Immunomodulating Lactobacilli in Chicken

Marjorie Koenen

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Immuunmodulerende lactobacillen in de kip

'With thanks to all sacrificed chickens'

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Introduction

The gastrointestinal tract

The gastro-intestinal (GI) tract of a chicken starts with the beak, followed by the esophagus and crop, proventriculus (glandular stomach), gizzard (muscular stomach), duodenum, ileum, a pair of blind elongated caeca, colon and ending in the cloaca. The GI-tract consists of a large, single cell layer of epithelial cells surface and a complex microflora that symbiotically interacts with the host. The surface is enlarged by the folds in the mucosal surface which are covered with villi, which in turn are covered by microvilli. For humans the surface of the skin is about 2 m^2, while the gut surface is 150-200m^2 (about the size of a tennis court). For chickens, these numbers are not available, but the ratio probably is also 100 times the skin surface. Villi are present throughout the small and large intestine. They are longest in the duodenum, but gradually shorten and thicken towards the colon. In the first part of the cloaca they are stumpy and rounded. Villi are present in the caeca also, becoming flattened toward the blind end.

Although the GI-tract is inside the body, the epithelial cell layer forms the border between inside and outside the body and is thus continuously in contact with the external environment. The GI-tract has conflicting functions: active transport of nutrients and water while keeping bacteria, viruses, toxins and potentially antigenic food molecules outside. Transport is arranged by diffusion, facilitated diffusion and active transport. The barrier is formed by the combination of a confluent single epithelial cell layer covered with mucus on the lumen side and a supportive underlying connective tissue layer. They form the border between the internal and external milieu, integrated with specific and non-specific immunological defense mechanisms. The gut-associated lymphoid tissue (GALT) comprises all cells and tissues along the alimentary tract from beak to cloaca. It includes organized structures like cecal tonsils (situated near the ileocolonic junction), Meckel's diverticulum (or diverticulum vitellinum, formed around the connection between yolk sac and embryonic intestine, situated at the jejunum), lymphoid aggregates and single cells in the lamina propria and epithelium (137).

The epithelial wall of the intestine of the chicken is similar to that of mammals, although Peyer's patches (in organized, clear structures as in mammals) are absent (18). Furthermore chickens duodenal glands are absent and the submucosa is extremely thin (7). M-cells exist in the chicken, but their phenotype and function are less well distinguished from neighbor epithelial cells than observed in mammals (138). All epithelial cells are capable of absorption from the lumen (137). The running time of food through the GI-tract is 10 times shorter in chickens than in humans. In humans the total transit time through the gastrointestinal tract is between 55-72 hours (162), while in chickens it is around 6 hours: gizzard 1 hour 6 min; duodenum 6 min; jejunum 1 hour; ileum 1 hour 20 min; caeca 1 hour 15 min; colon + cloaca 42 min (254).

Chicken

In the past, chickens have been selected for improved feed conversion and rapid growth or for high production of eggs. This has led to two different phenotypes of chickens: broiler- and layer type chickens. Because of this selection for economically important production traits, these chickens differ in body weight gain and maximum life span. Recently data became available that these chicken types also differ in immune system because of genetic differences. Growth of broiler type chickens has been markedly increased. The modern broiler type chicken weighs almost 5 times as much as the random-bred chicken from 45 years ago and this is for 85-90% because of genetic selection. Another cause of the increased body weight is the improved nutrition, which increased growth performance with 20-30%) (109, 110). Modern heavier broiler type chickens and turkeys have a less well developed immunocompetence (46, 148, 163, 164), disease resistance (312) and exhibit a propensity for skeletal and metabolic disorders (109, 110, 141). Therefore, concern has risen with respect to their ability to mount an immune response to the whole array of pathogens as encountered in poultry breeding.

More recently, the public opinion is turning around from focusing on cheap broiler meat to awareness about the welfare of the animals. This is leading to the development of more so called biological, ecological and organic farming systems, where the animals are living in an environment where they can behave naturally. However, they lack the strict hygienic measurements and antibiotics to prevent pathogenic and parasitic infections. Although the welfare of the animals is apparently increased in the eyes of the general public and customers, the environmental threats outside is an increased risk for the health of the chickens and therewith for the consumers. The risk of viruses (e.g. Avian influenza), bacteria (e.g. Salmonella and Campylobacter) and parasites (e.g. Eimeria) may be increased enormously. The chickens that are used in the intensive farming nowadays are especially bred for fast growth or egg production under hygienic circumstances. When chickens are held organically, more robust chickens will be necessary. In addition, antibiotics as feed additives will be prohibited per January 2006. Fear for possible transmission of antibiotic resistance from animals to humans and increased awareness of consumer safety have led to this prohibition. Antibiotics will be allowed for therapeutical purposes only (77). Increased disease resistance and a well-developed immune system will be even more important when antibiotics are removed from the feed and/or when chickens will be held organically.

Chicken immune system

The immune system is part of the host's defense against infection. The chicken immune system is similar to the immune system of mammals. Although lymph nodes do not occur in the chicken, diffuse lymphatic tissue and lymphatic nodules are widespread (8). The immune system can be divided in innate (or natural)

immunity and acquired (or adaptive) immunity, each composed of cellular and humoral elements. Both forms of immunity are functionally integrated with each other. The function of the adaptive immune system is based on specific antigen recognition, enabling specific responses, and memory, enabling faster responses upon subsequent encounters. The innate response is mainly based on pattern recognition (conserved motifs) and receptor recognition and is therefore fast, although it does not specifically recognize individual pathogens. The lack of specificity is more than compensated for by the speed and level of response, which is much faster (minutes rather than days) than that of the adaptive immune response (133, 172). Phagocyte recognition and uptake of pathogens involves pattern-recognition molecules such as the macrophage mannose receptor, scavenger receptors and Toll-like receptors (19, 103, 184).

Table 1 Innate versus acquired immunity (172)

Innate immunity	Acquired immunity
Immune response is antigen independent	Immune response is antigen dependent
Immediate maximal response	Lag time exposure -> maximal response
Group specific (sequence, motive, structure)	Antigen-specific
No immunological memory	Exposure results in immunological memory
Involves myeloid cells (platelets, eosinophils, neutrophils, basophils, mast cells ->macrophage, monocytes)	Involves lymphocytes (B cell ->plasma cell, T cell (helper, cytotoxic, suppressor)

Innate immunity

The innate immune response is the first line of defense against pathogens. The elements of the innate immune system include:

- anatomical barriers (skin, mucosal epithelia);
- secretory molecules (antimicrobial substances as lysozyme, complement, peroxidase, lactoferrin, defensins);
- cytokines
  - pro inflammatory: Interleukin-1 (IL-1), tumor necrosis factor (TNF), interferon-gamma (IFN-γ), IL-12, IL-18 and granulocyte-macrophage colony stimulating factor (GM-CSF);
  - anti-inflammatory : IL-4, IL-10, IFN-α;
- cellular components (inflammatory reaction as accumulation of macrophages, monocytes)
- resident flora (colonization resistance).

Until recently, only a few avian cytokines have been characterized and potential applications of cytokine detection and measurements have remained limited to mammals. Classic approaches (e.g. producing antibodies) to identify cytokine genes in birds proved difficult because the sequence conservation is generally low. As new technology and high throughput sequencing became available, this situation changed quickly in the last few years. The whole genome of the chicken is just identified and will be published soon. Recently the identification of genes for the avian homologues of interferon-alpha/beta (IFN- α, β and γ, various interleukins, and several chemokines took place. From the initial data on the biochemical properties of these molecules, the image is emerging that avian and mammalian cytokines may perform similar tasks, although their primary structures in most cases are remarkably different (270). Recently in our lab lightcycler RT-PCR assays have been set up in chicken to determine relative concentrations of mRNA of IFN-γ, IL-2, IL-6 and IL-18 (25). Expanding this technique to more interleukins could be helpful for identifying immune responses in tissue of the chicken, e.g. the gut.

Acquired immunity

An acquired immune response is the development or enhancement of antigen-specific defense mechanisms in response to a stimulus. Immune responses are associated with antigen presenting cells and two major subpopulations of lymphocytes, namely B lymphocytes and T lymphocytes (172). The main feature of the adaptive immune system is to discriminate between self- and non-self antigens. The antigen specific adaptive immune system has two important effector mechanisms to attack pathogens. One is based on the formation of immunoglobulins by B cells and is called humoral immune response. The other, the cellular response, is based on a great variety of cells each with their own individual effector mechanism. Examples of these cells are cytotoxic T cells that kill infected or tumor cells. T cells may produce an enormous variety of soluble molecules that function as messengers between cells of the immune system or can directly kill pathogens. The communication signals between the cells may be proteins such as lymphokines, cytokines and chemokines (126). These bring about a complex response, mutual affecting, with cellular dialogues and feedback mechanisms, resulting in an enormous response reserve that guarantees an optimal enhancing effect of both the humoral and cellular responses (75).

An antigen specific response leaves the host with specific immunological memory, enabling it to respond more effectively to re-exposure to the same stimulus. The response to a second round of infection is more rapid than to the primary infection because of the activation of existing memory B and T cells.

