Molecular and physiological studies on the functionality of probiotic lactobacilli

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Hiermit erkläre ich, dass ich die vorliegende Arbeit selbständig angefertigt und keine anderen als die angegebenen Quellen und Hilfsmitteln verwendet habe.

Karlsruhe, 20. September 2006 María Guadalupe Vizoso Pinto

Para mi amado Jan...

Cuando parece que el camino se encajona,
Una pequeña pausa y tu calma,
Tu mirada amorosa y tu mano amiga,
Bastan para que se abran miles de nuevos caminos,
Para que la tormenta pase y el olor a tierra mojada regocije el alma.
No creo en los imprescindibles,
Sin vos también lo hubiera logrado, asi soy de testaruda,
Pero cómo, cuándo y dónde seguramente no hubiera sido
Feliz, ahora y aqui contigo.

Para mi famila...

Porque las distancias no existen para el amor,
Porque ni el mar ni el tiempo alcanzan para no sentirlos cerca,
Porque me dieron la fortaleza simentada en principios,
El abrazo tierno, llanto y risa compartidos,
Las raíces y las alas...

ZUSAMMENFASSUNG

Probiotika sind bakterielle Präparate, die – wenn sie in ausreichender Menge eingenommen werden – die Gesundheit eines Wirtes verbessern, indem sie die Mikrobiota bestimmter Habitate dieses Wirtes verändern. In den letzten Jahren expandierte der Markt für Probiotika, aber es besteht noch immer keine Einigung über die Wirkungsmechanismen dieser Produkte. Die meisten probiotischen Eigenschaften scheinen stammspezifisch zu sein. Es besteht demnach Bedarf an neuen und gut charakterisierten Stämmen, ebenso wie an der Aufdeckung ihrer funktionellen Wirkungsweisen. Ziel dieser Studie war es, Stämme von *Lactobacillus* aus traditionellen, fermentierten afrikanischen Produkten sowie aus dem Stuhl von Säuglingen als mögliche Probiotika *in vitro* zu isolieren, zu identifizieren und zu charakterisieren. Ein bestehendes Model einer Magen-Darm-Passage wurde optimiert und verwendet um 30 *Lactobacillus*-Isolate auf ihre Überlebensfähigkeiten unter simulierten gastrointestinalen Bedingungen zu testen. Sieben Stämme mit guten Überlebensfähigkeiten wurden selektiert und mit Hilfe von pheno- und genotypischen Methoden identifiziert. All diese Stämme besaßen Gallensalzhydrolase-Aktivität, welches es Ihnen ermöglichen könnte in der Galle zu überleben und möglicherweise Cholesterol zu verwerten. Einige Stämme besaßen β-Galaktosidase-Aktivität, die zur Linderung Laktoseintoleranz beitragen könnte. Manche dieser Stämme waren in der Lage, auf dem präbiotischen Zucker Raftiline® ST zu wachsen, welches die Entwicklung eines synbiotischen Produktes ermöglichen könnte. Darüber hinaus konnten einige Stämme das Wachstum von Krankheitserregern aus Nahrungsmitteln durch die Bildung organischer Säuren und H_2O_2 hemmen; auch vermochten sie, mit Pathogenen zu koaggregieren, welches die Ansiedlung von Pathogenen durch Blockade ihrer Adhäsionsplätze im Darm verhindern könnte. Die Stämme waren zudem in der Lage, an Darmzellen anzuhaften, ein Zeichen dafür, dass sie zumindest vorübergehend im Darm verbleiben, und somit ihre probiotischen Wirkungen ausüben können. Schließlich regten einige Stämme mittels Lipoteichonsäure die Sezernierung von IL-8 aus Darmzellen (HT29-Zellen) an. Diese sanfte Stimulation könnte dazu beitragen, den Wirt in einen Zustand „immunologischer Wachsamkeit" zu versetzen. Mit Hilfe der Durchflusszytomerie konnte somit erstmals eine Heraufregulierung von TLR2 und TLR5 in Darmzellen durch Milchsäurebakterien nachgewiesen werden. Dieser Mechanismus ermöglicht neue Einblicke in die Kommunikation zwischen Bakterien und Darmzellen und unterstreicht die Rolle von Enterozyten als „Wachtposten". *L. johnsonii* BFE 6128, *L. plantarum* BFE 1685 and *L. rhamnosus* GG steigerten die angeborene Immunantwort von HT29-Zellen auf *S.* Typhimurium, indem sie auf synergistische Weise den IL-8-Spiegel auf Protein- und mRNA-Ebene erhöhten. Unter Anwendung von real-time RT-PCR-basierten Arrays, wurde die Veränderung der TLR-Signalkaskade durch *L. johnsonii* BFE 6128 und *S.* Typhimurium eingehend untersucht. Auf mRNA-Ebene wurde zudem die Expression von TLR und anderer, in den Wirkungspfad von TLR involvierter Moleküle, verschiedentlich verändert. Diese Beobachtung eröffnet neue Möglichkeiten für die Entwicklung probiotischer Produkte, da sie darauf hindeutet, dass bestimmte probiotische Stämme die Immunantwort auf Krankheitserreger intensivieren können. Andererseits sind Stämme mit diesen Eigenschaften möglicherweise nicht geeignet um Darmentzündungen zu behandeln, da sie die Entzündung selbst verstärken könnten.
Zusammenfassend liefert diese Studie eine Begründung für die weitere Verwendung von vier der selektierten *Lactobacillus*-Stämme (*L. plantarum* BFE 5759, BFE 1684 and BFE 1685 sowie *L. johnsonii* BFE 6128) als Probiotika für therapeutische oder Krankheitsvorbeugende Zwecke.

ABSTRACT

Probiotics are bacterial supplements, which, when applied in sufficient numbers, improve the health of the host by modifying the microbiota of a particular compartment of this host. The market of probiotic products has expanded in the last years, but there is still no consensus about the mechanisms of action of these products. Most probiotic effects appear to be strain specific. Thus, there is a need for new and well characterised strains, as well as the elucidation of their mode of functional action. The objective of this study was to isolate, identify and characterise *Lactobacillus* strains from traditional African fermented milk products and children's faeces as potential probiotic candidates *in vitro*. A model of the stomach-duodenum passage was optimised and used to screen 30 *Lactobacillus* isolates for their survival under simulated gastrointestinal conditions. Seven strains showing good survival were selected, and identified by phenotypic and genotypic methods. All strains possessed bile salt hydrolase, as an indicator of survival to bile and of possible cholesterol utilisation, and some also hydrolysed lactose, which may serve for alleviating lactose intolerance. Some of these strains were able to grow on the prebiotic sugar Raftiline® ST, which would allow the formulation of a synbiotic product. Furthermore, some strains could inhibit the growth of food borne pathogens by producing organic acids and H_2O_2 , and also coaggregated with pathogens, which may enhance their removal in the gut by blocking their adhesion sites. The strains also adhered well to intestinal cells, indicating that they may at least transiently remain in the gut to exert their probiotic effects. They also inhibited adhesion of food borne pathogens. Furthermore, some strains stimulated secretion of IL-8 by intestinal cells (HT29 cell line) via their lipoteichoic acids. This low stimulation may help maintaining the host in a state of immunologic alert. This is the first time that an up-regulation of TLR2 and TLR5 in intestinal epithelial cells by lactobacilli was reported, as determined by flow cytometry. This mechanism provides new insight in the communication between bacteria and intestinal epithelial cells, and underlines the sentinel role of enterocytes. *L. johnsonii* BFE 6128, *L. plantarum* BFE 1685 and *L. rhamnosus* GG enhanced the innate response of HT29 cells to *S.* Typhimurium by synergistically increasing IL-8 levels at the protein and mRNA levels. By means of real-time RT-PCR-based arrays, the modulation of TLR pathways by *L. johnsonii* BFE 6128 and *S.* Typhimurium was further investigated. The expression of TLR and other molecules involved in TLR pathways were also differentially modulated at the mRNA level. This finding opens new possibilities for probiotic development as it indicates that certain probiotic strains may act as adjuvants to increase responses against pathogens. On the other hand, strains with these characteristics may not be suitable for treatment of inflammatory bowel disease as they may potentiate the inflammatory response. In conclusion, this study provides a rationale for the further use of four of the selected *Lactobacillus* strains (*L. plantarum* BFE 5759, BFE 1684 and BFE 1685 as well as *L. johnsonii* BFE 6128) as probiotics for therapeutic or disease preventive purposes.

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CONTENTS

1 INTRODUCTION

The microbiota of the gastrointestinal tract (GIT) is one of the most complex and concentrated groups of organisms in nature (O'Sullivan, 1999) and it can be considered as a functionally active organ (Hart et al., 2002). Each segment of the gastrointestinal tract has a particular microbial population. This study focuses on bacteria, normally found in the small intestine, which may positively influence the health state of the host. The microbiota of the small intestine is less stable than that of the colon (Bezkorovainy, 2001), and thus, more susceptible to modifications. The composition of these bacterial populations is influenced by several factors such as diet, genetic background, age, gender, hygiene practises, administration of antibiotics and the physiological state of the host (Holzapfel & Schillinger, 2002; Isolauri & Salminen, 2005; Tlaskalova-Hogenova et al., 2005).

1.1 Development of the human gut microbiota

During birth, the first bacteria stemming from the mother's vaginal and faecal bacterial populations colonise the gastrointestinal tract of the newborn (Holzapfel et al., 1998). Afterwards, genetic factors, but also environmental factors such as the type of milk (breast milk or formula) that the newborn receives, determine the composition of the intestinal microbiota (McCracken & Lorenz, 2001; Isolauri & Salminen, 2005). *Bifidobacterium* (*B.*) spp. and to a lesser extend *Lactobacillus* (*L.*) spp. are the main genera constituting the intestinal microbiota of healthy breastfed infants. The most common lactobacilli constituting the gut microbiota of breastfed infants are *L. acidophilus*, *L. gasseri* and *L. johnsonii*, which belong to the so-called *L. acidophilus*-group (Vaughan et al., 2002). Upon introduction of solid food, the differences observed between breastfed and formula-fed infants disappear, while the numbers of *Clostridium, Bacteroides, Escherichia* (*E.*) *coli* and enterococci increase in both groups. After the first year of life, the diversity of the microbiota increases, and it is especially during this time when host-microbe interactions are most vital. During this period, the newborn, who comes from a sterile environment, establishes the first contact with a huge antigen challenge. This implies a strong stimulatory effect for the maturation of the gut associated lymphoid tissue (GALT) (Isolauri & Salminen, 2005). In addition, the composition of the intestinal microbiota seems to influence the development of autoimmune and atopic diseases (Matricardi & Bonini, 2000). Bjorksten et al. (2001) and Watanabe et al. (2003) observed that children with atopic dermatitis have differences in the bacterial composition of

the gut, with increased numbers of clostridia and *Staphylococcus* (*S.*) *aureus,* and reduced numbers of lactic acid bacteria and bifidobacteria.

Apart from genetic and environmental factors (air pollution, dietary changes), the increased hygiene and life standards associated with western lifestyles have been related to the development of atopic and autoimmune diseases in the so-called 'hygiene hypothesis' (Strachan, 1989). The 'hygiene hypothesis' relates the increased incidence, severity and prevalence of atopic dermatitis in the past 30-40 years to the decreased exposure to microbial antigens associated with a western lifestyle (Bloomfield et al., 2006). In Finland, where there has been an extreme rise in paediatric allergy, diet supplementation with *Lactobacillus* (*L.*) *rhamnosus* GG has been implemented to prevent and reduce the severity of atopic dermatitis (Baker, 2006).

1.2 Microbial balance

Experiments with germ-free and conventional mice have shown that the microbiota strongly influences many biochemical, physiologic and immunologic characteristics of the host. In fact, gnotobiotic animals have a lower metabolic rate, high susceptibility to pathogens, vitamin deficiencies, less efficient digestion and limited intestinal cell growth (Tannock, 2001; Hart et al., 2002). Microbial balance is the key factor that determines whether substances in the intestine are converted into compounds that are beneficial or detrimental to the host (Percival, 1997). If bacteria expressing virulent properties predominate, the host will suffer from disease (e.g. cancer, geriatric diseases, diarrhoea, constipation). In contrast, if the resident microbiota has predominantly healthy properties (support of the immune system, production of short chain fatty acids and vitamins such as vit. K and vit. B_{12}, and protection of overgrowth and/or infection by pathogens) this would help maintain a state of good health. Manipulation of this balance for therapy or as a preventive measure is a challenge, which makes the use of probiotics an interesting tool as an alternative to antibiotics. Another way of influencing the microbial balance in the gut is by means of naturally occurring or synthetic sugars, that are normally indigestible in the human gut, but that are selectively used as a carbon source for growth and metabolism by certain colonic bacteria. These substrates should stimulate growth of *Bifidobacterium* spp. and *Lactobacillus* spp., but not of potential pathogens such as toxin producing clostridia, proteolytic bacteroides and toxigenic *E. coli*. Substances which fulfil these requisites, such as inuline, fructooligosaccharides and lactulose, are called prebiotics. Preparations combining both pro- and prebiotics for obtaining synergistic effects are termed synbiotics (Schrezenmeir & de Vrese, 2001; Bielecka et al., 2002; Holzapfel & Schillinger, 2002).

1.3 Probiotics

1.3.1 Definition

The hypothesis that a shift of the microbial balance towards the beneficial properties would improve health was postulated at the beginning of the 20th century by Metchnikoff. He related the longevity of the Caucasians to the consumption of fermented milk products (Metchnikoff, 1907). But the first documentation about health promoting effects of fermented milk dates back to a Persian version of the Old Testament (*Genesis* 18:8) that states that 'Abraham owed his longevity to the consumption of sour milk'. Kollath in 1953 and Vergio in 1954 were probably the first to introduce the term 'Probiotic' (Holzapfel & Schillinger, 2002). The definition of probiotics has been modified and improved several times; a consensus definition has been stated by Havenaar and Huis in't Veld (1992): 'A preparation of, or a product containing viable, defined microorganisms in sufficient numbers, which alter the microflora (by implantation or colonisation) in a compartment of the host and by that exert beneficial health effects in this host'.

1.3.2 Postulated mechanisms of action

Probiotic strains have beneficial effects on the host by controlling undesirable micro-organisms and by modulating the immune system (McCracken & Gaskins, 1999; Fuller & Perdigon, 2003). However, despite the efforts to elucidate their mechanism of action, it is still not well understood how probiotics work (Tannock, 2002).

There are several postulated mechanisms through which probiotics exert their beneficial effects in the host (Fig. 1.1). Most of the mechanisms have been studied *in vitro* because of the complexity of the gut ecosystem and the numerous interactions taking place in the gut (bacteria-bacteria, nutrients-bacteria, nutrients-epithelium, epithelium-bacteria, epithelium-immune system and bacteria-immune system). Some *in vitro* experiments can be extrapolated to the *in vivo* situation, as shown in several studies that compare *in vitro* results with *in vivo* studies (Jacobsen et al., 1999; Cesena et al., 2001). Animal models are also useful tools; nevertheless, they have substantial differences in the anatomy of the gastrointestinal tract and MALT (mucosa associated lymphoid tissue) and in the microbial composition of the gut microbiota, when compared with human beings. In addition, some strains show host-specificity for adherence and exerting their health effects (Morelli, 2000).

Some of the mechanisms through which probiotics may antagonise pathogens include production of antimicrobial compounds such as lactic acid, acetic acid, hydrogen peroxide and bacteriocins (Alakomi et al., 2000; Annuk et al., 2003). Other functional properties to

characterise probiotics are their ability to modulate immune responses and to adhere to gut tissues (Holzapfel & Schillinger, 2002) (Fig. 1.1).

The ability to adhere to the intestinal epithelium is one of the main criteria for selecting new probiotic strains, as this property allows strains to remain, at least transiently, in the intestinal tract, and exert their probiotic effects such as excluding pathogenic bacteria by competing for adhesion sites (Alander et al., 1999; Blum et al., 1999; Saarela et al., 2000).

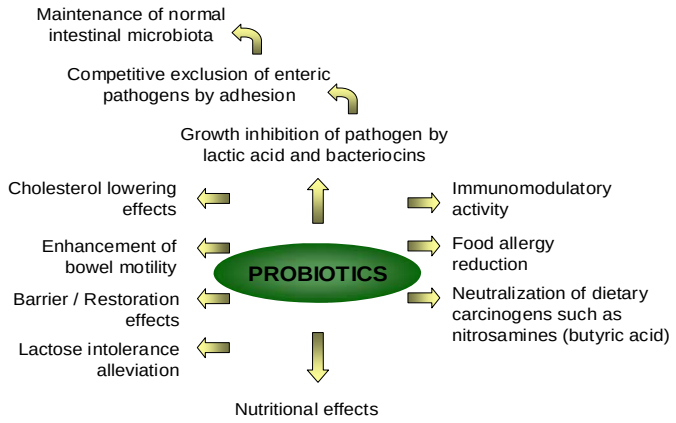


Fig. 1.1 Postulated mechanisms of action of probiotics.

Lactic acid bacteria have been shown to reduce the symptoms of lactose intolerance. Lactose, a disaccharide composed of glucose and galactose, is the main sugar present in milk and needs to be cleaved to the monosaccharides in order to be absorbed in the small intestine. This enzymatic hydrolysis is catalysed by lactase (*β*-galactosidase), an enzyme present in the brush border of the enterocytes in childhood. Some adults still express this enzyme and can benefit from milk as a protein and calcium source, but the expression of this enzyme generally decreases with age and is, in some cases, completely lost (Szilagyi, 2002). In individuals with low lactase contents, lactose cannot be absorbed and it can be used as fermentable substrate by the intestinal microbiota. In addition, water accumulates in the intestinal lumen due to lactose osmotic properties. As a result, the patient may suffer from bloating, flatulence, pain, nausea and even diarrhoea (Hove et al., 1999; de Vrese et al., 2000). The consumption of yogurt and fermented milk containing lactic acid bacteria has been proven to alleviate lactose maldigestion symptoms because of the lower lactose content, and its hydrolysis by microbial *β*-galactosidase (Gilliland & Kim, 1984; Mustapha et al., 1997). This enzyme is sensitive to low pH and, therefore, bacteria surviving low pH protect the enzyme from activity loss until it reaches its site of action, which is the small intestine (Zarate et al., 2000). On the other hand, bacteria sensitive to bile salts present a membrane with increased permeability, which allows two events which may or may not be exclusive: one is the transport of the substrate at higher

rates into the bacterial cell, and the other one is the increased release of the bacterial enzyme into the lumen. Probiotic bacteria, with higher resistance to bile salts, release less amounts of enzyme into the lumen. Therefore, yogurt starter cultures which do not survive the effect of bile, seem to be more effective in alleviation of lactose intolerance (de Vrese et al., 2000).

Another desirable property of some probiotic strains is their ability to reduce cholesterol levels (De Smet et al., 1998; du Toit et al., 1998). This property has been partly related to bile salt hydrolase (Bsh), which deconjugates bile salts by releasing the amino acid (taurine or glycine) bound to the side chain of the steroid core. Deconjugated bile salts are less soluble, and thus easily excreted via faeces, resulting in decreased reabsorption and recirculation of bile salts into the liver. Consequently, more cholesterol is needed for *de novo* synthesis of bile salts (Usman & Hosono, 1999). Bsh activity has also been related to the ability of some bacteria to survive bile and colonise the intestine (De Smet et al., 1995; Klaenhammer & Kullen, 1999), but this hypothesis is controversial because others have found that deconjugated salts are more toxic than their conjugated counterparts (Grill et al., 2000). In humans, excess bile deconjugation may lead to adverse effects such as steatorrhea and formation of secondary bile salts, which are toxic and/or mutagenic (Marteau et al., 1995).

Probiotics have also been shown to modulate the immune system at different levels. They may have anti- and/or pro-inflammatory properties. Some strains have shown to influence the adaptive immunity, whereas others affect the innate immunity (Fuller & Perdigon, 2000). The immunomodulatory activities of probiotics are presented in section 1.4.

1.3.3 New probiotic strains and sources of isolation

There is still no consensus about the need for viability of probiotics to exert health effects (Gopal et al., 2001). Some studies maintain that the viability of probiotic strains is necessary for stimulation of the gut associated immune system (Lammers et al., 2002) or for anti-genotoxic effects (Galdeano & Perdigon, 2004; Ma et al., 2004). Others have shown that heat-killed probiotics (Pool-Zobel et al., 1996), or specific components derived from probiotic strains such as DNA (Nagy et al., 2005) or bacterial cell wall (Lammers et al., 2002; Jijon et al., 2004), are responsible for some immunomodulatory effects. If this is the case, and live microorganisms are not necessary to obtain the desired effects, a revision of the definition of probiotics or the development of a new concept defining these probiotic-derived components will be needed.

A general agreement, among those in favour of the classical definition of probiotics, refers to the need of live microorganisms exerting health-promoting effects, and thus, the need of survival to host conditions to reach the site of action. The general criteria for selection of

strains to be used as probiotics include: safety and origin of the bacteria, their tolerance to the hostile conditions of the stomach and the small intestine, and their ability to adhere to gut epithelial tissue (Davidkova et al., 1992; Tannock, 2002). The first step in the choice of new microbial strains to be used as probiotics should be their safety and origin. GRAS (generally recognised as safe) microorganisms include *Lactobacillus* spp. and *Bifidobacterium* spp., which are bacteria with a long history of safe use as they have been consumed by humans for centuries. Selection of other microorganisms must include expensive and time consuming short- and long-term toxicological studies (Morelli, 2000; Chesson et al., 2002). Havenaar et al. (1992) considered that the origin of the strain regarding host species and location specificity play an important role if colonisation is essential for achieving the desired effect of the probiotic. In this study, strains from human origin and from fermented milks were compared in their *in vitro* probiotic potential.

The bacterial isolates screened to be selected as potential probiotics in this study were isolated from African traditionally fermented milk products (Kwerionik and Kule naoto) and human faeces. Of special interest because of their background were the strains isolated from 'Kule naoto', a traditional fermented milk that was spontaneously fermented in gourds made out of dried calabash by the Maasai people (Chesson et al., 2002). The Maasai are a semi-nomadic and pastoral tribe living in Kenya and northern Tanzania. Traditionally, the Maasai rely on meat, milk and blood (only in special ceremonies) from cattle for protein and caloric needs (Mathara et al., 1996; 2004). The Maasai consume Kule naoto daily, because of its flavour and its supposed health-promoting properties. As a matter of fact, they believe that this product alleviates constipation and prevents diarrhoea (McAdams Wright, 2006). Interestingly, in a study of cholesteremia conducted by Mann and Spoerry (1974) in a tribe of Maasai, serum cholesterol level of Maasai men decreased after consumption of large amounts of milk fermented with a wild *Lactobacillus* strain.

1.3.4 Safety considerations

Even though *Lactobacillus* spp. belong to risk group 1 organisms (European Food Safety Authority, 2004), which include biological agents that are unlikely to cause human or animal disease, it is important to assess the safety of those microorganisms intended for use as food additives. Because of the serious concerns about the increasing level of resistance to antibiotics in regular use in human medicine, one of the aspects which needs to be analysed is antibiotic resistance. Probiotic strains as well as bacteria used in food fermentations may harbour resistance genes, which can be transferred to pathogenic bacteria (Havenaar et al., 1992; Danielsen & Wind, 2003; Franz et al., 2005). Therefore, probiotic strains intended for

the market should be screened for transferable resistance genes. A report by the Scientific Committee for Animal Nutrition (SCAN) recommended to the European Union commission (Teuber, 1999) that the absence of transferable resistance genes should be considered as an important pre-requisite for approval of probiotics.

1.3.5 Site of action of probiotics: the small intestine

The small intestine is a long tube of about 3-5 m in length; it begins with the duodenum, continues with the jejunum and ends with the ileum. Its main functions are digestion of food and absorption of water, electrolytes and nutrients necessary for cellular growth of the whole organism. In order to assure a maximal contact with the digested food in a limited place, the intestine presents different surface-increasing strategies: circular folds, intestinal villi and microvilli. This makes the gut mucosa (with a surface of approximately 200 m^2) the largest area of the body in contact with the environment (Holzapfel et al. 1998).

The intestinal epithelium is a highly organised, single-cell layer covering the interface between tissues and the intestinal lumen. This monolayer is mainly constituted of enterocytes, which are the cells responsible for taking up nutrients, Paneth cells, which secrete the mucus bathing the epithelium, and intra-epithelial lymphocytes, which are part of the MALT. Yet, all epithelial cells arise from common non-differentiated precursors present in the epithelium (Brandtzaeg, 1995). This monolayer is constantly being renewed as epithelial cells undergo a lifecycle, which starts in the deep of the crypts, where they arise, continues with their differentiation and migration towards the tip of the villi and ends with apoptosis and exfolliation (Stadnyk, 1994; Turner, 2003; Dommett et al., 2005). This cycle takes about 3 to 5 days in humans and allows epithelial self-renewal. Because of this turn over, the gut surface is covered by dead and exfoliating cells, which provide together with the mucus, bathing the cells, and the nutrients passing through the lumen an excellent growth substrate for microorganisms (Stadnyk, 2002; Tlaskalova-Hogenova et al., 2005).

1.4 Tolerance and immunity in the gut

Mucosal surfaces are constantly exposed to foreign substances and microorganisms from the environment; indeed, they are the most common gate of entry for pathogens. All mucosal surfaces have similar organised lymphoid tissue collections and are characterised by the local presence of IgA. They all share anatomical and functional characteristics, and form the so-called MALT (Hill, 1995).

Mucosal surfaces are connected with each other; stimulation in one anatomical site also induces immunity at other mucosal sites. This process occurs because lymphocytes activated

at one mucosal site migrate and relocalise selectively in other mucosal sites ("mucosal homing") (Roux et al., 2003). In contrast to other tissues, the intestinal mucosa must react against threatening microorganisms, but it must simultaneously tolerate the presence of a myriad of commensal microorganisms and their by-products. This presents an immunological paradox, which is still not fully understood. The non-responsiveness to harmless food antigens and the generation of an adaptive immune response to potentially dangerous microorganisms are both vital physiological processes essential for the effective function of the intestinal epithelial barrier (Mowat & Viney, 1997; Roux et al., 2003).

The usual response to innocuous antigens is the induction of local and systemic tolerance, known as oral tolerance. Oral tolerance can be defined as the systemic unresponsiveness to previously ingested antigens when encountered in the parenteral route (Brandtzaeg, 1998; Strobel & Mowat, 1998; Nagler-Anderson, 2000). This phenomenon may be exploited for the immunotherapy of autoimmune and inflammatory diseases, but also presents a hindrance in the development of mucosal vaccines. Inappropriate responses to harmless food and other luminal (e.g., bacterial) antigens are likely to play a role in the induction of food allergy and inflammatory bowel disease (IBD) (Strobel & Mowat, 1998).

Innate immunity is also probably involved in the developing of tolerance in the gut. In contrast to other epithelia, such as the pulmonary epithelium or uroepithelium, which react potently to both pathogens and commensals, the intestinal epithelium seems to be able to distinguish them. This discrimination ability is disturbed in IBD as it is shown by the immunological reactivity of patients to their own commensal microbiota (Strobel & Mowat, 1998; Faria & Weiner, 2005).

Some members of the family of pattern-recognition receptors (PRRs), e.g. Toll-like receptors (TLRs), can be expressed by intestinal epithelial cells. Nevertheless, they are poorly responsive to a variety of TLR2 and TLR4 ligands (e.g. peptidoglycan and lipopolysaccharide, respectively) when compared to professional immune cells such as dendritic cells or macrophages. This seems to be partly due to the low expression of both receptors in enterocytes and to the presence of high levels of Toll inhibitory protein (Tollip) (Ghosh et al., 2004; Ohkusa et al., 2004).

As most pathogens enter the body through mucosae, a strong immune response is required to protect these tissues. Therefore, inflammation is the initial and usually adequate phase of the immune response against a potentially dangerous agent (Melmed et al., 2003). As a short-term survival strategy, some pathogen virulence factors alter function and/or functionality of host proteins and so pathogens can colonise, enter or survive in the host cell. As a long-term survival strategy, pathogens can modify host gene transcription in order to evade the immune

system and keep their niches hospitable (Neish, 2003; Beutler, 2004b). Many pathogens are able to suppress the inflammatory process, overcoming host defences, as a critical feature of their pathogenicity (Rosenberger et al., 2001). As an example, *Porphyromonas gingivalis*, an oral pathogen, is able to 'paralyse' chemokine production during infection, and this could serve as pathogenic mechanism (Wilson et al., 1998; Neish, 2003). *Yersinia enterocolitica* is another example of a pathogen able to evade, to some extent, the initiation of an inflammatory response. *Yersinia enterocolitica* appears to decrease the amount of IL-8 released by cells, as IL-8 levels induced by this microorganism in intestinal epithelial cells were significantly lower than those induced by other invasive strains (Darveau et al., 1998). Some strains of *Yersinia*, *Bordetella* and *Salmonella* are also able to inhibit the NF-κB pathway at different levels, leading either to apoptosis of the infected cell or to a blockade of the innate immune responses (Schulte et al., 1996).

1.4.1 Bridging innate immunity and adaptive immunity

The acquired or adaptive immunity is characterised by the exquisite antigen-specificity caused by re-arrangement of the genes encoding T-cell receptors and immunoglobulins. All higher vertebrates are able to mount specific immune responses against infectious agents. Protozoa also have to deal with microbes; they do it efficiently even though they lack this specific immunity. This is possible thanks to the innate immune system, which is present in the whole animal kingdom. Innate immunity is specifically inherited in the genome, recognises a wide spectrum of pathogens and activates an immediate answer. 'Innate' and 'adaptive' immune systems are more an academic classification than two separate systems. Therefore, it is very difficult to decide where the one ends and the other begins. They are connected at different stages and, to a greater extent, cells of the adaptive immune system depend on the signals and cells of the innate immune system to get activated (Collier-Hyams & Neish, 2005).

The GIT possesses physical and chemical barriers which have to be overcome before a microorganism infects the host. These barriers represent obstacles for both potential probiotics and pathogens. Indeed, the presence of lysozyme in saliva, the low pH in the stomach, the presence of proteolytic enzymes, the abrupt pH change towards the intestine, the detergent action of bile salts, the antimicrobial peptides (defensins) secreted by Paneth cells, the mucus bathing the intestinal epithelium, the continuous peristaltic movement and the presence of normal microbiota are most of the times enough to prevent infection by foreign microorganisms (Hoffmann et al., 1999; Janeway & Medzhitov, 2002; Kaufmann et al., 2004; Schütt & Bröker, 2006). If these barriers are overcome, a pathogen infects the host, and

defences of another kind have to be mobilised in order to prevent further progress of infection into disease. Before the elucidation of the sensing pathways involved in the innate immune response, there was a lack of knowledge concerning what exactly happens during the first twelve hours that the adaptive immune system needs for acting, expanding and generating memory cells for long-term responses (clonal expansion). Now it is clear that when the immune system contacts the pathogen for the first time, the innate arm protects the host during the time needed for the clonal expansion of the adaptive and more specific arm to occur (McCracken & Lorenz, 2001; Turner, 2003; Dommett et al., 2005). That is why the innate immune response is critical for the immediate combat of invading pathogens at their port of entry (skin or mucosae).

During a first infection, the recognition of the threat can take from seconds to hours (Janeway et al., 2002). The tissue macrophages engulf the intruder and destroy it with an arsenal of enzymes and toxic reactive oxygen species. The complement system, present in extracellular liquids, can be activated trough the classical or alternative pathways at the surface of the pathogens either opsonising them, which increases phagocytosis, or directly lysing them by forming channels through their cell walls and/or membranes. If these measures are not enough, the phagocytes (and other cells such as epithelial cells, too) send warning signals to initiate the process called inflammation. The molecular mediators and regulators of immune responses are proteins called cytokines (CK) which mediate cell-to-cell communication. Chemokines (CHKs) are special CKs which augment permeability of blood vessels, induce the expression of adhesion molecules by endothelial cells and serve as chemo-attractants that recruit professional immune cells from circulation to the site of infection. Interleukin-8 (IL-8), a potent neutrophil attractant and activator, is one of the first messengers released (Beutler, 2004b). Natural killer cells also express IL-8 receptors (Morohashi et al., 1995) and can migrate in response to IL-8 (Campbell et al., 2001).

Neutrophils are short-lived effector cells of crucial importance for the resolution of infection. Upon activation by CKs and CHKs such as IL-8, they become potent phagocytes with an arsenal of ‘weapons’ to effectively destroy pathogens (Moser, 2004; Seiler et al., 2004; Schütt & Bröker, 2006). Their importance is reflected by the fact that the absence of this cellular subset (neutrophils) causes a severe immunodeficiency (Gewirtz et al., 2003; Hayashi et al., 2003; Kuijpers & Roos, 2004). In turn, neutrophils further secrete other soluble mediators, which activate other immune cells, such as lymphocytes, and the process undergoes a positive feed-back. As result of an effective inflammatory process, the invader is eliminated and the tissue damage is locally restricted. This complex, non-specific reaction to

deleterious stimuli is characterised by erythema (redness), edema (swelling), pain and fever (heat) at the site of infection.

Macrophages play a central role as nexus between innate and adaptive immunity (Beutler, 2004b). They are, together with dendritic and mast cells, professional antigen-presenting cells (APCs). After destroying the pathogen, they process it and present the antigens (digested peptides) in the context of class II major histocompatibility complex molecules (MHC-II) to T lymphocytes. If the necessary co-stimulatory stimuli are present, T cells secrete IL-2 and proliferate (clonal expansion). T-cytotoxic cells ($CD8^+$) attack and destroy cells invaded by pathogens. Activation of naïve $CD4^+$ T cells leads to differentiation into either a Th1 or a Th2 phenotype. Th1 cells mediate immunity towards pathogens, especially those adapted to intracellular life cycles, and release IFN-γ, TNF-α and IL-12. Th2 cells are involved in responses against parasites (especially helminths) and produce IL-4, IL-5 and IL-10 and IL-15 inducing IgE and eosinophil-mediated destruction of the pathogen (Mosmann & Coffman, 1989). The differentiation into a Th1 or a Th2 phenotype occurs early in the immune response and is crucial for the clinical course of infection. The mechanisms of this differentiation process are still not fully elucidated and it is also possible that the canonical Th1 and Th2 global phenotypes only represent two polar extremes of all possible single phenotypes (Kelso, 1995).

Vertebrates rely on innate and adaptive defences to cope with invasive microorganisms, which would parasitise or eventually kill them (Hoffmann & Dutton, 1971). Both arms interact and cooperate very closely, but activation of innate immunity is a pre-requisite for the induction of adaptive immunity (Hoffmann et al., 1999). The recognition of the importance of innate immunity arose after the discovery that innate immune cells discriminate between non-self and self, and have a high degree of specificity (Akira & Takeda, 2004; Beutler, 2004b). Toll-like receptors are to a great extent responsible for this discrimination capacity. The revolutionary finding of these receptors, and their key role in the innate afferent (sensing) arm, has changed the way of thinking about pathogenesis and treatment of cancers, infectious, immune and allergic diseases (Janeway & Medzhitov, 2002).

1.4.2 Toll-like receptors

Toll-like receptors belong to a family of PRRs. They recognise foreign microbial products and are responsible for the initiation of host defence responses in all multicellular organisms investigated to date (Akira & Takeda, 2004). Two main advances in understanding innate immunity were first, the discovery that a protein called ‘Toll’ involved in embryogenesis of *Drosophila* also played a role in innate immunity against fungi (Aderem & Ulevitch, 2000;

O'Neill, 2004) and second, the finding of similar receptors in mice (Lemaitre et al., 1996). Until now 10 TLR homologues have been found in humans. They do not participate in embryogenesis, but all of them sense microbial molecules (Fig. 1.2).

TLRs and interleukin-1 receptors (IL-1 Rs) share a conserved region of about 200 amino acids called the Toll/IL-1R (TIR) domain. In the extracellular domain, TLRs contain 19-25 tandem copies of leucine rich repeats (LRRs), which form a horseshoe structure. This concave structure is thought to be responsible for ligand recognition. When the microbial derived ligand binds the LRR, the TIR domain initiates a complex enzyme cascade (see Fig. 1.2) including adaptor proteins like myeloid differentiation factor (MyD88), IL-1R-associated kinase (IRAK), tumour necrosis factor (TNF) receptor-associated factor (TRAF6), several mitogen-activated protein (MAP) kinases and inhibitor κB kinases (IKKs) (Poltorak et al., 1998; Beutler, 2004a).

This cascade ends up in the activation of the transcription factor NF-κB, which regulates the inducible expression of key pro-inflammatory mediators such as cytokines, chemokines and cationic antimicrobial peptides (defensins) (Aderem & Ulevitch, 2000; Janssens & Beyaert, 2003).

NF-κB dimmers are kept inactive in the cytoplasm by IKKs. TLRs cascades lead to the inactivation of the IKKs by phosphorylation, ubiquitination and proteasome-mediated degradation, resulting in the release of NF-κB. In turn, NF-κB translocates into the nucleus and induces NF-κB-dependent gene expression. Many of these genes are also up-stream activators of NF-κB, which implies a positive feedback for the amplification of the inflammatory response. The mechanistic details of NF-κB pathways are still not fully elucidated (Schnare et al., 2006).

TLRs influence or control the activation of adaptive immunity in several ways, such as regulation of dendritic cell maturation, induction of expression of CKs and co-stimulatory proteins and reversal of tolerance (van Duin et al., 2006). It also seems that TLR favours Th1 polarisation of the adaptive immune response (Trinchieri, 2003).

The discovery of TLRs has opened new possibilities for treatment and prevention of disease. Autoimmunity, sterile inflammation, sepsis and vaccination against malaria or HIV are only a few of the still not resolved health problems in which TLR targeting would be possibly applied (Ulevitch, 2004; van Duin et al., 2006). Nevertheless, there is still a long way to go before it is discovered when, how (i.e. blocking or stimulating) and what should be targeted.

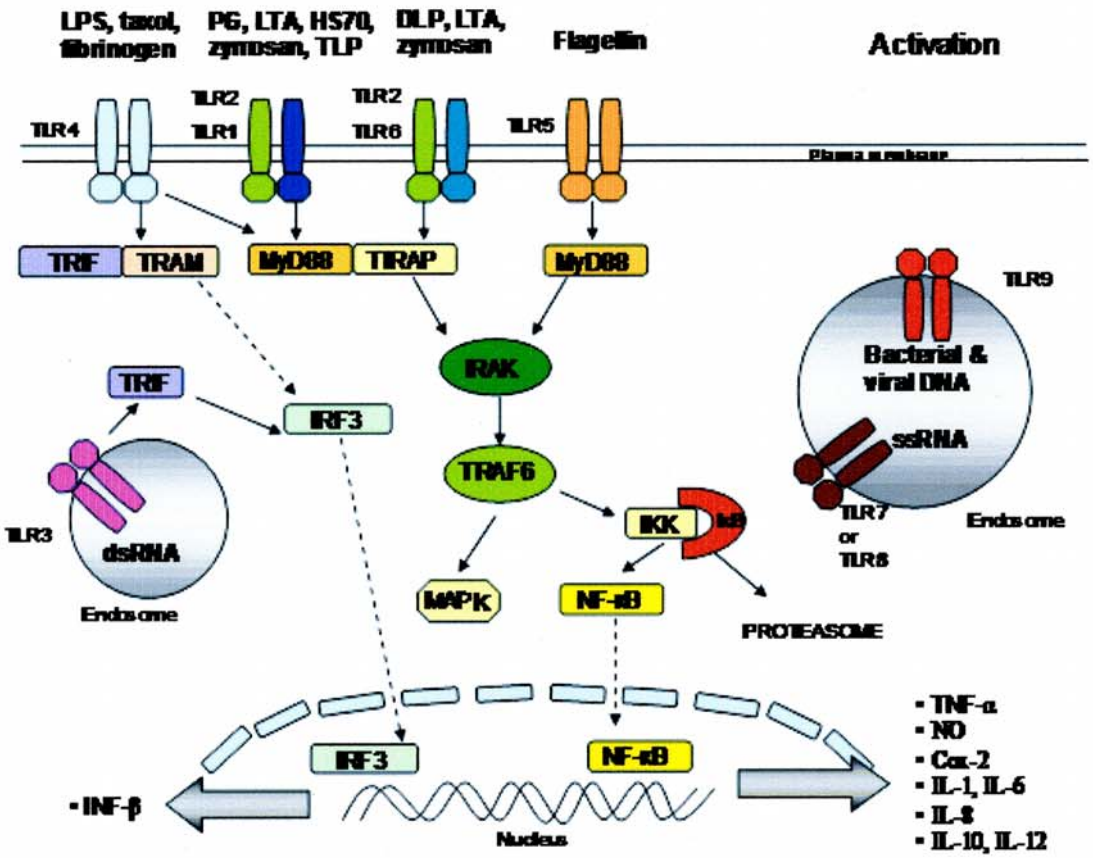


Fig. 1.2 Overview of simplified main TLR signalling pathways. Most TLRs signal through MyD88. TLR3, TLR4 and the TLR2 subfamily (TLR1, TLR2 and TLR6) also engage TIRAP and TLR3 also engages TRIF. Activated TLRs associate with a cytoplasmatic adaptor molecule, e.g. MyD88, through interaction between their TIR domains. Some of these adaptors may activate a serine-threonine kinase, IRAK. Subsequently, another adaptor molecule, TRAF6, is activated and in turn activates the MAPK kinases and the IKK complex (Junk pathways can also be activated, not shown). The IKK complex induces phosporylation of IκB, which renders IκB competent for being ubiquitinated and degraded. Its degradation liberates NF-κB, which in turn translocates to the nucleus and induces expression of target genes that encode pro-inflammatory mediators. Dashed arrows indicate translocation into the nucleus. LPS, lipopolysaccharide; PG, peptidoglycan; LTA, lipoteichoic acids; HS70, heat-shock protein 70; TLP, triacylated lipopeptides; DLP, diacylated lipopeptides; ssRNA, single-stranded RNA; dsRNA, double-stranded RNA; MyD88, myeloid differentiation factor 88; TRIF, Toll receptor associated activator of interferon; TIRAP, Toll-interleukin-1 receptor-associated protein; TRAM, Toll receptor-associated molecule; IRAK, IL-1 receptor-associated kinases, TRAF6, tumor necrosis factor receptor-associated factor 6; IKK, IκB kinase; IκB, inhibitor of NF-κB; NF-κB, Nuclear factor κB; NO; nitro-oxygenase; Cox-2, inducible Cycle-oxigenase. Adapted from Beutler (2004a), Ulevitch (2004), Cario and Podolsky (2005) and van Duin et al. (2006).

1.4.3 The enterocyte as sentinel of the gut

The main function of the intestinal epithelial cell (IEC) is the absorption of nutrients, electrolytes and water. Enterocytes build a single monolayer which is the only barrier which separates our body from the ecosystem of approximately 10^{14} bacteria of near 400 species residing in our intestines (Natoli et al., 2005). But the intestinal epithelium is not only a

physical barrier. Because of their strategically located anatomical situation, it also plays an active and central role in the so-called 'trilogue' between luminal bacteria, epithelium and professional immune cells in the lamina propria. IECs scan the gut environment for microbial threats, interpret signals from luminal bacteria, transmit this information to professional immune cells in the lamina propria and thereby cooperate with the GALT to mount a response to certain antigenic stimuli (O'Sullivan, 1999). Because of the continual exposure to the gut microbiota, the intestinal epithelium maintains, under normal conditions, a state of controlled or physiologic inflammation (Collier-Hyams & Neish, 2005; Shao et al., 2005). It is clear that commensal bacteria should not elicit as intense an inflammatory response as a pathogen; however, some investigators (Kagnoff, 2003; Jijon et al., 2004; Rakoff-Nahoum et al., 2004) have shown that a constant TLR stimulation may be necessary for intestinal health. Gewirtz et al. (2004) also pointed out that some degree of low level 'surveillance' NF-κB activation may be physiological.

One of the features that make IEC apt for this role as 'transducer' is the expression of receptors and antigen-presenting molecules. The receptors allow IECs to sense microbial components. They belong to the family of pattern recognition receptors (PRRs) and include lectins, adhesins, members of the nucleotide-binding oligomerization domain (NOD) family and the Toll-like receptor (TLR) family (Gewirtz et al., 2003). The antigen presenting molecules belong both to the classical (HLA-II, HLA-I) and non-classical types (e.g.: CD1d β2-m associated and non-β2-m associated) (Cario & Podolsky, 2000; Cario, 2005). These molecules enable direct communication with adjacent immune cells of the lamina propria. It was assumed that presentation of antigen trough IECs leads to anergy because of lack of co-stimulatory signals. This was only until non-typical co-stimulatory molecules (e.g.: CD58, CD86, ICAM-I), normally present in dendritic cells, were found in IECs, demonstrating once again that the rules of the mucosal immune system are different from those of the systemic immune system (Dotan & Mayer, 2003; Shi & Walker, 2004; Shao et al., 2005). These data strongly suggest that IECs may function as important APCs in the mucosal immune system.

IECs are able to further communicate with cells of the immune system indirectly by releasing cytokines. In response to pathogens, IECs secrete pro-inflammatory cytokines that recruit pro-inflammatory cells from circulation. Apart from that, they also actively participate in the defence against infection by secreting antimicrobial peptides called defensins (Dotan & Mayer, 2003). The expression of both cytokines and defensins is regulated by TLR signalling (Ogle et al., 2002).

The expression of β-defensin-2 is regulated by TLR4- and TLR2-dependent pathways (Vora et al., 2004). O'Neil et al. (1999) demonstrated that IEC up-regulate β-defensin-2

expression in response to IL-1α and invasive enteric bacteria. Expression of TLRs is increased in response to Th1 cytokines in IBD mucosa. This increased expression of TLRs may enhance reactivity to bacteria resulting in chemokine expression, recruitment of inflammatory cells, and secretion of antimicrobial peptides (Vora et al., 2004). The Crohn's disease associated mutations in CARD15/NOD2 (a protein with antimicrobial properties highly expressed in Paneth cells) result in decreased responsiveness to bacterial muramyldipeptide. Some studies have also suggested that deficiency in antimicrobial peptide (e.g. defensins) expression may contribute to the pathogenesis of Crohn's disease (Abreu et al., 2002; Fellermann et al., 2003; Vora et al., 2004). Thus, defects in the innate immune response to pathogens or commensal bacteria may contribute to the pathogenesis of IBD.

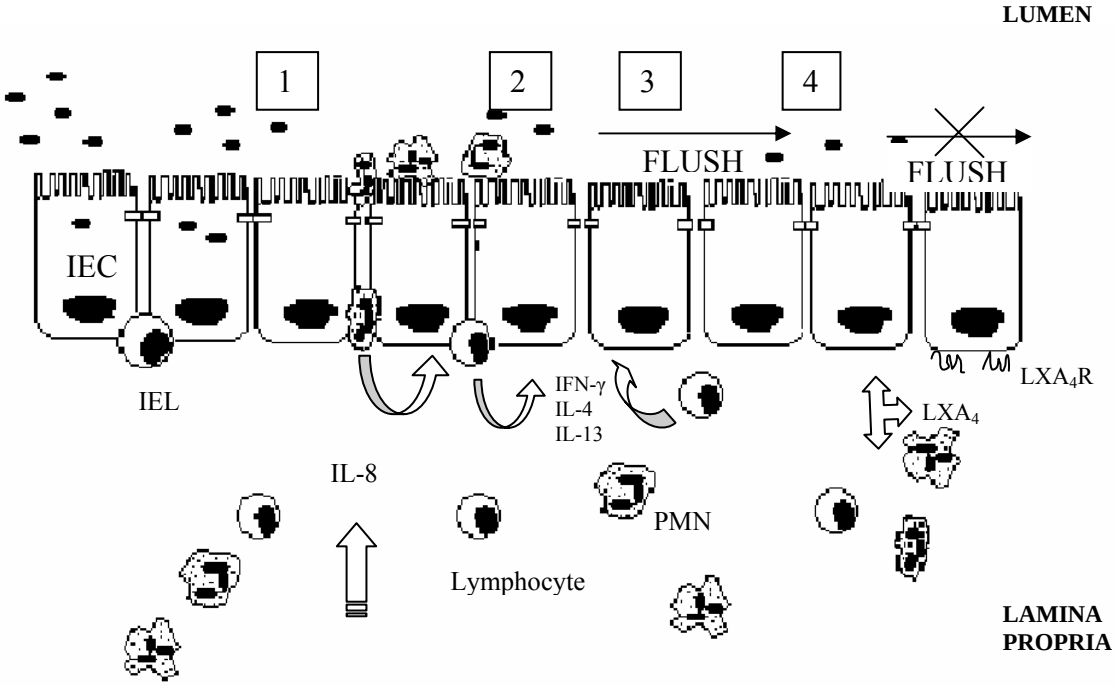


Fig. 1.3 Phases on acute epithelial infection. (1) IECs are central in the coordination of the response to infection. They elicit the first warning signals (e.g.: IL-8) to recruit neutrophils (PMN). (2) PMNs migrate from neighbouring vessels attracted by the IL-8 gradient and transmigrate through the epithelium to the lumen, where they induce secretion of Cl^- and fluid transport, which flushes the surface of the epithelium. (3) Other epithelium-derived CKs (e.g.: TNF-α) synergises with CKs secreted in the lamina propria to accelerate fluid transport. In phase (4), CKs released by intraepithelial lymphocytes (IEL) and lamina propria lymphocytes, initiate attenuation of PMNs accumulation. Adapted from Colgan et al. (2003).

A putative model was suggested by Colgan et al. (2003) to summarise and highlight the key role of IECs and cytokines in the development and resolution of inflammation (Fig. 1.3). Taken together, all these data underline the central role of the enterocytes in orchestrating the response to microorganisms.

1.5 Probiotics and their effect in the immune system

As mentioned before, the microbial load during the first days of life is very important as it primes the immune system for the antigens encountered later in life. If microbial stimuli are poor, there will be a defective immune maturation leading to disregulation of the innate and acquired immune systems. The mechanisms by which microorganisms may influence maturation of the host immune response and immune diseases are still poorly understood.

Probiotics have been shown to influence both innate and adaptive immunity. Some strains have been shown to increase phagocytosis and to modify CK production on different cell populations (Erickson & Hubbard, 2000; Haller et al., 2000; McCracken et al., 2002; Wallace et al., 2003). The type of modulation required to down-regulate the chronic inflammation in Crohn's disease is quite different from the one required for treatment of allergy or gastroenteritis. In inflammatory bowel disease there is an enhanced Th1 response against the own microbiota, which is characterised by high levels of pro-inflammatory CKs, e.g. IL-12. Therefore, probiotics able to increase levels of downregulating CKs such as IL-10, may be used as therapeutic supplements for treatment of IBD. In the case of cancer, the opposite may be true. IL-12 enhances the cytotoxicity mediated by NK cells against tumours (Trinchieri, 1994), which may be relevant for those lactobacilli with claimed anti-tumour activity (Kato et al., 1994). Some pathogens are able to induce IL-10, which inhibits neutrophil phagocytic and bactericidal activity as a strategy to evade host defences (Fiorentino et al., 1991; Laichalk et al., 1996). In this setting, probiotics able to enhance the inflammatory immune response (with recruitment of neutrophils, activation of macrophages and NK cells for example by inducing IL-12 production) would be more effective for clearance of the infective agent (Hessle et al., 1999; Ogawa et al., 2006). In mice, *L. casei* Shirota feeding induced a Th1 rather than a Th2 response, and this provides a means for reducing IgE when co-fed with the appropriate antigen (Matsuzaki & Chin, 2000). Most of the studies have been done on models of pathologies such as chronic inflammation, allergies, tumours and gastroenteritis. Healthy people, the most important target group of probiotics in the market, have been somewhat neglected as there are only few studies done on healthy volunteers, which can be explained by the strict safety and human ethic requirements for such studies.

Less exploited but very promising fields are the use of probiotic strains or some of their components as adjuvants for vaccines, as well as the use of genetically modified strains for delivering regulatory molecules (e.g. IL-10) or encoding proteins from pathogenic microorganisms for immunisation (Pouwels et al., 1998; Nagy et al., 2005).

Probiotic products represent a relatively economical source for boosting the immune system and covering some nutritional requirements of children in developing countries, who

suffer from immunodeficiency associated with malnutrition, and for whom gastroenteric infections are among the main causes of morbidity. In addition, children in developed countries suffering from the consequences of living in a 'too clean environment' (see 1.1.1) may also benefit from the consumption of these products.

Some rare clinical reports associate *Lactobacillus* strains with endocarditis and formation of abscesses (Maskell & Pead, 1992; Harty et al., 1994). Therefore, the characterisation and safety assessment of new strains and their effect on healthy as well as on immuno-deficient patients is very important in order to avoid any negative interaction.

