A comparison of the effect of selected lactic acid bacteria on cytokine secretio from monocytes and macrophages in vitro

En sammenligning av effekten av utvalgte melkesyrebakterier på cytokinsekresjon fra monocytter og makrofager in vitro

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Master Thesis

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Abstract

Lactic acid bacteria (LAB) are commonly ingested with many types of food. Some strains have been shown to have beneficial health effects when ingested, of which a few have been given the term “probiotic”. Probiotics are defined by the Food and Agriculture Organization and World Health Organization (FAO/WHO) as “live microorganisms which when administered in adequate amounts confer a health benefit on the host”. Previous studies have shown that probiotics may improve human health through immune modulation by inducing cytokine secretion. Therefore, the aim of this thesis was to compare the cytokine secretion from human immune cell lines exposed to the 7 selected LAB: *Lactobacillus plantarum* MF1298, NC8 and 299v, *L. reuteri* DSM 20016, DSM 17938 and mm4-1a, and *L. rhamnosus* GG. Furthermore, the effect of putative bacterial surface proteins on the immune response were also investigated using *L. reuteri* mutants.

The secretion of interleukin (IL-) 6, IL-8, IL-10 and tumor necrosis factor (TNF) α from the human monocytic THP-1 cell line and THP-1 derived macrophages was investigated following 6 h co-culture with LAB.

The results showed that *L. reuteri* strains stimulated a high secretion of cytokines compared to the other strains, while *L. plantarum* NC8 and *L. rhamnosus* GG induced low secretion of cytokines compared to the other strains. The tendency to induce cytokine secretion was overall the same for the live LAB as for the UV-inactivated LAB. However, UV-inactivated *L. rhamnosus* GG induced higher levels of secreted cytokines than the live form. Of the putative bacterial surface proteins tested, a protein essential for adhesion to intestinal epithelial cells (IEC) was not important for the cytokine secretion from THP-1 cells. However, sortase activity showed a possible importance for the induction of IL-8 secretion.

Sammendrag

Melkesyrebakterier (MSB) blir ofte inntatt i mange typer mat. Noen varianter har vist seg å kunne gi gunstige helseeffekter ved inntak, hvorav noen få har fått betegnelsen "probiotiske". Probiotika er av «Food and Agriculture Organization» og Verdens helseorganisasjon (FAO / WHO) definert som "levende mikroorganismer som ved inntak i tilstrekkelige mengder gir verten en helsegevinst". Tidligere studier har vist at probiotika kan forbedre menneskers helse gjennom immunmodulering, ved å indusere cytokinsekresjon. Målet med denne avhandlingen var derfor å sammenligne cytokinsekresjon fra humane immuncellelinjer inkubert med 7 utvalgte MSB: *Lactobacillus plantarum* MF1298, NC8 og 299v, *L. reuteri* DSM 20016, DSM 17938 og mm4-1a, og *L. rhamnosus* GG. Videre ble effekten av antatte bakterielle overflateproteiner på immunresponsen, undersøkt ved hjelp av *L. reuteri*-mutanter.

Sekresjon av interleukin (IL) 6, ble IL-8, IL-10 og tumor nekrose faktor (TNF) α fra den humane monocytiske cellelinjen THP-1 og makrofager differensiert fra THP-1 celler ble undersøkt etter 6 t inkubasjon med MSB.

Resultatene viste at *L. reuteri*-stammene stimulerte høy sekresjon av cytokiner i forhold til de andre stammene, mens *L. plantarum* NC8 og *L. rhamnosus* GG induserte lav sekresjon av cytokiner i forhold til de andre stammene. Tendensen til å indusere cytokinsekresjon var generelt den samme for levende MSB som for UV-inaktiverte MSB. Unntaket var UV-inaktiverte *L. rhamnosus* GG som induserte høyere nivåer av sekrerte cytokiner enn i levende form. Av de antatte bakterielle overflateproteiner testet, var et protein avgjørende for adhesjon til intestinale epitelceller ikke viktig for cytokinsekresjon fra THP-1celler. Imidlertid viste sortase aktivitet en mulig betydning for induksjon av IL-8 sekresjon.

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Marte Taraldrud

1 Introduction

The main focus of this thesis was the cytokine secretion from the human immune cell lines THP-1 and THP-1 derived macrophages exposed to 7 selected lactic acid bacteria (LAB). The LAB used were *Lactobacillus plantarum* MF1298, NC8 and 299v, *L. reuteri* DSM 20016, DSM 17938 and mm4-1a and *L. rhamnosus* GG. 3 of the strains used were commercially available probiotics (*L. plantarum* 299v, *L. reuteri* DSM 17938 and *L. rhamnosus* GG).

1.1 Lactic acid bacteria

The LAB are a group of bacteria that have some morphological, metabolic and physiological characteristics in common (Axelsson 1998). They produce lactic acid as the major end product during fermentation of carbohydrates, and are in general Gram-positive, non-spore forming, catalase-negative cocci or rods that grow anaerobically (Holzapfel et al. 2001). The LAB are widespread in nature, and they are found in humans and animals (oral cavity, gastrointestinal tract (GIT) and vagina, breast milk and skin), as well as in dairy and fermented products.

The species of LAB used in this thesis all belong to the genus *Lactobacillus*. The genus *Lactobacillus* is the largest of the genera included in the LAB, and the definition of this genus is essentially rod-shaped LAB (Axelsson 1998). This definition explains why the genus consists of such a large number of heterogeneous species (Axelsson 1998). They are, however, divided further into three groups: (1) The obligately homofermentative, (2) the facultatively heterofermentative, and (3) the obligately heterofermentative strains (Stiles & Holzapfel 1997), depending on the presence or absence of key enzymes involved in metabolism of carbohydrates (Axelsson 1998). The homofermentative strains metabolize carbohydrates fermentatively producing lactic acid as the major end-product, while the heterofermentative strains produce lactic acid as a significant component in a mixture of end-products (Stiles & Holzapfel 1997).

In food production LAB are used as starter cultures in many products, such as fermented dairy products, sausages and fermented vegetables (Mäyrä-Mäkinen & Bigret 1998). LAB produce lactic acid, which lowers the pH in products, creating an environment favorable for LAB (Mäyrä-Mäkinen & Bigret 1998). This will in turn suppress the growth of food spoiling bacteria, thus making the product microbiologically stable (Mäyrä-Mäkinen & Bigret 1998).

1.1.1 Probiotic bacteria

Probiotics are defined as “live microorganisms which when administered in adequate amounts confer a health benefit on the host” (FAO/WHO 2001). Probiotic strains should be of human origin, resistant to acid and bile in order to survive passage through the GIT, be able to adhere to human intestinal cells, be able to colonize the human gut (at least temporarily), have antagonistic effects against cariogenic and pathogenic bacteria, and be safe for human consumption (Lee & Salminen 1995). For probiotics used in food industry good technical properties for large scale cultivation, acceptable shelf life and contribution to good taste is essential (Ouwehand et al. 2002).

The gut microbiota is essential for shaping and maintaining normal mucosal immunity (Collado et al. 2009; Rakoff-Nahoum et al. 2004). The intestinal microbiota also helps provide a barrier against other bacteria (e.g. potential pathogens) by production of microbial components as well as competition for nutrients and binding sites (Ouwehand 2007). Probiotic bacteria have been suggested to promote human health by the inhibition of pathogens, improvement of the epithelial barrier function, and modulation of host immune responses (Lebeer et al. 2008). Good adherence capacity is generally a desirable attribute in probiotic lactobacilli, as it may promote the gut residence time, exclude pathogens, and interact with host cells for the protection of epithelial cells or initiate immune modulation (Servin 2004). Exported proteins (proteins that within their sequences contain export signals and surface-retention domains) are found to mediate adhesion to intestinal components (Sanchez et al. 2008). LPXTG-motif, C-terminal membrane anchor protein, and sortase coding gene are examples of proteins and genes that are important for bacterial adhesion (Velez et al. 2007). LPXTG is a sequence motif (where X is any amino acid), and is present in the C-terminal part in many surface-associated proteins (Navarre & Schneewind 1999). The enzyme sortase recognizes the LPXTG motif, and cleaves between the T and G residues (Velez et al. 2007), and covalently links the threonine carboxyl group to amino groups supplied by the cell wall cross bridges of peptidoglycan precursors, which in turn yields a surface protein linked to peptidoglycan, incorporated into the envelope and displayed on the microbial surface (Marraffini et al. 2006). For review of other surface-associated proteins and enzymes see Boekhorst et al. (2006), Sanchez et al. (2008), and Velez et al. (2007).

Due to the high demands from the European Union (EU) on nutrition and health claims made for food (EU 2006), which is enforced by the European Food Safety Authority (EFSA), as of today no probiotic strains are approved for health claims. There are many reasons for this:

Studies have been performed with different strains (or not defined strains), with different design and different concentrations of probiotics. Thus, the total amount of evidence is not sufficiently to validate a health claim. The trials concerning probiotics should be well-defined studies and well-designed clinical trials that are double-blind, placebo controlled, randomized and the results should be confirmed by different groups (Lee & Salminen 1995). Even though no products containing probiotics are approved for health claims, there is a good marked for these products. The manufacturers are allowed to make general statements (e.g. "beneficial for the digestion and immune system") but not specific statements (e.g. "lowers the cholesterol", "increases the secretion of IL-10"), and this seem to be good enough for the consumers.

Some effects of probiotics are more solidly established by a large amount of (well designed and well conducted) studies, while other effects still need a lot more research. Probiotics have been shown to reduce the prevalence and duration of various types of diarrhea (e.g. duration of acute diarrhea in children by approximately 1 day (Francavilla et al. 2012; Huang et al. 2002) and prevention of antibiotics-associated diarrhea (Butler et al. 2012; Hempel et al. 2012)) and reduce the risk of necrotizing enterocolitis in preterm infants (Alfaleh et al. 2010; Alfaleh et al. 2011; Deshpande et al. 2010; Guthmann et al. 2010). On the other hand, many suggested effects still require more studies, such as treatment of irritable bowel syndrome (Hoveyda et al. 2009; McFarland & Dublin 2008; Moayyedi et al. 2010; Nikfar et al. 2008), early prevention of allergic disease (Szajewska 2012), treatment of Crohn's disease (CD) (Jonkers et al. 2012) and cholesterol-lowering effect (Kumar et al. 2012).

In the following section some selected studies done on the commercially available probiotics used in this study are described.

Lactobacillus plantarum 299v is a probiotic bacterium owned by the Swedish company Probi AB (Lund, Sweden). It is used in the product brand ProViva, and has been extensively tested. Binding of enteropathogenic *Escherichia coli* (EPEC) to epithelial cells *in vitro* was shown to be decreased when *L. plantarum* 299v was administered to the cells before EPEC, which in turn reduced the secretory response (from the epithelial cells) to EPEC (Michail & Abernathy 2002). In another study, *L. plantarum* 299v was shown to inhibit the adhesion of EPEC to intestinal epithelial cells (IEC) by inducing mucin production resulting in limited access to IEC (Mack et al. 2003). Two studies have shown that consumption of *L. plantarum* 299v temporarily increased the amount of lactobacilli in the fecal flora (Goossens et al. 2003;

Goossens et al. 2006). Furthermore, *L. plantarum* 299v, when given to patients with irritable bowel syndrome (IBS), seemed to ease their symptoms (Niedzielin et al. 2001).

Lactobacillus reuteri DSM 17938 is a probiotic bacterium owned by the Swedish company BioGaia AB (Stockholm, Sweden) and is in Norway used in the product brand BioQ produced by Q-meieriene AS (Norway). There have been many studies examining the effects of this strain. Studies have shown that *L. reuteri* DSM 17938 given to infants with colic seems to alleviate the symptoms compared with placebo treatment (Brown 2011; Cabana 2011; Savino et al. 2010). The strain has also been shown to increase the bowel movement of infants with chronic constipation, compared to the placebo group (Coccorullo et al. 2010). Compared to placebo treatment *L. reuteri* DSM 17938 reduced the frequency, duration and the recurring rate of acute diarrhea in children (Francavilla et al. 2012). On the other hand, *L. reuteri* DSM 17938 had no effect compared with placebo treatment regarding the overall incidence of nosocomial (hospital-acquired infection) diarrhea in hospitalized children (Wanke & Szajewska 2012).