Microflora
Normal chicken gut flora

Chickens are born with a sterile intestinal tract. Under natural conditions, immediately after birth bacteria originating from the maternal feces, the environment and the diet will colonize the GI-tract. This results in a diverse microbial population in the GI-tract that is balanced with the maternal environment. As a result, after the first colonization, dependent on the stability of the composition of this initial microflora, new bacterial species will have more difficulties to colonize (colonization resistance). In the common intensive breeding where chicken generations are strictly separated, there is no contact between the newly hatched chicken and the adult microflora of the mother hen. Therefore any bacteria from the environment might colonize the intestinal tract (290). It is suggested that lack of contact results in an imperfect composition and a delayed development of the intestinal microflora. In the modern housing situation, enterococci and lactobacilli dominate the crop, duodenum and ileum of broiler type chickens in the first week of life while coliforms, enterococci and lactobacilli dominate the caeca. Thereafter, a complex microflora with mostly obligate anaerobic bacteria starts to dominate the caeca (11, 260, 292) while lactobacilli dominate the crop, duodenum and ileum. After two to three weeks the microflora is established in the intestine of broiler type chickens (51) while under natural conditions this takes only a couple of days. The bacterial contents of chickens intestine is an enormous biomass that contains up to 10^{14} bacterial cells. It is estimated that this microflora comprises over 400 bacteria species. In chickens the most complex flora exists in the caeca. It contains up to 10^{11} viable bacteria per gram content. This most probably is the result of the low local flow rate (caeca are emptied 2-4 times per day and therefore resemble batch culture). Of the cecal species 99% are obligatory anaerobic species and about 10% are lactobacilli and bifidobacteria (12, 297). Until now, many species could not be grown in culture, but can now be detected with new PCR techniques (124, 125, 147, 246, 261, 281).

The normal flora consists of autochthonous flora (or indigenous flora). This indigenous flora consists of resident micro-organisms present in all communities of a particular animal species, the allochtonous flora, or harmless transient microflora, and microorganisms that are transiently present such as pathogens. Hence not all species represented in the indigenous flora of the chicken are present in all members of a single flock (248). Opportunistic bacteria are also a part of the microflora and under specific conditions these can be harmful. Indigenous flora bacteria can be harmless, harmful and everything in between.

The bacteria of the microflora and the host have multiple interactions and maintain a dynamic balance with each other (figure 1). The flora is quite stable although it adapts in overall metabolic activity and relative proportion of microorganism species to nutrient changes. This balance can also be modulated by medicines (antibiotics),

intruding microorganisms, host related factors (age, immunity, hormones), stress, feed (bacteria, growth enhancers) or environmental factors (125).

Function of the microflora

The gut flora has several functions which involve the digestion and health of the host. The flora assists in degradation of certain food components such as the predigestion of 'non'-digestible poly- and oligosaccharide fibers, production of vitamins B and K (20), stimulation of priming and development of the immune system, induction of GI tract peristalsis and intestinal mucosal integrity, conversion of dietary (pre)carcinogens to non-carcinogens (100), production of digestive and protective enzymes, production of antimicrobial substances, forming a barrier against pathogenic and opportunistic micro-organisms. Some of these beneficial effects also have a negative counterpart in converting dietary non-carcinogens to (pre)carcinogens (100), in ecological disruptions that lead to intestinal overgrowth by indigenous bacteria and opportunistic infections that lead to translocation over the GI tract wall (22, 122).

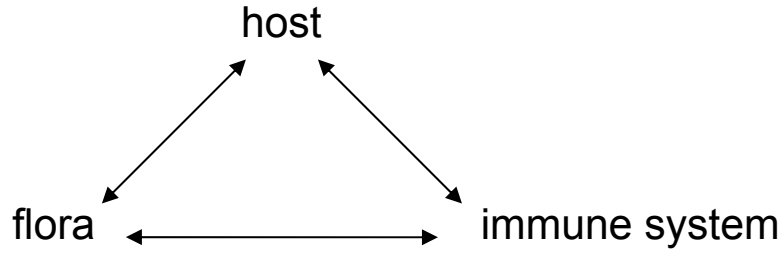

Figure 1. Flora, host and immune system have multiple interactions with each other

Since newly hatched chickens separated from their mother or other older animals do not have the chance to pick up a normal healthy adult microflora they are more vulnerable to pathogens and infections. Chicken feeding starts with storing food temporarily in the crop. The crop is colonized by lactobacilli and during food storing lactobacilli from the crop are continuously added to the food (88). Locally, these bacteria can create a micromilieu that is not favorable for pathogens and thereby lower the infection pressure of a pathogen. After leaving the crop the lactobacilli enter the rest of the GI-tract with the food. Probiotic lactobacilli might be able to help develop and stabilize the microflora of newly hatched chickens.

Interactions of the gut flora
Microflora interactions with the gut

Although research has focused mainly on intestinal pathogens that may cause local and systemic infections, most intestinal microflora bacteria are beneficial to the host

(123, 238). Beneficial functions attributed to intestinal bacteria include positive effects on the morphology, physiology, biochemistry and immune system of the host. This includes development of the gut wall, intestinal villi and lamina propria (20), induction of intestinal angiogenesis (271), nutrient processing (309), production of vitamin B complex and vitamin K (20), development of GALT (274), induction of oral tolerance (276), mucosal immunity (277)and diversification of the pre-immune Antibody repertoire (160, 238). In addition, the microflora seems to play a significant role in the generation of immunocompetent cells during development and maintenance of the intestinal homeostasis and prevention of inflammation (24, 31, 48, 121). It is hypothesized, and several recent publications are strengthening this hypothesis, that the lack of proper interactions between bacteria and the host contributes to the prevalence of allergies and Crohn's disease in developed countries (240, 273).

Host interactions with the microflora

Although the microflora has a considerable influence on the host, the host also plays a role in composition and magnitude of the microflora. The major host factors influencing the flora (122) are the intestinal pH; secretions such as immunoglobulins, bile-salts, enzymes; motility as speed, peristalsis; physiology (compartmentalization) and excretions of cells such as mucins and bacteriocidins produced by Paneth cells and other tissue exudates. Changes in these factors may be related to changes in the physiological conditions of the host (aging, stress, and health status), composition of the diet and environmental conditions (e.g. contamination with pathogens, use of pharmological products). In this way the digestion (e.g. pH, substrate availability, redox potential, transit time, flow of enteric fluid, IgA secretion, etc.) may be modulated and thereby the microflora (122).

The immune system is known to regulate the composition of the microflora. During pregnancy or in the egg a coating of maternal antibodies is formed along the gut. This antibody coating influences the colonization of the gut by bacteria. The antibodies bind microorganisms, thereby preventing their adhesion to the gut, so that they are selectively washed-out. Because of this selection of microorganisms the immune system is exposed to a selection of microorganisms to which tolerance is established and the specific immune system (IgA, T- and B cells) is selectively triggered by this selection. The repertoire of antibodies provided by the mother is therefore first influencing the selection of microorganisms and the presentation of the microorganisms to the immune system. After that the microorganisms, to which animals are exposed to, seem to determine the balance of the microflora later in life. For the Lactobacillus flora the environment is an important factor. This flora is more defined by the housing than by host genetics (65).

The process of primary bacterial colonization in the gut is also dependent on the nature of the individual's innate carbohydrate repertoire. If these first bacteria

posses glycolytic activities, they will modify the carbohydrate repertoire, thereby encouraging new bacteria to establish and others to be lost (162). In conclusion, the microflora is influenced by the bacterial flora of the mother and the maternal antibodies, the primary bacteria the individual is exposed to, the carbohydrate expression in the host (host genetics) and influx of bacteria from the environment.

Interaction of microflora with pathogens

Several mechanisms have been proposed by which the resident microflora inhibits the colonization of invading bacteria in the intestine. After passing the protective mucus layer, indigenous bacteria adhere to specific glycoprotein receptors on intestinal epithelial cells. The resulting barrier of microflora, which is firmly attached to the mucosa, together with the mucus layer, prevents contact and colonization by pathogenic organisms together with the mucus layer. Mucosal attachment is a prerequisite for successful colonization and possible translocation of a pathogen. Without adhesion, bacterial multiplication is generally inadequate to compensate for the wash out of bacteria with the flow of intestinal content (35). The microflora interacts with pathogens and other microorganisms in several ways:

- synergy (metabolic co-operation, growth factors and vitamin excretion, changes to oxidation-reduction potential, pH, O_2tension);
- antagonism/stimulation (short chain fatty acids, lactic acid, amines; changes to oxidation-reduction potential, pH, O_2tension; antimicrobial components (H_2O_2, bacteriocidins and acids), siderophores; nutritional requirements, etc.) (35, 101, 115, 122);
- competition: two or more microbial types in rivalry for a factor in the gut-ecosystem that is not present in sufficient quantities to satisfy the demands of all of the inhabitants. This could be competition for nutrients, space, etc.;

The prevention of pathogenic colonization by excluding other microbes than the resident flora by competition and antagonism (e.g. by lactobacilli) is called colonization resistance (20, 201).

Lactobacillus

Lactobacilli are gram positive, rod shaped lactic acid bacteria. Although there are some exceptions, most of the Lactobacillus strains are not pathogenic and lactobacilli are major constituents of the human and animal gut. Because of the safe use in food for centuries, they have the GRAS-status and are frequently used in bioprocessing and preservation of food and feed (e.g. yogurt, kefir, milks, cheeses, breads, wines, meats, sauerkraut, olives, pickles and silage). Nowadays lactobacilli are mainly known for their health stimulating properties. The importance of lactobacilli for human health was first recognized by Metchnikoff at the beginning of the 20th century (186). He suggested that harmful effects of undesired bacteria could be overcome by establishing a new balance between intestinal bacteria, through ingestion of lactobacilli or fermented products made using these organisms.

He attributed the long, healthy lives of Bulgarian peasants to their consumption of fermented milk products containing 'Bulgarian Bacillus' (now known as *Lactobacillus bulgaricus*).