1.6 Probiotics and acute gastroenteritis

Acute diarrhoea causes significant morbidity and mortality all over the world. The main etiological agents are viruses such as rotaviruses, caliciviruses, adenoviruses, Norwalk and Norwalk-like viruses and, to a lesser extent, enteropathogens. Several clinical studies have revealed that administration of probiotics such as *L. rhamnosus* GG reduces the duration of diarrhoea (Majamaa et al., 1995; Isolauri et al., 2001; Guandalini, 2002). When used as a preventive therapy in healthy infants, the consumption of probiotics reduces the number of episodes as well as their duration (Saavedra et al., 1994). These favourable clinical effects have been partly explained by the improvement of the intestinal immunological barrier, especially IgA responses by *L. rhamnosus* GG (Isolauri et al., 1993).

Bacterial infections of the gastrointestinal tract also cause diarrhoea. Probiotics could thus be used as a preventive measure, as support therapy and sometimes also as an alternative to the use of antibiotics. The strains with these preventive effects should fulfil some of the following requirements: produce antimicrobial metabolites, compete for nutrients, adhere to mucus and/or intestinal cells, and modulate the gut associated lymphoid tissue (Lee & Puong, 2002; Resta-Lenert & Barrett, 2003; Collado et al., 2005; Sherman et al., 2005). In experiments with mice, it has been shown that *L. casei*, *L. delbrueckii* and *S. thermophilus* are able to protect against *Salmonella enterica* serovar Typhimurium by inducing an increase in the sIgA levels and in the number of IgA producing plasma cells (Perdigon et al., 1991; 1995).

1.7 Study objective

Over the last decades, there has been a continuous increase in the consumption of functional foods and the market of these products is flourishing. There are already many studies about the effects of probiotics, prebiotics and synbiotics on health, but there are still a lot of unanswered questions. Not all probiotic bacteria act in the same way, their ability to inhibit pathogens and to modulate the immune system (see 1.4) are quite different and these differences are not related to the genus or the species but appear to be strain specific. Therefore, and because of safety reasons, for each single strain there should first be an intensive *in vitro* investigation of probiotic properties, which report a functional effect that justify further *in vivo* studies, which are associated with high costs and ethical requirements.

The general objective of this work was to find new probiotic *Lactobacillus* candidates to be used in functional fermented milks and to characterise the strains *in vitro* as an initial part in their development in order to elucidate their possible mechanisms of probiotic action. The partial aims of this work were:

- To isolate *Lactobacillus* strains from traditional African fermented milks and children's faeces, select those strains able to survive under simulated gastrointestinal conditions and accurately identify them by phenotypic and genotypic methods via a polyphasic taxonomic approach.
- To screen the strains for technological and probiotic properties: antimicrobial activities against pathogens, presence of β-galactosidase and bile salt hydrolase, and utilisation of prebiotics for a possible application as synbiotics.
- To study the surface characteristics of selected strains in order to investigate their possible interaction with epithelial cells via adhesion or aggregation mechanisms.
- To investigate the ability of selected strains to adhere to epithelial cells and assess the effect of such adhesion properties on possible competitive exclusion of pathogens.
- To determine whether there is a modulation by *Lactobacillus* strains or their components (LTAs, DNA; cell wall) of pathways involved in innate immunity in intestinal epithelial cells. To study the influence of these strains on innate recognition and/or responses towards *S.* Typhimurium in intestinal cells by investigating the RNA and protein expression levels.
- Based on the an evaluation of these results, to recommend probiotic strains for further probiotic development.

2 MATERIALS AND METHODS

2.1 Culture media

ABTS-Medium for determination H_2O_2 production. Composition (g/l): Fish extract 10.0; tryptone 10.0; yeast extract 5.0; Tween® 80 1.0; dipotassium hydrogen phosphate 2.0; diammonium hydrogen citrate 2.0; magnesium sulphate 0.2, manganese sulphate 0.05, sodium acetate 5.0, D(+)-glucose 15.0; agar 13.0; pH 6.5. Supplemented with 0.5 mM ABTS (2,2-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid, Boehringer, Mannheim) and 0.3 U/ml horse-radish peroxidase (Sigma, Taufkirchen).

Basal medium for sugar fermentation test (API50CH; Biomerieux, Nürtingen). Composition (g/l): Peptone from casein 5.0; meat extract 5.0; yeast extract 5.0; dipotassium hydrogen phosphate 2.0; Tween® 80 1.0; magnesium sulphate 0.2; manganese sulphate 0.05.

Basal medium for prebiotic fermentation test of probiotic strains. Composition (g/l): Peptone from casein 5.0; meat extract 5.0; yeast extract 5.0; dipotassium hydrogen phosphate 2.0; Tween® 80 1.0; magnesium sulphate 0.2; manganese sulphate 0.05.

DMEM used for culturing of HT29 colon carcinoma cells. Dulbecco's modified Eagle's medium (Invitrogen Technologies, Karlsruhe).

M17 broth (Difco, Heidelberg) for testing lactose utilisation. Composition (g/l): Peptone from soymeal 5.0; peptone from meat 2.5; peptone from casein 2.5; yeast extract 2.5, meat extract 5.0; lactose monohydrate 5.0; ascorbic acid 0.5; sodium β-glycerophosphate 19.0 and magnesium sulphate 0.25.

MacConkey agar (Oxoid, Wesel). Composition (g/l): Peptone 20.0; lactose 10.0; bile salts 1.5, NaCl 5.0; neutral red, 0.03; crystal violet 0.001; agar, 15.0.

MRS (De Man, Rogosa and Sharpe) broth (Merck, Darmstadt). Composition (g/l): Peptone from casein 10.0; meat extract 8.0; yeast extract 4.0; D(+)-glucose 20.0; dipotassium hydrogen phosphate 2.0; Tween® 80 1.0; diammonium hydrogen citrate 2.0; sodium acetate 5.0; magnesium sulphate 0.2; manganese sulphate 0.04.

MRS agar: MRS broth containing 15.0 g/l agar.

MRS-Arginine broth. Composition (g/l): universal peptone 10.0; yeast extract 5.0; D(+)-glucose 0.5; dipotassium hydrogen phosphate 2.0; Tween® 80 1.0; diammonium hydrogen citrate 20.0; sodium acetate 5.0; magnesium sulphate 0.1; manganese sulphate 0.05; arginine 3; pH 7.0 ±0.1.

PALCAM agar supplemented with *Listeria* selective supplement (Oxoid). Composition (g/l): Peptone 23.0; starch 1.0; sodium chloride 5.0; agar 13.0; yeast extract 3.0; D(-)-mannitol 10.0; ammonium iron (III) citrate 0.5; esculin 0.8; glucose 0.5; lithium chloride 15.0; phenol red 0.08. pH: 7.2±0.2. The sterile medium was cooled to 45-50°C and supplemented with 5.0 mg/l polymixin-B-sulphate; 10.0 mg/l ceftacidim and 2.5 mg/l acriflavine.

Rogosa agar (Oxoid). Composition (g/l): Peptone from casein 10.0; yeast extract 5.0; D(+)-glucose 20.0; potassium dihydrogen phosphate 6.0; ammonium citrate 2.0; Tween® 80 1.0; sodium acetate 15.0; magnesium sulphate 0.575; iron (II) sulphate 0.034; manganese sulphate 0.12; agar-agar 15.0; pH adjusted to 5.5 with acetic acid 96% (v/v). This medium was not autoclaved.

***Salmonella/Shigella* agar (Oxoid).** Composition (g/l): Peptone 10.0; lactose 10.0; ox bile 8.5; sodium citrate 10.0; sodium thiosulphate 8.5; ammonium iron(III) citrate 1.0; brilliant green 0.0003; neutral red 0.025; agar 12.0. This medium should not be autoclaved. Oxoid Ltd. Usage: for the isolation of S*almonellae* and *Shigellae*.

Standard I broth (Merck). Composition (g/l): Peptone 15.0; yeast extract 3.0; sodium chloride 6.0; D(+)-glucose 1.0; agar (not present in the broth) 15.0. Merck, Darmstadt.

Standard I agar (Merck). Standard I broth containing 15 g/l agar.

2.2 Buffers and solutions

2.2.1 Buffers and solutions used in molecular biology

Denaturing solution. 4 M guanidinium isothiocyanate (Ambion, Huntigdon, Cambridgeshire) 25 mM sodium citrate (0.75 M; pH 7; Sigma); 0.1 M β-mercaptoethanol (Sigma); 0.5 % N-lauryl sarcosinate (Sigma).

DEPC-H_2O. DEPC (diethylpyrocarbonate, Sigma) was added to redistilled water to a concentration of 0.1 % to eliminate RNases. The solution was agitated overnight and then autoclaved to eliminate DEPC. DEPC-H_2O is free of RNases.

EDTA (0.5 M). 181.6 g EDTA.$2H_2O$ in 800 ml distilled water, adjusted to pH 8 with approx. 20 g NaOH to solubilise the EDTA and then adjusted to 1 l.

GES solution. 5 M guanidinium thiocyanate (Sigma); 100 mM EDTA (Roth, Karlsruhe); 0.5% Sarkosyl; pH 8.

Loading buffer. 2.5 mg/ml bromophenol blue dye, 50 % (v/v) glycerol in 1 x TE (pH 8.9).

MOPS (10 x) buffer. 41.8 g 3 -(N-morpholino)-propane sulfonic acid (Sigma) was dissolved in 800 ml DEPC-H_2O and adjusted to pH 7.0 with NaOH. After this, 16.6 ml 3 M sodium acetate (prepared in DEPC- H_2O) and 20 ml 0.5 M EDTA (prepared in DEPC- H_2O, pH 8.0) were added and the final volume was adjusted to 1 l. The buffer was sterilised by filtration (0.2 µm) and kept protected from light at RT.

Sample buffer for RNA electrophoresis. 2 µl 10 x MOPS, 3.5 µl formaldehyde (Merck), 10 µl deionised formamide (Sigma), 1 µl 400 ng/µl ethidium bromide (Roth). Daily prepared.

TE (10 x). Composition in (g/l): Tris-HCl, 121.1; Boric acid (Roth), 61.83; EDTA, 0.76; final pH 8.

TERMLS. 10 mM EDTA, 0.2 g/l D(+)glucose; 0.015 g/ml lysozyme (L3790, Sigma); 100 U/ml mutanolysin (M9901, Sigma); 25 µg/ml RNase (R6513, Sigma).

Tris-HCl. 121.1 g Tris base (Merck) dissolved in 800 ml H_2O, adjusted to pH 8 with approx. 42 ml HCl, adjusted to 1 l and autoclaved.

2.2.2 Buffers and solutions required for sandwich ELISA

Activation reagents for TGF-β. 1 N HCl and 1.2 N NaOH/0.5 M Hepes (Sigma).

Blocking buffer for detection of TGF-β. 5 % Tween®20, 5 % sucrose in PBS pH 7.2-7.4. Freshly prepared.

Blocking buffer. 1 % Bovine serum albumin (BSA) (Roche, Mannheim, Germany) in 0.05% Tween®20 in PBS pH 7.2-7.4. Freshly prepared.

Reagent diluent for detection of TGF-β. 1.4 % delipidised bovine serum (Opticlear, Biocell laboratories), 0.05% Tween®20 in PBS pH 7.2-7.4. Freshly prepared.

Reagent diluent for IL-10 and TNF-α detection. PBS. (See 2.2.4)

Reagent diluent for IL-8 detection. 0.1 % BSA, 0.05 % Tween®20 in Tris-buffer saline (20 mM Trizma base, 150 mM NaCl) pH 7.2-7.4. The Tris-buffer can be autoclaved, the reagent diluent was freshly prepared.

Reagent diluent. 1 % BSA in PBS, pH 7.2-7.4. Freshly prepared

Stop solution. 1 N H_2SO_4.

Substrate solution. 1:1 mixture of colour reagent A (H_2O_2) and colour reagent B (Tetramethylbenzidine) from R&D Systems.

Wash buffer. 0.05 % Tween®20 (Sigma) in PBS pH 7.2-7.4.

Working solution of streptavidin-horse radish peroxidase. A 1:400 dilution of streptavidin in the corresponding reagent diluent was used.

2.2.3 Buffers and solutions required for FACS (fluorescence activated cell sorter) analysis

Antibodies. Monoclonal antibody to TLR9 FITC labelled (0.5 µg/µl, no. IMG-305C, Biomol, Hamburg), monoclonal antibody to TLR5 PE labelled (0.5 µg/µl, no. IMG-663D,

Biomol), mouse antibody to human TLR2 FITC labelled (supplied as 100 tests in 1 ml PBS/NaN_3, no. 11000-09, Biozol, Eching), mouse IgG_{2a} istotype control PE-labelled antibody (supplied as 100 tests in 1 ml PBS/NaN_3, no. 0103-09, Biozol) and mouse IgG_1 isotype control FITC-labelled antibody (supplied as 100 tests in 1 ml PBS/NaN_3, no. MCA982F, Serotech, Oxford).

Cleaning and transport solutions for FACS. FACS Clean, FACS Flow and FACS Rinse (BD Biosciences).

Lysis solution. FACS lysing solution (10 x) dilute 1:10 in steril dH_2O. (BD Biosciences, Heidelberg).

Saponin buffer. Wash buffer added with 0.5% saponin (Sigma).

Wash buffer. 1 %BSA, 0.1 % NaN_3 PBS.

2.2.4 Other buffers and solutions

Artificial duodenum solution. 6.4 g/ l $NaHCO_3$, 0.239 g/l KCl, 1.28 g/l NaCl, 0.5 % bile salts (Oxgall, Merck) and 0.1 % pancreatin (no. 77160, Fluka, Taufkirchen).

Disruption buffer. 0.05M Tris-HCl pH 8.0 containing 1 mM $MgCl_2$, 0.2 mM dithiothreitol and 50 µg/ml of each RNAse and DNAse (no. R6513 and 31135, respectively) (Sigma).

Electrolyte solution. 6.2 g/l NaCl, 2.2 g/l KCl, 0.22 g/l $CaCl_2$ and 1.2 g/l $NaHCO_3$, pH 2.5.

Phosphate buffered saline (PBS). 8 g NaCl, 0.2 g KCl, 1.44 g Na_2HPO_4 and 0.24 g KH_2PO_4 (adjusted to pH 7.4 with 1 N HCl) dissolved in 1 l water and autoclaved. For cell cultures endotoxin-free PBS was purchased from Gibco, Invitrogen.

Quarter-strength Ringer (QSR) solution. Prepared by dissolving tablets as indicated by the supplier (Merck) and diluting 1:4 in dH_2O. Solutions were autoclaved and stored at 8°C.

2.3 Isolation, selection and identification of *Lactobacillus* strains

2.3.1 Isolation of bacterial strains

One gram of freshly collected faeces from a 3-year-old and a 4-year-old child were suspended (10^{-1} dilution) in 9 ml quarter-strength Ringer solution (QRS) and diluted further in a ten-fold dilution series with QRS. One hundred microlitre aliquots of suitable ten-fold dilutions were spread plated onto Rogosa and MRS agar to enumerate bacteria and isolate predominant colonies. Plates were incubated anaerobically (Anaerocult®, Merck) for 48 h at 37°C. Several randomly selected colonies were picked from plates of the highest dilution of each sample. The morphology of strains was checked by microscopy (1000x magnification) and twenty strains with rod-shaped morphology were further sub-cultured in MRS broth and streaked out three times to check for purity. Stock cultures were kept at -80°C in MRS broth containing 20 % (v/v) glycerol.

2.3.2 Strain selection and identification

Gram-positive, catalase-negative rods from infant faeces were selected for further studies to determine if they were able to survive a successive passage through solutions mimicking saliva, gastric juice and intestinal juice (see below). In addition, strains deposited in the BFEL culture collection, which were previously isolated from two traditional fermented milk products 'Kule naoto' and 'Kwerionik' from Kenya and Uganda, respectively, were also used in this study (Table 2.1). Strains with good survival rates were further identified using phenotypic and genotypic characterisation and identification techniques as described below.

2.3.3 Phenotypic characterisation

2.3.3.1 Growth at different temperatures

Overnight cultures were inoculated (1 %) in MRS broth and incubated in a water bath at 15°C and 45°C for 5 days. Turbidity indicated growth.

2.3.3.2 Production of gas from glucose

Overnight cultures were inoculated (1 %) in MRS broth (ca. 1 x 10^6 CFU/ml) in an assay tube containing an inverted Durham's tube and incubated at 37°C for 48 h. The presence of gas in the Durham's tube indicated a heterofermentative metabolism.

Table 2.1 Strains used in this study.

Genus	Species	Strain	Origin	Use/Reference
Lactobacillus (*L.*)	spp.	BFE 5759	Kwerionik	This study. Isolates deposited by Dr. J. Nakavuma.
		BFE 5878	Kule naoto	This study. Isolates deposited by Dr. J. M. Mathara.
		BFE 5092	Kule naoto	This study. Isolates deposited by Dr. J. M. Mathara.
		BFE 6128	Kule naoto	This study. Isolates deposited by Dr. J. M. Mathara.
		BFE 6154	Kule naoto	This study. Isolates deposited by Dr. J. M. Mathara.
		BFE 1684	Human faeces	This study.
		BFE 1685	Human faeces	This study.
	johnsonii	LA1 (BFE663)	Nestlé	Good gastrointestinal survival, diminishes *H. pylori* colonisation and improves oral vaccination (Link-Amster et al., 1994; Salminen et al., 1998; Felley et al., 2001; Holzapfel et al., 2001).
	rhamnosus	GG	Valio Ltd.	Reduces duration of rotavirus and traveller's diarrhoea, immunomodulation, treatment of allergies, improvement of IBD (Kaila et al., 1992; Guandalini, 2000; Gupta et al., 2000; Kalliomaki et al., 2001). High binding to IEC (Blum et al., 1999).
	paracasei	BFE 675	Danone	Low binding to IEC (Tuomola & Salminen, 1998).
	casei	DSM20011	DSMZ	Type strain.
	casei	Shirota (BFE 688)	Yakult	Low binding to IEC (Tuomola & Salminen, 1998). Immunmodulation (Yasui et al., 1999).
	sakei	DSM20017	DSMZ	Arginine positive control strain.
Enterococcus (*Ent.*)	*faecalis*	DSM20409	DSMZ	Indicator strain for agar spot test.
	faecalis	FAIR-E155	BFEL	Bsh-positive control strain. (Franz et al., 2001)
	faecium	DSM13590	DSMZ	Pathogenic strain, vancomycin resistant (Klein, 2003). Indicator strain for agar spot test. Assays of inhibition of adhesion.
	faecium	FAIR-E179	BFEL	Bsh-negative control strain. (Franz et al., 2001)
Listeria (*List.*)	*innocua*	WS2258	Weihenstephan Inst.	Indicator strain for agar spot test.
	monocytogenes	Scott A	BFEL	Indicator strain for agar spot test. Coaggregation test. Assays of inhibition of adhesion.
Staphylococcus (*S.*)	*aureus*	ATCC 25923	ATCC	Coaggregation test. Clinical isolate
Streptococcus	*mutans*	DSM6178	DSMZ	Indicator strain for agar spot test.
Escherichia	*coli*	ATCC 43895	ATCC	Indicator strain for agar spot test.
(*E.*)	*coli*	ATCC 25922	ATCC	Clinical isolate. Enterotoxigenic strain. Assays of inhibition of adhesion. Coaggregation test.
Salmonella (*S.*)	*enterica* serovar Typhimurium	S 5489	BFFF	Indicator strain for agar spot test.
	enterica serovar Typhimurium	ATCC 14028	ATCC	Clinical isolate. Pathogenic strain. Assays of inhibition of adhesion. Coaggregation test.

2.3.3.3 Hydrolysis of arginine

Overnight cultures were inoculated (1 %) in MRS-arginine broth at 37°C for 24-72 h. For detection of ammonia, 100 µl were spotted onto a white spotting tile and an equal volume of Nessler's reagent was added. If ammonia was present, a dark orange colour appeared immediately. *L. sakei* DSM 20017 (Table 2.1) was used as an arginine positive control strain.

2.3.3.4 Presence of *meso*-diaminopimelic acid (*m*DAP) in the cell walls

One and a half millilitre of an overnight culture was centrifuged (7,500 x *g*, 10 min; Megafuge 1.0 R, Heraeus) in Eppendorf tubes, resuspended in 200 µl 4 N HCl and hydrolysed at 100°C in a heating block overnight in tightly closed tubes. HCl was removed by gently streaming nitrogen at 40 to 50°C into the solution for 1 h. The dry residue was collected in a drop of water and spotted onto a thin layer chromatography plate (pre-coated cellulose plastic sheets 20 cm x 20 cm, Merck, no. 5577). A *m*DAP standard (5 mg/ml) was also spotted as a positive control. The ascending one-dimensional chromatography was run in a solvent solution containing methanol:pyridine:10 N HCl: water (32:4:1:7). After drying, the chromatograms were developed with an acidic ninhydrin spray (0.5 % wt/v ninhydrin in 1-butanol:acetic acid [13:1]) and heated at 100°C in an oven for 5 min. mDAP is characterised by a low R_f (retention factor) and an olive green colour which changes to yellow with time and light exposure.

2.3.3.5 Determination of lactic acid enantiomers produced

Lactic acid enantiomers were determined using an enzymatic kit (Boehringer Mannheim) based on the oxidation of D-lactate or L-lactate into pyruvate in the presence of NAD^+, which in turn reduces to NADH, by the corresponding enzymes D-lactate-dehydrogenase or L-lactate-dehydrogenase. The equilibrium of these reactions lies on the side of lactate. By trapping pyruvate in a subsequent reaction catalysed by glutamate pyruvate transaminase in the presence of L-glutamate, the equilibrium can be shifted in favour of pyruvate and NADH. The amount of NADH formed in the mentioned reactions is stoichiometric to the amounts of lactic acid oxidised by the lactate-dehydrogenases. The increase of NADH was determined spectrophotometrically (spectrophotometer Bioad SmartSpec™ Plus, Munich) by measuring the increase in light absorbance at 340 nm over time.

2.3.3.6 Sugar fermentation profiles

Overnight cultures grown in MRS broth were washed twice in QRS (7,500 x *g*, 10 min) and resuspended in basal medium for API 50 CH (bioMeriéux, Nürtingen). The API 50 CH strips consist of 50 microtubes containing dehydrated carbohydrates and its derivatives (heterosides, polyalcohols, uronic acids). The microtubes are inoculated with the bacterial suspension, which rehydrates the substrates. When fermentation occurred, it was revealed by a color change in the tube, caused by the anaerobic production of acid which is detected by the pH indicator present in the medium. The first tube, which does not contain any substrate, was used as negative control. Fermentation profiles were determined according to manufacturer's instructions.

2.3.4 Genotypic characterisation

2.3.4.1 Isolation of genomic DNA from bacteria

The total genomic DNA of selected *Lactobacillus* strains was isolated according to the guanidinium isothiocyanate method of Pitcher et al. (1989) as modified by Björkroth and Korkeala (1996) for Gram-positive bacteria. Briefly, 5 ml of an overnight culture (grown in MRS) were centrifuged (17,860 x *g*, 10 min) and the pellet was resuspended in 1.5 ml 1 x TE containing 0.5 % NaCl and centrifuged again. The pellet was resuspended in 100 µl TERMLS (see 2.2.1) and incubated at 37°C for an hour. To degrade bacterial peptidoglycan, cells were lysed and protein denatured by adding 500 µl GES to the suspension and gently mixing by inversion. The preparation was incubated on ice for 5 min after which 250 µl 7.5 M ammonium acetate was added and the tubes were gently inverted before incubating for 10 min and centrifuging at 15,000 x *g* for 10 min. The upper phase was carefully removed and placed in a new Eppendorf tube. The DNA was precipitated by adding 460 µl ice cold 2-propanol and centrifuging at 15,000 x *g* for 10 min at 4 °C. The supernatant was discarded and two washes with 400 µl ethanol followed. The pellet was vacuum-dried (Vacuum-drier, Eppendorf, Hamburg) at 45°C for about 3-5 min. The DNA was resuspended in 200 µl of 10 mM Tris-HCl (pH 8).

2.3.4.2 Determination of DNA and RNA concentration and purity

The concentration of DNA and RNA in solution was determined spectrophotometrically by measuring the light absorbance *(*A) at 260 nm in UV-cuvettes (VWR International, Bruchsal). An A_{260} of 1 corresponds to approximately 50 µg/ml of dsDNA and 40 µg/ml of ssRNA (Sambrook et al., 1989). The ratio between the readings at

260 nm and 280 nm (A_{260}/A_{280}) provides information on the purity of the nucleic acid, with pure preparations of DNA and RNA having A_{260}/A_{280} ratios of 1.7 to 2.0. Appropriate dilutions were prepared for measurements. DNA solution was diluted 1:200 and RNA was diluted 1:100 before measurements. Aliquots were stored at -20°C and -80°C until use.

2.3.4.3 Rep-PCR

Rep-PCR using the primer GTG5 (5`-GTG GTG GTG GTG GTG-3`) was used for identification of strains according to methods as previously described by Gevers et al. (2001). DNA was amplified in a 50-µl volume containing 100 ng template, 1 x *Taq* DNA polymerase buffer (Amersham Pharmacia, Freiburg, Germany), 200 µM dNTPs (BioRad, München, Germany), 50 pM primer GTG5, 4 % dimethylsulphoxide (Sigma) and 1.5 U *Taq* DNA polymerase (Amersham Pharmacia). PCR products were separated by electrophoresis on 1.8 % (wt/v) agarose gels in 1 x TBE puffer (Saambrook et al., 1989). Rep-PCR products were subjected to electrophoresis for 16.5 h at 48 V. The gels were stained with ethidium bromide and gel images were captured using the Fluorchem Imager 5500 system (Alpha Innotech, USA). The digitised images were normalised and subsequently analysed using the Bionumerics (version 2.5) software package (Applied Maths, Sint-Martens-Latem, Belgium). Groupings of the rep-PCR fingerprints were performed by means of the Pearson product-moment correlation coefficient (*r*) and the unweighted pair-group method using arithmetic averages clustering algorithm (UPGMA)(Sneath & Sokal, 1973).

2.3.4.4 16 S rDNA sequence

The almost complete 16S rDNA of selected strains was amplified by PCR using the primers 16Sseqfw (5'-AGA GTT TGA TCM TGG CTC AG-3') and 16Sseqrev (5'-GGN TAC CTT GTT ACG ACT TC-3') corresponding to positions 8 to 27 and 1511 to 1491 of the 16S rDNA gene of *E. coli* (Brosius et al., 1978), respectively. DNA was amplified in 32 cycles (94°C, 1 min; 56°C, 1 min, 72°C, 2 min) in a 50 µl reaction volume containing *Taq* DNA polymerase (Amersham Pharmacia), 1 x polymerase buffer (Amersham Pharmacia), primers and dNTPs at the same concentrations as described for rep-PCR. The PCR products were cleaned using quantum prep PCR clean columns (see below) and sequenced bidirectionally at GATC Biotech (GATC, Konstanz, Germany).

2.3.4.5 Analytical scale purification of PCR products

Quantum Prep PCR Kleen spin columns (BioRad) were used to purify PCR products. The resin contained in the column was resuspended by vortexing for 5 s. The column was placed in a 2 ml wash tube and spun for 1 min in a micro-centrifuge at 735 x *g*. After this, the column was placed in a clean 1.5 ml collection tube and 25-100 µl sample was applied at the top centre of the column without disturbing the resin. The tube was spun again at 735 x *g* for 2 minutes. The purified PCR product was recovered in the bottom of the collection tube.

2.4 Testing *Lactobacillus* strains for *in vitro* probiotic properties

2.4.1 Studies on resistance to gastrointestinal conditions

2.4.1.1 Evaluation of parameters which affect survival of probiotic strains during simulated stomach passage

The Plackett–Burman (PB) statistical design was used to select parameters that can affect the survival of probiotic strains during simulated stomach passage. This statistical design allows the identification of variables responsible for main effects in a model study with relatively few experiments (Plackett & Burman, 1946).

For testing five variables at two levels in their various combinations, theoretically 25 tests would have to be done. The PB design, however, allows to determine the significance of these parameters on bacterial survival in only eight experiments (Plackett & Burman, 1946). The PB matrix for planning the eight experiments is shown in Table 2.2.

Table 2.2 Plackett-Burman matrix for 8 experiments

Run no.	A	B	C	D	E	F	G
1	+	-	-	+	-	+	+
2	+	+	-	-	+	-	+
3	+	+	+	-	-	+	-
4	-	+	+	+	-	-	+
5	+	-	+	+	+	-	-
6	-	+	-	+	+	+	-
7	-	-	+	-	+	+	+
8	-	-	-	-	-	-	-

A to G: indicate the variables. + or - : indicate one of the two levels at which each variable is tested. Run no.: indicates the experiment number.

The variables tested included: protective effect of 15 % (w/v) skim milk (Merck), medium composition (MRS or electrolyte solution), presence of lysozyme (100 ppm; Merck), presence of pepsin (0.3%; Fluka Biochemika, Steinheim), and pH of the medium. Each variable was tested at two levels: either presence or absence of each enzyme, two different pH values, using MRS broth or an electrolyte solution (see 2.2.4) resembling artificial gastric solution and finally, the probiotic candidates were either inoculated into skim milk as food matrix or into MRS broth (Table 2.3). In order to allow calculation of variance and standard error, two variables defined as "dummy" because they do not really exist, were used following the PB design (Stowe & Mayer, 1966; Lavilla et al., 1998; Fonseca et al., 2001).

Table 2.3 Variables used in the PB-design

Code	A	B	C	D	E	F	G
Variable/ Level	Protective effect of milk	Solution 2	Lysozyme	pH	Dummy	Pepsin	Dummy
-	MRS	MRS	absent	2.5	-	absent	-
+	Milk	Saline solution	present	3.0	-	present	-

L. johnsonii LA1 (BFE 663) was used as reference strain (Table 2.1), because of its well documented tolerance to gastrointestinal conditions (Salminen et al., 1998; Holzapfel et al., 2001), while other reference strains were not used. Milk or MRS broth were inoculated with an overnight culture to reach a final concentration of approximately 5×10^6 to 1×10^7 CFU/ml. One millilitre samples were taken at the beginning of each assay (T0) and after one hour (T60). The response was calculated as the difference between the log units of the initial and final counts (inhibition: log CFU/ml_{T0}−log CFU/ml_{T60}). The effect of each individual variable, the variance and the standard error were calculated as described by Stowe and Mayer (1966) and shown below.

The effect of each variable on the inhibition of *L. johnsonii* LA1 can be calculated as the difference between the mean of the inhibition values for the four experiments at the (+) level and the mean of the inhibition values for the experiments at the (-) level. As an example, the equation for calculation of the effect of variable A is shown below:

$$E_A = [I_{exp1} + I_{exp2} + I_{exp3} + I_{exp5}] / 4 - [I_{exp4} + I_{exp6} + I_{exp7} + I_{exp8}] / 4$$

E_A: Effect of variable A

$I_{expx:}$ inhibiton (log CFU/ml $_{T0}$ – log CFU/ml $_{T60}$) in experiment no. i

i: 1 to 8

The variance is calculated as the average of the squares of the inhibition values when analysing the dummy variables (E and G). The standard error is equal to the square root of the variance (Stowe and Meyer, 1966).

$$\text{Variance} = (E_E + E_G) / 2 \qquad \text{S.E.:} \sqrt{\text{variance}}$$

The significance of variable A was tested by means of the t-test for individual effects, which allows the evaluation of the probability (*P*) of finding an effect merely by chance.

$$T = E_A / \text{S.E.}$$

Probability (*P*) can be read from distribution tables using the calculated T values. If *P* is sufficiently small, it can be assumed that the inhibition was caused by the change of levels of variable A. The same calculations were also done for each variable (A, B, C, D and F). For this kind of test, a 85 % confidence level is considered significant (Stowe & Mayer, 1966; Lavilla et al., 1998; Fonseca et al., 2001).

2.4.1.2 Survival of *Lactobacillus* strains after the successive passages through artificial saliva, gastric juice and intestinal juice

The parameters determined as important for bacterial survival in the PB design were adopted in a model stomach/intestinal passage experiment in order to compare the survival of potential probiotic strains. Reconstituted skim milk (15 % wt/v, Merck) was inoculated with approximately 2 x 10^8 CFU/ml of an overnight culture. A 1-ml aliquot was removed, serially diluted in QSR (ten-fold dilution series) and spread-plated onto MRS agar to determine the CFU/ml at time 0. To simulate the dilution and possible hydrolysis of bacteria in the human oral cavity, the suspension was diluted 1:1 in a sterile electrolyte solution (see 2.2.4) to which lysozyme was added to a final concentration of 100 ppm, and incubated for 5 min at 37°C. The sample was subsequently diluted 3:5 with an artificial gastric fluid, consisting of the electrolyte solution mentioned above, adjusted to pH 2.5 and to which pepsin (no. 76190, Fluka) was added to a final concentration of 0.3 %. If required, the pH was readjusted to pH 2.5 with 5 N HCl. After 1 h of incubation at 37°C, another 1-ml aliquot was removed, serially

diluted in QSR and spread-plated onto MRS agar. To simulate the dilution in the small intestine, the remaining volume was diluted 1:4 using an artificial duodenal secretion (pH 7.2, see 2.2.4) (Zarate et al., 2000; Fernandez et al., 2003). One-millilitre aliquots were again removed after 2 and 3 h, serially diluted in QSR and spread-plated onto MRS agar to determine the CFU/ml in duplicate. Experiments were conducted in triplicate. Only strains which survived this passage were further characterised.

2.4.1.3 Resistance to 0.4 % phenol

Some aromatic amino acids derived from dietary or endogenously produced proteins can be deaminated in the gut by bacteria leading to the formation of phenols (Suskovic et al., 1997). These compounds can exert a bacteriostatic effect against some *Lactobacillus* strains. Thus, testing for the resistance to phenol may generate further information on the potential for survival of lactobacilli in gastrointestinal conditions (Xanthopoulos et al., 2000). Therefore, the ability of *Lactobacillus* strains to grow in the presence of phenol by inoculating cultures (1 % of an overnight culture) in MRS broth with and without 0.4 % phenol was tested. Serial dilutions were spread-plated (100 µl aliquots) onto MRS agar at time 0 and after 24 h of incubation at 37°C to enumerate surviving bacteria as described by Xanthopoulos et al. (2000).

2.4.2 Determination of antimicrobial potential of probiotic strains

2.4.2.1 Production of H_2O_2

Ten microlitres of overnight cultures were spotted onto ABTS-agar plates (see 2.1) and incubated anaerobically at 37°C for 72 h. After anaerobic incubation, plates were exposed to the atmosphere. Oxidative coloration of ABTS by H_2O_2 was visible as a violet halo surrounding the colony of H_2O_2 producer *Lactobacillus* strains, indicating H_2O_2 production (Marshall, 1979).

2.4.2.2 Screening for antagonistic activity

The agar spot test as described by Schillinger and Lücke (1987) was used for screening the antagonistic activity of the selected *Lactobacillus* strains. Ten microlitres of an overnight lactobacilli culture were spotted onto modified MRS agar (2 g/l glucose and 13 g/l agar) and incubated at 37°C for 24 h. These plates were overlayered with MRS soft agar (7.5 g/l agar) inoculated with ca. 1 x 10^8 CFU/ml of indicator strains (shown in Table 2.1).

The agar spot test method of Uhlman (1992) was used to test whether the inhibition zones observed in the screening for antagonistic activity were due to bacteriocin production or as a result of acid inhibition. Briefly, cell-free neutralised supernatants were obtained from overnight producer cultures grown in MRS broth at 37°C. After centrifuging the culture at 7,200 x *g* for 10 min, the supernatants were neutralised with sterile 5 M NaOH and then boiled for five min to inactivate residual viable cells. The supernatants were tested against the same indicator strains mentioned above.

2.4.3 Bile salt hydrolase activity

Bile salt hydrolase (Bsh) activity of the cultures was detected using the procedure described by du Toit et al. (1998). Briefly, ten microliters of overnight cultures were spotted onto MRS agar plates supplemented with 0.5 % (wt/v) taurodeoxycholic acid sodium salt (T0875, Sigma) and 0.37 g/l of $CaCl_2$. The plates were incubated anaerobically for 72 h, after which those strains with a white precipitation zone surrounding the colony were considered as positive (du Toit et al., 1998; Franz et al., 2001).

2.4.4 Production of β-galactosidase

The *o*-nitrophenyl-β-D-galactopyranoside (ONPG) substrate (N1127, Sigma) was used to determine β-galactosidase activity as described by Zárate et al. (2000), with modifications as described below. Those strains able to grow in M17 medium (Merck), which contains lactose as only carbon source, were harvested by centrifugation and washed twice in phosphate buffered saline (PBS) at pH 7.4. Strains growing on M17 were thought to have β-galactosidase activity, which enables utilisation of lactose and hence growth on this type of medium. The samples were adjusted to an A_{580nm} of 1.0. Hundred-µl aliquots of each of the bacterial suspensions were incubated in the presence of 2 mmol/l ONPG for 40 min in a water bath at 37°C. The reaction was stopped by addition of 1ml 0.25 M Na_2CO_3. The samples were centrifuged at 7,200 ×*g* for 10 min at 4°C and the supernatants were recovered to measure the A_{420nm}. A standard curve was obtained with *o*-nitro-phenol (ONP, Sigma) (concentrations of 0.05 to 0.5 µmol/ml in 0.05 µmol/ml increments). In order to compare the activity of those strains able to hydrolyse ONPG, cell-free extracts were prepared by disruption using a French pressure cell (SLM Aminco, SLM Instruments Inc., Lorch, Germany). Briefly, 10 ml of overnight cultures grown in M17 broth were harvested by centrifugation (7,200 × *g* at 4°C for 10 min) washed twice with buffer KH_2PO_4/Na_2HPO_4 (50 mM pH 7.25) and resuspended in the same buffer. The cell suspension was passed through the French pressure cell at 1,000 psi

pressure for at least two times. Cell debris was separated by centrifugation at 10,000 × *g* at 4°C for 10 min. Cell extracts were kept on ice until incubation with the substrate as previously described. Protein contents were determined by the method of Bradford (1976), using bovine serum albumin (Roche, Mannheim) as standard. One enzymatic unit was defined as the micromoles of ONP liberated from ONPG per milligram of protein and per min.

2.4.5 Growth on prebiotics

Overnight cultures grown in MRS were washed twice in QSR (750 x *g*) and inoculated into basal medium containing either 1 % wt/v prebiotic sugars or 1 % wt/v prebiotics together with 0.1 % wt/v glucose. The following prebiotics from Orafti (Belgium) were used: Raftilose®P95 (> 93 % oligofructose, 2-8 polymerisation degree, ≤ 6.8 % content of glucose + fructose + sucrose); Raftiline®LS (> 99 % oligofrustose, 2-60 polymerisation degree, ≤ 1 % content of glucose + fructose + sucrose); Raftiline®ST (> 90 % oligofructose, >10 polymerisation degree, ≤ 4 % content of glucose + fructose and ≤ 8 % + sucrose) and Raftiline®HP (> 99.5 % oligofructose, 2-60 ploymerisation degree, ≤ 0.5 % content of glucose + fructose + sucrose). One hundred µl of each suspension was added to each well of a 100-well-Biocreen plate and incubated at 37°C to determine growth kinetics. The A_{580nm} was measured automatically every 15 min in an automatic spectrophotometer plate reader (Bioscreen C200, Applied Biosystems).

2.5 Safety considerations: antibiotic resistance profiles of *Lactobacillus* strains

The selected strains were investigated for their antibiotic resistance profile using the E-test (Viva Diagnostika, Cologne, Germany) using MRS agar and anaerobic incubation conditions and following the manufacturer's instructions. The minimum inhibitory concentration (MIC) values used to determine whether strains were susceptible or resistant were those as suggested by the scientific Commission on Animal Nutrition (SCAN) for *Lactobacillus* spp. (Chesson et al., 2002).

2.6 Adhesion properties of selected *Lactobacillus* strains

2.6.1 Microbial adhesion to solvents

Microbial adhesion to solvents was measured using the methods described by Rosenberg et al. (1980) and Bellon-Fontaine et al. (1996) with slight modifications. Overnight cultures of lactobacilli were harvested by centrifugation at 5,000 x *g* for 5 min, washed twice

in PBS (pH 7.4), resuspended and diluted in PBS to reach an absorbance (A_0) of approx. 0.5 at 580 nm. Two ml of test solvent were added to 2 ml of the suspension and mixed by vortexing for one minute. The three test solvents used were n-hexadecane (Sigma), which is an apolar solvent, chloroform (Merck), which is a monopolar and acid solvent, and ethyl acetate (Merck), which is a monopolar and basic solvent. The aqueous phase was taken after 20 min incubation at RT and its absorbance at 580 nm was measured (A_1). The percentage of bacterial adhesion to solvent was calculated as $(1 - A_1/A_0) \times 100$.

2.6.2 Aggregation

The auto-aggregation assay was performed as described by Del Re et al. (2000) with modifications. Overnight cultures were washed as described above and the cells were suspended in either PBS pH 7.4, PBS pH 4.0, supernatant of bacteria grown overnight in MRS or in neutralised supernatant (neutralised with 1 N HCl). Two ml of each of these suspensions were vortexed for 10 s and auto-aggregation was determined after 2 h at RT. For determination of auto-aggregation, 100 µl were removed from the top of the suspension and were transferred to a cuvette containing 900 µl PBS pH 7.4. The absorbance (A_1) was measured at 580 nm. The auto-aggregation percentage is expressed as: $(1 - A_1/A_0) \times 100$, where A_0 represents the absorbance at time = 0.

2.6.3 Coaggregation

Overnight cultures of lactobacilli and pathogenic strains (Table 2.1) were washed twice with PBS pH 7.4. To each well of a 24-well microtray (Costar, VWR Int.), 500 µl of the *Lactobacillus* suspension (10^9 CFU/ml) and 500 µl of the pathogen suspension (10^9 CFU/ml) were added. After mixing, the trays were incubated at 37°C with shaking at 100 rpm for 2 h. The scoring system of 0 to 4 (0, for no coaggregation, 1 for small and dispersed clumps, 2 for medium-sized and dispersed clumps, 3 for abundant and medium sized clumps and 4 for very big clumps and clear supernatant) as described by Reid et al. (1988) was used. Each suspension was examined for aggregation microscopically at 200x and 400x magnification with an inverted microscope, though clumping at score levels of 3 and 4 could be seen macroscopically.

2.6.4 Lactobacilli adhesion to HT29 cells

Overnight cultures (2 ml) were washed twice with the same volume of PBS pH 7.4 and the bacterial cells were resuspended and diluted to a concentration of 2×10^8 CFU/ml in DMEM. Prior to the adhesion assays, confluent HT29 cell monolayers (see section 2.8.1)

grown on 8-well-chamber slides (Falcon, VWR Int.) were washed twice with PBS (pH 7.4) to remove cellular debris, FCS and antibiotics. Bacterial suspensions (2 x 10^8 CFU/ml, 363 µl per chamber) were added to the monolayers (approx. 7 x 10^5 HT29 cells, resulting in a ratio of 100 bacterial cells per HT29 cell) and incubated for one hour at 37°C and 5 % CO_2. After incubation, the cells were washed three times with 1 ml PBS (pH 7.4) to remove non-adherent bacteria. Slides were allowed to air dry for 30 min at RT, fixed with methanol (100 %, Merck) for 10 min and Gram stained (Del Re et al., 2000; Gopal et al., 2001; Nam et al., 2002). Each chamber measured 28 microscopic fields in length and 18 microscopic fields in width. Nine photographs were taken with a Leitz Camerasystem for automatic microscopy (Leitz, Wetzlar, Germany) fitted to a Leitz Aristoplan microscope. Photographs from nine different length-width co-ordinates (for different chamber wells the same coordinates were used) were taken at 1000x magnification. All HT29 cells, as well as attached bacteria, were counted and the number of adherent bacteria per 100 HT29 cells was calculated. Adherence of strains was classified according to the criteria reported by Del Re et al. (2000) as follows: non-adhesive, fewer than 5 bacteria per 100 cells; adhesive, 6 to 40 bacteria to 100 cells; strongly adhesive, more than 40 bacteria per 100 cells.

2.6.5 Competitive exclusion: adhesion inhibition assays

Changes in adherence to epithelial cells of *List. monocytogenes* Scott A, *Ent. faecium* DSM 13590, *S.* Typhimurium ATCC 14028 and *E. coli* ATCC 25922 (Table 2.1) were determined using an *in vitro* epithelial binding assay. HT29 cells were grown to 100 % confluence on 24-well plates (Falcon, VWR Int., Bruchsal) and washed twice with 1 ml PBS/well (pH 7.4). One ml of a suspension of approx. 1 x 10^8 CFU/ml lactobacilli in DMEM (without FCS and without antibiotics) was added to the wells and incubated for an hour at 37°C and 5% CO_2 to allow adhesion of the probiotic candidates. Control wells without lactobacilli were treated in the same way and incubated with DMEM without bacteria. Following the one hour incubation, *List. monocytogenes* Scott A, *Ent. faecium* DSM 13590, *S.* Typhimurium ATCC 14028 or *E. coli* ATCC 25922 were inoculated to the wells (1 x 10^5 CFU/well) and incubated under the same conditions. After a further hour incubation, non-adherent bacteria were removed by washing three times with 1 ml PBS (pH 7.4). Cells with adherent bacteria were lysed with 1.5 ml of 1 % (v/v) Triton 100-X (Merck), serial diluted and plated on to differential media (Palcam Agar for *List. monocytogenes* with *Listeria* selective supplement, MacConkey Agar for *E. coli*, *Salmonella-Shigella* Agar for *S.* Typhimurium. and MRS supplemented with 64 µg/ml erythromycin (for *Ent. faecium*).

2.7 Interaction of *Lactobacillus* strains with intestinal epithelial cells: activation of innate responses

2.7.1 Co-culture of HT29 cells with bacteria for induction of cytokines

In order to see if the selected *Lactobacillus* strains and control strains were able to induce secretion of cytokines by HT29 cells, bacterial suspensions and HT29 cells were co-cultured for 24 h at 37°C and 5 % CO_2.

HT29 cells (1 x 10^6 per well) were seeded in 6-well-plates (Falcon, VWR International) and allowed to attach and grow for 48 h. Sub-confluent monolayers (because it was shown in previous pilot studies that this was the optimal condition for studying CK induction) were washed twice with endotoxin-free PBS pH 7.4 before adding new culture medium without FCS or antibiotics to the control wells, and 2 ml of bacterial suspensions containing 5 x 10^6 CFU/ml to trial wells. These co-cultures were incubated for 24 h after which supernatants were collected, centrifuged and stored at –80°C until assayed

2.7.2 Co-culture of HT29 cells with bacteria, LPS, LTA and TNF- α for induction of cytokines

In a similar experimental set as indicated in 2.7.1, HT29 cells were treated with bacteria (same conditions as above) and 0.1 µg/ml LPS from *E. coli* (no. L4516, Sigma), 0.1µg/ml LTA from *S. aureus* (no. L2515, Sigma) or 0.1 µg/ml TNF-α (no. C63720, PromoKine, Heidelberg). These co-cultures were incubated for 24 h after which supernatants were collected, centrifuged and stored at –80°C until assayed.

2.7.3 Co-culture of HT29 cells with bacterial cell components, supernatant and bacteria pre-treated with proteinase K and KIO_4

In a similar experimental set as in 2.7.2, HT29 cells were treated with bacterial crude cell wall extracts, cell walls isolated with SDS, lipoteichoic acids, DNA, the sterile-filtered supernatant of bacteria grown for 24 h in DMEM. HT29 cultures were incubated for 24 h, after which supernatants were collected, centrifuged and stored at –80°C until assayed (see details below).

When isolating cellular components, one-way endotoxin free material or material treated with 1 N NaOH (for elimination of endotoxin) was used in order to avoid possible contaminations with endotoxin.

2.7.3.1 Isolation of crude cell walls from selected *Lactobacillus* strains

When disrupting Gram-positive bacteria with the French pressure cell, the cell walls retain functional fragments of cytoplasmic membrane and lipoteichoic acid. Since this disruption process does not shear nucleic acids, it is essential to treat the broken cell suspension with nucleases before purification of cell walls (Hancock & Poxton, 1988)

Harvested bacteria were washed twice in ice-cold 0.9 % NaCl and frozen overnight at -20°C. The thawed bacteria were resuspended in approx. 30 ml disruption buffer (see 2.2) to a final concentration of 20-30 % wet wt/v. The cell suspension was passed through the French pressure cell (SLM Aminco) at 1000 psi. Two passages were necessary to provide adequate disruption. Unbroken bacteria were removed by centrifugation at 17,000 x *g* for 1 h at 0°C and washed twice with the disruption buffer added with 0.2 M KCl. Cell walls were frozen at -80°C and lyophilised for 24 h. A 0.1 µg/ml fresh suspension was prepared in DMEM before performing the assays as described in 2.10.3.

2.7.3.2 Isolation of cell walls with SDS

Crude cell wall extracts were obtained as mentioned above and treated with boiling SDS to simultaneously inactivate autolysins and remove contaminating membrane, proteins, LTA and cytoplasmic material as described below.

The crude wall was suspended in the minimum volume possible of cold water and poured into approx.20 ml boiling 4 % SDS to a final concentration of 30 mg dry w/ml, with stirring. The suspension was kept in a boiling water bath for 15 min before recovering the walls by ultracentrifugation (45,000 x *g* for 15 min at 20°C). If the pellet remained pigmented or inhomogeneous, the SDS treatment was repeated. The wall pellet was washed repeatedly, by resuspension and centrifugation, first with 0.9 % NaCl and then with water at 20°C to remove traces of SDS. After washing, SDS-treated wall was suspended in 0.01 M Tris-HCl, pH 8.2, to 30 mg wt/ml, for trypsin digestion.

The wall suspension was mixed with trypsin to 40 U/ml and incubated with gentle mixing at 30°C overnight, with the addition of a drop of toluene to prevent microbial contamination.

Walls were recovered by ultracentrifugation (45,000 x *g* for 15 min) and washed once with boiling 1 % SDS to remove insoluble peptides. The residue was washed repeatedly with water. Cell walls were lyophilised and a 0.1 µg/ml fresh suspension was prepared in DMEM before performing the assay as explained in 2.7.3.

2.7.3.3 Isolation of LTA

Bacteria were grown in 2 l MRS and harvested by centrifugation at 10,000 x *g* for 10 min at 4°C. Pellets were washed twice in PBS and lyophilised. The lyophilisates were brought to the laboratory group of Dr. Corinna Hermann at the 'Forschungszentrum für den wissenschaftlichen Nachwuchs', University of Constance. The purification protocol (Morath et al., 2002) consisted in adding an equal volume of n-butanol (Merck) to a defrosted suspension of bacteria (in 100 ml PBS) under stirring for 30 min at RT. After centrifugation at 13,000 x *g* for 20 min, there were two phases: an upper organic and a lower aquatic phase. The aquatic phase, which contained the LTAs, was transferred to a new tube and lyophilised. The lyophilisate was resuspended in starting chromatographic buffer (15 % n-propanol in 0.1 M ammonium acetate, pH 4.7) and centrifuged at 45,000 x *g* for 15 min. The supernatant was filtered (0.22 µm) and applied onto an octyl-Sepharose column for a hydrophobic interaction chromatography. After loading the column (2.5 x 11 cm), a linear elution gradient (15 % to 60 % n-propanol in 0.1 M ammonium acetate, pH 4.7) was used. Eight-ml fractions were collected at a flow rate of 0.5 ml/min and analysed for LTA-phosphate (Ames, 1966). LTA containing fractions were pooled and lyophilised. This lyophilisate was again resuspended in starting buffer and applied onto a previously equilibrated column (1 x 5 cm, equilibrated with 35 % n-propanol in 0.1 ammonium acetate, pH 4.7). The column was eluted at a flow rate of 0.5 ml/min. After elution of the first 20 ml starting buffer, a linear salt gradient was started (0.1 M to 0.8 M ammonium acetate). Each collected aliquot was measured for phosphate (Ames, 1966) and for absorption at 260 nm; aliquots with no absorption at 260 nm but high levels of phosphate contained LTA. These LTA-containing aliquots were lyophilised and kept frozen at -80 °C until use. During the whole procedure extreme care was taken in order not to contaminate the samples with exogenous endotoxin. Therefore endotoxin-free water, endotoxin-free one-way material or materials treated with 1 N NaOH were used.

2.7.3.4 Isolation of DNA

Bacterial DNA was isolated using the same procedure explained in section 2.3.4.1 but at a larger scale. The DNA precipitate was resuspended in 200 µl ddH_2O and added to 3 ml 1 x TE. The DNA solution was transferred to the pre-cooled French pressure cell and disrupted by 1000 psi. DNA solutions were passed 2-3 times through the press. Short-chain DNA was recovered by precipitating with 2 volumes of ethanol and 1/9 vol of ammonium acetate overnight. Tubes were centrifuged (10,000 x *g* for 11 min at 4°C) and the supernatant was discarded. The precipitate was washed twice with ice-cold ethanol as explained in section

2.3.4.1. Stock DNA solutions containing 250 µg/ml were prepared. A volume of 8 µl of these solutions was added to the cell cultures as explained before (2.7.3) to reach a final concentration of 1 µg/ml.

