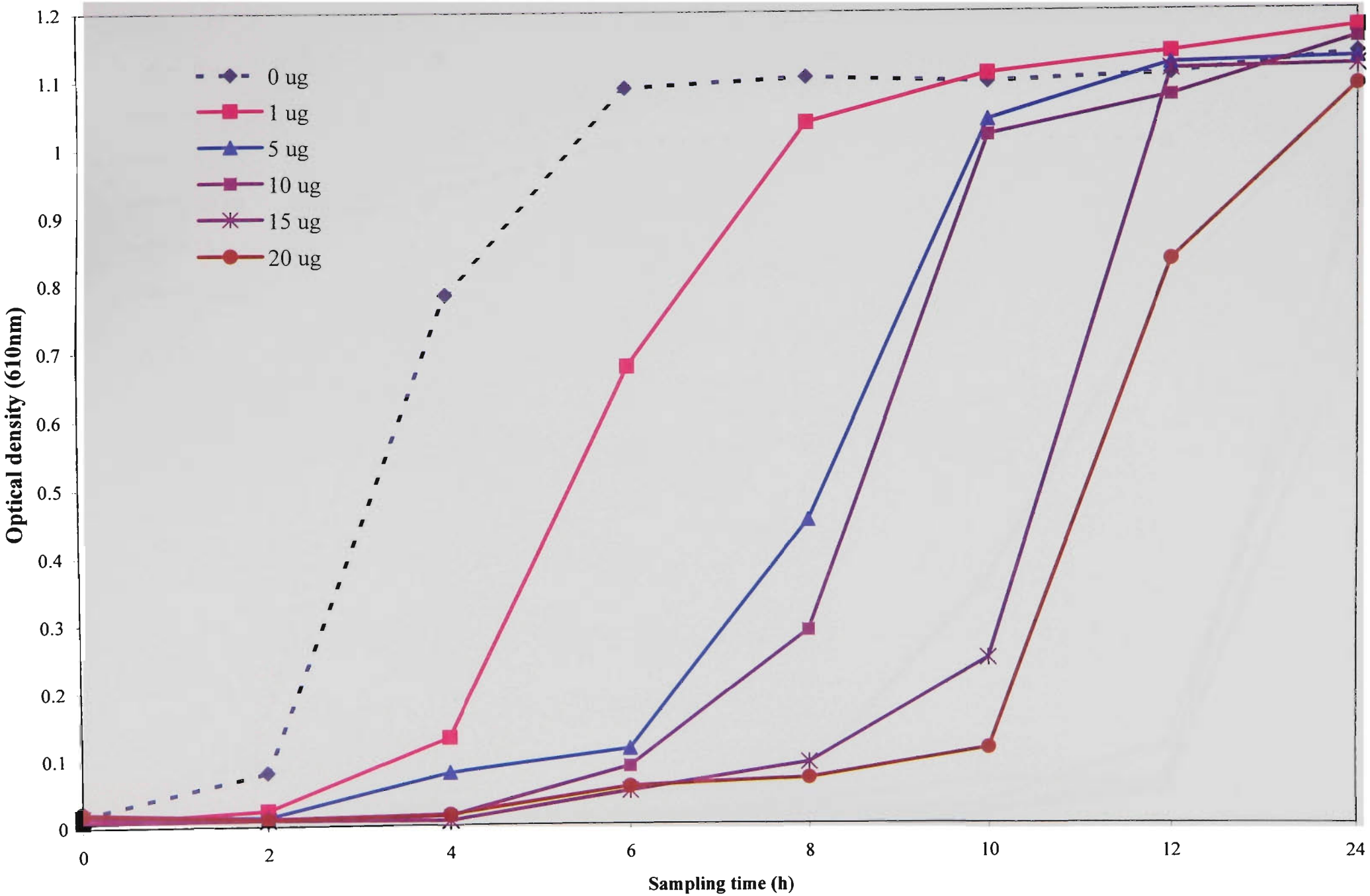

Figure 6.22: Optical density of VRE (A) subjected to increasing concentrations of nisin (ug/ml) (Mean values, n = 3)