Probiotics are live bacterial food supplements, which beneficially affect the host by improving its intestinal balance (87). In the seventies of the 20th century probiotics were presented as miracle bacteria with all kinds of positive effects on the health of the host. These positive effects varied from improvement of feed-conversion rate in farm animals (88), overall improvement of well-being (194), shorten viral diarrhea (128), positive effect on cancer, prevention of cancer, lowering blood cholesterol levels (58), enhancing disease resistance (236), treatment and prevention of allergies (129), and improving the immune response (54, 217). As a result of all these positive effects probiotic bacteria were expected to be super-bacteria and expectations with respect to application ran high. This resulted in many investigations. Subsequently, also disappointing results were reported. These were in part the result of expectations with respect to beneficial effect on certain functions while the probiotics that were subject of research had not been selected for the investigated purpose. Therefore, the interest in probiotics declined during the seventies and eighties. In the nineties the interest in probiotics revived. New techniques and insights renewed the vision on probiotics. It became apparent that a single probiotic strain could never meet all these expectations and that the positive effects should be adapted to these new insights. In addition, it became apparent that a probiotic strain could have a specified positive effect in one host and not in another.

Probiotics can have a positive effect on the health of the host, but the effect often is small and limited to the combination of Lactobacillus strain and host in the investigation that reports the effect. It also became clear that the properties of the strains of probiotic bacteria had to be well characterized. The nature of the modulating effects of the strains also needed refined description, so as to allow attributing certain effects to selected strains. It became apparent that different strains exerted different sets of effects (62, 111, 169).

Chicken feed contains relatively low, non-therapeutical concentrations of antibiotics because of their growth promoting and antibacterial effect. The long term use of antibiotics could lead to resistance to certain groups of antibiotics which could possibly end up in the human food chain and there form a danger to humans.

Especially during the last few decades, extensive research on the positive effects of Lactobacillus strains has been carried out, in search of health and/or nutritional benefits for humans and animals by oral administration (168). Still, the exact role of lactobacilli in the enhancement of health is not fully understood. The more recent research is carried out for the greater part more precisely with well-defined strains and sometimes even with randomized placebo controlled human studies. A list of

proposed effects of orally administered probiotics is shown in table 2. Apparently, the beneficial effects of probiotic bacteria are strain dependent and largely based on the improvement of the intestinal barrier in terms of competitive exclusion, production of inhibitory substances, and stimulation of innate and acquired immunity (69, 199, 208). For instance, it has been shown that various Lactobacillus strains induce the production of certain cytokines in the host that direct particular immune responses (167, 169). Despite many studies in which lactobacilli interacted with immunocompetent cells in *in vitro* assays (reviewed by Maassen (168)) the exact nature of the physical interaction of lactobacilli with the immune system *in vivo* is still largely unknown. It appears that orally administered lactobacilli are taken up by M-cells or dendritic cells (237) in the small intestine or by intestinal epithelial cells and there interact with the immune system.

Table 2 Short overview of various probiotic microorganisms and the suggested health effects of probiotics in animals and humans

Described probiotic bacteria species	Suggested health-promoting effects of probiotics. (Reviews: 69, 168, 199, 208, 209, 255)
L. acidophilus (58, 247, 313)	Reduced diarrhea (128, 170)
L. casei (16, 63)	Enhanced protection against intestinal infections (31, 91, 236, 278)
L. rhamnosus (94, 98, 224, 253)	Colonization prevention (6, 71)
L. plantarum (41, 192)	Prevention of cancer (233)
L. johnsonii (66, 288)	Anti-allergy activity (129, 194)
L. reuteri (48, 197, 241, 282)	Anti-auto-immune activity (99, 146, 179)
L. paracasei (150, 169)	Improvement of immune responses (54, 62, 166, 217-219)
B. breve (6, 111, 310)	Improved lactose digestion (4, 38, 81)
B. longum (97, 111)	Modulation of intestinal flora (37, 140)
B. bifidum (228)	Improved gut physiology (81)
B. lactis (47, 98)	

Recent progress in molecular microbiology opens the possibilities to identify strains precisely as well as microorganisms in the microflora, which until now can not be cultured. New techniques as microchips and -arrays (genomics, proteomics) provide opportunities to reveal the host interactions by which probiotics modulate the immune system.

Probiotics and the immune system

Most exogenous microorganisms used as probiotics are taken up orally and arrive via the stomach in the gut. There, the probiotics interact with other bacteria of the microflora and the intestinal epithelium. Interactions with the epithelial cells of the gut wall may lead to various bioactive effects. Ideally, the normal, undamaged gut forms a barrier through which particles such as living bacteria, viruses and living parasites cannot pass. The gut wall with its layer of mucus is thereby preventing

systemic infection and disease. From numerous studies, it has become clear that most particulate matter such as bacteria is taken up rapidly by M- cells or antigen presenting cells as dendritic cells (DCs) (154, 196, 237). These cells are covered with small microvilli. Although M-cells are actually a highly specialized version of gut epithelial cells they may behave like macrophages. Within a few hours after uptake of particles from the gut, M-cells have transferred and presented their contents to specialized immunologically active cells (145, 154). DCs can sample through the intestinal barrier without disrupting it (237).

The particulate matter that is taken up by M-cells is probably recognized by antigen-presenting cells and presented to both B and T cells. These cells become stimulated and start expressing surface molecules on their cell wall indicating their activated state. In mammals this triggers these cells to migrate and leave the Peyer's patches with the efferent lymph towards the nearest draining mesenteric lymph node (53, 145, 196). In chickens, which lack lymph nodes or similar structures, activated cells migrate via the blood towards the spleen. Upon arrival in the draining lymph node and spleen, the T- and B cells together with the antigen presenting cell establish themselves as 'ménage a trois' in and around the germinal centers. Germinal centers are follicles engaged in an immune response. The ensuing immune response is indicated by the cognate physical interaction between antibody producing B cells and cytokine producing T cells. The activated T cells leave the lymph node or spleen, enter the bloodstream, and migrate back to the villi where they take up residence in the lamina propria and between the epithelial cells (as intra-epithelial lymphocytes) at the mucosal surface lining the gut towards the lumen. The cells producing cytokines and antibodies are mainly located inside the villi in the lamina propria.

The T cells in the lamina propria are mainly producing B cell stimulating cytokines whereas the B cells are producing vast amounts of IgA that is secreted in such a form and way that it can cover the entire inner surface of the gut. This secretory IgA coating with its multiple binding sites is a formidable defense against pathogens due to its high binding (although low affinity) and agglutinating capacity. The repertoire of IgA is broad and allows a rather promiscuous interaction with microflora and pathogens. Natural antibodies, i.e. IgA that binds to pathogens non-specifically, are produced independent on the specific immune response. But specific IgA, generated in response to an infection/immune encounter, is even more effective in clearing pathogens from the gut (39, 53, 145, 196).

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Lactobacilli as probiotics in chicken feeds

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Abstract

In the current intensive live stock industry the gut flora can be disturbed as a result of several stressors that affect the intestinal tract. New food safety regulations demand to replace antimicrobial additives in poultry feed and still produce healthy poultry. Probiotic micro-organisms like lactic acid bacteria administered with the feed may contribute to a strategy to comply with these demands. Lactobacilli have been shown to stimulate immunity, increase colonization resistance and increase competitive exclusion. This review highlights some effects of probiotics and their working mechanisms. It is concluded that still more effort should be directed towards the mechanisms behind immune stimulating properties, their contribution to resistance against enteric infections, and the selection of probiotic strains.

Intestinal flora and probiotics

The bacterial contents of the animals intestine is estimated to contain over 400 bacteria species; 99% of these species are obligatory anaerobic species and about 10% are lactobacilli and bifidobacteria (12, 297). These bacteria and their host have multiple interactions and maintain a dynamic balance with each other. The flora is quite stable and it adapts in overall metabolic activity and relative proportion of micro-organism species to nutrient changes. However, this balance can be modulated by antibiotics, intruding micro-organisms, host related factors, feed or environmental factors.

In the current intensive live stock industry this microbial intestinal balance is affected by stressors, such as composition of feed, hygienic conditions, infection pressure, and fast growth and high production yields achieved by genetic selection. In order to realize increased growth rates and to suppress pathogenic bacteria, antibiotic feed additives are commonly added in animal husbandry. The continuous use of relatively high amounts of antibiotics in animal feed may lead to contamination of the food chain with residues of antibiotics and antibiotic resistant micro-organisms, including pathogens. In addition, the possible transmission of antibiotic resistance from animal to humans together with increased awareness of consumer safety has lead to prohibiting these feed additives and antibiotics will be allowed for therapeutical purposes only (77).

The working mechanism of the antibiotic feed supplements is not exactly known. Part of its actions includes the bacteriostatic influence on specific constituents of the microflora. Probiotic feed supplementation can repair deficiencies in the composition and/or activity of the gut flora and to provide the type of microflora which contributes to the host disease resistance (89).
The most frequently used definition of a probiotic is: a live microbial feed supplement that beneficially affects the host by improving its intestinal microbial balance (87).

In humans and mice probiotics have been shown to have several beneficial properties. They contribute to resistance against pathogens, modulate the immune system, strengthen the gut mucosal barrier, regulate gut motility, control intestinal infections, reduce inflammatory reactions, reduce allergic effects, reduce mutagenic and tumorogenic activity, have nutritional advantages, control serum cholesterol levels and modulate effects on allergy and autoimmune disease (61, 180, 214, 243, 300). Examples of these probiotics are *L. casei*, *L. casei* Shirota, *L. acidophilus*, *L. rhamnosus* GG, *Bifidobacterium bifidum*.