2.7.4 Flow cytometric detection of TLRs in non-stimulated HT29 cells and HT29 cells stimulated with lactobacilli

Both TLR2 and TLR5 are transmembrane proteins present in the plasma membrane, whereas TLR9, also a transmembrane protein, is present in the endosomic membranes of certain cells, i.e. an intracellular receptor. For that reason, the detection of TLR9 includes an extra step for permeabilisation of the cells to allow access of the antibody to TLR9 (see below). All buffers and solutions needed for detection of TLRs by means of a fluorescence activated cell sorter (FACS) are described in section 2.2.3.

HT29 cells (1 x 10^6 cells/well) were allowed to attach and grow in 6-well plates for 48 h until subconfluency and were co-incubated with suspensions (1 x 10^7 CFU/well) of *L. rhamnosus* GG, *L. johnsonii* BFE 6128 or *L. plantarum* BFE 1685 or *S.* Typhimurium for 20 h, after which cells were washed in PBS and incubated for 16 h with DMEM (10 % FCS and 1 % penicillin / streptomycin).

Single cell suspensions were prepared by adding 250 µl trypsin/EDTA per well and incubating for 3-5 min to detach monolayers and obtain single HT29 cells. A volume of 1.8 ml DMEM (10 % FCS ad 1 % P/S) was added to stop trypsin activity. Cell suspensions were centrifuged (200 x *g* for 5 min), the supernatant was aspirated and new medium was added. Cell counts were determined in a Neubauer chamber. 1 x 10^6 HT29 cells were transferred to FACS tubes, centrifuged (200 x *g* for 5 min) and the supernatant was aspirated. Hundred µl PBS were added to the tubes to re-suspend the cell pellet. An individual tube containing 1 x 10^6 cells was prepared for each particular antibody staining. HT29 cells were fixed by adding 2 ml 'lysis' solution (see 2.2.3), shortly vortexed and incubated for 10 min at RT protected from light. HT29 cells were centrifuged (200 x *g* for 5 min) and washed with 2 ml wash buffer. For staining steps, 10 µl of antibody to TLR2 (no amount in µg specified by the manufacturer), 5 µl of antibody to TLR5 (=2.5 µg), 5 µl antibody to TLR9 (=2.5µg) or 7 µl of the isotypes controls (IgG_1-FITC for TLR9-Ab or IgG_2-PE for TLR2-Ab and TLR5-ab) were added to each fixed cell suspension and incubated at RT for 1 h protected from light. Cells were centrifuged (200 x *g* for 5 min) and washed with 2 ml wash buffer twice. The supernatant was aspirated and the cells were resuspended in 500 µl wash buffer. The labelled cell suspensions were vortexed for 5 sec and passed through the flow cell of a flow cytometer

(FACS, Calibur fluorescence associated cell sorter, BD Biosciences). Twenty thousand events (cells) were analysed in each run. Each cell passes individually through a highly focused laser beam of the flow cytometer. The fluorochrome of each labelled monoclonal antibody attached to the cell is excited by the laser light and emits light of a certain wavelength. The cells also scatter light at multiple angles. Photodetectors placed a forward angle and at right angles to the axis of the laser beam collect the emitted or scattered light. Forward and right angle scatter signals, and the fluorochrome signal can be detected from each cell (multiparametric analysis). For FITC-coupled antibodies, channel FL1 (absorption maximum 485 nm and emission minimum 501 nm) was used. For PE-coupled antibodies, channel FL2 (absorption maximum 488 nm and emission minimum 578 nm) was used. The intensity of the specific fluorescence can be directly related to expression of the target receptor being investigated.

For TLR9 staining the same procedure as described above was used with an additional permeabilisation step, which was done before staining the cells. This step consisted in suspending the cells in 190 µl saponin buffer (see 2.2.3) and incubating cells at RT for 20 min protected from light. As permeabilisation with saponin is a reversible phenomenon, all subsequent wash and staining steps were done in saponin buffer.

Three experiments were done; a representative histogram (obtained with the software provided with the instrument) of one such experiment is presented in the results section.

2.7.5 Treatment of HT29 cells with lactobacilli before challenge with *S.* Typhimurium

The aim of these experiments was to determine if treatment of HT29 cells with selected lactobacilli (*L. johnsonii* BFE6128 and *L. plantarum* BFE 1685) or a known probiotic strain (*L. rhamnosus* GG) changed the way in which HT29 cells respond to challenge with a pathogenic *S.* Typhimurium strain. In the first experimental set, cells were treated with lactobacilli or medium without bacteria for 2 h, followed by a four-hour treatment with medium without bacteria. In the second experimental set, cells were treated also for 2 h with lactobacilli or medium without bacteria but cells were afterwards challenged with *S.* Typhimurium ATCC 14028 for 4 h.

HT29 cells grown under the same conditions as explained in 2.7.1 were treated with 2 ml of suspensions containing 5 x 10^6 CFU/ml of *L. rhamnosus* GG, *L. plantarum* BFE1685 and *L. johnsonii* BFE6128 in DMEM or DMEM alone (negative control). After 2 h incubation (37°C, 5% CO_2), wells were washed twice with PBS and culture media (DMEM without penicillin/streptomycin nor serum) was added to the wells and incubated for further 4 h (1st experimental set) or 2 ml of a 5 x 10^6 CFU/ml (=1 x 10^7/well) suspension of *S.* Typhimurium

(2nd experimental set). Cell supernatants were collected, centrifuged to separate bacteria and freezed at -80 °C for later measurement of IL-8 and LDH. The supernatant pH was immediately measured after collecting the sample with pH strips (Merck). Total RNA was also immediately isolated from the cells by means of a modified 'Single step' protocol as described below.

2.7.6 Treatment of HT29 cells with *L. johnsonii* BFE 6128 before challenge with *S.* Typhimurium components

The aim of these experiments was to determine the components of *S.* Typhimurium to which *L. johnsonii* BFE 6128 enhanced the response of HT29 cells. Cells were treated with *L. johnsonii* BFE 6128 (1 x 10^7 CFU/well) for 2 h, followed by a four-hour treatment with medium containing different components of *S.* Typhimurium. The following components were tested: flagellin (no. tlrl-dtfla, Invivogen, San Diego) at a final concentration of 0.1 µg/ml; LPS form *S.* Typhimurium (no. L6143, Sigma); DNA from *S.* Typhimurium (isolated in this study as described in section 2.7.3.4) at a final concentration of 0.1 µg/ml and ODN2006 (no. tlrl-hodnb, Invivogen) at a final concentration of 1µM.

2.8 Cell culture conditions and isolation of peripheral blood mononuclear cells

2.8.1 Culturing of HT29 cells

The enterocyte-like HT29 cell line was obtained from the German collection of micro-organisms and cell cultures (DSMZ, Braunschweig, Germany). This cell line expresses certain differentiation features which are characteristic of mature intestinal cells: tight junctions and a typical brush border. Cells were routinely cultured in T_{80} culture flasks (Nunclon TM Δ Surface, Nunc, Roskilde) containing DMEM supplemented with 10 % (v/v) heat-inactivated (56°C, 30 min) fetal calf serum (FCS, PAA Laboratories GmbH, Cölbe) and which contained 100 U/ml penicillin G (Invitrogen) and 100 µg/ml streptomycin sulphate (Invitrogen). Cultures were incubated at 37°C under 5 % CO_2. The medium was changed every other day and cells were sub-cultured every 4 to 8 days. All media, solutions and enzymes given to the cells are pre-warmed to 37°C before use.

In order to disrupt the monolayer and detach the cells from the plastic surface of 75 cm^2 culture flasks, cells were treated with 1 ml trypsin/EDTA (0.25 % trypsin with EDTA 4Na; Invitrogen, Karlsruhe) for 3-5 min at 37°C. This incubation time is critical because a longer incubation time would be detrimental for the cells as trypsin also attacks the cell plasma membrane. In order to facilitate the activity of this proteolytic enzyme, cells were pre-

washed with PBS to eliminate serum remnants. To stop trypsin activity, cells were suspended in fresh medium containing serum. Cells were washed once, by centrifuging (200 x *g*, 5 min, 37°C) to eliminate trypsin containing medium, and the pellet was resuspended in fresh medium. Cells cannot be vortexed and therefore the careful mixing with the pipette is crucial for disaggregating cell clumps.

To determine the number of cells per ml, 20 µl of a cell suspension were mixed with 40 µl of a trypan blue solution (diluted 1:3 in PBS; Sigma-Aldrich). This vital stain allows the differentiation between dead (blue stained) and live (refractive) cells. Cells were counted under the microscope (200x) using a modified Neubauer chamber.

2.8.2 Isolation of peripheral blood mononuclear cells (PBMC)

PBMC constitutively express TLRs (Zarember & Godowski, 2002; Hayashi et al., 2003). Therefore, PBMC were isolated in order to extract their RNA as control for TLR expression.

Seven ml heparinised venous whole blood collected from healthy volunteers were carefully mixed with 3 ml PBS in 15 ml-polystyrene tubes at room temperature (RT). Four ml Ficoll-Hypaque (Sigma), which has a specific density of 1.077 g/l were carefully overlayered with 10 ml of the whole blood-PBS suspension. The tubes were centrifuged at 400 x *g* for 10 min at RT. The interfacial layer containing the PBMC was carefully removed with a sterile Pasteur pipette and collected in a new tube. RNA was isolated using the same procedure as for HT29 cells (see 2.10.2).

2.8.3 Cell vitality assays

2.8.3.1 LDH cytotoxicity test

Lactate dehydrogenase (LDH) is a stable cytoplasmatic enzyme in eukaryotic cells which is released to the cell culture supernatant only upon damage of the plasma membrane. The activity of this enzyme was measured in the collected supernatants with a cytotoxicity detection kit (Roche, Mannheim, Germany) using a spectrophotometric microtiter plate reader (ThermoMax). After incubating HT29 cells with lactobacilli, LPS, LTA, other bacterial components or TNF-α, supernatants were collected. Hundred µl of each cell-free supernatant were mixed in a 96-well microtiter plate with 100 µl substrate mixture. This mixture is provided in the kit and contains a catalyst (diaphorase/NAD^+) and a dye solution [2-*p*-(iodophenyl)-phenyltetrazolium chloride and sodium lactate]. Two enzymatic reactions take place. In the first one, LDH reduces NAD^+ to NADH by oxidation of lactate to pyruvate; in

the second reaction the catalyst (diaphorase) transfers H/H^+ to the tetrazolium salt, which turns from pale yellow into red. The formazan dye can be spectrophotometrically quantified at λ=500 nm.

2.8.3.2 MTT Test

In parallel experiments, the vitality of HT29 cells treated with bacterial suspensions was studied by means of a MTT test which relies on the reduction of 3-(4,5-dimethyldiazol-2-yl)-2,5-diphenyl tetrazolium bromide (no. M5655, Sigma) by the metabolically active mitochondrial dehydrogenases into formazan salts. HT29 cells grown in 96-well plates to 80-90 % confluence were treated with 100 µl of a suspension of each probiotic strain (5 x 10^6 CFU/ml) for 24 h. At the end of the treatment period, cells were washed with PBS three times and 100 µl of MTT (0.5 mg/ml in DMEM) were added to each well at 37°C for 4 h protected from light. Formazan salts were dissolved in 100 µl of a solution containing 10 % (v/v) SDS and 0.3 % (v/v) HCl and measured colorimetrically at 560 nm.

2.9 Cytokine ELISA

Culture supernatants were analysed for IL-1, IL-8, IL-6, MCP-1, and TGF-β, by means of sandwich ELISA with the capture antibodies, detection antibodies, standards and streptavidin-horse radish peroxidase provided by DuoSet® (R&D Systems Europe, Abingdon, U.K). In addition, culture supernatants were also analysed for IL-10 and TNF-α by means of sandwich ELISA with the capture antibodies, detection antibodies, standards and streptadividin-horse radish peroxidase provided by ELISA CytoSets® (Biosource Europe S.A., Nivelles, Belgium). All solutions not provided in the kits are listed in section 2.2.2. The procedure used is described below.

Capture antibody was diluted to the working concentration in PBS without carrier protein or in reagent diluent as suggested by the supplier for each antibody. A 96-well microplate was coated with 100 µl of the diluted capture antibody. Wells were decanted and washed 3 times with wash buffer. The liquid was completely removed every time to assure good washing performance. After the last wash, liquid had to be completely removed by inverting the plate and blotting it against clean paper towels. Plates were then blocked by adding 300 µl of blocking buffer into each well and incubated for 2 h at RT. After this, plates were washed as described above.

For TGF-β, a further activation step is needed. 160-µl samples were treated with 32 µl HCl. After 10 min incubation, 32 µl of Hepes/NaOH were added to neutralise the samples.

Hundred µl of samples or standards diluted in the corresponding reagent diluent were added to each well, and incubated for 2 h at RT. An eight point standard curve was made in duplicate using 2-fold serial dilutions in reagent diluent. For calibration curves the following highest concentrations were 2,000 pg/ml for IL-10, IL-8, TNF-α and TGF-β, 600 pg/ml for IL-6, and 250 pg/ml for IL-1. Plates were washed again as indicated above.

Hundred µl of detection antibody, diluted in reagent diluent according to the supplier's suggestions for each case, was added to each well and incubated for 2 h at RT after which plates were washed as indicated above. Then, 100 µl of working solution of streptavidin were added to each well and plates were incubated for 20 min at RT protected from light. Plates were then washed again as previously indicated and 100 µl of substrate solution were added to each well and incubated for 20 min at RT protected from light. Next, 100 µl of stop solution were added to each well. Immediately afterwards, the absorbance of each well was measured by using a microplate reader (ThermoMax, Molecular Devices, MWG Biotech, Freiburg) set to 450 nm with wave length correction set at 570 nm. All samples were measured in duplicate. For each set of samples assayed, a 4 parameter logistic standard curve was generated.

2.10 Transcriptomics

2.10.1 Working with RNA

RNases are very stable omnipresent enzymes and therefore, careful handling of RNA containing samples is necessary to avoid losses of RNA. All glass material was baked at 200 °C before use. Plastic ware was treated with RNase Away® (Roth, Karlsruhe) for 10-15 min to eliminate contaminating RNases and washed before use with DEPC-H_2O. Prior use, gel box, combs and chambers were thoroughly cleaned with RNase Away® or 3 % H_2O_2 for 15 min, rinsed with DEPC-H_2O and dried with ethanol. All working surfaces were treated with RNase Away® before use. Gloves were used in all cases as skin contains RNases.

2.10.2 Isolation of RNA from HT-29 cells by a modified 'Single-step' method

Chomczynski and Sacchi (1987) established a method for isolation of RNA from eukaryotic cells which was later modified by Meltzer et al. (1990). When trying this protocol, genomic DNA traces could still be detected. Therefore, a second treatment with DNase (Recombinant Bovine RNase free DNase 1**;** Ambion, Huntigdon, Cambridgeshire) to

hydrolyse genomic DNA and a second phenol-chloroform (125:24:1, pH 4.3-4.7; Sigma-Aldrich, Taufkirchen) treatment, two precipitation steps with isopropanol (Roth) and two wash steps with ethanol (Merck) were added to the modified protocol.

HT29 cells were grown in 6-well plates (as indicated before), the medium covering the cells was aspirated and the cells were lysed by adding 500 µl of the ice cold denaturing solution (see 2.2.1) and kept for about 5 min at RT. Cell lysates were homogenised at least 10 times with a one-way syringe and needle by aspiration and discharge of the homogenates. Homogenates were transferred into Eppendorf tubes, 650 µl of acidic phenol-chloroform-isoamylalcohol (Sigma) was given to the tubes, mixed by end-over-end rotation for at least 30 s and placed on ice for 15 min (in between steps, samples were kept on ice). The emulsion was centrifuged (11,000 x *g*, 20 min, 4 ºC) to accelerate the separation of phases. The upper aqueous phase containing RNA was carefully removed in order not to disturb the interphase where proteins, and DNA are trapped and transferred into a new Eppendorf tube carefully. An equal volume of ice cold 100 % isopropanol was added, and the tubes were kept at -20°C overnight to precipitate RNA. Tubes were centrifuged (11,000 x *g*, 20 min, 4 ºC), the supernatant was aspirated and the RNA precipitate was washed twice with 700 µl 75% ethanol. The RNA precipitate was collected by centrifugation (12,000 x *g*, 20 min, 4 ºC). Ethanol was removed with a disposable pipette tip and dried in a vacuum extractor. RNA was dissolved in 90 µl DEPC-H_2O. The RNA solution was treated for 30 min with RNase-free DNase I (Ambion Inc.) at 37°C (90 µl RNA solution, 4 µl 2 U/µl DNase and 10 µl 10x DNase I buffer) to eliminate possible DNA contamination. DNase I buffer was supplied with the enzyme and contained 100 mM Tris-HCl pH 7.5, 25 mM $MgCl_2$ and 5 mM $CaCl_2$. The DNA digestion was stopped by addition of 396 µl denaturing solution (4°C). A phenol-chloroform-isoamylalcohol extraction was repeated to eliminate DNase traces. As indicated above, RNA was precipitated again by addition of isopropanol, washed twice with ethanol, allowed to dry and resuspended in 50 µl DEPC-H_2O. RNA concentration was measured as explained in 2.3.4.2. Aliquots adjusted to 400 ng/µl were stored at -80°C until use.

2.10.3 RNA gel electrophoresis under denaturing conditions

RNA electrophoresis under denaturing conditions in formaldehyde was performed according to Schulte et al. (1994) using the MOPS buffer system. RNA under these conditions is fully denatured and migrates according to the $\log_{10}$ of its molecular weight.

For 100 ml of a 1.2 % agarose gel, 1.2 g agarose in 5 ml 10 x MOPS (see 2.2.1) and 75 ml DEPC-H2O were melted in a microwave. After cooling to approximately 60°C (hand hot) 15

ml formaldehyde were added. The samples (4.5 µl of aliquots containing 400 ng/ml) were given to 16.5 µl sample buffer for RNA (see 2.2.1) and incubated for 15 min at 65°C. Immediately after, the samples were cooled by placing them on ice for 2 min. Before loading, 2 µl loading buffer was added. Samples were loaded onto the gel and electrophoresed 90 min at 90 V in 1 x MOPS buffer until the leading bromophenol blue dye front had migrated approximately three quarters of the length of the gel. Because of the formation of toxic gases through evaporation of formaldehyde, the gel had to be run in a fume hood. Visualisation of RNA was achieved by irradiation with UV light and photographed with a video documentation system (Fluor Chem 5500, Alpha Innotech Corporation, Biozym scientific GmbH, Oldendorf).

mRNA quality has historically been assessed by electrophoresis of total RNA stained with ethidium bromide. This method relies on the assumption that rRNA quality and quantity reflect that of the underlying mRNA population. Total RNA preparations from human cells contain 95 % rRNA (28S, 18S and 5S). Quality indicators are the absence of fluorescence in the wells, the absence of smearing and the presence of two bands being the 28S RNA band twice as fluorescent as the 18S RNA one (Fig. 2.1) (Sambrook et al., 1989).

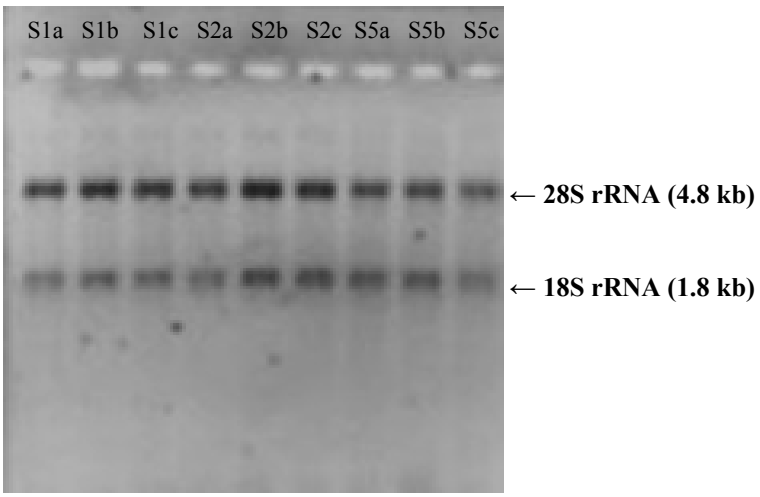


Fig. 2.1 RNA electrophoresis. S1: RNA from HT29 cells incubated with DMEM. S2: RNA from HT29 cells incubated with *S.* Typhimurium and S5: HT29 cells incubated with *L. johnsonii* BFE 6128 as explained in section 2.7.5.

2.10.4 One step protocol for RT-PCR

Retrotranscriptase PCR was performed with the Ready-to-go™ kit (Amersham Biosciences, Freiburg), which utilizes Moloney Murine Leukemia Virus (M-MuLV) and *Taq* polymerase to generate a PCR product from an RNA template. When brought to a final volume of 50 µl, each individual RT-PCR reaction contained 2 U of *Taq* DNA polymerase,

10 mM Tris-HCl (pH 9.0), 60 mM KCl, 1.5 mM $MgCl_2$, 200 µM of each dNTP, M-MuLV Reverse Transcriptase (concentration not provided by the supplier), RNAguard™ (ribonuclease inhibitor) and stabilisers, including RNase/DNase-free BSA. In each reaction a first-strand DNA synthesis was primed with an oligo(dT)primer (1 µg/µl) and the target gene was primed with a gene-specific primer (10 pmol/µl; Thermo Electron GmbH Biopolymers, Ulm). The thermal cycling parameters are specified in Table 2.4

Table 2.4 Thermal cycling parameters for RT-PCR

	Temperature (°C)	Time	Step
1.	42	30 min	Reverse transcription
2.	95	7 min	Retrotranscriptase inactivation
3.	95	45 sec	
4.	Annealing T° *	45 sec	30-40 PCR-cycles
5.	72	1 min	
6.	8		End

* Annealing temperatures are given for each individual pair of primers in Table 2.5.

Table 2.5 Primers of the target genes amplified by RT-PCR

Name, annealing temperature and size of amplicon	Fw primer	Rw primer	Source
HBD 2 (51 ºC) 254 bp	5′ CCA GCC ATC AGC CAT GAG GGT 3′	5′ GGA GGC CTT TCT GAA TCC GCA 3′	(O'Neil et al., 1999)
IL8 (52 ºC) 220 bp	5′ TTG GCA GCC TTC CTG ATT TCT 3′	5′ TTT CCT TGG GGT CCA GAC AGA 3′	(Li et al., 1998)
GAPDH (54 ºC) 306 bp	5′ CGG AGT CAA CGG ATT TGG TCG TAT 3′	5′ ACG CTT CTC CAT GGT GGT GAA GAC 3′	(Otte et al., 2004)
TLR2 (50 ºC) 638 bp	5′ CGC CAG CAA ATT ACC TGT GTG 3′	5′ CTG AGC CTC GTC CAT GGG CCA CTC C 3′	(Schaefer et al., 2004)
TLR4 (50 ºC) 449 bp	5′ TGC AAT GGA TCA AGG ACC AGA GGC 3′	5′ GTG CTG GGA CAC CAC AAC AAT CAC C 3′	(Schaefer et al., 2004)
TLR9 (50 ºC) 511 bp	5′ GCG AGA TGA GGA TGC CCT GCC CTA CG 3′	5′ TTC GGC CGT GGG TCC CTG GCA GAA G 3′	(Schaefer et al., 2004)

2.10.5 Real-time RT-PCR

Real-time PCR is one of the most sensitive and reliable quantitative methods for gene expression analysis. Data derived from real-time RT-PCR are quantified absolutely and relatively. Relative quantification is sufficient for most pathological and physiological studies. This method relies on the comparison between expression of a target gene in a control sample and the expression of the same target gene in reference samples (Yuan et al., 2006). The experimental system used in this study involved a control sample (RNA from cells incubated

in DMEM) and seven treatment samples (RNA from cells incubated as explained in 2.7.5 and summarised in Table 2.6). Real time RT-PCR was done in duplicate for each sample. The experimental set was done in triplicate. The experimental system also included the investigation of target genes (e.g. TLR2, TLR9, TLR4) and a reference gene for internal control (a housekeeping gene, here GAPDH*)* for each sample.

Table 2.6 Experimental set for gene expression studies

Sample name	Pre-treatment (2h)	Post-treatment (4h)
S1 (control)	DMEM	DMEM
S2	DMEM	*S.* Typhimurium
S3	*L. rhamnosus* GG	DMEM
S4	*L. rhamnosus* GG	*S.* Typhimurium
S5	*L. johnsonii* BFE 6128	DMEM
S6	*L. johnsonii* BFE 6128	*S.* Typhimurium
S7	*L. plantarum* BFE 1685	DMEM
S8	*L. plantarum* BFE 1685	*S.* Typhimurium

2.10.5.1 Synthesis of cDNA

cDNA was synthesised with the Omniscript Transcriptase Kit (Qiagen). Each RT-PCR reaction contained 2 µl 10 x RT Buffer, 2 µl dNTP mix, 2 µl oligo(dT) primer (10 pmol/µl), 1 µl RNase inhibitor (9.8 U/µl; diluted 1:4 in RT Buffer), 1µl Omniscript Reverse Transcriptase, 4 µl RNA-free water and 8 µl (400 ng/µl) RNA template. The reaction took place at 37°C for 1 h.

2.10.5.2 Real time-PCR general protocol

Real time-PCR was performed in MicroAmp Optical Tubes (sealed with ABIPrism optical caps, both from Applied Biosystems, Darmstadt) using a reaction volume of 25 µl and a real-time PCR kit (SYBR Green PCR Core Reagents, Applied Biosystems, Darmstadt). Each reaction consisted in 1 µl RT-PCR product (obtained as indicated in 2.10.5.1), 2.5 µl 10x SYBR Green PCR Buffer, 3 µl 25 mM MCl_2, 2 µl dNTP-mix, 1 µl of each forward and reverse primers (10 pmol/µl), 14.125 µl DEPC-H_2O, 0.25 AmpErase UNG and 0.125 µl Ampli*Taq* gold. Master mixes were prepared on ice and templates were added last. All reagents were supplied by the kit, with exception of primers (Table 2.7) and templates. The reactions were performed with the programme shown in Table 2.8 in a thermal cycler with fluorescence detection (GeneAmp® 5700 Sequence Detection System, Applied Biosystems).

A no template control (NTC) as well as a no reverse transcriptase control (NRT) were used to check for contamination of the master mix with foreign nucleic acids or for the presence of genomic DNA in RNA samples, respectively.

Table 2.7 Primers used for real-time RT-PCR.

Name, annealing temperature and size	Fw primer	Rev primer	Source
Int GAPDH (55 ºC) 207 bp	5′ CTG GAA GAT GGT GAT GGG AT 3′	5′ GGA TTT GGT CGT ATT GGG CG 3′	This study
Int TLR 2b (54 ºC) 300 bp	5′ GCA GAA GCG CTG GGG AAT GG 3′	5′ GGA TGC CTA CTG GGT GGA GAA 3′	This study
Int TLR 4b (53 ºC) 182 bp	5′ GGT GGA AGT TGA ACG AAT GG 3′	5′ CCA GCA AGA AGC ATCAGG TG 3′	This study
Int TLR9b (54 ºC) 132 bp	5′ GAG CGC AGT GGC AGA CTG GGT G 3′	5′ CAC AGG TTC TCA AAG AGG GT 3′	This study

Table 2.8 Real-time PCR programm

	Temperature	Time	Reaction
1	50°C	2 min	AmpErase UNG activity
2	95°C	10 min	Inactivation of RT-Transcriptase Ampli*Taq* Gold activation
4	95°C	20 sec	
5	See annealing temperature for each primer pair in Table 3.67	20 sec	Real-Time PCR (35-40cycles)
6	72°C	30 sec	

2.10.5.3 Calibration curves for real time PCR

TLRs are known to be expressed constitutively in PBMC (peripheral blood mononuclear cells). For this reason, PBMC were isolated from blood (2.8.2) for RNA isolation and further amplification of the mRNA coding for TLR2, TLR4, TLR9 and GAPDH. These RNA molecules were amplified with the ready-to-go one step RT-PCR. The products were purified using Quantum Prep® PCR Kleen Spin columns (See 2.3.4.5). The purified RT-PCR products were sent to GATC Biotech (Constance) for sequencing. The

identities of these products were confirmed and the sizes also correlated with the expected amplicon size.

The RNA concentration of the purified RT-PCR products was determined spectrophotometrically by measuring the A_{260nm} of a 1:40 dilution (see 2.3.4.2). A suspension containing 10^7 copies/µl of each target gene was prepared and 10-fold serial dilutions were made until 10^2 copies/µl. A real-time PCR was done using this serial dilution and a calibration cure was constructed by relating the C_t and the log of the number of copies as shown in Fig. 2.2.

The threshold cycle (C_t) for each well was calculated using the instrument's software.

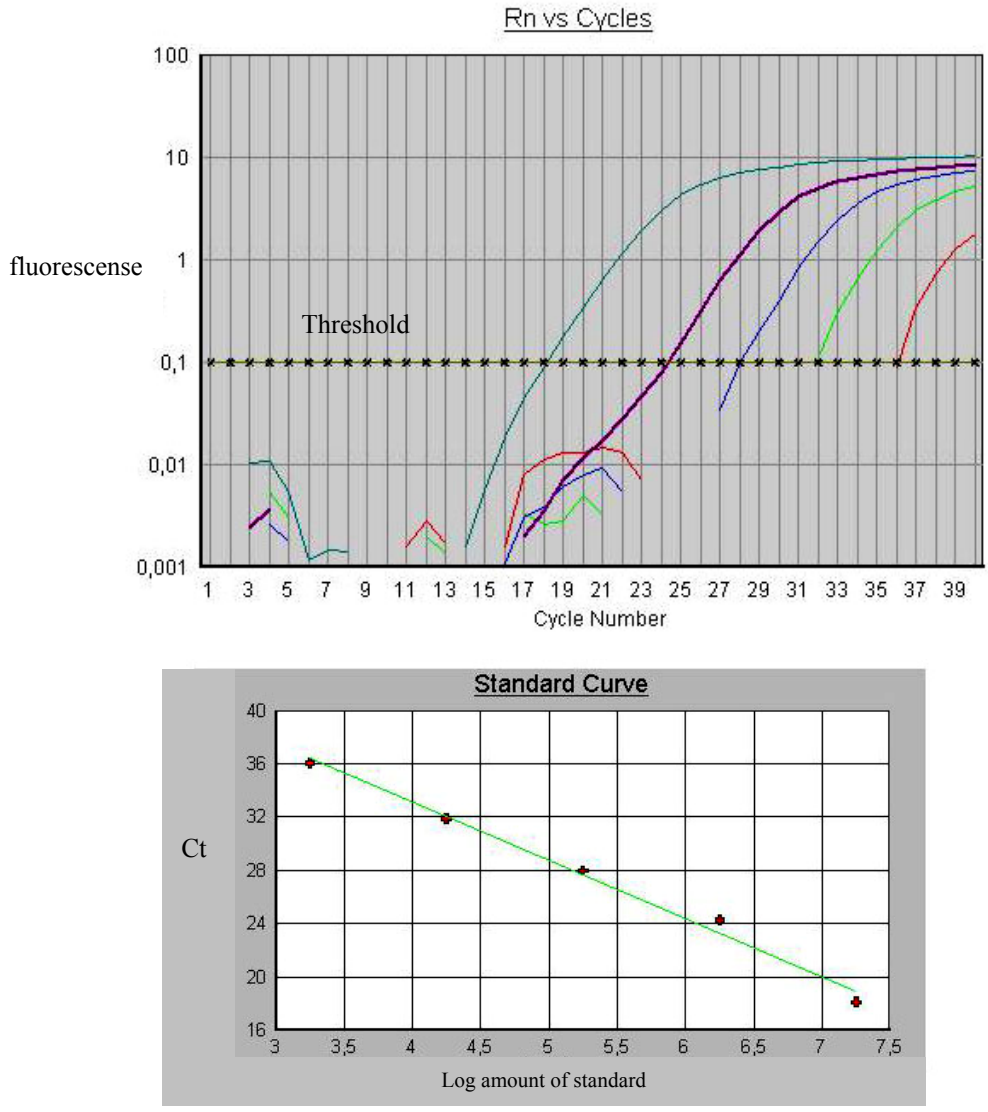


Fig. 2.2 Real-time RT-PCR calibration curve for TLR9. The C_t was plotted in function of the log of numbers of copies of template.

2.10.6 RT^2*Profiler*® PCR Array

The modulation of expression of genes involved in TLR pathways in HT29 cells stimulated with DMEM (negative control) or with *L. johnsonii* BFE 6128 alone, *S.* Typhimurium alone or after a pre-treatment with *L. johnsonii* BFE 6128 (experiment set as explained in 2.7.5) was investigated using a PCR-based TLR-pathway-focused gene expression profiler (no. APH-018A, SuperArray, Biomol) and SuperArray's RT^2 Real Time® SYBR Green PCR kit.

First strand cDNA synthesis was done using Omniscript reverse transcriptase as explained before (2.10.5.1). The cDNA synthesis reaction was diluted 1:5 (20 µl cDNA synthesis reaction + 80 µl DEPC-H_2O) and kept on ice until use.

The array consisted of a 96-well plate with 84 wells each containing specific primer pairs to detect 84 different target genes and 4 different control genes (housekeeping genes). In addition, 5 wells contained primers to detect ACTB gene expression that were used to make the standard curves to calculate the PCR efficiency of each array. Two extra wells, each containing a primer pair to detect housekeeping genes, were used for the NTC and NRC. For detection of target and control gene expression, a 1 x array master mix was prepared by mixing 1,255 µl 2 x SuperArray PCR master mix, 98 µl diluted 1st cDNA strand reaction and 1,127 DEPC-H_2O. Then, 25 µl of this array master mix were added to each well. The standard curve for ACTB was prepared by preparing 10-fold serial dilutions in the array master mix.

The plate was carefully sealed with the corresponding optical thin-wall 8-cap strips provided by the kit. All these steps were done on ice. To remove bubbles, plates were shortly centrifuged. The following real-time PCR programme was run: 1 cycle: 95 °C (necessary to activate the HotStart DNA polymerase), 10 min; 40 cycles: 95°C, 15 sec and 60°C 1 min.

The threshold cycle (C_t) for each well was calculated using the instrument's software.

Genes included in the RT^{2}Profiler PCR®Array are described in Table 2.9.

Table 2.9 Genes investigated by means of RT²Profiler® PCR Array

Group	Symbol	UniGene	RefSeq	Description	Gene Name
Adaptors and interacting proteins	BTK	Hs.159494	NM_000061	Bruton agammaglobulinemia tyrosine kinase	AGMX1/AT
	CD14	Hs.163867	NM_000591	CD14 antigen	CD14
	HMGB1	Hs.434102	NM_002128	High-mobility group box 1	DKFZp686A04236/HMG1
	HRAS	Hs.37003	NM_005343	V-Ha-ras Harvey rat sarcoma viral oncogene homolog	HRAS1/K-ras
	HSPA1A	Hs.520028	NM_005345	Heat shock 70kDa protein 1A	HSP70-1/HSP72
	HSPD1	Hs.471014	NM_002156	Heat shock 60kDa protein 1 (chaperonin)	CPN60/GROEL
	LY86 (MD-1)	Hs.567394	NM_004271	Lymphocyte antigen 86	MD-1
	LY96 (MD-2)	Hs.69328	NM_015364	Lymphocyte antigen 96	MD-2
	MAPK8IP3	Hs.207763	NM_015133	Mitogen-activated protein kinase 8 interacting protein 3	DKFZp762N1113/JIP3
	MYD88	Hs.82116	NM_002468	Myeloid differentiation primary response gene (88)	MyD88
	PELI1	Hs.7886	NM_020651	Pellino homolog 1 (Drosophila)	pellino
	RIPK2	Hs.103755	NM_003821	Receptor-interacting serine-threonine kinase 2	CARD3/CARDIAK
	SARM1	Hs.532781	NM_015077	Sterile alpha and TIR motif containing 1	SAMD2/SARM
	TICAM2	Hs.278391	NM_021649	Toll-like receptor adaptor molecule 2	TICAM-2/TIRAP3
	TIRAP	Hs.537126	NM_052887	Toll-interleukin 1 receptor (TIR) domain containing adaptor protein	Mal/wyatt
	TOLLIP	Hs.368527	NM_019009	Toll interacting protein	IL-1RAcPIP
	TICAM1 (TRIF?)	Hs.29344	NM_014261	Toll-like receptor adaptor molecule 1	PRVTIRB/TICAM-1
Effectors	CASP8	Hs.369736	NM_001228	Caspase 8, apoptosis-related cysteine peptidase	FLICE/MACH
	EIF2AK2	Hs.131431	NM_002759	Eukaryotic translation initiation factor 2-alpha kinase 2	EIF2AK1/PKR
	FADD	Hs.86131	NM_003824	Fas (TNFRSF6)-associated via death domain	GIG3/MORT1
	IRAK1	Hs.522819	NM_001569	Interleukin-1 receptor-associated kinase 1	IRAK/pelle
	IRAK2	Hs.449207	NM_001570	Interleukin-1 receptor-associated kinase 2	IRAK-2
	MAP3K7	Hs.485968	NM_003188	Mitogen-activated protein kinase kinase kinase 7	TAK1/TGF1a
	MAP3K7IP1	Hs.507681	NM_006116	Mitogen-activated protein kinase kinase kinase 7 interacting protein 1	TAB1
	NR2C2	Hs.549074	NM_003298	Nuclear receptor subfamily 2, group C, member 2	TAK1/TR2R1
	PPARA	Hs.103110	NM_005036	Peroxisome proliferative activated receptor, alpha	NR1C1/PPAR
	PRKRA	Hs.405537	NM_003690	Protein kinase, interferon-inducible double stranded RNA dependent activator	HSD14/PACT
	SITPEC	Hs.515146	NM_016581	Signaling intermediate in Toll pathway, evolutionarily conserved	ECSIT
	TRAF6	Hs.444172	NM_004620	TNF receptor-associated factor 6	MGC:3310/RNF85
	UBE2N	Hs.524630	NM_003348	Ubiquitin-conjugating enzyme E2N (UBC13 homolog, yeast)	UBC13/UbcH-ben
	UBE2V1	Hs.420529	NM_021988	Ubiquitin-conjugating enzyme E2 variant 1	CIR1/CROC-1
House-keeping genes	HPRT1	Hs.412707	NM_000194	Hypoxanthine phosphoribosyltransferase 1 (Lesch-Nyhan syndrome)	HGPRT/HPRT
	RPL13A	Hs.546356	NM_012423	Ribosomal protein L13a	RPL13A
	GAPDH	Hs.544577	NM_002046	Glyceraldehyde-3-phosphate dehydrogenase	G3PD/GAPD
	ACTB	Hs.520640	NM_001101	Actin, beta	b-Actin
IRF Pathway	CXCL10	Hs.413924	NM_001565	Chemokine (C-X-C motif) ligand 10	C7/IFI10
	IFNA1	Hs.37026	NM_024013	Interferon, alpha 1	IFL/IFN
	IFNB1	Hs.93177	NM_002176	Interferon, beta 1, fibroblast	IFB/IFF
	IFNG	Hs.856	NM_000619	Interferon, gamma	IFG/IFI
	IRF1	Hs.436061	NM_002198	Interferon regulatory factor 1	IRF-1/MAR
	IRF3	Hs.75254	NM_001571	Interferon regulatory factor 3	IRF-3
	TBK1	Hs.505874	NM_013254	TANK-binding kinase 1	NAK/T2K

JNK/p38 pathway	ELK1	Hs.181128	NM_005229	ELK1, member of ETS oncogene family	Elk1
	FOS	Hs.25647	NM_005252	V-fos FBJ murine osteosarcoma viral oncogene homolog	c-fos
	JUN	Hs.525704	NM_002228	V-jun sarcoma virus 17 oncogene homolog (avian)	AP1
	MAP2K3	Hs.514012	NM_002756	Mitogen-activated protein kinase kinase 3	MAPKK3/MEK3
	MAP2K4	Hs.514681	NM_003010	Mitogen-activated protein kinase kinase 4	JNKK/JNKK1
	MAP3K1	Hs.508461	XM_042066	Mitogen-activated protein kinase kinase kinase 1	MAPKKK1/MEKK
	MAPK8	Hs.522924	NM_002750	Mitogen-activated protein kinase 8	JNK/JNK1
NF/IL6 pathway	CLEC4E	Hs.236516	NM_014358	C-type lectin domain family 4, member E	CLECSF9/MINCLE
	PTGS2	Hs.196384	NM_000963	Prostaglandin-endoperoxide synthase 2 (prostaglandin G/H synthase and cyclooxygenase)	COX-2/COX2
NF-κB pathway	CCL2	Hs.303649	NM_002982	Chemokine (C-C motif) ligand 2	GDCF-2/GDCF-2 HC11
	CHUK	Hs.198998	NM_001278	Conserved helix-loop-helix ubiquitous kinase	IKBKA/IKK-alpha
	CSF2	Hs.1349	NM_000758	Colony stimulating factor 2 (granulocyte-macrophage)	GMCSF
	CSF3	Hs.2233	NM_000759	Colony stimulating factor 3 (granulocyte)	G-CSF/GCSF
	IFNA1	Hs.37026	NM_024013	Interferon, alpha 1	IFL/IFN
	IFNB1	Hs.93177	NM_002176	Interferon, beta 1, fibroblast	IFB/IFF
	IFNG	Hs.856	NM_000619	Interferon, gamma	IFG/IFI
	IKBKB	Hs.413513	NM_001556	Inhibitor of kappa light polypeptide gene enhancer in B-cells, kinase beta	IKK-beta/IKK2
	IL10	Hs.193717	NM_000572	Interleukin 10	CSIF/IL-10
	IL12A	Hs.673	NM_000882	Interleukin 12A (natural killer cell stimulatory factor 1, cytotoxic lymphocyte maturation factor 1, p35)	CLMF/IL-12A
	IL1A	Hs.1722	NM_000575	Interleukin 1, alpha	IL-1A/IL1
	IL1B	Hs.126256	NM_000576	Interleukin 1, beta	IL-1/IL1-BETA
	IL2	Hs.89679	NM_000586	Interleukin 2	IL-2/TCGF
	IL6	Hs.512234	NM_000600	Interleukin 6 (interferon, beta 2)	BSF2/HGF
	IL8	Hs.624	NM_000584	Interleukin 8	3-10C/AMCF-I
	LTA	Hs.36	NM_000595	Lymphotoxin alpha (TNF superfamily, member 1)	LT/TNFB
	MAP3K1	Hs.508461	XM_042066	Mitogen-activated protein kinase kinase kinase 1	MAPKKK1/MEKK
	MAP4K4	Hs.431550	NM_004834	Mitogen-activated protein kinase kinase kinase kinase 4	FLH21957/HGK
	NFKB1	Hs.431926	NM_003998	Nuclear factor of kappa light polypeptide gene enhancer in B-cells 1 (p105)	DKFZp686C01211/EBP-1
	NFKB2	Hs.73090	NM_002502	Nuclear factor of kappa light polypeptide gene enhancer in B-cells 2 (p49/p100)	LYT-10/LYT10
	NFKBIA	Hs.81328	NM_020529	Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha	IKBA/MAD-3
	NFKBIL1	Hs.2764	NM_005007	Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor-like 1	IKBL/LST1
	NFRKB	Hs.530539	NM_006165	Nuclear factor related to kappaB binding protein	DKFZp547B2013
	REL	Hs.370620	NM_002908	V-rel reticuloendotheliosis viral oncogene homolog (avian)	C-Rel
	RELA	Hs.502875	NM_021975	V-rel reticuloendotheliosis viral oncogene homolog A, nuclear factor of kappa light polypeptide gene enhancer in B-cells 3, p65 (avian)	NFKB3
	TNF	Hs.241570	NM_000594	Tumor necrosis factor (TNF superfamily, member 2)	DIF/TNF-alpha
	TNFRSF1A	Hs.279594	NM_001065	Tumor necrosis factor receptor superfamily, member 1A	CD120a/FPF
Regulation of adaptive immunity	CD80	Hs.838	NM_005191	CD80 antigen (CD28 antigen ligand 1, B7-1 antigen)	CD28LG/CD28LG1
	CD86	Hs.171182	NM_006889	CD86 antigen (CD28 antigen ligand 2, B7-2 antigen)	B7-2/B70

	RIPK2	Hs.103755	NM_003821	Receptor-interacting serine-threonine kinase 2	CARD3/CARDIAK
	TRAF6	Hs.444172	NM_004620	TNF receptor-associated factor 6	MGC:3310/RNF85
	CD180	Hs.87205	NM_005582	CD180 antigen	LY64/Ly78
	SIGIRR	Hs.501624	NM_021805	Single immunoglobulin and toll-interleukin 1 receptor (TIR) domain	TIR8
	TLR1	Hs.111805	NM_003263	Toll-like receptor 1	DKFZp547I0610/DKFZp564I0682
	TLR2	Hs.519033	NM_003264	Toll-like receptor 2	TIL4
	TLR3	Hs.29499	NM_003265	Toll-like receptor 3	TLR3
TLRs	TLR4	Hs.174312	NM_003266	Toll-like receptor 4	TOLL/hToll
	TLR5	Hs.114408	NM_003268	Toll-like receptor 5	TIL3
	TLR6	Hs.366986	NM_006068	Toll-like receptor 6	TLR6
	TLR7	Hs.443036	NM_016562	Toll-like receptor 7	TLR7
	TLR8	Hs.272410	NM_016610	Toll-like receptor 8	MGC119599
	TLR9	Hs.87968	NM_017442	Toll-like receptor 9	TLR9
	TLR10	Hs.120551	NM_030956	Toll-like receptor 10	MGC104967

2.10.7 Data analysis of gene expression modulation detected with the RT^2*Profiler*® PCR Array: the ΔΔCt method

The NTC tested for DNA contamination in the PCR system, while the NRT control tested for contamination of the original RNA with genomic DNA. If no DNA was present, both threshold cycles should be greater than 35. Thus, if a PCR product was formed reaching the detection threshold at a cycle <35, it could be assumed that the sample was contaminated with DNA.

For calculation purposes, all Ct values reported as greater than 35 or as N/A (not detected) were arbitrarily set to 35. The replicates of the same gene measured in three different arrays were used to calculate the average Ct.

ΔCt for each pathway-focused gene in each treatment group was calculated as follows:

$$\Delta Ct_{(control\ group)} = \text{average target gene Ct} - \text{average of housekeeping genes Ct}$$

for control group (HT29 cells treated only with DMEM)

$$\Delta Ct_{(treatment\ group)} = \text{average target Ct} - \text{average of housekeeping genes Ct}$$

for treatment group (e.g. HT29 cells pre-treated with DMEM or *L. johnsonii* BFE 6128 and treated with *S.* Typhimurium or DMEM)

The ΔΔCt for each gene across to PCR arrays was calculated as follows:

$$\Delta\Delta Ct = \Delta Ct_{(treatment\ group)} - \Delta Ct_{(control\ group)}$$

The fold-changes in expression (increases or decreases) for each gene from control group to treatment group was calculated as: $\mathbf{2^{-\Delta\Delta Ct}}$

Statistical analysis was done by comparing the $\Delta Ct_{(control\ group)}$ and $\Delta Ct_{(treatment)}$. Two non-parametrical tests were used: the Wilcoxon test for two-sample comparisons as suggested by Yuan et al. (2006) and the Kruskal-Wallis, which is identical to the Wilcoxon test but for multiple comparisons. Both tests were done with the SAS 9.1 software.

2.11 Statistical analysis

Data are expressed as mean ± standard error of the mean of at least three experiments done in duplicate. Statistical significance between groups was assessed by 2-way ANOVA followed by Dunnett's test for multiple comparisons to a control. In some cases, the t-test for multiple comparisons was used. For analysis of real-time data non-parametrical tests were used. Probability values *P* of <0.05 or <0.01 were taken as criteria for a significant difference as indicated in each case. Statistical analyses were performed using SAS® 9.1 Software.

3 RESULTS

3.1 Survival of lactobacilli under gastrointestinal conditions

3.1.1 Effect of parameters which affect survival of probiotic strains during a simulated stomach passage

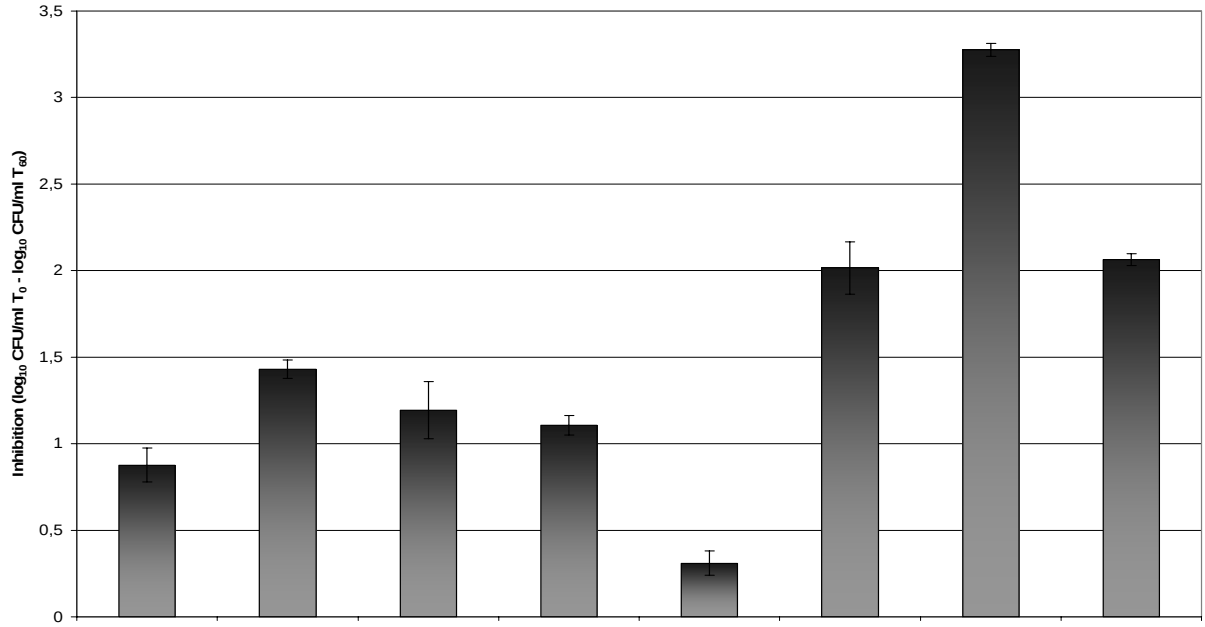
The most commonly used tests for selection of bacteria to be used as probiotics are resistance to acid and to bile. These tests are mostly done in MRS broth pH 2.0 to 3.5 and in MRS broth with bile salts (Chesson et al., 2002). In order to see which parameters should be included in an *in vitro* test of simulated stomach conditions, the Plackett-Burman design was used to test the effect of the presence of milk, the media in which the bacteria are tested, the pH and the presence of enzymes on survival of a commercial control strain (*L. johnsonii* LA-1) with known good survival to gastrointestinal conditions. The analytical results of the PB-test are summarised in Table 3.1. Confidence values corresponding to the calculated t-value with two degrees of freedom were taken from test tables (Richter, 2002).

Table 3.1 Analysis of the effect of variables in the PB-design

Variable				Effect	t-test	Confidence level
Code	**Designation**	**Low (-)**	**High (+)**		**t**	**%**
A	Protective effect of Milk	inoculum in MRS	inoculum in milk	-0,581	-0.581	95
B	Solution 2	MRS	Saline solution	-0.097	-0.097	n.s.
C	Lysozyme	no	yes	-0.496	-2.666	90
D	Effect of final pH	2.5	3.0	-0.457	-2.456	90
E	Dummy	-	-	0.224	1.204	75
F	Pepsin	no	yes	0.306	1.645	87.5
G	Dummy	-	-	0.138	0.742	n.s.
Veff= 0.03461				**S.E. Eff=** 0.186038		

n.s.: not significant

According to Stowe & Mayer (1966) and Bull et al. (1990), confidence levels greater than 85 % are acceptable for this approach. As shown in Table 3.1, both dummy variables (experiments code E and G) were not significant, which ereas the other dummy showed significance only within a 75 % confidence level. These values reflected a low experimental error. The use of either an artificial gastric electrolyte solution (solution 2) or MRS did not play a significant role in the viability (confidence level < 85 %, Table 3.1). Lysozyme, more acidic conditions (pH 2.5), pepsin and the presence of milk did have a highly significant influence (relative significance > 90 %, Table 3.1) on the viability of the strain and were therefore selected as important variables to be included in a model stomach passage survival trial for the potential probiotic strains *in vitro*. The effect of these variables correlated well with the inhibition data shown in Fig. 3.1.



