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Probiotic lactobacilli in experimental persistent *Salmonella* infection

UNIVERSITY OF TARTU
PRESS

Department of Microbiology, University of Tartu, Estonia

Dissertation has been accepted for the commencement of the degree of Doctor of Philosophiae on January 16th, 2013 by the Council of the Faculty of Medicine, University of Tartu, Estonia

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Commencement: April 5th, 2013

Publication of this dissertation is granted by University of Tartu

This research was supported by the European Regional Development Fund and

ISSN 1024–395X
ISBN 978–9949–32–233–6 (print)
ISBN 978–9949–32–234–3 (pdf)

University of Tartu Press
www.tyk.ee
Order No. 55

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LIST OF ORIGINAL PUBLICATIONS

1. **Truusalu K**; Naaber P; Kullisaar T; Tamm H; Mikelsaar R; Zilmer K; Rehema A; Zilmer M; Mikelsaar M. The influence of antibacterial and antioxidative probiotic lactobacilli on gut mucosa in a mouse model of Salmonella infection. Microbial Ecology in Health and Disease. 2004 16JJ:180–187.
2. **Truusalu K**, Mikelsaar RH, Naaber P, Karki T, Kullisaar T, Zilmer M, Mikelsaar M. Eradication of *Salmonella* Typhimurium infection in a murine model of typhoid fever with the combination of probiotic *Lactobacillus fermentum* ME-3 and ofloxacin. BMC Microbiol. 2008 Aug 4; 8:132.
3. **Truusalu K**, Kullisaar T, Hütt P, Mahlapuu R, Aunapuu M, Arend A, Zilmer M, Mikelsaar RH, Mikelsaar M. Immunological, antioxidative, and morphological response in combined treatment of ofloxacin and *Lactobacillus fermentum* ME-3 probiotic in *Salmonella* Typhimurium murine model. APMIS. 2010 Nov;11, 8:864–72.
4. Mikelsaar M, Songisepp E, Smidt I, Stsepetova J, Zilmer M, Hütt P, **Truusalu K**, Kilk K. Isolated *Lactobacillus plantarum* strain Inducia DSM 21379 as probiotic that enhances natural immunity and food products and medicinal preparations comprising it. Priority date 13.05.2008 EE 200800027. Estonian Patent EE 05341; European patent EP 2288 360 B1.

Dissertant's contribution:
In paper I: performed animal studies and microbiological tests, participated in preparation of the manuscript.
In papers II and III: attended in designing the studies, performed animal studies, microbiological tests, data analysis and wrote the papers.
In paper IV: performed the animal study.

ABBREVIATIONS

API CHL50	Analytical Profile Index of 50 Carbohydrates for *Lactobacillus*
CFU	colony forming unit
CD	cluster of differentiation
CWDB	cell wall deficient bacteria
DC	dendritic cell
DNA	desoxyribonucleinic acid
DSM	Deutsche Sammlung von Mikroorganismen und Zellkulturen
E-test	ellipsoid test
FAE	follicle associated epithelia
FAO	Food and Agriculture Organization of the United Nations
FHEL	facultatively heterofermentative lactobacilli
GALT	gut associated lymphoid tissue
GSH	reduced glutathione
GSSG	oxidised glutathione
IEC	intestinal epithelial cells
IL	interleukin
INF-γ	interferon gamma
LAB	lactic acid bacteria
LA-test	linolenic acid test
LPO	lipid peroxidation
LPS	lipopolysaccharide
MDA	malondialdehyde
MHC	major histocompatibility complex
MRS	de Man-Rogosa-Sharpe agar
NADPH	reduced nicotineamide dinucloetide phosphate
NCCLS	National Committe for Clinical Laboratory Standards
NLR	Nucleotide Oligomerization Domain receptors
OFL	ofloxacin
OHEL	obligately homofermentative lactobacilli
OHOL	obligately heterofermentative lactobacilli
OR	odds ratio
OxS	oxidative stress
PAMP	pathogen associated molecular pattern
PBS	phosphate buffered saline
PP	Peyer`s patches
PRR	pattern recognition receptor
PUFA	polyunsaturated fatty acid
ROS	reactive oxygen species
RNS	reactive nitrogen species
RS	reactive species
sIgA	secretory immunoglobulin A

SseI	protein secreted by *S*. Typhi
Sp.	species
TAA	total antioxidative activity
TAS	total antioxidative status
TLR	toll-like receptor
TNF-α	tumor necrosis factor alpha
XLD	xylose lysine deoxylate
WHO	World Health Organisation

1. INTRODUCTION

Infection includes several bacteriological, morphological, biochemical, and immunological processes between pathogenic bacteria, host, and commensal bacteria.

Pathogenic bacteria are defined by their inherent ability to cross anatomical barriers due to specific virulence factors, inhabit tissue sites, breach host defences that ordinarily limit the survival, or replication of commensal bacteria (Falkow, 2006). The distinction between the pathogen and the commensal is not always easy as some commensals may cause disease in certain conditions, whereas some pathogens can persist without any disease symptoms.

In the majority of infections the interaction between the bacteria and the host`s immune system eradicates the invading bacteria. If some pathogens survive, they cause persistent infection (Young *et al*., 2002¸ Rhen *et al*., 2003). This may lead to recurring infections with high epidemiological burden and/or morbidity of patients or carriage of the particular microbe.

Salmonella Typhi causes enteric fever in many parts of the developing world, especially in Asia and North Africa (Andrews-Polymenis *et al*., 2010), despite the decline in the overall incidence of typhoid fever due to a specific typhoid fever control program, economic development, and improved sanitation.

In treatment of infectious diseases, the discovery of antibiotics more than 70 years ago significantly increased the survival of patients suffering from bacterial infections. Today we have met problems concerning antibiotic resistance, *e.g.* multidrug resistance and resistance to nalidixic acid and fluoroquinolone among *Salmonella* serotypes responsible for typhoid fever have been reported (Chau *et al*., 2007). Until recently it was evident that the antibiotic resistance is due to overuse of antibiotics that enables the migration of resistance-carring plasmids and integrons. However, D'Costa and colleagues demonstrated with targeted metagenomic analyses of ancient DNA from 30000-year-old Beringian permafrost sediments that antibiotic resistance is a natural phenomenon that predates use of antibiotics (D'Costa *et al*., 2011). Though antibiotic treatment is targeted against bacterial pathogens it also alters the gut microbial ecology, resulting in selective removal of commensals and changes in the colonization resistance. The antibiotic-induced disturbances in microbiota composition are mostly temporary, returning to its original composition within 2 months (Gerritsen *et al*., 2011). Administration of indigenous non-pathogenic bacteria is an option for restoring the colonization resistance and microbiota composition.

Lactobacillus spp. strains belong to the category of organisms classified as generally regarded as safe for food and medical application (FAO/WHO, 2002). Probiotics are defined as live microorganisms which, when administered in adequate numbers result in a health benefit for the host. In clinical trials the consumption of probiotic food containing different lactic acid bacteria has shown several scientifically established and/or clinically proven health effects in preventing particular infections and non-infectious disorders (Minocha, 2009; Kullisaar *et al*., 2003; Songisepp *et al*., 2004; Mikelsaar, Zilmer *et al*., 2009; Floch *et al*., 2011). However, there are few studies showing the mechanisms of the beneficial effect of different probiotics in experimental *Salmonella* infections (Ashara *et al.,* 2010).

We have developed a mouse model of persistent *Salmonella* Typhimurium infection with granulomas that resembles *Salmonella* Typhi infection in humans. Our aim was to test the microbiological, morphological, biochemical and immunological effect of the probiotic *Lactobacillus fermentum* ME-3 on the *Salmonella* Typhimurium persistent infection and to clarify the impact of the probiotic *Lactobacillus plantarum* Inducia in murine model.

2. REVIEW OF LITERATURE

2.1. Essence of persistent infection

Persistent infection is a specific phase in the pathogenesis of infection rather than a fortuitous imbalance in the host–pathogen interaction (Rhen *et al.*, 2003). It represents the evolved selection for balancing host and microbial interests, resulting in an equilibrium that is long-term but not necessarily stable forever (Blazer *et al.*, 2007). From the microbial perspective, persistent infection is essential for microbial survival in nature (Monack, 2012). Some pathogenic bacteria are capable of maintaining infections in mammalian hosts even in the presence of inflammation, specific antimicrobial mechanisms, and adaptive immune response giving rise to persistent infection. The persistent infection can manifest as acute or chronic disease or can be clinically asymptomatic with the potential to be reactivated. For instance, *Salmonella* Typhi causes systemic typhoid fever that involves the colonization of the reticuloendothelial system (Monack *et al.,* 2004).

2.1.1. Persisters

The essence of persistent infection may be associated with persister cells. Despite the early discovery of persisters by Bigger already in 1944, several aspects of persisters are still obscure.

Persisters are dormant variants of regular cells that form stochastically in microbial populations and are highly tolerant to antibiotics (Dorr *et al.*, 2009, Lewis, 2010). Dormant cells have a global slowdown of metabolic processes and do not divide (Jayaraman and Wood, 2008). The production of persisters depends on growth stage: it starts in the early exponential, increases in the mid-exponential and reaches a maximum of 1% of cells in the stationary phase (Keren *et al.*, 2004). Persisters pre-exist in microbial population prior to the addition of antibiotics (Balaban *et al.*, 2004).

Persister cells constitute a small fraction of the population. They are transiently refractory to killing, without having acquired resistance through genetic modification (Keren *et al.*, 2004; Fauvart *et al.*, 2011). The microbes that survive antibiotic treatment are able to give rise to a population sensitive to treatment, and the next microbial population again contains a small proportion of persister cells. This discriminates persister cells from resistant mutants. While resistant mutants are stable with inheritable properties, persistence is a reversible microbial phenomenon (Fauvart *et al.*, 2011).

2.1.2. CWDB and L-forms

Under certain conditions bacteria can spontaneously or by induction, lose part or all of their cell wall resulting in osmosis-sensitive cells known as cell wall deficient bacteria (CWDB). CWDB-s that are capable of specific “fried egg” growth on specialized solid media are termed L-forms (Elliott and Lambert, 2001).

Despite the discovery of L-form bacteria already in 1935, the molecular mechanisms underlying L-form formation and survival have remained obscure. L-form formation and survival is associated with pathways and genes involved in cell envelope stress, DNA repair, iron homeostasis, outer membrane biogenesis, and drug efflux/ABC transporters (Glover *et al.*, 2009). These findings suggest a relationship between L-forms and persisters. It has been suggested that the usage of antibiotics may generate CWDB. For instance, beta-lactams and glycopeptides damage bacteria by inhibiting cell wall murein synthesis and CWDB are generated before the bacteria die. They have an increased ability to uptake DNA by transformation (Woo *et al.*, 2003; Onwuamaegbu *et al.*, 2005; Allan *et al.*, 2009).

2.2. Persistence and the immune response

The location of bacteria inside the host cell during persistent infection is crucial for its success as a pathogen. Intracellular compartment is relatively safe, if bacteria can avoid phagolysosomal fusion as it protects bacteria from immune recognition and serum mediated extracellular killing by the host. It has been demonstrated earlier that although majority of the bacteria are intracellular in *Salmonella* persistence model of mice, some bacteria may still be located in the extracellular compartment (Eswarappa, 2009). Persistent microbes have successful strategies to thwart host responses sufficiently to gain a niche. Many microbial adaptations have been recognized, involving stealth, antigenic variation, and immune response. The known mechanisms of persistence in different microbes may vary, *e.g.* in *S.* Typhi infection the low expression of stimuli for innate responses and in *Escherichia coli* K1 the molecular mimicry have been described (Rhen *et al.*, 2003).

Moreover, in persistent infections the modifications of intra-vacular environment of the cell through reduced surface antigen presentation or the control of apoptotic pathways are involved. Some bacteria can modify the structure of Toll-like receptors (TLR) binding components, for example, *Salmonella* modify their lipid A composition under different growth conditions (Rhen *et al.*, 2003). This seemingly complicates the recognition of the pathogen and its clearance by immune mechanisms. Recently it was demonstrated that to subvert the immune system *Salmonella* secrets the protein SseI into dendritic cells (DC), which normally play a role in immune activation (Ruby and Monack, 2011). The bacterial protein interferes with the migration of infected cells to lymphoid tissues by specifically binding to the cell-migration regulator. This prevents normal DC migration, limits presentation of *Salmonella* antigens and naive T-cell priming, and thereby inhibits adaptive immunity (McLaughlin *et al.*, 2009).

2.3. Infections due to *S.* Typhi and *S.* Typhimurium

2.3.1. Taxonomy

Salmonella is a genus of the family *Enterobacteriaceae.* According to contemporary classification, the genus *Salmonella* contains only two species, *Salmonella bongori* and *Salmonella enterica,* but there are more than 2,500 serotypes of *S. enterica* due to diverse surface structures of antigens: somatic O antigens, the carbohydrate component of lipopolysaccharide (LPS), and flagellar H antigens (Andrews-Polymenis *et al.*, 2010). According to the current CDC nomenclature system, the full taxonomic designation of *Salmonella enterica* subspecies *enterica* serotype Typhimurium can be abbreviated as *Salmonella* Typhimurium and similarly *S.* Typhi.

A major virulence factor of *Salmonella enterica* is LPS, and strains with reduced LPS expression show poor growth under stress conditions and express lowered virulence (Netea *et al.*, 2009). *Salmonella* sp. can modify their lipid A composition under different growth conditions (Rhen *et al.*, 2003), thus changing the structure of TLR binding components and becoming unrecognizable by innate immune mechanisms.

Different serotypes of *Salmonella enterica* are responsible for human diseases ranging from gastroenteritis to systemic infections.

2.3.2. Pathogenesis of *S.* Typhi infection

S. enterica Typhi, the etiologic agent of typhoid fever, infects only humans. After oral ingestion of a facultative intracellular *S.* Typhi, it enters the host through microfold (M) cells, which are specialized epithelial cells that sample intestinal antigens and transport them to lymphoid cells in the underlying Peyer's patches (PP), specialized lymphoid tissue in the small intestine (Jones *et al.*, 1994). After penetration through the epithelial barrier, the phagocytes in lamina propria ingest *S.* Typhi. In order for the infection to extend beyond the intestinal mucosa, facultatively intracellular *S.* Typhi is able to survive and replicate in macrophages and thus elude the adaptive immune response (Haraga *et al.,* 2008). *S.* Typhi pathogenity island 7 encodes functions for the production and export of the Vi-capsular polysaccharide antigen. The latter is important in evading detection of *S.* Typhi by TLR-4 (Monack 2012). Tissue DCs take up microbial antigens; migrate to regional lymph nodes, and present processed microbial antigens to naïve CD4 T cells. *S.* Typhi secretes protein SseI into DCs (Ruby and Monack 2011). This prevents normal dendritic-cell migration, limits presentation of Salmonella antigens and naive T-cell priming, and thereby inhibits adaptive immunity (McLaughlin *et al.*, 2009).