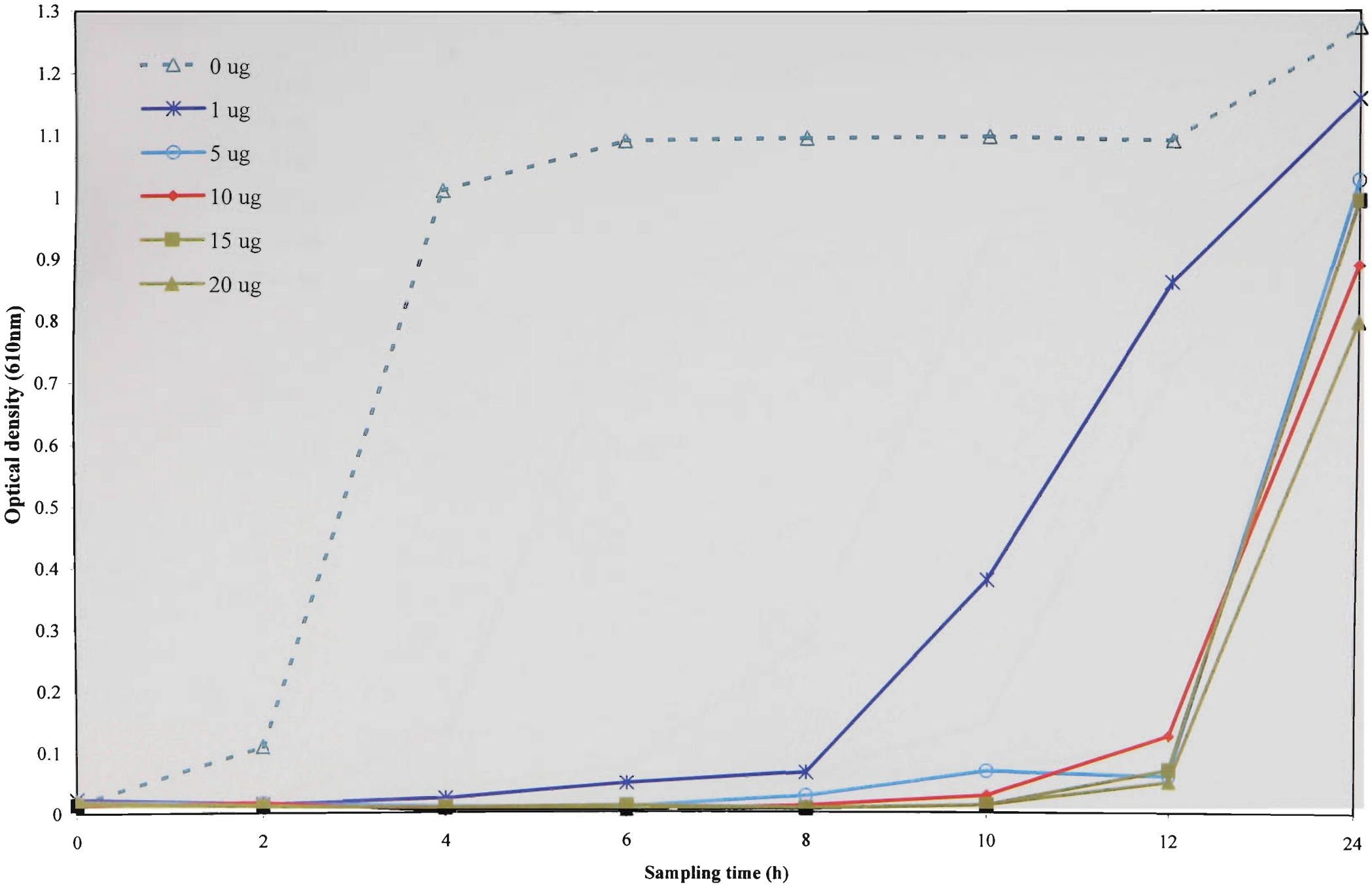

Figure 6.23: Optical density of VRE (C) subjected to increasing concentrations of nisin (ug/ml) (Mean values, n = 3)