For probiotics for farm animals (already on the market), claims have been made including increased animal growth rate, improved feed conversion, better absorption of nutrients, provision of essential nutrients, increased egg production, improved

milk production in dairy cows and improved health (88, 89). As a result of the reduced rate of bacterial infections, improved quality of eggs and meat is claimed in addition. There is much variation between these features and therefore it is important to realize that not all properties are represented in one probiotic micro-organism. For farm animals these probiotic properties of lactobacilli have both health and welfare aspects and as a consequence economic aspects.

When antibiotic additives are removed from the feed, probiotic micro-organisms can be applied to guide the balance into a host favorable condition. These bacteria can stimulate innate and acquired immune functions (299), prevent pathogens from colonization, enhance defense by antagonistic interactions (e.g. production of anti-microbial factors (70)) and improve the health status of the animal. Through these properties probiotics can, at least in part, replace antibiotic feed supplements.

Functional criteria to select probiotic micro-organisms

For proper selection, the capabilities of the specific probiotics should be known. Probiotics should at least have one or more of the following properties (78, 80, 104, 174, 207, 208, 280):

- adapted to survive local conditions in the digestive system, such as acidic conditions in the stomach, digestive enzymes, bile salts and other bacterial interactions
- demonstrate non-pathogenic behavior;
- be able to influence metabolic activities of microflora components;
- modulate immune responses;
- adhere (might be temporarily) to the intestinal mucosa;
- produce anti-microbial substances.

According to Berg and Salminen and our own experience each potential probiotic strain should be assessed independently, as extrapolation from data of closely related strains or hosts is not acceptable. These strains can show large variations in properties, e.g. health-stimulating properties, strength of the effect obtained and fermentation pathways (21, 243, 245).

Probiotic lactobacilli

Lactobacilli have the longest history as probiotics and are still among the most common ingredients used in animal feed for notably calves, pigs, and poultry. In addition, lactobacilli are presently known for their health stimulating properties. Lactobacilli form a substantial proportion of the gastro-intestinal (GI)-tract microflora when pigs and poultry are maintained under optimal animal husbandry conditions (297). The choice for lactobacilli as probiotics seems obvious, especially when animals are confronted with non-optimal conditions as in young animals which have to grow rapidly or the early weaning as in piglets (279). Because of their harmless

character lactobacilli have the GRAS-status, which indicates that they are generally regarded as safe. Lactobacilli are already frequently used in classic and modern preservation of food and feed and biotechnology.

The importance of lactobacilli for human health was first recognized by Metchnikoff at the beginning of the last century (186). He suggested that harmful effects of undesired GI-tract bacteria could be overcome by establishing a shift in the balance between intestinal bacteria, through ingestion of fermented milk products containing high amounts of lactic acid bacteria.

Some of the positive effects of lactobacilli are mediated by non-immune components such as modulation of vitamin production, enzymes, and antibiotics. Other positive effects are immune system related, especially modulation of the mucosal immune system seems to play an important role (122). Enhancing protection against infections could be induced by activation of local innate immune defense effector functions (e.g. macrophages) and/or to support of the specific local response against infectious micro-organisms by upregulation of IgA production.

Lactobacilli in chicken

In chickens the passage of feed and other ingested material through the GI-tract is relatively fast (2-5 hours (40)). This is not only of importance for the digestion and absorption, but it is also of importance with respect to the administration of lactobacilli with probiotic characteristics. Because of this short passage time probiotics in chicken have a short time to exert their actions and therefore good adhesion or even colonization characteristics play an important role. Probiotic strains that are poorly adhering or colonizing may be added to feed, e.g. fermented feed, and guarantee in such a way that the strain is continuously present.

An important site for colonization of lactobacilli is the crop. Within 24 hours after the newly hatched chick starts eating, a layer of lactobacilli (1 to 3 cells thick) is formed in the crop (85). After feeding, the pH in the crop decreases and the concentration of lactobacilli is high (10^8-10^9 cfu/g wet weight) (86). This indicates that the feed in the crop will be inoculated by lactobacilli. Lactobacilli hardly multiply in the small intestine therefore the inoculation via the crop is important to maintain the level of lactobacilli in the jejunum (86). In the caeca the number of lactobacilli is 10^8 cfu per gram, but the total number of obligatory anaerobe bacteria is at least 10 times higher (11). This localization is probably the result of the slow flow rate, caeca are emptied 2-4 times per day and probably enough bacteria stay in the caeca to inoculate new caecum contents, and the complexity of the cecal flora. Many bacterial species including pathogens like *Salmonella* spp. can frequently be cultured from the cecal content (12). Therefore this preferential localization seems to be non-specific (15).

Immune stimulation by probiotics in chicken

Probiotics can stimulate both the humoral and the cellular immune system. In an animal experiment groups of 10 layer type chickens were administered orally for 5 days with 10^9 cfu/day of a *Lactobacillus* strain and the control group received buffer instead of lactobacilli. On day 5 all animals were immunized with a suboptimal amount of a model antigen, TNP-KLH, and after 10 days blood samples were collected for serum. These samples were analyzed or specific IgM and IgG anti TNP titer. The results are presented in figure 1.

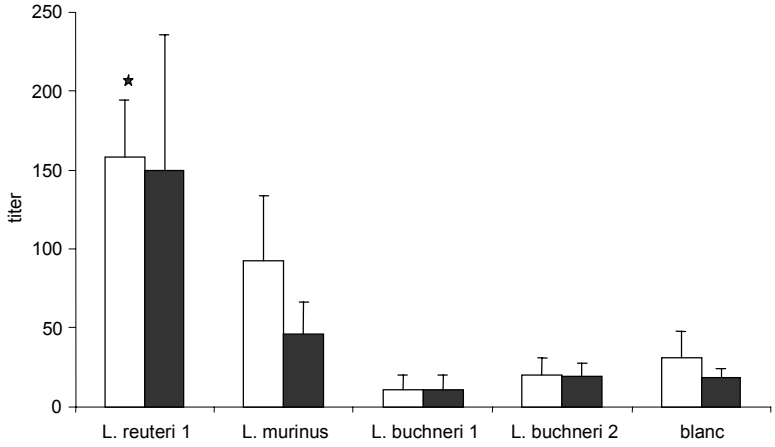

*Figure 1. Specific IgM (white bars) and IgG (black bars) anti TNP antibody titers (+ S.E.M.) in layer type chickens (n=10) orally fed with 4 different Lactobacillus strains or buffer (blanc) at 10 days after priming with TNP-KLH. * indicates statistically significant higher than blanc (p<0.05).*

The group, which was fed with *L. reuteri*, had a higher specific antibody titer than the blanc group ($p<0.05$). *L. murinus* also gives higher specific antibody titers, but not significantly. Both *L. buchneri* strains don't have an effect on the specific antibody titer. This experiment is demonstrated that Lactobacilli can have an adjuvant effect on the humoral immune response in chickens.

We developed an *in vitro* test to screen lactobacilli on probiotic activity in layer type chickens. The test is based on a lymphocyte proliferation assay. A single cell suspension of chicken spleen is enriched over Ficoll and lymphocytes are incubated for 68 hours at 41°C with a suboptimal concentration Concanavalin A (ConA) and lactobacilli in 3 different percentages compared to the number of cells in the test. The last 4 hours of incubation ^{3}[H]-thymidine is added and the built in radioactivity is

measured in a beta-counter. The proliferation index is calculated by the following formula:

$$\frac{[\#\text{ cpm cells + suboptimal conc. ConA + lactobacilli}] - [\#\text{ cpm lactobacilli}]}{[\#\text{ cpm cells + suboptimal conc. ConA}]} * 100\%$$

The 3 *L. reuteri* strains, one *L. buchneri*, *L. murinus* and one *L. casei* strain have a positive effect on the proliferation of spleen lymphocytes *in vitro*. *L. buchneri* 2, *L. plantarum* and *L. casei* 2 have no effect on the proliferation of the spleen lymphocytes. From this *in vitro* experiment it is clear that different strains of lactobacilli give different proliferation indexes. *In vitro* pre-screening of lactobacilli in this way is feasible. It is also clear that strains from the same species do not have the same effect on the proliferation and thus it is necessary to evaluate every strain on probiotic properties in order to know if a particular strain has these properties.

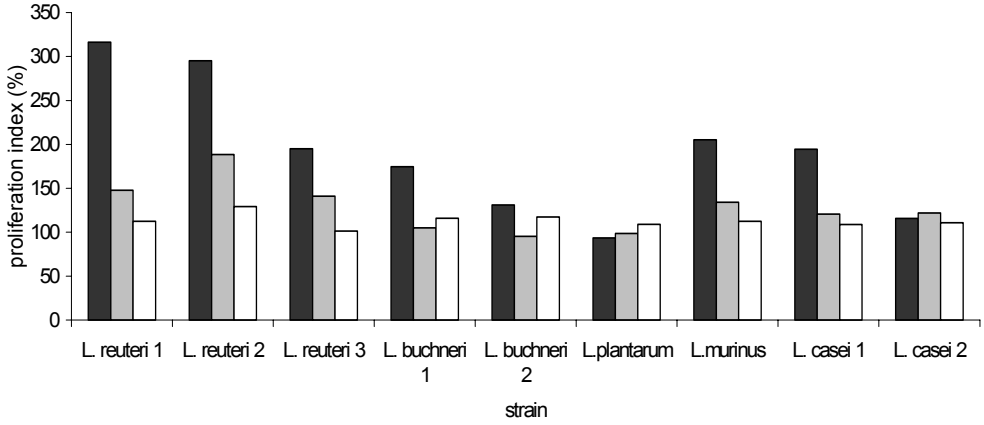

Figure 2. Nine different Lactobacillus strains in three concentrations (in percentage bacteria to spleen cells: black 10%, grey 1% and white 0.1%) were evaluated in vitro on chicken spleen lymphocytes. The extra proliferation by lactobacilli is shown in the proliferation index.