The activated CD4 T cells leave the lymph node and migrate to the focus of the infection, secreting soluble mediators. At the same time, inflammatory phagocytes are attracted to the site of microbial invasion in a process mediated by cytokines causing up-regulation of adhesion molecules on leukocytes and endothelial cells (Nix *et al.*, 2007; Silva-Herzog and Detweiler, 2008). *S.* Typhi reaches bloodstream and spreads to the reticuloendothelial system, including spleen, liver and bone marrow. Following the accumulation and activation of macrophages by Th-1 cytokines INF-γ, IL-12 and TNF-α, the inflammatory lesion may take a granulomatous form.

There are several possibilities for the outcome of *S.* Typhi infection. Approximately 5% of the diseased persons with acute typhoid fever progress to an asymptomatic chronic infection. They will suffer from life-long carriage of *S.* Typhi in the gallbladder from where bacteria reach intestines via the bile duct, with periodical excretion in stools. These individuals intermittenly shed the pathogen into community sewers and thereby serve as a reservoir for dissemination to naïve hosts (Parry *et al.*, 2002). For example, Mary Mallone ("bloody Mary"), the first identified healthy typhoid carrier, infected at least 57 people in New York City before she was confined to lifelong quarantine in 1907 (Tischler and McKinney, 2010).

Recently, a correlation between the presence of gallstones and *S.* Typhi carriage was demonstrated. Namely, *S.* Typhi forms bile-mediated biofilms on human gallstones and cholesterol coated surfaces which facilitate the gallbladder colonization (Crawford *et al.*, 2010). Furthermore, the carriers of *S.* Typhi are at risk of developing malignancies in the gastric and the hepatobiliar tract (Blaser and Kirschner, 2007). It has been demonstrated that significantly high Vi-antigen positivity (29.4%) was observed in patients with gallbladder carcinoma (Shukla *et al.*, 2000).

2.3.2.1. Granuloma formation

The formation of granulomas is a response to chronic inflammatory stimuli either of infectious origin (*e.g.* intracellular microorganisms) or inert material (*e.g.* silica). It is a nodular organized aggregation of mononuclear inflammatory cells or a collection of modified macrophages surrounded by a fibrotic rim of lymphocytes containing multinucleated giant cells. Granulomas associated with infection are commonly understood to be a protective form of delayed-type hypersensitivity that leads to the control of the expansion of infection (Sneller, 2002).

Microscopically, typhoid lesions consist predominantly of histiocytes, lymphocytes, and plasma cells. The typhoid nodules may occur in several organs, *e.g.* the bone marrow, liver, spleen, and in the ileum or mesenteric lymph nodes (Bharadwaj *et al.*, 2009).

2.3.3. *S.* Typhimurium infection

As *S.* Typhi is restricted to humans, there are no suitable animal models with the particular microbe. In order to study the pathogenesis of typhoid fever pathogenesis, *S.* Typhimurium has been used in a murine model of systemic infection mimicking persistence observed in *S.* Typhi carriers (Monack *et al.*, 2004; Andrews-Polymenis *et al.*, 2010). In humans, opposite to *S. enterica* serovar Typhi, *S. enterica* serovar Typhimurium does not reach beyond the lamina propria and therefore causes self-limiting gastroenteritis and requires treatment only in immunosuppressed patients. The infection in mice resembles typhoid fever-like systemic infection in humans (Hudault *et al.*, 1997; Monack *et al.*, 2004). Similarly, after colonization and invasion of the ileum of mice, *S.* Typhimurium proliferates within the reticuloendothelial system, *incl.* liver and spleen (Thygesen *et al.*, 2000).

S. Typhi and *S.* Typhimurium share many of the virulence factors important for gastroenteritis, including flagella. The difference is in about 10% of their genes, including mutations in over 200 of *S.* Typhi genes. Interestingly, most of the genes involved in intestinal colonization identified in *S.* Typhimurium are inactivated in *S.* Typhi (Sabbagh *et al.*, 2010).

2.4. Oxidative stress

Oxidation is a gain of oxygen or a loss of electrons, whereas reduction is a loss of oxygen or a gain in electrons. Oxidative stress (OxS) is a serious imbalance between the generations of reactive oxygen species and antioxidant protection in favour of the former, causing excessive oxidative damage (Halliwell, 2011). Oxidative stress is a disruption of redox signaling and control (Jones, 2006).

OxS is considered to play a pivotal role in the pathogenesis of aging and several degenerative diseases, atherosclerosis, cardiovascular diseases, type 2 diabetes, and cancer (Masella *et al.*, 2005). *S.* Typhimurium-mediated intestinal infection is accompanied by an increased generation of reactive oxygen species (ROS), which may induce the lipid peroxidation of the enterocyte membrane, thereby leading to a loss of cell viability (Mehta *et al.*, 1998). Though the cellular damage favours the generalisation of the infection, the role of OxS in the development of persistent infection process needs to be clarified.

2.4.1. Pro-oxidants

Pro-oxidants are products of normal cellular metabolism. They are either free radicals of reactive oxygen species (ROS) or reactive non-radical particles (H_2O_2, HOCl, O_3) with either beneficial or deleterious influence.

Beneficial effects of ROS occur at low/moderate concentrations and involve physiological roles in energy production, in phagocytosis, in intercellular signalling systems, and in cell growth. Besides, ROS can be generated as a result of intracellular metabolism of foreign compounds, toxins or drugs by cytochrome P450, mono-oxygenases, or due to exposure to environmental factors, such as excessive iron salts or UV radiation (Masella *et al.*, 2005).

At high concentrations, ROS can be important mediators of damage to cell structures, nucleic acids, lipids and proteins (Valko *et al.*, 2007).

Thus, abnormal formation of the reactive species leads to the damage of lipids, proteins, nucleic acids and carbohydrates of cells and tissues, and causes an imbalance in the pro-oxidants/antioxidants system.

2.4.2. Lipid peroxidation

Lipid peroxidation involves a chain reaction where free radicals remove electrons from the lipids of membranes to surrounding cells and organelles such as mitochondria, lysosomes, and peroxisomes (Halliwell and Gutterridge, 1999, Gutteridge and Halliwell, 2010). This affects polyunsaturated fatty acids (PUFAs), which are responsible for the maintenance of physiologically important membrane properties, including fluidity and permeability. Peroxidation products of PUFAs, such as malonedialdehyde (MDA) and alkenals, are also mutagenic and cytotoxic and can damage membrane proteins. Increased production of ROS also oxidizes unsaturated fatty acids of cell membranes and produces lipid hydroperoxides by initiating a chain reaction (Halliwell and Chirico, 1993). This leads to changes in cell membranes which result in tissue damage (Baker and He, 1991).

Still, the role of LPO in persistent infections is obscure and needs further investigation.

2.4.3. Antioxidants

By definition, antioxidant is “any substance that delays, prevents or removes oxidative damage to a target molecule” (Gutteridge and Halliwell, 2010). Defence mechanisms against free radical-induced oxidative stress involve antioxidants. Enzymatic antioxidant defences include superoxide dismutase, glutathione peroxidase, catalase and heme oxygenase. Non-enzymatic anti-oxidants are glutathione (GSH), ascorbic acid (vitamin C), α-tocopherol (vitamin E), carotenoids, and flavonoids, blood albumine, uric acid, and bilirubin (Halliwell, 2011).

2.4.3.1. Glutathione

Glutathione is a cysteine-containing tripeptide that exists either in the reduced (GSH) or in the oxidized (GSSG) form. Under normal cellular redox conditions, the major portion is in reduced form and is distributed in mammals’ red blood cells, liver, spleen, pancreas, kidneys, eyes and intestinal cells, while GSSG cellular level is maintained at less than 1% of the total glutathione via rapid reduction back to GSH by glutathione reductase (Zilmer *et al.* 2005).

GSH has several biological functions. It is the major non-enzymatic antioxidant of intracellular redox homeostasis; is involved in the restoration of of thiol groups of proteins and coenzyme A, and is required for the stabilization of cell membranes, for the synthesis of proteins, nucleic acids, leukotriens and prostaglandins (Masella *et al.*, 2005, Zilmer *et al.* 2005). In the presence of oxidative stress, GSH concentration rapidly decreases while GSSG increases due to the reduction of peroxides or as a result of free radical scavenging. The redox state of the cell is linked to iron and copper redox couple and is maintained within strict physiological limits. It has been suggested that iron regulation ensures depletion of free intracellular iron; however, *in vivo*, under stress conditions, *e.g.* infection, an excess of superoxide releases “free iron” from iron-containing molecules (Peran *et al.*, 2006; Halliwell, 2009; Niki, 2010).

2.5. Immunity in infection

The immune response is a redox regulated process; the activation of T lymphocytes is significantly enhanced by ROS or by a shift in intracellular glutathione redox state (Halliwell, 2011). Vertebrates have evolved two complementary systems to detect and clear pathogens: the innate and the adaptive immune system. Persistence is established after an acute infection period involving activation of both the innate and acquired immune system (Young *et al.*, 2002). Persistent infection may continue for a long time due to constant modulation of the immune system and/or the microbe. The primary function of the immune system is to protect the host from the harmful insults of microorganisms. The pattern-recognition receptors (PRRs) are expressed by many cell types, including macrophages, monocytes, DCs, neutrophils, and epithelial cells.They allow the early detection of pathogens directly at the site of the infection (Medzhitov and Janeway, 2002). PRRs recognize conserved microbial signatures termed pathogen-associated molecular patterns (PAMPs) (Janeway, 1992).

Toll-like receptors (TLRs) are the most intensely studied of PRRs. So far, there are 10 members of the human and 13 members of the mouse TLR family that have been identified. TLR1-TLR10 are similar in humans and mice, although TLR10 is not functional in mice due to a retroviral insertion. TLRs 11–13 are not present in humans. Thus, despite some species-specific receptors, most members are similar in mammals. Once activated by PAMPs, the TLRs induce different signalling cascades leading to the activation of the transcription factors and interferon-regulatory factor.

TLR activation results in the production of antimicrobial peptides, inflammatory cytokines and chemokines, *e.g.* TNF-α, IL-1 and costimulatory adhesion molecules, as well as in the upregulation of major histocompatibility complexes (MHCs). Besides TLR, two other families of PRRs have been described: the NLRs (NOD-like receptors) and the RLHs (RIG-like helicases). Unlike TLRs, these families consist of soluble proteins that give the cytoplasm signs of intracellular pathogens (Martinon *et al.*, 2009).

Although both arms of the immune system (innate and adaptive) have distinct functions, there is interplay between these systems (*i.e.*, components of the innate immune system influence the adaptive immune system and *vice versa*).

Opsonisation of the respective microbes facilitates phagocytosis by phagocytes. During phagocytosis, small peptides bind to MHC class II proteins. The adaptive immune system exerts highly specific responses to microbes by producing antibodies from B cells or through the generation of killer or helper T lymphocytes, resulting in life-long immunological memory (Yamamoto and Takeda, 2010). T and B lymphocytes recognize the antigen specific sites. B lymphocytes recognize a membrane, proliferate and differentiate into antibody producing plasma cells. T lymphocytes are divided into: T-helper, regulatory, and cytotoxic cells (Mileti *et al.*, 2009). Two types of T helper cells are produced in the thymus, the Th1 cells that help the CD8+ pre-cytotoxic cells to differentiate into cytotoxic T cells, and Th2 cells that help B cells differentiate into plasma cells, which secrete antibodies. Activated Tc cells are involved in destruction of cells infected with intracellular microorganisms (Yamamoto and Takeda, 2010). Almost all immune cells secrete cytokines.

2.5.1. Cytokines

Cytokines are 15 to 44 kD sized glycoproteins functioning as signal molecules between different immune cells. Thirty-five different cytokines have been described to date.

Pro-inflammatory cytokines are generally produced by activated immune cells and enhance the inflammatory reactions, while anti-inflammatory cytokines inhibit the activated cells. Monocytes and macrophages produce initially pro-inflammatory cytokines: IL-1, TNF-α, INF-γ *etc*.

We chose for investigation the pro-inflammatory (TNF-α and INF-γ) and anti-inflammatory cytokines (IL-10) as they have been earlier associated with persistent infection and granuloma formation (Monack *et al.,* 2004; Sachinami *et al.,* 2006). Moreover, the modulation of these cytokines is considered to be one of the principal mechanisms of protection against gastroenteric infections by probiotic lactobacilli though exact pathways and cells involved are not clear yet (Mileti *et al.*, 2009).

TNF-α is a pro-inflammatory cytokine that induces activation and recruitment of neutrophils and produces intestinal epithelial barrier dysfunction, contributing to the entry and colonization of pathogenic bacteria usually excluded from the subepithelial mucosa (Castillo *et al.*, 2011). It is produced by several types of cells, but especially by macrophages. TNF-α is considered to be a major early mediator in the systemic inflammatory response syndrome observed during Gram-negative sepsis (Sakaguchi *et al.*, 2006). Since TNF-α exerts its effects on almost every cell and organ within the body, the production of TNF-α is strictly regulated during infection (Castillo *et al.*, 2011).

INF-γ is produced predominantly by natural killer cells as a part of the innate immune response, and by CD4 Th1 and CD8 Tc lymphocyte. IL-12 and IL-18, secreted by activated macrophages, act both independently and synergistically on natural killer cells and helper T cells to induce the production of further INF-γ, which activates the macrophages through a positive feedback loop. It contributes to the activation of macrophages to promote the effective killing of pathogens that can survive within them (Castillo *et al.*, 2011).

IL-10 is produced in a broad variety of cells, including Th2 cells, regulatory T cells, DCs, B cells, and macrophages (Mosser and Zhang, 2008). IL-10 is required to maintain the IgA (+) B cell population (Castillo *et al.*, 2011).

2.5.2. Gut as immune organ

The gut immune system can be divided into three major compartments: organized gut-associated lymphoid tissue (GALT), the mucosal lamina propria, and the epithelium.

The GALT consists of both isolated and aggregated lymphoid follicles and is one of the largest lymphoid organs, containing up to 80% of the cells within the immune system (Bezirtzoglou and Stavropoulou, 2011). Aggregated lymphoid follicles were named Peyer's patches (PP) after their detailed description by the Swiss pathologist Johann Conrad Peyer in 1677. PPs are composed of aggregated lymphoid follicles surrounded by a particular epithelium, follicle-associated epithelium (FAE) that forms the interface between the GALT and the luminal microenvironment. FAE contains specialized cells named M (microfold) cells. M cells are able to transport luminal material, like soluble proteins, antigens, viruses and bacteria, toward the underlying immune cells that activate or inhibit the immune response, leading to either tolerance or systemic immune cell response (Siebers and Finlay, 1996). M cells express an IgA-specific receptor on their apical surfaces that mediate the transepithelial transport of sIgA from the intestinal lumen to underlying gut-associated organized lymphoid tissues (Mantis *et al.*, 2002).

The cellular composition of the FAE (*i.e.* the proportion of enterocytes and M cells) may be modulated by bacteria present in the gut lumen. Namely, pathogenic bacteria may increase the number of M-cells within the FAE (Savidge *et al.*, 1991).