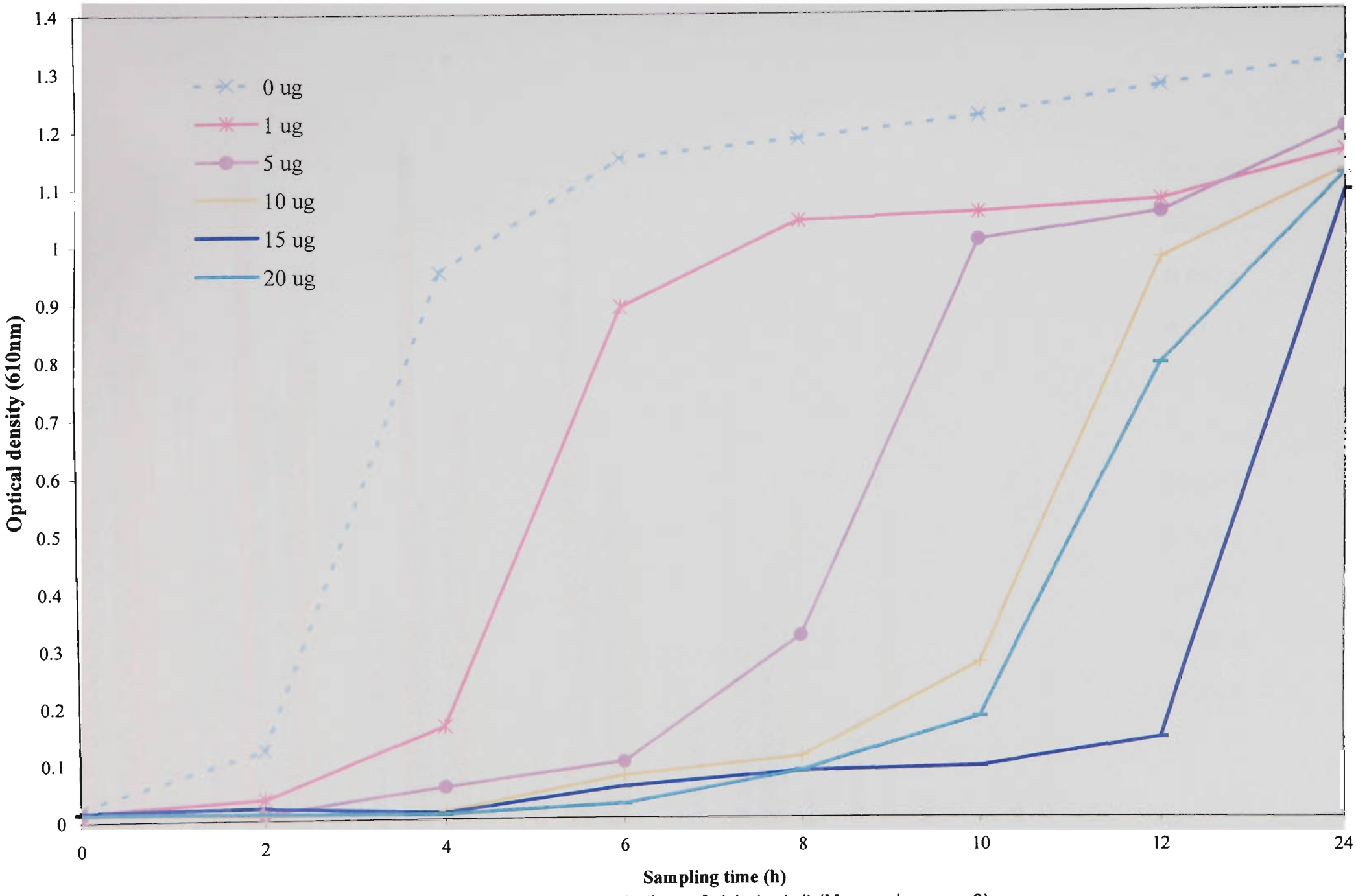

Figure 6.24: Optical density of VRE (E) subjected to increasing concentrations of nisin (ug/ml) (Mean values, n = 3)