Protection against intestinal colonization

Probiotics, which have a protective effect against the colonization by pathogenic bacteria, can be divided into cecal flora and other complex flora's, anaerobes, and lactobacilli. In commercial conditions for breeding the hygienic measures and the separation of the young from the adult bird prevent the chick from uptake and ingestion of the adult intestinal flora. As the adult flora is thought to contribute to

resistance of chicks to colonization with *Salmonella*, Nurmi and Rantala were the first who treated chickens with cecal flora (204, 234). By such treatments the colonization level of *Campylobacter* (265), *E. coli* (264, 304), and *Clostridium* was reduced (76).

Goren *et al*. suggested that in protective bacterial mixtures anaerobes are essential for protection (102). Lactobacilli are facultative but not strict anaerobes and thus it is questionable whether lactobacilli alone can protect chickens from colonization with pathogens. This may explain why studies on *Salmonella* prevention with lactobacilli are not consistent. Several authors found that a single strain of *Lactobacillus* or a simple mixture of lactobacilli was unable to protect chickens against *Salmonella* colonization (3, 205, 263, 305). Others have found suppressive effects of lactic acid bacteria on the colonization level of enterococci (231, 232, 285), *Enterobacteriaceae* (86, 190, 231), *Salmonella* spp. (302, 303), and *Campylobacter* (195).

At least four mechanisms are postulated to explain the working mechanism of lactic acid bacteria in prevention of colonization by opportunistic pathogens like *E. coli*, enterococci and *Salmonella*e.
First, competition for nutrients. The probiotic consumes the available and necessary nutrients so it competes for nutrients with pathogens (82, 202).

Second, adhesion to the mucosa. The ability to adhere to the intestinal epithelium is generally considered to be the most important property to prevent e.g. *Salmonella* colonization (80, 183, 272). Adhesion of lactobacilli can prevent colonization of pathogens by competition for adhesion receptors with the pathogens (304); forming a bacterial mat that prevents pathogens to adhere to epithelial receptors or preventing the specific binding to cell receptors by steric hindrance or by masking the cell receptor (43, 83, 262, 264, 268). Moreover, adhesion is considered to be a prerequisite for successful colonization of the probiotic strain(80). However, Rada *et al*. used a *Lactobacillus* strain that had good colonization characteristics, which was however poorly adhering (232). For lowering the level of colonization of *Campylobacter*, microaerophilic or obligatory anaerobic bacteria with a preference of the mucus layer of the epithelial surface in the caecum might be essential (3, 38), because this is the niche colonized by *Campylobacter* (17).

Third, coaggregation. By binding of the *Lactobacillus* to the pathogen, the pathogen can subsequently not bind to epithelial receptors (139, 268). Coaggregating bacteria can facilitate colonization of other probiotic lactobacilli (105).

Fourth, bactericidal substances. Lactobacilli can produce substances like hydrogen peroxide (H_2O_2(96)), and lactic acids or other volatile fatty acids that are toxic for e.g. *Salmonella* (2, 252). The anti-microbial activity of lactic acid may be the result of decreased pH in and the specific toxic effects of the un-dissociated molecule (2).

Following dissociation of short chain organic acids, the un-dissociated acid molecules enter cells, dissociate into anions and protons, and causes the pH of the cytoplasm to decrease. Some lactobacilli, *L. reuteri*, *L. acidophilus* and *L. plantarum*, produce antibacterial substances or even bacteriocidins like reuterin, acidophilin, lactocidin, and lactolin (267). This is a heterogeneous group of substances produced by many bacteria, as reviewed extensively elsewhere (235, 269).

Future research of probiotics

Although different specific mechanisms have been postulated, it is still not elucidated how lactic acid bacteria influence the dynamic ecosystem of the gut. Because multiple factors contribute to the diversity of the ecosystem when in optimal balance, multiple factors, and also multiple organisms of the microbiota will be responsible for pathogen exclusion (161).

It has been established in rodents that lactic acid bacteria given per os can significantly affect both the systemic and mucosa associated immune response (78, 222). However, thus far specific enhancement of immune-reactivity has only been demonstrated for vaccines and model antigens. Though the stimulation of immunity may be significantly improved, it will only be protective if the levels of specific immunity that are reached are sufficiently high to counteract bacterial or viral infections. Although promising results exist in rodents, this has not been confirmed in farm animals yet (89). Lactobacilli still may be of importance in animal husbandry. Probiotics stimulate the individual resistance against e.g. *Salmonella* colonization. Although this resistance may be insufficient to totally protect a chicken against colonization, the sum of all decreased susceptibilities of individual chickens can lead to sufficient resistance of the total flock.

Continued progress in probiotic research will require improved understanding of host intestinal physiology, its relationship with intestinal microbes and the mechanism by which these bacteria influence the immune system. For example, identification of host cell-surface molecules that serve as receptors for microbial colonization may allow screening for desirable probiotic organisms (182).

A serious limitation in studying the gut flora composition is that the majority of the intestinal micro-organisms cannot be cultured by traditional *in vitro* methods. Several *in vitro* models can help to predict the fate of ingested strains: these systems consist of simple models to test sensitivity to acid or bile (122). Dynamic models of the stomach and small intestine, which simulate the *in vivo* gastrointestinal environment, have been developed and validated and these may become excellent accessory tools to conventional *in vitro* methods. These models simulate the dynamics of the transit and secretions in the gastrointestinal tract. The applications of these dynamic models include following the survival and interaction

of the indigenous microflora, probiotic, pathogenic, and genetically modified micro-organisms (173, 175).

As probiotics are living micro-organisms, they may in theory be responsible for side-effects, e.g. systemic infections (in single case studies in catheterized severely immunocompromised patients only). In over-dose situations the risk exists of deleterious metabolic activities such as induction of diarrhea and intestinal lesions. The adjuvant side-effects may be based on enhanced unwanted responses such as fever or arthritis induced by peptidoglycans. Another type of risk could be gene transfer. However, genetically modified probiotics are not available for feed use and therefore their theoretical adverse effects need no consideration.

Lactic acid bacteria display a wide range both of natural and antibiotic sensitivity and resistance. In most cases antibiotic resistance is not transmissible, but represents an intrinsic species or genus specific characteristic of the organisms. Although plasmid-linked antibiotic resistances are not common among lactic acid bacteria, they cannot be excluded and safety implications should be taken into consideration. Strains harboring resistance plasmids should not be used either as human or animal probiotics. Checking the ability of a proposed probiotic strain to act as a donor of antibiotic resistance genes may be a precaution, in particular in the case of animal feeding, where use of antibiotics as growth promoters apparently creates a selective advantage for resistance factors (244).

The features claimed for lactic acid bacteria overweigh their possible risks. The immune-stimulation and colonization resistance features may be useful in poultry husbandry. Food safety demands urge to replace antimicrobial feed additives and to produce healthy and *Salmonella*- and *Campylobacter*- free poultry. Lactic acid bacteria added with the feed may be a good strategy to comply with present and future regulations.

During the last decades a lot of research has been done on the effect of probiotics in rodents, humans, and also farm animals. However, still more effort must be invested to elucidate the immune stimulating properties, find the mechanisms causing resistance against enteric infections, and to select probiotic strains.

3

Introduction to the experimental work

Introduction to the experimental work

The aim of our study was to find Lactobacillus strains with probiotic immunomodulating properties. To select strains with these capabilities we optimized a model in which immunomodulation by lactobacilli could be tested in chickens (chapter 4), and a screening method to find immunoprobiotic strains within the existing pool of Lactobacillus strains (chapter 5). The selected strains were tested on their immunomodulating capacities *in vivo* (chapter 6). The two most capable strains were tested in a relevant infection model, Salmonella infection (chapter 7).

The quest for immunomodulating probiotics in chicken started with testing the models that could be used for selecting bacteria with probiotic properties. In order to detect immunoprobiotic lactobacilli an *in vivo* immunomodulation model was a necessary tool. In chapter 4, the TNP-KLH immunization model was modified in order to detect immunomodulation. Intravenous immunization was selected from the different routes tested (intravenous, intramuscular, ocular, subcutaneous) to be used in the subsequent experiments. Stimulation of the immune response should be detectable in the model. Therefore the baseline immune response to the antigen should be way below maximal. When the chickens were immunized with a suboptimal amount of TNP-KLH, immune stimulating probiotic lactobacilli could notably enhance the cellular and humoral anti-TNP-KLH response. In the experiments, it became clear that although broilers and layers have developed differently with regard to their physiology. This did not only affect meat or egg production, but also the immune response to TNP-KLH. The broilers seem to be specialized in IgM response only, while layers responded broader, resulting in higher IgG and cellular responses to TNP-KLH, while the IgM response was not low either.

In order to find immunomodulating probiotics an *in vitro* assay was set up for lymphocyte proliferation (chapter 5). In this assay, the cells are not only stimulated by mitogens or antigens, but also by mitogen (ConA) in combination with lactobacilli. The assay was validated by two *in vivo* experiments. In the *in vitro* assay spleen cells from 6-week old layer type chickens were stimulated with a suboptimal concentration of ConA (in order to be able to measure stimulation of proliferation) and the lactic acid bacterium strain to be tested. The stimulating effect of a strain was measured by extra proliferation by the strain compared to the proliferation by the suboptimal ConA concentration alone. The inter-chicken-variation made it necessary to incorporate a reference strain to which all results could be compared. With these referenced values, comparing of data obtained with spleen cells from different animals and performed on different days became possible. The *in vitro* proliferation enhancement and the *in vivo* humoral immune response enhancement were found to be related to antigen presenting cells like macrophages and dendritic cells. The lactobacilli activated these cells to produce interleukins, which stimulated the T cells to proliferate. These activated T cells supported more B cells to produce immunoglobulins. With this assay, only five

strains out of almost 100 seemed to have a potential immunomodulating effect in the chicken type tested.