Lactobacillus rhamnosus GG is a probiotic strain owned by the Finnish company Valio, and is probably the worlds most investigated LAB. In Norway the strain can be found in the Biola products produced by TINE®. A study showed that *L. rhamnosus* GG inhibits the adhesion of EPEC to IEC by binding strongly to the epithelial cells and up-regulating the mucin production, thus making it difficult for the EPEC to adhere to the epithelial cells (Mack et al. 2003). Another study showed that *L. rhamnosus* GG could alleviate the intestinal inflammation in infants with atopic eczema/dermatitis syndrome and suspected cows milk allergy, by decreasing intestinal inflammatory markers (e.g. TNF-α) (Viljanen et al. 2005). Long term consumption of *L. rhamnosus* GG may reduce respiratory infections among children in day care according to a seven month long study (Hatakka et al. 2001). Furthermore, prophylactic intake of *L. rhamnosus* GG have been shown to reduce the risk of nosocomial diarrhea in infants (Szajewska et al. 2001), and to control diarrhea in undernourished children at increased risk of diarrhea (Oberhelman et al. 1999).

1.2 The gastrointestinal tract

The GIT consists of the stomach where food is mechanically broken down and chemical digestion of proteins begins, and the small and large intestines where further digestion takes place and nutrients, water and salts are absorbed (Saladin 2010). The GIT has the important task of digesting and absorbing nutrients in order to meet the metabolic requirements and

demands for human growth and development, and to provide protective host defense against the constant presence of food antigens and microorganisms in the lumen of the gut (Singh et al. 2009). Gastric acid, saliva, mucus, gut flora, peristalsis, IEC and intracellular junctional complexes all provide protection against potentially harmful agents in the GIT (Singh et al. 2009). The bacterial flora of the GIT mainly resides in the large intestine (approximately 1×10^{12} cfu/g contents), but there are micro-organisms present throughout the entire GIT (Cummings et al. 2004). The commensal bacteria in the gut are essential for shaping and maintaining normal mucosal immunity (Kelly et al. 2005).

1.2.1 Gut-associated lymphoid tissue

The gut-associated lymphoid tissue (GALT) consists of Peyers patches (PP), the appendix and many isolated lymphoid follicles (ILF) (Shi & Walker 2004). The PP are mainly located in the small intestinal distal ileum, and it is where the initiation of immune responses occurs (Cummings et al. 2004). The PP and ILF are composed of specialized follicle-associated epithelium, which contains microfold (M) cells, a sub epithelial dome rich in dendritic cells (DCs), and B-cell follicle(s) that contain germinal centers (Fagarasan & Honjo 2003). In GCs differentiation of the follicular B cells can efficiently take place (Fagarasan & Honjo 2003). In addition, the ILF contains T-cells and macrophages (Delcenserie et al. 2008). The M cells do not have brush border, their function is the transport of antigens across the epithelium to the lymphoid follicle and the antigen presenting cells that resides there (Artis 2008).

1.3 The immune system

The immune system consists of an innate and an adaptive part. The innate immune system is non-specific and can be triggered by preserved parts on microorganisms, while the adaptive immune system is specific and develops memory as it encounters infectious agents and foreign antigens (Cummings et al. 2004). This thesis describes the effect of LAB on immune cells *in vitro*, thus the following sections will focus on the immune system and bacteria.

1.3.1 The innate immune system

The innate immune system has barriers to prevent microbial threats from invading our body: Physical/structural barriers such as the epithelial linings of the skin and mucosae, mucus, ciliary function and peristalsis, chemical factors such as pH of bodily fluids, antimicrobial peptides and proteins, and phagocytic cells, e.g. macrophages and DCs (Cummings et al.

2004). In addition, the complement proteins, acute phase reactants, natural killer (NK)-cells, phagocytes, and cytokines are included in the innate immune system (Kekkonen 2008).

When our body is invaded by pathogens, the cells of the innate immune system act as a first line of defense (Delcenserie et al. 2008). The cells of the innate immune system have receptors that recognize conserved components on microorganisms (e.g. bacterial cilia or lipopolysaccharide (LPS)), generally called pathogen-associated molecular patterns (PAMPs) (Cummings et al. 2004; Harris et al. 2006). Since these structures are also found on commensal and non-pathogenic microorganisms the term microbe-associated molecular patterns (MAMPs) are commonly used (Neish 2009; Wells et al. 2010). Pattern recognition receptors (PRRs) are the cellular receptors of the innate immune system that recognize MAMPs (Harris et al. 2006). Many of them belong to the so-called Toll-like receptors (TLRs), which is mainly expressed by the DCs and macrophages, but also by other cell types such as B cells and epithelial cells (Cummings et al. 2004). The nucleotide-oligomerization domain (NOD)-like receptors (NLRs) is another family of PRRs, and is expressed on a broad range of tissue types, including intestinal cells (Wells et al. 2011). A key characteristic of the innate immune system is the speed of the response (Delcenserie et al. 2008). As commensal bacteria in the gut have MAMPs on their cell surface, they have the potential to activate immune responses trough PRRs such as TLRs and NLRs (Goto & Kiyono 2012).

Among the phagocytic cells are the monocytes and macrophages (Delcenserie et al. 2008). Macrophages are monocytes that have migrated from blood to tissue, and depending on the tissue the macrophage migrates to, they display different patterns of surface molecules (Ziegler-Heitbrock 2007). Macrophages also have the ability to present antigen to certain T-cells (Birmingham et al. 1982). Antigen presenting cells can phagocytize an antigen and display fragments of it on its surface, in order for other cells of the immune system to recognize the antigen (Cummings et al. 2004). In this thesis a human monocyte cell line (THP-1) and macrophages derived from this cell line have been used, and the cytokine profiles from these after co-culture with LAB have been examined.

1.3.2 The adaptive immune system

In the adaptive immune system the most important cells and mechanisms are B-lymphocytes, T-helper cells (Th1, Th2, Th3), cytotoxic T-cells, regulatory T-cells (T-reg), production of antibodies and cytokines (Kekkonen 2008). B- and T-lymphocytes (B- and T-cells) have specialized receptors that can bind and identify antigens (Andersen et al. 2006). When an

antigen binds, the cells may become activated and differentiate into effector-cells, which in turn is responsible for fighting the microbial threats (Cummings et al. 2004; Delcenserie et al. 2008).

Lymphocytes

The B- and T-cells are equipped with receptors that have the ability to distinguish between foreign structures and the bodys own (Cummings et al. 2004). One of the main differences between the B-and T-cells and the defensive cells in the innate immune system (e.g. macrophages) is that while a macrophage has PRRs, a lymphocyte has specificity for one particular antigen alone (Cummings et al. 2004). When a lymphocyte is stimulated, this leads to an increase in the cells volume, preparing them for division in order to make more lymphocytes with the same type of receptors and ability to recognize and react with the specific antigen (Cummings et al. 2004). Furthermore, binding of antigen leads to further development of effector cells, which are more specialized than the original lymphocytes (Delcenserie et al. 2008).

The antigen receptor of B-cells is a membrane bound version of antibody molecules, and when bound to antigen this can lead to stimulation (Cummings et al. 2004). Stimulation differentiate the B-cell into plasma cells (secreting immunoglobulins (Ig)) that have the same specificity as the membrane bound receptors on the initial, stimulated B-cell (Cummings et al. 2004). The B-cells are primarily equipped to recognize and fight extracellular microorganisms and compounds (Cummings et al. 2004). Memory B-cells give the host the ability to provide more effective immune responses upon secondary infections with an antigen it has encountered before (Delcenserie et al. 2008).

The T-cells monitor the intracellular environment (Delcenserie et al. 2008), and there are mainly 4 different types of T-cells: The cytotoxic T-cells (also called killer T-cells, carry out the attack on enemy cells (Kekkonen 2008)), T-helper cells (involved in both humoral and cellular immunity (Delcenserie et al. 2008)), regulatory T-cells (limits the immune response by blocking the activity of other T-cells and by secreting the anti-inflammatory cytokine IL-10 (Beissert et al. 2006), some regulatory T-cells are also important in preventing autoimmune diseases (Beissert et al. 2006)) and memory T-cells (responsible for the memory in cellular immunity (Saladin 2010)) (Kekkonen 2008).

1.3.3 Inflammation

Inflammation is characterized by redness, heat, swelling, and pain (Kekkonen 2008). Redness and heat are caused by the increased blood flow to the inflammation site, swelling by the accumulation of fluids, and pain is caused by the swelling (Hakansson & Molin 2011). Inflammation can be triggered by infections, decomposition of body tissue by trauma (e.g., surgery or accidents) and allergy or autoimmunity (Hakansson & Molin 2011). The inflammation in allergy is triggered by the adaptive immune system which reacts to different types of harmless compounds in the environment, e.g., pollen, while in autoimmunity, the inflammation is caused by the adaptive immune system attacking the bodys own cells and tissue (Hakansson & Molin 2011).

1.4 Cytokines

Cytokines are polypeptide messenger compounds that stimulate cellular differentiation, cellular growth, and functional development (Cummings et al. 2004). Cytokines include interleukins (IL), interferons, chemokines, colony-stimulating factors and many growth factors (Scheller et al. 2011). They regulate hematopoiesis, immune reactions, inflammatory reactions, and vascular reactions, and both stimulatory and inhibitory cytokines play important roles in the function of endothelial cells, smooth muscle cells, macrophages, and T-cells (Kofler et al. 2005). The cytokines do not function as effector molecules on their own, but have an effect after binding to specific surface receptors on the membrane of cells (Kekkonen 2008). Cytokines have autocrine (on the producing cell) or paracrine (on neighbor cells) effects (Kekkonen 2008).

The cytokines measured in this thesis are described in more detail in the following sections.

1.4.1 Interleukin 6

Alternative names for IL-6 are interferon β2, B-cell stimulatory factor-2 (BSF2), hepatocyte stimulatory factor and plasmacytoma/hybridoma growth factor (Akdis et al. 2011; Schwab et al. 1991). Endothelial cells, fibroblast, monocytes and macrophages produce IL-6 during systemic inflammation in response to different stimuli (IL-1, IL-17 and TNF-α) (Akdis et al. 2011). In chronic inflammation, the T-cells produce IL-6 (Naugler & Karin 2008). The main target cells are hepatocytes, leukocytes, T-cells, B-cells and hematopoietic cells (Akdis et al. 2011). IL-6 is a multifunctional cytokine with many effects (Akdis et al. 2011). It is involved in regulation of immune responses, acute-phase responses, hematopoiesis and inflammation

(Akdis et al. 2011). The levels of IL-6 are low under normal conditions, but during stress the levels of IL-6 in serum rise quickly (Naugler & Karin 2008). In innate immunity, IL-6 directs leukocyte trafficking and activation (Hurst et al. 2001), and induces production of acute-phase proteins by hepatocytes (Gauldie et al. 1987). Some studies have shown that IL-6 may act as an anti-inflammatory mediator by suppressing LPS induced production of IL-1 and TNF in macrophages *in vitro*, and in LPS-treated mice *in vivo* (Barton & Jackson 1993; Schindler et al. 1990), but IL-6 is mostly regarded as a pro-inflammatory cytokine (Scheller et al. 2011). IL-6 has been shown to induce fever (LeMay et al. 1990; Sakata et al. 1991). It has also been demonstrated that IL-6 is responsible for T-cell proliferation (Uyttenhove et al. 1988) and the final maturation of B-cells into Ig-secreting plasma cells (Hirano et al. 1985). Studies have shown that IL-6 may have both autocrine (Schwab et al. 1991) and paracrine (Klein et al. 1989) effects. IL-10 has been shown to inhibit the production of IL-6 (de Waal Malefyt et al. 1991).