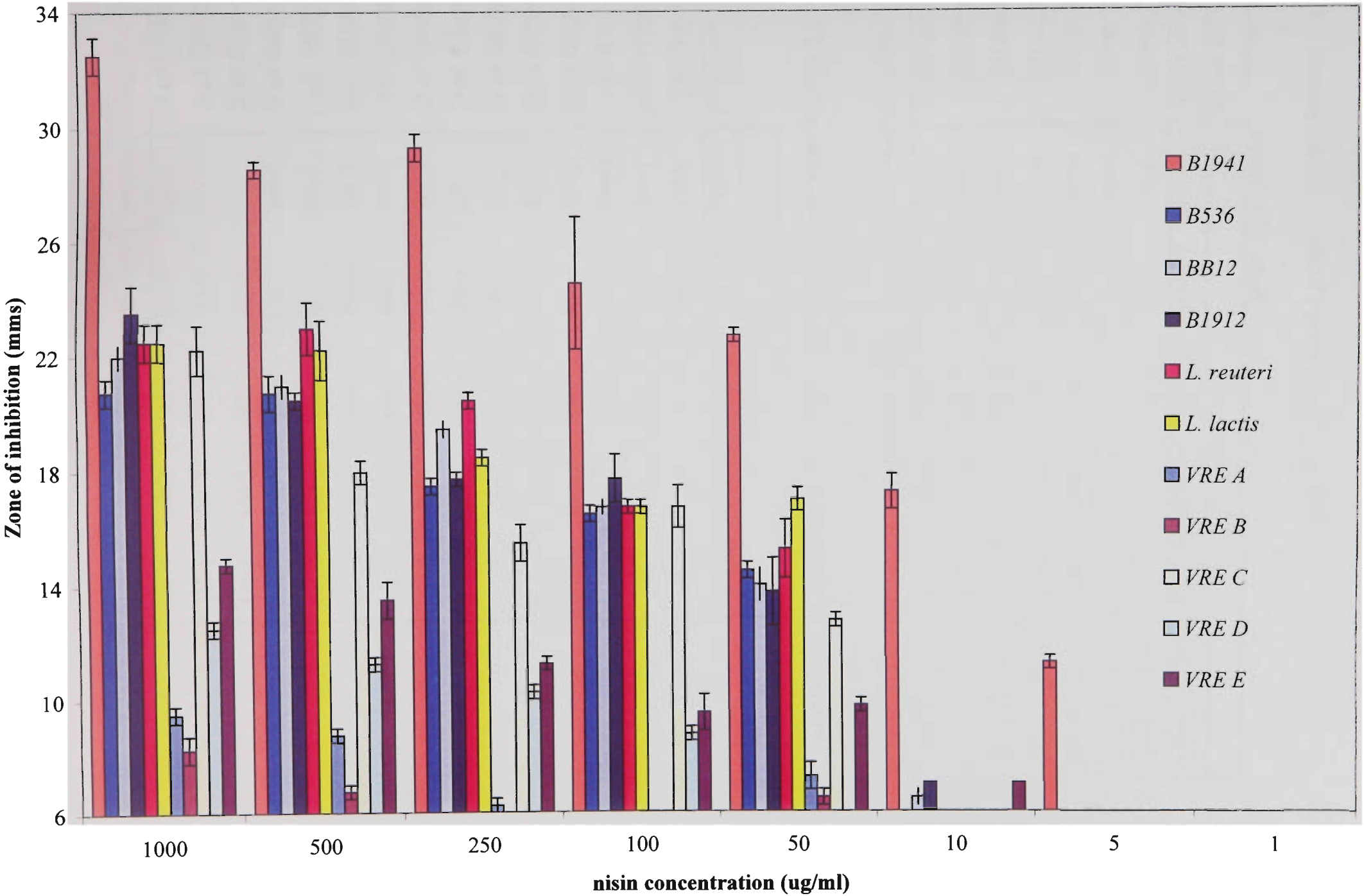

Figure 6.25. Inhibition of probiotic bacteria and VRE strains using different concentrations of nisin using well diffusion inhibition tests (Mean ± SE, n = 4)

Table 6.1. Overlay inhibition tests of 2 probiotic and 5 VRE strains

Probiotic producer strain	**Target VRE strain**						
	Overlay strain						
Producer strain	***L. reuteri***	***L. lactis***	***VRE(A)***	***VRE(B)***	***VRE(C)***	***VRE(D)***	***VRE(E)***
L. reuteri	NA	(-ve)	(-ve)	(-ve)	(-ve)	(-ve)	(-ve)
L. lactis	(-ve)	NA	(-ve)	(-ve)	(-ve)	(-ve)	(-ve)
VRE (A)	(+ve)	(+ve)	NA	(+ve)	(-ve)	(+ve)	(-ve)
VRE (B)	(+ve)	(+ve)	(+ve)	NA	(+ve)	(+ve)	(-ve)
VRE (C)	(+ve)	(+ve)	(-ve)	(-ve)	NA	(+ve)	(-ve)
VRE (D)	(+ve)	(-ve)	(-ve)	(-ve)	(-ve)	NA	(-ve)
VRE (E)	(+ve)	(-ve)	(-ve)	(-ve)	(-ve)	(-ve)	NA

(+ve) = inhibition observed, (-ve) = inhibition not observed

Table 6.2. Overlay inhibition tests of 5 VRE strains at different incubation temperatures

	Overlay strain				
Producer strain	***VRE(A)***	***VRE(B)***	***VRE(C)***	***VRE(D)***	***VRE(E)***
VRE(A) 30 C	(-ve)	(-ve)	(-ve)	(-ve)	(-ve)
VRE(A) 37 C	(-ve)	(-ve)	(-ve)	(-ve)	(-ve)
VRE(A) 45 C	(-ve)	(-ve)	(-ve)	(-ve)	(-ve)
VRE(B) 30 C	(-ve)	(-ve)	(-ve)	(-ve)	(-ve)
VRE(B) 37 C	(-ve)	(-ve)	(-ve)	(-ve)	(-ve)
VRE(B) 45 C	(-ve)	(-ve)	(-ve)	(-ve)	(-ve)
VRE(C) 30 C	(-ve)	(-ve)	(-ve)	(-ve)	(-ve)
VRE(C) 37 C	(-ve)	(-ve)	(-ve)	(-ve)	(-ve)
VRE(C) 45 C	(-ve)	(-ve)	(-ve)	(-ve)	(-ve)
VRE(D) 30 C	(-ve)	(-ve)	(-ve)	(-ve)	(-ve)
VRE(D) 37 C	(-ve)	(-ve)	(-ve)	(-ve)	(-ve)
VRE(D) 45 C	(-ve)	(-ve)	(-ve)	(-ve)	(-ve)
VRE(E) 30 C	(-ve)	(-ve)	(-ve)	(-ve)	(-ve)
VRE(E) 37 C	(-ve)	(-ve)	(-ve)	(-ve)	(-ve)
VRE(E) 45 C	(-ve)	(-ve)	(-ve)	(-ve)	(-ve)

(+ve) = inhibition observed, (-ve) = inhibition not observed

Table 6.3. Spot on lawn inhibition test using 3 probiotic strain supernatant against 5 VRE strains