It has been shown earlier that in mouse PP exhibit about 60% of B-cells, 25% of T-cells, 10% of dendritic cells and less than 5% of macrophages or polymorphonuclear neutrophil (Jung *et al.*, 2010)

Cell composition and cytokine production may affect the function of PP.

Pro- and anti-inflammatory cytokines are known to modulate intestinal paracellular permeability. INF-γ, TNF-α, and IL-4 act on the membrane receptors of epithelial cells and increase tight junction permeability (Barreau *et al.*, 2007).

The activation of naïve T cells takes place in the GALT where differentiation of the activated lymphocytes occurs in PP, from where the lymphocytes circulate to the peripheral circulation. The gut immune system has a dual role: it provides defence against infectious agents, but also induces tolerance to harmless microbial antigens encountered in the gut. Oral tolerance is a major compartment of peripheral tolerance and its control of the immune response is not necessarily restricted locally but may include systemic effects (Vaarala, 2003).

Intestinal epithelial cells (IEC) are important in the presentation of oral antigens and in the regulation of intestinal immune responses. The changes in the epithelial structure of gut influence the intestinal immune system and *vice versa*. Permeability controls the amount and quality of antigenic exposure, *e.g.* dose and size of antigens encountered in the gut immune system. Activation of gut immune cells resulting in the secretion of cytokines may cause epithelial injury. Intestinal microbes have been suggested to be important regulators of the function and development of immune and epithelial cells.

2.6. Lactobacilli

Lactobacillus spp. belongs to a heterogeneous group of lactic acid bacteria (LAB). It includes about 20 genera within the phylum *Firmicutes.*

LAB are divided into homolactic or heterolactic fermentation groups according to carbohydrate fermentation patterns (Kandler, 1986). In homolactic fermentation the end product of glycolyse is lactic acid. The majority of LAB are responsible for heterolactic fermentation. During pentose-phosphate pathway besides lactic acid, several organic acids, *e.g.* acetic, succinic, accompanied with ethanol and CO_2, are produced.

The bacteria from genus *Lactobacillus* are gram-positive, acid-tolerant, non-spore forming rods. *Lactobacilli* sp. is the biggest group among LAB including 135 species and 27 subspecies (Bernardeau *et al.*, 2008). These numbers have been periodically re-evaluated due to the application of new genome-based molecular methods. The amount of lactobacilli has been estimated at almost 1% of colonic microbiota in humans (Louis *et al.*, 2007), it varies due to several different factors, *e.g.* diet, environment and host individual properties (Stsepetova *et al.*, 2011).

Lactobacillus strains are present in the gastrointestinal tract of 70% of humans consuming a Western-like diet according to culture-based methods (Heilig *et al.*, 2002). Ahmed and his colleagues have studied gut mucosal bacterial communities of 26 patients undergoing emergency resection of the large bowel with real-time PCR. They found that the terminal ileum had higher bacterial cell densities than the colon and that overall bacterial numbers were generally similar within the ascending, transverse, and descending colon (Ahmed *et al.*, 2007).

The dominant species differ between the mucosa-associated and fecal microbiota, and in an individual, the microbiota is relatively stable along the distal digestive tract (Mikelsaar *et al.*, 1987; Lepage *et al.*, 2005).

Lactobacillus is a very heterogeneous genus, encompassing species with a large variety of phenotypic, biochemical, and physiological properties. Obligately homofermentative (OHOL) lactobacilli, *e.g. L. delbrueckii* and *L. acidophilus,* produce lactic acid as a major end product from glucose, and grow at 45°C but not at 15°C. Facultatively heterofermentative lactobacilli (FHEL) *L. casei* and *L. plantarum* grow at 15°C and show variable growth at 45°C. They can produce acetate, if O_2 is present. Obligatively heterofermentative lactobacilli (OHEL), *e.g. L. fermentum, L. brevis,* produce lactic acid from glucose, along with CO_2 and ethanol (Annuk *et al.*, 2003; Hutt *et al.*, 2006).

Lactobacilli belong to the category of organisms classified as generally regarded as safe – GRAS for food and medical application (FAO/WHO, 2002).

2.6.1. Probiotics

Probiotics are defined as live microorganisms which, when administered in adequate amounts, beneficially affect the health of the host (FAO/WHO, 2002). The word "probiotic" is derived from the Greek words "pro" and "biotikos," meaning "for life".

Several microbial genera have been used as probiotics, *e.g. Bifidobacteria, Lactobacilli, Enterococci,* and yeasts.

Cell wall molecules of gram-positive LAB are composed of a thick peptidoglycan layer, proteins, teichoic acids, and polysaccharides. After interaction with host receptors and induction of signalling pathways, probiotic effects result. The main cell wall macromolecules have a similar basic architecture between species, but various modifications, such as glycosylation, can contribute to the strain-specific properties of probiotics (Lebeer *et al.*, 2010).

Specific health effects attributed to probiotics that have been investigated include alleviation of diarrheal illness, constipation, urogenital infections, atopic diseases, and neonatal necrotizing enterocolitis (Kontiokari *et al.*, 2003; Reid and Bruce, 2006, Gerritsen *et al.*, 2011). Though probiotics are generally safe, they should be used with caution in patients who have lowered immunity or integrity of the intestinal mucosa. There are three theoretical concerns regarding the safety of probiotics: 1) the occurrence of disease, such as bacteremia or endocarditis; 2) toxic or metabolic effects on the gastrointestinal tract; and 3) the transfer of antibiotic resistance in the gastrointestinal microbiota (Snydman, 2008).

2.6.2. Mechanisms of action of probiotic lactobacilli

The exact mode of how probiotics act is scarcely known. Lactobacilli can influence the host by using different mechanisms depending on strain and species-specific properties. Their efficacy has been studied concerning defence against infectious and non-infectious lesions.

First, in the case of infective agents, probiotic microorganisms may exert their action through a modulation of the intestinal microbiota, which may result from competitive metabolic interactions with potential pathogens. Lactic and non-lactic acids, and hydrogen peroxide enable to inhibit or kill pathogens (Annuk *et al.*, 2003; Hütt *et al.,* 2006). Furthermore, lactobacilli produce bacteriocins at the end of the exponential growth-phase (Montalban-Lopez *et al.*, 2012). They are ribosomally synthesized proteins with variable molecular weight, genetic origin, biochemical properties, and mode of action. The bactericidial influence is selective for prokaryotes and varies depending on the producing species. Producer strains are immune to their own bacteriocins because they possess genes that encode immunity mechanisms, which enable a distinction between "self" and "non-self" (Pessione, 2012).

Second, in the gut lactobacilli seemingly compete with the pathogen for the adhesion sites, nutrients and antagonistic relations (Hutt *et al.*, 2006; Stsepetova *et al.*, 2011). Recently, McNulty and his colleagues demonstrated that probiotic strains are able to change the metabolic pathways of the carbohydrates of indigenous microbiota (McNulty *et al.*, 2011).

Third, probiotics have been proposed to modulate host defenses by influencing the intestinal immune system by increasing phagocytosis, modifying cytokine production, or enhancing IgA production (Alakomi *et al.*, 2000; Castillo *et al.*, 2011). Immune stimulation has been suggested to underlie the anti-infection and anti-carcinogenic effects of lactic acid bacteria (Gill *et al.*, 2000). At intracellular level it has been shown that probiotic strains can inhibit NF-kappa B activation (Petrof *et al.*, 2004).

Fourth, positive affection to the intestinal barrier function by fortifying the epithelial tight junctions has also been postulated (Doron *et al.*, 2005).

By using these abovementioned mechanisms, probiotics can not only potentially modulate the intestinal microbiota composition, but also prevent pathogenic bacterial overgrowth.

3. AIMS OF THE STUDY

We tested the hypothesis of whether probiotic *Lactobacillus* sp. as adjunct to antimicrobial treatment could help to resolve persistent *S.* Typhimurium infection in mice and studied the possible mechanisms behind it.

The goal of the present study was to detect the effect of lactobacilli of human origin (the probiotic strain *Lactobacillus fermentum* ME-3 DSM 14241 and *Lactobacillus acidophilus* E1) on microbiological, histological, biochemical, and immunological status of mice in *Salmonella* Typhimurium persistent infection model, and to detect the impact of the probiotic strain *Lactobacillus plantarum* Inducia DSM 21379 on total count of lactobacilli and immunological response in the gut of mice.

The following objectives were set:

1. To detect viable *S.* Typhimurium in the blood, liver, spleen, and gut; total count of lactobacilli in gut; granulomas in the liver and spleen; oxidative stress related indices (LPO, GSSG/GSH), and cytokines (TNF-α, INF-γ and IL-10) in the liver and gut of mice infected with *S.* Typhimurium.

2. To test the influence of two selected *Lactobacillus* spp strains of human origin, probiotic strain *L. fermentum* ME-3 and *L. acidophilus* E-1, on the *Salmonella enterica* serovar Typhimurium infection in mice by detecting salmonella, the total count of lactobacilli, morphologic changes in the liver and spleen, and oxidative stress related indices.

3. To determine the impact of *Lactobacillus plantarum* Inducia on the total count of intestinal lactobacilli and the response of gut-associated lymphoid tissue of the ileum and colon of healthy mice after 30 days of intake of cheese containing *L. plantarum* Inducia.

4. To detect if the *L. fermentum* ME-3 in combination with ofloxacin would influence the viability of *S.* Typhimurium in the blood, liver and spleen, the development of liver and spleen granulomas and the indices of oxidative stress in the mucosa of the ileum.

5. To assess the profile of pro- and anti-inflammatory cytokines in the gut and liver evoked by the addition of *L. fermentum* ME-3 to ofloxacin treatment in the persistent infection model of *S.* Typhimurium.

4. MATERIAL AND METHODS

4.1. Study design

For the experimental infection in studies I–III we applied a murine model of *S.* Typhimurium infection (Santos *et al.*, 2001; Tsolis *et al.*, 2011). A total of 193 (47, 72, 54 and 20 in studies I–IV, respectively) NIH line conventional male mice (Kuopio, Finland), who were 6 weeks old at the beginning of the experiments, were used. The mice were divided into separately housed groups depending on the following treatments. All animal experiments were approved by the Committee of Animal Experiments of Estonian Ministry of Agriculture (06 03505, 07 04679, 06 08560).

A commercial diet R-70 (Lactamin, Sweden) and tap water *ad libitum* were available throughout studies I–III. Daily feeding was similar in study IV, while at night 10 mice were administered cheese (4,4g per mouse) containing *L. plantarum* Inducia 2×10^8 cfu/g and another 10 mice from control group received cheese without *L. plantarum* Inducia. The faecal material was collected before the experiment and then on the 10-th and 15-th day of the experiment per cage. After 30 days the 20 mice were sacrificed by cervical dislocation.

Experimental infection studies (I–III) included control groups treated with 0.5 ml of sterile PBS orally by intragastric gavage (7, 11, and 6 mice, respectively) and groups challenged with a clinical isolate of *Salmonella* Typhimurium (16, 22 and 12 mice, respectively) (Table 1, Fig. 1–3).

The deaths of mice were registered and all surviving animals were sacrificed using cervical dislocation either on the 5th (study III) or on the 10th day (studies I–III) following *S.* Typhimurium administration. The autopsy was performed under sterile conditions using a Class II microbiological safety cabinet (Jouan, France). Bacteriological tests from heart blood (10 μl), liver, spleen and gut were were carried out immediately, while samples for biochemical testing were maintained at -80^0C for up to three months before testing. The samples for histological investigation were collected from the liver, spleen and ileum, placed into 10% formaldehyde for fixation and processed further for paraffin embedding prior to hematoxylin and eosin staining.

In study I, the impact of the administration of *L. acidophilus* E-1 and probiotic *L. fermentum* ME-3 on experimental *S.* Typhimurium infection was evaluated in Gr 3 (n=14). Namely, for 5 consecutive days before and 10 days after challenging the mice with *S.* Typhimurium, the aforementioned lactobacilli were added to ultra-pasteurized milk. The mice of Gr 4 (n=10) were fed with ultra-pasteurized milk fermented with *L. acidophilus* E-1 and *L. fermentum* ME-3 for 15 consecutive days and served as a positive control group.

Table 1. Study groups and study designs presented in papers I–IV

No. of experimental animals	Study description	Presented in paper
47 NIH male mice Gr 1 PBS (n=7) Gr 2 *S*. Typhimurium (n=16) Gr 3 *L. acidophilus* E-1 and *L. fermentum* ME-3 for 5 days prior challenge with *S*. Typhimurium 10 days after (n=14) Gr 4 *L. acidophilus* and *L. fermentum* ME-3 for 15 days (n=10)	Intervention study Elaboration of the persistent *S.* Typhimurium mouse model. Evaluation of the impact of *L. fermentum* ME-3 and *L. acidophilus* E-1 on microbiological, morphological and OxS-indicative markers of gut mucosa (Figure 1).	I
72 NIH male mice Gr 1 *S*. Typhimurium (n=22) Gr 2 *S*. Typhimurium and ofloxacin; (n=13) Gr 3 *S*. Typhimurium and *L. fermentum* ME-3 (n=13) Gr 4 *S*.Typhimurium and ofloxacin and *L. fermentum* ME-3 (n=13) Gr 5 PBS (n=11)	Intervention study Detection of microbiological, antioxidative and morphological response in combined treatment of ofloxacin and *L. fermentum* ME-3 in *S.* Typhimurium murine model (Figure 2).	II
54 NIH male mice Gr 1 *S.* Typhimurium (n=12) Gr 2 *S*.Typhimurium and ofloxacin (n=12) Gr 3 *S*.Typhimurium, ofloxacin and *L. fermentum* ME-3 (n=12) Gr 4 *L. fermentum* ME-3 (n=12) Gr 5 PBS (n=6)	Intervention study Detection of microbiological, immunological, antioxidative and morphological response in combined treatment of ofloxacin and *L. fermentum* ME-3 in *S.* Typhimurium murine model (Figure 3).	III
20 NIH male mice Gr 1 Control cheese without *L. plantarum* Inducia (n=10) Gr 2 Cheese containing *L. plantarum* Inducia (n=10)	Intervention study To determine the impact of administration of *Lactobacillus plantarum* Inducia on the gut-associated lymphoid tissue and total count of lactobacilli in gut of mice (Figure 4).	IV

In study II and III we aimed to detect the impact of combined treatment of ofloxacin and *L. fermentum* ME-3 in *S.* Typhimurium murine model. Therefore, *L. fermentum* in drinking water was added to ofloxacin (OFL) treatment of the experimental *S.* Typhimurium infection. OFL (Hoechst, Germany) at doses of 20 mg/kg (Fu *et al.*, 1990) was diluted in 0.5 ml of PBS and given intragastrically by a sterile syringe with a blunt-ended tube once daily. 48 hours after the challenge with *S.* Typhimurium, mice were treated either with OFL Gr 2 of study II and III (13 and 12 mice, respectively) or with the combination of OFL and *L. fermentum* ME-3 (13 in Gr 4 of study II and 12 mice in Gr 3 of study III). Gr 3 mice (n=13) in study II received *L. fermentum* ME-3 48 hours after challenge with *S.* Typhimurium for 8 days, while Gr 4 mice (n=12) in study III were administered *L. fermentum* ME-3.