1.4.2 Interleukin 8

IL-8 is a pro-inflammatory cytokine, also known as CXCL8 (Akdis et al. 2011) and monocyte-derived neutrophil chemotactic factor (MDNCF) (Yoshimura et al. 1987). IL-8 belongs to the chemokines, a group of structurally related, small, mostly basic molecules (Zlotnik & Yoshie 2000). Among other effects, IL-8 can induce the directional migration of many cell types, including neutrophils, monocytes, T-cells, basophils and fibroblasts (Taub et al. 1993). A variety of cells secrete IL-8, including fibroblasts (Burke et al. 2008; Fredriksson et al. 2003), skeletal muscle cells (Chan et al. 2004), smooth muscle cells (Issa et al. 2008), monocytes (Bhattacharyya et al. 2002), macrophages (Lin et al. 2008), T cells, neutrophils, NK-cells, endothelial cells, epithelial cells, tumor cells (Mukaida et al. 2003) and mast cells (Burke et al. 2008). IL-8 production can be induced by IL-1, IL-2, IL-3 and TNF-α (Seitz et al. 1991). A major effector function of IL-8 is the recruitment of neutrophils to an infection or injury site (Matsushima et al. 1988). IL-8 can also recruit NK cells to sites of viral infection (Burke et al. 2008). Elevated levels of IL-8 have been detected in patients with rheumatoid arthritis (RA) (Seitz et al. 1991) and patients with *Helicobacter pylori*-infection (Holck et al. 2003). IL-10 has been shown to inhibit production of IL-8 (de Waal Malefyt et al. 1991).

1.4.3 Interleukin 10

IL-10 has immunosuppressive effects, and protects the host from autoimmune diseases and exaggerated inflammatory responses to microbial infections (Akdis et al. 2011). IL-10 was

originally named cytokine synthesis inhibitory factor (CSIF), as it inhibits cytokines such as interferon (INF)-γ (Fiorentino et al. 1989), IL-1α, tumor necrosis factor (TNF)-α, GM-CSF, G-CSF, IL-1β, IL-6 and IL-8 (de Waal Malefyt et al. 1991). IL-10 is produced by monocytes (de Waal Malefyt et al. 1991), DCs, T-cells (Nagalakshmi et al. 2004), B-cells (Benjamin et al. 1992), macrophages (Okamoto et al. 2011) and NK cells (Wolk et al. 2002). IL-10s primary purpose is to limit cytokine- and chemokine production in mainly macrophages (Akdis et al. 2011), monocytes (Sabat et al. 2010) and DCs (Akdis et al. 2011). In monocytes/macrophages IL-10 inhibits antigen presentation, influences the cells to release anti-inflammatory mediators, and enhances the inhibitory, tolerance-inducing and scavenger functions (Sabat et al. 2010).

IL-10 is produced at high levels relatively late in the immune response compared to some of the pro-inflammatory cytokines (e.g. IL-6 and IL-8) (de Waal Malefyt et al. 1991). The pro-inflammatory cytokines peaks early in the immune response (4 - 8 h after stimulation) while IL-10 peak 24 - 48 h after stimulation (de Waal Malefyt et al. 1991). Other target cells include B-cells (Wakkach et al. 2000) which are differentiated into antibody-secreting/plasma cells (Akdis et al. 2011), NK cells, and T-cells (Wolk et al. 2002).

IL-10 plays an important role in disease (Sabat et al. 2010). Over-production of IL-10 can result in growth of tumors and undesired immunosuppressive effects (Sabat et al. 2010). Examples of this kind of diseases are systemic lupus erythematosus (Grondal et al. 1999; Llorente et al. 2000) and Epstein-Barr virus-associated lymphomas (Stewart et al. 1994). A relative or absolute IL-10 deficiency will result in a continuous activation of the immune response (Sabat et al. 2010). Examples of these types of disease are psoriasis (Asadullah et al. 1998), RA (Sheff et al. 1994) and CD (van Montfrans et al. 1998).

1.4.4 Tumor Necrosis Factor α

TNF-α, also called TNF and cachectin, is a pleiotropic pro-inflammatory cytokine (Wang et al. 2003) and it is mainly produced by activated macrophages and T-cells (De Paepe et al. 2012). Other TNF-α producing cells include (among others) monocytes, mast cells, NK cells, smooth muscle cells, tumor cells (Wang et al. 2003), endothelial cells (ten Hagen et al. 2008) and fibroblasts (Roberts et al. 2011). TNF-α is released in response to inflammatory stimuli and cytokines, including peptidoglycan, LPS and other bacterial components (Roberts et al. 2011). Since systemic overproduction of TNF-α activates inflammatory responses to infection and injury, mediates hypotension, diffuse coagulation and gives widespread tissue damage,

the expression of TNF-α is tightly controlled (Wang et al. 2003). TNF-α can induce both apoptosis and cell survival, and it has been shown that systemic administration of TNF-α causes well established subcutaneous tumors to undergo necrosis (ten Hagen et al. 2008). TNF-α has been demonstrated to have a growth inhibitory effect on SV40-transformed human mammary epithelial cells, and a cytotoxic effect on breast cancer cell lines, but there was no effect on normal human mammary epithelial cells *in vitro* (Dealtry et al. 1987). TNF-α have also been shown to have a cytostatic effect on hepatoma cells, while it had little effect on non tumorgenic liver cells (Motoo et al. 1986). TNF-α is inhibited by IL-10 (de Waal Malefyt et al. 1991; Sheff et al. 1994).

2 Objectives

The major aims of this thesis were:

- To compare immune stimulatory effects of selected strains of LAB on the monocytic THP-1 cell line and THP-1 derived macrophages.
- To compare the effect of live LAB versus UV-inactivated LAB.
- To investigate the effect of putative bacterial surface proteins on the immune response of THP-1 cells by help of *L. reuteri* mm4-1a mutants.

The selected LAB strains include *L. plantarum* MF1298, NC8 and 299v, *L. reuteri* DSM 20016, DSM 17938 and mm4-1a and *L. rhamnosus* GG.

The goal of this thesis was to focus on similarities and differences between the strains, not to find a new probiotic bacterium or define what a good probiotic bacterium is.

Some studies indicate that LAB not necessarily have to be alive to administer a positive health effect (Kataria et al. 2009). Thus, the effect of live and Ultraviolet (UV) inactivated LAB were compared in this thesis.

3 Materials

3.1 Chemicals and reagents

Chemicals and reagents	Supplier
2-mercanptoethanol 50 mM	Gibco®, Life technologies, Grand Island, NY
3,3`,5,5`-Tetramethylbenzidine	Sigma-Aldrich, St. Louis, MO
Biotin Purified Mouse Anti-Human IL-8	BD Pharmingen, Franklin Lake, NJ
Biotin Purified Mouse Anti-Human TNF-α	BD Pharmingen, Franklin Lake, NJ
Biotin Purified Rat Anti-Human and viral IL-10	BD Pharmingen, Franklin Lake, NJ
Biotin Purified Rat Anti-Human IL-6	BD Pharmingen, Franklin Lake, NJ
Bovine Serum Albumin (BSA)	Sigma-Aldrich, St. Louis, MO
Brain Heart Infusion (BHI)-agar	Oxoid Limited, Hampshire, UK
Brain Heart Infusion (BHI)-broth	Oxoid Limited, Hampshire, UK
$C_6H_8O_7$ x H_2O	Merck KGaA, Darmstadt, Germany
De man, Rogosa, Sharpe (MRS) agar	Oxoid Limited, Hampshire, UK
De man, Rogosa, Sharpe (MRS) broth	Oxoid Limited, Hampshire, UK
Dimetylsulfoksid (DMSO)	Sigma-Aldrich, St. Louis, MO
Dulbecco's Phosphate Buffered Saline (DPBS)	Sigma-Aldrich, St. Louis, MO

Fetal Bovine Serum (FBS)	Gibco®, Life technologies, Grand Island, NY
Gentamicin 10 mg/mL	Sigma-Aldrich, St. Louis, MO
Glycerol 87 %	Merck KGaA, Darmstadt, Germany
H_2O_2 30 %	Sigma-Aldrich, St. Louis, MO
H_2SO_4	Merck KGaA, Darmstadt, Germany
High Performance ELISA (HPE) buffer	Sanquin, Amsterdam, The Netherlands
KCl	Merck KGaA, Darmstadt, Germany
KH_2PO_4	Merck KGaA, Darmstadt, Germany
L-Glutamine 200 mM	Gibco®, Life technologies, Grand Island, NY
Lipopolysaccarid (LPS), from *Escherichia coli* 055:B5	Sigma-Aldrich, St. Louis, MO
Na_2CO_3	Merck KGaA, Darmstadt, Germany
Na_2HPO_4	Merck KGaA, Darmstadt, Germany
NaCl	Merck KGaA, Darmstadt, Germany
$NaHCO_3$	Merck KGaA, Darmstadt, Germany

Penicillin/Streptomycin (P/S) 100 U/mL, 100 µg/mL	Gibco®, Life technologies, Grand Island, NY
Phorbol 12-myristate 13-acetate (PMA)	Sigma-Aldrich, St. Louis, MO
Purified Mouse Anti-Human IL-8	BD Pharmingen, Franklin Lake, NJ
Purified Mouse Anti-Human TNF-α	BD Pharmingen, Franklin Lake, NJ
Purified Rat Anti-Human and viral IL-10	BD Pharmingen, Franklin Lake, NJ
Purified Rat Anti-Human IL-6	BD Pharmingen, Franklin Lake, NJ
Recombinant human IL-10	BD Pharmingen, Franklin Lake, NJ
Recombinant human IL-6	BD Pharmingen, Franklin Lake, NJ
Recombinant human IL-8	BD Pharmingen, Franklin Lake, NJ
Recombinant human TNF-α	BD Pharmingen, Franklin Lake, NJ
RPMI 1640 culture medium	Gibco®, Life technologies, Grand Island, NY
Sterile Dulbecco's Phosphate Buffered Saline (SDPBS)	Gibco®, Life technologies, Grand Island, NY
Streptavidin-HRP(Sav-HRP)	BD Pharmingen, Franklin Lake, NJ
Tween® 20	Sigma-Aldrich, St. Louis, MO

3.2 Equipment

Equipment	Supplier
353003-Tissue Culture Dish	Becton Dickingson Labware, Franklin Lake, NJ
AnaeroGen™ 2.5 L	Oxoid Limited., Hampshire, UK
AnaeroGen™ 3.5 L	Oxoid Limited., Hampshire, UK
Barseal™	Nunc, Roskilde, Denmark
Centrifuge	Thermo Electron Corporation, Waltham, MA
Centrifuge tubes 15 ml	VWR International, Radnor, PA
Centrifuge tubes 50 ml	VWR International, Radnor, PA
Certoclave	LGA, Nürnberg, Germany
Eppendorf tubes 1.5 ml	Sarstedt, Nümbrecht, Germany
MaxiSorp™ ELISA plates	Nunc, Roskilde, Denmark
Multi-channel automatic pipette	Biohit, Helsinki, Finland
Multiwell™, 12-well tissue culture plate	Becton Dickingson Labware, Franklin Lake, NJ
Pipetboy	Integra Biosciences, Zizers, Switzerland
Platform shaker	Stuart scientific, Chelsford Essex, UK
ProtoCOL2, colony counter	Synbiosis, Cambridge, UK
SPECTROstar^{nano}	BMG LABTECH, Offenburg, Germany

Sterile 1.8 ml Cryo Pure Tubes	Sarstedt, Nümbrecht, Germany
Sterile petri dishes	VWR International, Radnor, PA,
Sterile Single pipettes 5 ml	VWR International, Radnor, PA,

3.3 Software

Software	**Supplier**
GraphPad Prism 5.02	GraphPad Software, Inc, La Jolla, CA
SPECTROstar^{nano}Mars	BMG LABTECH, Offenburg, Germany

3.4 Bacteria

Bacteria	**Supplier and references/origin**
Escherichia coli K12	ATCC 47076
Lactobacillus plantarum 299v (DSM 9843)	Sourdough. ProViva brand of probiotic products. (Johansson et al. 1993)
Lactobacillus plantarum MF1298	Norwegian mutton salami (Klingberg et al. 2005)
Lactobacillus plantarum NC8	Grass silage (Shrago et al. 1986)
Lactobacillus reuteri DSM 17938	Plasmid cured variant of ATCC 55730. Human breast milk. (Rosander et al. 2008).