		Supernatant dilution								
Probiotic producer strain	Target VRE strain	1×10^{-0}	1×10^{-1}	1×10^{-2}	1×10^{-3}	1×10^{-4}	1×10^{-5}	1×10^{-6}	1×10^{-7}	1×10^{-8}
L. reuteri	*A*	non-obs	non-obs	non-obs	non-obs	non-obs	non-obs	non-obs	non-obs	non-obs
L. reuteri	*B*	non-obs	non-obs	non-obs	non-obs	non-obs	non-obs	non-obs	non-obs	non-obs
L. reuteri	*C*	non-obs	non-obs	non-obs	non-obs	non-obs	non-obs	non-obs	non-obs	non-obs
L. reuteri	*D*	non-obs	non-obs	non-obs	non-obs	non-obs	non-obs	non-obs	non-obs	non-obs
L. reuteri	*E*	non-obs	non-obs	non-obs	non-obs	non-obs	non-obs	non-obs	non-obs	non-obs
L. lactis	*A*	non-obs	non-obs	non-obs	non-obs	non-obs	non-obs	non-obs	non-obs	non-obs
L. lactis	*B*	non-obs	non-obs	non-obs	non-obs	non-obs	non-obs	non-obs	non-obs	non-obs
L. lactis	*C*	non-obs	non-obs	non-obs	non-obs	non-obs	non-obs	non-obs	non-obs	non-obs
L. lactis	*D*	non-obs	non-obs	non-obs	non-obs	non-obs	non-obs	non-obs	non-obs	non-obs
L. lactis	*E*	non-obs	non-obs	non-obs	non-obs	non-obs	non-obs	non-obs	non-obs	non-obs
B. longum	*A*	non-obs	non-obs	non-obs	non-obs	non-obs	non-obs	non-obs	non-obs	non-obs
B. longum	*B*	non-obs	non-obs	non-obs	non-obs	non-obs	non-obs	non-obs	non-obs	non-obs
B. longum	*C*	non-obs	non-obs	non-obs	non-obs	non-obs	non-obs	non-obs	non-obs	non-obs
B. longum	*D*	non-obs	non-obs	non-obs	non-obs	non-obs	non-obs	non-obs	non-obs	non-obs
B. longum	*E*	non-obs	non-obs	non-obs	non-obs	non-obs	non-obs	non-obs	non-obs	non-obs

non-obs = no zones of inhibition were observed after 24 h incubation

Table 6.4. Inhibition of 5 VRE strains using concentrated supernatant of *L. lactis* and *L. reuteri*

		Permeate			Retentate		
Probiotic strain	Target VRE	Control	<10Kda	<10Kda(pH 6)	Control	>10KDa	>10KDa(pH 6)
L. reuteri	A	(-ve)	(-ve)	(-ve)	(-ve)	(-ve)	(-ve)
L. reuteri	B	(-ve)	(-ve)	(-ve)	(-ve)	(-ve)	(-ve)
L. reuteri	C	(-ve)	(-ve)	(-ve)	(-ve)	(-ve)	(-ve)
L. reuteri	D	(-ve)	(-ve)	(-ve)	(-ve)	(-ve)	(-ve)
L. reuteri	E	(-ve)	(-ve)	(-ve)	(-ve)	(-ve)	(-ve)
L. lactis	A	(-ve)	(-ve)	(-ve)	(-ve)	(-ve)	(-ve)
L. lactis	B	(-ve)	(-ve)	(-ve)	(-ve)	(-ve)	(-ve)
L. lactis	C	(-ve)	(-ve)	(-ve)	(-ve)	(-ve)	(-ve)
L. lactis	D	(-ve)	(-ve)	(-ve)	(-ve)	(-ve)	(-ve)
L. lactis	E	(-ve)	(-ve)	(-ve)	(-ve)	(-ve)	(-ve)

(-ve) = no Inhibition observed using well diffusion tests

Table 6.5. Inhibition of VRE (A) using serially diluted concetrated retentate of *L. reuteri* supernatant

		Dilution					
Probiotic strain	pH	1×10^{-2}	1×10^{-3}	1×10^{-4}	1×10^{-5}	1×10^{-6}	Inhibition
L. reuteri	6	(-ve)	(-ve)	(-ve)	(-ve)	(-ve)	non-obs
L. reuteri	4.51	(-ve)	(-ve)	(-ve)	(-ve)	(-ve)	non-obs

(-ve)= no inhibition was observed for any well diffusion test

6.5. Conclusion

The inhibitory ability of probiotic bacteria especially bifidobacteria towards vancomycin-resistant enterococci (VRE) has important clinical implications. The inhibition of VRE strains was observed by VRE and probiotic supernatant using well-diffusion tests, co-culturing with live probiotic cultures and dose-dependent freeze-dried probiotic preparations (VSL#3™), modification of medium pH (especially low pH) and dose-dependent addition of nisin (5-1000μg/ml).

However, VRE shows rapid adaptation to bacteriocin (nisin), but not to pH adjusted growth media and during co-culturing with probiotic strains. These observations suggest that probiotic-induced suppression of VRE *in vivo* might be further enhanced by additional approaches to lowering gastrointestinal pH (such as co-ingestion of fermentable substrates). In addition, a greater collection of probiotic strains need to be further assessed and critically evaluated by clinical research on patients colonised and infected with VRE.

7.0. OVERALL CONCLUSIONS

7.1. Inhibition of pathogenic bacteria by *Bifidobacterium* spp

Bifidobacterium infantis 1912, *B. longum* 1941, *B. longum* BB536 and *B. pseudolongum* 20099 were able to inhibit eleven distinctive strains of pathogenic and putrefactive microorganisms as measured by well-diffusion inhibition assays and during growth curves with supernatants obtained from bifidobacteria. The inhibition of pathogenic and putrefactive bacteria by the *Bifidobacterium* spp. was due to the lowering of the pH (pH 3.83 to 4.04) of the supernatant obtained from the growth medium. However, after the pH of the supernatant was adjusted to pH 6.0 using 5N NaOH, the inhibition of pathogenic bacterial growth and zones of inhibition were minimal. The antimicrobial effects of *Bifidobacterium* were generally attributed to the decrease of pH resulting from production of variable concentrations of lactic and acetic acids produced as a result of fermentation of glucose. The measured acetic acid production from *Bifidobacterium* spp. ranged from 55.4 to 39.8 mM, while lactic acid concentrations ranged from 33.2 to 17.1 mM.