Run N°	1	2	3	4	5	6	7	8
Milk	+	+	+	-	+	-	-	-
Solution 2 (pH 2.5)	MRS	Saline Solution	Saline Solution	Saline Solution	MRS	Saline Solution	MRS	MRS
Lysozyme	-	-	+	+	+	-	+	-
Final pH	3	2.5	2.5	3	3	3	2.5	2.5
Pepsin	+	-	+	-	-	+	+	-

Fig. 3.1 Effect of different variables (presence of milk, lysozyme and pepsin, medium composition and pH) on survival expressed as growth inhibition ($\log_{10}$ CFU/ml T_0 − $\log_{10}$ CFU/ml T_{60}) of *L. johnsonii* LA1 for 1 h in artificial gastric juice medium. Eight experiments were conducted according to the Plackett–Burman design. The variables used in each run are specified on the abscise. Error bars indicate standard error.

From run no. 7, it can be clearly seen that the significant variables like absence of milk, presence of lysozyme, pepsin and pH 2.5 resulted in the greatest inhibition, i.e. lowest survival. The use of MRS broth instead of milk (Fig. 3.1), combined with the absence of pepsin and pH set at 3.0, clearly resulted in lowest inhibition (highest survival) even though lysozyme was present (run no. 5, Fig. 3.1).

3.1.2 Effect of simulated gastric and intestinal passage on the viability of lactobacilli

Thirty isolates from children's faeces, 'Kule naoto' and 'Kwerionik' were screened for their survival under simulated gastro-intestinal conditions. Two *L. johnsonii* and five *L. plantarum* strains with good survival properties were chosen for further studies of probiotic properties (see 3.2 for identification results), all other strains, which did not survive gastro-intestinal conditions, were not further investigated. The effect of the successive passages through artificial saliva and gastric duodenum juices on the viability of the *L. johnsonii* strains is shown in Figure 3.2 A. Strain BFE 6154 showed the highest level of survival with 93 % of cells surviving simulated gastric and intestinal transit, whereas only 31.3 % and 11.0 % of *L. johnsonii* strain LA1 (BFE 663, a control strain with known good survival to gastrointestinal conditions) and strain BFE 6128 cells survived these conditions. The highest decline in the live counts seemed to be due to the effect of gastric juice (determined after one h incubation with simulated acid gastric juice and before addition of artificial duodenum juice), because in the following stages of incubation, the counts did not change more than approx. 1 log unit. In the case of the positive control strain, *L. johnsonii* LA1 (BFE 663), its tolerance towards gastric juice was higher than that of *L. johnsonii* BFE 6128, but the strain BFE 663 was more sensitive to artificial duodenum juice. As a result, only 31.3 % of the initial inoculum of *L. johnsonii* LA1 survived both conditions (Fig. 3.2 A). In the case of the strains belonging to the *L. plantarum* group, a high variability in their survival was noted. Some strains presented the best survival rates in this study, even higher than those observed for the *L. johnsonii* strains, for e.g., *L. plantarum* BFE 1684 with 98 % survival. *L. plantarum* K5A included in Fig. 3.2 B as a negative control and other strains (results not shown) did not survive the simulated gastric and intestinal conditions at all. These strains were therefore not chosen for further study.

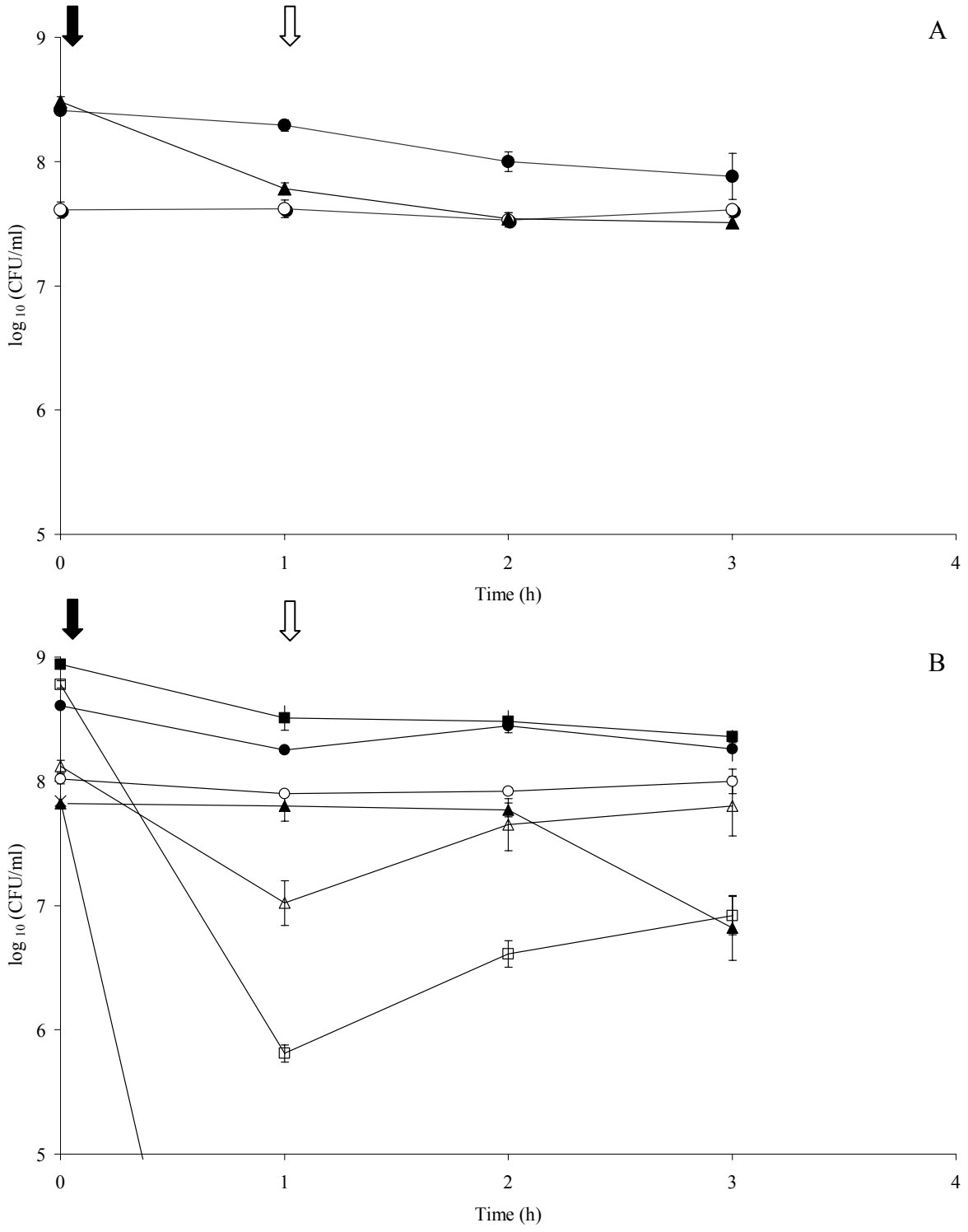


Fig. 3.2 Effect of simulated gastric and intestinal transit on viability of *L. johnsonii* and *L. plantarum* strains. A*: L. johnsonii* strains: BFE 663 (LA1) (●), BFE 6154 (○), BFE 6128 (▲). B: *L. plantarum* strains: BFE 5759 (●), BFE 1684 (○), BFE 1685 (▲), BFE 5092 (Δ), ATCC 8014 (■), BFE 5878 (□) and K5A (x). Black and white arrows indicate addition of simulated gastric juice at time 0 and simulated duodenum juice after 1 h, respectively. Error bars indicate standard error.

3.1.3 Resistance of *Lactobacillus* strains to 0.4 % phenol

Resistance to phenol was tested as an additional indicator for survival under intestinal conditions (Xanthopoulos et al., 2000). Three reference strains (*L. plantarum* ATCC 1684, *L. johnsonii* LA-1 and *L. paracasei* BFE 675) and the seven probiotic candidate strains (*L. plantarum* BFE 5759, BFE 5878, BFE 1684 and BFE 1685 and *L. johnsonii* BFE 6154 and BFE 6128, Table 3.2), which had survived the *in vitro* gastrointestinal passage as described above (3.1.2), were tested and showed different degrees of sensitivity towards phenol. *L. johnsonii* BFE 663 (LA1) and BFE 6128 were completely inhibited after 24 h, whereas *L. johnsonii* BFE 6154 was considerably inhibited (a reduction of approx. 3 log units CFU/ml) (Table 3.2). All *L. plantarum* strains were less sensitive to phenol. Four of the six strains tolerated 0.4 % phenol for 24 h, as their numbers did not decrease (initial inoculum of approx. log 7.6 to 8.0). However, none of the strains was able to grow in the presence of phenol during this incubation time (Table 3.2).

Table 3.2 Resistance of *Lactobacillus* strains to 0.4 % phenol

		Viable counts[a] (log_{10} CFU/ml)		
		MRS Broth + 0.4%phenol		
	Strain	T_0	T_{24}	Inhibition[b]
Probiotic candidates				
L. plantarum	BFE 5759	7.67±0.06	6.28±0.03	1.39
	BFE 5878	8,02±0.01	7.80±0.19	0.21
	BFE 5092	8.23±0.05	8.18±0.10	0.04
	BFE 1684	8.03±0.07	6.69±0.01	1.33
	BFE 1685	7.98±0.02	7.88±0.02	0.10
L. johnsonii	BFE 6128	7.71±0.03	0.00±0.00	7.71
	BFE 6154	7.74±0.03	4.72±0.01	3.02
Reference strains				
L. plantarum	ATCC 8014	7.58±0.05	7.14±0.07	0.44
L. johnsonii	BFE 663	7.92±0.11	0.00±0.00	7.92
L. paracasei	BFE 675	8.21±0.03	6.93±0.02	1.29

[a]Log means counts of three trials (average±S.E.)

[b]Inhibition = Log_{10} (initial population) - Log_{10} (final population)

3.2 Selection of strains and identification

Gram-positive, catalase-negative and rod-shaped bacteria which were able to survive the passage through simulated gastric and duodenum juice in the *in vitro* gastrointestinal model, were further identified, and their probiotic characteristics were studied.

The selected strains were identified to the species-level by both phenotypic and genotypic investigations. The only two strains (BFE 1684 and BFE 1685) selected for further studies which stemmed from children's faeces showed phenotypic properties typical of *Lactobacillus plantarum* strains. Accordingly, these bacteria were rod-shaped, produced DL-lactate, possessed *m*DAP in the cell walls and did not produce gas from glucose fermentation (Table 3.3). These strains were able to ferment ribose, a pentose sugar, which implies that the strains were facultatively heterofermentative lactobacilli. In addition, three strains from African fermented dairy products (BFE 5878, BFE 5759 and BFE 5092) also showed similar characteristics, indicating that these could also be characterised as presumptive *L. plantarum* strains. These three strains not only fermented ribose, but also an additional pentose sugar, i.e., arabinose (Table 3.3). Two other strains (*L. johnsonii* BFE 6154 and BFE 6128) which stemmed from 'Kule naoto' exhibited characteristics typical of lactobacilli of the *L. acidophilus*-group. These strains did not produce gas from glucose and did not ferment the pentose sugars tested (Table 3.3), indicating that they were obligately homofermentative lactobacilli. They did not grow in MRS broth at 15°C but at 45°C. Furthermore, they did not contain *m*DAP in the cell wall and produced DL- lactate (Table 3.3).

Analysis of the rep-PCR fingerprints showed that the presumptive *L. plantarum* strains (BFE 1684, BFE 1685, BFE 5878, BFE 5759, BFE 5092) clustered very closely at a correlation value r = 73.5 %, together with the *L. plantarum* DSM 20174^T type strain and the *L. plantarum* ATCC 8014 and DSM 20686 reference strains, confirming their characterisation as *L. plantarum* (Fig. 3.3). The 16S rDNA gene of one of these strains (BFE 1685) was sequenced and showed 99.9 % identity in the nucleotide sequence to the 16S rDNA gene of *L. plantarum* DSM 20174^T (result not shown). This also confirmed the identification of these strains grouping together in the rep-cluster as *L. plantarum*. The strains BFE 6128 and BFE 6154 isolated from African fermented milk grouped together with *L. johnsonii* DSM 10533^T type strain at r = 83.2 % (Fig. 3.3), indicating that these strains could also be identified as *L. johnsonii*.

Table 3.3 Phenotypic grouping and key characteristics of *Lactobacillus* strains (only those relevant for obligately homofermentative and facultatively heterofermentative lactobacilli are included):

Group A[a]

Strain	Gas from Glucose	*m*DAP	Lactic acid Isomer	NH_3 from Arginine	Growth (°C) 15/45	Carbohydrates fermented											
						Amygdalin	Cellobiose	Galactose	Lactose	Maltose	Mannitol	Mannose	Melibiose	Raffinose	Salicin	Sucrose	Trehalose
L. johnsonii																	
BFE 6128	-	-	DL	-	-/+	+	+	-	-	+	-	-	-	-	+	+	+
BFE 6154	-	-	DL	-	-/+	+	+	-	-	+	-	-	-	-	+	+	+

Group B[b]

Strain	Gas from Glucose	*m*DAP	Lactic acid Isomer	NH_3 from Arginine	Growth 15 / 45 (°C)	Carbohydrates fermented													
						Amygdalin	D-Arabinose	L-Arabinose	Cellobiose	Esculin	Gluconate	Mannitol	Melezitose	Melibiose	Ribose	Raffinose	Sorbitol	Sucrose	Xylose
L. plantarum																			
BFE 1684	-	+	DL	-	+/+	+	-	+	+	+	+	+	+	+	+	-	+	-	-
BFE 1685	-	+	DL	-	+/-	+	-	-	+	+	+	+	+	-	+	-	-	+	-
BFE 5878	-	+	DL	-	+/-	+	-	+	+	+	+	+	+	+	+	+	+	-	-
BFE 5759	-	+	DL	-	+/-	+	-	+	+	+	+	+	+	+	+	+	+	-	-
BFE 5092	-	+	DL	-	+/-	+	-	+	+	+	+	+	+	+	+	+	+	+	-

[a]: obligately homofermentative

[b]: facultatively heterofermentative

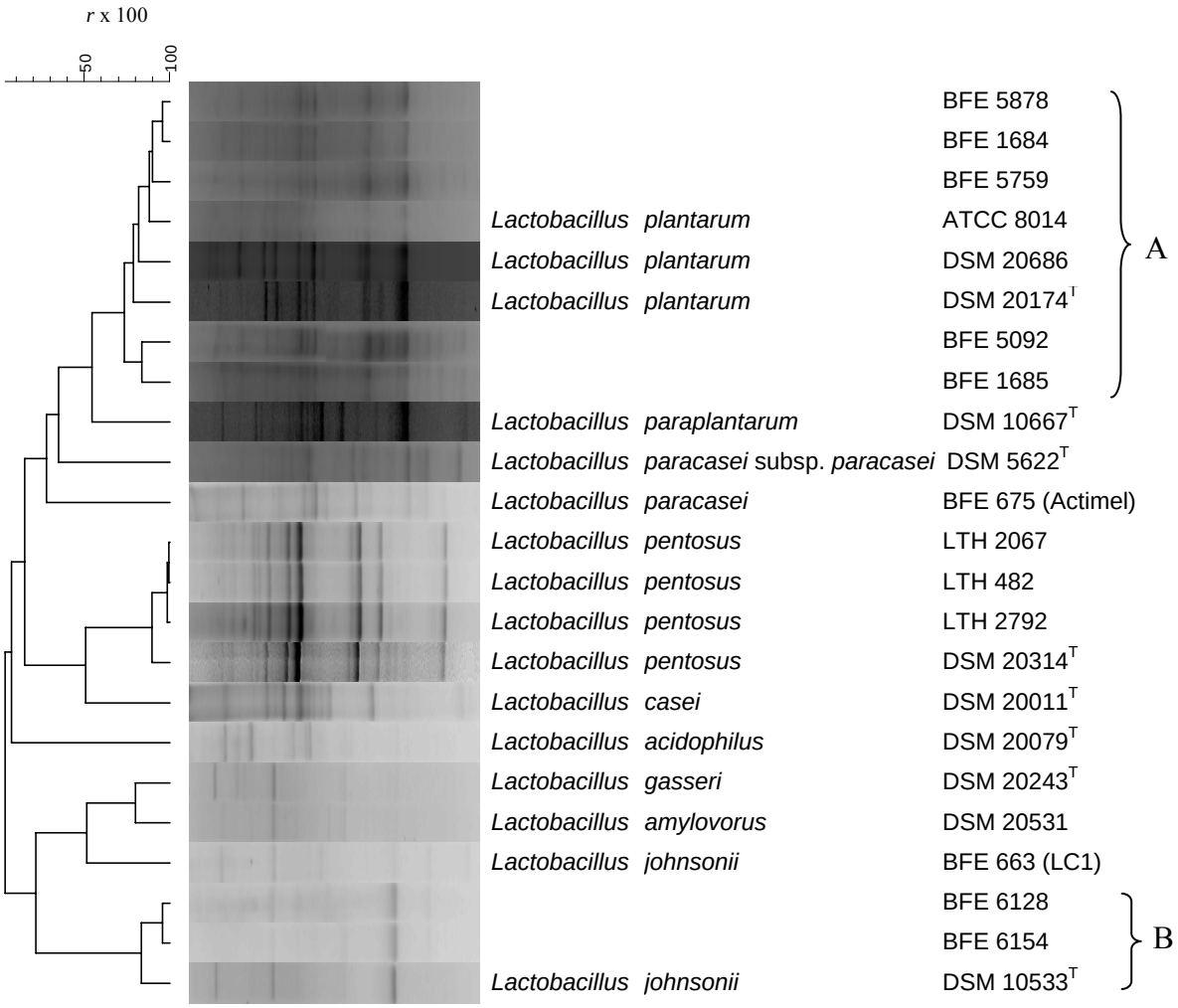


Fig. 3.3 Dendrogram obtained by UPGMA clustering of correlation value *r* of rep-PCR fingerprint patterns of potentially probiotic isolates and appropriate reference strains obtained with primer GTG5. ^{T}: Type strain. A: *L. plantarum* cluster, B: *L. johnsonii* cluster.

In addition, strains BFE 6128 and BFE 6154 also grouped with *L. johnsonii* LA1, *L. amylovorus* DSM 20531 and *L. gasseri* DSM 20243^{T}, which indicates the close relationship of these species as they, together with *L. acidophilus, L. gallinarum* and *L. crispatus,* make up the *L. acidophilus*-group. The sequencing of the 16S rDNA gene of one of the two strains (BFE 6128) confirmed this identification, as this strain showed 99.5 % similarity in the nucleotide sequence when compared to the *L. johnsonii* DSM10533^{T} type strain 16S rDNA sequence (result not shown).

3.3 Antagonistic activity against pathogens

In the agar spot test, the indicator strains, *Salmonella* Typhimurium DSM 5489, *E. coli* ATCC 43895, *List. innocua* WS 2258, *Ent. faecalis* DSM 20409, *Ent. faecium* DSM 13590, showed weak to strong inhibition (zone of inhibition of more than 1 mm from edge of producer colony up to 10 mm) as listed in Table 3.4. *Streptococcus mutans* DSM 6178 was not inhibited at all by three of the *L. plantarum* strains tested, inhibited weakly by two *L. plantarum* strains, and medium to strongly inhibited by the *L. johnsonii* strains tested (Table 3.4). The indicator strains belonging to the *Lactobacillus* genus were weakly or not inhibited (inhibition zone of less than 2 mm). The inhibitory activity was not due to bacteriocin production, because neutralised, cell-free supernatant of the producer culture did not exhibit any antimicrobial activity when compared to the effect of live cells in the agar spot test. Therefore, the inhibitory activity observed probably depended on production and diffusion of organic acids into the medium. Hydrogen peroxide could hypothetically also act as an inhibitory substance, but the incubation of the plates under anaerobic conditions discards this as a possible cause for the observed inhibition.

Production of H_2O_2 under aerobic conditions was also investigated. Only two of the *L. plantarum* strains tested (BFE 5878 and BFE 1684), but both *L. johnsonii* strains were able to produce H_2O_2 (Table 3.5). The reference probiotic strain, *L. johnsonii* LA1 (BFE 663), used as a control in investigations of probiotic properties, did not produce H_2O_2.

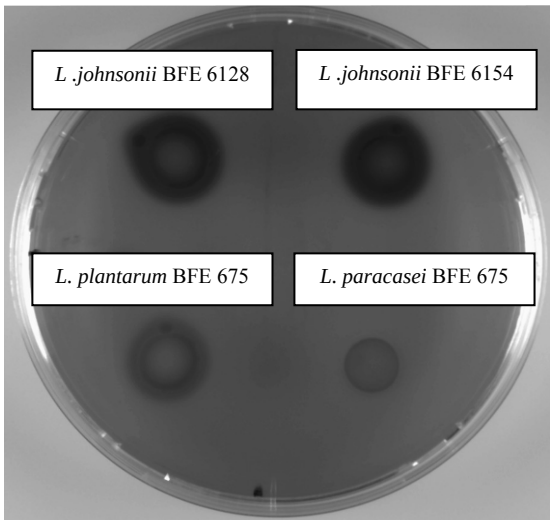


Fig. 3.5 Production of H_2O_2 by *Lactobacillus* spp. under aerobic conditions. Overnight cultures were spotted onto ABTS-agar plates containing 0.5 mM 2,2´-azino-bis(3-ethylbenzthiazoline-6)-sulfonic acid and 0.3 units/ml horse radish peroxidase. Positive strains were surrounded by a purple halo. *L. paracasei* BFE 675 did not produce H_2O_2 as indicated by the absence of a purple zone surrounding the colony.

Table 3.4 Agar spot test for detection of antagonistic activity[a]

Probiotic candidates	Strains	*S.* Typhimurium DSM 5489	*E. coli* ATCC 43895	*List. innocua* WS 2258	*Ent. faecium* DSM 13590	*Ent. faecalis* DSM 20409	*Streptococcus mutans* DSM 6178	*L. johnsonii* BFE 663	*L. paracasei* BFE 675	*L. casei* DSM 20011
L. plantarum	BFE 1684	8	4	6	10	14	0	0	0	2
	BFE 1685	6	4	8	6	20	2	2	0	2
	BFE 5092	6	2	6	6	12	2	0	2	0
	BFE 5878	10	4	4	8	12	0	0	0	2
	BFE 5759	10	4	6	10	14	0	0	0	2
L. johnsonii	BFE 6128	13	4	8	6	8	6	2	0	2
	BFE 6154	8	2	10	4	12	12	2	0	0

[a]: Inhibitory activity expressed as zone of inhibition surrounding the colony in mm.

3.4 Bile salt hydrolase and β-galactosidase activities

All seven candidate probiotic strains were tested for their ability to hydrolyse the sodium salt of taurodeoxycholic acid. Bsh positive strains were surrounded by a white precipitation zone as shown in Fig. 3.6. All strains possessed bile salt hydrolase activity (Table 3.5), and in connection with this, it was noted that all these strains also exhibited high resistance to duodenum juice containing 0.5 % bile salts in the gastrointestinal passage model, which may be connected with this Bsh activity as suggested by some authors (De Smet et al., 1995; De Smet et al., 1998; De Boever et al., 2000).

Not all the strains in this study were capable of growth on M17 agar medium (Table 3.5). Only those strains which were able to grow on lactose as the sole C source tested on this medium were further investigated for β-galactosidase activity. The highest activity (9.3 $\mu mol.ml^{-1} .min^{-1}$) was determined for the reference strain *L. plantarum* ATCC 8014 (Table 3.5). This strain was followed by the human faecal isolate *L. plantarum* BFE 1684, which also exhibited a high β-galactosidase activity of 8.3 $\mu mol.ml^{-1}.min^{-1}$. *L. johnsonii* strains, on the other hand, were not capable of growth in M17 medium containing lactose as sole C source (Table 3.5).

Fig. 3.6 Examples of a Bsh-negative strain, *L. paracasei* BFE 675, and a Bsh-positive strain, *L. plantarum* BFE 5759, showing a large zone of white precipitation around the colony.

Table 3.5 H_2O_2 production and enzymatic activities of reference strains and selected potentially probiotic *L. plantarum* and *L. johnsonii* strains

Strains		H_2O_2 production	β-galactosidase		Bsh
			Growth in lactose as C source	β-galactosidase activity (µmoles.ml^{-1}.min^{-1}	
Probiotic candidates					
L. plantarum	BFE 5092	-	+	<D.L[a]	+
	BFE 5878	+	+	<D.L.	+
	BFE 5759	-	+	5.95	+
	BFE 1684	+	+	8.31	+
	BFE 1685	-	-	n.d.	+
L. johnsonii	BFE 6154	+	-	n.d.	+
	BFE 6128	+	-	n.d.	+
Reference strains					
L. plantarum	ATCC8014	-	+	9.3	+
L. johnsonii	BFE 663	-	-	n.d.	+
L. paracasei	BFE 675	-	+	0.08	-

[a]: <D.L.: lower than the detection level. n.d.: not determined.

3.5 Antibiotic Resistance

All strains were resistant to streptomycin and gentamicin, as well as to ciprofloxacin (Table 3.6). In contrast, all strains were considered as susceptible to the antibiotics erythromycin, ampicillin, penicillin, benzylpenicillin, tetracycline and chloramphenicol (Table 3.6), when using the MIC breakpoint values as suggested by SCAN (Chesson et al., 2002).

As an example, the E-test results for determination of antibiotic resistance of *L. johnsonii* BFE 6128 is shown in Fig. 3.7.

Table 3.6 Antibiotic Resistance of reference strains and selected potentially probiotic *L. plantarum* and *L. johnsonii* strains

Strains		Minimum inhibitory concentration (µg/ml)							
		EM	**GM**	**AB**	**TC**	**CL**	**SM**	**CI**	**PG**
Probiotic candidates									
L. plantarum	BFE 5092	1.0	256	0.064	6.0	2.0	256	32	0.38
	BFE 5878	1.0	256	0.064	6.0	2.0	256	32	0.38
	BFE 5759	1.0	128	0.032	3	2	256	32	0.75
	BFE 1684	0.75	48	1.0	0.25	1.5	128	8	0.125
	BFE 1685	0.75	256	0.064	3	2	256	32	0.25
L. johnsonii	BFE 6128	1.0	256	0.047	4	1.5	256	32	0.19
	BFE 6154	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Reference strains									
L. johnsonii	BFE 663	0.25	256	0.19	0.25	1.5	32	32	0.064
L. paracasei	BFE 675	0.5	24	0.38	0.75	2	42	12	0.125
Breakpoint value[a]		4	1	2	16	16	16	4	2

EM: Erythromycin, GM: Gentamicin, AB: Ampicillin, TC: Tetracycline, CL: Cloramphenicol, SM: Streptomycin, CI: Ciprofloxacin, PG: Benzylpenicillin. n.d.: not determined.

[a]*:* breakpoints according to SCAN (2002).

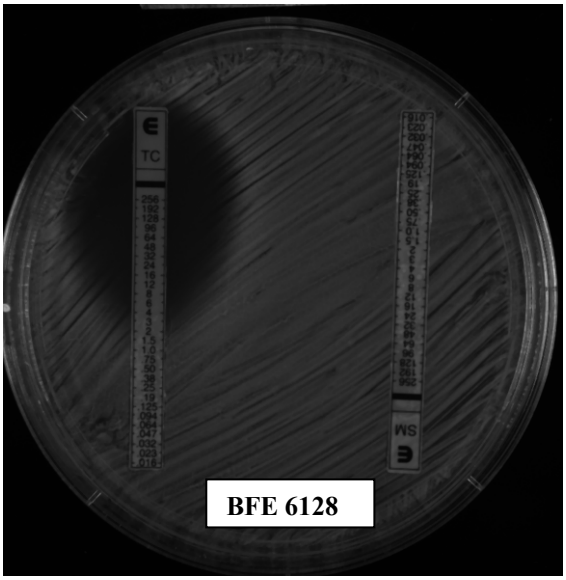


Fig. 3.7 E-test for determination of antibiotic resistance of *L. johnsonii* BFE 6128. This strain was sensitive to tetracycline (left) but resistant to streptomycin (right).

3.6 Growth of selected probiotic candidates on prebiotics

Prebiotics are oligosaccharides which selectively enhance the growth of beneficial inhabitants of the gut, such as *Lactobacillus* spp. and *Bifidobacterium* spp. These bacterial species are able to utilise these sugars, which otherwise cannot be digested in the intestine by the human host. To see if the selected *Lactobacillus* strains of this study were able to utilise commercial prebiotics, the growth of these strains on a minimal medium containing prebiotics as main carbon source was monitored. In parallel, the growth of these strains in medium supplemented with both glucose and prebiotics was also tested in order to see if the presence of glucose in limited concentration favoured the growth on prebiotics, and to evaluate the effect of residual sugars (2-12 % wt/v) present in the prebiotic preparations. Basal medium (BM) was supplemented with 1 % wt/v prebiotic sugar and therefore the highest concentration of fructose, glucose or sucrose present in the supplemented medium was approximately 0.1 % wt/v. A control growth curve was done in basal medium supplemented with 0.1 % glucose in order to determine if the growth observed with the prebiotic sugars was due to the residual glucose present.

3.6.1 Growth of *L. johnsonii* probiotic candidate strains on prebiotics

L. johnsonii BFE 6128 and BFE 6154 strains were apparently able to grow on Raftiline ST, Raftiline LS and Raftilose (Fig. 3.8), because the growth curves obtained when these oligosaccharides were added to basal medium (BM) reached a higher A_{580nm} than on BM alone (Fig. 3.8). But when comparing with the growth observed when the BM was supplemented with 0.1 % glucose, the highest A_{580nm} of approx. 0.5 obtained on Raftilose and Raftiline LS alone were also obtained when grown on BM containing 0.1 % glucose. Therefore, it may be assumed that the growth on these prebiotic sugars was actually due to growth on the residual sugars. When medium was supplemented with Raftiline ST with and without 0.1 % glucose, the growth of both *L. johnsonii* strains was higher then the growth on the respective control curves as shown by the highest A_{580nm} of approx. 0.7 and 0.9, respectively.

The presence of glucose in limited concentration did not change the behaviour towards the prebiotic sugars tested. The only observed change was on growth on Raftilose, where the second lag phase disappeared when glucose (in limited conc.) was present. Nevertheless the higher growth observed with respect to the growth on Rafitlose + 0.1 % glucose may again be

explained as a result of the residual sugars. In conclusion, only Raftiline ST favoured growth of the *L. johnsonii* strains tested.

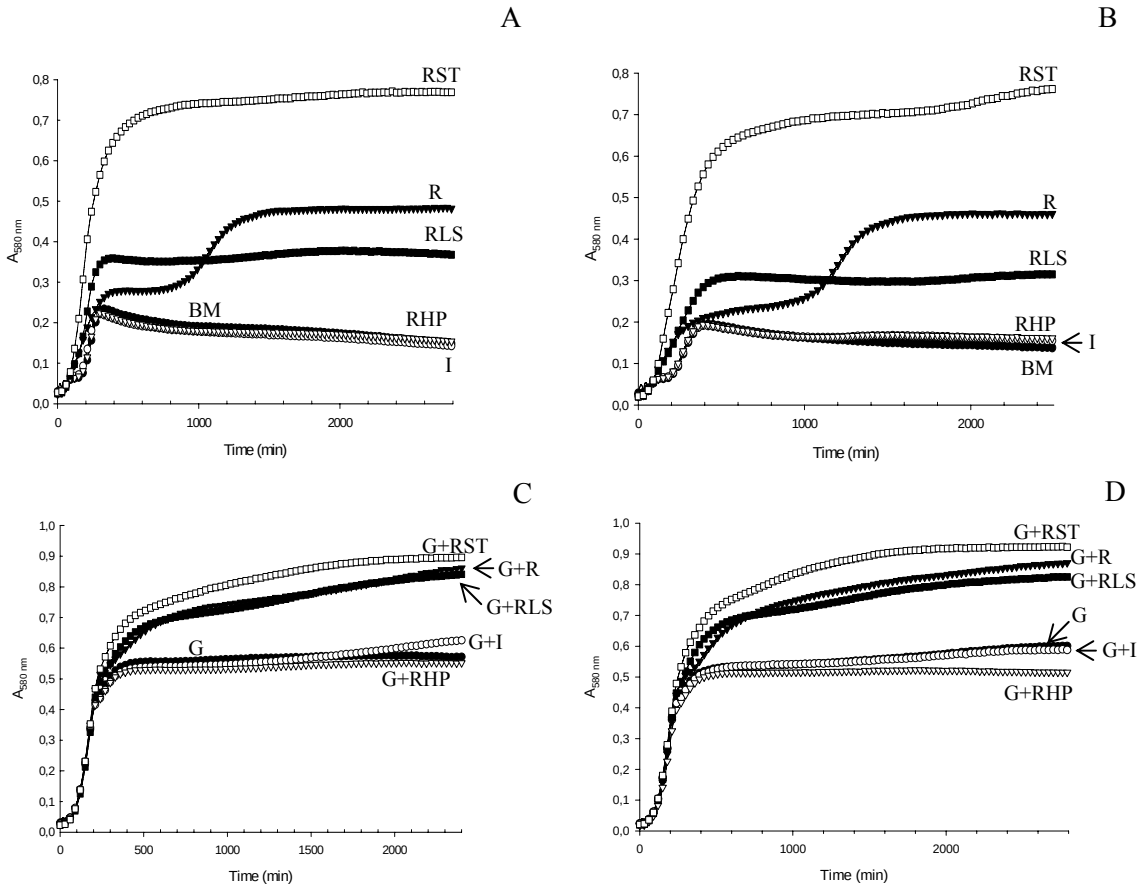


Fig. 3.8 Growth of *L. johnsonii* strains on prebiotics. A) *L. johnsonii* BFE 6128 on basal medium (BM) supplemented with 1 % wt/v prebiotics. B) *L. johnsonii* BFE 6154 on basal medium (BM) supplemented with 1 % wt/v prebiotics. (●), medium alone (BM); (○), BM + Inuline (I); (▼), BM + Raftilose (R); (▽), Raftiline HP (RHP); (■), Raftiline LS (RLS) and (□), Raftiline ST (RST). C) Growth of *L. johnsonii* BFE 6154 on basal medium (BM) supplemented with 0.1 % glucose and 1 % wt/v prebiotics. D) Growth of *L. johnsonii* BFE 6128 on basal medium (BM) supplemented with 0.1 % glucose (Gluc) and 1 % wt/v prebiotics. (●), basal medium +Gluc (G); (○), BM + Inuline + Gluc (I+G); (▼), BM + Raftilose + Gluc (R+G); (▽), Raftiline HP + Gluc (G+RHP); (■), Raftiline LS + Gluc (RLS+G) and (□), Raftiline ST + Gluc (RST+G).

3.6.2 Growth of *L. plantarum* probiotic candidates on prebiotics

In general, *L. plantarum* strains did not grow well on prebiotic sugars, but not all strains presented the same behaviour and therefore are grouped in three different figures.

L. plantarum BFE 5759 and BFE 1685 had a similar behaviour towards utilisation of prebiotic sugars. Both strains were not able to grow on Raftilose, Inuline, Raftiline HP nor

Raftline LS (Fig. 3.9). Only growth on Raftiline ST was superior to growth on 0.1 % glucose indicating that growth was supported by Raftiline ST and not by the residual sugars. When glucose was present, the utilization of prebiotic sugars did not change (Fig. 3.9).

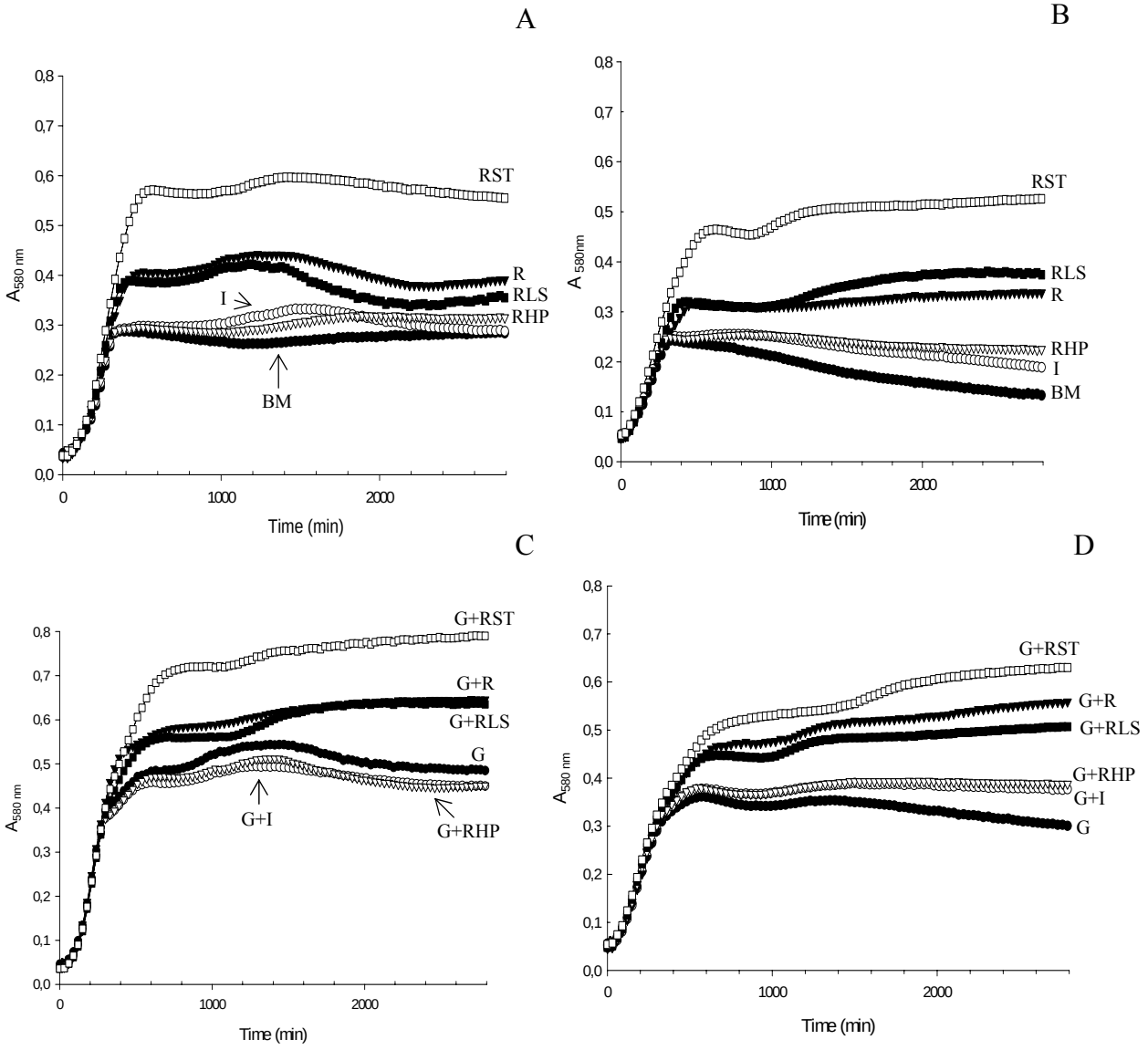


Fig. 3.9 Growth of *L. plantarum* strains on prebiotics. A) *L. plantarum* BFE 5759 on basal medium (BM) supplemented with 1 % wt/v prebiotics. B) *L. plantarum* BFE 1685 on basal medium (BM) supplemented with 1 % wt/v prebiotics. (●), medium alone (BM); (○), BM + Inuline (I); (▼), BM + Raftilose (R); (▽), Raftiline HP (RHP); (■), Raftiline LS (RLS) and (□), Raftiline ST (RST). C) Growth of *L. plantarum* BFE 5759 on basal medium (BM) supplemented with 0.1 % glucose and 1 % wt/v prebiotics. D) Growth of *L. plantarum* BFE 1685 on basal medium (BM) supplemented with 0.1 % glucose (Gluc) and 1 % wt/v prebiotics. (●), basal medium +Gluc (G); (○), BM + Inuline + Gluc (I+G); (▼), BM + Raftilose + Gluc (R+G); (▽), Raftiline HP + Gluc (G+RHP); (■), Raftiline LS + Gluc (RLS+G) and (□), Raftiline ST + Gluc (RST+G).

In Fig. 3.10 it is shown that neither *L. plantarum* BFE 5092 nor BFE 5878 were able to utilize none of the prebiotic sugars tested, as growth was not higher than that observed on 0.1 % glucose. When both, the corresponding prebiotic sugar and 0.1 % glucose were present there were no changes in utilization of prebiotic sugars.

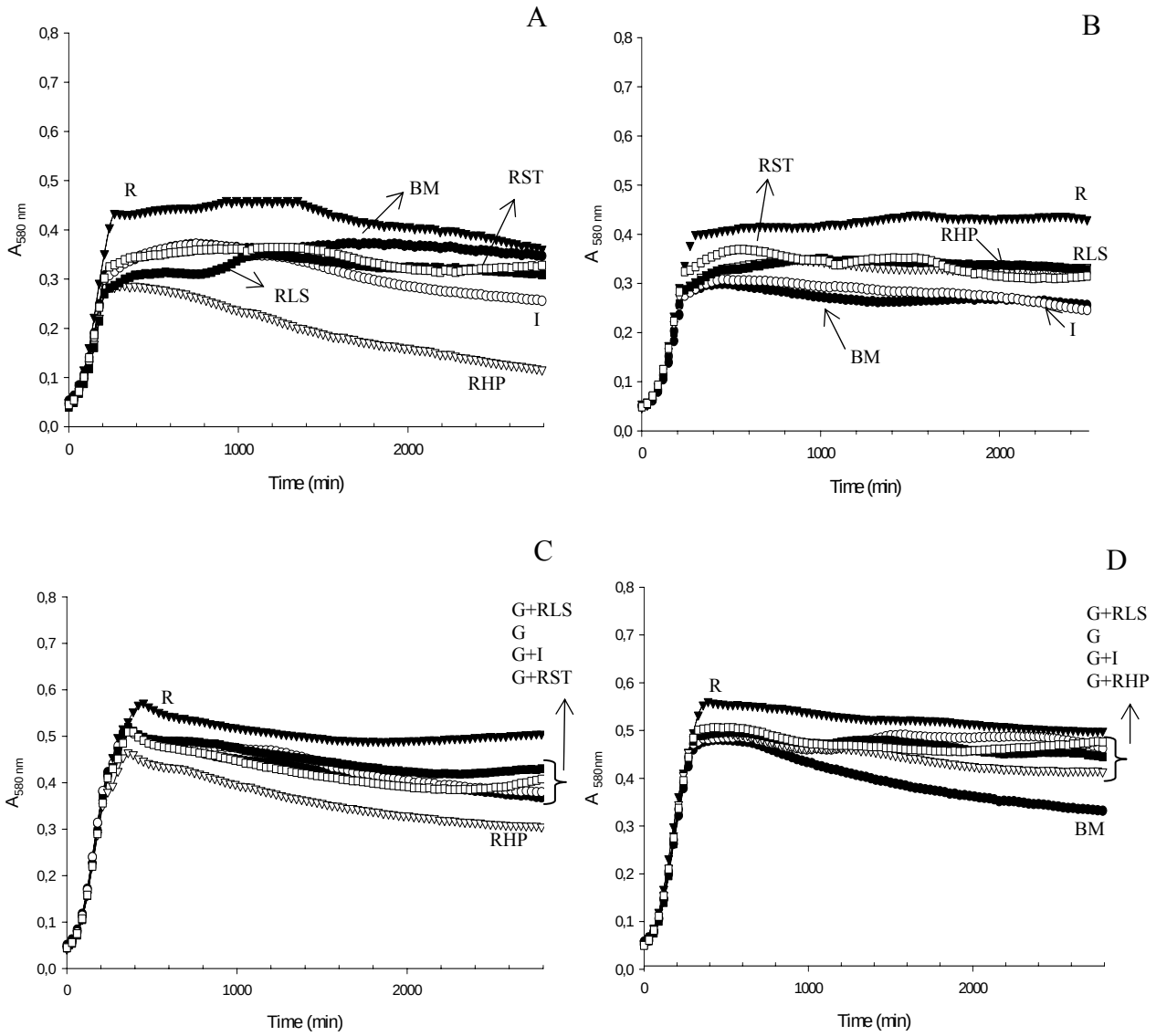


Fig. 3.10 Growth of *L. plantarum* strains on prebiotics. A) *L. plantarum* BFE 5092 on basal medium (BM) supplemented with 1 % wt/v prebiotics. B) *L. plantarum* BFE 5878 on basal medium (BM) supplemented with 1 % wt/v prebiotics. (●), medium alone (BM); (○), BM + Inuline (I); (▼), BM + Raftilose (R); (▽), Raftiline HP (RHP); (■), Raftiline LS (RLS) and (□), Raftiline ST (RST). C) Growth of *L. plantarum* BFE 5092 on basal medium (BM) supplemented with 0.1 % glucose and 1 % wt/v prebiotics. D) Growth of *L. plantarum* BFE 5878 on basal medium (BM) supplemented with 0.1 % glucose (Gluc) and 1 % wt/v prebiotics. (●), basal medium +Gluc (G); (○), BM + Inuline + Gluc (I+G); (▼), BM + Raftilose + Gluc (R+G); (▽), Raftiline HP + Gluc (G+RHP); (■), Raftiline LS + Gluc (RLS+G) and (□), Raftiline ST + Gluc (RST+G).

The only prebiotic sugar *L. plantarum* BFE 1684 appeared to be capable of utilising was Raftiline ST (Fig. 3.11) similar to the case of *L. plantarum* BFE 1685 and BFE 5759 (Fig. 3.9). Nevertheless, the growth of the strain did not reach the same A_{580nm}, and it seemed to be diauxic as indicated by the second lag phase, which may be due to the need of enzymes, which may need to be synthesised *de novo*.

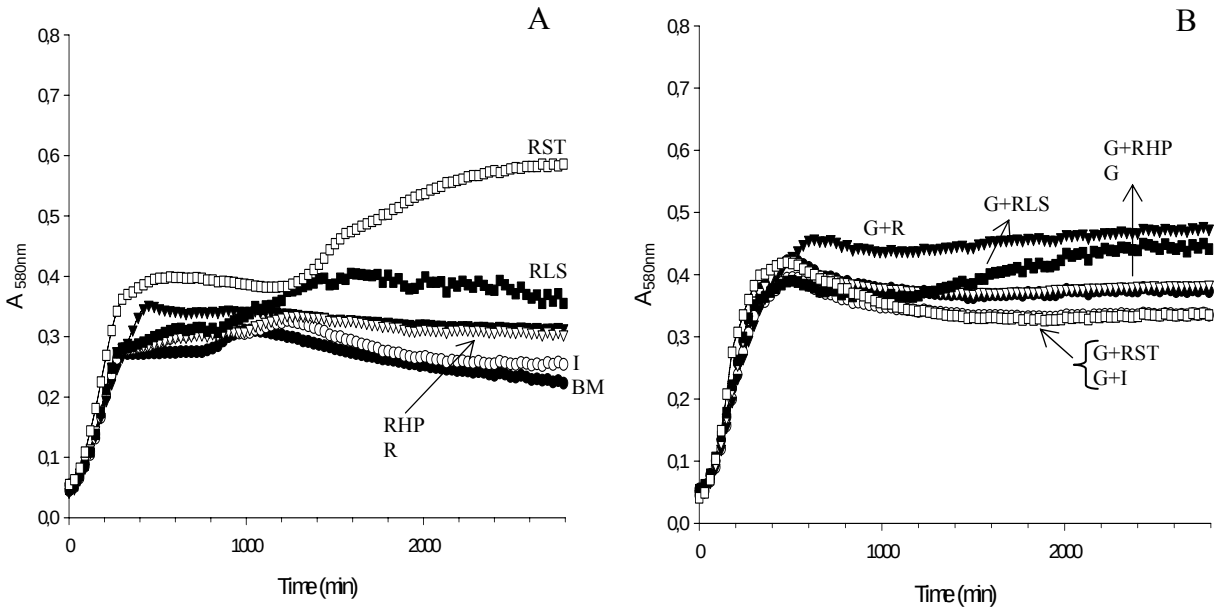


Fig. 3.11 Growth of *L. plantarum* BFE 1684 on basal medium (BM) supplemented with 1 % wt/v prebiotics. (●), medium alone (BM); (○), BM + Inuline; (▼), BM + Raftilose; (▽), Raftiline HP; (■), Raftiline LS and (□), Raftiline LT. B) Growth of L. plantarum BFE 1684 on basal medium (BM) supplemented with 0.1 % glucose and 1 % wt/v prebiotics. (●), basal medium +Gluc ; (○), BM + Inuline + Gluc; (▼), BM + Raftilose + Gluc; (▽), Raftiline HP + Gluc; (■), Raftiline LS + Gluc and (□), Raftiline ST + Gluc.

3.7 Adhesive properties

3.7.1 Microbial adhesion to solvents

The use of three solvents allowed the evaluation of the hydrophobic/hydrophilic cell surface properties of lactobacilli and their Lewis acid-base (electron donor and acceptor) characteristics (Pelletier et al., 1997; Briandet et al., 1999). As shown in Table 3.7, *L. plantarum* strains BFE 5878, BFE 5092, BFE 5759 and ATCC 8014 and *L. rhamnosus* GG had a low partitioning percentage in the apolar solvent n-hexadecane, indicating that these strains possess a hydrophilic surface. *L. plantarum* BFE 1684 and BFE 1685, *L. johnsonii* BFE 6128, BFE 6154 and LA-1 (BFE 663), *L. paracasei* BFE 675 and *L. casei* Shirota (BFE 688) were characterised by a high affinity to n-hexadecane, indicating the hydrophobic nature of their surfaces (Table 3.7).

In order to measure the Lewis acid-base properties of the bacterial surface, two solvents (chloroform and ethyl acetate) with the same van der Waals properties as n-hexadecane (Pelletier et al., 1997) were used in order to be sure that the affinity for each solvent tested was not due to van der Waals forces.

The results of the microbial adhesion to chloroform showed that four *L. plantarum* strains (BFE 5878, BFE 5092, BFE 5759 and ATCC 8014) had a low affinity for this acidic solvent, whereas all other strains had a strong affinity for it.

When ethyl acetate, an electron donor, was employed for determining the microbial adhesion to solvents, adhesion values were noticeably high (ranging from 36.3 to 79.2%) for *L. johnsonii* strains BFE 663, BFE 6128 and BFE 6154. This indicates that these bacteria also have an acidic surface character. *L. plantarum* 1684 and *L. rhamnosus* GG also showed a relatively high adherence value (30 %) to ethyl acetate. The rest of the strains adhered poorly to this strongly basic solvent, with values ranging from 0 to 16.8 %, reflecting the non-acidic character of these strains.

Table 3.7 Adhesion of potential probiotic *Lactobacillus* strains and *Lactobacillus* reference strains to HT29 cells and to solvents

Strains	Origin	Adhesion index[a]	% Adhesion (±S.E.) to:		
			n-Hexadecane	Chloroform	Ethyl Acetate
Probiotic candidates					
L. plantarum					
BFE 1684	Infant faeces	45.9±5.9*	60.8±4.4	55.5±4.6	30.4±4.3
BFE 1685	Infant faeces	69.1±8.8*	67.6±1.8	77.6±3.4	8.7±3.4
BFE 5878	Kule naoto	55.5±4.6*	14.7±1.8	20.6±0.7	16.8±0.7
BFE 5092	Kule naoto	62.7±6.8*	12.0±2.3	22.0±0.7	6.1±1.4
BFE 5759	Kwerionik	3.7±0.7	7.3±0.4	21.1±0.5	8.3±1.1
L. johnsonii					
BFE 6128	Kule naoto	100[d]±n.d.*	68.1±3.7	90.0±1.0	79.2±0.8
BFE 6154	Kule naoto	100[d]±n.d.*	79.7±8.7	66.2±5.8	50.4±4.7
Reference strains					
L. johnsonii BFE 663[b]	LA-1 Nestlé	23.0±3.22*	74.5±3.6	99.9±0.3	36.3±0.1
L. plantarum ATCC 8014	ATCC	24.7±2.6*	5.7±0.6	11.6±1.2	0±0.0
L. rhamnosus GG[b]	Valio Ltd.	33.85±4.79*	18.5±1.9	67.1±5.5	30.0±0.8
L. paracasei BFE 675[c]	Actimel®, Danone	2.38±0.48	59.6±1.7	100.0±0.3	0±0.0
L. casei Shirota BFE 688[c]	Yakult®	2.62±0.5	40.3±6.8	65.3±0.1	0±0.0

[a] Adhesion Index: mean ± S.E. (Standard Error) (n=4). Number of bacteria adhering to 100 HT29 cells. [b] Positive control strains. [c] Negative control strains. [d] More than 100 bacteria per 100 HT29 cells were not possible to count, therefore they were given an adhesion index of 100 and no S.E. was calculated; n.d.: Not determined. [e] Means ± standard error of two measurements of three separate experiments.* Significantly different (ANOVA, Dunnet's t-test, $P<0.05$) from the negative control *L. casei* Shirota.