In chapter 6 immunomodulation by probiotic bacteria, selected by the *in vivo* model and the *in vitro* assay is described. Immunomodulation was found as enhancement of the humoral (in serum) and cellular immune responses (proliferation of spleen cells and uptake and killing of Salmonella by monocytes of spleen, caecum and ileum). The immunomodulation was shown to be dependent on the Lactobacillus strain used, its interaction with the host immune system, the dosage and administration schedule of the lactobacilli and the age of the host.

L. paracasei and *L. brevis* seemed to be the most promising immunomodulating strains in chickens. Therefore these strains were tested in an infection model employing Salmonella enteritidis infection (chapter 7). The effects of the probiotic lactobacilli on the Salmonella infection and the physiological effects in the gut and the immune response were monitored. Our hypothesis was that the selected probiotics could have a positive effect on disease resistance because of the combination of competitive exclusion and immunomodulation. The infection was reduced by the Lactobacillus strains tested (faster clearance of the systemic infection), as was the immune response. The latter was found both locally (interleukins in the gut) and systemically (humoral and cellular immune responses). Infection was less severe due to the use of the probiotic lactobacilli, the strains did not stop the shedding of Salmonella from the infected chickens or prevented colonization of the chickens.

In chapter 8, the background of immunomodulating probiotics in chickens is discussed in relation to the data presented in the chapters 4 to 7.

4

Immunological differences between layer- and broiler- type chickens

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Abstract

In commercial poultry husbandry alternatives for the use of antibiotics and vaccines are under investigation, which preferably have to be applicable for both layer- and broiler-type chickens. There are indications that the defense mechanisms vary between layer- and broiler-type chickens. Therefore, the difference in immune response between layer- and broiler-type chickens of the same age was investigated, using TNP-KLH (trinitrophenyl-conjugated keyhole limpet hemocyanin) as antigen without adjuvant. First different routes of immunization (intravenously, intramuscular, subcutaneous and ocular) were examined to find out which immunization route gives the highest antibody titers. The intravenous immunization route resulted in higher TNP-specific antibody responses than the other immunization routes tested and therefore this immunization route was used in both following experiments. In order to investigate the optimal dose of antigen needed for immunization, a dose-response curve in broiler- and layer-type chickens was completed. The humoral immune response was measured in serum by a TNP-specific ELISA and the *in vitro* cellular immune response by an antigen-specific lymphocyte proliferation assay.

The antibody response of layer- and broiler-type chickens appeared to differ, not only in optimal dose and response, but also in kinetics of the response itself. Broiler chickens generated higher IgM anti-TNP titers whereas layer-type chickens generated higher IgG anti-TNP titers. This specific antibody response in broiler-type chickens did not last as long as in layer-type chickens. The TNP-specific cellular immune response was detectable in layer-type chickens, but not in broilers. Both types generate a non-specific cellular immune response, although this response in broilers is lower than in layer-type chickens.

From these results we conclude that broilers primarily respond to TNP-KLH with a high IgM antibody response whereas layer-type chickens respond with a high IgG response. In addition, the cellular response of layer-type chickens is much higher than the response of broilers. The results suggest that broilers are specialized in the production of a strong short-term humoral response and layer-type chickens in a long-term humoral response in combination with a strong cellular response, which is in conformity with their life expectancy.

Introduction

In the past, chickens have genetically been selected for improved feed conversion and rapid growth or production of eggs. This has led to two different types of chickens: broilers and layer-type chickens. As a consequence of this selection for economically important production traits, these chickens differ in body weight gain, duration of life and, recently data are becoming available, also in immune system caused by genetic differences. Therefore concern has risen with respect to their ability to mount an immune response to the whole array of pathogens as encountered in poultry breeding. In organic animal husbandry the animals are kept in a more natural environment without the strict hygienic measurements and antibiotics to prevent pathogenic and parasitic infections. When antibiotics are removed from the feed or if chickens will be held organically the capacity to mount immune responses will be even more important.

Several publications are available on the relation between performance traits and immunological responses (32, 159, 206, 226, 230, 258, 293). A negative correlation was found between weight and antibody response (total antibody response and specific anti-sheep red blood cell (SRBC)) in broilers. It was concluded that the genetic and possibly nutritional changes in broiler chickens have put faster growing broilers in a disadvantageous situation in terms of humoral immune function. This correlation was also demonstrated in layer chickens: a layer-type chicken line selected for low antibody responses to SRBC had significantly higher 4-week body weights than either the unselected control or the high antibody response line (259). In addition, broiler-type chickens of a relatively low body weight line were reported to have high and long-lasting anti-SRBC titers after primary immunization as compared to those of a heavy body weight line (191). Although layer-type chickens have also undergone selective breeding, it seems that this selection has less influenced their immune system, although only few data are available about the effect of the selection for egg production on this type of chicken (258).

From literature data it is quite impossible to compare the immunological competence of layer- and broiler-type chickens, as the conditions in most reports differ with respect to type and dose of antigen, route of immunization, age of the chickens, etc. So far, broiler- and layer-type chickens have never been compared for their immune responsiveness under identical circumstances. Therefore, we investigated whether the immune system of layer-type chickens differs from that of broiler chickens under similar circumstances at similar age. As prototype for layer chickens we have chosen White Leghorn and as a typical broiler type Ross 508. For immunization, we used an artificial antigen that chickens have never been exposed to before, trinitrophenyl-conjugated KLH (TNP-KLH). TNP-KLH is a non-replicating, thymus-dependent antigen, which is used without an adjuvant. Interaction with maternal antibodies against this antigen is not possible, which ensures that no differences exist between broilers and layer-type chickens concerning responses to

TNP-KLH. Because the immunization route for non-replicating or dead antigens influences the humoral and cellular immune response significantly in chickens (200, 294) and mice (284), we first investigated which immunization route (intravenous, ocular, intramuscular or subcutaneous) resulted in the optimal antibody responses. Subsequently we compared the humoral response to different concentrations of TNP-KLH (administered intravenously) in broiler- (Ross) and layer- (White Leghorn) type chickens. Finally we compared the specific (TNP-KLH) and non-specific (ConA) cellular response of the two types of chickens after intravenous immunization with TNP-KLH.

Materials and methods

Animals and husbandry

Eggs of broiler (Ross, Pronk B.V. Meppel, the Netherlands and Praktijkonderzoek pluimvee, Beekbergen, the Netherlands) and layer-type (White Leghorn, Charles River) chickens were bred and kept under routine specific pathogen free (SPF) conditions. Feed ('Opfokkruimel 2775', Hope Farms B.V., Woerden, the Netherlands) and water were available ad libitum and the boxes had wood chips on the ground. The conditions in the boxes were as follows: temperature 25 °C, lightcycle 16 hours light and 8 hours dark and humidity 55%. The animals were numbered individually and held together in groups. Layers and broilers were held in the same room but in different pens.

Immunization and experimental design

Antigen

2,4,6-Trinitrophenyl (TNP; Eastman Kodak, Rochester NY, USA) was conjugated to KLH (Sigma, St. Louis, USA) as described previously (49).

Influence of immunization route

At 4 weeks of age, four groups of 10 SPF layer-type chickens (White Leghorn) were immunized with 20 µg TNP-KLH in 0.2 ml 0.9% NaCl by different routes: intravenous (in the wing vein), intramuscular (in the thigh muscles), subcutaneous (in the neck) or ocular (eye drop). The relatively low dose of 20 µg was chosen to better evaluate the efficacy of the four routes of administration. On day 7 and 10 after first immunization (day 7 and 10 p.p.) blood samples were collected for serum. At 21 days after first immunization, the chickens were immunized again with the same dose and via the same route as the first immunization. On days 7 and 10 after second immunization (day 7 and 10 p.b.) blood samples were collected for serum.

Background titers and proliferation

At four weeks of age, one group of 17 layer-type chickens was intravenously immunized with 20 µg TNP-KLH in 0.2 ml 0.9% NaCl. Four weeks after first immunization the chickens were immunized again with 20 µg TNP-KLH via the same route. Another group of 15 layer-type chickens was not immunized. On day 5, 7 and 10 after first immunization and day 5, 7, 10 and 14 after secondary immunization serum samples were taken from all chickens. One week after primary immunization LPA was performed on spleen cells of six chickens.

Comparative studies
Antigen dose and response kinetics

At 4 weeks of age, groups of six chickens of broiler- or layer-type were intravenously immunized with 10, 33, 100, 333 or 1000 µg TNP-KLH in 0.5 ml 0.9% NaCl. At 6, 10 and 14 days after first immunization two chickens of each group were used to obtain serum for detection of TNP-specific antibodies.

Specific T cell proliferation

Broiler chickens (n=15) and layer-type chickens (n=15) were divided in six groups (group A-B-C broilers and group D-E-F layer-type chickens) of five animals each. On day 14 after hatch groups A, B, D and E were primed by intravenous immunization with 500 µg TNP-KLH in 0.2 ml 0.9% NaCl. On day 35 after hatch group A and D were immunized again (iv) with 500 µg TNP-KLH in 0.2 ml 0.9% NaCl and group C and F were primed with 500 µg TNP-KLH in 0.2 ml 0.9% NaCl. At this same day chickens of groups B and E were used for lymphocyte proliferation assays (LPA) on peripheral blood and spleen. On day 42 and 49 after hatch (= 7 and 14 days after the last immunization) LPA were performed on peripheral blood and spleen of chickens of group A, D, C and F.