	Commercially available probiotic bacteria.
Lactobacillus reuteri DSM 20016	Type strain. Human intestine (Kandler et al. 1980)
Lactobacillus reuteri mm4-1a (ATCC PTA 6475)	Human breast milk (Oh et al. 2009)
Lactobacillus reuteri mm4-1a S1065_E	Mutant of *Lactobacillus reuteri* mm4-1a (ATCC PTA 6475), nonsense mutation in gene equivalent to LAR_0044*, C-terminal membrane anchor protein (van Pijkeren & Britton 2012)
Lactobacillus reuteri mm4-1a 130_A	Mutant of *Lactobacillus reuteri* mm4-1a (ATCC PTA 6475), nonsense mutation in gene equivalent to LAR_0227*, sortase (van Pijkeren & Britton 2012)
Lactobacillus reuteri mm4-1a 1696_H2	Mutant of *Lactobacillus reuteri* mm4-1a (ATCC PTA 6475), nonsense mutation in gene equivalent to LAR_0813*, LPXTG protein, amidase (van Pijkeren & Britton 2012)
Lactobacillus reuteri mm4-1a S241_E	Mutant of *Lactobacillus reuteri* mm4-1a (ATCC PTA 6475), nonsense mutation in gene equivalent to LAR_0958*, repeated LPXTG protein (van

	Pijkeren & Britton 2012)
Lactobacillus reuteri mm4-1a S655_H	Mutant of *Lactobacillus reuteri* mm4-1a (ATCC PTA 6475), nonsense mutation in gene equivalent to LAR_0983*, LPXTG (van Pijkeren & Britton 2012)
Lactobacillus reuteri mm4-1a S647_E	Mutant of *Lactobacillus reuteri* mm4-1a (ATCC PTA 6475), nonsense mutation in gene equivalent to LAR_0989*, LPXTG protein, part of Rib motif (van Pijkeren & Britton 2012)
Lactobacillus rhamnosus GG (ATCC 53103)	Human intestine (Silva et al. 1987)

ATCC, American Type Culture Collection

DSM, Deutsche Sammlung von Mikroorganismen

*****locus tags from sequenced strain *L. reuteri* JCM1112

3.5 Buffer

Buffer	**Preparation**
0.05 M Substrate buffer	7.3 g Na_2HPO_4
	5.1 g citric acid
	1 l dH_2O
	pH adjusted to 5
	autoclaved before use
10×Phosphate Buffered Saline (PBS)	80 g NaCl

	2 g KCl 20 g Na_2HPO_4 4 g KH_2PO_4 pH adjusted to 7.2 – 7.3 to 1 l of dH_2O
Blocking buffer	1×PBS with 5% Bovine Serum Albumin (BSA)
Coating buffer	Solution A: 1.06 g Na_2CO_3 in 100 ml dH_2O Solution B: 1.68 g of $NaHCO_3$ in 200 ml dH_2O Mix solution A and solution B to obtain a pH of 9.6
High Performance ELISA (HPE) buffer	Diluted 1:7 in dH_2O
Substrate	One tablet 3,3`,5,5`-Tetramethylbenzidine was dissolved in 1 ml dimetylsulfoksid (DMSO). Immediately before use, 9 ml of substrate buffer and 2 µl of H_2O_2 were added. The substrate was protected from light.
Washing buffer	1×PBS with 0.01% Tween® 20

4 Methods

4.1 Agar/broth

MRS- and BHI-agar/broth was prepared as described by the supplier. The powder was suspended in deionized water and certoclaved. The MRS- and BHI-agar was poured in sterile petri dishes in a sterile bench, and allowed to cool down completely before packing in plastic bags and storing at 4 °C. The MRS- and BHI-broth was cooled down on the bench and stored in room temperature.

4.2 Lactic acid bacteria

To make a stock of the bacteria for the experiments 1.5 ml of overnight bacteria culture were mixed with 300 µl 87 % glycerol in sterile 1.8 ml Cryo Pure tubes and stored at -80 °C.

When needed for experiments bacteria were taken up two days before experiments and grown anaerobically on MRS-agar plates in a 2.5 l container with an anaerobic sachet at 37 °C. On the day of the experiment all the preparation except the centrifugation, (adjusting to 1×10^8 cfu/ml) and measuring of OD was done in a sterile bench.

L. reuteri mm4-1a (ATCC PTA 6475) mutants were a kind gift from Jan-Peter van Pijkeren at the Michigan state University (MI), and made as previously described (van Pijkeren & Britton 2012).

4.3 Preparing the bacteria for experiments

Bacteria were scraped from MRS-agar in 5 ml of DPBS, and transferred with a pipet boy to separate 15 ml plastic tubes. The tubes were centrifuged at 3000 rpm for 10 min. The supernatant was discarded and the pellet re-suspended in 5 ml sterile DPBS (hereafter referred to as bacterial concentrate). The bacterial concentrate was then used to adjust the optical density (OD) (Table 1) to achieve a concentration of 1×10^8 cfu/ml. The OD for each individual strain was adjusted at 600 nm as shown in Table 1. This solution was centrifuged at 3000 rpm for 10 min, the supernatant discarded and the pellet was re-suspended in a tenth of the volume of sterile DPBS to up-concentrate the bacteria to 1×10^9 cfu/ml. The *E. coli* K12 was grown overnight in 5 ml BHI-broth at 37 °C before experiments. The over-night culture was then centrifuged, the supernatant discarded, and the pellet re-suspended in 5 ml DPBS.

To control the bacterial concentration used in each experiment, the bacterial suspension was plated on MRS-agar plates by the use of a WASP spiral plater and incubated anaerobically at 37 °C for 48 h. The bacterial suspension of *E. coli* K12 was plated on BHI-agar plates and incubated anaerobically at 37 °C for 24 h. After the incubation time the colonies on the plates were counted using a colony counter.

Table 1. OD-adjustment of the LAB.

Strain	**OD_{600} (10^8 cfu/ml)**
L. reuteri DSM 20016	0.605
L. reuteri DSM 17938	0.655
L. plantarum MF1298	0.750
L. plantarum NC8	0.425
L. plantarum 299v	0.695
L. rhamnosus GG	0.940
L. reuteri mm4-la (ATCC PTA 6475)	0.635

4.4 Preparing UV-inactivated bacteria

Live bacterial suspension of 1×10^9 cfu/ml was spread out in tissue culture dishes, put on a swinging board at 20 RPM in an opaque box with an UV-lamp inside. The bacteria were radiated with UV-light for 20 min. The suspensions were aliquoted at 500 µl in 1.5 ml eppendorf-tubes and frozen at -80 °C. To investigate whether the UV-inactivation had successfully killed the bacteria, the bacterial suspension was plated on MRS-agar plates by the use of a WASP spiral plater and incubated anaerobically at 37 °C for 48 h. Only suspensions with confirmed dead bacteria were used in the experiments.

4.5 Maintenance of THP-1 cells

The human monocytic leukemia cell line THP-1 was grown in RPMI 1640 culture medium supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin and 100 µg/ml streptomycin (P/S), 2 mM L-Glutamine and 0.005 mM 2-mercanptoethanol at 37 °C and 5% CO_2 in a humidified atmosphere. Cells were sub-cultured three times per week. As the cells

change their properties after long periods in culture, the cells were discarded after 25 passages and replaced by frozen stocks.

4.6 Cell experiments

24 h prior to experiments, THP-1 cells were seeded out in 12-well tissue culture plates, at the concentration 3×10^5 cells/ml.

To differentiate the THP-1 cells to macrophages 100 ng/ml Phorbol 12-myristate 13-acetate (PMA) diluted in cell culture medium was added to 1×10^6 cells/ml in 12-well tissue culture plates immediately after the cells were seeded out (Zhou et al. 2010). PMA targets protein kinase C (PKC) which is involved in the regulation of cell differentiation and other cellular functions (reviewed by Schwende et al. (1996)). The differentiation was started 48 h prior to experiments. On the day of the experiment with macrophages, the cell culture medium with PMA was removed, and the cells were washed once with sterile Dulbecco's Phosphate Buffered Saline (SDPBS), 1 ml/well. Afterwards new RPMI 1640 cell culture medium (containing 10% FBS, (100 U/ml/100 µg/ml P/S depending on the experiment), 2 mM L-Glutamine and 0.005 mM 2-mercanptoethanol) without PMA was added to the wells, 1.5 ml/well. This was done immediately before the experiment started.

The cells were exposed to different concentrations of LAB, and for different incubation times. The cells were also exposed to *E. coli* K12 and LPS from *E. coli* O55B:5 at different concentrations.

Based on pilot experiments, the test concentration of bacteria was set to 1×10^8 cfu/ml and the test incubation time was set to 6 h.

4.7 Enzyme-Linked Immunosorbent Assay

The cytokine concentrations in the cell culture supernatants were determined using enzyme-linked immunosorbent assay (ELISA).

4.7.1 The method

The day before running the ELISA, MaxiSorp™ ELISA plates were coated with an antibody against the cytokine to be measured. Table 2 shows the concentration of the antibodies used.

The antibodies were diluted in coating buffer. After adding of the coating solution the plate was sealed and incubated overnight at 4 °C.

Table 2. Coating antibody end concentration.

Antibody	End concentration
IL-6	2 µg/ml
IL-8	1 µg/ml
IL-10	1 µg/ml
TNF-α	2 µg/ml

Before experiments, the plates were washed 3 times with washing buffer using a plate washer, Wellwash AC. If not otherwise stated in the following description, the plate washer was used in the washing steps. After washing, the plates were incubated with 70 µl of blocking solution per well on a swinging board for at least 1 h. All incubation hereafter was carried out on the swinging board at room temperature.

Before adding the samples, the plate was washed five times with washing buffer (100 µl/well). The samples were diluted in HPE-buffer (Table 3 and Table 4).

Table 3. Dilution of samples from THP-1 cells.

Cytokine	Control	Samples	LPS and *E. coli* K12
TNF-α	1:1	1:5	1:5
IL-8	1:1	1:200	1:40
IL-10	1:1	1:1	1:1

Table 4. Dilution of samples from macrophages.

Cytokine	Control	Samples	LPS and *E. coli* K12
TNF-α	1:1	1:100	1:100
IL-6	1:1	1:1	1:20
IL-8	1:100	1:2000	1:500
IL-10	1:1	1:1	1:1

The standard (recombinant human IL-6, IL-8, IL-10 and TNF-α) was diluted in 1:1 mixture of HPE-buffer and cell culture medium to the right concentrations (Table 5).

Table 5. Standard curves and concentrations.

	IL-6	**IL-8**	**IL-10**	**TNF-α**
S1	0 pg/ml	0 pg/ml	0 pg/ml	0 pg/ml
S2	1.4 pg/ml	2 pg/ml	4.1 pg/ml	4.1 pg/ml
S3	4 pg/ml	6 pg/ml	12.3 pg/ml	12.3 pg/ml
S4	12 pg/ml	18 pg/ml	37 pg/ml	37 pg/ml
S5	37 pg/ml	55.5 pg/ml	111.1 pg/ml	111.1 pg/ml
S6	111.1 pg/ml	166.6 pg/ml	333.3 pg/ml	333.3 pg/ml
S7	333.3 pg/ml	500 pg/ml	1000 pg/ml	1000 pg/ml
S8	1000 pg/ml	1500 pg/ml	3000 pg/ml	3000 pg/ml
S9	3000 pg/ml	3000 pg/ml	5000 pg/ml	5000 pg/ml
Control	50 pg/ml	50 pg/ml	50 pg/ml	50 pg/ml

The standards were added in duplicate, 50 µl/well. The samples were analyzed in triplicate, 50 µl/well. The control was added last and in six wells, 50 µl/well. The plate was then incubated for 1.5 h.

The samples were removed from the plate with a multi-channel pipette. The tips were changed for every new sample. Afterwards, the plate was washed six times manually with a multi-channel automatic pipette. The detection antibody, diluted in HPE-buffer, was added, 50 µl/well. The concentration of detection antibodies are shown in Table 6.

Table 6. Detection antibody concentration.

Antibody	Concentration
IL-6	1 µg/ml
IL-8	1 µg/ml
IL-10	0.25 µg/ml
TNF-α	0.5 µg/ml

The plate was incubated for 1 h, and washed five times with the washing buffer, 100 µl/well. 0.6 µl HRP was added to 6 ml HPE buffer, and 50 µl of the solution was added to each well. The plate was protected from light and incubated for 30 min.

Following this, the plate was washed 5 times with washing buffer. The plate was soaked with the washing buffer for at least 30 sec between each wash steps. The 30 sec soak is important to remove all the excess HRP.