7.2. Selective enumeration of *Bifidobacterium* spp

MRS agar containing filter-sterilised 0.05% L-cysteine·hydrochloride and NNLP solution was suitable for the selective enumeration of *Bifidobacterium* spp. and in co-culturing experiments with pathogenic bacteria. However, the preparation of NNLP medium requires considerable time and numerous ingredients.

7.3. Growth, viability and activity of *Bifidobacterium* spp. in skim milk containing prebiotics

The growth, activity and viability of five strains of bifidobacteria in skim milk were dependent on the prebiotic present as well as the strain of *Bifidobacterium*. Doubling time was significantly decreased with the addition of prebiotics lactulose, raftilose and inulin, although Hi-maize™ gave higher doubling times than the control. This was due to Hi-maize™ being a highly cross-linked starch requiring the action of more enzymes to break down as compared to non-cross linked raftilose and inulin.

Fermentation of the four prebiotics with the five strains of *Bifidobacterium* was assessed. The average ratio of acetic to lactic acid was variable from 3.3:1 (Hi-maize™) to 1.9:1 (inulin). The addition of prebiotics improved viability of bifidobacteria over 4 wks in refrigerated storage ranging from 75.34% (*B. animalis* with Hi-maize™) to 23.65% (*B. animalis* with raftilose). The pH of fermented samples after 4 wks of refrigeration ranged from pH 4.07 (*B. longum* 1941 with inulin) to pH 4.34 (*B. animalis* Bb12 with raftilose). The effect of prebiotics would be more obvious in the *in vivo* situation where prebiotics reach the colon unabsorbed and are selectively utilized by bifidobacteria. Hence, a combination of an appropriate *Bifidobacterium* strain with a specific prebiotic would be a feasible approach in administering the beneficial bacteria.

7.4. Viability of freeze-dried bifidobacteria at various temperatures during prolonged storage

The long term storage of freeze-dried *B. longum* 1941 and BB536 containing inulin was assessed at frozen (-18°C), refrigerated (4°C) and room temperatures (20°C). Over 20 months, the viability was maximal when capsules containing freeze-dried bifidobacteria were

stored at −18°C, while bifidobacterial counts were significantly reduced ($p<0.05$) at the refrigerated temperature (4°C). Storage at room temperature (20°C) reduced the viable counts significantly ($p<0.05$) and no bifidobacteria were detected after 5 months. Although commercial and laboratory probiotic preparations showed a general trend of decline in bacterial counts over time when placed at similar storage temperatures, commercial probiotic products showed a reduced rate of decrease in *Bifidobacterium* viability as compared to laboratory probiotic preparations.

7.5. Effects of feeding *Bifidobacterium longum* and inulin on some gastrointestinal indices in human volunteers

Interest in consumption of probiotic and prebiotics (indigestible oligosaccharides) to improve human gastrointestinal health is increasing. Consumption of beneficial probiotic bacteria combined with oligosaccharides may provide enhanced gastrointestinal benefits and improvements in internal health. The objective of this study was to evaluate the effectiveness of administering *Bifidobacterium longum* 1941 or *B. longum* BB536 and inulin to healthy, adult volunteers over 2-wk to observe changes in gastrointestinal indices (bacterial counts in stool, stool defecation frequency and consistency, and in organic acids, β-glucuronidase and β-glucosidase enzyme concentration, pH and moisture). A randomised, double-blind and placebo-controlled parallel group comparison was carried out. Subjects were randomly assigned to receive 25 mg of freeze-dried bacterial preparation containing $\geq 1 \times 10^{10}$ cfu/g of either *B. longum* 1941 and 475 mg inulin (n = 10), *B. longum* BB536 and 475 mg inulin (n = 10) or a placebo containing 475 mg inulin (n = 10). Efficacy was based on comparison of initial values of gastrointestinal indices with final values. No significant difference between the baseline and the final reading among the three treatment groups was observed on bacterial counts, defecation frequency, stool consistency, pH, enzyme and organic acid concentrations

or moisture content of stools. However, levels of butyric acid increased after subjects consumed probiotic capsules.

The feeding of *B. longum* 1941 and *B. longum* BB536 with inulin appears to be safe for human consumption. The subjects did not experience any cases of illness or being unwell during the feeding trial period. The bacterial counts, defecation frequency and colour, moisture content, organic acid profile of stool and faecal enzyme concentration showed no significant difference from the placebo group. Furthermore, the subjects did not experience any gastrointestinal discomfort and there was slight to marked improvement in well-being of most subjects participating in the study. The absence of significant differences on several gastrointestinal indices may be due to subjects taken part in the study being relatively healthy and the relatively short duration of the study.

7.6. *In vitro* inhibition of vancomycin-resistant enterococcus (VRE) by probiotic bacteria

Seven unique probiotic strains including *Bifidobacterium*, commercial probiotic preparations, pH modification of media and addition of nisin were investigated for their inhibition effect against eleven strains of *Enterococcus faecium* and nine *Enterococcus faecalis* (VRE). The twenty strains of VRE were isolated from infected patients and environmental samples and had variable MIC (8 to 64 μg/ml) resistance towards vancomycin.