3.7.2 Adhesion of *Lactobacillus* strains to HT29 cells

Potentially probiotic *Lactobacillus* strains were investigated for their ability to adhere to the human intestinal epithelial HT29 cells. In addition, four commercial probiotic strains, previously well characterised for their adhesion properties (Tuomola et al., 1999; Gopal et al., 2001) as well as a *L. plantarum* strain with unknown adhesion properties were used as reference strains. *L. rhamnosus* GG and *L. johnsonii* (LA-1) served as positive control strains, while *L. paracasei* (Actimel, Danone) and *L. casei* Shirota (Yakult Ltd.) were used as negative control strains and were described as being either adhesive or with a low and non-specific binding capability, respectively (Tuomola et al., 1999; Gopal et al., 2001). After Gram staining of the slides, quantification of adherent bacterial strains, clearly visible as dark purple rods on a pale pink cell background, was possible (Fig. 3.12).

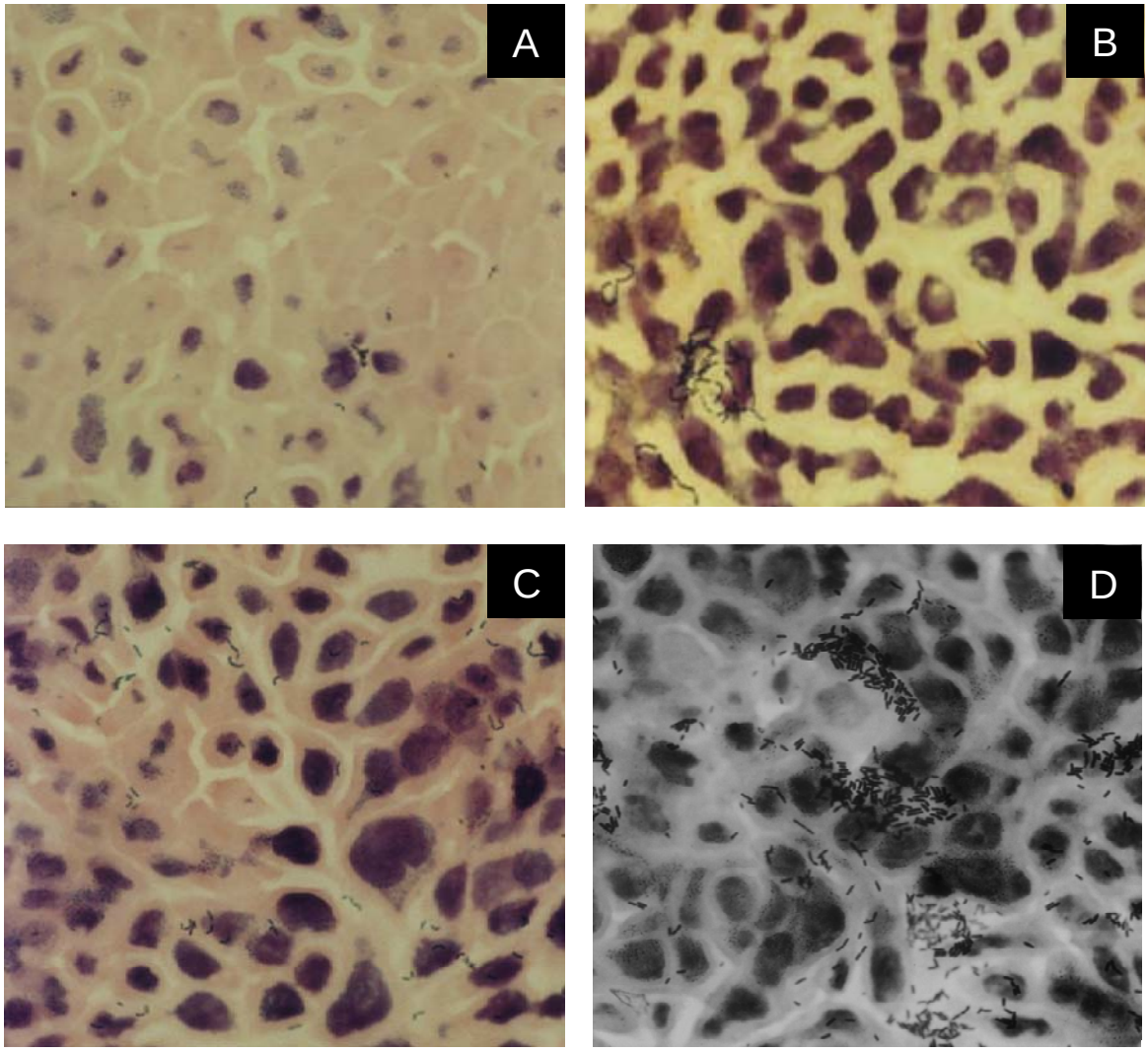


Fig. 3.12 Adhesion of lactobacilli to HT29 cells. **A:** *L. casei* Shirota BFE 688 (negative control strain), B: *L. rhamnosus* GG (positive control strain), **C:** *L. plantarum* BFE 1685, **D:** *L. johnsonii* BFE 6128. 1000x magnification.

L. plantarum BFE 5759 could be characterised as non-adhesive, as its adherence values did not differ significantly from those of the negative control (*L. casei* Shirota, $P>0.05$, Table 3.7). *L. plantarum* ATCC 8014 showed moderate adherence with values differing significantly from those of the negative control (*L. casei* Shirota, $P<0.05$). All other probiotic candidates were classified as strongly adhesive, and they all showed significantly higher adherence values when compared to the negative control ($P<0.05$). Interestingly, the *L. johnsonii* strains BFE 6128 and BFE 6154 isolated from Maasai milk (Kule naoto) showed a much higher adhesive index than the positive control strains *L. johnsonii* LA-1 and *L. rhamnosus* GG, which are described in the literature to adhere well to intestinal epithelial cells (Lee et al., 2000).

3.7.3 Competitive exclusion: effect of adherent *Lactobacillus* strains on the adhesion of pathogens to HT29 cells

L. plantarum BFE 1685 and *L. johnsonii* BFE 6128 were selected for competitive exclusion studies with pathogens because of their strong adhesion to HT29 cells (3.7.2, Table 3.7). As control strains, the adhesive *L. rhamnosus* GG and the non-adhesive *L. casei* Shirota (BFE 688) were used. In these experiments, HT29 cells were pre-incubated with lactobacilli (5×10^8 CFU/ml) before addition of enterotoxigenic *E. coli* ATCC 25922, the vancomycin resistant *Ent. faecium* DSM 13590, *List. monocytogenes* Scott A or *S.* Typhimurium ATCC 14028. Adhesion of *E. coli* was significantly ($P<0.01$) reduced when HT29 cells were pre-treated with *L. rhamnosus* GG or *L. johnsonii* BFE 6128, and completely abolished when cells were pre-treated with *L. plantarum* BFE 1685. On the other hand, only *L. plantarum* BFE 1685 was able to completely inhibit ($P<0.01$) *Ent. faecium* DSM 13590 adhesion, whereas neither *L. johnsonii* BFE 6128 nor the reference strains (*L. rhamnosus* GG, *L. casei* Shirota BFE 688) tested were able to influence the adhesion of this *Enterococcus* strain (Fig. 3.13).

List. monocytogenes Scott A adhesion was significantly ($P<0.01$) reduced by all tested strains, except for the non-adherent *L. casei* Shirota (BFE 688). This latter strain did not influence the adhesion of *E. coli*, *Ent. faecium* or *List. monocytogenes* strains used in this study, but it slightly reduced ($P<0.05$) the adherence of *S.* Typhimurium ATCC 14028 cells. *S.* Typhimurium adhesion was also significantly reduced by the presence of *L. plantarum* BFE 1685 cells (Fig. 3.13).

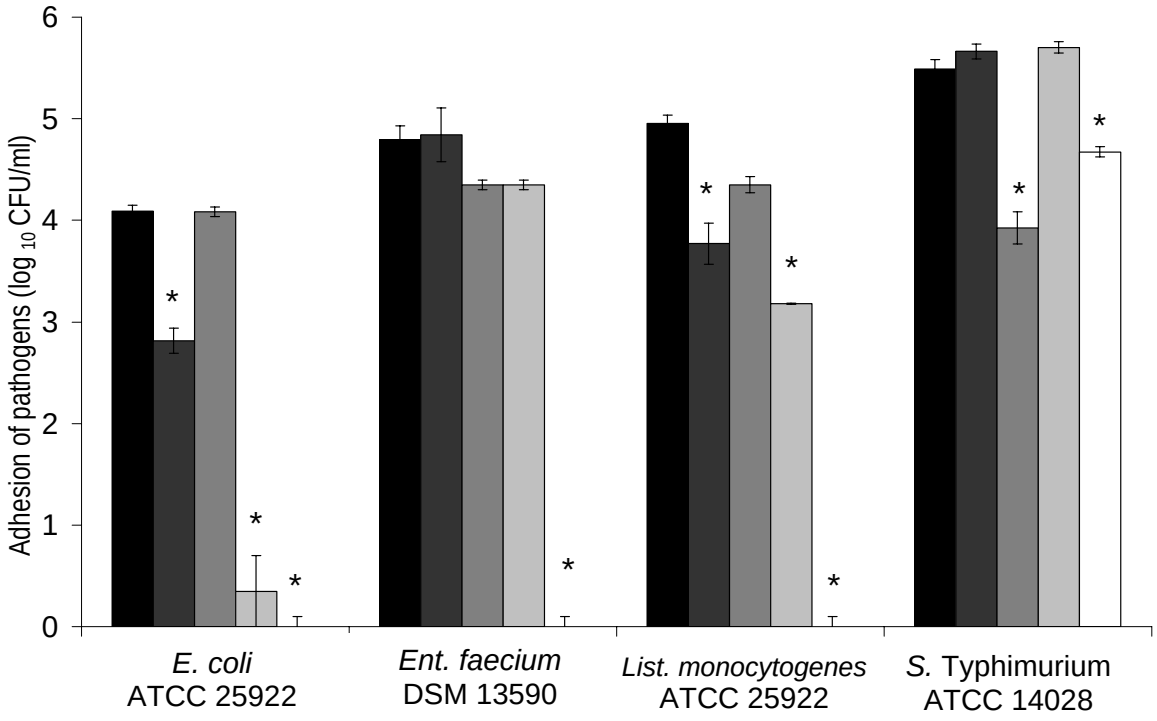


Fig. 3.13 Adhesion of pathogens to HT29 cells alone or after pre-treatment of HT29 cells with potentially probiotic or reference *Lactobacillus* spp. strains. Results are expressed as means ± S.E. of at least four experiments. *Significantly decreased binding when compared to binding on wells without lactobacilli ($P<0.05$, ANOVA, Dunnett's t test). Binding of the pathogen alone ■, or after pre-treatment of HT29 cells with *L. rhamnosus* GG ■, *L. casei* Shirota BFE 688 ■, *L. johnsonii* BFE 6128 ■ or *L. plantarum* BFE 1685 □.

3.7.4 Auto-aggregation of *Lactobacillus* strains

Auto-aggregation was studied on the basis of the sedimentation characteristics of the strains because when cells aggregate, they sediment and clear the supernatant. The non-adherent reference strains *L. paracasei* BFE 675 and *L. casei* BFE 688 showed aggregation values of less than 20 % (Fig. 3.14), whereas the adherent reference strains *L. johnsonii* LA-1 (BFE 663, Fig. 3.14) and *L. rhamnosus* GG auto-aggregated with a value higher than 20 %. Although these strains are both known to adhere well to intestinal epithelial cells, a clear difference in auto-aggregation could be observed as *L. johnsonii* BFE 663 showed much higher auto-aggregation values (more than 80 %), while those of *L. rhamnosus* GG were below 40 % (Fig. 3.14). A variation in aggregation values obtained using acidic (pH 4.0) and neutral (pH 7.4) PBS was not noted. Only in the case of *L. johnsonii* strains BFE 6154 and BFE 6128, the auto-aggregation was enhanced when tested in their overnight spent

supernatants and it was noticeably higher than that observed in PBS at pH 4.0 (Fig. 3.14). Measurements of auto-aggregation on neutralised supernatants was not possible because most of the strains continued growing, which led to an increase in A_{580nm}, resulting in negative auto-aggregation values (data not shown).

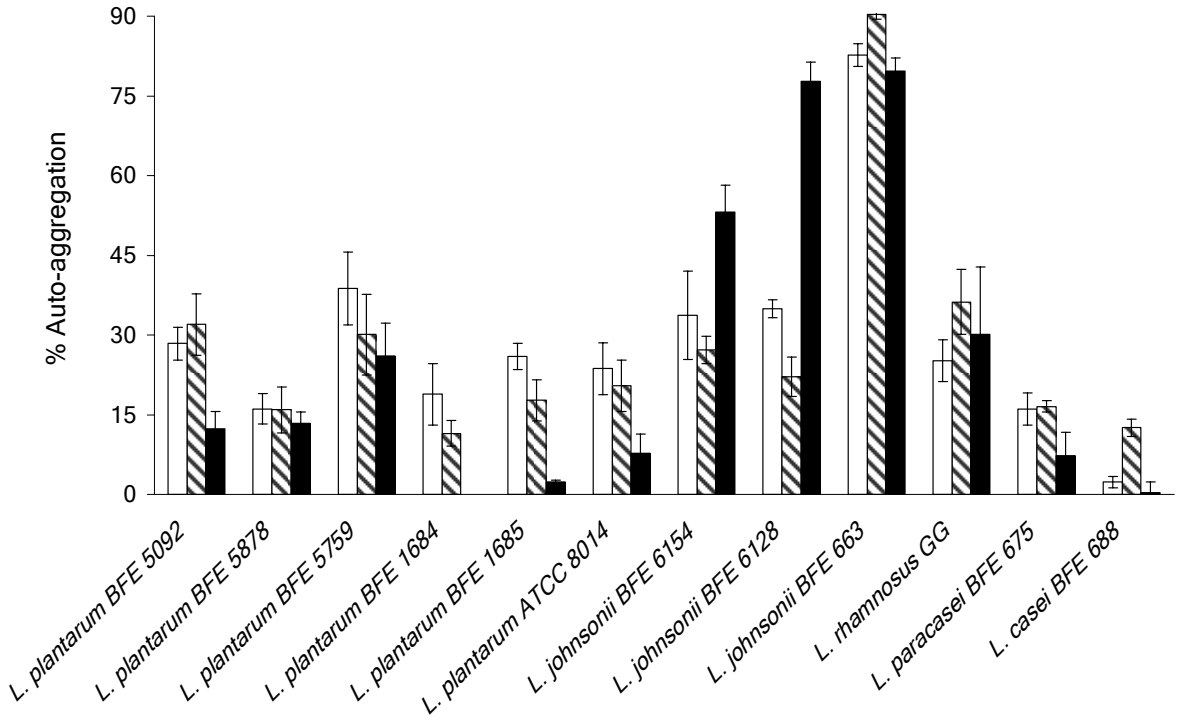


Fig. 3.14 Auto-aggregation of potentially probiotic and reference *Lactobacillus* strains. Overnight cultures were washed and resuspended in PBS pH 7.4 (□), in PBS pH 4 (▨) or in MRS spent culture supernatant (■). Absorbance (580 nm) was measured at the beginning (A_0) and after a 2-hour-incubation (A_1) at 23°C. Auto-aggregation % was calculated as: $(1 - A_1/A_0) \times 100$.

3.7.5 Coaggregation of *Lactobacillus* strains with foodborne pathogens

The scoring system used for the coaggregation test is illustrated in Fig. 3.15. *E. coli* ATCC 25922 and *S.* Typhimurium ATCC 14028 were able to auto-aggregate with scores of 4 and 3, respectively (Table 3.8). For coaggregation of pathogens with *Lactobacillus* spp., the scores were reduced by at least one unit in all cases, except for *L. johnsonii* BFE 6128 and BFE 6154 strains, which coaggregated with *S.* Typhimurium with a score of 3 (Table 3.8). After Gram staining, it was verified by light microscopy that aggregates comprised Gram-positive as well as Gram-negative bacteria.

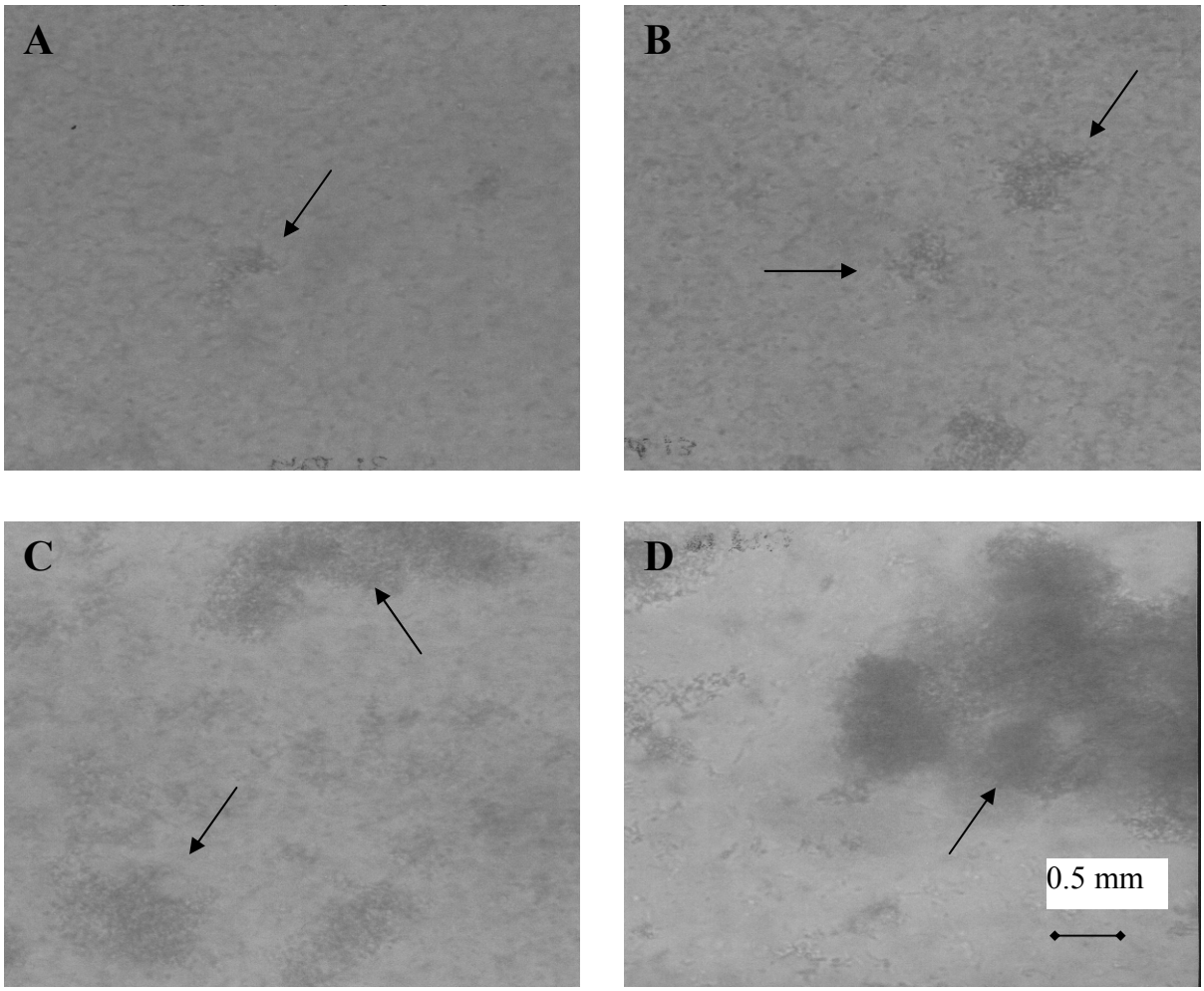


Fig. 3.15 Scoring system for the coaggregation assay. Organisms used to illustrate the different scores were *S.* Typhimurium ATCC 14028 together with *L. plantarum* BFE 1685 (A) *S.* Typhimurium ATCC 14028 together with *L. plantarum* BFE 5092 and 10 % spent supernatant from *L. plantarum* BFE 5092 grown in MRS broth at 37°C overnight (B), *L. johnsonii* BFE 6128 together with *L. monocytogenes* Scott A (C) and *S.* Typhimurium ATCC 14028 together with *L. johnsonii* BFE 6128 and 10% supernatant from *L. johnsonii* BFE 6128 grown in MRS broth (D). Photographs were taken on a Zeiss inverted microscope (200x magnification). The photographs show scores 1 to 4 (1 for small and dispersed clumps in some microscopic fields (A), 2 for medium-sized and dispersed clumps (B), 3 for abundant and medium-sized clumps with turbid supernatant (C) and 4 for very big clumps and clear supernatant (D). Scores 3 and 4 were also macroscopically visible.

Three probiotic reference strains (*L. rhamnosus* GG, *L. casei* Shirota and *L. paracasei* BFE 675) as well as *L. plantarum* ATCC 8014 and *L. johnsonii* BFE 6128 and BFE 6154 were able to coaggregate with *List. monocytogenes* Scott A with a higher score than that obtained when *List. monocytogenes* auto-aggregated (Table 3.8). *L. plantarum* strains coaggregated with *S. aureus* ATCC 25923 to varying degrees. Two of the reference probiotic strains *L.*

johnsonii LA-1 and *L. casei* Shirota did not coaggregate at all, whereas *L. johnsonii* BFE 6128 and BFE 6154 coaggregated with *S. aureus* with a score of 4 (Table 3.8).

When spent culture supernatant of overnight cultures of lactobacilli was added to the coaggregation system (1:10) with *List. monocytogenes* and *S.* Typhimurium, the coaggregation with *L. plantarum* strains BFE 5878, BFE 5092, BFE 5759, BFE 1685 and BFE 1684 and *L. johnsonii* BFE 6128 increased by one unit (result not shown). In that way, *L. johnsonii* BFE 6128 reached the highest score (4) in coaggregation with these foodborne pathogen strains.

Table 3.8 Coaggregation[a] of potential probiotic *Lactobacillus* strains with intestinal pathogens

Strain		*List. monocytogenes* Scott A	*S. aureus* ATCC 25923	*E. coli* ATCC 25922	*S.* Typhimurium ATCC 14028
Probiotic candidates					
L. plantarum	BFE 5878	1	1	3	1
	BFE 5092	1	2	3	1
	BFE 5759	1	2	3	1-2
	BFE 1684	1-2	1	3	1-2
	BFE 1685	1	2	3	1
L. johnsonii	BFE 6154	3	4	3	3
	BFE 6128	3	4	3	3
Reference strains					
L. johnsonii	BFE 663	1-2	0	3	2
L. rhamnosus	GG	3	1	3	2
L. casei Shirota	BFE 688	3	0	3	2
L. paracasei	BFE 675	3	2	3	2
L. plantarum	ATCC 8014	3	3	3	2
Foodborne pathogens					
List. monocytogenes	Scott A	2	-	-	-
S. aureus	ATCC 25923	-	0-1	-	-
E. coli	ATCC 25922	-	-	4	-
S. Typhimurium	ATCC 14028	-	-	-	3

[a] Plates were observed with an inverted microscope with a 20 x objective. Scoring system adopted for the coaggregation assay: 0, no clumps; 1, small and dispersed clumps in some microscopic fields; 2, medium-sized and dispersed clumps, 3, abundant and medium-sized clumps with turbid supernatant; 4, very big clumps with a clear supernatant (3 and 4 also macroscopically visible).

3.8 Interaction of selected probiotic candidate strains with HT29 cells

3.8.1 HT29 cell viability in coculture experiments

None of the probiotic candidate and control strains tested (inoculum: 1 x 10^7 CFU/well, ratio: two bacteria to one HT29 cell) significantly modified (P>0.05) the vitality of HT29 cells after 24 h incubation, when compared to the negative control (cells cultivated with DMEM alone), as assessed by the release of lactate dehydrogenase into the supernatant. Bacterial LDH was not released into the medium as corroborated with supernatants of bacteria grown on DMEM without HT29 cells. LDH concentration in supernatant of cell monolayers disrupted with 2 % Triton-X were considered as 100 % cytotoxicity and used to calculate vitality (Fig. 3.16).

When HT29 cells were infected with *S*. Typhimurium (inoculum: 1 x 10^7 CFU/well, ratio: two bacteria to one HT29 cell) for a period of 24 h, the HT29 cells died as indicated by a 96-100 % cytotoxicity as determined by measuring LDH released in the supernatant by necrotic cells (Fig. 3.16). The monolayers also detached during the washing steps and the pH of the medium was acid as indicated by the shift of the pH indicator present in DMEM (phenol red). When infection lasted only 4 h, as in the experiments of section 3.9, there were no significant changes in vitality of HT29 cells or in the pH of the medium.

A similar experiment on cytotoxicity of bacterial strains and components to HT29 was done using the MTT assay test (Fig. 3.17). Neither the probiotic candidates, nor the reference strains significantly affected HT29 cells vitality (P<0.05). The commercial microbial components and TNF-α were not cytotoxic, at least at the concentration used in these assays.

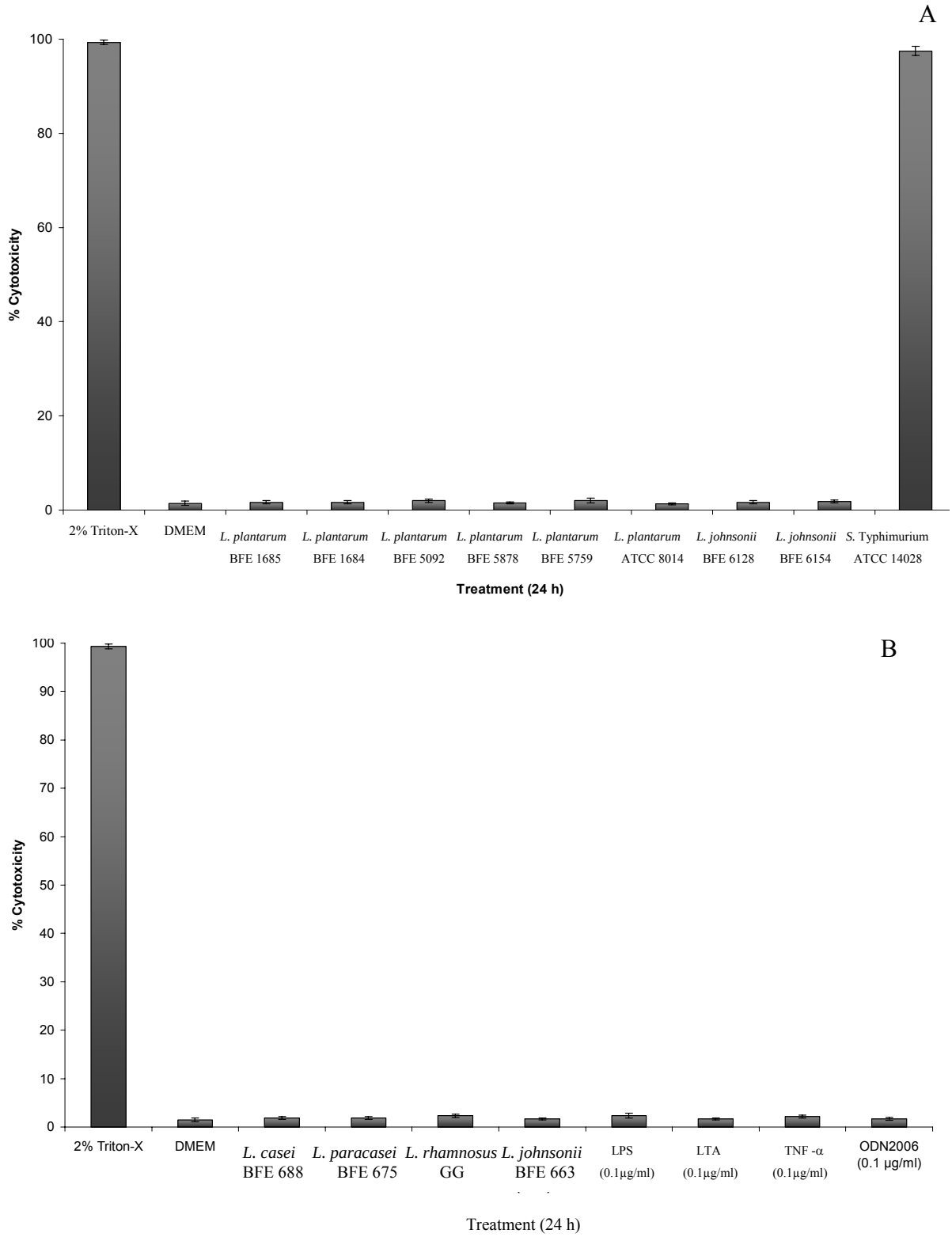


Fig. 3.16 Cytotoxicity of bacterial cultures, LPS, LTA, TNF-α and ODN2006 to HT29 cells expressed as % cytotoxicity observed after 24 h treatment. A: Probiotic candidates and *S.* Typhimurium. B: reference strains, commercial LPS, LTA, ODN2006 and TNF-α. Cells treated with 2 % Triton-X were used as a positive control for 100 % cytotoxicity and cells treated with medium alone (DMEM) as a negative control.

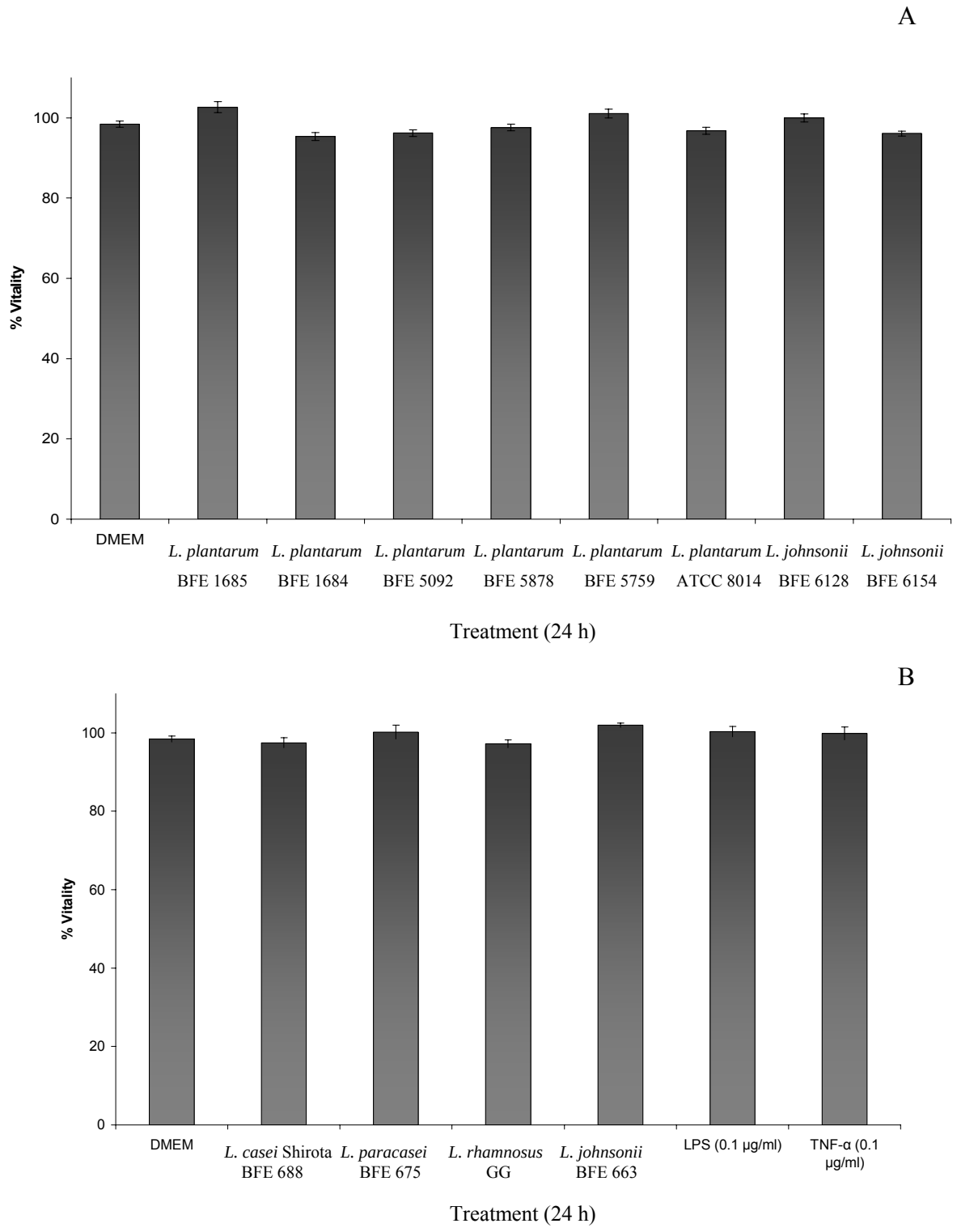


Fig. 3.17 Cytotoxicity of bacterial cultures, LPS and TNF-α to HT29 cells expressed as % vitality observed after 24 h treatment. A: probiotic candidates; B: Control strains, LPS and TNF-α. The vitality of HT29 cells treated with bacterial suspensions was studied by means of the MTT test.

3.8.2 Modulation of cytokine secretion by HT29 cells

Seven potentially probiotic candidate strains and three reference strains (*L. rhamnosus* GG, *L. paracasei* BFE 675 and *L. casei* Shirota BFE 688) were tested for their ability to induce cytokine secretion in intestinal epithelial cells. IL-1, IL-6, MCP-1, IL-10 and TNF-α could not be detected in the supernatants treated with either bacteria or with commercially obtained lipopolysaccharide (LPS, 0.1 µg/ml). TGF-β was detected, but it was secreted constitutively and no significant (P>0.01) difference could be demonstrated between the 24-hour-supernatant of the control (cells grown with DMEM without FCS and antibiotics) and probiotic-treated samples (5 x 10^6 CFU/ml in DMEM without FCS and antibiotics). Two *L. plantarum* strains (BFE 5759 and BFE 1685) and one *L. johnsonii* strain (BFE 6128) significantly (P<0.01) induced the secretion of IL-8 when compared to the control without bacteria (Fig. 3.18). Nevertheless, when cells were incubated for 24 h together with LPS alone (0.1 µg/ml), the levels of this chemokine were much higher (264.02 ± 10.28 pg/ml; Fig. 3.19). The two well-known probiotics *L. rhamnosus* GG and *L. paracasei* BFE 675 (Actimel®, Danone) on the other hand, down-regulated the release of IL-8 as shown in Fig. 3.18. The other *Lactobacillus* strains neither reduced nor enhanced the secretion of this chemokine by HT29 cells significantly.

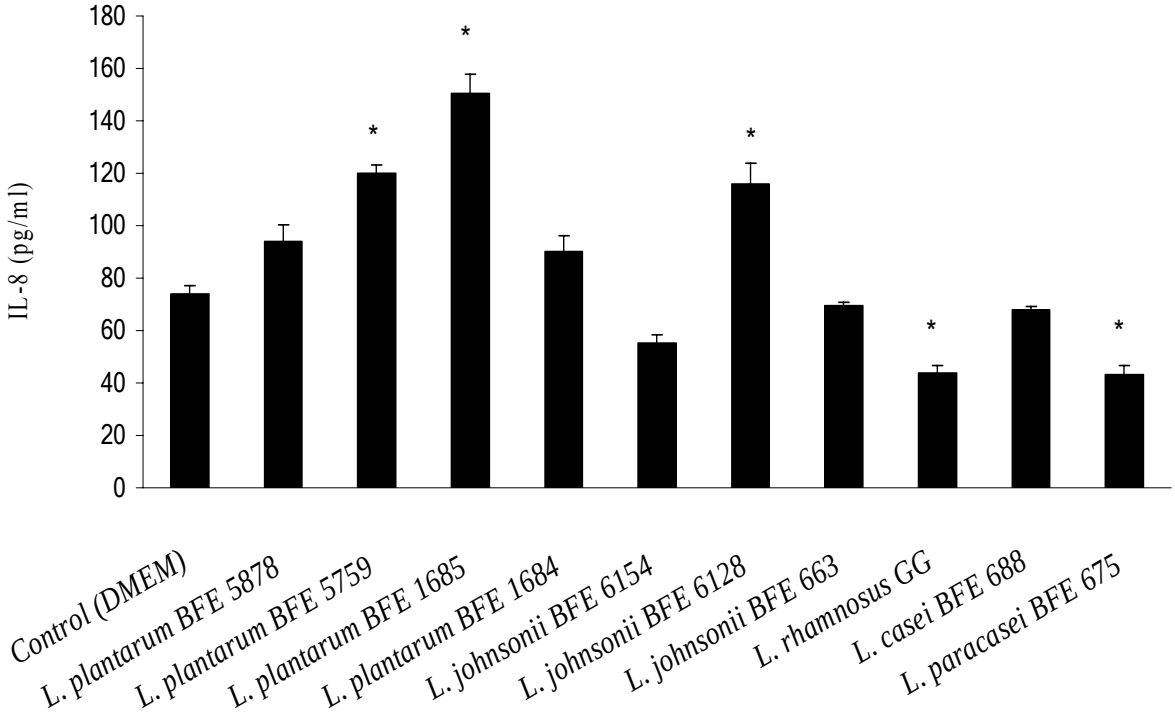


Fig. 3.18 Induction of IL-8 secretion (pg/ml) in HT29 epithelial cells by lactobacilli as determined by enzyme-linked immunosorbent assay. Values are means ± S.E. of at least three experiments. *Significantly different from the control (P<0.01 ANOVA, Dunnett's t-test).

Some of the strains were further tested to determine wether they exerted an effect on the response of HT29 cells to LPS (0.1 μg/ml). Indeed, *L. plantarum* BFE 1685, *L. plantarum* BFE 5878 and *L. johnsonii* BFE 6128 enhanced the response of HT29 cells to LPS in a synergistically way (Fig. 3.19), when incubated together for 24 h. The well studied commercial probiotic strain *L. rhamnosus* GG and the potentially probiotic candidates, *L. plantarum* BFE 1685 and *L. johnsonii* BFE 6128, were selected for further studies on the interaction of lactobacilli with enterocytes based on their IL-8 co-stimulatory properties.

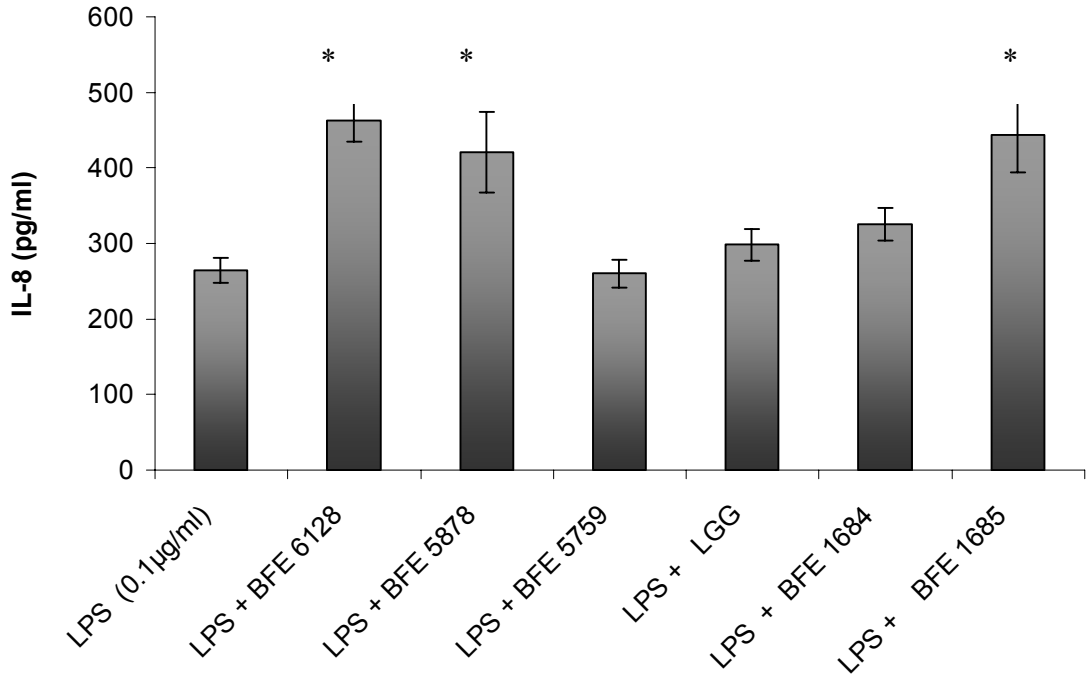


Fig. 3.19 Effect of co-stimulation of HT29 cells with LPS and lactobacilli on IL-8 production by HT29 cells. 1 x 10^7 CFU/well and 0.1 μg/ml LPS (from *E. coli*) were given to HT29 cells and incubated for 24 h. Supernatants were collected; centrifuged and IL-8 levels were determined by ELISA. Values are means ± S.E. of at least three separate experiments. Cells incubated only with 0.1 μg/ml LPS were considered as control. **P*<0.05, ANOVA, Dunnett's t-test.

3.8.3 Identification of probiotic cellular components responsible for IL-8 induction on HT29 cells after 24 h

Purified cell walls, LTAs and DNA, as well as sterile-filtered spent supernatant (of 24-hour bacterial cultures grown in DMEM), lactic acid and cells treated with proteinase K or potassium periodate, were tested for their ability to induce IL-8 secretion by HT29 cells after 24 h incubation as described in section 2.10.3. IL-8 levels (pg/ml) induced by the different bacterial components are shown in Fig. 3.20, 3.21 and 3.22. Statistical differences when

compared to the untreated control (cells treated with DMEM) were determined by means of ANOVA and Dunnett's t-test ($P<0.05$).

LTAs isolated from *L. rhamnosus* GG induced a higher production of IL-8 ($P<0.05$) by HT29 cells than the untreated cells (cells treated with DMEM) as shown in Fig. 3.20. The sterile-filtered supernatants of 24-hour-old *L. rhamnosus* GG cultures grown in DMEM were also able to induce higher levels of IL-8 secretion ($P<0.05$), which contrasted with the previously shown results of the effect of live *L. rhamnosus* GG (Fig. 3.18). None of the other components tested, significantly influenced IL-8 secretion.

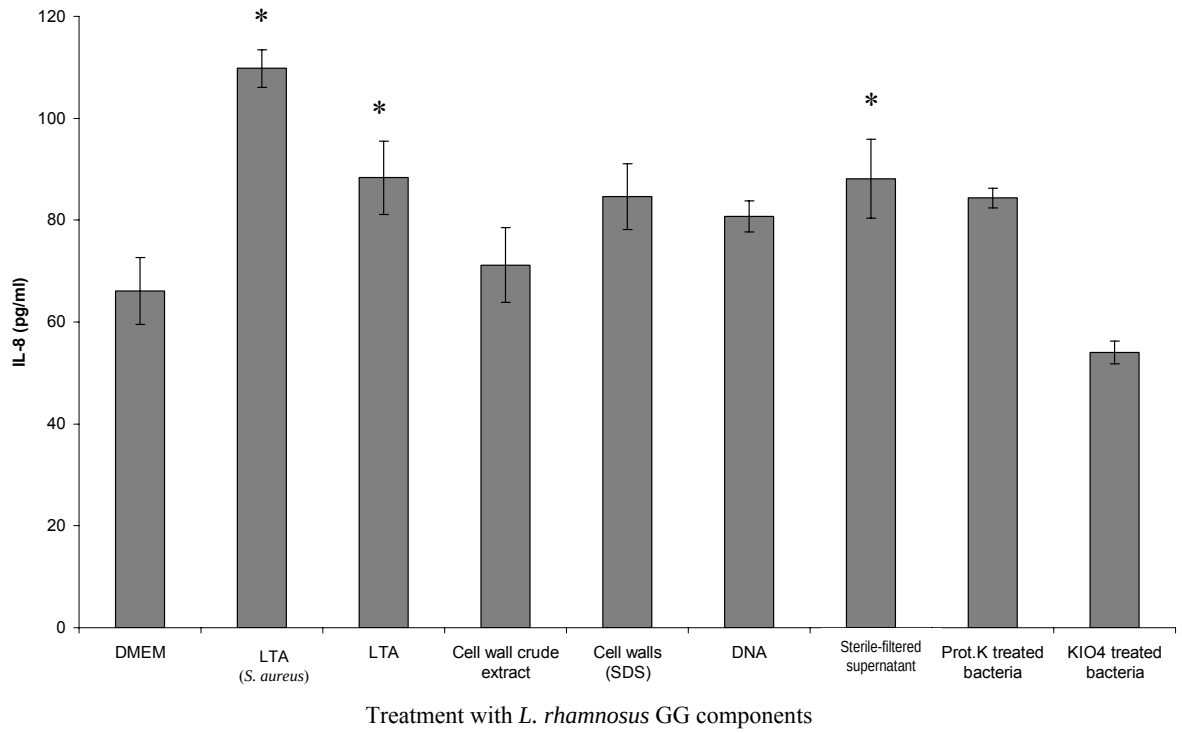


Fig. 3.20 Effect of *L. rhamnosus* GG cellular components on IL-8 production by HT29 cells. Cells incubated only with medium were used as the negative control (basal secretion of IL-8). *S. aureus* LTA was used as a positive control for induction of IL-8 on intestinal epithelial cells. **P*<0.05, ANOVA, Dunnett's t-test.

In the case of *L. plantarum* BFE 1685, LTAs did not influence the secretion of IL-8 significantly. Other cellular components (crude cell wall extracts, DNA) and cells treated with proteinase K or KIO_4 did not exert an effect on IL-8 secretion, either. Interestingly, the filtered supernatant of *L. plantarum* 24-hour cultures significantly influenced the secretion of IL-8 by HT29 cells (Fig. 3.21).

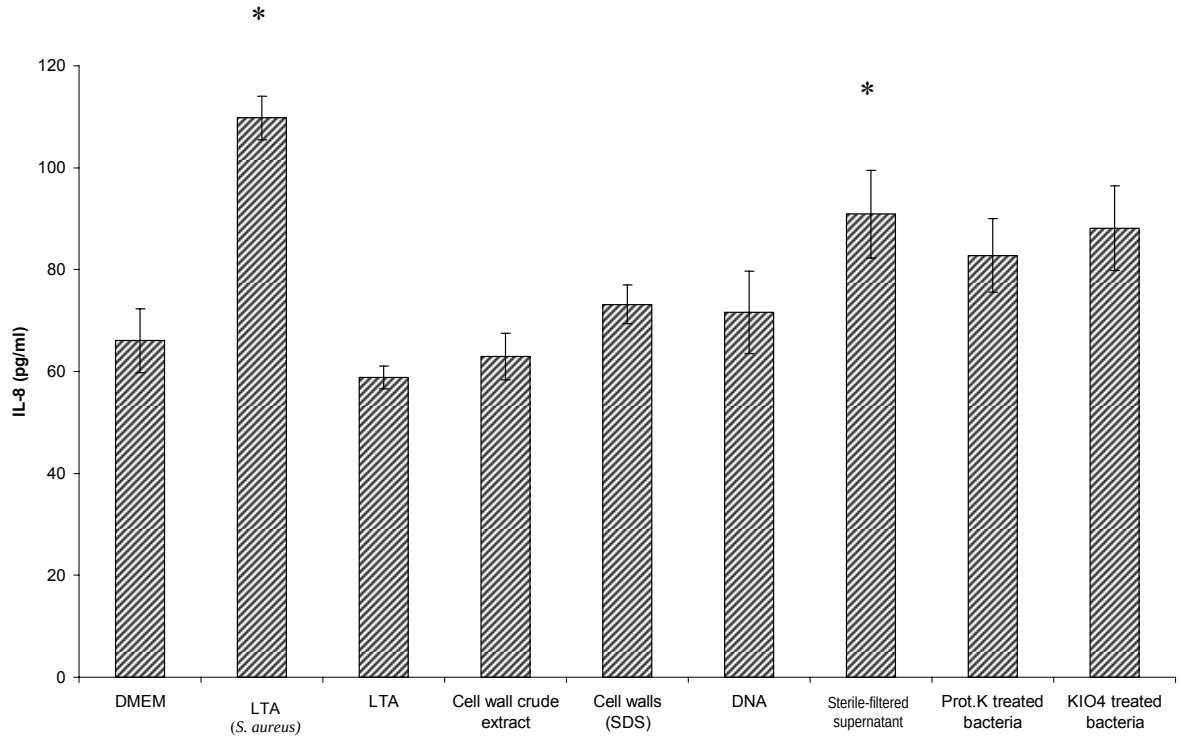


Fig. 3.21 Effect of *L. plantarum* BFE 1685 cellular components on IL-8 (pg/ml) production by HT29 cells. Cells incubated only with medium were used as negative control (basal secretion of IL-8). *S. aureus* LTA was used as a positive control for induction of IL-8 on intestinal epithelial cells. **P*<0.05, ANOVA, Dunnett's t-test.

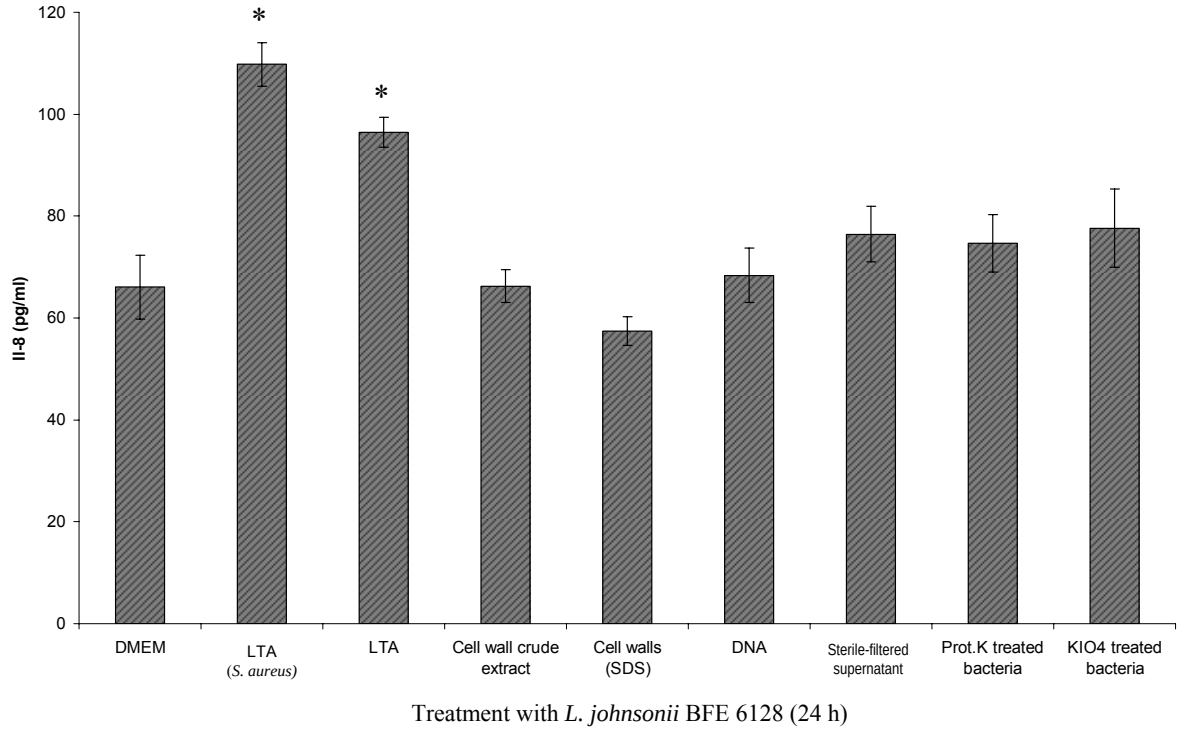


Fig. 3.22 Effect of *L. johnsonii* BFE 6128 cellular components on IL-8 (pg/ml) production by HT29 cells. Cells incubated only with medium were used as negative control (basal secretion of IL-8). *S. aureus* LTA was used as a positive control for induction of IL-8 on intestinal epithelial cells. **P*<0.05, ANOVA, Dunnett's t-test.

On the other hand, LTAs isolated from *L. johnsonii* BFE 6128 significantly ($P<0.05$) increased IL-8 secretion by HT29 cells. In contrast to the other two strains, *L. johnsonii* BFE 6128 spent supernatant did not exert any effects on the levels of IL-8. Other bacterial and cells treated with proteinase K or KIO_4 components tested did not modulate IL-8 secretion (Fig. 3.22).