50 µl of substrate solution was added to each well. The plate was protected from light and incubated for 10 min. The reaction was stopped with 2 N H_2SO_4, 50 µl/well. The absorbance was measured at 450 nm using the SPECTROstar^{Nano}. The detection limit for the cytokines were 1.4 pg/ml for IL-6, 2 pg/ml for IL-8, and 4.1 pg/ml for IL-10 and TNF-α.

The data from the plate reader was imported into a computing program, Mars. The deviating values were deleted. The mean values were then imported to a work sheet in excel and the secretion was calculated according to the dilution factors.

4.8 Statistics

Statistics were performed in GraphPad Prism version 5.02 for windows. To test if the data were normally distributed, the Kolmogorov-Smirnov (KS) Normality test was performed. As the vast majority of the data were normally distributed, we assumed that the data was normally distributed. The data were analyzed with one-way Analysis Of Variance (ANOVA), and Dunnets Test was used as a post-hoc test. Statistical significant difference was set to $p < 0.05$ for all analysis. The data is presented as mean ± standard error of the mean (SEM). The figures were created in GraphPad Prism.

5. Results

THP-1 cells and macrophages were stimulated with *L. plantarum* MF1298, NC8 and 299v, *L. reuteri* DSM 20016, DSM 17938 and mm4-1a, *L. rhamnosus* GG, live mutant strains of *L. reuteri* mm4-1a (only THP-1 cells), live *E. coli* K12 (1×10^8 cfu/ml) and LPS (100 and 1000 pg/ml). In addition, basal secretion (cell medium, control) from non-treated cells was also measured. LPS and *E. coli* K12 were used as positive controls because they are known to induce cytokine secretion (Chanput et al. 2010; Parlesak et al. 2004).

Both live and UV-inactivated LAB were included in the experiments as LAB might exert an effect even when they are dead (Kataria et al. 2009). The use of UV-inactivated LAB gives important information about the true effect of the bacteria as problems with bacterial growth and production of metabolites are eliminated.

The effect of selected LAB on the cytokine secretion from THP-1 cells and macrophages were tested and measured with ELISA. The IL-8, IL-10 and TNF-α secretion were measurable in both THP-1 cells and macrophages, while IL-6 was only secreted in measurable amounts by the macrophages. The results are presents in this chapter.

5.1 Pilot experiments to optimize the test system

The experiments were optimized to ensure the right LAB concentration, incubation time, and presence of antibiotics. All pilot experiments were performed with THP-1 cells.

The effects of 4 different LAB concentrations (1×10^4, 1×10^6, 1×10^7 and 1×10^8) were tested in order to find the optimal concentration for stimulation of cytokine secretion (data not shown). Based on the measured cytokine secretion, 1×10^8 cfu/ml was set as the optimal concentration, because lower concentrations did not induce cytokine secretion detectable by the ELISA (data not shown). We did not test higher concentrations of LAB, as that is not a realistic number of bacteria that survives the hostile environment of the human gut (Johansson et al. 1993; Vesa et al. 2000).

Two different incubation times were tested for co-culture between THP-1 cells and LAB: 6 h and 24 h. Based on the results (data not shown) a co-culture time of 6 h were chosen as it gave a good response and TNF-α secretion decreased after 6 h. Furthermore, it is easier to control the bacterial growth and the production of metabolites with short incubation time. An incubation time of 24 h can increase the possibility for secondary effects on the cells, as the

secreted cytokines might bind to receptors on the cells and elicit a stimulating effect (autocrine effect).

In order to prevent uncontrolled growth of the live LAB during co-culture and adverse effects of acid production, antibiotics were used. P/S was first tried out, but some strains still grew (data not shown). In order to inhibit the growth but not kill the live bacteria, gentamicin was tested. Three different concentrations were tested, 50, 100 and 150 µg/ml, and only the highest concentration of gentamicin inhibited the bacterial growth sufficiently (data not shown). The tendency of cytokine secretion was the same with both antibiotics (data not shown).

5.2 Cytokine secretion by THP-1 cells exposed to live lactic acid bacteria

The secretion of IL-8, IL-10 and TNF-α from THP-1 cells exposed to live LAB, *E. coli* K12 and LPS (100 and 1000 pg/ml) for 6 h is shown in Figure 1.

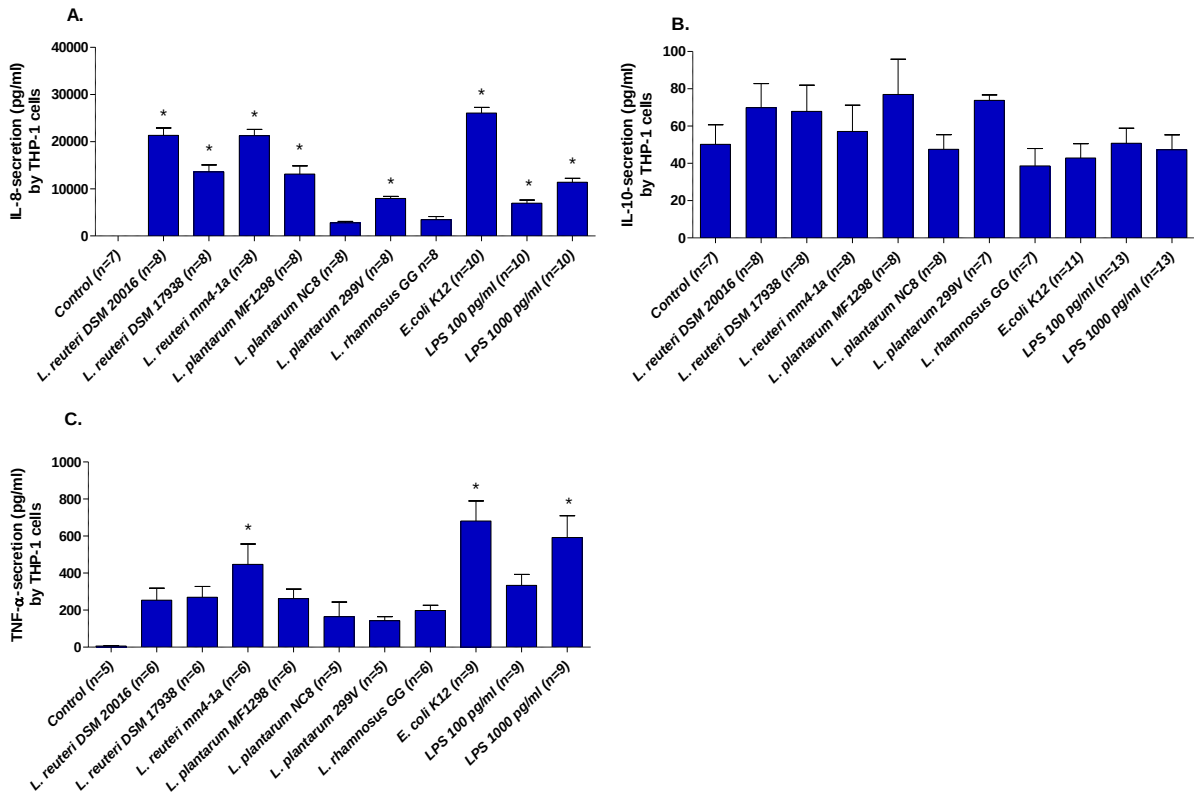

Figure 1. ***Cytokine secretion after stimulation with selected live LAB****. Secretion of IL-8 (A), IL-10 (B) and TNF-α (C) by THP-1 cells after 6 h co-culture with live LAB, E. coli K12, and LPS in original cell culture medium with gentamicin. Data are presented as mean ± SEM from 3-7 experiments performed in duplicate. * indicate $p \leq 0.05$ (ANOVA with Dunnets post hoc test, treatment vs. control).*

The same tendency was observed for IL-8 and TNF-α secretion: *E. coli* K12 stimulated a higher cytokine secretion than the live LAB, and the 1000 pg/ml LPS stimulated higher cytokine secretion than the 100 pg/ml LPS (Fig. 1A and 1C).

All the live LAB, except *L. plantarum* NC8 and *L. rhamnosus* GG, stimulated the THP-1 cells to secrete statistically significant more IL-8 than the basal secretion (Fig. 1A). *E. coli* K12 and LPS (both concentrations) also stimulates the THP-1 cells to secrete statistically significant more IL-8 than the basal secretion. Of the LAB, the strains *L. reuteri* DSM 20016 and mm4-1a stimulated the THP-1 cells to secrete the highest level of IL-8.

There was no statistically significant difference in IL-10 secretion from the THP-1 cells between the live LAB, *E. coli* K12, LPS (both concentrations) and the basal secretion (Fig. 1B).

Of the 7 strains of live LAB, only *L. reuteri* mm4-1a stimulated the THP-1 cells to secrete statistically significant more TNF-α than the basal secretion (Fig. 1C). *E. coli* K12 and 1000 pg/ml LPS also stimulated the THP-1 cells to secrete statistically significant more TNF-α than the basal secretion (Fig. 1C).

5.3 Cytokine secretion by THP-1 cells exposed to UV-inactivated lactic acid bacteria

The secretion of IL-8, IL-10, and TNF-α from THP-1 cells following exposure to UV-inactivated LAB, and LPS 100 and 1000 pg/ml for 6 h is shown in Figure 2.

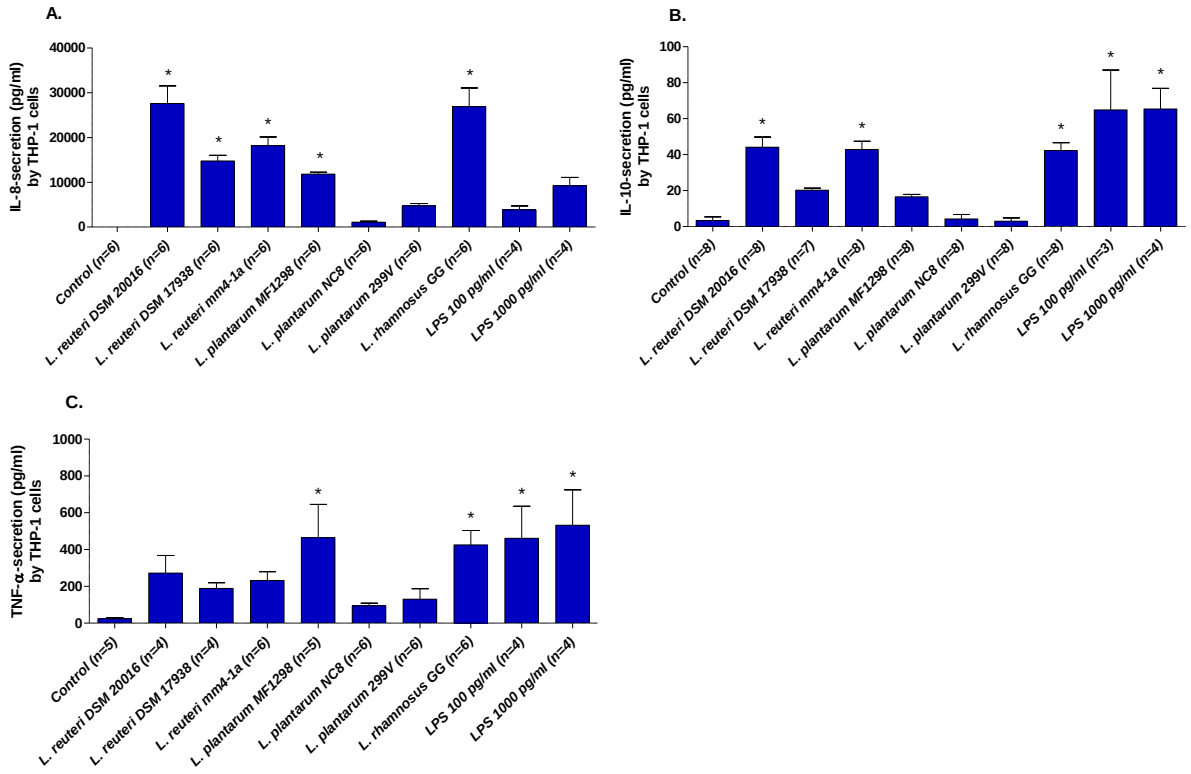

Figure 2. ***Cytokine secretion after stimulation with selected UV-inactivated LAB.*** *Secretion of IL-8 (A), IL-10 (B) and TNF-α (C) by THP-1 cells after 6 h co-culture with UV-inactivated LAB and LPS in original cell culture medium with P/S. Data are presented as mean ± SEM from 2-4 experiments performed in duplicate. * indicate $p \leq 0.05$ (ANOVA with Dunnets post hoc test, treatment vs. control).*

L. rhamnosus GG was the only UV-inactivated strain that stimulated the THP-1 cells to secrete statistically significant higher levels of all the cytokines compared to the basal secretion (Fig. 2A-C).