Determination of TNP-specific humoral and cellular response
Antibody determination by ELISA

Serum antibodies to TNP were determined by means of a direct ELISA as described previously (136). Briefly, TNP-bovine serum albumin (BSA, Sigma, St. Louis USA) was coated overnight in 96-well high binding ELISA plates (Greiner, Nürtingen, Germany). Titrated sera were incubated for 1 hour at room temperature in a twofold dilution. The isotype specific responses were determined with mouse monoclonal antibodies CVI-ChIgM-59.7, specific for chicken IgM, and CVI-ChIgG-47.3, specific for chicken IgG (23, 135). Detection was performed with rabbit-anti-mouse-HRPO (DAKO A/S, Glostrup, Denmark) and the substrate tetramethylbenzidine (0.1 mg/ml) and H_2O_2 (0.005 % v/v). Extinction was measured at 450 nm. Antibody titers were calculated as the dilution of the sample giving an

extinction value of 1 above the background. Geometric mean titers (GMT) of individual 2-log titers, S.E.M. and antilog (2^{GMT}) values were calculated.

Lymphocyte proliferation assay

Spleens were removed aseptically and homogenized directly. Single cell suspensions of splenocytes were prepared by crushing individual spleens through 70 µm nylon cell strainers (Becton Dickinson, Franklin Lakes, NJ USA). Heparinized blood was diluted two times in PBS. The mononuclear cells of spleen and blood were enriched over a Ficoll-Paque gradient (Amersham Pharmacia Biotech, Uppsala Sweden) and washed three times in PBS. 10^6 viable cells per well were incubated 68 hours in a humidified incubator at 41°C with 5% CO_2 in 200 µl RPMI 1640 (Gibco, Life Technologies) containing 1% normal chicken serum (Gibco, Life Technologies) and antibiotics in flat bottom 96-well plates (Costar, Corning Inc., Corning NY, USA). The cells are incubated with different concentrations of ConA (40 and 80 µg/ml) or TNP-KLH (1.5, 5, 15 and 50 µg/ml) in order to measure (re)stimulation *in vitro*. For ConA after 68 hours and for TNP-KLH after 92 hours 0.4 µCi/well ^{3}[H]-thymidine (Amersham Pharmacia Biotech, United Kingdom) was added and 4 hours later the plates are harvested onto fiberglass filters and counted by liquid scintillation spectroscopy (Betaplate, Wallac Oy, Turku, Finland).

Statistical analysis

All statistical analyses were carried out with Student's *t*-test and are shown wherever appropriate. Differences between groups with *P*-values > 0.05 were considered not to be significant.

Results
Influence of immunization route

We investigated four immunization routes to find out which of these resulted in optimal antibody responses after immunization with TNP-KLH. Table 1 shows TNP-specific IgM and IgG antibody titers in sera of layer-type chickens on day 7 and 10 after first immunization and day 7 and 10 after second immunization.

After first immunization the IgM responses were not high and therefore the differences between the routes were not pronounced. The immunizations via ocular and intravenous routes resulted in significant higher IgM titers than the intramuscular immunization route on day 7. At this day the subcutaneous route is not significantly different from the other routes. On day 10 the serum IgM responses following intramuscular and subcutaneous routes were still as high as on day 7, while IgM titers after intravenous and ocular immunization started to decrease. After second immunization the intravenous immunization route resulted in the significantly highest IgM and IgG titers at both 7 and 10 days.

Table 1 IgM and IgG anti-TNP titers in layer-type chickens after immunization via different immunization routes[a,b].

Immunization route	7 days after first immunization	10 days after first immunization	7 days after second immunization	10 days after second immunization
IgM				
Intravenous	289 *(51)*[a]	146 *(21)*[b]	15040 *(3340)*[a]	5706 *(1252)*[a]
Intramuscular	161 *(17)*[b]	165 *(39)*[b]	377 *(119)*[b]	1619 *(807)*[b]
Subcutaneous	285 *(84)*	319 *(91)*[a]	566 *(261)*[b]	519 *(321)*[b]
Ocular	343 *(62)*[a]	254 *(38)*[a]	205 *(31)*[b]	178 *(31)*[b]
IgG				
Intravenous	119 *(31)*[a]	11 *(3)*[a]	3716 *(1155)*[a]	30901 *(13443)*[a]
Intramuscular	45 *(9)*[b]	5 *(1)*[b]	180 *(63)*[b]	1556 *(628)*[b]
Subcutaneous	60 *(16)*[b]	7 *(2)*[a]	125 *(30)*[b]	234 *(97)*[c]
Ocular	60 *(38)*[b]	5 *(3)*[b]	37 *(15)*[c]	28 *(13)*[d]

[a] Means (S.E.M.) from serum samples taken on days 7 and 10 after first and second immunization.
[b] Different subscripts in the same column are significantly different (at least $P < 0.05$).

The IgG titers were low after first immunization. The intravenous route results in a significant higher IgG response on day 7 after first immunization. On day 10 after first immunization the intravenous route is significantly higher than the intramuscular and ocular routes.

The highest IgG titers were generated on day 10 after second immunization and the IgM titer peaked depending on the route: on day 7 the intravenous route and on day 10 the intramuscular route generated the highest titer. IgM titers generated after ocular and subcutaneous immunization did not result in a peak. These titers stayed at the same level on day 7 and day 10.

Comparative studies
Background titers and proliferation

The background titer against TNP-KLH in chickens was compared to the titers in chickens that were immunized with this antigen. In tables 2 and 3 it is shown that the chickens that were not immunized with TNP-KLH, neither had a specific antibody-response nor a specific cellular response against TNP-KLH.

Table 2 IgM and IgG anti-TNP titers in layer-type chickens after first and second immunization with TNP-KLH[a].

		IgM		IgG	
		Control	Immunized	Control	Immunized
	Day 0	18 *(4)*	30 *(5)*	3 *(11)*	4 *(2)*
First immunization	Day 5	44 *(3)*	2114 *(730)*	1 *(1)*	372 *(165)*
	Day 7	65 *(8)*	1492 *(408)*	6 *(1)*	425 *(143)*
	Day 10	41 *(3)*	295 *(77)*	7 *(2)*	397 *(94)*
Second immunization	Day 5	53 *(4)*	12388 *(3114)*	9 *(3)*	6639 *(1651)*
	Day 7	45 *(5)*	5407 *(1458)*	10 *(5)*	4697 *(1055)*
	Day 10	42 *(5)*	882 *(452)*	45 *(15)*	1652 *(355)*
	Day 14	38 *(1)*	446 *(208)*	29 *(21)*	1205 *(273)*

[a] *Mean titers (S.E.M.) from serum samples taken on days 5, 7 and 10 after first immunization (iv) and days 5, 7, 10 and 14 after second immunization (iv).*

Table 3 Proliferation (cpm) of spleen T cells of layer-type chickens after restimulation with TNP-KLH or ConA[a].

	Medium	TNP-KLH	ConA
Immunized	388 *(246)*	2844 *(1380)*	5469 *(8630)*
Control	162 *(38)*	516 *(215)*	54120 *(10012)*

[a] *Proliferation assays were performed 1 week after second immunization with TNP-KLH (iv) and the control group was not immunized.*

Antigen dose and response kinetics

The humoral immune response was tested after intravenous immunization with several doses of TNP-KLH in broiler- and layer-type chickens. Responses were measured on day 6, 10 and 14 after immunization. Figures 1a-d show the specific IgM and IgG antibody titers of layer- and broiler-type chickens after first immunization (iv) with different doses (3, 10, 33, 100, 333 and 1000 µg) TNP-KLH.

Broilers generated higher IgM titers than the layer-type chickens. Both chicken types had the highest IgM antibody titer on day 6 after immunization with 333 µg TNP-KLH (figures 1a and b). For broilers the IgM titer after the highest TNP-KLH immunization declined over a few days while for layer-type chickens the IgM titer seemed to disappear earlier.

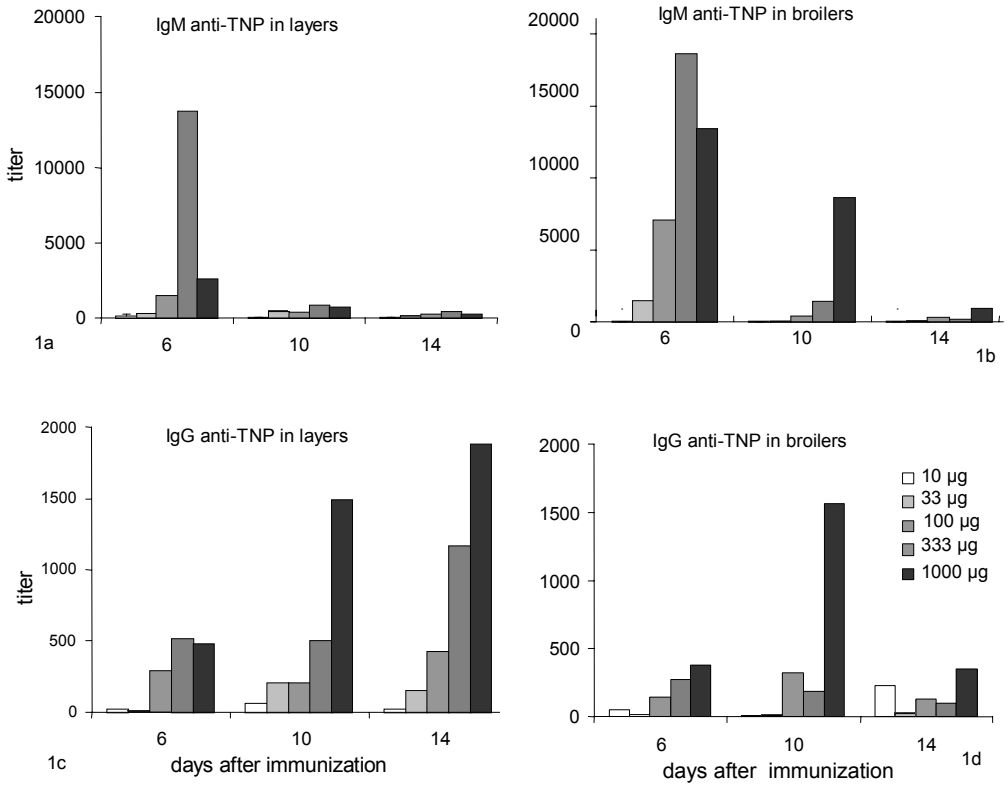

Figure 1. a) IgM anti-TNP titer in layer-type chickens; b) IgM anti-TNP titer in broilers; c) IgG anti-TNP titer in layer-type chickens; d) IgG anti-TNP titer in broilers