3.8.4 TNF-α sensitises HT29 cells to probiotic lactobacilli

To test the response of enterocytes to probiotic strains in an inflammatory context, the effect of pre-treatment of enterocytes with TNF-α on the response to *L. plantarum* BFE 1685, *L. johnsonii* BFE 6128 and *L. rhamnosus* GG was tested. As shown before in Fig. 3.16 and 3.17, neither lactobacilli nor TNF-α affected HT29 cell viability over a period of 24 h.

HT29 cells were pre-treated with TNF-α (0.1μg/ml) for 2 h, washed twice with PBS and stimulated with lactobacilli (1 x 10^7 CFU/well). The three lactobacilli strains tested exerted a much stronger response on the secretion of IL-8 (Fig. 3.23) than on cells without TNF-α pre-treatment (Fig. 3.18). The levels of IL-8 secreted into the medium by HT29 cells were also much higher than those of IL-8 induced by TNF-α alone (Fig. 3.23).

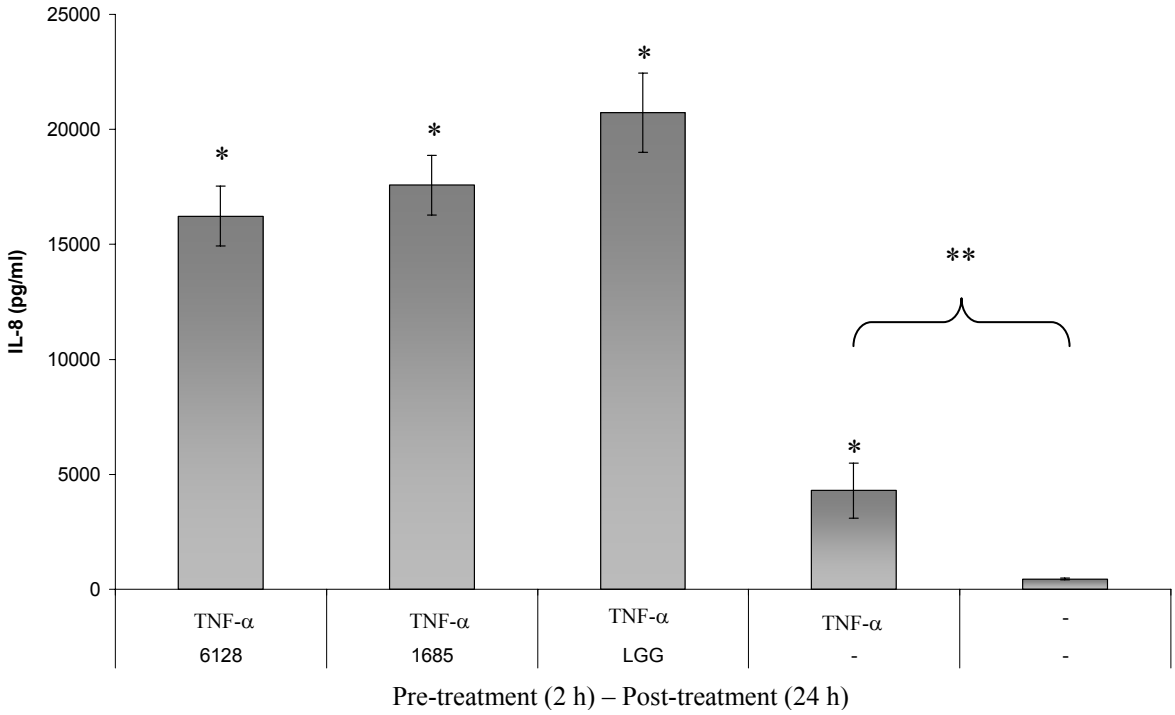


Fig. 3.23 Effect of TNF-α on the response of HT29 cells (IL-8 secretion in pg/ml) to selected lactobacilli [(*L. johnsonii* BFE 6128 (6128), *L. plantarum* BFE 1685 (1685) or *L. rhamnosus* GG (LGG)] strains**.** Cells pre-treated with TNF-α, washed and treated with medium served as control. **P*<0.05, ANOVA, Dunnett's t-test. Cells treated only with TNF-α also induced an increased in IL-8 when compared to cells pre-treated and treated only with medium (DMEM). ***P*<0.05, ANOVA, t-test.

3.8.5 Effect of lactobacilli on gene expression in HT29 cells

3.8.5.1 RT-PCR investigation of TLR2-, TLR4-, TLR9- and HBD-2-gene expression modulation in HT29 cells by lactobacilli

Intestinal epithelial cells were stimulated with *L. rhamnosus* GG, *L. johnsonii* BFE 6128 or *L. plantarum* BFE 1685 (1 x 10^7 CFU/well) for 2 h, washed and incubated with medium for further 4 h. Non-stimulated cells were treated in the same way but incubated only with medium (DMEM). After incubation, RNA was isolated and the expression of TLR2, TLR4 and TLR9 was investigated by means of RT-PCR using the primers listed in Table 2.4 and the RT-PCR programme as indicated in Table 2.2.

From the previous results shown in this study, it could be hypothesised that lactobacilli may be recognised by their LTAs through TLR2 expression. In the literature, there are some discrepancies in the reports of TLR2 expression in intestinal epithelial primary cultures and cell lines (Cario & Podolsky, 2000; Hausmann et al., 2002; Melmed et al., 2003; Furrie et al., 2005). Therefore, it was important to determine if the cell line used in this study was able to express TLR2. As shown in Fig. 3.24, the expected RT-PCR product could not be detected with this method, neither in untreated cells nor in cells treated with the *Lactobacillus* spp. strains used in this study.

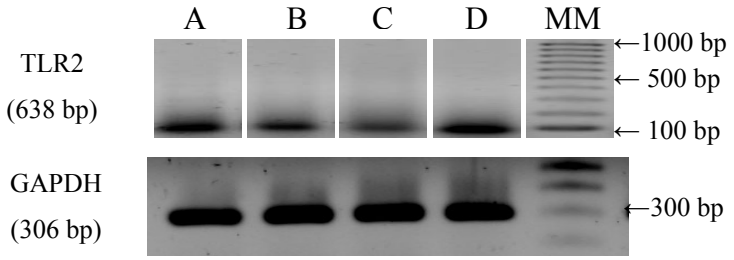


Fig. 3.24 Expression of TLR2 in HT29 cells as determined by RT-PCR using TLR2 specific primers. Cells remained unstimulated (A) or were stimulated with 1 x 10^7 CFU/well of *L. rhamnosus* GG (B), *L. johnsonii* BFE 6128 (C) or *L. plantarum* BFE 1685 (D)**.** mRNA was analysed by RT-PCR. MM: molecular marker. The expected amplicon sizes are show in parenthesis under the corresponding gene. The bands shown with the TLR2 experiments corresponded to primer or primer-dimer bands. GAPDH was amplified as control house-keeping gene.

HT29 cells responded to LPS with an increase in IL-8 secretion. LPS is known to be recognised by TLR4 but also by CD14 and LBP (lipopolysaccharide binding protein). To confirm that HT29 cells expressed TLR4, the expression of this receptor was investigated by RT-PCR as shown in Fig. 3.25. As mentioned before, some *Lactobacillus* strains seemed to synergise with LPS to increase IL-8 when compared to cells not treated with lactobacilli. Thus, it was of interest to determine if TLR4 was expressed in HT29 cells, and to see if their

levels were modulated by the presence of lactobacilli. The RT-PCR method is a qualitative method, but it can also be used in a semi-quantitative way to estimate if there are variations in the expression levels of the target gene. As shown in Fig. 3.25, it seems that TLR4 is constitutively expressed by HT29 cells, because TLR4 mRNA was detected in non-stimulated as well as in stimulated cells. Nevertheless, the intensity of the bands differed, which may suggest differences in the levels of expression. A more sensitive method such as real-time RT-PCR was used later to confirm this (section 3.8.5.2).

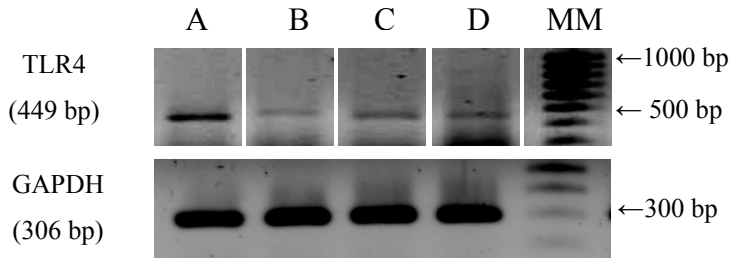


Fig. 3.25 Expression of TLR4 in HT29 cells as determined by RT-PCR using TLR4 specific primers. Cells remained unstimulated (A) or were stimulated with 1 x 10^7 CFU/well of *L. rhamnosus* GG (B), *L. johnsonii* BFE 6128 (C) or *L. plantarum* BFE 1685 (D)**.** mRNA was analysed by RT-PCR. MM: molecular marker. The expected amplicon sizes are show in parenthesis under the corresponding gene. GAPDH was amplified as control house-keeping gene.

Some studies have reported that DNA from probiotic strains is responsible for some of their immune effects (Lammers et al., 2003; Jijon et al., 2004; Rachmilewitz et al., 2004). The DNA samples of the three strains investigated here were not able to induce an increase on IL-8 levels secreted by HT29 cells (Fig. 3.20 to 3.22). The expression of TLR9 was investigated by RT-PCR in order to see if this lack of response was due to the absence of TLR9 expression in the HT29 cell line. In Fig. 3.26, it is shown that TLR9 is not expressed in non-treated cells and it is induced in cells treated with whole cells of *Lactobacillus* strains.

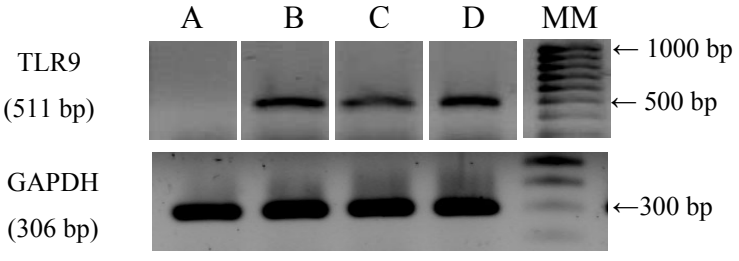


Fig. 3.26 Expression of TLR9 in HT29 cells as determined by RT-PCR using TLR9 specific primers. Cells remained unstimulated (A) or were stimulated with 1 x 10^7 CFU/well of *L. rhamnosus* GG (B), *L. johnsonii* BFE 6128 (C) or *L. plantarum* BFE 1685 (D)**.** mRNA was analysed by RT-PCR. MM: molecular marker. The expected amplicon sizes are show in parenthesis under the corresponding gene. GAPDH was amplified as control house-keeping gene.

HBD-2 is expressed in epithelial cells as a result of the activation of TLR-pathways (Vora et al., 2004). By means of RT-PCR it was not possible to detect the expression of HBD-2 neither in unstimulated nor in lactobacilli-treated HT29 cells (data not shown). It could therefore be inferred that HBD-2 is not expressed by HT29 cell line, at least under the conditions tested in this study.

3.8.5.2 Real-time RT-PCR determination of modulation of TLR2-,TLR4-, TLR9- and HBD-2-gene expression in HT29 cells by probiotic and potentially probiotic lactobacilli

As already mentioned, conventional RT-PCR is not quantitative. Therefore, the more sensitive and appropriate method for quantifying gene expression is the real-time RT-PCR method. For the calibration curves, 10-fold serial dilution series of TLR2, TLR4 and TLR9 amplicons were used. These amplicons were obtained by amplifying mRNA isolated from PBMC which express these genes constitutively. Internal primers were design to amplify a smaller fragment (included in these amplicons) during real-time RT-PCR using the cDNA obtained by RT-PCR as template. Both the initial RT-PCR products and the amplicons amplified with the second pair of primers (Table 2.6) were purified with Quantum Prep PCR Kleen spin columns (see 2.3.4.5) and sequenced at GATC Biotech. The sequences of all the DNA products correlated with the expected sequences of the target genes.

Real-time RT-PCR has a much higher sensitivity than conventional RT-PCR. With this technique, it was possible to detect the presence of TLR2 mRNA in HT29 cells. Furthermore, the levels of TLR2 were significantly higher ($P<0.05$) in cells stimulated with lactobacilli than in non-stimulated cells (Fig. 3.27). This finding suggests a possible up-regulation of this gene by lactobacilli.

When TLR4 expression was investigated, no differences were observed in the expression levels of this receptor neither in non-stimulated nor in lactobacilli-stimulated cells (Fig. 3.28).

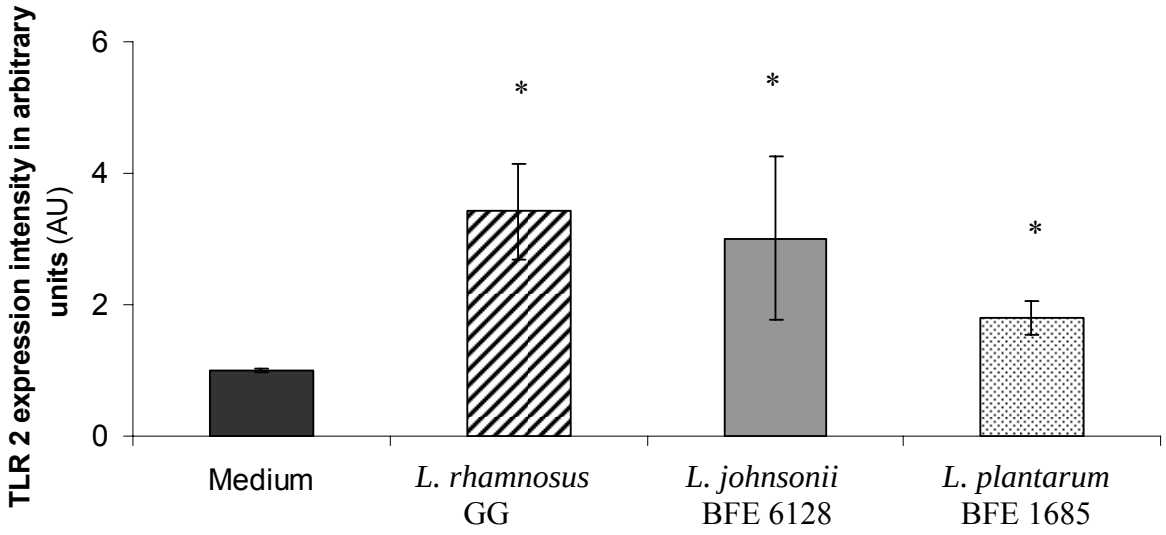


Fig. 3.27 Expression of TLR2 (AU) in HT29 cells as determined by quantitative PCR. Cells remained unstimulated (A) or were stimulated with 1 x 10^7 CFU/well of *L. rhamnosus* GG (B), *L. johnsonii* BFE 6128 (C) or *L. plantarum* BFE 1685 (D)**.** mRNA was analysed by real-time RT-PCR. Data represent results of three independent experiments done in duplicate. **P*<0.05, ANOVA, Dunnett's t-test for comparisons with a control (A).

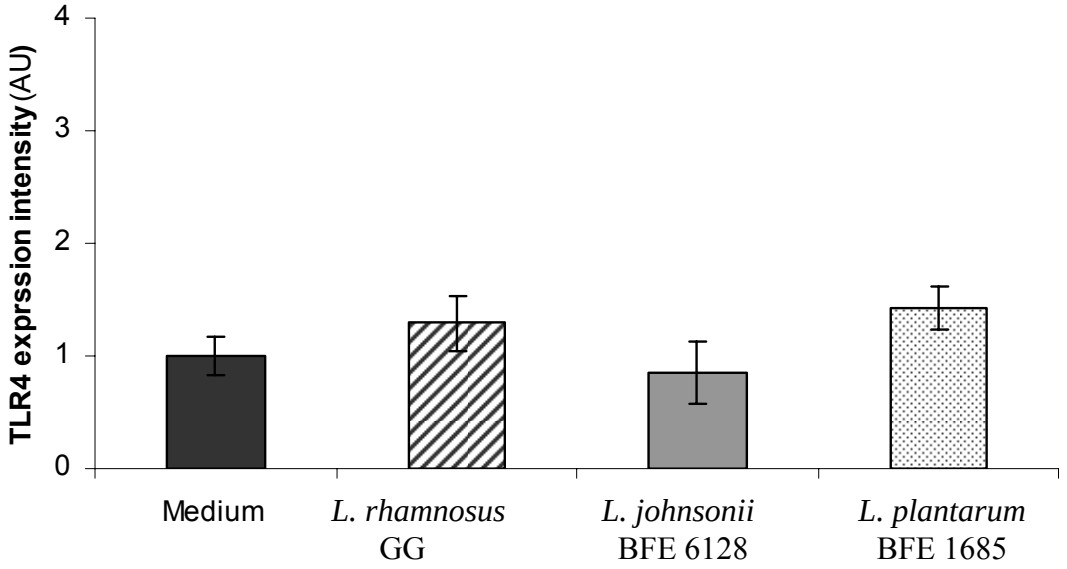


Fig. 3.28 Expression of TLR4 (AU) in HT29 cells as determined by quantitative PCR. Cells remained unstimulated (A) or were stimulated with 1 x 10^7 CFU/well of *L. rhamnosus* GG (B), *L. johnsonii* BFE 6128 (C) or *L. plantarum* BFE 1685 (D)**.** mRNA was analysed by real-time RT-PCR. Data represent results of three independent experiments done in duplicate. *P*<0.05, ANOVA, Dunnettt's t-test for comparisons with a control (A).

In the case of TLR9 gene expression, the presence of lactobacilli significantly (P<0.05) up-regulated the levels of mRNA coding for this receptor as shown in Fig. 3.29. Not all the strains exerted this effect with the same intensity. *L. johnsonii* BFE 6128 up-regulated the expression of TLR9 approx. 11 folds, whereas *L. rhamnosus* GG and *L. plantarum* BFE 1685 approx. 5 and 3 fold, respectively.

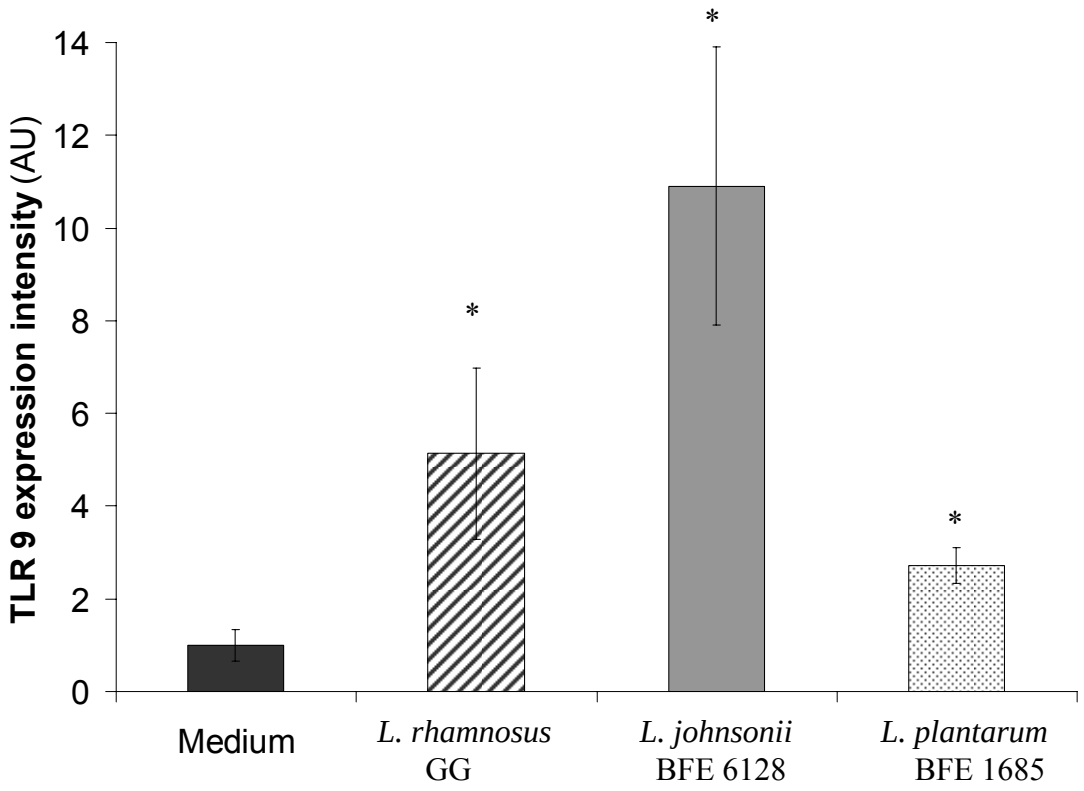


Fig. 3.29 Expression of TLR9 (AU) in HT29 cells as determined by quantitative PCR. Cells remained unstimulated (A) or were stimulated with 1 x 10^7 CFU/well of *L. rhamnosus* GG (B), *L. johnsonii* BFE 6128 (C) or *L. plantarum* BFE 1685 (D)**.** mRNA was analysed by real-time RT-PCR. Data represent results of three independent experiments done in duplicate. P<0.05, ANOVA, Dunnett's t-test for comparisons with a control (A).

3.8.6 Effect of *L. johnsonii* BFE 6128, *L. plantarum* BFE 1685 and *L. rhamnosus* GG on TLR2, TLR5 and TLR9 expression at the protein level in HT29 cells

3.8.6.1 TLR2, TLR5 and TLR9 expression by HT29 cells without prior stimulation with lactobacilli

Based on the previous real-time PCR results (3.8.5), it was interesting to determine if the changes in expression seen at the mRNA level after stimulation with lactobacilli corresponded to expression changes at the protein level. In these experiments, TLR5 was

investigated instead of TLR4 because it is known that flagellated bacteria (including both Gram-positive and Gram-negative flagellated bacteria) are recognised by this receptor and may initiate the activation of intestinal cells through binding to TLR5. The effects of lactobacilli on the response of HT29 cells to *S.* Typhimurium (a flagellated bacterium) is described below in section 3.9.

TLR2 and TLR5 are known to be located in the cytoplasmatic membranes of cells which express these receptors. TLR9 has an intracellular location; it is associated with endosomic membranes. For this reason, the method used for detection of TLR9 included a permeabilisation step to allow the entry of the detection antibody in the cell to bind to its target ligand. In each FACS run 20.000 events (cells passing detector) were measured. Fig 3.30 A, B and C show the overlayed histograms obtained by marking cells with the specific antibodies against each TLR and with the corresponding isotype-control antibodies to discard unspecific binding.

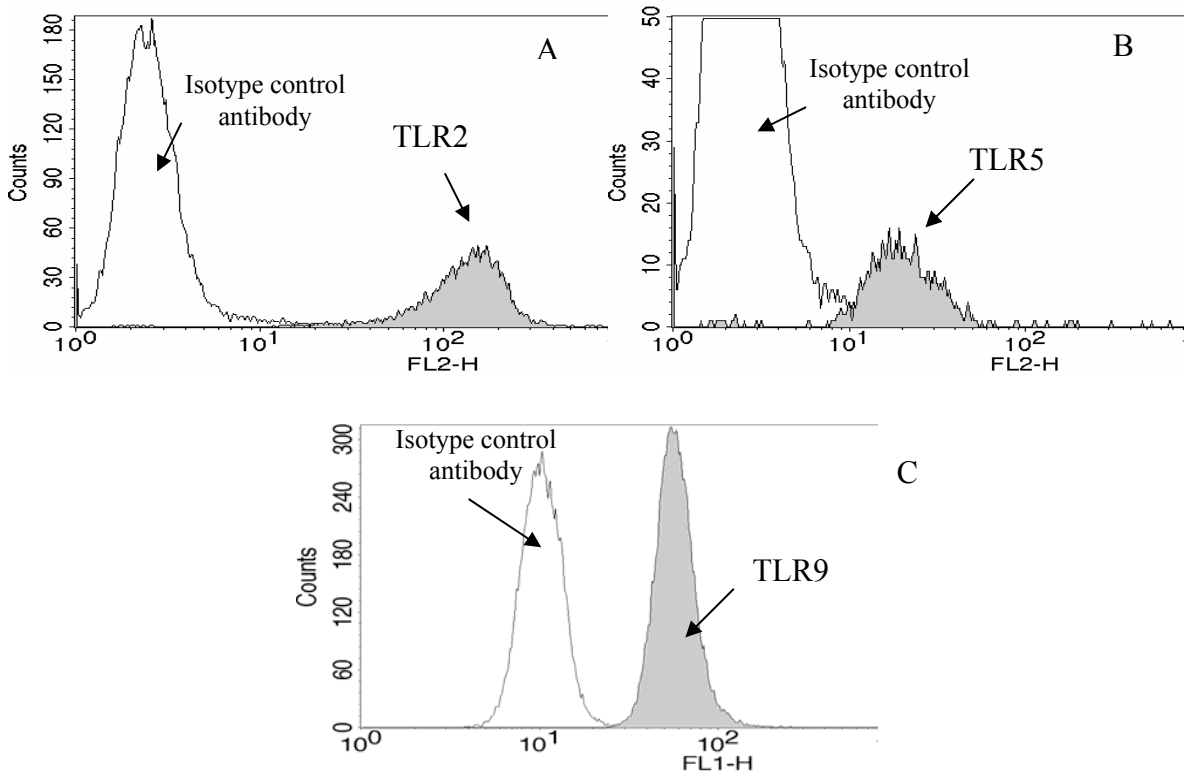


Fig. 3.30 Cell surface expression of TLR2 and TLR5, and intracellular expression of TLR9 in HT29 cells as determined by FACS by labelling HT29 cells with PE-conjugated antibodies against TLR2 or TLR5 and with FITC-conjugated antibody against TLR9 (filled peaks). Isotype antibodies were used as controls to discard non-specific binding (open peaks).

3.8.6.2 Modulation of TLR2, TLR5 and TLR9 protein expression in HT29 cells by potentially probiotic lactobacilli

L. rhamnosus GG and *L. plantarum* BFE 1685, but not *L. johnsonii* BFE 6128 were able to increase the TLR2 levels expressed on the membrane of HT29 cells. Fig. 3.31 A and C show the increase in mean fluorescence intensity on treated cells as indicated by the shift of the peaks to the right, and an increase in the number of cells expressing this receptor. When stimulated with *L. johnsonii* BFE 6128, there was a minimal increase in fluorescence intensity, though insignificant, as indicated by the slight shift of the histogram to the right.

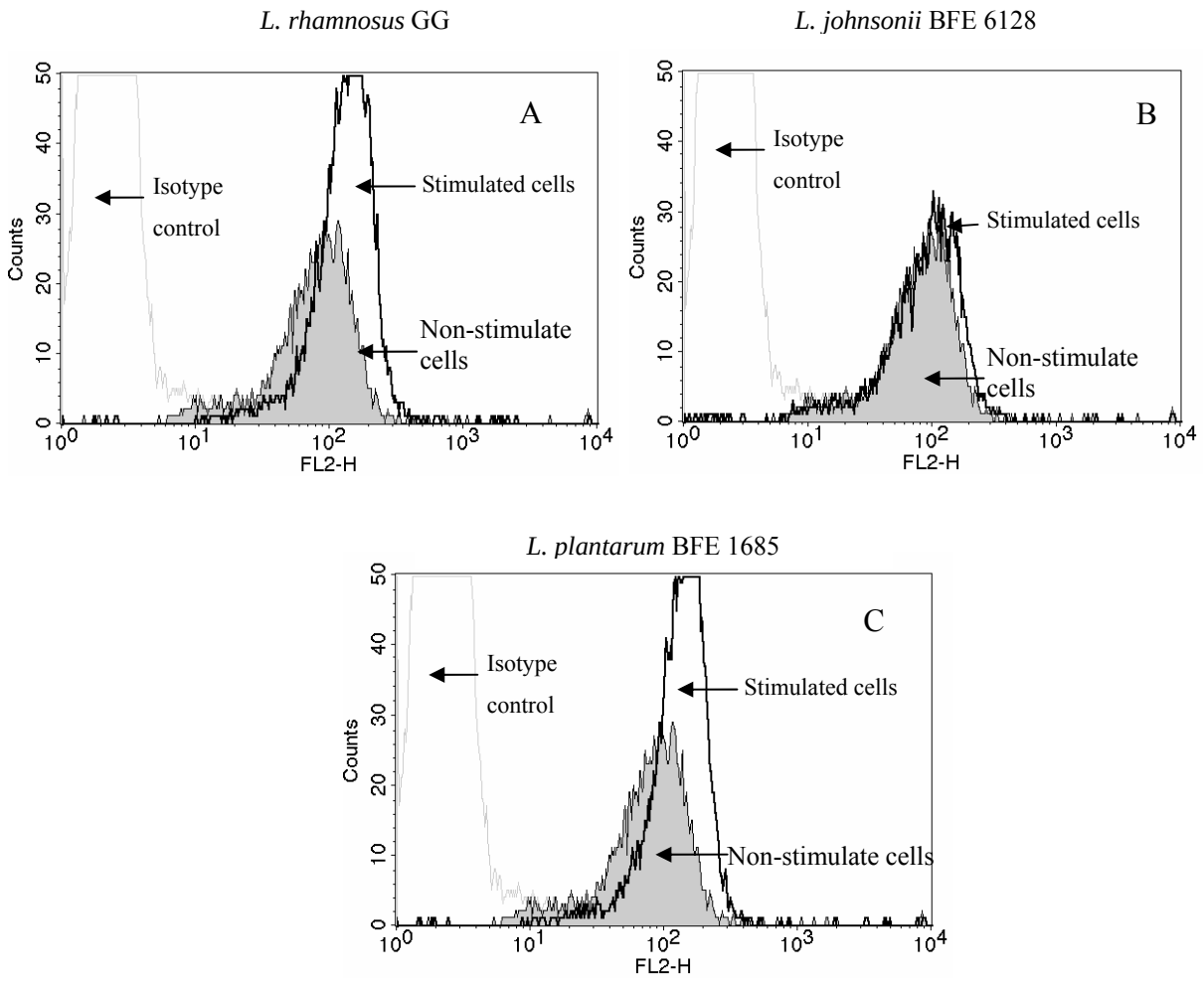


Fig. 3.31 Modulation of cell surface expression of TLR2 in HT29 cells as determined by labeling cells with TLR2 antibody and measuring fluorescence by FACS. The filled peaks indicate the background level of expression of TLR2 in HT29 cells. The histogram isotype-antibody (grey-lined open peaks on the left) was also overlaid onto the histogram. Three experiments with similar results were performed. This figure is representative of one such experiment.

Again, *L. rhamnosus* GG and *L. plantarum* BFE 1685 but not *L. johnsonii* BFE 6128 were able to increase the TLR5 levels expressed on the membrane of HT29 cells. Fig. 3.32 A and C show the increase in mean fluorescence intensity on treated cells as indicated by the shift of the peaks to the right.

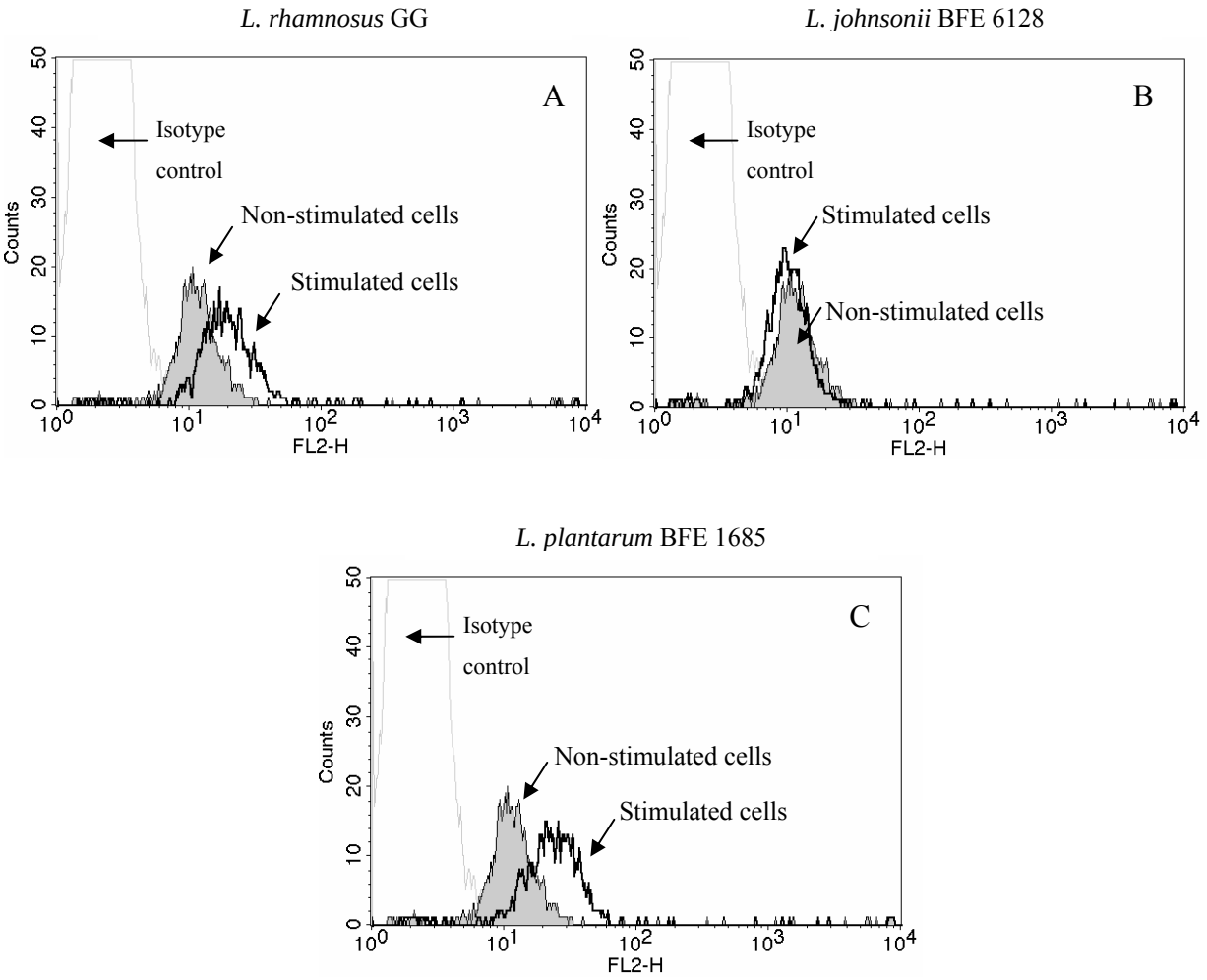


Fig. 3.32 Modulation of cell surface expression of TLR5 in HT29 cells as determined by labeling cells with PE-conjugated TLR5 antibody and measuring fluorescence by FACS. The filled peaks indicate the background level of expression of TLR5 in HT29 cells. The histogram isotype-antibody (grey-lined open peaks on the left) was also overlaid onto the histograms. Three experiments with similar results were performed. This figure is representative of one such experiment.

None of the strains modulated the expression of TLR9 at protein level under the conditions tested, as shown in Fig. 3.33 A, B and C, where the peaks of stimulated and non-stimulated cells overlapped thus indicating no increase in expression.

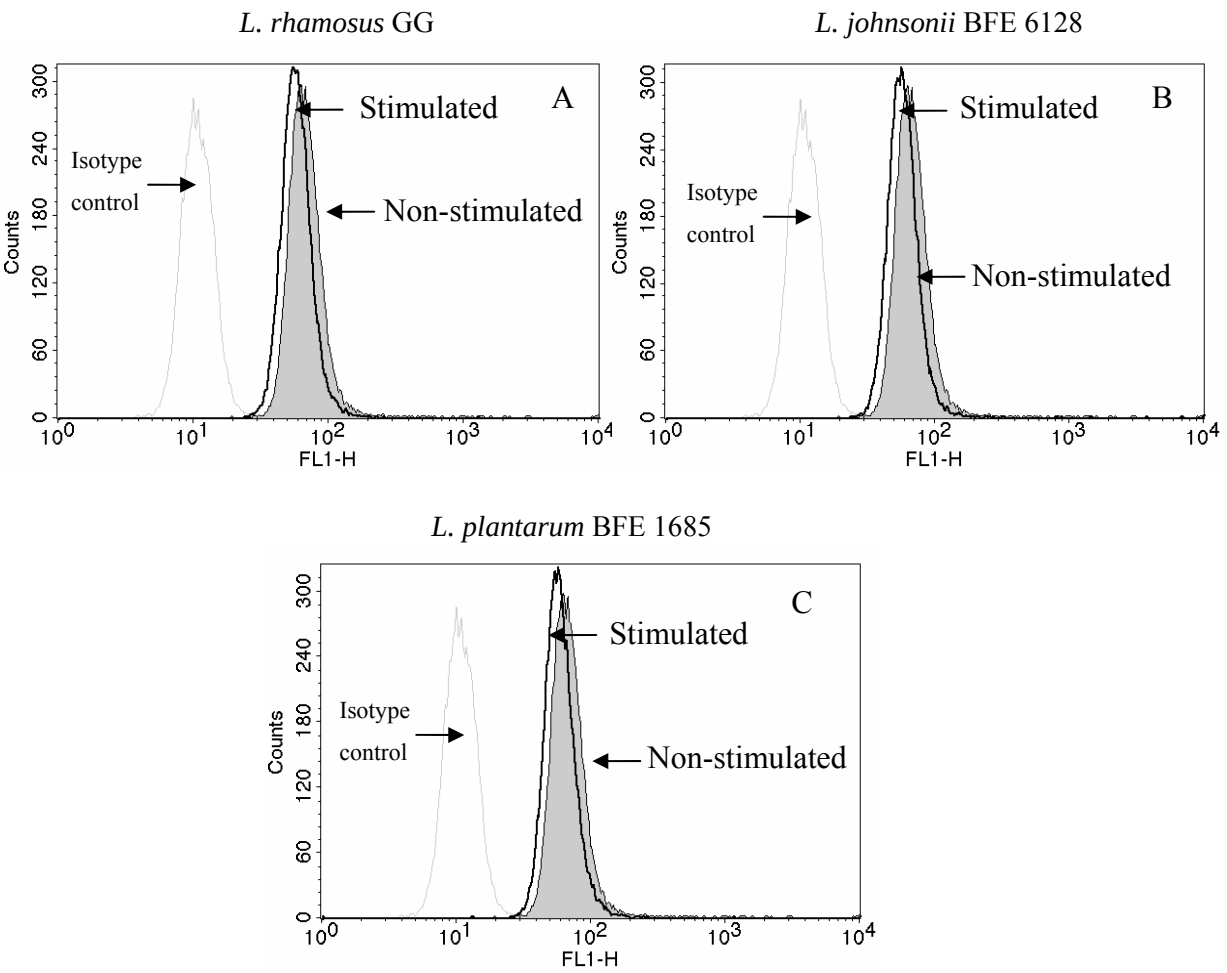


Fig. 3.33 Modulation of intracellular expression of TLR9 in HT29 cells as determined by labeling cells with FITC-conjugated TLR9 antibody and measuring fluorescence by FACS. The filled peaks indicate the background level of expression of TLR9 in HT29 cells. The histogram isotype-antibody (grey-lined open peaks on the left) was also overlaid onto the histograms. Three experiments with similar results were performed. This figure is representative of one such experiment.

3.9 Modulation of HT29 cell responses to *S.* Typhimurium by probiotics

3.9.1 Effect of lactobacilli pre-treatment of HT29 cells on IL-8 secretion by HT29 cells infected with *S.* Typhimurium

Because of the results showing an up-regulation of TLRs by lactobacilli, it was hypothesised that the strains tested in this study may have an influence on the recognition of an enteric pathogen by increasing the amount of these pattern recognition receptors by modulating their gene expression. *S.* Typhimurium was chosen as model pathogen because of its known invasive potential, and because it may be recognised at different levels with

different TLRs, i.e. LPS by TLR4, flagellin by TLR5 and CpG motifs in its bacterial DNA by TLR9. In order to determine if *Lactobacillus* strains were able to modulate the response of enterocytes to a pathogen, HT29 cells were incubated with 5 x 10^6 CFU/ml (corresponding to a ratio of approx. 5 bacteria to 1 eukaryotic cell) for 2 h. After this time, cells were washed with PBS twice, infected with 5 x 10^6 CFU/ml *S.* Typhimurium and incubated for further 4 h. For comparisons, control HT29 cells were treated only with medium (DMEM) for 2 h, washed twice with PBS and infected with *S.* Typhimurium as described in section 2.6.5.

IL-8, whose *de novo* synthesis is induced by NF-κB as a result of TLR pathways activation, was determined in the supernatants as a measure of the responsiveness of IEC to bacteria. All three *Lactobacillus* strains (*L. rhamnosus* GG, *L. johnsonii* BFE 6128 and *L. plantarum* BFE 1685) tested induced a significant ($P<0.05$) increase in the IL-8 level secreted by HT29 cells when compared with the control (cells pre-treated only with DMEM) as shown in Fig.3.34 A.

Lactobacilli enhanced the response of HT29 cells to *S.* Typhimurium and this was not due to an additive effect, as the levels of IL-8 induced by the *Lactobacillus* strains after 2 h incubation, was negligible (ca. 8.0±2.0 pg/ml) and did not significantly differ from the non-treated cells as shown in Fig. 3.34 B. This experiment was done in parallel with the one shown in Fig. 3.34 A and HT29 cells were subjected to the same washing steps indicated before, in order to maintain the same conditions.

This effect may be connected to the increased expression of TLR5, which recognises the main TLR-ligand of *S.* Typhimurium: flagellin, observed in HT29 cells stimulated with *L. rhamnosus* GG or *L. plantarum* BFE 1685 (Fig. 3.32 A and C). In the case of *L. johnsonii* BFE 6128, there must be other mechanisms for the observed IL-8 expression increase, which may explain this effect, as the expression of TLR5 at protein level was not influenced, at least under the conditions tested here (Fig. 3.32 B). Therefore, the effect of cellular components of *S.* Typhimurium on *L. johnsonii* BFE 6128 pre-treated HT29 cells was investigated next.

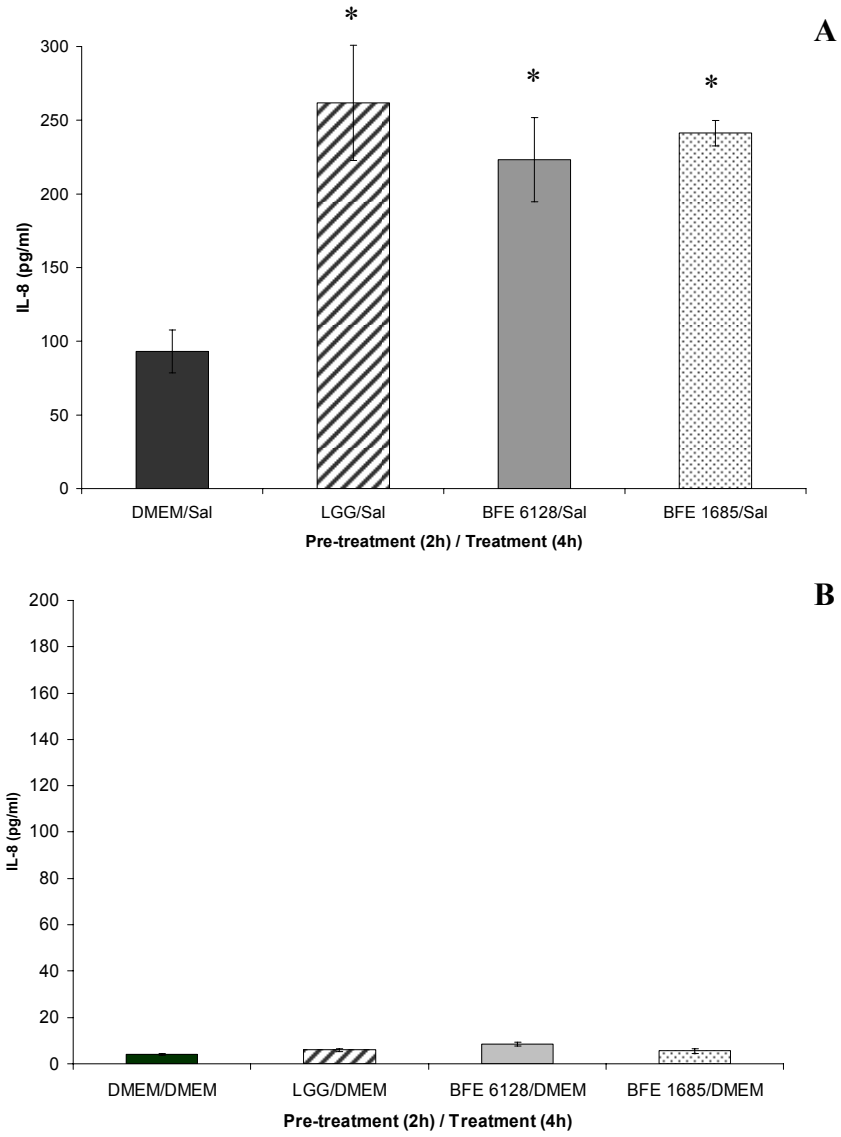


Fig. 3.34 Effect of probiotics on the response of HT29 cell in IL-8 production (pg/ml) after infection with *S.* Typhimurium. A: HT29 cells pre-treated for 2 h with a commercial probiotic strain LGG (*L. rhamnosus* GG) or probiotic candidates: BFE 6128 (*L. johnsonii*) or BFE 1685 (*L. plantarum*) were washed with PBS and treated with *S.* Typhimurium for 4 h. B: Pre-treatment of HT29 cell with 5 x 10^7 CFU/well of LGG, BFE 6128 or BFE 1685 for 2 h after which cells were washed with PBS and incubated with DMEM for 4 h. (n=6). *$P<0.05$, ANOVA, Dunnett's t-test.

3.9.2 *L. johnsonii* BFE 6128 increases the IL-8 response of HT29 cells to cellular components of *S.* Typhimurium

It was shown that *L. johnsonii* BFE 6128 was able to induce an increased IL-8 response of HT29 cells to *S.* Typhimurium (Fig. 3.34 A). The interrelationship between IL-8 and TLR pathways has been described in Fig. 1.2. Therefore, the enhanced IL-8 secreted by HT29 cells should somehow be related to the TLR pathways. As already mentioned, this effect could not be explained by an induction of increased expression of TLR receptors at protein level, as this strain did not increase the expression of TLR5 nor TLR9 (Fig. 3.29 B and 3.30 B). At the mRNA level, this strain also did not influence the TLR4 expression levels, when investigated by RT-PCR (Fig. 3.21) but influenced TLR9 mRNA levels. Another possibility to approach this question could be the identification of the putative ligand stemming from *S.* Typhimurium to which HT29 cells are sensitised by *L. johnsonii* BFE 6128.

Thus, in an attempt to identify the component of *S.* Typhimurium to which *L. johnsonii* BFE 6128 enhanced the innate response of HT29 cells, cellular components from *S.* Typhimurium, which are known TLR-ligands i.e. LPS, flagellin, and DNA, were tested on HT29 cells pre-treated with strain *L. johnsonii* BFE 6128 or pre-treated only with DMEM. ANOVA followed by the t-test were done to determine if the differences in IL-8 secretion of HT29 cells pre-treated with strain BFE 6128 and HT29 cells pre-treated with medium to the mentioned TLR-ligands were significant, as shown in Fig. 3.35. Neither the response to LPS nor to DNA from *S.* Typhimurium were significantly ($P>0.05$) influenced by the pre-treatment with *L. johnsonii* BFE 6128 when compared with cells pre-incubated with DMEM. The well known TLR9-ligand ODN2006, which is an unmethylated CpG rich sequence that stimulates immune cells by binding to TLR9 (Vollmer et al., 2004; Dalpke et al., 2005; Roda et al., 2005), did not exert a significant effect on HT29 cells either.

Nevertheless, pre-treatment of cells with *L. johnsonii* BFE 6128 enhanced the response to both whole cells of S. Typhimurium and to flagellin.

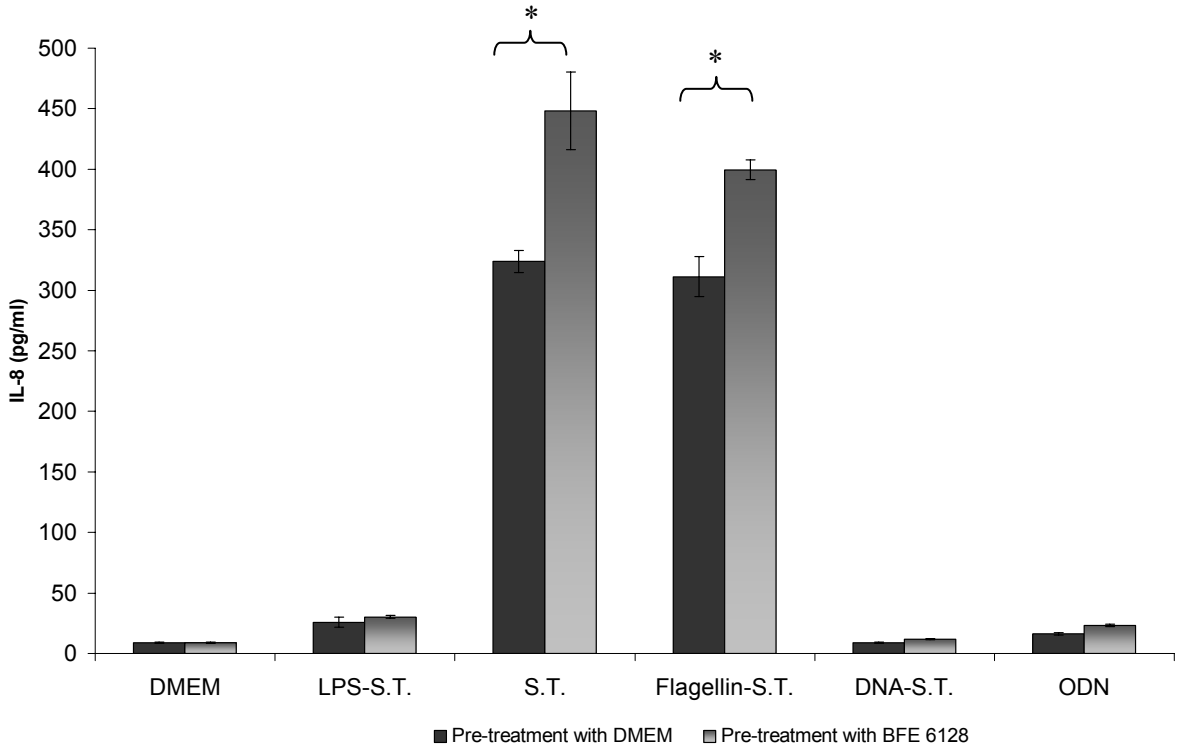


Fig. 3.35 Effect of *L. johnsonii* BFE 6128 on the response of HT29 cells measured as IL-8 production (pg/ml) after infection with *S.* Typhimurium or its cellular components. HT29 cells pre-treated for 2 h only with medium (black bars) or with *L. johnsonii* BFE 6128 (grey bars) after this, cells were washed with PBS and treated with *S.* Typhimurium (S.T.) or their components: LPS (LPS-S.T.), flagellin (Flagellin-S.T.) or DNA (DNA-S.T.) for 4 h. ODN2006 (ODN) which is an unmethylated, CpG rich sequence, was also tested. $*P<0.05$, ANOVA, t-test.

3.9.3 Transcriptomics: modulation of genes involved in TLR pathways

These experiments were done to test whether *L. johnsonii* BFE 6128 was able to modulate the expression of other TLRs or molecules involved in TLR pathways, which may help explaining the observed increased response of HT29 cells pre-treated with this strain to *S.* Typhimurium (Fig. 3.31 A). For this reason, HT29 cells were incubated with *L. johnsonii* BFE 6128 (5 x 10^6 CFU/ml) for 2 h. After this time, cells were washed with PBS twice, infected with 5 x 10^6 CFU/ml *S.* Typhimurium and incubated for further 4 h. HT29 cells treated only with medium (control cells), only with *L. johnsonii* BFE 6128 or only treated with *S.* Typhimurium were also analysed (see 2.6.1). At the end of the incubation period, RNA was isolated and expression of genes involved in TLR pathways was quantified by real-time RT-PCR-based arrays.

Modulation of genes is shown as up- or down-regulation (Fig. 3.36 to 3.43 and Table 3.9) with respect to the control (HT29 cells treated only with DMEM) as calculated with the ΔΔCt method (see 2.9.7).