Except for the strains *L. plantarum* NC8 and 299v, all the UV-inactivated LAB stimulated the THP-1 cells to secrete statistically significant higher levels of IL-8 compared to the basal secretion (Fig. 2A). No statistically significant effect was observed following incubation with LPS (both concentrations).

Figure 2B shows that UV-inactivated *L. reuteri* DSM 20016, mm4-1a, *L. rhamnosus* GG and both concentrations of LPS stimulated the THP-1 cells to secrete statistically significant more IL-10 than the basal secretion. The UV-inactivated *L. plantarum* NC8 and *L. plantarum* 299v resulted in low levels (corresponding to the basal secretion) of IL-10 (Fig. 2B).

The UV-inactivated *L. plantarum* MF1298 and *L. rhamnosus* GG were the only UV-inactivated strains that stimulated the THP-1 cells to secrete statistically significant more

TNF-α than the basal secretion (Fig. 2C). Furthermore, incubation with LPS (both concentrations) stimulated the THP-1 cells to secrete more TNF-α than the basal secretion (Fig. 2C).

5.4 Cytokine secretion by macrophages exposed to live lactic acid bacteria

Figure 3 shows the secretion of IL-6, IL-8, IL-10 and TNF-α from macrophages exposed to live LAB, *E. coli* K12 and LPS (100 and 1000 pg/ml) for 6 h.

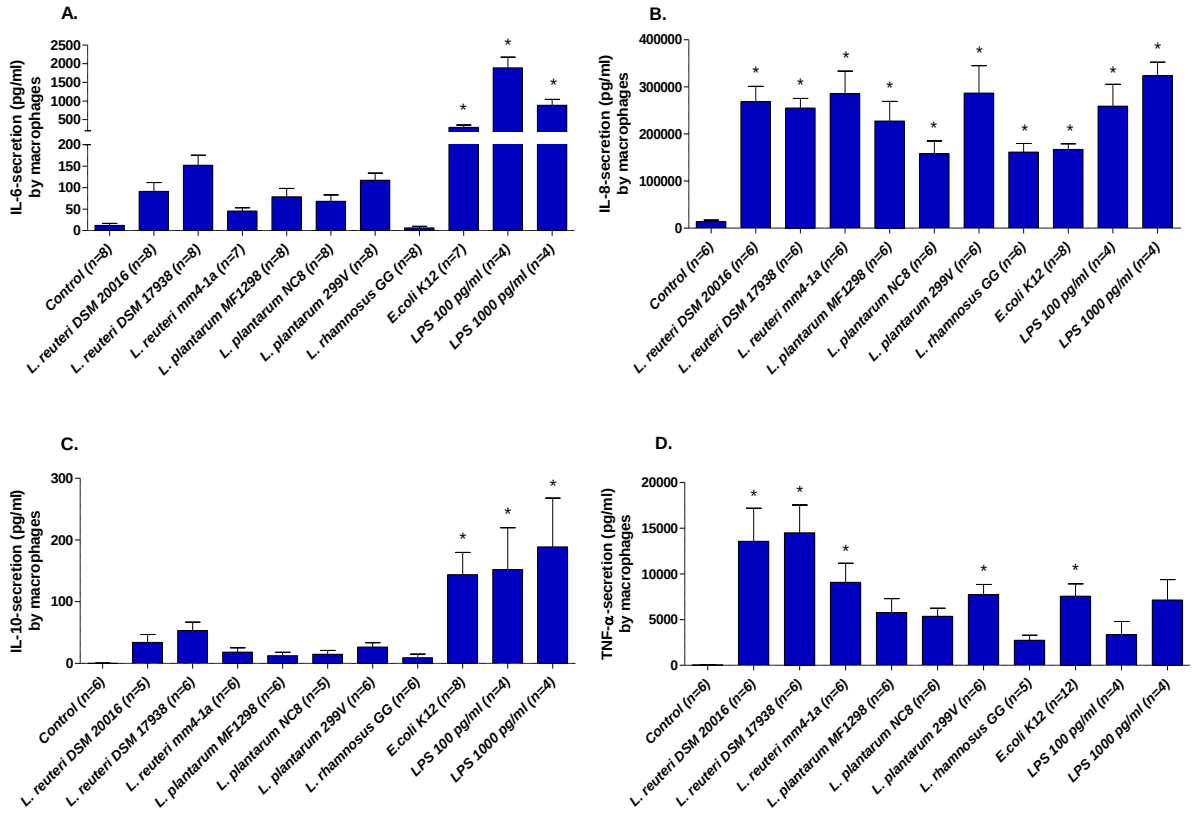

Figure 3. ***Cytokine secretion after stimulation with selected live LAB.*** *Secretion of IL-6 (A), IL-8 (B), IL-10(C) and TNF-α (D) by macrophages after 6 h co-culture with live LAB, E. coli K12 and LPS in original cell culture medium with gentamicin. Data are presented as mean ± SEM from 2-6 experiments performed in duplicate. * indicate $p \leq 0.05$ (ANOVA with Dunnets post hoc test, treatment vs. control).*

LPS and *E. coli* K12 stimulated the macrophages to secrete statistically significant higher levels of all cytokines compared to the basal secretion (Fig. 3A-C).

No statistically significant effect was observed for IL-6 secretion after exposure to live LAB. However, *L. reuteri* DSM 17938 showed a tendency to induce a higher IL-6 secretion than the other LAB (Fig. 3A).

All the live bacteria stimulated the macrophages to secrete statistically significant higher levels of IL-8 than the basal secretion (Fig. 3B). The strains *L. plantarum* NC8, *L. rhamnosus* GG and *E. coli* K12 had the least effect on the IL-8 secretion (Fig. 3B).

Figure 3C shows that none of the live LAB had a statistically significant effect on the IL-10 secretion from the macrophages, but *L. reuteri* DSM 17938 revealed a tendency to stimulate a higher IL-10 secretion than the other live LAB.

The *L. reuteri* strains, *L. plantarum* 299v and *E. coli* K12 stimulated the macrophages to secrete statistically significant higher levels of TNF-α compared to the basal secretion (Fig. 3D). *L. plantarum* MF1298, NC8 and *L. rhamnosus* GG had no statistically significant effect but showed a tendency to induce a higher TNF-α secretion than the basal secretion (Fig. 3D).

5.5 Cytokine secretion by macrophages exposed to UV-inactivated lactic acid bacteria

Figure 4 shows IL-6, IL-8, IL-10, and TNF-α secretion from macrophages after exposure to UV-inactivated LAB and LPS (100 and 1000 pg/ml) for 6 h.

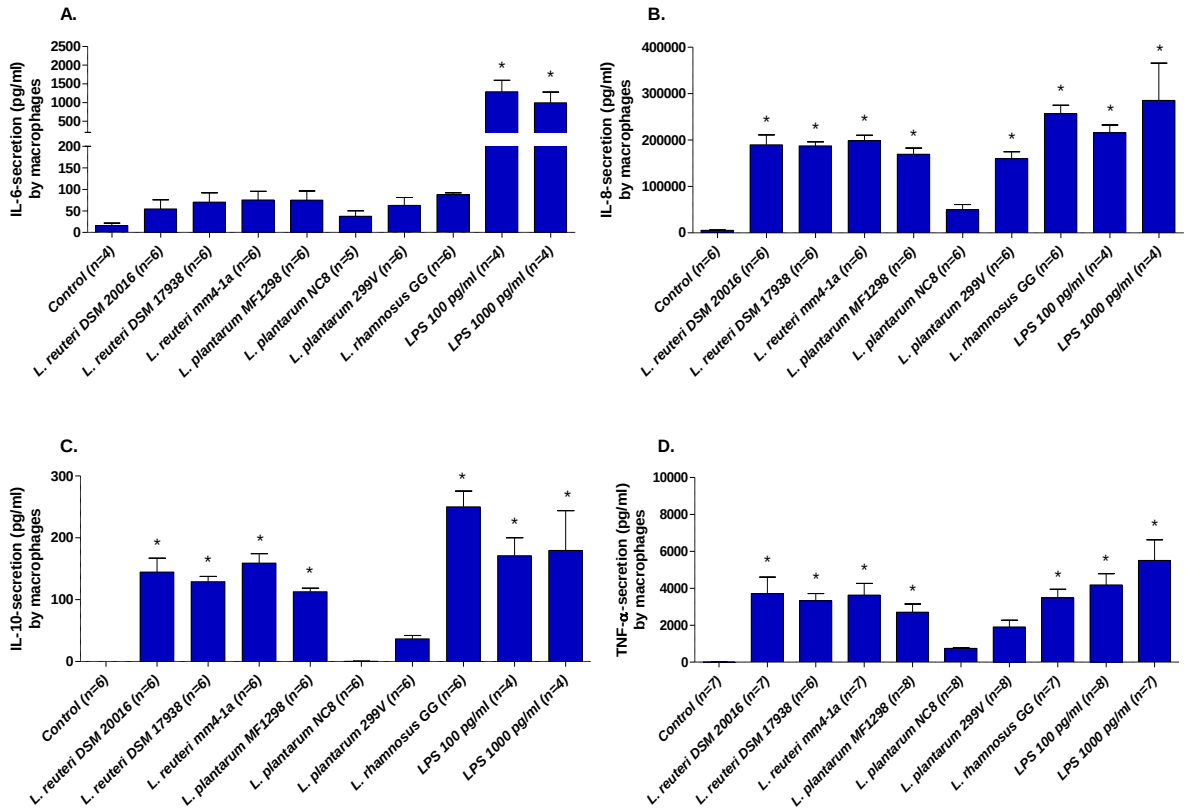

Figure 4. ***Cytokine secretion after stimulation with selected UV-inactivated LAB.*** *Secretion of IL-6 (A), IL-8 (B), IL-10 (C) and TNF-α (D) by macrophages after 6 h co-culture with UV-inactivated LAB and LPS in original cell culture medium with P/S. Data are presented as mean ± SEM from 2-4 experiments performed in duplicate. * indicate $p \leq 0.05$ (ANOVA with Dunnets post hoc test, treatment vs. control).*

Figure 4 shows that LPS (both concentrations) stimulate the macrophages to secrete statistically significant higher levels of all the cytokines compared to the basal secretion. Furthermore, UV-inactivated *L. plantarum* NC8 had hardly any effect on the secretion of the measured cytokines (Fig. 4A-D).

All the UV-inactivated LAB showed a tendency to stimulate to a small increase in the level of IL-6 from the macrophages, none being statistically significant different from the basal secretion (Fig. 4A). However, co-culture with LPS (both concentrations) induced massive IL-6 secretion (Fig. 4A).

All the UV-inactivated LAB, except for *L. plantarum* NC8, stimulated the macrophages to secrete statistically significant more IL-8 than the basal secretion, and at approximately the same levels as LPS (Fig. 4B). *L. plantarum* NC8 induced low levels of IL-8 (Fig. 4B).

All the UV-inactivated LAB, except for *L. plantarum* NC8 and 299v, stimulated the macrophages to secrete statistically significant more IL-10 than the basal secretion. With the exception of *L. rhamnosus* GG, they all stimulated lower secretion of IL-10 than LPS (both concentrations) (Fig. 4C). *L. plantarum* NC8 and 299v stimulated the macrophages to secrete low levels of IL-10 (Fig. 4C).

LPS (both concentrations) and all the UV-inactivated LAB, except *L. plantarum* NC8 and 299v, stimulated the macrophages to secrete statistically significant more TNF-α than the basal secretion, with no major differences between the UV-inactivated strains (Fig. 4D). *L. plantarum* NC8 and 299v induced low levels of TNF-α secretion from macrophages (Fig. 4D). However, they showed a tendency to stimulate the macrophages to produce more TNF-α than the basal secretion (Fig. 4D).