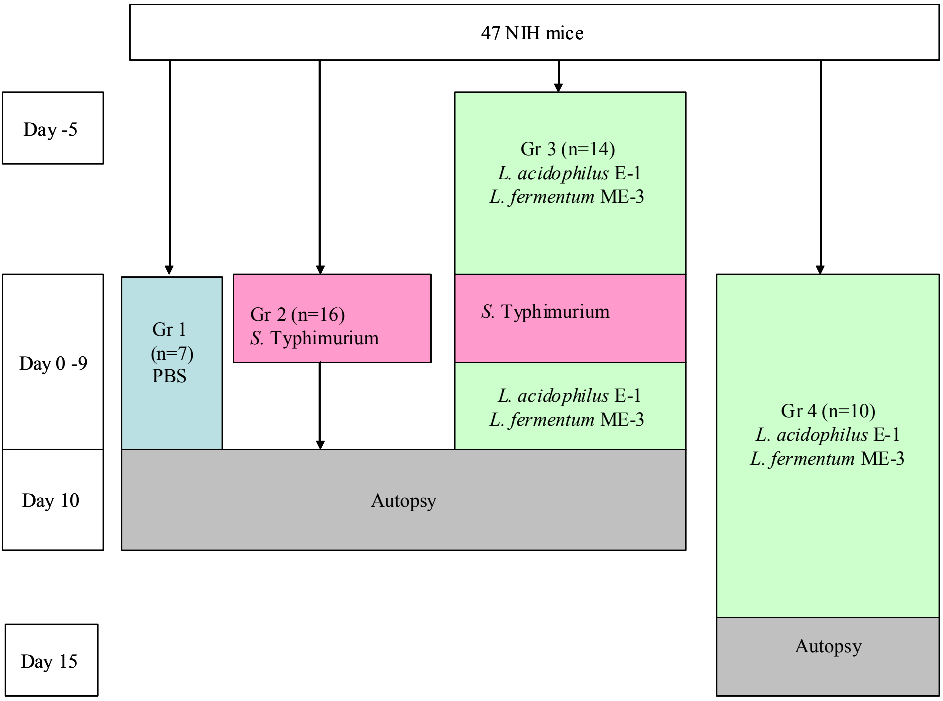

Figure 1. Design of study I.

47 NIH male mice were divided into four groups. Gr 1 (n=7) mice were PBS treated for 10 days. Gr 2 (n=16) mice were infected with *S.* Typhimurium on Day 0. Gr 3 (n=14) mice were pre-treated with *L. fermentum* ME-3 and *L. acidophilus* E-1 for 5 days before the challenge with *S.* Typhimurium. The administration of lactobacilli was continued up to Day 10. Gr 4 (n=10) received aforementioned lactobacilli for 15 days.

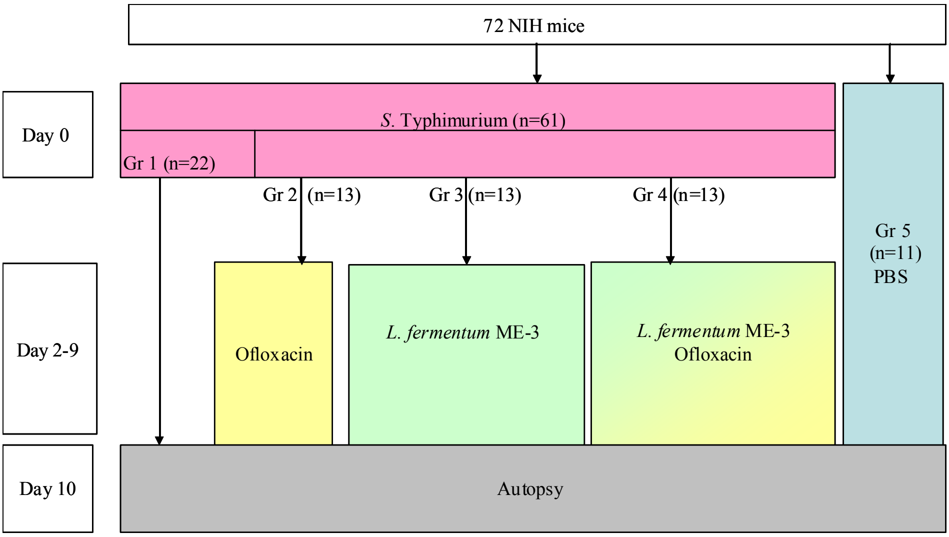

Figure 2. Design of study II.

72 NIH male mice were divided into 5 groups. Gr 1 (n=22) mice were challenged with *S.* Typhimurium on Day 0. Gr 2 (n=13) mice were infected with *S.* Typhimurium on Day 0 and, 48 hours after that, treated with ofloxacin daily for 8 days. Gr 3 (n=13) mice were infected with *S.* Typhimurium on Day 0 and 48 hours after that were treated with *L. fermentum* ME-3 continuously in drinking water for 8 days. Gr 4 (n=13) mice were infected with *S.* Typhimurium on Day 0 and 48 hours after that treated with ofloxacin daily and *L. fermentum* ME-3 continuously in drinking water for 8 days. Gr 5 mice (n=11) received PBS via intragastric gavage once daily for 10 days.

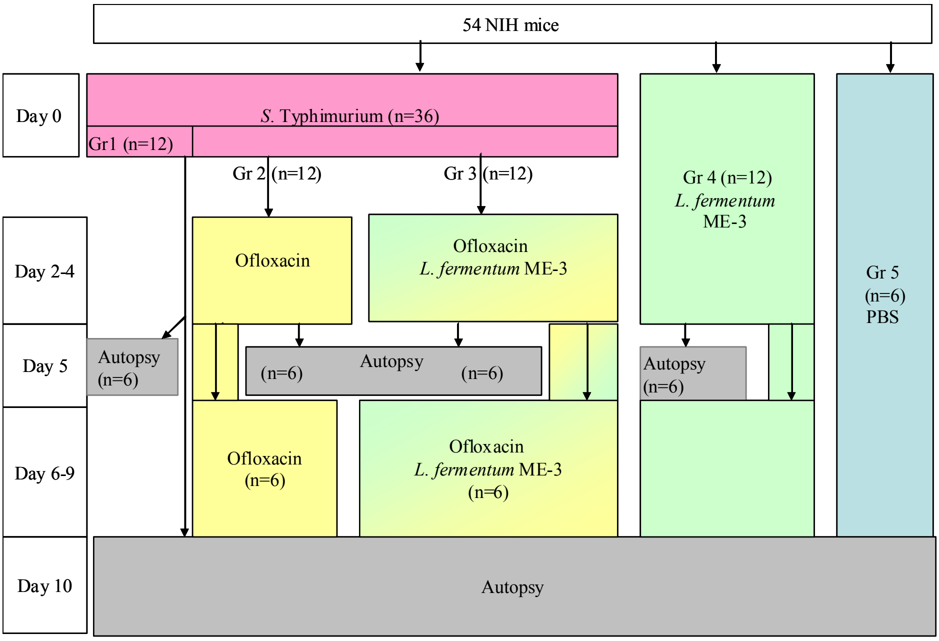

Figure 3. Design of study III.

54 NIH male mice were divided into 5 groups. Autopsy was performed at two time points in Gr 1-Gr 4 mice. Half of the mice (n=6) were sacrificed on Day 5 and the other half (n=6) on Day 10. Gr 1 (n=12) mice were challenged with *S.* Typhimurium on Day 0. Gr 2 (n=12) mice were infected with *S.* Typhimurium on Day 0 and, 48 hours after that, treated with ofloxacin (OFL) daily. Gr 3 (n=12) mice were infected with *S.* Typhimurium on Day 0 and 48 hours after that treated with *L. fermentum* ME-3 continuously in drinking water OFL daily. Gr 4 (n=12) mice were administered *L. fermentum* ME-3 continuously in drinking water for 8 days. Gr 5 mice (n=6) received PBS via intragasric gavage once daily for 10 days.

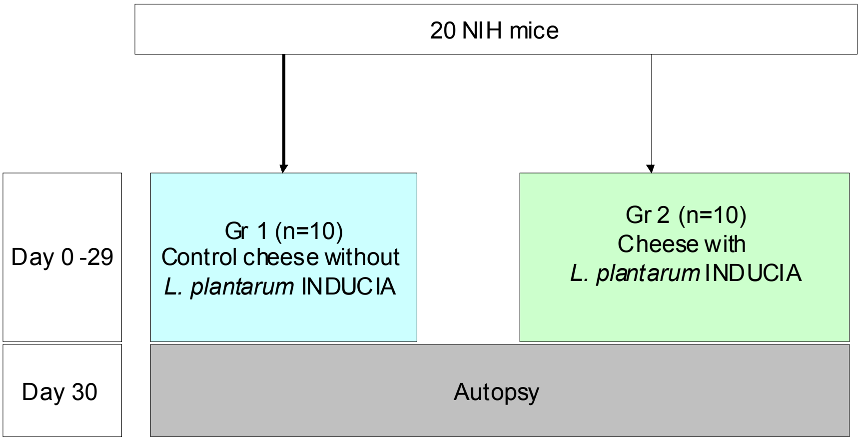

Figure 4. Design of study IV.

20 NIH mice were divided into 2 groups. Gr 1 (n=10) mice were administered cheese containing *L. plantarum* Inducia and Gr 2 (n=10) received control cheese without *L. plantarum* Inducia for 29 days.

4.2. Microbial strains

4.2.1. *Salmonella* Typhimurium

The clinical isolate of *Salmonella enterica* serovar Typhimurium was kindly provided by the Estonian Health Board Laboratory of Microbiology. After cultivation on blood agar for 24 h at 37ºC, the colonies were suspended in PBS, and adjusted to the concentration of 5×10^4 CFU/ml. The mice were inoculated intragastrically with a single 0.5 ml dose of the *S.* Typhimurium suspension (5×10^4 CFU/ml) using a sterile syringe with a blunt-ended tube. As we aimed to study a persistent infection we applied the adjusted minimal dose of *S.* Typhimurium from preliminary experiments.

4.2.2. Lactobacilli

All three *Lactobacillus* strains used in our studies were isolated from the faecal samples of Estonian children during a comparative study of the gut microbiota of Estonian and Swedish children (Mikelsaar *et al.,* 2002).

These strains were identified as *L. fermentum, L. acidophilus* and *L. plantarum* by API 50 CHL (bioMérieux, France) and internal transcribed spacer polymerase chain reaction (Annuk *et al.,* 2003). Two strains are patented and deposited in DSM: *L. plantarum* Inducia DSM 21379 and *L. fermentum* ME-3 DSM 14241.

L. fermentum ME-3 demonstrated a high antagonistic activity against *S.* Typhimurium (inhibition zone of 13–15 mm) and a total antioxidative activity (TAA) value (29±0.7%) *in vitro*, while the *L. acidophilus* E-1 had minimal antagonistic activity against *S.* Typhimurium (inhibition zone of 0–2 mm) and a low-grade antioxidativity (TAA value as 8±3%) (Kullisaar *et al.*, 2002).

In our study I, *L. fermentum* ME-3 and *L. acidophilus* E-1 strains were cultivated separately in de Man-Rogosa-Sharpe (MRS) broth (Oxoid, UK) at 37ºC for 24 h in a 10% CO_2 environment. The strains of both lactobacilli in equal volumes (5×10^7 CFU/ml) were added to ultra-pasteurized milk and fermentation was carried out at 37ºC for 48 h in a 10% CO_2 environment. The product was divided into daily portions for the whole experiment and maintained at –20ºC until administration to mice. There was no antagonism between these two strains of lactobacilli *in vitro.*

In studies II and III, the lyophilized *L. fermentum* ME-3 (Probiotical s.r.l, Novara, Italy) was suspended in PBS to a final concentration of 5×10^7CFU/ml. During the experiments each mouse consumed approximately 5 ml of *L. fermentum* ME-3 containing PBS, and received 2.5×10^8 CFU of lactobacilli daily.

In study IV, we applied probiotic *L. plantarum* Inducia DSM 21379 in concentration of 2×10^8CFU/g of cheese. Total antioxidative activity (TAA) and total antioxidative status (TAS) of *Lactobacillus plantarum* Inducia DSM 21379 demonstrated high values (26±1.2% and 0.13±0.04, respectively).

4.3. Antibacterial susceptibility testing

In study II, the value of minimal inhibitory concentration (MIC) of ofloxacin to *S.* Typhimurium on Mueller-Hinton media (Oxoid, UK) was measured by the E-test (Oxoid, UK).

The combinative effect of OFL and *L. fermentum* ME-3 against *S.* Typhimurium was evaluated by two following *in vitro* tests. First, in the overlay test, 10 ml of the MRS agar (Oxoid, UK) containing 10^8 CFU/ml of *L. fermentum* ME-3 was poured onto plates and incubated in 10% CO_2 at 37°C for 48 h. E- test was applied after overlay with 5 ml 1.0% (w/w) Isosensitest agar (Oxoid, UK), inoculation with *S.* Typhimurium in concentration of 10^8CFU/ml, and incubation in microaerobic conditions at 37°C for 24 h. Second, in the dilution test two-fold serial dilutions of OFL in broth were prepared. *S.* Typhimurium and *L. fermentum* ME-3 solutions were adjusted according to the 0.5 McFarland turbidity standard and 10 µl of the suspension was placed into the broth (Nutrient broth No2 Oxoid, UK) containing OFL and the minimal bactericidial values were detected by plating. All susceptibility tests were performed in duplicate.

4.4. Bacteriology

At autopsy 10 µl of the heart blood was cultured in thioglycolate broth (Oxoid, UK) and after 24 hours onto Bismuth Sulphite agar and 5% blood agar (study I), McConkey agar (study II) and XLD (study III) (Oxoid, UK) for detection of *S.* Typhimurium and on the de Man-Rogosa-Sharpe (MRS) (Oxoid, UK) for lactobacilli. The samples of liver, spleen and intestine were weighed, homogenized with sterile glass powder, serially diluted (10^{-2}–10^{-7}) in PBS (pH 7.2), and 0.1 ml of each 10-fold dilution was seeded on the aforementioned media. The incubation was performed both at 37ºC for 24 h in an aerobic environment (*Salmonella*) or in a 10% CO_2 environment 48 hours (*Lactobacillus*). The particular characteristic colonies were counted, identified at the genus level and the counts of bacteria were assessed. The detection level of the bacteria was 2 log CFU/ml for blood, 2 log for liver and 1.7 log CFU/g for intestinal samples (study I), while in study II ≥3.0 log CFU/g, respectively. The total counts of lactobacilli were calculated as CFU/mg.