Yuan et al. (2006) described four statistical approaches to analyse, present and compare real-time PCR data. As a non-parametric alternative to the t-test, the Wilcoxon test for comparisons between two samples to analyse the pools of ΔCt values was used. In this study, the Kruskal-Wallis test, which is an alternative to the Wilcoxon test, was also used for comparisons among multiple samples. Using realistically small sample sizes, the free-distribution Wilcoxon test is a more robust and appropriate alternative to the t-test (Yuan et al., 2006). The primary assumption by calculating ΔCt is that the additive effect of concentration, gene and replicate can be adjusted by subtracting Ct number of reference gene (in this case the mean of the Ct value of four housekeeping genes) from that of target gene (See equations in section 2.11.6.2). The ΔCt for each treatment and control (cells treated only with medium) can therefore be subject to both the Kruskal-Wallis test and the Wilcoxon test, which will yield the estimation of ΔΔCt. The results presented here were derived from testing the null hypothesis, i.e. that ΔΔCt is equal to 0. If $P<0.05$, it means that the treatment had a significant effect on the expression of the target gene.

The quality controls used were the no template control (NTC), which allowed the evaluation of possible contaminations of the master mix and the no reverse transcription control (NRT), which allowed testing the absence of genomic DNA contamination. Table 2.9 (in the material and methods section) shows the genes included in the real-time based RT-PCR array, Table 3.9 in this section shows relevant modulated genes.

As a result of the statistical analyses, it became evident that the deviations among replicates by analysing the expression of some target genes resulted in a non-significant difference between expression of these genes in control cells (treated only with medium) compared to treated cells as in the case of the CD14 and LY-86 genes (Fig. 3.36), while for others a significant difference of expression could be determined as for example for BTK or TICAM1 genes (Fig. 3.36). The lack of significance by the modulation of certain genes with an up- or down-regulation higher than 2-fold (Table 3.9), may have been a result of the too low sample size (n= 3 for each treatment). As a result, the discrimination sensitivity of the statistical test is affected and no significant differences in expression levels can be established. This could be improved for some of such genes if a greater sample size was used. Unfortunately, due to the prohibitive cost of these arrays this was not possible in this study.

Nevertheless, for some target genes a significant difference could be noted with some important results becoming clear.

When HT29 cells were pre-treated with medium and afterwards infected with *S.* Typhimurium, a lot of the genes analysed were down-regulated, though not significantly. On the other hand, when HT29 cells were pre-treated with *L. johnsonii* BFE 6128 and afterwards infected with *S.* Typhimurium most of the genes were up-regulated, seventeen of which were significantly ($P<0.05$) up-regulated. This could indicate that the pre-treatment with *L. johnsonii* BFE 6128 of intestinal epithelial cells (HT29 cells) sensitises the cells to initiate signal transductions, which lead to the enhanced transcription of genes involved in innate immunity that may help responding to the pathogenic strain.

Table 3.9 Up- or down-regulation[a] of genes involved in TLR pathways in HT29 cells by *L. johnsonii* BFE 6128, *S.* Typhimurium or both

Treatment		**DMEM (2h) + *S.* Typhimurium (4h)**	***L. johnsonii* BFE 6128 (2h) + *S.* Typhimurium (4h)**	***L. johnsonii* BFE 6128 (2h) + DMEM (4h)**
Grouping	Gene Symbol	Fold up- or down-regulation	Fold up- or down-regulation	Fold up- or down-regulation
Adaptors and interacting proteins	BTK	-1.14	4.92[b]	7.23[c]
	CD14	-1.28	1.12	2.46
	LY86 (MD-1)	-1.41	4.71	9.70
	LY96 (MD-2)	-1.69	2.00	5.38
	MAPK8IP3	2.37	3.01	2.33
	MYD88	-1.08	-1.13	1.14
	PELI1	1.46	1.78	-1.65
	RIPK2	1.67	2.27	-1.61
	TICAM1	1.36	1.84	1.34
IRF Pathway	CXCL10	1.66	2.72	-1.74
	IFNA1	1.22	2.00	2.92
	IRF1	1.87	2.65	-2.20
JNK/p38 pathway	FOS	3.46	7.84	-2.51
	JUN	1.95	3.36	-1.85
	MAP3K1	1.12	1.14	-1.40
NF/IL6 pathway	CLEC4E	-1.79	3.16	7.54
	PTGS2	1.51	1.39	-2.23
NF-κB pathway	CCL2	-1.25	2.80	3.75
	CSF2	2.95	10.14	-2.97
	CSF3	1.89	2.65	6.81
	IFNA1	1.22	2.00	2.92
	IL6	-2.83	-1.42	1.80
	IL8	11.38	54.37	-8.75
	IL10	-1.77	3.68	7.58
	LTA	2.28	2.97	9.66
	NFKB1	1.24	1.42	-1.50
	NFKB2	1.46	2.50	-1.78
	NFKBIA	1.57	2.52	-1.91
	TNF	4.44	10.69	4.03

Effectors	IRAK1	-1.29			-21.04			1.07		
	IRAK2	1.92			3.02			-2.22		
	PPARA	1.16			1.33			-1.23		
Regulation of adaptive immunity	CD80	1.03			7.65			10.64		
	CD86	-1.36			2.28			6.41		
	RIPK2	1.67			2.27			-1.61		
TLRs	CD180	-1.46			3.56			10.94		
	TLR10	-2.29			1.35			2.48		
	TLR2	-1.13			3.97			8.76		
	TLR5	-1.63			1.21			3.93		
	TLR7	-3.14			-1.06			5.74		
	TLR9	2.21			2.12			4.77		
Total number of genes	Up-regulated	2	8	15	17	15	3	3	18	3
	Down-regulated	0	3	12	0	1	3	1	5	10

[a] Up- (positive values) or down-regulation (negative values) in comparison to the control (HT29 cells treated only with DMEM); [b] light grey (notably regulated up- or down- regulation >2 fold but $P>0.05$); [c] dark grey significantly regulated ($P<0.05$) according to the results of the Kruskal-Wallis and Wilcoxon tests.

In the group of genes coding for adaptors and modulating proteins (Fig. 3.36), *L. johnsonii* BFE 6128 alone was only able to significantly ($P<0.05$) up-regulate the Bruton agammaglobulinemia tyrosine kinase (BTK) gene and the Toll-like receptor adaptor molecule 1 (TICAM1) gene. When cells were pre-treated with *L. johnsonii* BFE 6128 and afterwards treated with *S.* Typhimurium, Mitogen-activated protein kinase 8 interacting protein 3 (MAPK8IP3), Pellino homolog 1 (Drosophila) (PELI1), Receptor-interacting serine-threonine kinase 2 (RIPK2) and TICAM1 genes were significantly ($P<0.05$) up-regulated. *S.* Typhimurium alone only significantly up-regulated RIPK2 (Fig. 3.36 and Table 3.9).

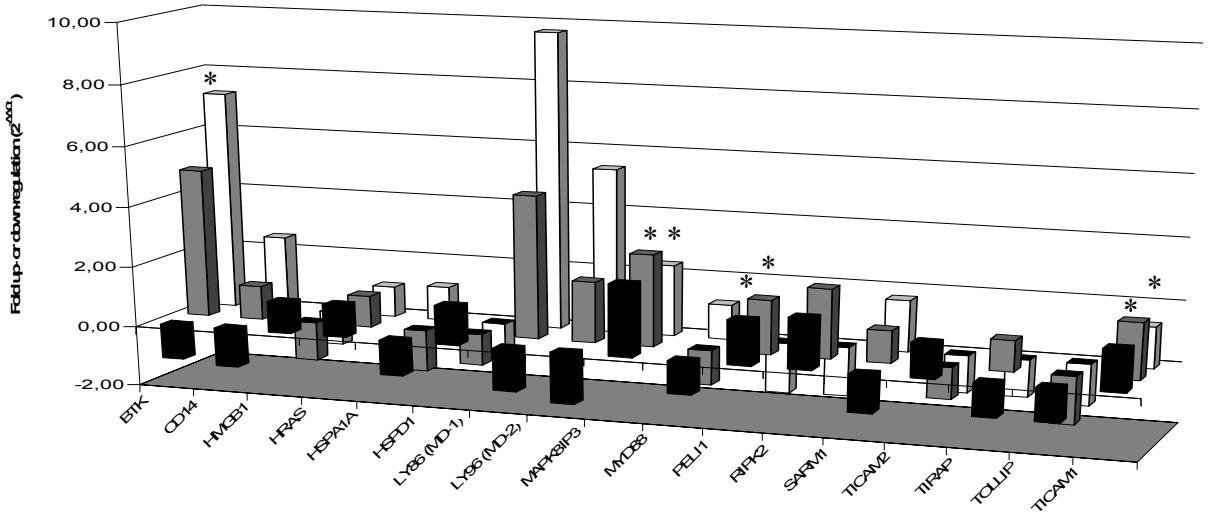


Fig. 3.36 Modulation of expression of genes coding for adaptors and interacting proteins involved in TLR pathways. RNA was isolated from HT29 cells treated only with medium (control cells), from cells pre-treated with *L. johnsonii* BFE 6128 for 2 h and followed with a 4-h incubation with medium (white bars), from cells pre-treated with *L. johnsonii* BFE 6128 and followed by a 4-h treatment with *S.* Typhimurium (grey bars), and from

cells pre-treated with medium for 2 h and followed by a 4-h-incubation with *S.* Typhimurium (black bars). **P*<0.05, Kruskal-Wallis test and Wilcoxon test.

For the interferon regulatory factor (IRF) pathway, two genes, chemokine (C-X-C motif) ligand 10 (CXCL10) and interferon regulatory factor 3 (IRF3), belonging to the IRF pathway were significantly up-regulated (P<0.05), as shown in Fig. 3.37.

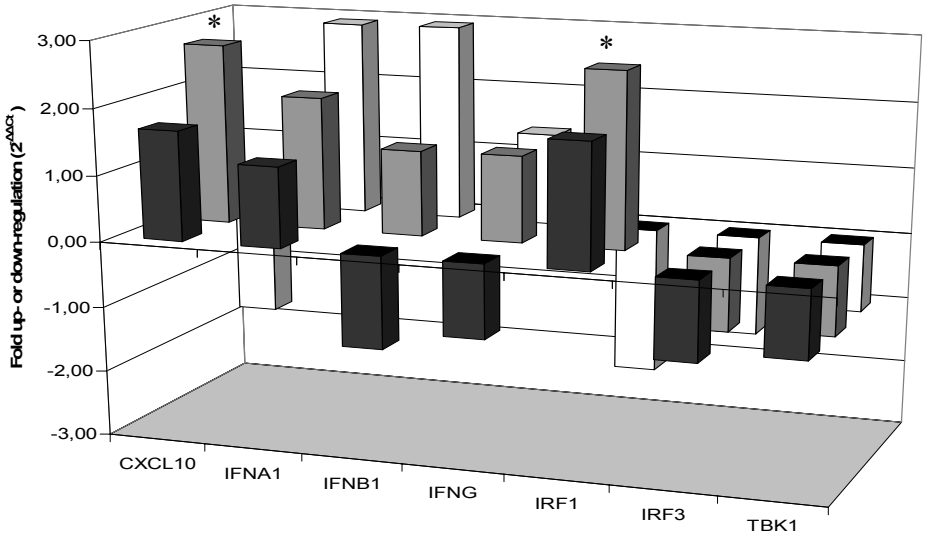


Fig. 3.37 Modulation of genes involved in the IRF pathway. RNA was isolated from HT29 cells treated only with medium (control cells), from cells pre-treated with *L. johnsonii* BFE 6128 for 2 h and followed with a 4-h incubation with medium (white bars), from cells pre-treated with *L. johnsonii* BFE 6128 and followed by a 4-h treatment with *S.* Typhimurium (grey bars), and from cells pre-treated with medium for 2 h and followed by a 4-h-incubation with *S*. Typhimurium (black bars). **P*<0.05, Kruskal-Wallis, Wilcoxon test.

For the JNK/p38 pathway, the V-fos FBJ murine osteosarcoma viral oncogene homolog (FOS) was down-regulated by *L. johnsonii* BFE 6128, but up-regulated (*P*<0.05, Fig. 3.38) when both *L. johnsonii* BFE 6128 and *S*. Typhimurium were given to the HT29 cells (Fig. 3.38).

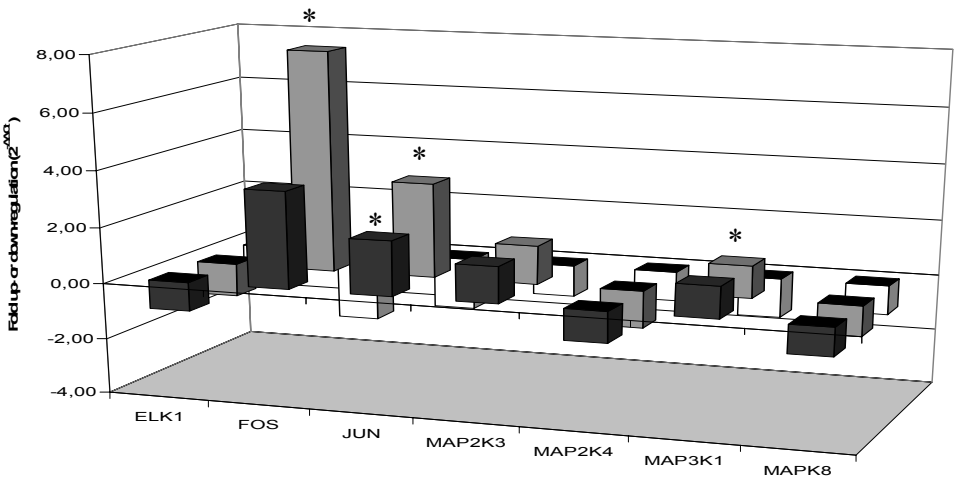


Fig. 3.38 Modulation of genes involved in the IRF pathway (B). RNA was isolated from HT29 cells treated only with medium (control cells), from cells pre-treated with *L. johnsonii* BFE 6128 for 2 h and followed with a 4-h incubation with medium (white bars), from cells pre-treated with *L. johnsonii* BFE 6128 and followed by a

4-h treatment with *S.* Typhimurium (grey bars), and from cells pre-treated with medium for 2 h and followed by a 4-h-incubation with *S.* Typhimurium (black bars). **P*<0.05, Kruskal-Wallis and Wilcoxon test.

V-jun sarcoma virus 17 oncogene homolog (avian) (JUN) and Mitogen-activated protein kinase kinase kinase 1 (MAP3K1) both belonging to the JNK/p38 pathway, were also up-regulated (*P*<0.05) when cells were pre-treated with *L. johnsonii* BFE 6128 and then infected with *S.* Typhimurium (Fig. 3.38).

Only one gene of the NF/IL-6 pathway was significantly modulated when compared with the untreated cells (Table 3.9). This gene was prostaglandin-endoperoxide synthase 2 (prostaglandin G/H synthase and cyclo-oxygenase) also known as COX-2 (PTGS2), which was up-regulated (Fig. 3.39) when HT29 cells were pre-treated with *L. johnsonii* BFE 6128 and followed by a 4-h treatment with *S.* Typhimurium, but it was down-regulated when cells were treated only with the probiotic candidate *L. johnsonii* BFE 6128 (Fig. 3.39).

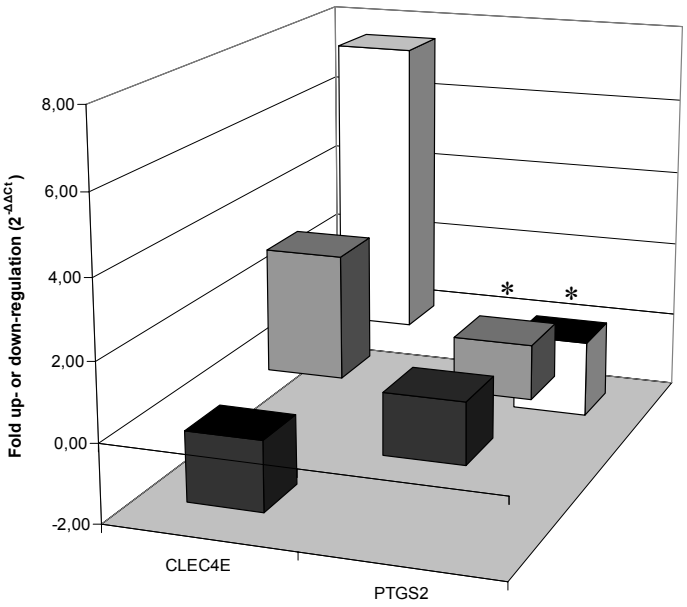


Fig. 3.39 Modulation of genes involved in the NF-IL6 pathway. RNA was isolated from HT29 cells treated only with medium (control cells), from cells pre-treated with *L. johnsonii* BFE 6128 for 2 h and followed with a 4-h incubation with medium (white bars), from cells pre-treated with *L. johnsonii* BFE 6128 and followed by a 4-h treatment with *S.* Typhimurium (grey bars), and from cells pre-treated with medium for 2 h and followed by a 4-h-incubation with *S.* Typhimurium (black bars). **P*<0.05, Kruskal-Wallis test, Wilcoxon test.

For the nuclear-factor κB pathway, three genes, i.e. granulocyte-macrophage colony stimulating factor 2 (CSF2) gene, interleukin-8 (IL-8) gene and TNF-α were significantly (*P*<0.05) up-regulated (Fig. 3.40, Table 3.9) when HT29 cells were pre-treated with *L. johnsonii* BFE 6128 and infected with *S.* Typhimurium. In general, it can be seen that the pre-treatment of HT29 cells with the probiotic candidate *L. johnsonii* BFE 6128 induced a higher expression of genes involved in the NF-κB pathway in response to *S.* Typhimurium. When naïve HT 29 cells were infected with this pathogenic strain, these same genes were also up-

regulated but to a much lesser extent (Fig. 3.40 and Table 3.9). The same phenomenon was observed by other genes such as CCL2, CSF3, IFNA1, NFKB1 and NFKB2 also involved in these pathway but with a significance $P<0.05$.

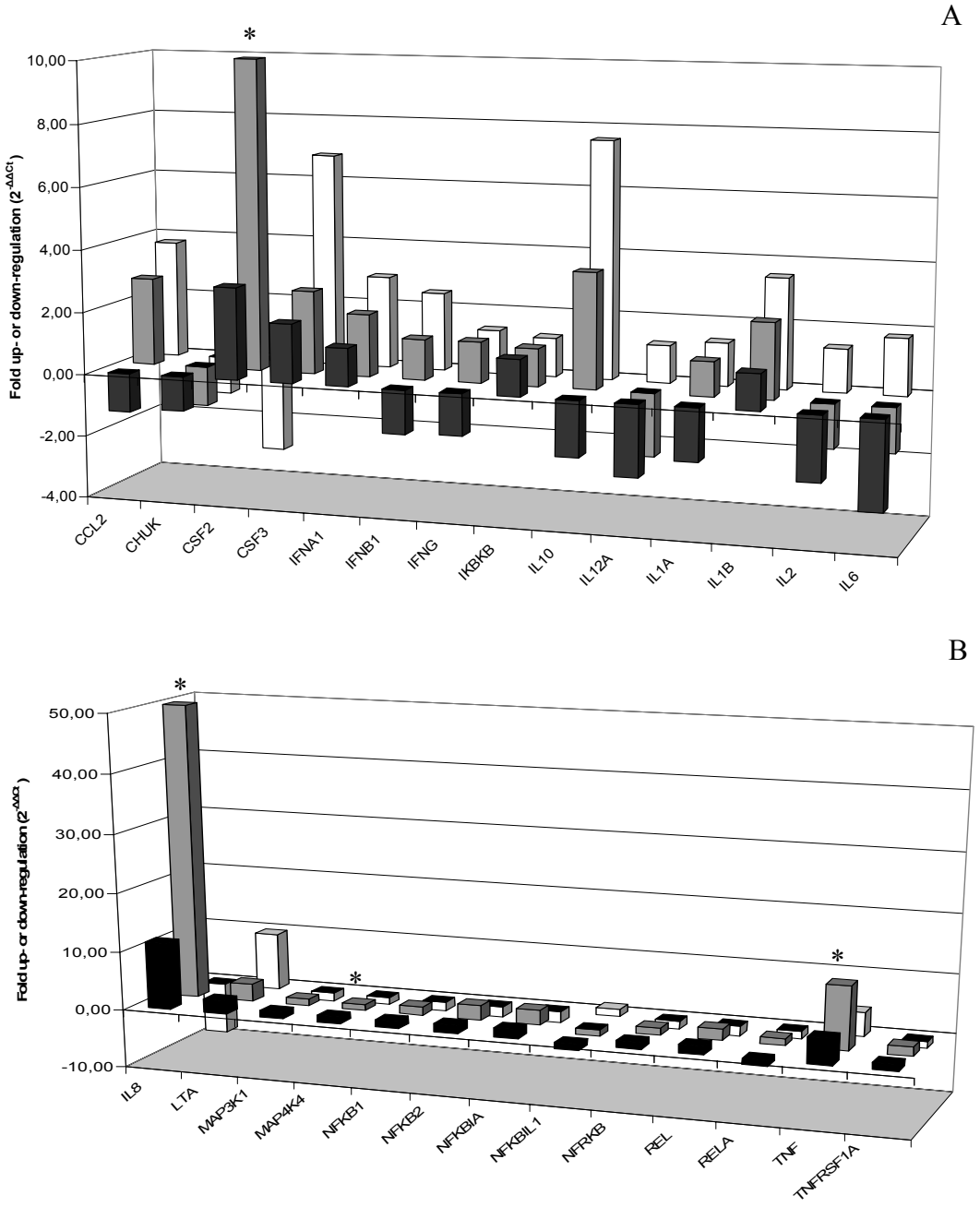


Fig. 3.40 Modulation of genes involved in the NF-KB pathway (A and B). RNA was isolated from HT29 cells treated only with medium (control cells), from cells pre-treated with *L. johnsonii* BFE 6128 for 2 h and followed with a 4-h incubation with medium (white bars), from cells pre-treated with *L. johnsonii* BFE 6128 and followed by a 4-h treatment with *S.* Typhimurium (grey bars), and from cells pre-treated with medium for 2 h and followed by a 4-h incubation with *S.* Typhimurium (black bars). **P*<0.05, Kruskal-Wallis test, Wilcoxon test.

Among effector genes associated to TLR pathways (Fig. 3.41), only two genes were significantly ($P<0.05$) modulated, and only when HT29 cells were treated with both strains. Interleukin-1 receptor-associated kinase 2 (IRAK2) and peroxisome proliferative activated

receptor alpha (PPARA) were significantly (P<0.05) up-regulated. IRAK1 gene was markedly down-regultated when HT29 cells were treated with both lactobacilli and *S.* Typhimurium, but the significance of this effect was P<0.05.

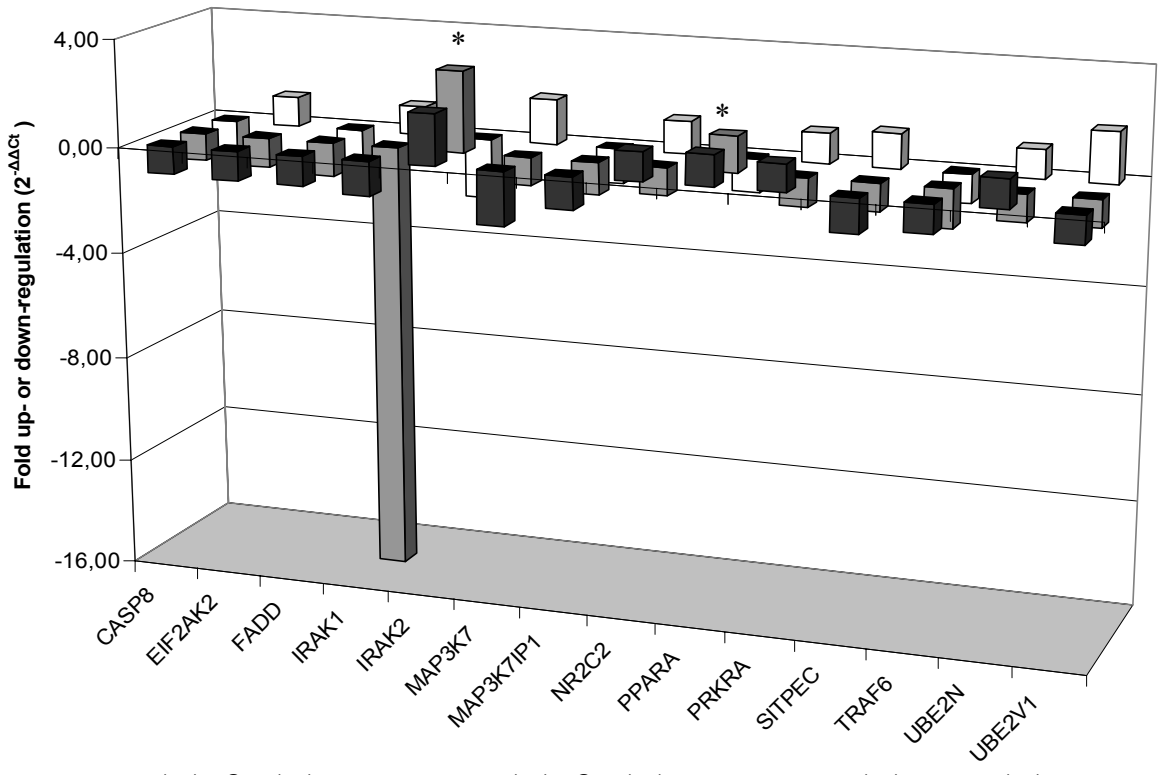


Fig. 3.41 Modulation of effector genes involved in the TLR pathways. RNA was isolated from HT29 cells treated only with medium (control cells), from cells pre-treated with *L. johnsonii* BFE 6128 for 2 h and followed with a 4-h incubation with medium (white bars), from cells pre-treated with *L. johnsonii* BFE 6128 and followed by a 4-h treatment with *S.* Typhimurium (grey bars), and from cells pre-treated with medium for 2 h and followed by a 4-h incubation with *S.* Typhimurium (black bars). *P<0.05, Kruskal-Wallis test, Wilcoxon test.

The only gene involved in regulation of the adaptive immune response which was significantly (P<0.05) up-regulated by *S.* Typhimurium alone and when cells had been pre-treated with *L. johnsonii* BFE 6128 followed by a treatment with *S.* Typhimurium was RIPK2 (Fig. 3.42). This gene is also included in the group of adaptors and modulating proteins. Nevertheless, it is worth mentioning that two genes involved in this pathway which code for non-classical antigen presenting molecules CD80 and CD86 were also up-regulated, though not significantly (P>0.05) by *L. johnsonii* BFE 6128 alone and by *L. johnsonii* BFE 6128 followed by a treatment with *S.* Typhimurium (Table 3.9).

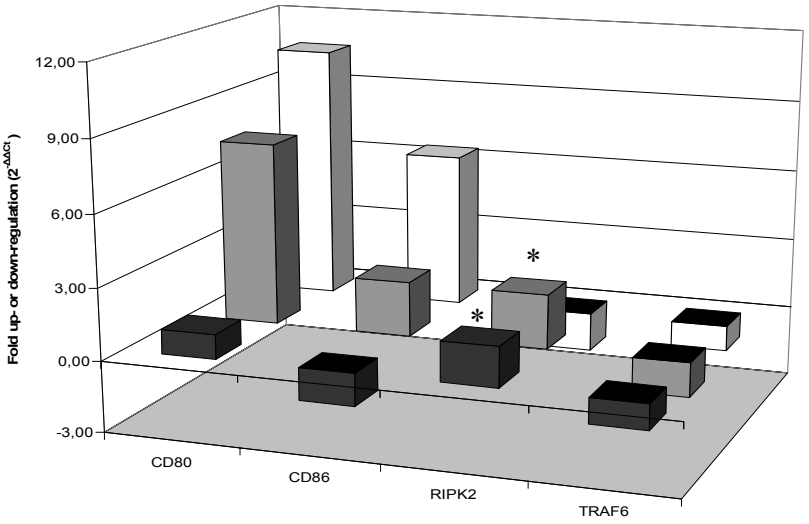


Fig. 3.42 Modulation of genes involved in the adaptive immunity. RNA was isolated from HT29 cells treated only with medium (control cells), from cells pre-treated with *L. johnsonii* BFE 6128 for 2 h and followed with a 4-h incubation with medium (white bars), from cells pre-treated with *L. johnsonii* BFE 6128 and followed by a 4-h treatment with *S.* Typhimurium (grey bars), and from cells pre-treated with medium for 2 h and followed by a 4-h incubation with *S.* Typhimurium (black bars). **P*<0.05, Kruskal-Wallis test, Wilcoxon test.

For the TLR pathways, the CD180 gene was also significantly up-regulated (Fig. 3.42, Table 3.9) when both strains were present. Most TLRs were up-regulated in HT29 cells treated with *L. johnsonii* BFE 6128 alone or followed by exposure with *S.* Typhmurium. In contrast, *S.* Typhimurium alone down-regulated most of the TLRs. Nevertheless, the significance of these results was *P*>0.05, which can be attributed to the low number of replicates as mentioned above.

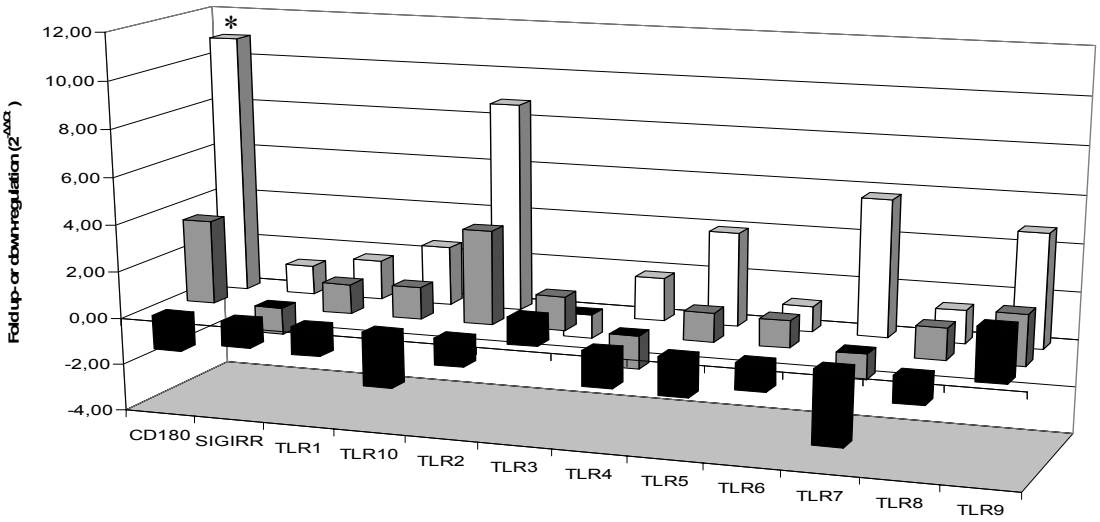


Fig. 3.43 Modulation of TLR and TLR-related genes. RNA was isolated from HT29 cells treated only with medium (control cells), from cells pre-treated with *L. johnsonii* BFE 6128 for 2 h and followed with a 4-h incubation with medium (white bars), from cells pre-treated with *L. johnsonii* BFE 6128 and followed by a 4-h treatment with *S.* Typhimurium (grey bars), and from cells pre-treated with medium for 2 h and followed by a 4-h-incubation with *S.* Typhimurium (black bars). **P*<0.05, Kruskal-Wallis test, Wilcoxon test.

Moreover, the up-regulation of TLRs correlates with the up-regulation of TLR2 and TLR9 by lactobacilli described before (Fig. 3. 42) as determined by real-time RT-PCR with own-designed primers.

4 DISCUSSION

The first criterion of this study for selection of new potentially probiotic strains was the ability of the strains to survive under simulated gastrointestinal conditions (Table 4.1). This ability should ensure that these strains reach the small intestine, which is the intended site of action. According to the guidelines for the evaluation of probiotics in food reported by a Joint FAO/WHO working group (Chesson et al., 2002), two of the currently most widely used *in vitro* tests are resistance to gastric acidity and bile salts, as based on both survival and growth studies. The predictability of these *in vitro* tests is limited, but the use of sophisticated and dynamic, computer models of the gastrointestinal tract, like the one developed by Marteau et al. (1997), is beyond the scope of most laboratories. Most of the reported studies have been done in acidified MRS (pH 2, 2.5, 3 and 3,5) (Chou & Weimer, 1999; Kociubinski et al., 1999; Xanthopoulos et al., 2000; Saito, 2004) and in MRS broth containing 0.15 to 0.5 % bile salts (Chou & Weimer, 1999; Zarate et al., 2000; Fernandez et al., 2003). Rather than evaluating the effect of each component in separate experiments, it should be important to evaluate all components (enzymes, low pH, bile salts, duodenum secrete and food vehicle) in one system, as the two successive stresses of stomach transit and small intestinal transit might interact and thereby affect the survival of the strains in a synergistic way. On the other hand, acidified MRS broth does not contain the enzymes released by gastric cells nor by the pancreas, and MRS broth represents the optimum medium for growth of lactobacilli.

Therefore, the Plackett-Burman design was used in this study to determine which variables really affect the viability of bacteria in a simple *in vitro* model of the stomach, which was later to be combined with the passage through simulated intestinal conditions. According to the analysis of this design, it is necessary not only to test the tolerance to low pH, but also the action of enzymes like pepsin and lysozyme. It is also important to consider the food vehicle in which the probiotic would be included, as it might exert a protective role and enhance the viability of bacteria. For this reason, not only was the effect of pH, but also that of pepsin and lysozyme included in the stomach passage model. After one hour incubation under simulated gastric conditions, a further treatment followed, with simulated intestinal fluids containing bile salts and pancreatin, which is a pool of pancreatic enzymes (amylases, trypsin, lipase, ribonuclease and proteases). In this way, both gastric and intestinal stresses were successfully combined in a simulated *in vitro* gastrointestinal model.

Table 4.1 Probiotic properties of novel *Lactobacillus* isolates

Probiotic candidates		Probiotic properties																
		Selection criterion	Safety		Technological and functional properties						Adhesive properties							Stimulation of innate immunity	
Phenotypic and genoptypic identification	Strain	Survival under gastrointestinal conditions	Transferable antibiotic resistance	Origin	Resistance to 0.4 % phenol	Antagonistic activity against food-borne pathogens	H_2O_2	Bsh	β-galactosidase	Growth on the prebiotic sugar Raftiline ST	Cell surface hydrophobicity	Cell surface acidic character	Cell surface basic character	Adhesion to intestinal epithelial cells (HT29)	Competitive exclusion of intestinal pathogens	Auto-aggregation	Coaggregation with food-borne pathogens	Induction of IL-8 by HT29 cells	Cytotoxicity on HT29 cells
L. plantarum	BFE 1684	+++[a]	-	HF[e]	++[b]	++	Y[h]	Y	Y	+	+++	++		++	n.d.[j]	+	++	N	-
	BFE 1685	+++	-	HF	++	++	N[i]	Y	N	++	+++	+++	+	+++	+++	+	++	Y	-
	BFE 5759	+++	-	Kw[f]	++	++	N	Y	Y	++	++	+	+	-	n.d.	++	++	Y	-
	BFE5878	+++	-	KN[g]	++	++	Y	Y	N	-	++	+	+	++	n.d.	+	-	N	-
	BFE 5092	+++	-	KN	++	++	N	Y	N	-	+	+	+	+++	n.d.	++	+	n.d.	-
L. johnsonii	BFE 6128	+++	-	KN	-[d]	++	Y	Y	N	+++	+++	+++	+++	+++	++	+++	+++	Y	-
	BFE 6154	+++	-	KN	+[c]	++	Y	Y	N	+++	+++	+++	++	+++	n.d.	+++	+++	N	-

[a] +++: high, [b] ++: medium, [c]+: low, -[d]: none, [e]HF: Human faeces, [f]Kw: Kwerionik, [g]KN: Kule naoto, [h]Y: yes, [i]N: no, [j]n.d. not determined.

Resistance to bile salts is considered an important property in strains to be used as probiotics, however, there is still no consensus about the precise concentration to which the selected strain should be tolerant. The physiological concentration of bile acids in the small intestine varies from 5 to 20 mmol/l (Hofmann, 1991). Therefore, a concentration of 0.5 % oxgall, equivalent to 12.25 mmol/l bile acids was used in this study, as was also done by Kociubinsky et al. (1999). This concentration is higher than the ones (0.3 % and 0.15 %) which have previously been used by other investigators of bacterial probiotic properties (Chou & Weimer, 1999; Zarate et al., 2000; Fernandez et al., 2003).

Using this *in vitro* simulated stomach-duodenum passage, only a few of the 30 strains tested, including the reference probiotic strain *L. johnsonii* LA1 (BFE 663), were able to survive the passage under the conditions of this study. *L. johnsonii* LA1 was used because of its known good survival to gastrointestinal conditions *in vivo* (Holzapfel et al., 1998; Salminen et al., 1998). The fact that only seven strains (Table 4.1) out of thirty isolates tested together with this reference strain survived the passage through this model validates the model, since gastrointestinal conditions are considered to be detrimental to most food-associated lactic acid bacteria, and because the probiotic control strain indicated survival under the conditions tested. Actually, the conditions selected in this *in vitro* model may have been even harsher than in the real situation, where the gastric pH after a meal is of approx. 3, and there is a continual removal of the bile salts, leading to fluctuating and often lower bile levels. However, this was based on a 'worst case' scenario, to determine the most stress resistant strains. As already pointed out by various workers in the field (Havenaar et al., 1992; McCracken & Gaskins, 1999; Bezkorovainy, 2001; Dunne et al., 2001), as well as a Joint FAO/WHO working group (Chesson et al., 2002), *in vitro* studies can only partially mimic the actual *in situ* conditions in the gut ecosystem. Nevertheless, such *in vitro* systems are powerful tools especially for screening numerous samples.

Another test for resistance to intestinal conditions used in this study was resistance to 0.4 % phenol (Table 4.1). Phenols may be formed in the intestine as a result of the bacterial deamination of some aromatic amino acids derived from dietary and endogenous proteins. These compounds are known to have bacteriostatic properties at least *in vitro* (Suskovic et al., 1997). Hence, if they also present this bacteriostatic activity *in vivo,* bacteria tolerant to phenols may have more chances of survival than those which are not. In contrast to the high phenol resistance (resistance to 0.4 % phenol) which was previously reported by Xanthopoulus et al. (2000) and Suskovi et al. (1997) for *L. acidophilus* strains, the *L. johnsonii* strains in this study were highly sensitive towards this compound. This was despite

the fact that these bacteria are physiologically and phylogenetically closely related. In contrast, the results of this study on the phenol resistance of *L. plantarum* strains suggest that the latter were generally moderately tolerant. The significance of the resistance to phenol has not been correlated with *in vivo* data yet.

Seven new potentially probiotic isolates were selected because of their good survival properties for further characterisation (Table 4.1). The selected strains were successfully characterised using both phenotypic and genotypic methods, five strains as *L. plantarum,* and the other two as *L. johnsonii*. These strains were further investigated for their probiotic properties as discussed below. Two *L. plantarum* strains (BFE 1684 and 1685) stemmed from faeces of healthy children, indicating the safe origin of the strains and also the ability of these strains to survive the passage through the harsh conditions in the gastrointestinal tract (Table 4.1). The other selected strains (two *L. johnsonii* and three *L. plantarum*) stemmed from African traditional fermented milk products (Table 4.1). In a previous study conducted by Mathara et al. (2004), *L. plantarum* was the dominating species present in 'kule naoto', the Maasai traditional fermented milk in Kenya. Although this species has been mainly associated to plant-based food fermentations (Holzapfel & Wood, 1995; Stiles & Holzapfel, 1997), it has also been reported to be part of the microbiota of African traditional fermented milks (Isono et al., 1994; Beukes et al., 2001), and of traditional fermented milks and cheeses from Argentina (Medina et al., 2001). *L. plantarum* is also a normal inhabitant of the gut (Tannock, 1999), and therefore it is not surprising, that there are strains able to survive well under gastrointestinal conditions. The strain *L. plantarum* 299v, which was originally isolated from the human intestine, is a known probiotic strain that is commercialised mainly in Sweden in a lactic acid fermented oatmeal gruel, that is mixed in a fruit drink (Molin, 2001; Pathmakanthan et al., 2004). This could be also an interesting alternative for developing a probiotic product with no milk or milk constituents using the *L. plantarum* strains characterised in this study.

The two selected *L. johnsonii* strains were isolated from Kule naoto, but they were present in low numbers in this product and were not a representative important and predominant species in the microbiota of this fermented milk (Mathara et al., 2004). They had been previously characterised as *L. acidophilus* (Mathara et al., 2004), but in this study it was clearly shown that although they showed phenotypic properties typical of *L. acidophilus*, they actually belong to *L. johnsonii* species, as assessed by more accurate genotypical methods. *L. johnsonii* also belongs to the normal microbiota of the intestine and is one of the first species, after *Bifidobacterium* spp., which colonises the gut of newborns (Vaughan et al., 2002). The

strain *L. johnsonii* LA-1, which had been isolated from the human intestine, is a probiotic strain used world-wide and which has proven gastrointestinal survival (Salminen et al., 1998; Felley et al., 2001; Holzapfel et al., 2001). Although the *L. johnsonii* strains of this study have not been isolated from humans, they originate from a traditional 'domesticated' environment, and associated with fermented milk, they would e most suitable for development of probiotic products.

A major requirement for probiotic strains is that they should be safe for human consumption. In order to assure the safety of bacteria used in food, the European Food Safety Authority (EFSA) has initiated a 'Qualified Presumption of Safety' concept which, similar to the GRAS system in the USA, has the purpose of allowing strains with long history of safe use and status to enter the market without extensive testing requirements ((EFSA), 2004). Antibiotic resistance is one of the safety concerns included in the QPS concept to determine a strain's QPS status. The reason for this is the hypothesis that food bacteria may act as reservoirs of antibiotic resistance genes (Chesson et al., 2002; Danielsen & Wind, 2003; Franz et al., 2005). In a survey of 62 *Lactobacillus* starter strains, Danielsen and Wind (2003) found a high level of resistance to aminoglycosides for all investigated lactobacilli. Similar results were also found by Charteris et al. (1998) and Katla et al. (2001). These authors concluded that resistance to aminoglycosides among lactobacilli is a natural, i.e. intrinsic trait, which has been attributed to the absence of a cytochrome-mediated electron transport, that mediates uptake of aminoglycosides (Charteris et al., 2001). Danielsen and Wind (2003) also suggested that resistance to ciprofloxacin appeared to constitute a natural or intrinsic resistance. Considering these data, the only antibiotic resistances which were observed for the strains in this study, i.e. resistance towards ciprofloxacin and aminoglycosides, were considered to be intrinsic or natural resistances. Conversely, the strains selected in this study did not contain any of the transferable, acquired resistances which are known to occur among LAB and include resistances towards e.g., chloramphenicol, erythromycin and tetracycline (Danielsen & Wind, 2003). Therefore, the strains selected in this study do not pose a risk for consumers with respect to antibiotic resistance transfer to other gut-associated bacteria (Table 4.1).

Some variability was noted in the pathogen inhibition profiles of the *Lactobacillus* strains tested. All the probiotic candidates of this study were able to inhibit at least to some extent some pathogenic strains associated with food, as indicated by the results of the agar spot test (Table 4.1). This antagonistic activity was not due to the production of a bacteriocin, because they did not inhibited related strains and the neutralised supernatants did not show inhibitory activity. As the plates were incubated anaerobically, the antimicrobial effect was not thought

to be due to H_2O_2 production. Thus, the inhibition of the indicator strains most probably was due to the production of organic acids. Under aerobic conditions, both *L. johnsonii* strains BFE 6128 and BFE 6154 strains and *L. plantarum* strains BFE 1684 and BFE 5878 were able to produce H_2O_2, showing that this is a strain-specific trait, because other strains belonging to the same species did not produce H_2O_2 (Table 4.1). Production of this antimicrobial compound by lactobacilli has been described before (Annuk et al., 2003; Servin, 2004) but its specific role is still unclear. H_2O_2-producing lactobacilli are especially found in the vagina of healthy women and the presence of these bacteria have been associated with a lower frequency of vaginosis as they can, for example, inhibit gonococci (Servin, 2004). H_2O_2-producing lactobacilli able to coaggregate with pathogens may exert an antagonistic effect in the close proximity, resulting from the coaggregate formation. Therefore, it would be interesting to test *in vivo* if the lactobacilli selected in this study also display these properties (coaggregation and H_2O_2 production) in the vaginal microenvironment. The significance of this property in the small intestine has not been reported yet, but we hypothesise that this could be possible in a transitional segment of the GIT, such as the duodenum, where the conditions are microaerophilic, rather than anaerobic.

Whether Bsh activity is correlated with high tolerance to bile salts is still under debate. While some researchers sustain that deconjugation of bile salts might be a detoxification mechanism of vital importance to the *Lactobacillus* cell, and thus play an important role during colonisation of the gastrointestinal tract (Tannock et al., 1989; De Smet et al., 1995; Usman & Hosono, 1999; De Boever et al., 2000), others infer that the higher toxicity of the deconjugated salts might more strongly affect the viability of the bacterium (Grill et al. 2000). Bsh activity is a controversial probiotic property, particularly because the primary bile salts can be hydroxylated by 7α-hydroxylating bacteria in the gut (e.g. clostridia) and thus converted to secondary bile salts which are pro-carcinogenic (Marteau et al., 1995). Tanaka et al. (1999) have found a correlation between the habitat of lactic acid bacteria species and the presence of Bsh activity. Thus, lactic acid bacteria isolated from human intestine or faeces were Bsh positive, whereas those of food-origin were mostly Bsh-negative (Tanaka et al., 1999). In contrast, all selected strains of this study exhibited Bsh regardless of their origin (fermented milk or human faeces; Table 4.1). Bsh activity is considered a functional property, which has also been suggested to be important in lowering serum cholesterol levels (du Toit et al., 1998; Pereira et al., 2003). Accordingly, deconjugation of bile acids to primary bile salts by Bsh-positive bacteria leads to an increased demand of cholesterol from which bile salts are synthesised *de novo* in the liver, and thus may lead to decreased serum cholesterol. This has

been observed in short-term studies using Bsh positive enterococci or lactobacilli (De Smet et al., 1998; du Toit et al., 1998; Agerholm-Larsen et al., 2000), although the effect was unclear in long-term studies (Richelsen et al., 1996).

Interestingly, in a study of cholesteremia in a tribe of Maasai conducted by Mann and Spoerry (1974), serum cholesterol levels of Maasai men decreased after consumption of large amounts of milk fermented with a wild *Lactobacillus* strain. Though, the significance of those results is difficult to extrapolate to people with a typical western life-style diet, the Maasai have a quite different diet, which is mostly based on milk and fermented milk, as they rarely consume grain or fruit (McAdams Wright, 2006). Four of the strains selected in this study based on the property of good survival under gastrointestinal conditions stemmed from 'Kule naoto', the traditional fermented milk from the Maasai. The four isolated strains possessed Bsh activity, and therefore their potential for lowering cholesterol levels should be further investigated. The results presented in this study show that not only the isolates of the Maasai fermented milk, but all the isolates in this study, including the probiotic *L. johnsonii* LA1 control strain, were Bsh positive. This indicates that this property should be a valuable trait for these bacteria, which may be connected with either survival or colonisation in the gastrointestinal tract. However, the significance of their Bsh activity is not yet clear. Whether this trait may play a positive role in reduction of serum cholesterol levels, would need to be further investigated *in vivo*.

Lactose maldigestion may be improved with therapy, utilising bacteria from fermented milk products which contain the lactose cleaving enzyme β-galactosidase (Hove et al., 1999; Szilagyi, 2002). Probiotic bacteria such as some of the strains characterised in this study, which possess this activity (Table 4.1), might also be used for compensation of lactase insufficiency. Some authors sustain that yogurt bacteria are more efficient for treatment of lactose intolerance, because they do not survive gastrointestinal conditions as well as probiotic bacteria. Thus, the yoghurt bacteria release the lactase enzyme after disruption of the cell wall as a result of bile sensitivity in the small intestine (Gilliland & Kim, 1984; Schrezenmeir & de Vrese, 2001). On the other hand, Zarate et al. (2000) have reported that β-galactosidase activity was severely affected at pH 2. Therefore, if the enzyme is not release during the passage through the stomach, the activity of the enzyme is preserved until reaching its site of action in the small intestine. This is also supported by a study in humans on the improvement of lactose intolerance with fermented milks (Mustapha et al., 1997), where it was shown that a *L. acidophilus* strain, which exhibited the lowest β-galactosidase activity and lactose transport but the greatest bile and acid tolerance among the strains tested, was the

most effective in improving lactose digestion and tolerance. Thus, Mustapha et al. (1997) proposed that bile and acid tolerance may be other important factors to consider when *Lactobacillus* strains are selected for improving lactose digestion and tolerance. Another aspect is that lactose can also be used as carbon source for growth by β-galactosidase-positive bacteria, and it has therefore been proposed as a potential prebiotic sugar (Szilagyi, 2002). However, the use of this sugar as a prebiotic in persons with lactose intolerance would obviously not be recommended. In conclusion, strains such as *L. plantarum* BFE 5759 or BFE 1684 characterised in this study, with good tolerance to gastrointestinal conditions and high β-galactosidase activity, should be considered for further *in vivo* studies as they may contribute to alleviate symptoms of lactose intolerance.

From the seven probiotic candidates investigated in this study, only four (*L. johnsonii* BFE 6154 and BFE 6128 and *L. plantarum* BFE 1685 and BFE 5759, Table 4.1) were able to grow to some extent on the prebiotic sugar Raftiline® ST. A combination of these *Lactobacillus* strains and this compound could, therefore, be used for developing a synbiotic product, which would provide the probiotic effects of the selected strains and at the same time would support the growth of the lactobacilli contained in the product. In addition, Raftiline® ST would also contribute with the proven 'bifidogenic effect' of prebiotics in the gut (Bielecka et al., 2002).

Bacterial attachment to cells of the intestinal mucosa is considered a very important criterion for selection of probiotic strains, as it allows bacterial strains to at least prolong their transit time through the intestine, allowing them to exert their beneficial interactions or possibly colonise the intestine (Lehto & Salminen, 1997a; Blum et al., 1999). Intestinal epithelial cell lines have been used extensively to perform comparative studies of adhesion properties of probiotic strains (Lehto & Salminen, 1997a; Blum et al., 1999; Lee et al., 2000). The human colon carcinoma HT29 cell line used in this study was established in 1964 by Jorgen Fogh (Rousset, 1986). This cell line expresses two important differentiation features which are characteristic of mature intestinal cells: tight junctions and a typical brush border. When cells are grown in the absence of serum, they secrete lysozyme and half of the cells differentiate as goblet-like cells (Rousset, 1986). In addition, the time-course of the differentiation process, with exponentially dividing cells being undifferentiated and the differentiation taking place when cells stop dividing, mimics the situation found in the small intestine, with dividing crypt cells being undifferentiated and with the differentiation taking place during the crypt to villus migration of non-dividing cells. There are, of course, limitations to this model. These cells are not normal but malignant cells, and they do not

derive from the small intestine, but from the colon. In spite of these facts, these cells have been proven to be useful tools for the investigation of intestinal cell differentiation, function, the adhesion and invasion of bacteria and parasites, to name but a few (Rousset, 1986; Kerneis et al., 1992; Jung et al., 1995; Blum et al., 1999; Lammers et al., 2002; Lee et al., 2005). Although mucus-producing epithelial cells in tissue culture are available, this study focused on the cell-cell interaction of eukaryotic cells with bacteria from two different points of view, i.e. the binding interaction and the elicitation of a cytokine response as a result of this binding interaction. For this reason, mucus-secreting cells were not used, as it was also the case in most other binding studies, because of the possible interference of mucus with binding and signal transduction (Lehto & Salminen, 1997a; Blum et al., 1999; Del Re et al., 2000; Lee et al., 2000). However, the role of the mucus in inhibiting binding of bacteria to the epithelial cells should not be forgotten. There are several direct and indirect methods for studying adhesion potential *in vitro*, but there is still no consensus about the optimal method that can actually predict microbial adhesion *in vivo* (Blum et al., 1999). Nevertheless, it is widely accepted that the level of adhesion of a probiotic strain under a given assay condition (often expressed as percentage adhesion) does not constitute an absolute value and has to be evaluated in relation to non-adherent strains tested under the same conditions. In this and other studies (Tuomola & Salminen, 1998; Tuomola et al., 1999), the *L. casei* strain Shirota, known to adhere weakly to intestinal epithelial cells, was used as a negative control. Another relevant point when comparing the binding of different strains, is to maintain the amount of bacteria added to the system at similar levels, because the number of bound bacteria is influenced by the number of bacteria added to the assay (Tuomola & Salminen, 1998; Lee et al., 2000). Both factors, the inclusion of a negative control and the maintenance of the same inoculum of probiotic bacteria or probiotic candidates (as determined by plating) were considered in the adhesion assay in this study. The inclusion of strains with good adherence properties (*L. johnsonii* LA-1 and *L. rhamnosus* GG), which have also been shown to persist in the gastrointestinal tract (Holzapfel et al., 1998; Salminen et al., 1998; Alander et al., 1999) also contributed to validate the adhesion assay used in this study. Not all the strains tested in this study could be described as adherent strains (Table 4.1). However, two *L. johnsonii* strains (BFE 6128 and BFE 6154), isolated from Maasai milk were strongly adherent, as expressed by percentage values much higher than those observed for the commercial probiotic strains (*L. rhamnosus* GG and *L. johnsonii* LA-1) used as positive controls in this study.