In general, there was a highly variable level of inhibition of VRE by supernatant obtained from VRE and probiotic bacteria using well-diffusion tests and co-culturing with live probiotic cultures. Dose-dependent addition (0.01 g to 2 packets) of commercial freeze-dried probiotic (VSL#3) containing seven different probiotic strains with a concentration of 4.5×10^9 cells per gram showed significant levels of inhibition against VRE. Adjustment of the growth medium pH (2.0, 3.0, 4.0, 5.0, 6.0 and 7.0) using 5N HCl displayed the highest

levels of inhibition against VRE, whereby low pH of less than 4.0 resulted in significant decreases in VRE counts. The dose-dependent addition of nisin (1-1000μg/ml), a bacteriocin produced by *Lactococcus* subsp. *lactis* resulted in a significant inhibition at relative high concentrations, however, probiotic strains are more susceptible than VRE strains. The addition of nisin in media (1-20 μg/ml) with pure VRE strains produced a brief initial inhibition effect, while VRE showed quick adaptation to nisin in a 24 h period.

Overall, VRE showed rapid adaptation to bacteriocin, but not to low pH induced and during co-culturing with probiotic bacteria. Concentration of supernatant obtained from probiotic bacteria had no detectable inhibitory effect on the growth of VRE cells *in vitro*. These observations suggest that probiotic-induced suppression of VRE *in vivo* might be enhanced by additional approaches to lowering luminal pH such as co-ingestion of fermentable substrates.

8.0. FUTURE RESEARCH DIRECTIONS

As knowledge of probiotic bacteria increases globally, the demand for products containing probiotic bacteria have increased significantly over the last twenty years. There has been an increasing range of probiotic products produced of dairy origin such as yoghurt, fermented milks and other dairy products while consumption of tablets or capsules containing freeze-dried probiotic organisms have become increasingly popular due to their higher bacterial concentrations.

Further research into the fermentation of different types of prebiotics by probiotic bacteria is critical to establish which products produce the greatest synbiotic response. Research into the mechanisms as to why certain prebiotics provide a greater beneficial effect than others to particular probiotic strains would improve knowledge of the reactions that take place inside the gastrointestinal tract.

The selective enumeration of probiotic bacteria from human faecal samples is an important requirement of determining the efficacy of the action of probiotic bacteria in the human gastrointestinal tract. Successful development of selective media for isolation of anaerobic bacteria from human faecal samples is critical to understand the relationship between feeding probiotic and their survival in the gut and shedding in faeces.

While knowledge of some probiotic strains for their bile and acid tolerance has been ascertained as well as antimicrobial ability and anti-carcinogenic properties, further work is still needed on improving viability of probiotic strains in commercial products and determining the concentration of probiotic bacteria for daily consumption in order to achieve health benefits.

The inhibitory ability of probiotic bacteria especially bifidobacteria towards vancomycin-resistant enterococci (VRE) has important clinical implications. Due to the exponential increase in resistance to antibiotics by many bacterial species, research on probiotics is imperative to successfully demonstrate effectiveness in reduction and elimination of these strains from the human gastrointestinal tract.

Our work has shown that several probiotic strains are able to successfully inhibit VRE strains of both human and environmental origin *in vitro*. These strains need to be further assessed and critically evaluated by clinical research on patients colonised and infected with VRE. It may be useful to study which probiotic strain(s) combined with prebiotics are most successful in eliminating VRE *in vivo* without the use of various antibiotics, which cause disturbance in the balance of the gastrointestinal microflora.

9.0. REFERENCES

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10.0. APPENDIX

A1. Sample Consent Form for Subjects Involved in Research Certification by Subject

I, ...
of
(Address).....

certify that I am at least 18 years old and that I am voluntarily giving my consent to participate in the experiment entitled:

The effects of bifidobacteria and oligosaccharides on intestinal microflora.

Being conducted at Victoria University by:

Mr Frank Bruno (BSc, MSc)
Associate Professor Nagendra Shah (Principle Investigator)
Dr Lanka Lankaputhra (Co-investigator)

I certify that the objectives of the experiment, together with any risks to me associated with the procedures listed hereunder to be carried out in the experiment, have been fully explained to me by:

Mr Frank Bruno

and that I freely consent to participation involving the use on me of these procedures:

Procedures:

(i) Consumption of 2 capsules daily of freeze-dried bifidobacteria with an oligosaccharide (carbohydrate source) over a 14 day period.
(ii) Stool specimen collection before and after 2 weeks of consuming capsules.
(iii) Information given relating to age, height and weight.
(iv) Filling out a simple questionnaire concerning stool frequency and consistency.

I have been informed that the information I provide will be kept confidential. Any data collected relating to the samples provided will only be presented as an overall group result for publication which will be coded by a 3 digit code i.e. No. 123.

I also acknowledge the right to depart at any time from the study for any reason without any incrimination or liability and accept that any data collected from my samples will be omitted from the study's results by the investigators involved.