4.5. Morphological investigation

Morphological investigation was performed by Professor R.H. Mikelsaar and Dr. H. Tamme at the Department of Pathological Anatomy and Forensic Medicine, University of Tartu. Samples from the ileum, colon, liver and spleen were fixed in 10% formaldehyde and processed further for paraffin embedding. Tissue sections (approx. 5 µm) were stained with haematoxylin and eosin. Destructive and inflammatory signs, namely hyperaemia, necrosis, number of typhoid nodules and hyperplasia of Peyer's patches, were evaluated. The two pathologists evaluated coded slides in a blinded manner. The inflammatory changes were graded between 0 and 5, with 0 for no changes and 5 for severe changes in study I–II. The degree of necrosis was scored on a scale ranging from 1 to 3 (1 – weak, 2 – moderate, and 3 – strong) (study III). The hyperplasia of lymph follicles was evaluated similarly in study IV.

4.6. Biochemical assays

All biochemical studies were performed in collaboration with senior researchers T. Kullisaar, K. Zilmer, A. Rehemaa and R. Mahlapuu from the Department of Biochemistry, University of Tartu. The mucosa of ileum (study I–II), liver and mucosa of small intestine (study III) were obtained during autopsy and stored at –80°C for a maximum of three months. All biochemical indices were measured simultaneously after homogenisation in a 1.15% KCl solution (1:10).

4.6.1. Total antioxidative activity

Total antioxidative activity (TAA) was assessed using the linolenic acid test described by Kullisaar (Kullisaar *et al.*, 2002). It was expressed as the inhibition of the peroxidation of the linolenic acid (LA) standard by the sample, measured as a percentage. The high numerical value of TAA (>10%) indicates the high total antioxidative activity of the sample.

4.6.2. The indices of oxidative stress

4.6.2.1. Lipid peroxidation

Malondialdehyde (MDA) was used as an indicator of lipid peroxidation (LPO), and was measured using a commercial kit, Bioxytech LPO-586 (Oxis International, Catalog No. 21012). The assay is based on the reaction of a chromogenic reagent, N-methyl-2-Phenylindole, with malonedialdehyde (MDA) and hydroxynonenals at 45º C, yielding a stable chromophore with maximal absorbance at 586 nm. The results were calculated according to the kit formula and the tissue values were given in pmol/mg protein.

4.6.2.2. Glutathione redox status

Glutathione redox ratio was tested by measuring total glutathione and oxidized glutathione using the method described by Griffith (Griffith, 1980). The glutathione content was quantified by comparison with a standard curve generated using specific amounts of glutathione. The amount of reduced glutathione (GSH) and oxidised glutathione (GSSG) was expressed as µg/ml, and demonstrated as (GSSG/GSH).

4.6.3. Iron detection

A special kit for iron detection (Sigma 565, Sigma Diagnostics, USA) was used for assessment of iron levels and iron-binding capacities (Kaur *et al.*, 2001). All procedures were performed in triplicate. Iron concentration was calculated using the formula of the applied kit. Iron content was expressed as µmol/l. The percentage of saturation of iron-binding capacity (indicates the percent of bound iron) was calculated from data measured with the kit.

4.7. Immunological assays

The immunological studies were performed in collaboration with senior researcher T. Kullisaar from the Department of Biochemistry, University of Tartu.

4.7.1. Detection of INF-γ, IL-10, TNF-α

The mucosa of the small intestine and liver obtained at autopsy was processed to the cytokine Mouse Immunoassay (R&D Systems, USA). This assay employs the quantitative sandwitch enzyme immunoassay technique. We followed the recommendations from the manufacturer. The purified polyclonal antibodies specific for mouse INF-γ, IL-10, TNF-α were pre-coated onto a microplate. Standards, control, and samples were pipetted into the wells and any aforementioned mouse cytokines present were bound by the immobilized antibody. After washing away unbound substances, an enzyme-linked polyclonal antibody specific for mouse corresponding cytokines was added to the wells. Following a wash to remove any unbound antibody-enzyme reagent, a substrate solution was added to the wells. The enzyme reaction yielded a blue product that turned yellow when the Stop Solution was added. The intensity of the measured color was in proportion to the amount of mouse cytokines bound in the initial step. The sample values were then read off the standard curve.

4.8. Statistical analysis

Statistical analyses were performed using the SigmaStat (Jandel Scientific), and R 2.6.2 (a Language and Environment; http://www.r-project.org) software.

The Fisher exact test was applied in comparing categorical values, and the Mann-Whitney rank sum test for continuous variables. The tests were selected according to data distribution: the Student t-test for describing the continuous indices, the Mann-Whitney test was used for comparing unevenly distributed data. The p-values less than 0.05 were considered statistically significant. In the case of group comparisons Bonferroni correction was applied. (4 groups p-value 0.012). The OR values were calculated to describe the impact of the intake of *L. plantarum* containing cheese on the lymph follicles of the ileum and colon of mice in study IV.

Due to similarities in study design we summarized the results of microbiology, morphology and biochemistry of study II and study III.

The One-Way ANOVA with Bonferroni correction test was used in group comparisons if we compared six unmatched groups by analysing the results of LPO values. The Kruskal-Wallis One-Way Analysis of Variance on Ranks was used in analyzing the results of glutathione redox ratio. The group of mice infected with *S.* Typhimurium and treated with the combination of ofloxacin and lactobacilli was set as a reference in all comparisons.

A linear logistic regression model was applied to find the relationship between the presence of typhoid nodules in the liver and the values of cytokines in the small intestine and liver.

5. RESULTS AND DISCUSSION

5.1. Antibacterial susceptibility testing

The minimal inhibitory concentration (MIC) values of ofloxacin to *S*. Typhimurium were 0.19 µg/ml and 8 µg/ml to *L. fermentum* ME-3. After cocultivation of *S*. Typhimurium and *L. fermentum* ME-3 a six-fold decrease in the MIC values (0.032 µg/ml) of ofloxacin was observed. We considered *S*. Typhimurium susceptible to ofloxacin according to NCCLS quidelines as the MIC breakpoint value to ofloxacin is ≤2 µg/ml (Wayne, 2006).

5.2. The survival of mice

The survival of mice challenged with *S*. Typhimurium was high. Altogether four mice died (4/50; 8%).

Namely, 2/16 mice in *S.*Typhimurium challenged Gr 2 in study I died on Day 8 and 9 respectively and 2/22 in *S*. Typhimurium challenged Gr 1 from study II on Day 8. We excluded the data of deceased mice from further analysis. One mouse died in Gr 3 challenged with *S*. Typhimurium and pre-and post-treated with *L. fermentum* ME-3 and *L. acidophilus* in study I.

All mice from studies III and IV survived the experiment.

5.3. Microbiological data

5.3.1. Detection of *S.* Typhimurium

In study I, the number of mice with viable *S*. Typhimurium in the blood, liver and gut was similar in Gr 2 of mice infected with *S*. Typhimurium and in Gr 3 pre-treated with *L. fermentum* and *L. acidophilus* before challenge with *S*. Typhimurium.

The presence of *S*. Typhimurium was highest in the liver 12/14 in Gr 2 and 8/13 in Gr 3, in gut 7/14 and 6/13, and in heart blood 3/14 and 3/13, respectively.

Figure 5 depicts the results of study II and III. The number of mice with viable *S*. Typhimurium in the gut decreased in both ofloxacin containing treatment regimens. Namely, ofloxacin treatment ($p<0.006$) and its combination with *L. fermentum* ME-3 ($p<0.001$). However, the decrease in the number of mice with viable *S*. Typhimurium in the liver was observed only if *L. fermentum* was added to ofloxacin treatment ($p<0.001$) (Fig. 5).

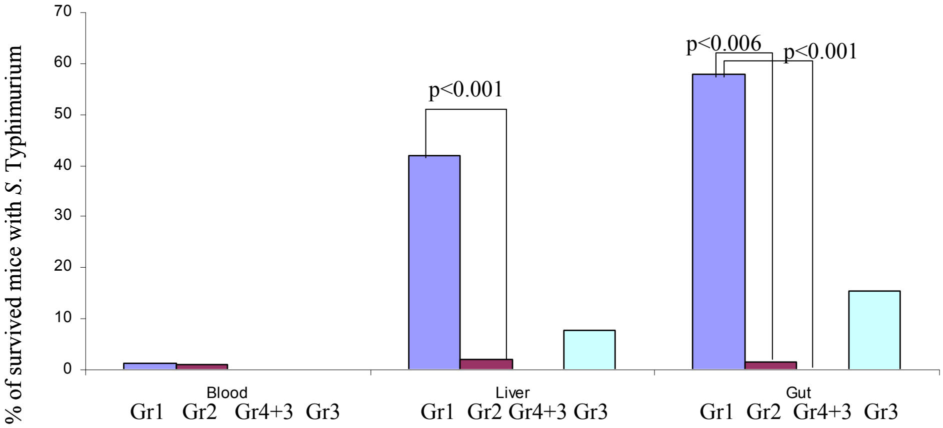

Figure 5. Proportion (%) of survived mice with viable *Salmonella* Typhimurium in the blood, liver and gut of mice groups challenged with *S.* Typhimurium (Summarized results of mice sacrificed on Day 10 from studies II and III). Gr 1 (n=26) indicates mice challenged with *S.* Typhimurium. Gr 2 (n= 19) stands for mice challenged with *S.* Typhimurium mice and treated with ofloxacin. Gr 4 from study II +Gr 3 from study III (n=19) received ofloxacin and *L. fermentum* ME-3 after challenge with *S.* Typhimurium. Gr 3 mice (n=13) from study II were administered *L. fermentum* ME-3 after *S.* Typhimurium (Fisher exact test).

5.3.2. Total count of lactobacilli in gut of mice

After application of three strains of lactobacilli of human origin from different fermentation groups: *L. fermentum* ME-3 belongs to OHEL group, *L. acidophilus* E-1 to OHOL, and *L.plantarum* Inducia to FHEL group, we found the unexpected results in the total count of lactobacilli (range and median in log cfu/g)

In study I, the intake of *L. fermentum* ME-3 and *L. acidophilus* E-1 for 15 days reduced the total count of lactobacilli in ileum if compared to the control group that received PBS (8.3–9.6/8.8 *vs.* 8.7–10.5/9.6, p<0.05 respectively). In studies II and III, no statistically significant alterations in the total number of lactobacilli were detected. In contrast to that, the increase of total count of lactobacilli was detected in the ileum and colon of mice after longer administration (30 days) of *L. plantarum* containing cheese in study IV (Table 2).

Still, the higher counts of intestinal lactobacilli were associated with absence of granulomas in the liver of *S.* Typhimurium experimental infection in study II. Namely, we found a statistically significant difference (p=0.002) when we compared the total intestinal lactobacilli counts from the mice with and without granulomas in the liver. Moreover, in study III the total number of lactobacilli in the small intestine of groups administered *L. fermentum* ME-3 (Gr 3 and Gr 4) was negatively correlated with the values of INF-γ (r=–0.422; p=0.039) and positively correlated with IL-10 (r=0.551; p=0.005).

Table 2. Total count of lactobacilli (log cfu/g) in faeces (days 0, 10 and 15) and in the ileum and colon (obtained at autopsy on Day 30) (range/median log cfu/g) of mice fed with cheese containing *L. plantarum* Inducia DSM 21379.

Faecal samples Days 0–15 Intestinal samples Day 30	Control group (cheese without *L. plantarum* Inducia DSM 21379 was administered) (n=10)	Test group (cheese containing *L. plantarum* Inducia DSM 21379 was administered) (n=10)
Day 0	6.7	7.6
Day 10	8.0	8.3
Day 15	7.0	8.0
Day 30 ileum	3.0–7.1/5.95*	6.3–7.7/6.95*
Day 30 colon	4.4–7.3/6.65**	6.9–7,8/7.45**

Student t-test *p= 0.001; **p<0.05

The differences in the total counts of lactobacilli in different experiments are probably due to *Lactobacillus* sp. strain specificity and the duration of intake of the strains. Namely, *L. plantarum* Inducia was administered for a longer period than in studies with *S.* Typhimurium infection (30 days *vs* 15 and 10 days).

Another important issue is the different carrier substances used for lactobacilli during separate studies: ultra-pastorized milk (study I), water (study II and III), and cheese (study IV). Hence, different microbial communities were involved, *e.g.* beside several starter strains the carrier cheese contains some nonstarter LAB strains that have survived pasteurization. Moreover, dietary calcium, present in milk, has been recognised to decrease colonisation and translocation of intestinal Gram-negative pathogens both in rats (Bovee-Oudenhoven *et al.*, 1999) and in humans (Bovee-Oudenhoven *et al.*, 2003). The dietary intake of calcium and phosphate results in the formation of a calcium phosphate complex in the proximal part of small intestine that adsorbs and precipitates luminal cytotoxic components, *e.g.* bile acids and fatty acids and reduces epithelial cell damage. This subsequently stimulates growth of endogenous lactobacilli (Bovee-Oudenhoven *et al.*, 1999), which exert antagonistic activity towards foodborne pathogens (Trautvetter *et al.*, 2011).

5.4. Morphological data

In study I, the number of mice with granulomas in the liver was similar in mice infected with *S.* Typhimurium of Gr 2 and in the ones of Gr 3 pretreated with *L. fermentum* and *L. acidophilus* (11/14 and 10/13, respectively).

Figure 6 depicts summarized results of mice sacrificed on Day 10 from studies II and III. The addition of *L. fermentum* ME-3 to ofloxacin decreased the number of mice with granulomas both in the liver (p<0.001), and in the spleen (p<0.001) if compared to the ones infected with *S.* Typhimurium. In the spleen, the ofloxacin treatment after *S.* Typhimurium challenge decreased the number of mice with granulomas compared to mice infected with *S.* Typhimurium (p<0.001) (Fig. 6).

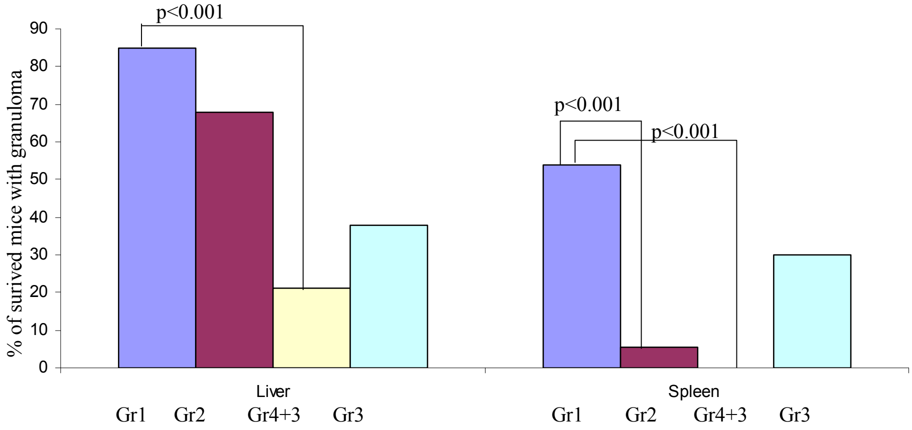

Figure 6. Proportion of survived mice with granuloma in the liver and/or spleen of mice groups challenged with *S.* Typhimurium (Summarized results of mice sacrificed on Day 10 from studies II and III). Gr 1 (n=26) indicates *S.* Typhimurium challenged mice. Gr 2 (n= 19) stands for mice challenged with *S.* Typhimurium and treated with ofloxacin. Gr 4 from study II +Gr 3 from study III (n=19) received ofloxacin and *L. fermentum* ME-3 after challenge with *S.* Typhimurium. Gr 3 mice (n=13) from study II were administered *L. fermentum* ME-3 after *S.* Typhimurium (Fisher exact test).