5.6 Cytokine secretion by THP-1 cells exposed to putative surface protein mutant strains of *L. reuteri* mm4-1a

As the levels of cytokine secretion after stimulation with selected live and UV-inactivated LAB correlates with the adhesion properties of the LAB (Jensen et al. 2012), it was desirable to investigate the effect of putative surface proteins on *L. reuteri* mm4-1a on the cytokine secretion from THP-1 cells. The experiments with *L. reuteri* mm4-1a mutants were only performed with THP-1 due to time-shortage.

Figure 5 shows IL-8, IL-10 and TNF-α secretion from THP-1 cells after stimulation with live *L. reuteri* mm4-1a and live mutant strains of *L. reuteri* mm4-1a for 6 h.

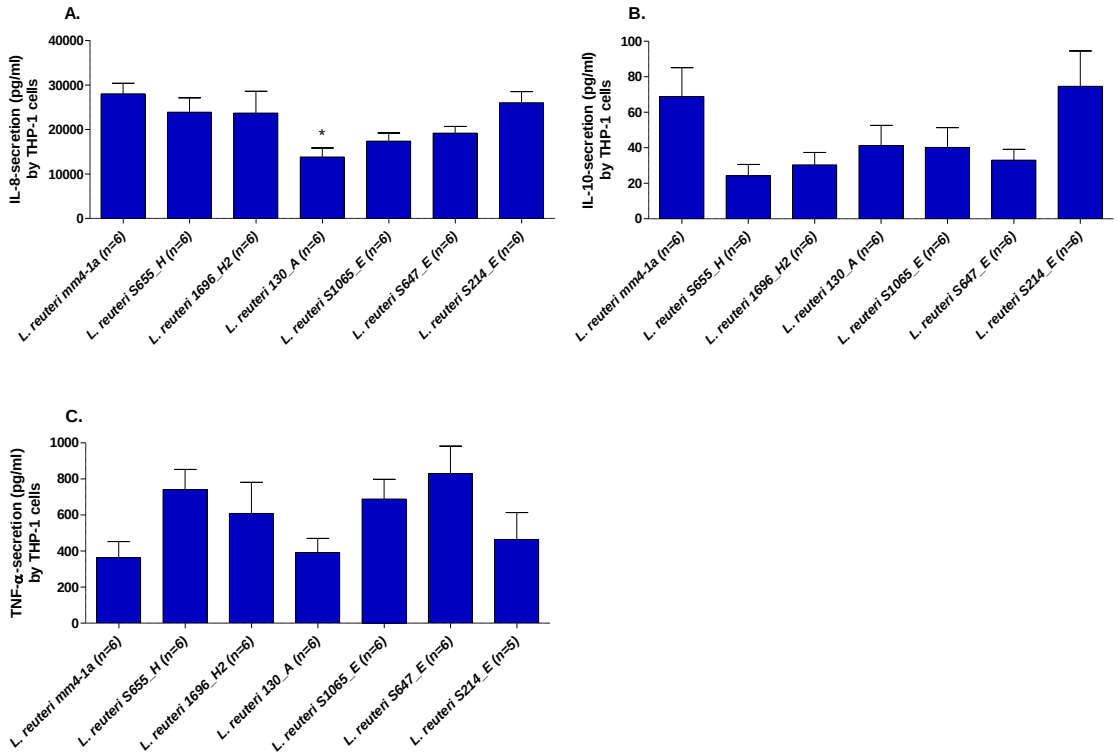

Figure 5. ***Cytokine secretion after stimulation with live mutant strains of L. reuteri mm4-1a and L. reuteri mm4-1a.*** *Secretion of IL-8 (A), IL-10 (B) and TNF-α (C) by THP-1 cells after 6 h co-culture with live mutant strains of L. reuteri mm4-1a and L. reuteri mm4-1a in original cell culture medium with gentamicin. Data are presented as mean ± SEM from 3 experiments performed in duplicate. * indicate $p \leq 0.05$ (ANOVA with Dunnets post hoc test, treatment vs. L. reuteri mm4-1a).*

The only statistically significant difference in Figure 5 was that incubation with *L. reuteri* 130_A resulted in a statistically significant lower secretion of IL-8 compared to the wild type strain *L. reuteri* mm4-1a (Fig. 5A). None of the other mutant strains induced statistically significant differences in secretion of IL-8, compared to the wild type *L. reuteri* mm4-1a (Fig. 5A). However, they revealed a possible tendency to lower secretion of IL-8 from THP-1 cells compared to the wild type strain *L. reuteri* mm4-1a, especially *L. reuteri* S1065_E and S647_E (Fig. 5A).

IL-10 secretion from THP-1 cells was about the same for mutant strain S214_E as for the wild type strain, whereas *L. reuteri* S655_H, 1696_H2, 130_A, S1065_E and S647_E showed a tendency to give a lower secretion of IL-10 from THP-1 cells compared to the wild type strain *L. reuteri* mm4-1a (Fig. B).

The mutant strains *L. reuteri* S655_H, 1696_H2, S1065_E and S647_E showed a tendency to stimulate the THP-1 cells to secrete more TNF-α than the wild type strain *L. reuteri* mm4-1a

(Fig. 5C). On contrary, *L. reuteri* 130_A and S214_E stimulated the THP-1 cells to secrete approximately the same levels of TNF-α as the wild type strain *L. reuteri* mm4-1a (Fig. 5C).

6. Discussion

In this study the focus has been on the immune stimulatory properties of selected Lactobacillus strains. The secretion of IL-6, IL-8, IL-10 and TNF-α from THP-1 cells and THP-1 derived macrophages after exposure to live and UV-inactivated LAB were investigated. The aim of the study was to compare the strains immunomodulatory properties and to compare the effect of live versus UV-inactivated bacteria. Furthermore, the cytokine secretion from THP-1 cells exposed to 6 mutant strains of *L. reuteri* mm4-1a was measured to investigate a possible correlation between putative surface proteins and cytokine secretion. The majority of other *in vitro* studies have used inactivated (e.g. UV, heat) or bacterial products (e.g. conditioned medium, fatty acids) from LAB to measure immunomodulatory properties. This study is one of few which investigate the effect of live LAB.

6.1 The Methods

The THP-1 cell line and macrophages were chosen because they elicit a measurable immune response when exposed to bacteria. THP-1 cells were chosen as they are well known to give similar results as peripheral blood mononuclear cells (PBMC) (Sharif et al. 2007) and are commonly used in the literature (Gonsalves & Kalra 2010; Lund et al. 2004; Zeng et al. 2010).

A test set-up like the one in this study, where one expose live human cell lines to live bacteria will naturally give variance because both the cells and LAB vary from one day to another. Reasons for this can be a variation in the number of both cells and bacteria due to manual procedures. Furthermore, differentiation from THP-1 cells to macrophages may differ from day to day, and well to well, resulting in cell cultures with a mixed state of differentiation. Moreover, the dilution of the supernatants before ELISA, and all the technical steps in the ELISA itself, may vary from experiment to experiment as the methods are hands-on. However, caution was taken to do the procedures as carefully and similar as possible. The ELISA method was adapted in the Nofima laboratory in collaboration with an experienced immunology group, and we trusted the method to be satisfactory. However, it seemed the method for the IL-8 ELISA was more reproducible than the IL-10 and TNF-α ELISA. It may have been beneficial to use some more time in optimizing the dilution of the antibodies for the ELISAs in question.

In general, the macrophages had higher levels of cytokine secretion than the THP-1 cells for IL-8 and TNF-α. A reason for this may be that the PMA used to differentiate THP-1 cells to macrophages induced macrophages to secrete higher levels of cytokines at the basal level. The basal secretion of IL-8 was 13.3 pg/ml from the THP-1 cells versus 8405.8 pg/ml from the macrophages. Similarly, the basal TNF-α secretion was also lower from THP-1 cells (6.1 pg/ml) than from macrophages (58.2 pg/ml). However, the basal secretion of IL-10 was higher from the THP-1 cells (50.2 pg/ml) than from the macrophages (0.3 pg/ml). IL-6 secretion was only measured from macrophages. In future studies it might be beneficial to allow the macrophages to rest a day or two in absence of PMA before experiments, in order to be sure that the PMA have no further effect on the macrophages besides the differentiation.

The co-culture time for the experiments was set to 6 h. Pilot experiments using 24 and 6 h incubation time was performed. In this study 6 h incubation time was chosen to measure the initial response of cytokines. If the incubation time had been longer the cells may have been affected by the cytokines secreted and ignite a response to the cytokines and not to the LAB. Chanput et al. (2010) show that from THP-1 cells stimulated with LPS the secretion of TNF-α and IL-10 peaked at 6 h, whereas the secretion of IL-6 and IL-8 kept on rising until 30 h after stimulation. In the same study, macrophages stimulated with LPS revealed increasing secretion of IL-6, IL-8 and IL-10 up to 18 h, while the secretion of TNF-α peaked at 6 h (Chanput et al. 2010).

Many studies have induced an inflammation response in THP-1 cells, in order to investigate whether LAB (or metabolites of LAB) have an inhibitory effect on the inflammation response (Jones et al. 2011; Kim et al. 2007; Kim et al. 2006; Lin et al. 2008; Thomas et al. 2012). In the current study, stimulating the THP-1 cells and macrophages by adding LPS 30 min after addition of LAB was tried out. However, the cytokine secretion from these experiments was high, and no differences between the strains could be detected (data not shown).

In this study, inactivation of LAB by UV radiation was chosen, as it is a more gentle way to inactivate the LAB thereby keeping the surface molecules intact (that may be degraded if heat is used). Stimulation with UV-inactivated bacteria will reveal whether the bacterium itself or the metabolites produced by the live bacterium is responsible for the induced cytokine secretion.

The pilot experiments and initial experiments were performed with UV-inactivated LAB, in the presence of the antibiotics P/S. During the initial experiments with live LAB problems

with growth of bacteria for some strains were experienced, killing or suppressing the viability of the human cells. To overcome this problem gentamicin was used as antibiotics in the cell medium during co-culture with live LAB. Gentamicin is commonly used in similar studies (Gaudana et al. 2010; Pathmakanthan et al. 2004). Interestingly, the use of gentamicin did not affect the cytokine secretion compared to P/S. Due to time constraints, the experiments initially performed with UV-inactivated bacteria and P/S were not repeated with gentamicin.

6.2 Cytokine secretion from THP-1 cells and macrophages

The main findings from co-cultures of live LAB with THP-1 cells or macrophages were that the three *L. reuteri* strains stimulated a high secretion of the cytokines compared to the other strains, while *L. rhamnosus* GG and *L. plantarum* NC8 induced low cytokine secretion compared to the other strains. In addition, the results from this study showed that bacteria belonging to the same species show the same tendencies in cytokine response. In general, not many studies are done with live LAB: Most are performed with inactivated or just metabolites of LAB. Furthermore, investigating the immune response *in vitro*, many studies have been performed with *L. rhamnosus* GG, some with *L. plantarum* 299v, while only a few have investigated the effect of L. *reuteri* DSM 17938.

Lactobacillus reuteri DSM 17938 is a commercially available probiotic bacterium which has been extensively tested in clinical trials and *in vivo* (see introduction for details). However, relatively few studies investigating the immune stimulatory properties of *L. reuteri* strains *in vitro* have been performed. In the present study, *L. reuteri* strains induced overall higher levels of secreted cytokines compared to the other strains investigated. Interestingly, *L. reuteri* strains have shown superior properties compared to other strains in *in vitro* experiments, such as good ability to survive gastric and intestinal juices *in vitro* (Jensen et al. 2012), to adhere to IEC *in vitro* (Christoffersen et al. 2012; Jensen et al. 2012; Wang et al. 2008), and to possibly strengthen the epithelial barrier in vitro (Jensen et al. 2012).

In general, the secreted levels of IL-6, IL-8 and TNF-α were higher than the levels of secreted IL-10. This can probably be explained by the nature of the cytokines: IL-6, IL-8 and TNF-α are pro-inflammatory cytokines and will be secreted early in the immune response, whereas IL-10 is an anti-inflammatory cytokine that will be secreted later in the immune response. This may explain the low levels of IL-10 that was measured after 6 h.