Signed:..... Date...../.../ 2001

Witness other than the experimenter:

..... Date...../.../ 2001

Contact number:_____

Age (years & months):_____

Height (cms)_____

Weight (kgs)_____

Subject Code:_____

Product Code:_____

Any queries about your participation in this project may be directed to the researcher (Frank Bruno pH 9216-8109 (AH) 0413 228 296). If you have any queries about the way you have been treated, you may contact the Secretary, University Human Research Ethics Committee, Victoria University, PO BOX 14428 MCMC, Melbourne, 8001 or telephone (03) 9688-4170.

A2. CONSISTENCY TABLE RELATING TO FEEDING TRIAL

SUBJECT CODE NO: _____

DATE: _____

PLEASE INDICATE LEVEL OF HARDNESS OF STOOL SAMPLE FOR EACH DAY BY GIVING A RATING FROM 1-5.

SUBJECT'S FAECAL CONSISTENCY SCALE RATINGS
1= Runny, very soft
2= Soft
3= Normal
4= Slightly firm
5= Hard
(Increments of 0.5 are allowed)

Day	Level of hardness	Any other comments?
1 (M)	(1 to 5)	
2 (T)	(1 to 5)	
3 (W)	(1 to 5)	
4 (T)	(1 to 5)	
5 (F)	(1 to 5)	
6 (S)	(1 to 5)	
7 (S)	(1 to 5)	
8 (M)	(1 to 5)	
9 (T)	(1 to 5)	
10 (W)	(1 to 5)	
11 (T)	(1 to 5)	
12 (F)	(1 to 5)	
13 (S)	(1 to 5)	
14 (S)	(1 to 5)	

* Please contact Frank if you have any further concerns

Contact details
Frank Bruno
PH (Office hours): 9216-8109
PH (Laboratory): 9216-8163

A3. DEFECATION FREQUENCY TABLE

Subject No :_____

Group No: _____

Code No: _____

Please can you indicate at what hour you go to the toilet by placing a cross (X) through the hour on the table matched by the date indicated. i.e. 4.35pm=4pm or 7.45am=7am

Day	HOUR																							
1	12am	1am	2am	3am	4am	5am	6am	7am	8am	9am	10am	11am	12pm	1pm	2pm	3pm	4pm	5pm	6pm	7pm	8pm	9pm	10pm	11pm
2	12am	1am	2am	3am	4am	5am	6am	7am	8am	9am	10am	11am	12pm	1pm	2pm	3pm	4pm	5pm	6pm	7pm	8pm	9pm	10pm	11pm
3	12am	1am	2am	3am	4am	5am	6am	7am	8am	9am	10am	11am	12pm	1pm	2pm	3pm	4pm	5pm	6pm	7pm	8pm	9pm	10pm	11pm
4	12am	1am	2am	3am	4am	5am	6am	7am	8am	9am	10am	11am	12pm	1pm	2pm	3pm	4pm	5pm	6pm	7pm	8pm	9pm	10pm	11pm
5	12am	1am	2am	3am	4am	5am	6am	7am	8am	9am	10am	11am	12pm	1pm	2pm	3pm	4pm	5pm	6pm	7pm	8pm	9pm	10pm	11pm
6	12am	1am	2am	3am	4am	5am	6am	7am	8am	9am	10am	11am	12pm	1pm	2pm	3pm	4pm	5pm	6pm	7pm	8pm	9pm	10pm	11pm
7	12am	1am	2am	3am	4am	5am	6am	7am	8am	9am	10am	11am	12pm	1pm	2pm	3pm	4pm	5pm	6pm	7pm	8pm	9pm	10pm	11pm
8	12am	1am	2am	3am	4am	5am	6am	7am	8am	9am	10am	11am	12pm	1pm	2pm	3pm	4pm	5pm	6pm	7pm	8pm	9pm	10pm	11pm
9	12am	1am	2am	3am	4am	5am	6am	7am	8am	9am	10am	11am	12pm	1pm	2pm	3pm	4pm	5pm	6pm	7pm	8pm	9pm	10pm	11pm
10	12am	1am	2am	3am	4am	5am	6am	7am	8am	9am	10am	11am	12pm	1pm	2pm	3pm	4pm	5pm	6pm	7pm	8pm	9pm	10pm	11pm
11	12am	1am	2am	3am	4am	5am	6am	7am	8am	9am	10am	11am	12pm	1pm	2pm	3pm	4pm	5pm	6pm	7pm	8pm	9pm	10pm	11pm
12	12am	1am	2am	3am	4am	5am	6am	7am	8am	9am	10am	11am	12pm	1pm	2pm	3pm	4pm	5pm	6pm	7pm	8pm	9pm	10pm	11pm
13	12am	1am	2am	3am	4am	5am	6am	7am	8am	9am	10am	11am	12pm	1pm	2pm	3pm	4pm	5pm	6pm	7pm	8pm	9pm	10pm	11pm
14	12am	1am	2am	3am	4am	5am	6am	7am	8am	9am	10am	11am	12pm	1pm	2pm	3pm	4pm	5pm	6pm	7pm	8pm	9pm	10pm	11pm