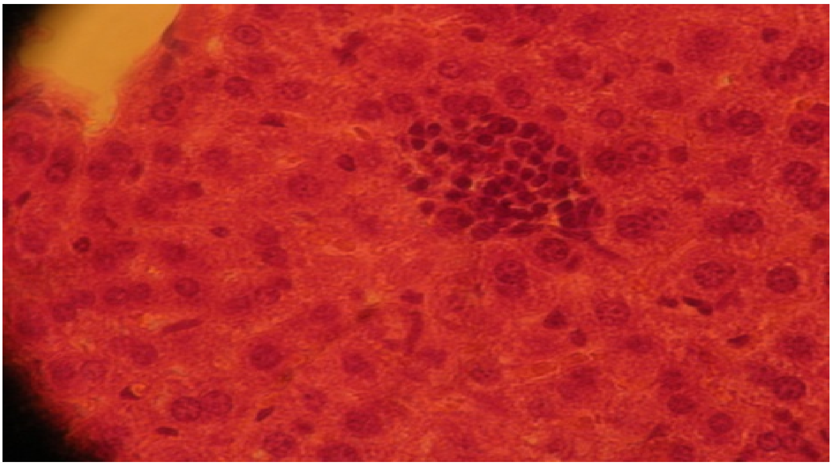

Figure 7. Histological sample (H&E,x400) of the liver granuloma from a mouse challenged with *S.* Typhimurium (Gr 2, study I).

Our results concerning the formation of granulomas in the liver and spleen are in accordance with an earlier study (Thygesen *et al.*, 2000). *Salmonella* Typhimurium was rapidly taken up by the reticuloendothelial system and induced reversible changes in the spleen of rats. The formation of granuloma and infiltration with macrophages was detected on Day 3 after the challenge with *S*. Typhiumurium (Thygesen *et al.*, 2000).

Next we evaluated the hyperplasia of lymphoid follicles in gut. According to scoring results, we found that all tested bacteria, *L. fermentum* ME-3, *L. acidophilus* E-1 and *S.* Typhimurium influenced the number of lymph follicles of gut in study I. The hyperplasia of lymph follicles increased in Gr 2 mice challenged with *S*. Typhimurium and Gr 4 mice administered *L. fermentum* ME-3 and *L. acidophilus* E-1 as compared to the mice of Gr 1 that received PBS ($p<0.01$; $p<0.05$, respectively).

Similarly, the intake of cheese containig *Lactobacillus plantarum* Inducia DSM 21379 during a month enhanced the count of lymphatic follicles in the ileum 3.5 times and in colon 6 times in comparison with control mice in study IV (Table 3). These results refer to the trend of enhancement of the defence capability of gut-associated lymphoid tissue by administration of probiotic lactobacilli.

Table 3. Number of mice with increased count of lymph follicles (scores 2 and 3) after administration of cheese containing *Lactobacillus plantarum* Inducia DSM 21379 for 30 days.

Mice group	Ileum	Colon
Animals consumed cheese containing *L. plantarum* Inducia DSM 21379	6/10	8/10
Control cheese consumed animals	3/10	4/10

In ileum OR 3,5 (95%CI 0.5–22.3)
In colon OR 6.0 (95%CI 0.8–44)

5.5. Biochemical indices

5.5.1. Study I

In study I, we evaluated the impact of *S*. Typhimurium on OxS indices, *i.e.* the lipid peroxidation, the ratio of oxidised and reduced glutathione, and saturation of Fe. The OxS indices increased significantly in mice challenged with *S*. Typhimurium as compared to the control animals receiving PBS ($p<0.01$; $p<0.05$, respectively) (Table 4).

Treatment of the mice with *L. fermentum* ME-3 and *L. acidophilus* E-1 before and during experimental infection had a positive influence on excessive OxS indicative parameters, except glutathione redox ratio. The reduction of

LPO, iron content, iron binding protein saturation, and the increase of the values of the gut mucosal TAA as compared to the same indices of *S*. Typhimurim challenged mice was found ($p<0.05$).

The mice not challenged with *S*. Typhimurium and administered *L. acidophilus* and *L. fermentum* ME-3 showed moderate changes in oxidative stress indices as compared to the PBS group: the elevated LPO ($p<0.01$).

Table 4. The mean values with standard deviations of LPO, TAA**,** GSSG/GSH, Fe, and saturation with Fe content in ileum mucosa in study I.

Mice groups	LPO (pmol/mg protein) in gut	TAA (%)	GSSG/GSH	Fe (μmol/L)	Saturation with Fe (%)
PBS	$109±6.7^{1/2/3}$	$38±0.5^{1/2}$	$0.12±0.02^{1/2}$	$10±6^{5}$	$15±6^{5}$
S. Typhimurium	$297±58^{1/4/5}$	$41±12^{5}$	$0.44±0.375^{1}$	$23±12^{5/6}$	$29±14^{5/6}$
L. fermentum ME-3+*L. acidophilus* E-1+ *S*. Typhimurium	$224±46^{2/5}$	$51±5^{1/\,3/5}$	$0.34±0.15^{2/3}$	$15±10^{6}$	$20±10^{6}$
L. fermentum ME-3+*L. acidophilus* E-1	$157±41^{3/4}$	$30±2^{2/3}$	$0.1±0.03^{3}$	11±5	18±8

Common numbers in superscription indicate statistically significant differences ($^{1,\,2,\,3,\,4}$ $p<0.01$; ^{5,6} $p<0.05$). All comparisons are performed between particular indices of different study groups.

5.5.2. Study II and III

We compared the results of LPO and GSSG/GSH in the gut in five groups with the group of mice infected with *S*. Typhimurium and thereafter treated with ofloxacin and *L. fermentum* ME-3 mice as a reference group. The indices of oxidative stress, *i.e.* the level of LPO and the ratio of oxidised and reduced glutathione were the highest in mice challenged with *S.* Typhimurium.

The ratio of oxidised and reduced glutathione decreased in the small intestine by combined ofloxacin and *L. fermentum* ME-3 treatment when compared to mice challenged with *S.* Typhimurium ($p<0.005$) (Fig. 8).

Similarly, the addition of *L. fermentum* ME-3 to ofloxacin treatment reduced the LPO values when compared to mice infected with *S.* Typhimurium ($p<0.005$), while the corresponding values of PBS group were lower ($p<0.005$) (Fig. 9).

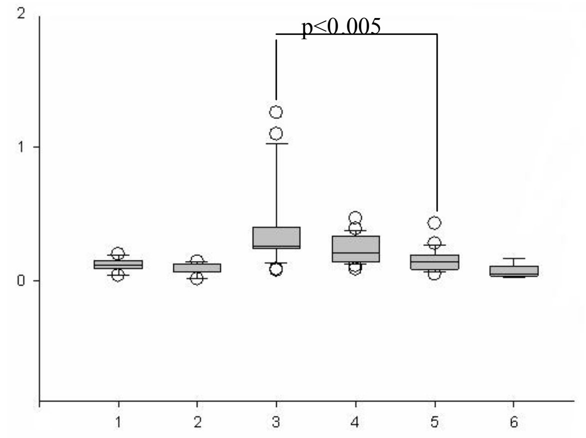

Figure 8. GSSG/GSH (range and median) in the gut of mice from study II and III. The numbers on x-axis indicate different mice groups. Gr 1 (n=17) mice received PBS by intragastric gavage. Gr 2 (n=12) study III mice were administered *L. fermentum* ME-3. Gr 3 mice (n=26) were challenged with *S.* Typhimurium. Gr 4 mice (n=19) were challenged with *S.* Typhimurium and treated with ofloxacin. Gr 5 mice (n=19) were challenged with *S.* Typhimurium and treated with ofloxacin and *L. fermentum* ME-3. Gr 6 mice (n=13) received *L. fermentum* ME-3 after challenge with *S.* Typhimurium. The Kruskal-Wallis One-Way Analysis of Variance on Ranks.

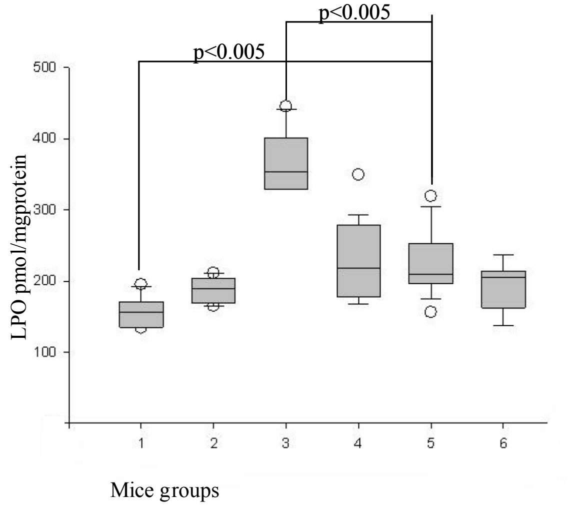

Figure 9. LPO (range and median) in the gut of the mice from study II and III. The numbers on x-axis indicate different mice groups. Gr 1 (n=17) mice received PBS by intragastric gavage. Gr 2 (n=12) study III mice were administered *L. fermentum* ME-3. Gr 3 mice (n=26) were challenged with *S.* Typhimurium. Gr 4 mice (n=19) were challenged with *S.* Typhimurium and treated with ofloxacin. Gr 5 mice (n=19) were challenged with *S.* Typhimurium and treated with ofloxacin and *L. fermentum* ME-3. Gr 6 mice (n=13) received *L. fermentum* ME-3 after challenge with *S.* Typhimurium. The Anova with Bonferroni correction was applied.

5.6. Immunological indices

5.6.1. Profile of cytokines in the small intestine and liver of mice challenged with *S.* Typhimurium on Day 5 and Day 10

The profile of cytokines was tested in study III. The values of pro-inflammatory cytokines INF-γ and TNF-α in the small intestine were the highest after inoculation with *S.* Typhimurium (Gr 1) at Day 10 (Table 5).

To find the impact of combined treatment to the profile of cytokines, the mice from Gr 3 (mice challenged with *S.* Typhimurium and treated with ofloxacin and *L. fermentum* ME-3) were set as a reference to all mice groups challenged with *S.* Typhimurium. The addition of *L. fermentum* to ofloxacin treatment reduced the values of the tested pro-inflammatory cytokines: INF-γ in the the liver on both days (p=0.04, p=0.002, respectively), and TNF-α on both days (p=0.03, 0.04 in the liver and 0.02, 0.004 in the small intestine) when compared to Gr 1. The values of anti-inflammatory IL-10 in the liver increased on both experimental days when compared to mice challenged with *S.* Typhimurium Gr 1 (p=0.01, 0.005, respectively).

The additive effect of the probiotic to ofloxacin was confirmed by the immunomodulatory response: decrease of the values of INF-γ and TNF-α on Day 5 in the liver (p=0.0007; p=0.02, respectively) when compared to Gr 2.

The presence of typhoid nodules in the liver was associated with high values of pro-inflammatory INF-γ in the liver on both tested days (p=0.002 and p=0.039, respectively) (Table 6). The absence of typhoid nodules was associated with high IL-10 values in the liver on Day 10 (p=0.001). Moreover, the degree of necrosis of the typhoid nodules in the liver was associated with the increase of TNF-α in the small intestine (R^2=0.18, p=0.002) in mice challenged with *S.* Typhimurium (data not shown).

Table 5. The concentration of cytokines (median; range) in the small intestine and liver of mice challenged with *S.* Typhimurium on Day 5 and Day 10.

Group n=6	Day of autopsy	INF-γ (pg/mg tissue)		TNF-α (pg/mg tissue)		IL-10 (pg/mg tissue)	
		small intestine	liver	small intestine	liver	small intestine	liver
Gr 1 *S.*Typhimurium	5	73 (56–78)	157 (88–298)^{a}	83 (27–148)^{a}	130 (95–272)^{a}	81 (72–126)	129 (66-202)^{a}
	10	78 (68–101)^{b}	124 (67–238)^{b}	156 (136–198)^{b}	161 (106–194)^{b}	74 (32–181)	165 (144–224)^{b}
Gr 2 *S.*Typhimurium+ ofloxacin	5	56 (16–86)	224 (42–533)^{a}	52.5 (15–116)	127 (42–196)^{a}	95 (59–121)	192 (111–210)
	10	55 (31–70)	92 (8–377)	69 (32–166)	120 (74–256)	82 (1–108)	235 (122–300)
Gr 3 *S.*Typhimurium+ Ofloxacin+ *L. fermentum* ME-3	5	53 (36–86)	60 (22–129)^{a}	33.5 (13–53)^{a}	52 (1–159)^{a}	89 (72–140)	223 (126–315)^{a}
	10	46 (30–69)^{b}	19 (2–24)^{b}	39 (28–200)^{b}	120(78–138)^{b}	68 (28–116)	265 (160–422)^{b}

Mann-Whitney test was applied.

[a] Significant differences ($p<0.05$) between Gr 3 *S.*Typhimurium+ofloxacin+*L. fermentum* ME3 and test groups on Day 5

[b] Significant differences ($p<0.05$) between Gr 3 *S.*Typhimurium+ofloxacin+*L. fermentum* ME3 and test groups on Day 10

Table 6. The median values and ranges of cytokines (pg/mg tissue) in liver in the presence /absence of liver typhoid nodules on Day 5 and Day 10 in mice challenged with *S.*Typhimurium.

Indices	Typhoid nodules		No typhoid nodules		p-values
	Day 5 (n=5)	Day 10 (n=13)	Day 5 (n=13)	Day 10 (n=5)	
INF-γ	272 (223–533)^{a}	113 (8–377)^{b}	113 (22–30)^{a}	26 (2–63)^{b}	^{a} 0.002 ^{b} 0.039
TNF-α	138 (95–196)	74 (10–256)	116 (10–272)	126 (80–134)	NS
IL-10	113 (108–210)	178 (22–300)^{c}	198 (66–315)	292 (238–422)^{c}	^{c} 0.001

Common letters indicate p- values. Mann-Whitney method was applied.

5.6.2. Profile of cytokines in the small intestine and liver of mice administered *L. fermentum* ME-3and mice of control group on Day 5 and Day 10

In non-infected mice the administration of *L. fermentum* ME-3 reduced the amount of pro-inflammatory cytokine TNF-α in the small intestine and liver on Day 5 (p=0.015, 0.002) and increased anti-inflammatory cytokine IL-10 in the small intestine (p=0.004) and liver on Day 5 (p=0.004) and Day 10 (p=0.014) as compared to control group mice (Fig. 10 and 11). The increase of INF-γ was detected on Days 5 and 10 (p=0.001, p<0.001) in the liver though no shifts were found in the gut. Further, the total number of lactobacilli in the small intestine of mice groups who were administered *L. fermentum* ME-3 was in a negative correlation (r=–0.422; p=0.039) with the values of INF-γ and in a positive correlation with IL-10 (r=0.551; p=0.005).