It has been demonstrated that immune stimulation in the gut from commensal bacteria is advantageous for the development of important gut defenses (Rakoff-Nahoum et al. 2004). Furthermore, it is obvious that LAB should not elicit as strong immune response as the positive controls LPS and *E. coli* K12 used in this study. In the current study, LPS (both concentrations) induced statistically significant higher secretion of IL-6 compared to the control, and compared to the LAB (both live and UV-inactivated) the LPS induced a much higher IL-6 secretion. The same was the case for *E. coli* K12. The secretion of TNF-α from THP-1 cells induced by the positive controls was overall higher than the TNF-α secretion induced by the LAB. The secretion of TNF-α from the macrophages showed overall no big difference between the secretion induced by the LAB and *E. coli* K12 and LPS. There were no big differences between the LAB and the positive controls regarding the secretion of IL-8 and IL-10. IL-10 secretion from macrophages stimulated with *E. coli* K12 and LPS induced statistically significant higher secretion from the macrophages compared to the basal secretion.

6.2.1 Cytokine secretion from THP-1 cells and macrophages incubated with live lactic acid bacteria

The immune stimulatory properties of live LAB were mostly the same in THP-1 cells and macrophages. The main difference was that the *L. reuteri* DSM 20016 and DSM 17938 and *L. plantarum* 299v induced lower TNF-α secretion in THP-1 cells compared to the macrophages.

This study has shown that all the live LAB induced statistically significant higher levels of IL-8 from the macrophages compared to the basal secretion. Furthermore, all the live strains induced statistically significant higher IL-8 secretion from THP-1 cells compared to the basal secretion, except for *L. plantarum* NC8 and *L. rhamnosus* GG. Not many studies have been performed to investigate the immune effect of live bacteria on cell lines. Zhang et al. (2005)showed that Caco-2 cells exposed to a high dose (1×10^7 cfu/ml) of live *L. rhamnosus* GG induced a higher secretion of IL-8 than the control (cells exposed to cell culture medium), while lower doses did not induce any difference in the secretion of IL-8 compared to the control. Vizoso Pinto et al. (2007) observed that live *L. rhamnosus* GG down-regulated IL-8 cytokine secretion, whereas two *L. plantarum* strains induced increased secretion of IL-8 from HT-29 cells compared to control (HT-29 cells incubated with only cell culture medium). Furthermore, the IL-6, IL-10 and TNF-α secretion could not be determined in the supernatants from the cells treated with the live bacteria (Vizoso Pinto et al. 2007). On contrary, another

study done by Vizoso Pinto et al. (2009) on HT-29 cells treated with live *L. rhamnosus* GG, show that the bacterium did not induce statistically significant higher secretion of IL-8 compared to the control (cells exposed to cell culture medium).

In the current study, THP-1 cells and macrophages stimulated with live LAB did not have statistically significant different IL-10 secretion compared to the basal secretion, and there were no major differences between the strains. The findings in the current study correlates with a study conducted with live *L. rhamnosus* GG and DCs which demonstrated no statistically significant effect of the bacterium on the secretion of IL-10 (Elmadfa et al. 2010). On contrary, Kim et al. (2006) found elevated levels of IL-10 from mouse bone marrow-derived immortalized macrophages (BMDIM) after stimulation with *L. rhamnosus* GG, compared to the control (cell culture medium).

In this study live *L. rhamnosus* GG induced the lowest TNF-α secretion from macrophages compared to the other LAB, although still higher than the basal secretion. Another study also demonstrated that live *L. rhamnosus* GG in comparison to other bacteria was a poor inducer of TNF-α secretion in a BMDIM (Kim et al. 2006). The positive control *E. coli* K12 induced statistically significant higher TNF-α secretion from macrophages compared to the basal secretion in the current study. Kim et al. (2006) demonstrated the same, as BMDIM exposed to a live strain of *E. coli* secreted much higher levels of TNF-α compared to the control (cell medium).

The difference between the results from the current study and other studies could be due to the use of different cell lines, and differences between laboratory practice for growing both bacteria and human cell lines. In addition, both bacteria and human cells may change properties over time during culture in growth medium. Furthermore, different concentrations of bacteria and human cells may also be factors contributing to different results.

6.2.2 Cytokine secretion from THP-1 cells and macrophages incubated with UV-inactivated lactic acid bacteria

As reviewed by Kataria et al. (2009) probiotic LAB may be effective even as heat-killed, UV-inactivated or fragmented. The results from the current study revealed no major differences between live bacteria and UV-inactivated bacteria in their ability to induce cytokine production in THP-1 cells/macrophages, with the exception of *L. rhamnosus* GG. UV-

inactivated *L. rhamnosus* GG induced higher cytokine secretion compared to live *L. rhamnosus* GG. The reason for this is unknown, and needs further investigations.

The results in this study showed that with the exception of IL-8 secretion from macrophages, the UV-inactivated *L. plantarum* 299v did not induce the cells to secrete statistically significant more cytokines than the basal secretion. Contrary to this, Christensen et al. (2002) showed that in murine DCs exposed to inactivated *L. plantarum* 299v, the IL-6, IL-10 and TNF-α secretion was higher than in control cells (cell culture medium).

In the present study, UV-inactivated *L. rhamnosus* GG gave statistically significant higher IL-8 secretion compared to basal secretion when tested on both THP-1 cells and macrophages. However, Zhang et al. (2005) found that Caco-2 cells treated with heat-killed *L. rhamnosus* GG secreted slightly elevated levels of IL-8 compared to control cells (cell culture medium). Furthermore, another study done on HT-29 cells demonstrated that heat-killed *L. rhamnosus* GG induced the cells to secrete statistically significant less IL-8 than the control (Wallace et al. 2003).

In the current study UV-inactivated *L. rhamnosus* GG stimulated the THP-1 cells and macrophages to secrete statistically significant higher levels of IL-10 compared to the basal secretion. These results are in accordance with the results from another study where mononuclear cell cultures (from blood donors) exposed to two strains of UV-inactivated *L. rhamnosus* secreted statistically significant higher levels of IL-10 compared to the control (cell culture medium) (Hessle et al. 1999). Furthermore, the results from the current study showed that UV-inactivated *L. plantarum* strains overall induced low levels of IL-10. Hessle et al. (1999) also found that two strains of *L. plantarum* stimulated mononuclear cell cultures to secrete relatively low levels of IL-10 compared to the other strains. The UV-inactivated *L. plantarum* strains in the current study did not stimulate the THP-1 cells to secrete statistically significant more IL-10 than the basal secretion. A study done with a heat-killed *L. plantarum* strain on THP-1 cells also demonstrated the same (Kim et al. 2007). While THP-1 and macrophages with UV-inactivated *L. plantarum* 299v in the current study induced only low levels of IL-10, Christensen et al. (2002) demonstrated high levels of IL-10 secretion from DCs stimulated with heat-killed *L. plantarum* 299v compared to the control (cell culture medium).

UV-inactivated *L. rhamnosus* GG induced statistically significant higher levels of TNF-α than the basal secretion in both cell-types in the current study. The results from this study were

consistent with the results from another study done with a macrophage cell-line (RAW 264.7) where heat-killed *L. rhamnosus* GG stimulated to a lot higher secretion of TNF-α than the control (cell culture medium) (Lin et al. 2011). The current study shows that the three UV-inactivated *L. reuteri* strains induced statistical significantly higher levels of TNF-α than the basal secretion, from the macrophages. The findings from this study is in accordance with a study done with macrophages from mice, where it was shown that a strain of gamma irradiated *L. reuteri* induced a lot higher secretion of TNF-α compared to the control (non-stimulated macrophages) (Marcinkiewicz et al. 2007). In the current study the UV-inactivated *L. plantarum* strains stimulated the cells to secrete different amounts of TNF-α. *L. plantarum* MF1298 stimulated the cells to secrete statistically significant higher levels of TNF-α than the basal secretion, whereas *L. plantarum* NC8 and 299v did not. *L. plantarum* MF1298 has previously shown promising probiotic properties *in vitro* compared to other *L. plantarum* strains (Klingberg et al. 2005).

It was an aim to compare immune stimulation by UV-inactivated and live LAB, as it might lead to clues regarding the mechanisms for the immunological effects. The immunological effects from live LAB may be due to secreted metabolites, and surface proteins, whereas the immunological effects from UV-inactivated LAB may be caused by surface proteins as they are preserved in the process. The results did not show big differences between the effects from live LAB versus UV-inactivated LAB, indicating that the bacteria themselves and not their metabolites are responsible for the observed immune response.

6.3 The effect of putative bacterial surface proteins on cytokine secretion

The initial results with THP-1 cells and macrophages revealed overall a high cytokine secretion after stimulation with *L. reuteri* strains and a low level of cytokine secretion after stimulation with *L. plantarum* strains. It was recently shown that *L. reuteri* strains possess a very good adhesion capacity to IEC, whereas *L. plantarum* strains adheres poorly (Jensen et al. 2012). To investigate a possible connection between putative bacterial surface proteins and cytokine secretion, 6 *L. reuteri* mm4-1a mutants were studied: 4 LPXTG mutant strains, 1 C-terminal membrane anchor protein mutant strain and 1 sortase mutant strain. All mutant strains are knock-out mutants where the targeted proteins are not expressed, thus they are all putative surface protein mutants.

One of the LPXTG mutant strains (S214_E) has an almost complete loss of adhesion capacity to IEC (unpublished results). However, the results showed that the mutant strain S214_E lead

to approximately the same amount of secreted cytokines from THP-1 cells as the wild type strain, indicating that the surface protein that is important for adhesion to IEC is not important for the ability to induce cytokine secretion from THP-1 cells. However, it is likely that the mechanism for cytokine induction is different in IEC compared to THP-1 cells. Thus, the immune stimulatory effect of mutant strain S214_E should be tested on intestinal epithelial cell lines.

L. reuteri mm4-1a 130_A, the sortase mutant, induces statistically significant lower IL-8 secretion from THP-1 cells compared to the wild type. This indicates that sortase activity might be important for the induction of IL-8. The mechanism for this is unknown and should be further investigated.

7 Conclusion

The aims for this thesis were to compare selected strains of LAB in their ability to modulate the immune response of THP-1 cells and macrophages, to compare the effects of live versus UV-inactivated LAB, and to investigate the effect of putative bacterial surface proteins on the cytokine secretion from THP-1 cells.

The selected LAB strains induce different cytokine profiles, as measured in THP-1 and macrophages. The *L. reuteri* strains induced an overall higher cytokine secretion than the other strains investigated, whereas *L. plantarum* NC8 and live *L. rhamnosus* GG induced low cytokine secretion. Most of the UV-inactivated LAB had the same immune stimulatory capacity as the corresponding live bacteria, indicating that it is the bacteria themselves and not their metabolites that are responsible for the observed immune response. Interestingly, the UV-inactivated *L. rhamnosus* GG induced high levels of secreted cytokines compared to live *L. rhamnosus* GG. The reason for this difference is unknown and needs further testing.

Of the putative bacterial surface proteins tested, a protein which is essential for adhesion to IEC was not important for the cytokine secretion from THP-1 cells. However, sortase activity showed a possible importance for the induction of IL-8 secretion.

8 Future perspectives

The effect of live versus UV-inactivated *L. rhamnosus* GG on cytokine secretion should be further investigated as they gave different immune responses. Other methods for inactivating the LAB (eg. heat, other types of radiation) should be tested, as different methods may affect the LAB differently. Heat treatment may denaturize surface proteins on the LAB, while UV-inactivation does not affect the surface proteins this way. The different treatments may affect how the LAB are perceived by the cells of the immune system.

Furthermore, it would be interesting to test the effects of the LAB on peripheral blood mononuclear cells (PBMC) or on intestinal epithelial cell lines (like Caco-2) to investigate whether the cytokine secretion are comparable to the results obtained in this thesis. Testing the effects of the LAB on a sandwich system consisting of Caco-2 cells and PBMC for immune responses should also be performed as it would be more similar to the *in vivo* situation.

In addition, investigating more mutant strains of LAB with regard to cytokine secretion and putative surface proteins would also be interesting, as this could reveal the mechanisms for the immune stimulatory effect of probiotics.

9 References

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