Adhesion of bacteria to animal cells is a much more complex phenomenon than adhesion of bacteria to inanimate surfaces. This is because of the complexity of both microbial cell

surfaces and eukaryotic membranes, and the ability of living cells to regulate the expression of molecules on their surface in response to changes in the environment. Thus, this process involves non-specific, as well as specific ligand-receptor mechanisms (Gordon et al., 1985). Hydrophobic interactions contribute in the initial adhesion of numerous pathogens to tissues (Doyle, 2000). As early as 1924, Mudd and Mudd (1924) demonstrated that bacteria can vary considerably in their degree of hydrophobicity. Furthermore, it was suggested that bacteria with high hydrophobic surfaces may reversibly adhere to intestinal cells (van Loosdrecht et al., 1987; Del Re et al., 2000; Ehrmann et al., 2002). However, other authors have reported no correlation between hydrophobicity and adhesion (Conway & Reginald, 1989; Vinderola et al., 2004). The values of the microbial adhesion to solvents test obtained with n-hexadecane reflect the hydrophobicity of the bacterial surface, whereas the values obtained with chloroform and ethyl acetate in this test can be regarded as a measure of the electron donor (basic) and electron acceptor (acidic) characteristics of the cell walls, respectively (Bellon-Fontaine et al., 1996). The two selected *L. johnsonii* strains (BFE 6154 and BFE 6128) studied showed both a high affinity for the hydrophobic solvent n-hexadecane and for the polar solvents chloroform and ethyl acetate (Table 4.1). This indicates that the surface of these strains is able to simultaneously interact with charged (hydrophilic) and non-charged (hydrophobic) molecules. A relationship between adhesion to intestinal mucus and amphiphatic characteristics has been also described (Collado et al., 2006) very recently. Thus, it appears that this bivalent nature of the cell surface of the *L. johnsonii* strains of this study may point towards a high adhesion to intestinal epithelial cells, as indicated by the adhesion assay results presented above.

However, adsorption to surfaces is a non-selective process that is only a part of the multistep phenomenon of adhesion. Nevertheless, it is still important, as it brings two surfaces close enough to permit possible adhesins and cell receptors to interact with each other (Gordon et al., 1985; Abraham et al., 1999). A great variability in adhesion to HT29 cells was found among *L. plantarum* strains (Table 4.1). One of the strains (*L. plantarum* BFE 5759) was non-adhesive, another was moderately adhesive (*L. plantarum* ATCC 8014) and four strains (*L. plantarum* BFE 5878, BFE 5092, BFE 1685, and BFE 1684) were strongly adhesive. Strains which showed poor adhesion to HT29 cells (*L. paracasei* BFE 675, *L. casei* Shirota BFE 688 and *L. plantarum* BFE 5759) showed variable values of adhesion to n-hexadecane and chloroform, but all had low adhesion values to ethyl acetate. The non-adhesive *Lactobacillus* strains in this study, therefore, appeared to have a weak acidic surface character as indicated by an affinity for a basic solvent (ethyl acetate), a variable

hydrophobicity and a moderate to strong basic character (as indicated by an affinity for an acidic solvent such as chloroform). In conclusion, the characterisation of the physicochemical properties of a particular strain may help explaining how this particular strain interacts at the first stages of the multistep process of adhesion to the intestinal cell. A generalisation is not possible as it has been shown by this and other studies (van Loosdrecht et al., 1987; Conway & Reginald, 1989; Del Re et al., 2000; Ehrmann et al., 2002; Vinderola et al., 2004), that there are always exceptions to the rule and no absolute correlation between hydrophobicity and adhesion can be established. Most of these studies only studied the hydrophobic character of the cell surfaces, but did not investigate the hydrophilic properties (including its acid/base properties). In this study, it was shown that lactobacilli with amphiphilic surfaces adhered well to HT29 cells. Therefore, it may be inferred, that the amphiphilic character of the surfaces allows bacteria to be more versatile in their interactions with complex surfaces, such as eukaryotic membranes.

Pelletier et al. (1997) reported similarities among the physicochemical surface properties of strains of the same species and suggested the use of these properties in a taxonomic perspective for microbial classification. The results shown in this study disagree with this suggestion, as important variations in the surface properties of bacteria of the same species (e.g. *L. plantarum*) occurred.

Strains isolated from traditional fermented products were shown to be capable of adhering well to intestinal epithelial cells (HT29) in this study, indicating that not only human isolates possess this property. Strains of the same species showed different adhesion capability, confirming that adhesion is a strain-specific property as it has been suggested before (Jacobsen et al., 1999; Todoriki et al., 2001). In the case of *L. johnsonii* BFE 6128 and BFE 6154, this could be due to the fact that they not only adhered individually, as in the case of *L. johnsonii* LA-1, but also formed big clusters. Some of the *L. plantarum* strains adhered individually and others in pairs or short chains as could be observed microscopically in this study.

In addition, Del Re et al. (2000) and Kos et al. (2003) suggested that auto-aggregation can also be correlated to adhesion to intestinal epithelial cells. Cesena et al. (2001) have shown that the gastrointestinal persistence *in vivo,* as well as the adhesion to epithelial cells *in vitro,* was higher for a *L. crispatus* strain with an aggregating phenotype, than for its non-aggregating mutant. Auto-aggregation and coaggregation have also been related to the ability to interact closely with undesirable bacteria (Gusils et al., 1999; Ehrmann et al., 2002). In this study, strains of *L. johnsonii* auto-aggregated well and also adhered to intestinal epithelial

cells. *L. plantarum* strains generally auto-aggregated to a lesser extent than the *L. johnsonii* strains (Table 4.1). While most of the *L. plantarum* strains (BFE 1684, BFE 1685, BFE 5878 and BFE 5092) adhered well to HT29 cells, *L. plantarum* strain BFE 5759 was non-adherent, even though its auto-aggregation ability was similar to the other highly adherent *L. plantarum* strains. The non-adherent strains *L. paracasei* BFE 675 and *L. casei* Shirota (BFE 688) also did not auto-aggregate well. Some authors (Boris et al., 1998; Del Re et al., 2000; Ehrmann et al., 2002) have suggested that auto-aggregation of probiotic bacteria is strongly related to adhesion. Generally, the results of this study also suggested that the majority of bacteria which were able to auto-aggregate, also adhered well to epithelial cells, although this was not the case for *L. plantarum* strain BFE 5759. Thus, it may be speculated that auto-aggregation may indeed be related to adhesion, but this relationship may not be exclusive, because exceptions were noted. Since a general trend between auto-aggregation and the level of adherence to intestinal epithelial cells was suggested by the results of this study, the auto-aggregation test may serve as an indicator of potentially probiotic strains with good adherence ability in initial screening experiments. However, since it was clearly shown that there may be exceptions to this indicative ability, adherence studies in cell cultures remain of great importance and cannot be replaced by auto-aggregation tests.

Apart from possibly serving as an indicator to adherence ability, the auto-aggregation phenotype is an interesting probiotic property, as it plays an important role in colonisation of the oral cavity (Kolenbrander, 1995) and the urogenital tract (Boris et al., 1998), as well as in the gastrointestinal persistence of the microorganisms *in vivo* (Cesena et al., 2001). For *L. johnsonii* strains BFE 6154 and BFE 6128, auto-aggregation was enhanced when tested in their own overnight supernatant (at pH of approx. 4.0), and it was considerably higher than that observed in PBS at pH 4.0. This indicated that auto-aggregation was not a pH-dependent effect but was potentiated by the presence of an auto-aggregating factor in the supernatant, as it has been reported before for other lactobacilli (Schachtsiek et al., 2004). It has also been hypothesised, that one of the mechanisms through which lactic acid bacteria protect the host from infections is by their ability to coaggregate with intestinal pathogens and uropathogenic bacteria (Reid et al., 1988; Huis in't Veld et al., 1994). A strong inclination to auto-aggregation does not imply a strong coaggregation property, but it has been observed that strains with high coaggregation ability also show high auto-aggregation (Ehrmann et al., 2002). This was also supported by our findings, because the strains with the highest coaggregation scores also auto-aggregated strongly. However, exceptions were noted because *L. johnsonii* LA-1 (BFE 663), which had high auto-aggregation values, did not coaggregate

with *S. aureus* and only weakly with *List. monocytogenes*. Previous coaggregation studies have been limited to bacteria from human origin. Schachtsiek et al. (2004) were the first to report coaggregation of *Lactobacillus coryniformis*, a food- or feed-associated bacterium, with pathogens. Thus, *L. coryniformis* was able to coaggregate with *E. coli* K88, *Campylobacter jejuni* and *Campylobacter coli,* but not with *S.* Typhimurium, *Clostridium perfringens* and *List. monocytogenes*. In this study, the first *Lactobacillus johnsonii* strains of food origin able to coaggregate with human pathogens (*List. monocytogenes*, *S. aureus*, enterotoxigenic *E. coli* and *S.* Typhimurium) were reported (Table 4.1), which coaggregated to even a higher degree than the commercial probiotic strains *L. rhamnosus* GG, *L. johnsonii* LA-1 (BFE 663) and *L. casei* Shirota (BFE 675). Strains (especially the *L. plantarum* strains because of their inherent fermentative versatility) with this property could be of special interest to the food industry, because coaggregates may be formed in the food matrix, and thereby prevent the entrapped pathogens from adhering to host cells when ingested (Schachtsiek et al., 2004). On the other hand, increased numbers of *S. aureus* together with decreased numbers in lactobacilli and bifidobacteria in the intestinal microbiota of infants have been associated with atopic dermatitis (Bjorksten et al., 2001; Watanabe et al., 2003). For these reasons, consumption of products enriched with prebiotics and probiotic bacteria such as the strains presented in this study, would compensate the intestinal microbial imbalance (by increasing the numbers of lactobacilli and bifidobacteria) in patients with atopic dermatitis. Furthermore, it could also help excluding *S. aureus* through coaggregation, by preventing the pathogen to adhere to IEC and by inhibiting the pathogen by production of inhibitory substances such as organic acids in the close proximity resulting from the formation of the coaggregate. Another reason why exclusion of *S. aureus* would be beneficial is that nasal and intestinal carriage of *S. aureus* in hospitalised patients has been shown to be a risk for subsequent infections, especially the emergence of methicillin- and multiresistant *S. aureus* (Vesterlund et al., 2006). The use of probiotic strains, such as the lactobacilli characterised in this study, may also serve as a supportive or preventive treatment.

Diarrhoea acquired in developing countries is caused mainly by viruses and bacteria such as enterotoxigenic *E. coli*, *Campylobacter, Salmonella* spp. (non-typhoid) and *Shigella* spp. (Yates, 2005). If this acute disease is acquired by people travelling to these destinations, it is known as traveller's diarrhoea. In general, although this disease is rarely life-threatening, it results in significant morbidity, as it affects millions of people (Guandalini, 2002). Probiotic preparations have been suggested as preventive/supportive therapy and/or post-therapy after treatment with antibiotics, to help re-establishing the microbial balance in the GIT and so

prevent antibiotic-associated diarrhoea (Isolauri et al., 1991; Kaur et al., 2002; Yates, 2005). *Enterococcus* spp. on the other hand, have been recognised as major opportunistic pathogens causing bacteraemia, endocarditis, urinary tract infections in the hospital environment and they can act as potential recipients of vancomycin recipient genes (Franz et al., 1999; Bertuccini et al., 2002; Klein, 2003). Listeriosis is a food-borne disease, which is characterised by meningitis, septicaemia and foetal death (Gahan & Hill, 2005). *List. monocytogenes* is an intracellular parasite, and the GIT is thought to be the primary site for entry through epithelial cells (Finlay & Falkow, 1997; Gahan & Hill, 2005). Therefore, the inhibition of this invasion step would prevent the further translocation to the spleen and liver, where *List. monocytogenes* cells multiply, and the following bacteraemia. Because of this background, and because adhesion is considered the first step of pathogenicity (Finlay & Falkow, 1997), two selected *Lactobacillus* strains were tested for their ability to inhibit adhesion to intestinal cells of such important pathogens as vancomycin-resistant *Ent. faecium* DSM 13590, *S.* Typhimurium ATCC 14028 and *List. monocytogenes* Scott A, which cause considerably morbidity world-wide.

It is expected that a sufficient amount of a probiotic bacteria must be consumed so that the probiotic strains may exert their beneficial effect (Lee et al., 2000). It has also been shown that some probiotic strains able to inhibit adhesion of pathogens only do so, when present at a higher concentration than the pathogen itself (Mack et al., 1999; Lee et al., 2000). This can also be related to the fact that probiotic bacteria are present in probiotic formulations in high numbers, and pathogens usually occur in low numbers, and in spite of this, they cause disease as their infective dose is generally quite low. For these reasons, higher concentrations of lactobacilli were also used in the adhesion inhibition assays of this study. Similar to a previous report of an inhibition model with Caco-2 cells (Lehto & Salminen, 1997b), *L. rhamnosus* GG also did not inhibit *S.* Typhimurium adhesion to HT29 cells in this study. Two strongly adherent strains, *L. plantarum* BFE 1685 and *L. johnsonii* BFE 6128, however, were able to significantly reduce the adhesion of the human pathogens *E. coli* and *List. monocytogenes* in HT29 cell culture. In addition, *L. plantarum* BFE 1685 also reduced the adhesion of the *Ent. faecium* and *S.* Typhimurium strains used in this study. A slight inhibition of *S.* Typhimurium ATCC 14028 by the non-adhesive *L. casei* Shirota was also observed, which seemed not to depend on competitive exclusion for adhesion sites, because *L. casei* Shirota adhered poorly to HT29 cells. Instead, it might depend on another antagonistic mechanism such as the production of non-lactic acid molecules with antimicrobial properties as has been described before (Fayol-Messaoudi et al., 2005). The spectrum and magnitude of

the inhibition of adhesion, as well as the auto-aggregation and coaggregation properties of the selected *L. plantarum* and *L. johnsonii* strains (Table 4.1), suggest that the mechanisms involved in inhibition of adhesion are different. More studies are needed to elucidate if this is due to unspecific steric hindrance or a specific mechanism involving adhesins. Adhesion of pathogenic bacteria to mucosal surfaces is considered as the first step of intestinal infections (Finlay & Falkow, 1997). Nevertheless, the findings of this study demonstrate the ability of live lactobacilli, especially *L. plantarum* BFE 1685 and *L. johnsonii* BFE 6128, to interfere with pathogens through mechanisms such as production of organic acids and H_2O_2, coaggregion and competition for adhesion sites.

In general, both strains of human origin and strains isolated from food were able to resist simulated gastrointestinal conditions, inhibit pathogens, coaggregate with pathogens and inhibit their adhesion to IEC (Table 4.1). This may suggest that no host-specificity is needed for probiotic activity.

The intestinal mucosa is the main interface between the immune system and the intestinal lumen. It does not only function as a physical barrier, which is constantly exposed to exogenous compounds such as food, microorganisms and their metabolites, but it can also generate signals for communication with underlying mucosal immune inflammatory cells (Haller et al., 2000; Saegusa et al., 2004). Thus, antigen processing and signalling by epithelial cells are primary steps of innate and adaptive defence mechanisms in the earliest phases after invasion by pathogens. Non-pathogenic bacteria also elicit a response, but their mechanism of immune stimulation have been even less elucidated.

There are contradictory reports about the spectrum of cytokines secreted by intestinal epithelial cells (Stadnyk, 1994; Kolios et al., 1999; Stadnyk, 2002; Colgan et al., 2003). This could be a reflection of the fact that studies which reported secretion of cytokines such as IL-10 included intestinal mucosa isolated from biopsies. Such intestinal mucosa not only contains epithelial cells, but also different immune cell populations from the epithelial lining (such as intraepithelial lymphocytes, Fig. 1.3) and the lamina propria, which are difficult to separate from the thin enterocyte layer (Stadnyk, 2002). Vidal et al. (2002) reported TNF-α secretion by HT29 cells at very low levels (14 pg/ml) when stimulated with LPS. Jung et al. (1995) reported TNF-α secretion (5 to 188 pg/ml) by HT29 cells after exposure to invasive Gram-positive and –negative bacteria, but this CK was not secreted after stimulation with LPS. In this study, neither LPS stimulation of HT29 cells nor exposure to *Lactobacillus* strains induced TNF-α at the protein level.

IL-6 has been shown to be secreted by intestinal epithelial Caco-2 cells stimulated with bacteria (Hosoi et al., 2003), but IL-6 was not detected in supernatants of HT29 cells upon stimulation with the *Lactobacillus* strains nor LPS in this study. In addition, IL-6 secretion by HT29 cells has not been reported yet. Therefore, the absence of IL-6 secretion in this study may be due to inherent variations which occur from cell line to cell line in the repertoire and levels of cytokine secretion, which depend in part on the degree of cell differentiation (Böcker et al., 2000). In addition, they may also depend on bacterial species/strain specific effects, i.e. other strains not tested here may be able to induce IL-6.

Jung et al. (1995) showed that HT29 cells secreted MCP-1 in response to Gram-negative invasive pathogens and to *List. monocytogenes,* but not in response to LPS. None of the *Lactobacillus* strains of this study were able to induce the secretion of this CHK.

Under the conditions investigated in this study, it was demonstrated that some strains modulated IL-8 levels secreted by HT29 cells in a strain-specific fashion (Table 4.1). Thus, *L. johnsonii* BFE 6128 and *L. plantarum* BFE 1684 and BFE 5759 enhanced IL-8 secretion, but this was considered a weak enhancement when compared to the stimulatory effect observed with LPS under the same conditions. This IL-8 induction was not due to cytotoxicity effects, as HT29 cell vitality was not affected by lactobacilli under the conditions tested (Table 4.1). A similar weak IL-8 induction in intestinal epithelial cell cultures was also previously reported for the non-pathogenic *Bacillus subtilis* natto (Hosoi et al., 2003) and *S. cerevisiae* (Saegusa et al., 2004) bacterial and yeast strains isolated from food. He et al. (2002) also found that *Bifidobacterium* spp. of human origin stimulated other pro-inflammatory CKs in a macrophage cell line. In healthy individuals, such a weak immune stimulation might result in keeping the immune system alert and in a condition of readiness to react with an inflammatory response if challenged by pathogenic bacteria (Saegusa et al., 2004). He et al. (2002) have also suggested that the increase of non-pathogenic bacteria with pro-inflammatory properties, such as bifidobacteria, may contribute to the homeostasis of local immunity by preventing local inflammation from being over-suppressed by mainly opportunistic and endogenous infective bacteria, which have been found to strongly trigger anti-inflammatory CK production. This could be of special interest for aged people, who have a distinct aged-related intestinal microbiota with more facultative anaerobic Gram-negative bacteria (O'Sullivan, 1999). Nevertheless, it should not be forgotten that a much stronger induction of these pro-inflammatory CK by lactobacilli or bifidobacteria alone would destroy the balance that they need as normal inhabitants of the intestine to live in harmony with their host. In contrast, the typical reaction after invasion of epithelial cells by pathogens is (and

should be) in most of the cases a high increase of IL-8, which *in vivo* may serve as the signal for initiation of the acute inflammatory response to eliminate the threat (Schulte et al., 1996; Wilson et al., 1998). As already mentioned, pathogenic strains also induce in intestinal cells other pro-inflammatory CKs, all involved in initiation and amplification of the immune response (e.g. TNF-α, MCP-1, IL-6). It seems that the intestinal epithelium does not respond to all bacterial species in the same way, with some bacteria inducing a strong cytokine release, and others eliciting a mild or no response. Therefore, it could be hypothesised that the signals (a specific array of pro-inflammatory CKs) elicited by IEC to activate immune cells in the lamina propria may be differential and hence allow distinguishing between tolerance and reactivity.

However, cytokine responses of epithelial cells are also influenced by immune cells (Haller et al., 2000). Therefore, the modulation of the further immune reaction course, which involves innate and adaptive components, is generally undertaken by professional immune cells of the lamina propria. Accordingly, it is hard to predict the outcome of the observed IL-8 stimulation by lactobacilli on the further development of an immune response, especially if such interactions were tested in cell culture in the absence of immune cells of the lamina propria. As an example, the probiotic strain *E. coli* Nissle 1917, which exhibited anti-inflammatory properties in animal studies, actually induced pro-inflammatory IL-8 production by HT29 cells *in vitro* (Lammers et al., 2002). Therefore, the data of this study strongly suggest that *L. plantarum* BFE 1685 and BFE 5759, and *L. johnsonii* BFE 6128 strains interacted specifically with enterocytes without assistance from other specialised cells of the immune system. However, the further course of this immune stimulation cannot be predicted and should be determined in animal experiments or human volunteer studies.

It is well known that IL-8 secreted by intestinal epithelial cells recruits and activates neutrophils, basophiles, lymphocytes and monocytes in the adjacent lamina propria (Schulte et al., 1996; Campbell et al., 2001; Beutler, 2004b). This might lead to a pro-inflammatory state, which could be harmful when there is already a background of inflammation, as it is the case, for example, in intestinal bowel disease. To mimic a pro-inflammatory background, the ability of TNF-α (a potent pro-inflammatory CK) to modulate the IL-8 responses of HT29 cells to lactobacilli was studied. The results showed that IEC were indeed sensitised by this pro-inflammatory CK to respond to lactobacilli. In a similar experimental set up, Bai et al. (2004) showed that two probiotic strains reduced TNF-α induced IL-8 secretion by HT29 cells, but the authors did not consider the possibility that the decrease in IL-8 could have been a result of protease degradation, as it had been reported before (Horvat & Parmely, 1988;

Mintz et al., 1993). Ma et al. (2004) reported that live *L. reuteri* cells were essential for reducing TNF-α-induced IL-8 levels in Caco-2 cells. In contrast, Mc Cracken et al. (2002) demonstrated that TNF-α enhanced IL-8 response to *L. plantarum* 299v, but they found a discrepancy between RNA levels and protein levels. Hence, IL-8 mRNA levels were much higher in TNF-α pre-treated HT29 cells exposed to *L. plantarum* 299v than the IL-8 mRNA levels induced by TNF-α alone. At the protein level, they found the opposite. In this study, IL-8 protein levels were higher when TNF-α pre-treated HT29 cells were co-cultured with *L. plantarum* BFE 1685, *L. johnsonii* BFE 6128 and *L. rhamnosus* GG. These data are supported by a previous report, which showed that leukocytes sensitised Caco-2 cells to non-pathogenic *E. coli* and *L. sakei* increased the expression of IL-8 mRNA (among other CKs), because the principal factor responsible for this was identified as TNF-α produced by the underlying leukocytes (Haller et al., 2000). The *Lactobacillus* strains of this study, as well as the reference strain *L. rhamnosus* GG, would be therefore not be appropriate for treatment of IBD, as they enhance the pro-inflammatory activity of TNF-α. However, *in vivo* studies should be done to confirm this in IBD models, such as mice suffering from chemical-induced colitis (induced with oxazolone, trinitrobenzene sulfonic acid or dextran sodium sulphate) or spontaneous colitis (e.g. in IL-10 knockout mice) (Boirivant et al., 1998; Blumberg et al., 1999; Rachmilewitz et al., 2002).

When HT29 cells were co-cultured with lactobacilli and LPS, a synergistic effect was seen, as the levels of IL-8 were higher than those observed when HT29 cells were treated with LPS alone. Haza et al. (2004) have reported that *L. plantarum* CBL/J was also able to stimulate IL-8 production in the THP-1 human macrophage cell line. However, in contrast to the results presented here, IL-8 was not enhanced by incubation with LPS. Haza et al. (2004) also tested the influence of both *L. plantarum* CBL/J and LPS on the production of TNF-α and IL-1β by this macrophage cell line and found that the production of these CKs was enhanced.

According to the results of TLR4 mRNA expression, there was no relationship between the expression of TLR4, which is activated by LPS, and the effect of lactobacilli on IL-8 increased secretion in the presence of LPS. Thus, it is possible that lactobacilli influenced the secretion of this CHK at a post-transcriptional level, or by influencing the expression of other factors involved in TLR4 pathway.

Because of their adhesive properties, ability to exclude pathogens and stimulate IL-8 in HT29 cells, the two probiotic candidates (*L. plantarum* BFE 1685 and *L. johnsonii* BFE 6128) were further investigated to elucidate their immunostimulatory mechanisms. *L. rhamnosus*

GG was used in these experiments as reference strain. In an attempt to arrive at a better understanding of the structural requirements for induction of IL-8 in IEC, the ability of the lactobacilli cellular components (DNA, LTAs, cell walls) and pre-treated bacteria (proteinase K and KIO_4 treatments) to trigger the secreting response in HT29 cells was investigated. Whole cells, cell wall components, DNA and soluble factors from *L. rhamnosus* GG are known to elicit pro-inflammatory CKs in human macrophages (Matsuguchi et al., 2003; Pena & Versalovic, 2003; Iliev et al., 2005), but the effect of these components in intestinal epithelial cells has been less studied. Lammers et al. (2002) have shown that lactobacilli, including *L. rhamnosus* GG, and bifidobacteria did not induce nor inhibit IL-8 production by HT29/19A cells, whereas both cell debris and cell extracts from *E. coli* Nissle 1917 did. Peptidoglycan is the major structural component of Gram-positive bacterial cell walls, and was shown to induce localised inflammatory responses augmenting cell- and humoral mediated responses (Deng et al., 2004).

LTAs are membrane-associated amphiphilic macromolecules that are found in many Gram-positive bacteria and have been associated with modulation of autolysins, binding of cations for enzyme function and electromechanical properties of the cell wall (Debabov et al., 2000). LTA is also immunologically active (Tsutsui et al., 1991) and has been shown to induce secretion of IL-8 from human blood leukocytes (Deng et al., 2004). In this study, LTA derived from *L. rhamnosus* GG and *L. johnsonii* BFE 6128 triggered IL-8 secretion by HT29 cells to levels significantly different ($P<0.05$) from those produced by naïve cells. In this investigation, LTAs were extracted using a gentle extraction procedure, which preserves D-Ala dipeptides and thus yielding bioactive, pure LTA (Morath et al., 2002). LTA derived from *L. plantarum* BFE 1685 did not modify IL-8 levels secreted by HT29 cells, but its supernatant did. A hypothesis to explain this could be that the structural conformation of purified LTA differs from the conformation when LTA is inserted in the cells wall. Such conformation changes may sometimes influence the immunogenicity of LTA (Bhakdi et al., 1991).

It was also hypothesised that a soluble factor may exert such an effect, because supernatants of *L. rhamnosus* GG and *L. plantarum* BFE 1685 also induced IL-8 secretion. None of the cell walls extracts induced IL-8 significantly, indicating that the peptidoglycan of these strains was not responsible for the lactobacilli stimulation as LTA, membrane and proteins were removed during preparation of cell walls with SDS. On the other hand, during preparation of crude cell walls, LTA may have lost its immunogenic potential, as it has been noted earlier that fatty acids and/or ester-bound alanine are easily lost (Fischer et al., 1980). Bacteria treated with proteinase K or KIO_4 did not exert any effect on IL-8 induction by HT29

cells, either. KIO_4 is an oxidant of lipids which may have altered LTA structure. It is also possible that certain proteins immersed in the cell wall contributed to the IL-8 induction, because the treatment of *L. rhamnosus* GG, *L. plantarum* BFE 1685 and *L. johnsonii* BFE 6128 with proteinase K eliminated this effect.

It is well known that bacterial DNA containing numerous repeating unmethylated CpG motifs has an immunogenic potential (Takeshita et al., 2004; Pedersen et al., 2005). DNA from probiotic bacteria has been reported to modulate the immune function of murine and human intestinal epithelia (Jijon et al., 2004; Rachmilewitz et al., 2004) and DNA from yogurt starters has been shown to activate B cells (Kitazawa et al., 2003). Akhtar et al. (2003) reported that *E. coli* DNA induced IL-8 in human epithelial cell lines. For these reasons, HT29 cells were incubated with DNA from *L. rhamnosus* GG, *L. plantarum* BFE 1685 and *L. johnsonii* BFE 6128 to determine if DNA contributed to the IL-8 induction described above. However, this was not the case, as no differences were seen with the untreated cells.

At the mRNA level, whole live *L. rhamnosus* GG, *L. plantarum* BFE 1685 and *L. johnsonii* BFE 6128 induced IL-8 as measured by RT-PCR after 2 h coculture with HT29 cells, after which HT29 cells were washed and incubated with medium alone for 4 h. IEC (including Caco-2 and HT29 cell line models) produce antimicrobial peptides (defensins) under certain stimuli such as IL-1, LPS or invasive bacteria (O'Neil et al., 1999; Vora et al., 2004). HBD-2 is an inducible defensins, which may protect the intestinal epithelium from pathogen invasion and from potential invaders among the commensal microbiota. *E. coli* Nissle 1917, a probiotic bacterium, has been reported to induce HBD-2 in a Caco-2 cell line model (Wehkamp et al., 2004). The *Lactobacillus* probiotic candidates tested in this study did not induce HBD-2 expression in HT29 cells, this may have be due to an strain-specific effect only exerted by Gram-negative or invasive bacteria. Another possibility could have been that the sensibility of RT-PCR was not high enough.

TLRs are pattern recognition receptors which recognise microbial components and initiate an innate immune response. Contradictory reports about the expression of these receptors in IEC have been reported (Cario & Podolsky, 2000; Hausmann et al., 2002; Melmed et al., 2003; Otte et al., 2004; Furrie et al., 2005). HT29 cells in this study expressed all TLRs at the mRNA level, as investigated with the signal arrays. RT-PCR was not sensitive enough to detect TLR2 mRNA, but real time RT-PCR was. The expression of TLR2, TLR5 and TLR9 in the HT29 cell line was also confirmed at the protein level by FACS analysis. TLR2 expression results, together with the IL-8 stimulation potential of LTAs from *L.*

rhamnosus GG and *L. johnsonii* BFE 6128, strongly suggests that these bacteria stimulate innate responses in IEC via their LTAs.

It has been described that microbial stimuli affect the expression of their cognate TLR (Poltorak et al., 1998; Visintin et al., 2001; Hornung et al., 2002). Therefore, it was hypothesised in this study, that expression of TLR involved in the recognition of lactobacilli may be modulated upon stimulation with these strains. *L. rhamnosus* GG, *L. plantarum* BFE 1685 and *L. johnsonii* BFE 6128 regulated not only TLR2 but also TLR9 mRNA transcription, but they did not influence expression of the TLR4 gene. At protein level, two of the three tested strains, namely *L. rhamnosus* GG and *L. plantarum* BFE 1685, up-regulated TLR2 and TLR5 expression in HT29 cells.

This is the first time that a modulation of TLR expression by lactobacilli is shown in intestinal epithelial cells. This important finding could serve to explain the adjuvant properties of some probiotic lactobacilli (Perdigon et al., 1991; Iliev et al., 2005; Ogawa et al., 2005; Ogawa et al., 2006) and establish the basis for further studies on development of mucosa vaccine adjuvants, as it has been suggested that TLR signalling pathways are a promising mechanism for boosting vaccine responses (van Duin et al., 2006). On the other hand, it may also raise attention towards the indiscriminative use of probiotic products for treatment or support of IBD, as some strains, such as the strains in this study, may not be adequate for patients with a background of chronic inflammation. However, as it has been mentioned above, this should be first confirmed in *in vivo* models of inflammation.

L. johnsonii BFE 6128 was shown to also enhance the response of HT29 cells towards *S.* Typhimurium. Nevertheless, the increase in IL-8 could not be related to an increase in the number of TLR5 receptors (which recognise flagellin). Flagellin has been described as the main determinant of *Salmonella*-mediated NF-κB and pro-inflammatory signalling (Tallant et al., 2004). In order to determine if *L. johnsonii* BFE 6128 was able to enhance the recognition of a specific antigen by HT29 cells, different *S.* Typhimurium derived cellular components were tested. Surprinsingly, the increased IL-8 production was only observed in cells stimulated with flagellin and with whole *S.* Typhimurium cells. Thus, the enhancement of the secretion of this innate immunity molecule (IL-8) may have been regulated at other levels, different than by enhancing TLR5 expression. To further approach this question, RT-PCR based arrays were used to evaluate if there were other genes involved in TLR pathways which were modulated by lactobacilli.

Transcriptomic analysis clearly showed that the pre-treatment with *L. johnsonii* BFE 6128 followed by an infection with *S.* Typhimurium activated the transcription of several

genes involved in TLR and innate immunity pathways. This was not the case when cells treated with *S.* Typhimurium had not been previously treated with *L. johnsonii* BFE 6128, nor when HT29 cells were treated only with lactobacilli. Among the genes modulated when HT29 cells had been pre-treated with lactobacilli and then exposed to *S.* Typhimurium, there were genes coding for kinases involved in the transduction of signals upon TLR activation, for transcription factors which in turn induce the transcription of other target genes, for CHKs involved directly in the recruitment of neutrophils and lymphocytes, for non-traditional antigen presenting molecules and for TLR themselves.

TLR engagement results in activation of the mitogen-activated protein kinases (MAPKs), which, together with the NF-κB pathway, transduce extracellular signals to cellular responses. Activation of the MAPKs is mediated by a core kinase module comprised of MAP3K, MAP2K, and MAPK through sequential protein phosphorylation (Chi et al., 2006). MAP kinases are regulatory signal mediators in immunocytes, and play an important role in host innate responses. In addition, IL-8 release has been shown to depend in part on MAPK activation (Akhtar et al., 2003; Chang et al., 2006). In this study, it was shown that two genes coding for the MAP kinases, MAPK8IP3 and MAP3K1 were significantly up-regulated. RIPK2 and IRAK2 genes were also up-regulated. RIPK2 (also known as CCK2 or CARD3) is a serine/threonine kinase containing a caspase recruitment domain, which is involved in multiple receptor signalling pathways that are important for innate and adaptive immune responses, but which have not yet been fully elucidated. It has been shown that upon LPS stimulation, RIPK2 was transiently recruited to the TLR4 receptor complex and associated with key TLR signalling mediators IRAK1 and TRAF6. Furthermore, RIPK2 kinase activity was induced by LPS treatment, indicating that it is directly involved in the LPS/TLR4 signalling (Lu et al., 2005). IRAKs (IL-1 associated kinases) on the other hand, are also directly involved in TLR signalling pathways. The transcription of PELI1 (Pellino 1) was also up-regulated. Pellino 1 is an adaptor molecule which has been shown to interact with IRAK and modulate IL-1 and LPS signalling (Jiang et al., 2003; Schauvliege et al., 2006). Thus, the up-regulation of these genes involved in transduction of TLR signalling may have played a role in the increases of IL-8 observed when HT29 cells were pre-treated with lactobacilli and then, infected with *S.* Typhimurium.

Three transcription factors genes, FOS, IRF and NF-κB1 were also up-regulated. The early growth transcription factor FOS, together with other members such as JUN complexes (whose gene has also been up-regulated) plays a critical role in regulating IL-12 in dendritic cells (Agrawal et al., 2003; Dillon et al., 2004). The role of this transcription factor in IEC is

less well known. IRF is the essential transcription factor for induction of iNOS by IFN-γ in macrophages (Fritsche et al., 2003) but whose function has not yet been defined in IEC. NF-κB1 is one of the components of the NF-κB pathway, which is the main pathway which follows after TLR activation (Kawai & Akira, 2006) and leads to transcription of pro-inflammatory genes. Therefore, if their up-regulation by lactobacilli, when cells encounter a pathogen in IEC could be extrapolated to professional antigen presenting cells, such as dendritic cells, the observed up-regulation of genes may have consequences in the further development of the immune response by influencing IL-12 and iNOS production. Thus, the influence of these lactobacilli on signal transduction in dendritic cells should be further investigated.

It has been proposed, that IEC may also function as alternative antigen presenting cells as they express non-classical antigen presenting molecules (Shao et al., 2005). In this study, two genes coding for co-stimulatory molecules, CD80 and CD86, were notably up-regulated in HT29 cells by pre-treatment with lactobacilli and then followed by treatment with *S.* Typhimurium, though with $P>0.05$. Nevertheless, the up-regulation of this molecules in dendritic cells by lactobacilli and in other antigen presenting cells after TLR activation had been described before (Drakes et al., 2004; van Duin et al., 2006). Co-stimulatory molecule-mediated signalling improves vaccine efficiency (van Duin et al., 2006) and therefore the up-regulation of CD80 and CD86 genes may represent a mechanism for TLR-ligands and for lactobacilli for enhancing the effect of vaccines (Isolauri et al., 1995; Pouwels et al., 1998; Suzuki et al., 2004; Mota et al., 2006).

Intestinal epithelial cells are the first cells which encounter a potential pathogen. They are able to recognise it and transduce the signal and express CKs and CHKs, which serve as chemical messengers for the immune cells present in the lamina propria (Kolios et al., 1999; Stadnyk, 2002). In this study, pre-treatment of HT-29 cells with lactobacilli followed by infection with *S.* Typhimurium led to an increase in IL-8 at the protein level and to the up-regulation of other CHK and CK genes (CSF2, IL-8, CXCL10 and TNF-α) which also play a role in recruiting and activating professional immune cells. CSF2 (granulocyte-macrophage colony stimulating factor) prolongs the survival of neutrophils, monocytes and eosinophils, and increases the response of those cells to other pro-inflammatory agonists which can further amplify the inflammatory response (Wilson et al., 1998). It can also stimulate TNF-α and IL-1 by monocytes/macrophages (Jung et al., 1995) and influence the initial phase of antigen processing and presentation (Siegmund & Zeitz, 2004). IL-8 is a potent neutrophil attractant and activator, which may also influence and recruit other cells such as natural killers and

basophils (Morohashi et al., 1995; Campbell et al., 2001). The chemokine interferon IFN-γ-inducible protein-10 (IP-10/CXCL10) is a chemoattractant for various leukocyte subsets. Krathwohl and Anderson (2006) hypothesised that CXCL10 could stimulate dendritic cells to mature and cross-present exogenous antigen to T cells, resulting in a Th1-type immune response. TNF-α can stimulate IL-8 and MCP-1 production by monocytes/macrophages (Jung et al., 1995). Therefore, the up-regulation of these CK and CHK by pre-treatment with lactobacilli followed by infection with *S.* Typhimurium may serve for recruiting immune cells from the lamina propria to the infection site at the very early stages of infection to eliminate the threat. This may lead to prevention of diarrhoea caused by *S.* Typhimurium or to the shortening of diarrhoea duration, if the pathogen is eradicated before invasion has occurred.

In summary, *L. johnsonii* BFE 6128 sensitised intestinal epithelial cells for recognition of *S.* Typhimurium, and thereby up-regulated innate immune genes which may serve as link between the innate and adaptive immunity.

It has been previously reported that *L. rhamnosus* GG improved the immunogenicity of a rotavirus vaccine (Isolauri et al., 1995) and that serum and intestinal immune responses were promoted by lactobacilli (Isolauri et al., 1991; Kaila et al., 1992; Majamaa et al., 1995; Guandalini, 2002). There are also many claims about the immune stimulation effects of lactobacilli, which include both innate and adaptive immunity components (Kato et al., 1994; Isolauri et al., 2001; Perdigon et al., 2002; Ogawa et al., 2006), that have led to the hypothesis that lactobacilli may be proper carriers for antigen delivery (Pouwels et al., 1998; Scheppler et al., 2002; Mota et al., 2006). Thus, the up-regulation of pro-inflammatory mediators and TLR pathways-related molecules can contribute to elucidating the so-called 'immunoadjuvant' properties of lactobacilli, as well as their contribution to prevention and treatment of bacterial and viral diarrhoea. On the other hand, the modulation of TLR expression by lactobacilli may open new research directions towards the understanding and developing of new vaccines targeting TLR pathways.

5 CONCLUSIONS

In this study, seven strains were selected out of thirty isolates because of their survival ability under gastrointestinal conditions, and they were further characterised for their probiotic, safety and immunostimulating properties. It was shown that each strain presented individual characteristics, which may contribute to their 'probiotic' health-promoting effects. In the final evaluation of their properties, four strains, *L. plantarum* BFE 5759, *L. plantarum* BFE 1684, and *L. plantarum* BFE 1685 and *L. johnsonii* BFE 6128, are proposed for the further development of a multiple-strain fermented milk product containing Raftiline® ST would combine the beneficial effect of each probiotic candidate strain, as well as the inherent effect of this prebiotic sugar, as has been discussed above. These four strains were able to survive gastrointestinal conditions, which indicates that they may reach the site of action, the small intestines unharmed. Because of their metabolic properties, they would probably contribute to the reduction of cholesterol levels due to the presence of Bsh. In addition, two of the strains, namely *L. plantarum* BFE 5759 and BFE 1684, may also contribute to alleviation of lactose intolerance because of their β-galactosidase activity. The four selected strains are microorganisms belonging to the risk 1 group because of their long history of safe use and they did not have transferable antibiotic resistance, which implies their acceptability according to the guidelines of the European Food Safety Association. The selected lactobacilli are most promising probiotic candidates as based on their antimicrobial, coaggregative and adhesive properties, as they may inhibit or exclude food pathogens by different mechanisms. By a coaggregating mechanism with pathogens, lactobacilli may enhance their clearing from the gastrointestinal tract, thus preventing them from adhering to IEC and also inhibiting them in this micro-environment by producing organic acids and H_2O_2. Competitive exclusion of two of the selected strains, i.e. *L. plantarum* BFE 1685 and *L. johnsonii* BFE 6128, have been proven *in vitro* against representative food-borne pathogens, suggesting an important role in prevention of enteric infections.

The ability of these strains to stimulate the production of IL-8 at a low level with their LTAs, and possibly also with further un-identified factors, may contribute to maintaining the immune system in a state of alert for the possible encounter of pathogens.

Furthermore, the induction of TLR5 protein expression in IEC supports this hypothesis and may increase the IEC capacity of recognising invasive flagellated bacteria such as *S.* Typhimurium already at the early stages of infection. This would mobilise the innate immune

response before the pathogen has time to proliferate, gaining time for the adaptive immunity to be activated. The significance of increasing TLR2 expression should be further evaluated as it might contribute to intolerance by recognising normal inhabitants of the GIT. The experiments in this study on the influence of lactobacilli on the response of HT29 cells to *S.* Typhimurium clearly showed that *L. johnsonii* boosts innate immunity mechanisms against this pathogen. Thus, it contributes to the early response against this pathogen by recruiting and activating monocytes/macrophages and neutrophils as a result of the enhanced production of pro-inflammatory CHK and CKs as well as expression of co-stimulatory proteins.

However, the colonisation ability and immune stimulatory properties must be validated in further *in vivo* studies. Nevertheless, the importance of *in vitro* studies should underestimated, as they provide a controlled system for the study of underlying probiotic mechanisms and are unavoidable steps in the development of new therapeutic agents. On the other hand, the screening of multiple strains *in vivo* without previous knowledge of the potential effects and/or hazards of the bacteria being tested should neither be done nor is it practicable, first and foremost because of ethical reasons. This, together with the prohibitive high costs associated with animal or human studies, is the reason why initial selection studies on new probiotic strains are generally first done *in vitro*. After screening numerous strains for their functional properties, those strains displaying interesting probiotic properties are then chosen for further *in vivo* testing and probiotic product development.

The main objective of this study was to find new probiotic candidates to be used in functional fermented milks and the *in vitro* characterisation of selected strains following the criteria for selection of probiotic strains proposed by the a Joint FAO/WHO working group of experts in probiotics (Chesson et al., 2002). This objective and the associated aims were successfully achieved as ‘multifunctional’ strains were isolated and characterised. Last but not least, novel mechanisms involved in the innate immunomodulation were elucidated, providing new insights in the communication between bacteria and intestinal epithelial cells as well as underlying the sentinel role played by enterocytes. This is the first report on modulation of expression of TLRs at the mRNA and protein level by probiotic microorganisms in intestinal epithelial cells.

Therefore, in the final analysis, this study provides a rationale for the further use of the selected *Lactobacillus* strains (*L. plantarum* BFE 5759, BFE 1684 and BFE 1685, and *L. johnsonii* BFE 6128) as probiotics for therapeutic and preventive purposes.

5.1 Outlook

As the preliminary *in vitro* tests have shown a definite probiotic potential and immunostimulating effects for the lactobacilli selected and characterised in this study, these strains are apt to pass to the second step necessary to develop health-promoting products, i.e. *in vivo* animal models. If successful also in these models, it would be necessary to test if the effects seen *in vitro* and in animal models are still observed in humans.

Double-blind placebo controlled studies may provide further information on the beneficial effects of these strains with respect to lactose intolerance, cholesterol lowering, prevention of bacterial diarrhoea and immunostimulatory properties.

The possibility of using these *Lactobacillus* strains for development of probiotics to be use in other compartments of the body such as oral cavity, vagina or the skin should be considered.

To further investigate the mechanisms involved in innate immunity, the use of primary intestinal epithelial cells, as well as other cells isolated from human biopsies would provide more information about the modulation of TLR-related pathways. The use of reporter-gene systems and bacterial mutants would also contribute to gain more insight in these mechanisms.

The results concerning innate immunomodulation by lactobacilli open new research possibilities for the use of these bacteria as immunoadjuvants and development of mucosal vaccines.

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LIST OF ABBREVIATIONS

APC	antigen presenting cell
ATB	antibiotic
BSA	bovine serum albumin
Bsh	bile salt hydrolase
CD	cluster differentiation
CHK	chemokine
CK	cytokine
COX-2	inducible cycle-oxygenase
Ct	threshold cycle
DEPC	diethylpyrocarbonate
ds	double-stranded
FACS	fluorescence activated cell sorter
FCS	foetal calf serum
FITC	fluorescein isothiocyanate
GALT	gut associated lymphoid tissue
GIT	gastrointestinal tract
GRAS	generally recognised as safe
HBD-2	human β-defensin 2
HLA	human leukocyte antigen
IBD	intestinal bowel disease
IEC	intestinal epithelial cells
IEL	intra-epithelial lymphocytes
IFN	interferon
Ig	immunoglobulin
IKK	inhibitor κB kinase
IL	interleukin
IRAK	IL-1R-associated kinase
LAB	lactic acid bacteria
LDH	lactate dehydrogenase
LPS	lipopolysaccharide
LTA	lipoteichoic acid
MALT	mucosa associated lymphoid tissue
MAPK	mitogen-activated protein kinase
MCP-1	monocyte chemoattractant protein-1
MHC	major histocompatibility complex
MTT	3-(4,5-dimethyldiazol-2-yl)-2,5-diphenyl tetrazolium bromide
NF-κB	Nuclear factor κB
NK	natural killer
NRT	no reverse transcriptase control
NTC	no template control
PB	Plackett Burman
PBMC	peripheral blood mononuclear cells
PBS	phosphate buffered saline
PRR	pattern recognition receptor
QSR	quarter-strength Ringer solution
RT	room temperature
SCAN	Scientific Committee for Animal Nutrition
ss	single-stranded
TIR	Toll/IL-1R
TLR	Toll-like receptors
TNF	Tumour necrosis factor
Tollip	Toll inhibitory protein

LIST OF PUBLICATIONS

Peer review publications

- Vizoso Pinto, M.G., Schuster, T., Briviba, K., Watzl, B., Holzapfel, W.H. and Franz, C.M.A.P. Adhesive and chemokine stimulatory properties of potentially probiotic *Lactobacillus* strains. J Food Prot (In Press).

- Vizoso Pinto, M.G., Franz, C.M.A.P., Schillinger. U. and W.H. Holzapfel. 2006. Isolation, selection and identification of *Lactobacillus* spp. with *in vitro* probiotic properties from human faeces and traditional fermented products. Int J Food Microbiol 109:205-214.

- Vizoso Pinto, M.G., Pasteris, S.E. and Strasser de Saad, A.M. 2004. Glycerol catabolism by *Pediococcus pentosaceus* isolated from beer. Food Microbiol 21:111-118.

Manuscript in preparation

- Vizoso Pinto, M.G., Rodriguez, Manuel, Girrbach, S., Watzl, B., Holzapfel, W.H. and Franz, C.M.A.P. Novel potentially probiotic *Lactobacillus* strains sensitise HT29 intestinal epithelial cells to respond to *S.* Typhimurium.

Selection of posters and oral presentations at national and international scientific meetings

- *Lactobacillus* strains sensitise intestinal epithelial cells to respond to pathogenic bacteria Poster presentation at the International congress FoodMicro2006, Bologna, Italy, 2006.

- Induktion der Toll-Like Rezeptor Genexpression durch *Lactobacillus*- Stämme in HT29 Kolonkarzinomzellen. Poster. DGHM-Meeting. Suhl, Deutschland, 2006.

- Selection and identification of potentially probiotic strains from African traditional fermented products and faeces. Induction of IL-8 production from intestinal epithelial-like HT-29 cells. Poster. International Yakult Symposium, Ghent, Belgium, 2005.

- Aggregation and adhesion properties of novel probiotic *Lactobacillus* strains. Poster. International Yakult Symposium, Ghent, Belgium, 2005.

- Investigations on the probiotic activities of selected *Lactobacillus* strains. Oral Presentation. DGHM Meeting. Seeon, Germany. 2005.

- Screening of variables to optimise an *in vitro* model of the stomach-duodenum passage. Poster. DGHM Meeting. Suhl, Germany. 2004.

- Degradación de glicerol por la vía reductiva en *P. pentosaceus* CAg aislado de cerveza. Poster. XVIII Meeting of the Society of Biology, Tucumán, Argentina. 2001.

- Isolation and identification of lactic acid bacteria at different stages of the production of beer. Behaviour towards glycerol. Poster. MicroAl - 2000. Bs. As. Argentina. 2000.

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Education

03.2003-10.2006	Ph. D. at the University of Karlsruhe.
12.2002 -03.2003	German courses, DSH-Prüfung for admission to a German University at the Goethe Institute in Mannheim, Germany.
03.1995-03.2001	Graduation as biochemist at National University of Tucumán (U.N.T.),
02.1994-12.1994	Highschool student-exchange at the Auguste-Viktoria-Gymnasium in Flensburg, Germany.
03.1989-12.1993	High-school graduation with specialisation in Mathematics and Physics, at the 'Esc. Normal de lenguas vivas J. B. Alberdi' in Tucumán, Argentina.
03.1982-12.1988	Primary school in Neuquén and in Buenos Aires, Argentina.

Research and work experience

04.2003-09.2006	Physiologic and molecular studies on the functionality of lactobacilli with probiotic properties at the lab of Dr. C.M.A.P. Franz at IHT – Federal Research Centre for Nutrition and Food headed by Prof. Dr. W.H. Holzapfel.
01.2004-12.2004	Laboratory assistant (HiWi) at Technologie Zentrum Wasser, Karlsruhe, Germany.
04.2001-11.2002	Research assistant. Topic: Glycerol metabolism of *Pediococcus* spp. from beer in the lab of Prof. Dr. A.M. Strasser de Saad at the Microbiology Chair of the Faculty of Biochemistry, Chemistry and Pharmacy (U.N.T.).
03.1999-04.2001	Undergraduate research assistant. Topic: Glycerol metabolism of lactic acid bacteria from fermented beverages in the lab of Prof. Dr. A.M. Strasser de Saad at the Microbiology Chair of the Faculty of Biochemistry, Chemistry and Pharmacy (U.N.T.).

04.1999-03.2001	Lab undergraduate teaching assistant. Led undergraduate Microbiology laboratory sections. (U.N.T.)
05.1998-03.1999	Lab undergraduate teaching assistant. Led undergraduate Biology laboratory sections.(U.N.T.)

Internship

05.1998-10.1998	Undergraduate assistant at the 24-h emergency laboratory of the hospital 'Dr. C. Padilla' (once a week), Tucumán.

Scholarships

12.2002-10.2006	Grant scholarship to gifted individuals from the Konrad-Adenauer-Foundation, Germany.
04.2001-11.2002	Research scholarship for graduates from C.O.N.I.C.E.T., Argentina.
03.1999-04.2001	Research scholarship for undergraduate students from C.I.U.N.T., U.N.T., Tucumán, Argentina.

Languages

- Spanish. Native speaker.
- German. Very good.
  DSH Prüfung.
- English. Very Good.
  CAE. Certificate of advanced English, University of Cambridge, U.K.

PC-skills

- Microsoft Office: Word, Excel, Outlook, Power point. Very good.
- S.A.S. 8: Basic knowledge.
- Sigma Plot 9.0: Basic knowledge.
- DnaStar: Basic knowledge

Hobbies

Singing (choir and solo), guitar playing, reading, aerobics, Tango dancing.

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