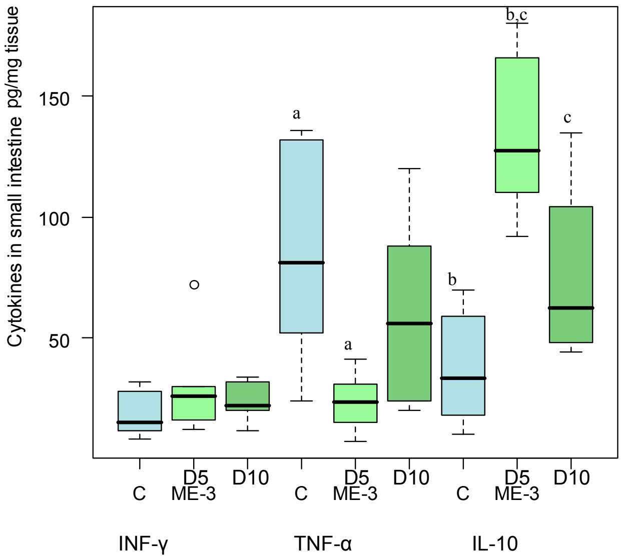

Figure 10. The concentration of INF-γ, TNF-α and IL-10 (range and median) in the small intestine of uninfected mice: control C with PBS and probiotic *L. fermentum* ME-3 (ME-3) on Day 5 is marked with light green and Day 10 is marked with dark green. Common letters indicate statistically significant differences: **a** – p=0.015, b – p=0.004, c – p=0.017

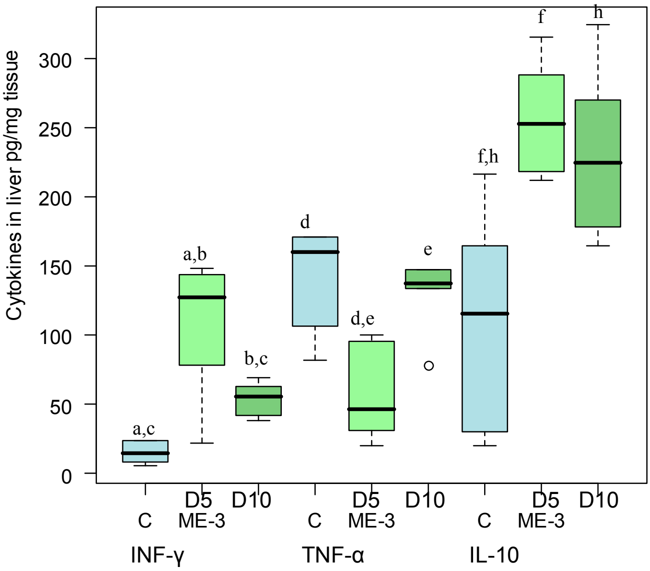

Figure 11. The concentration of INF-γ, TNF-α and IL-10 (range and median) in the liver of uninfected mice: control C with PBS is shown with blue and probiotic *L. fermentum* ME-3 (ME-3) on Day 5 is marked with light green and Day 10 is marked with dark green. Common letters indicate statistically significant differences: a – p=0.001, b – p=0.026, c – p= 0.002, d – p=0.002, e – p=0.004; f – p=0.014

6. GENERAL DISCUSSION

In our study the hypothesis concerning the additive value of probiotic *Lactobacillus* sp. to antimicrobial treatment was proved by the reduction of persistent *Salmonella* infection and the putative mechanisms behind it were assessed (Fig.12).

The persistence of infection was studied in *S.* Typhimurium experimental murine infection model. Experimentally induced *Salmonella* Typhimurium infection in mice and rats is a widely used model for typhoid fever in humans. Pathogenetically it resembles the acute phase of typhoid fever caused by *S.* Typhi (Monack *et al*., 2004; Andrews-Polymenis *et al*., 2010). The human and rodent diseases are similar: the ileum is the main site of bacterial colonization and invasion, and there is a bacterial proliferation and response with granulomas within the reticuloendothelial system (Naughton *et al*., 1996).

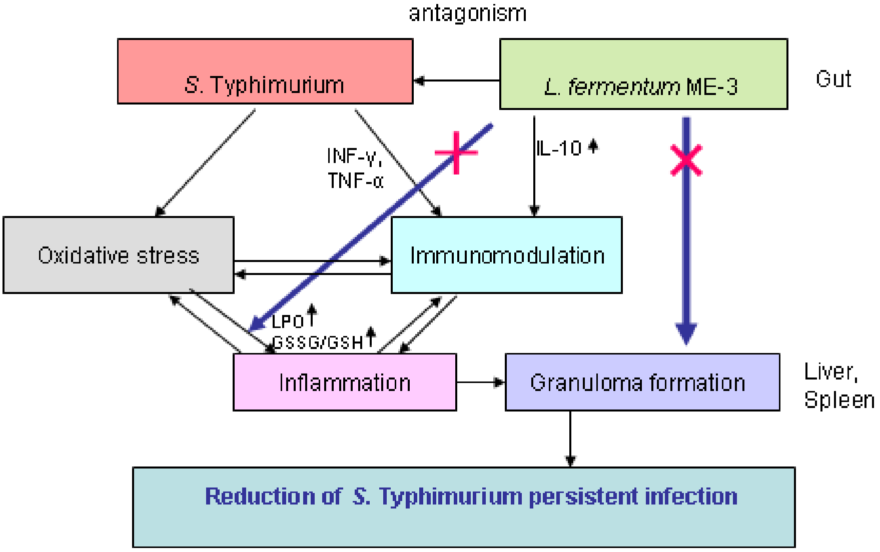

Figure 12. Summary of the detected microbiological, morphological, biochemical, and immunological effects. Arrows indicate detected effects of the tested microbes: *S.* Typhimurium, and *L. fermentum* ME-3.

6.1. Role of immunological and oxidative stress indices on the development of persisting *Salmonella* Typhimurium infection

The persistence of the infection was proved by the presence of granulomas in the liver and spleen on Day 5 and 10. It has been shown earlier that the majority of *S*. Typhimurium bacteria are localized within phagocytes in the granulomas in the liver or spleen (Clare *et al.*, 2003).

In order to characterize the impact of oxidative stress on persistent infection the following indices were detected: lipid peroxidation and glutathione redox ratio, iron concentration and saturation of iron. Our results indicated the presence of OxS in the developed persistent infection.

For detection of immunological impact we chose the pro-inflammatory cytokines INF-γ, TNF-α, and the anti-inflammatory IL-10. These cytokines have been previously studied in the persistence of the infection due to *S. enterica* serovar Typhimurium in mice (Sashinami *et al.*, 2006). The expression of pro-inflammatory cytokines is associated with the release of ROS and disruption of the total antioxidant response pathways (Murata *et al.*, 2002). This accordance was also found in our studies. The increased values of pro-inflammatory cytokines in infected mice were accompanied by the increased oxidative stress indicating values of LPO, GSSG/GSH, and Fe content, and low TAA in the small intestine.

In the initial stage of inflammation after invasion of *S*. Typhimurium, the pro-inflammatory cytokines (INF-γ, IL-1, IL-6, IL-12, and IL-18) are activated (Mittrucker *et al.*, 2000). Similarly, our data depicted the increased values of the pro-inflammatory cytokine INF-γ in the small intestine and in the liver on Day 5 of infection and persistence until Day 10. Moreover, the high values of INF-γ in the liver correlated with the presence of typhoid nodules.

TNF-α is involved in the formation and persistence of granulomas as well as in the regulation of NADPH oxidase-mediated killing of *Salmonella* Typhimurium by macrophages (Beal *et al.*, 2006). In our study III, the increase of the values of TNF-α in the gut seemingly reflect the inflammatory damage of intestinal mucosa in the small intestine, enabling the invasion of *S*. Typhimurium into the blood and organs. Also, on Day 10 of *S*. Typhimurium infection we found the association between the degree of necrosis of typhoid nodules in the liver and the increase in TNF-α in the small intestine in mice challenged with *S*. Typhimurium.

It has been shown earlier that the anti-inflammatory and regulatory cytokine IL-10 inhibits the production of reactive oxygen and reactive nitrogen species, and intermediates when macrophages are activated by INF-γ. Namely, it inhibits TNF-α and IL-12 production by macrophages and their stimulatory effect of INF-γ production by natural killer cells (Sashinami *et al.*, 2006). We found that the absence of typhoid nodules was associated with high IL-10 values in the liver on Day 10. We suggest that the high values of IL-10 may protract the development of liver granulomas and development of persistent infection.

6.2. The impact of lactobacilli on immunological and oxidative stress indicative indices of gut without and with infection

The immunomodulatory activity of probiotics is considered strain-specific and cannot be extrapolated to other genera or species (Dogi *et al.*, 2008). Similar trends were obtained in our study. We found that *L. fermentum* ME-3 increased the values of IL-10 in the mucosa of the small intestine and liver. When *L. acidophilus* E-1 and *L. fermentum* ME-3 were administered to mice in study I, the hyperplasia of lymph patches in small intestine was detected indicating the increased barrier function of gut mucosa. However, the counts of intestinal lactobacilli did not increase. The third tested probiotic *L. plantarum* Inducia also induced hyperplasia of lymph patches and after a longer one month period of administration the total counts of lactobacilli increased. Thus, the effect of probiotic bacteria on immunemodulation is beside strain-specificity also tightly bound to the duration of administration.

The effects of probiotics on the immune system are exerted through effects on antigen-presenting cells, such as macrophages and dendritic cells. Among the cytokines that are produced by these cells, particular attention has been paid to the probiotic control of the production of IL-12, which plays a central role in the activation of innate immunity, and IL-10, which, in contrast, acts to inhibit the inflammatory response (Foligne *et al.*, 2007).

In addition, *in vitro* studies have revealed that due to their effects on APCs, probiotics may affect the differentiation into Th cell subsets and the production of cytokines therein. It has been revealed that lactobacilli, such as *Lactobacillus casei* Shirota or *Lactobacillus reuteri* ATCC 23272, induce Th1 cells via the production of IL-12 generated by macrophages, and DCs (Mohamadzadeh and Klaenhammer, 2008) and *Bifidobacterium bifidum* W23 and *Bifidobacterium longum* W52 inhibit the production of cytokines generated by Th2 cells via the production of IL-10 generated by monocytes (Maassen *et al.*, 2000). The probiotic *L. casei* has considerable potential to induce IL-12 production and promote Th1 cell development (Chiba *et al.*, 2010).

Previous *ex vivo* experiments have reported the ability of *L. casei* and of *L. bulgaricus* to downregulate TNF-α production in colonic explants from patients with Crohn’s disease, thus supporting the possibilities for their future development in IBD therapy (Peran *et al.*, 2005). The suppression of this cytokine was found also in our model of systemic salmonella infection treated with probiotic *L. fermentum* ME-3.

Antioxidative and antagonistic activity of intestinal lactobacilli varies between species and strains (Achuthan, 2012). Thus, it can also be related to their fermentation type (Annuk *et al.*, 2003). For instance, the production of succinic acid by heterofermentative *L. fermentum* species supports the antioxidative effects (Stsepetova, 2011). Moreover, antioxidative *Lactobacillus* could modulate a redox state in colonic fermentation system, which is related to their free radical–scavenging ability or antibacterial effect (Sun *et al.*, 2010). The *L. fermentum* ME-3 strain we applied in our studies is characterized by a complete glutathione system: synthesis, uptake and redox turnover ability (Kullisaar *et al.*, 2010), and high TAA and TAS values of intact cells and lysates (Mikelsaar and Zilmer, 2009). At the same time, the antimicrobial effect against gastroenteritis causing *S.* Typhimurium, *S. sonnei*, and uropathogenic *E. coli* (Hutt *et al.*, 2006) is accompanied with the production of H_2O_2 (Mikelsaar, 2007). The antioxidative properties of *L. fermentum* ME-3 depend on the growth phase and environmental conditions (aerobic, anaerobic). The TAA values and production of H_2O_2 depends on the growth phase of *L. fermentum* ME-3. In the exponential growth phase the production of H_2O_2 increased and TAA decreased, in stationary phase H_2O_2 concentration remained stable and TAA values were at the highest level (Kullisaar *et al.,* 2010).

In our study I we applied two different lactobacilli of human intestinal origin: namely, *L. acidophilus* E-1 and *L. fermentum* ME-3. The administration of these lactobacilli decreased the total count of lactobacilli in the terminal ileum of mice; however, hyperplasia of lymph nodes was registered in the group of mice receiving both aforementioned lactobacilli. At the same time, the antioxidative potential of *L. fermentum* ME-3 influenced the gut mucosa by the reduction of iron level, and lipid peroxidation with simultaneous increase of total antioxidative activity and glutathione redox value. One explanation is probably the complete glutathione system of *L. fermentum* ME-3: synthesis, uptake and redox turnover ability (Kullisaar *et al.*, 2010). Antioxidative *Lactobacillus* could modulate redox state in gut fermentation systems, which is related to their free radical–scavenging ability or antibacterial effect (Sun *et al.*, 2010). DeLeBlanc *et al* 2010 determined the preventive and therapeutic effect of *L. casei* CRL 431 in protection against murine *Salmonella* Typhimurium infection. The results obtained demonstrated that 7 days of *L. casei* CRL 431 administration before infection decreased the severity of the infection with *Salmonella* Typhimurium due to the influence on the cells of the innate and adaptive immune response. Namely, the neutrophil infiltration decreased; the macrophage phagocytic activity was activated in different sites, and the number of IgA+cells in the lamina propria of the small intestine increased and correlated well with the increased release of s-IgA specific antibodies against the pathogen in the intestinal fluids (de LeBlanc and Ade, 2010).

Mileti *et al* 2009 compared the immunological properties of *Lactobacillus plantarum* NCIMB8826, *L. rhamnosus* GG (LGG), and *L. paracasei* B21060 against pathogenic *Salmonella* Typhimurium (SL1344) and found that the three strains exhibited different abilities to induce inflammatory cytokine production by DCs with *L. plantarum* being the most effective followed by LGG and *L. paracasei* (Mileti *et al.*, 2009).

L. fermentum ACA-DC 179 was a strong inducer of the anti-inflammatory regulatory cytokine IL-10 (Zoumpopoulou *et al.*, 2008). Additionally, when *L. fermentum* ACA-DC 179 was used in a Salmonella-infected mouse model, its administration revealed an *in vivo* anti-Salmonella activity. These results coincide with our results as *L. fermentum* ME-3 caused significant increase of IL-10 in study III.

6.3. Impact of the combination of lactobacilli and antibiotic on *S.* Typhimurium infection.

In our experimental infection, application of ofloxacin could not prevent the formation of granulomas in the liver and spleen of mice infected with *S.* Typhimurium, despite the fact that our applied S. Typhimurium was susceptible to ofloxacin. However, the antioxidative probiotic *L. fermentum* ME-3 combined with ofloxacin enhanced the eradication of experimental *S.* Typhimurium infection. *Salmonella* Typhimurium was eradicated from the blood, ileum and liver, the number of animals with liver and spleen granulomas decreased and the value of lipid peroxides in the ileum mucosa reduced. Moreover, we found that higher total counts of intestinal lactobacilli were associated with the absence of liver granulomas. The immunological response included a reduction of pro-inflammatory cytokines INF-γ, TNF-α and an increase in anti-inflammatory cytokine IL-10 in the livers of mice without typhoid nodules.

Several lactobacilli have proved efficacy in different diseases in experimental studies. Recently, Ashara and his colleagues have found that after oral infection with *S.* Typhimurium DT104 during fosfomycin treatment continuous administration of *Lactobacillus casei Shirota* inhibited the infection due to the increased concentration of organic acids and maintenance of lower pH in the intestine (Ashara *et al.,* 2010).

The probiotic bacteria *Lactobacillus plantarum 299v* given daily enterally to critically ill patients on broad-spectrum antibiotic therapy survived the passage through the gastrointestinal tract and colonized the rectal mucosa. Their administration increased the number of lactobacilli and reduced the number of *Enterobacteriaceae*. The authors concluded that *L. plantarum* 299v may have an effect on the mucosal barrier or even have a positive impact on the immune system (Klarin *et al.*, 2005). This finding coincides with our results of hyperplasia of the lymph nodes in the gut.

7. LIMITATIONS OF THE STUDY

The thesis has some limitations. First, we applied culture-based methods to estimate the total count of lactobacilli in the gut. Thus, we could not differentiate between the indigenous lactobacilli and the count of particular administered *Lactobacillus* strains.

Second, as we did not aim to detect the impact of ofloxacin, the group receiving only ofloxacin without salmonella infection was not included. In order to exclude its impact on biochemical and immunological indices this group would have been helpful.

Third, in study IV, the number of mice was too low to demonstrate a statistically significant difference though a 3.5 to 6 times increase of lymphatic nodules by administration of probiotic *L. plantarum* Inducia was apparent.

8. CONCLUSIONS

1. The developed persistent *S.*Typhimurium infection is characterized by presence of viable *S*. Typhimurium in the blood and organs. The presence of granulomas in the liver and spleen is accompanied by increased levels of OxS indices (LPO, GSSG/GSH) and increased values of pro-inflammatory cytokines (TNF-α and INF-γ) in the gut and liver.

2. The application of two lactobacilli of human origin, *L. fermentum* ME-3 with high antimicrobial and antioxidative potential *in vitro* and *L. acidophilus* E-1 for 10 days, neither eradicated *S.*Typhimurium nor prevented the developement of granulomas in the liver. An improvement of gut mucosal barrier due to decreased values of lipid peroxides and glutathione redox ratio was still detected.

3. The administration of cheese containing *Lactobacillus plantarum* Inducia for one month increased the count of intestinal lactobacilli and hyperplasia of the lymphatic follicles in the ileum and colon of healthy mice indicating immunomodulation of the intestinal mucosa by probiotic lactobacilli.

4. The combined treatment of ofloxacin and *L. fermentum* ME-3 increased the eradication of *S.* Typhimurium and reduced the prevalence of granulomas in the liver and was accompanied by decreased OxS indices in persistent typhoid model of mice. The higher total count of lactobacilli in the gut was associated with the absence of liver granulomas.

5. The effect of administration of combined treatment of ofloxacin and *L. fermentum* ME-3 in persistent *Salmonella* Typhimurium infection is demonstrated by the immunomodulation in the gut and liver. On Day 10 after challenge with *S.* Typhimurium, the reduction of pro-inflammatory cytokine INF-γ in the liver was accompanied with an increase of anti-inflammatory cytokine IL-10 in the mice without typhoid nodules in the liver.

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10. SUMMARY IN ESTONIAN

Probiootiliste laktobatsillide toime eksperimentaalsele persisteerivale salmonella infektsioonile: mikrobioloogilised, morfoloogilised, biokeemilised ja immunoloogilised efektid

Salmonella enterica serovar Typhi nakkus kulgeb generaliseerunud infektsioonina, mille suremus adekvaatse ravita on kõrge. Ka paranemisel võib kujuneda oluliseks probleemiks püsiva e. persisteeriva infektsiooni teke, mis kulgeb kas kroonilise infektsioonina, korduvate ägenemistega või sümptomivaba kandlusena. Viimatinimetatu on nakkuse leviku tõttu ohuks ümbritsevatele inimestele. Ajaloost on teada isik, kes nakatas 57 inimest, olles *Salmonella* Typhi kandja ilma haigunähtudeta. *S.* Typhi kandjatel on risk haigestuda maksa ja sapiteede kasvajatesse.

Kuigi *Salmonella* Typhi infektsiooni esinemissagedus Eestis on madal, on arengumaades endiselt raskusi selle infektsiooni profülaktika ja raviga, ohustades ka reisijaid neisse maadesse. *S.* Typhi tekitab kõhutüüfust ainult inimestel, seetõttu kasutatakse selle generaliseerunud infektsiooni uurimismudelina liinihiirte nakatamist *Salmonella enterica* serovar Typhimurium tüvedega.

Laktobatsillid e. piimhappe bakterid kuuluvad inimese soole normaalsesse mikroobikooslusesse. Probiootikumid on rahvusvahelise määratluse järgi elusad mikroobid, mis küllaldases annuses mõjuvad tervisele kasulikult. Probiootikumide toimemehhanismidena on kirjeldatud otsest mõju patogeenidele tänu vesinikperoksiidi, piimhappe, bakteriotsiinide tekkele, konkurentsi toitainetele, adhesiooni ning immuunsuse modulatsiooni. Mitmetel probiootilistel laktobatsillidel on täheldatud antagonistlikku toimet soolepatogeenidele, sh. salmonelladele, kuid puudub arusaam täpsematest toimemehhanismidest, eeskätt persisteerimisele kalduvate infektsioonide puhul.

Uurimistöö eesmärgid

Selgitada erinevate laktobatsillide võimalikke toimemehhanisme salmonellade persisteeriva infektsiooni suhtes ja teha kindlaks probiootilise *Lactobacillus plantarum* Inducia DSM 21379 mõju soole immuunsüsteemile.
Selleks püstitati järgnevad ülesanded:

1. Määrata *S.* Typhimuriumiga nakatatud hiirtel *S.* Typhimurium veres, maksas, põrnas ja sooles ning laktobatsillide üldhulk sooles; granuloomide olemasolu maksas ja põrnas ning oksüdatiivse stressiga seotud näitajad (LPO, GSSG/GSH) ja tsütokiinid (TNF-α, INF-γ ning IL-10).
2. Teha kindlaks inimpäritolu laktobatsillide *Lactobacillus fermentum* ME-3 ja *Lactobacillus acidophilus* E1 toime persisteerivale *Salmonella* Typhimurium infektsioonile hiire mudelis, määrates salmonella ja laktobatsillide üldhulga, samuti oksüdatiivse stressiga seotud näitajad ning maksa, põrna ja soole histoloogilised muutused.

3. Selgitada *Lactobacillus plantarum* Inducia toimet soole laktobatsillide üldhulgale ja soole limaskesta barjäärile tervetel hiirtel peale 30-päevast *L. plantarum* Inducia't sisaldava juustu söötmist.
4. Hinnata, kas *L. fermentum* ME-3 lisamine ofloksatsiin ravile mõjutab *S.* Typhimuriumi elulemust veres, maksas, põrnas ja sooles; maksa ja põrna granuloomide teket ning oksüdatiivse stressi näitajaid sooles.
5. Määrata pro- ja antiinflammatoorsete tsütokiinide tase sooles ja maksas *L. fermentum* ME-3 lisamisel ofloksatsiinravile persisteeriva *S.*Typhimurium infektsiooni korral hiirel.

Materjal ja meetodid

Salmonella Typhimuriumi ja laktobatsillide mikrobioloogilisteks uuringuteks kasutasime erinevaid söötmeid ja kasvutingimusi. Külvid tehti verest, maksast, põrnast, peen- ja jämesoolest.

Histoloogilised näitajad määrasime koostöös TÜ Patoloogilise anatoomia ja Kohtumeditsiini instituudiga. Katse lõpus loomad lahati, koe lõigud maksast, põrnast ja peen-ning jämesoolest värviti hematoksüliin eosiiniga, seejärel mikroskopeeriti ja hinnati destruktiivseid ja põletikunäitajad.

Oksüdatiivse stressi näitajad ja tsütokiinid määrasime koostöös TÜ Biokeemia instituudiga. Soole limaskestast ja maksast võetud proovidest analüüsiti oksüdatiivse stressi näitajaid: LPO, GSSG/GSH, raua hulk ning pro- ja antiinflammatoorsed tsütokiinid INF-γ, TNF- α ja IL-10.

Järeldused

1. Persisteerivat *S.*Typhimurium infektsiooni iseloomustas eluvõimeliste *S.* Typhimurium bakterite esinemine veres ja uuritud organites; granuloomide esinemine maksas ja põrnas, kõrgenenud oksüdatiivse stressi näitajad (LPO, GSSG/GSH) ja proinflammatoorsed tsütokiinid (TNF-α and INF-γ) sooles ja maksas.
2. Inimpäritolu laktobatsilli *in vitro* kõrgete antimikroobsete ja antioksüdantsete omadustega *L. fermentum* ME-3 ja *L. acidophilus* E-1 tüve koos manustamine 10 päeva jooksul ei eemaldanud *S.*Typhimuriumi. Siiski leiti soole limaskesta barjäärfunktsiooni paranemine oksüdatiivse stressi langetamise tõttu tänu lipiidperoksiidide ning oksüdeeritud ja redutseeritud glutatiooni suhte vähenemisele.
3. *Lactobacillus plantarum* Induciat sisaldava juustu manustamine ühe kuu jooksul põhjustas laktobatsillide hulga tõusu ning peen- ja jämesoole lümfifolliikulite hüperplaasia nakatamata hiirtel, mis näitab soole kaitsevõime paranemist.

4. Kombineeritud ravi ofloksatsiini ja *L. fermentum* ME-3-ga suurendas *Salmonella* Typhimuriumi eradikatsiooni, vähendas granuloome maksas ja oksüdatiivse stressi näitajaid persisteeriva *S.* Typhimurium infektsiooni hire mudelis. Leidsime, et sooles laktobatsillide kõrgema üldhulga korral puuduvad maksas granuloomid.
5. Kombineeritud ravi ofloksatsiini ja *L. fermentum* ME-3-ga soodustas immuunvastust sooles ja maksas. 10 päeva pärast vähenes proinflammatoorne tsütokiin INF-γ maksas ja sooles ja suurenes antiinflamatoorse IL-10 hulk maksas ilma granuloomideta hiirtel.

Seega näitavad meie uurimistulemused mikrobioloogilist, morfoloogilist, biokeemilist ja immunoloogilist efekti kasutades antibiootikumi (ofloksatsiin) ja probiootilise laktobatsilli (*Lactobacillus fermentum* ME-3) kombineeritud ravi persisteeriva *S.* Typhimurium infektsiooni korral hiire mudelis.

11. ACKNOWLEDGEMENTS

This study was carried out in the Department of Microbiology, and Department of Biochemistry of University of Tartu. The study was supported by grants from the Estonian Science Foundation (grants 0418, 0411, 0182555, 5042 and 6782), the Estonian Technology Agency (funding 07/2002, 01103).

The studies were based on teamwork with the contribution of several persons whom I wish to express my gratitude.

Professor Marika Mikelsaar, supervisor of my PhD studies, for her advice and inspiring scientific discussions and also for her support throughout my studies.

Professor Irja Lutsar, for her constructive criticism in the review processes of papers and the thesis.

I thank all the co-authors of my publications for their invaluable help. Associate Professor Tiiu Kullisaar, senior researcher Kersti Zilmer, Dr Aune Rehemaa, Dr. Riina Mahlapuu and Professor Mihkel Zilmer for biochemical and immunological investigations. Professor Raik-Hiio Mikelsaar, Hannes Tamm for morphological studies.

I am grateful to Heti Pisarev and Karolin Toompere for their help in the statistical analysis of the data.

I would like to thank all the co-workers of the Bio-Competence Centre of Healthy Dairy Products LLC for their support and encouragement.

My greatest thanks go to Kalle Kisand, Ursel Soomets, Paul Naaber, Siiri Kõljalg, Pirje Hütt, Imbi Smidt and Epp Sepp for the critical reading of the manuscript and for their valuable comments.

I sincerely thank the technical personnel of the Department of Microbiology.

I wish to thank all my friends for their interest and encouragement.

I thank my family for their support and patience.

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Naaber, Paul; Stsepetova, Jelena; Smidt, Imbi; Rätsep, Merle; Kõljalg, Siiri; Lõivukene, Krista; Jaanimäe, Liis; Löhr, Iren; Natås, Olav; **Truusalu, Kai**; Sepp, Epp (2011). Quantification of Clostridium difficile in Antibiotic-Associated Diarrhea Patients. Journal of Clinical Microbiology, (accepted)

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Patent:

"Isolated Lactobacillus plantarum strain Inducia DSM 21379 as probiotic that enhances natural immunity and food products and medicinal preparations comprising it"; Omanik: Tervisliku Piima Biotehnoloogiate Arenduskeskus OÜ; Autorid: Marika Mikelsaar, Epp Songisepp, Imbi Smidt, Jelena Stsepetova, Mihkel Zilmer, Pirje Hütt, **Kai Truusalu**, Kalle Kilk; Prioriteedinumber: P200800027; Prioriteedikuupäev: 13.05.2008

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Truusalu K, Mikelsaar RH, Naaber P, Karki T, Kullisaar T, Zilmer M, Mikelsaar M. Eradication of *Salmonella* Typhimurium infection in a murine model of typhoid fever with the combination of probiotic *Lactobacillus fermentum* ME-3 and ofloxacin. BMC Microbiol. 2008 Aug 4;8:132.

Truusalu K; Naaber P; Kullisaar T; Tamm H; Mikelsaar R; Zilmer K; Rehema A; Zilmer M; Mikelsaar M. The influence of antibacterial and antioxidative probiotic lactobacilli on gut mucosa in a mouse model of Salmonella infection. Microbial Ecology in Health and Disease. 2004 16JJ:180–187.

Patentsed leiutised:

Isoleeritud mikroorganismi tüvi *Lactobacillus plantarum* Inducia DSM 21379 kui organismi loomulikku kaitsevõimet tõstev probiootik, seda sisaldav toiduaine ja kompositsioon ning mikroorganismi kasutamine rakulist immuunsust tõstva ravimi valmistamiseks; Omanik: Tervisliku Piima Biotehnoloogiate Arenduskeskus; Autorid: Marika Mikelsaar, Epp Songisepp, Imbi Smidt, Jelena Štšepetova, Mihkel Zilmer, Pirje Hütt, **Kai Truusalu**, Kalle Kilk; Prioriteedinumber: P200800027; Prioriteedikuupäev: 13.05.2008

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