Layer-type chickens generated higher IgG titers than broilers on day 14 after first immunization with most doses of TNP-KLH (figure 1c). The IgG titer in layer-type chickens increased with higher concentrations of TNP-KLH (figure 1d). Broilers especially responded with IgG on day 10 after immunization with the highest concentration TNP-KLH used (1000 µg). This response level was comparable to the IgG response on day 10 to the same concentration of TNP-KLH in layer-type chickens. The IgG titer in layer-type chickens was increasing on days 10 and day 14, while the IgG titer decreased from day 10 towards day 14 in broiler-type chickens.

Antigen-specific and non-specific cellular response

From the results on the influence of antigen dose it appeared that the optimal response in both broiler- and layer-type chickens was found after immunization with doses between 333 and 1000 µg TNP-KLH. Therefore 500 µg TNP-KLH was used in the experiment to examine the lymphocyte proliferation in PBL and spleen.

Using PBL from TNP-KLH immunized chickens, no proliferation was induced by *in vitro* TNP-KLH restimulation as compared to the background (cells in medium). This was independent of the type of chicken or the concentration TNP-KLH used in this assay (data not shown).

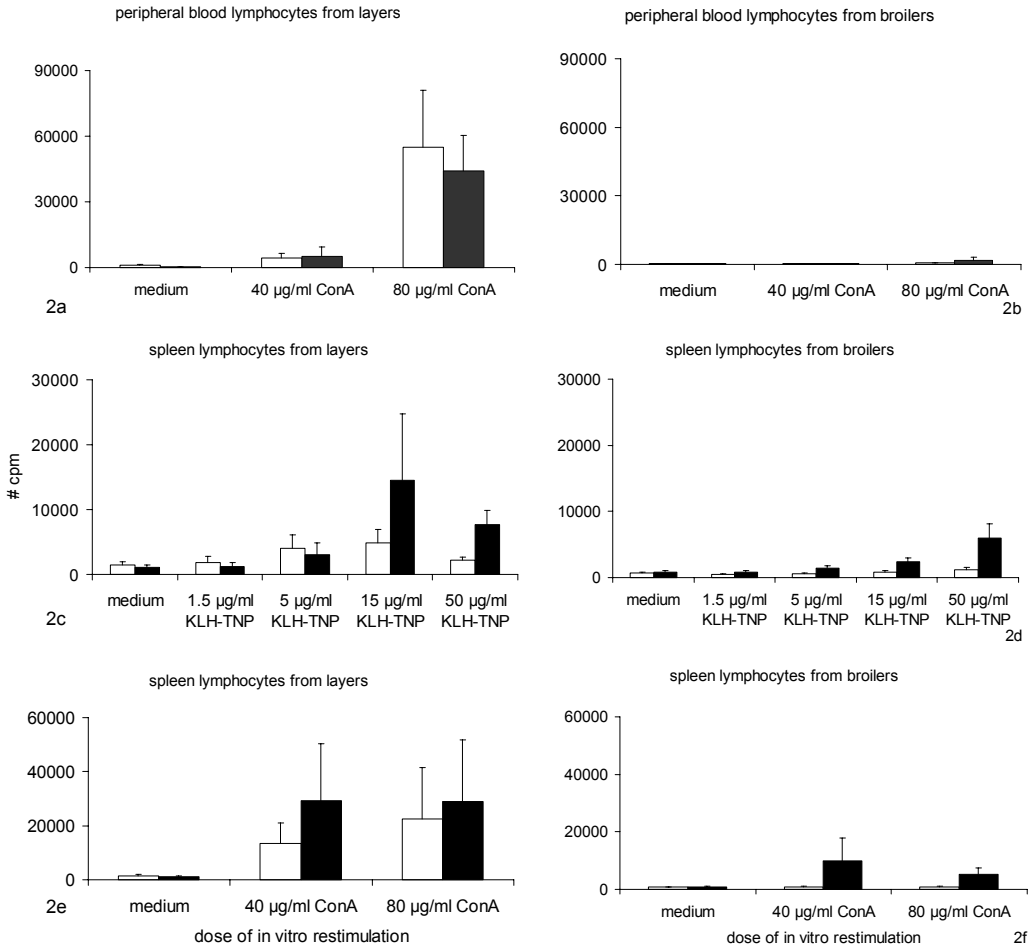

Figure 2. a) Proliferation (+S.E.M.) of peripheral blood lymphocytes from layer-type chickens and b) of broilers after stimulation with ConA. c) Proliferation (+S.E.M.) of spleen lymphocytes from layer-type chickens and d) of broilers after restimulation with TNP-KLH. e) Proliferation (+S.E.M.) of spleen lymphocytes from layer-type chickens and f) of broilers after stimulation with ConA. White bars: after first immunization; black bars: after second immunization with TNP-KLH.

The PBL did respond to ConA (figures 2a and b). Responses of PBL of layer-type chickens increased with a higher concentration of ConA. These lymphocytes proliferated after stimulation with 40 and 80 µg/ml ConA (respectively 1.2-4.8*10^3 and 44-54*10^3 net cpm). With 40 µg/ml ConA the broiler PBL could not be induced to proliferate above background, even after stimulation with 80 µg/ml ConA the lymphocytes of broilers did slightly proliferate. At this dose the proliferation of broiler PBL was low (200-1500 net cpm) as compared to the response of PBL of the layer-type chicken.

The specific proliferation induced by restimulation with TNP-KLH in spleen cells of layer-type chickens gave a dose-response curve with optimal restimulation concentration 15 µg/ml (figure 2 c). In addition, a clear first and second immunization effect was visible after restimulation with 15 and 50 µg/ml TNP-KLH, of which 15 µg/ml TNP-KLH induced the highest proliferation. In broilers, none of the concentrations used for *in vitro* restimulation induced proliferation after first immunization. After second immunization only *in vitro* restimulation with 50 µg/ml TNP-KLH induced proliferation in broilers (figure 2d).

Spleen lymphocytes of both types of chickens proliferated after stimulation with 40 and 80 µg/ml ConA (figures 2 e and f), although a big difference in response was observed. The spleen lymphocytes of layer-type chickens proliferated more than three times as much as the broiler lymphocytes after stimulation with ConA.

Discussion

The differences between layer- and broiler-type chickens with respect to immunological capacity were under identical circumstances investigated on B and T cell level using the artificial antigen TNP-KLH. For the model antigen TNP-KLH the intravenous immunization route induced the highest antibody titers in layer-type chickens. For this experiment applies what others previously found: the height of the antibody titer is influenced by the immunization route (284, 294). After first immunization all routes induced low titers whereas only the ocular route induced relatively high IgM titers. This route did not induce an IgG or second immunization response, though. This might be caused by the using the dose of 20 µg, perhaps being too low for this immunization route. We chose for this low dose of antigen for immunization in order to be able to determine modulation of the immune responses by for example probiotics or adjuvants. For the intramuscular and subcutaneous routes the dose of antigen used might be too low as well in order to get a proper antibody titer. The results of the subcutaneous immunization showed more variability in the response, which led to a higher standard deviation. The kinetics of the response after subcutaneous immunization showed a response that was higher on day 10 than on day 7, for all the other routes the reverse was observed. This route probably is less efficient for the antigen to be presented to the immune system than the other routes. The antibody response is decreasing rapidly after first

immunization, which is plausible to be caused by using the antigen without adjuvant. Without adjuvant the antigen can be immunized via the intravenous route, whereas antigen with adjuvant, as in commercially available vaccines, could be toxic when immunized in the veins. In conclusion, intravenous immunization with TNP-KLH induced the highest IgM and IgG titers after second immunization. The intravenous immunization route was therefore used in the following experiments.

Subsequently, the optimal dose of antigen was determined for the broiler- and layer-type chicken. The dose of TNP-KLH influenced the antibody response in both broilers and layer-type chickens. For IgM the optimum concentration was 333 µg TNP-KLH. The concentration of 1000 µg TNP-KLH had a negative influence on the antibody titer and is therefore too high. For IgG we found a linear dose-response curve in both type of chickens: the higher the dose, the higher the antibody response and 1000 µg inducing the highest titer. The optimum dose for intravenous immunization with TNP-KLH inducing both high IgM and IgG antibody titers is therefore between 333 and 1000 µg.

The kinetics of the specific IgM anti-TNP responses in layer- and broiler-type chicken was similar. The peak was on day 6 after immunization. The specific IgG anti-TNP response for layer-type chickens peaked between day 10 and 14. In contrast, broilers did not seem to generate a proper specific IgG response, because this response was only appreciable with the highest immunization dose on day 10 after immunization. In summary, broilers seemed to generate a good IgM response, but a poor IgG response. The layer-type chickens generated an IgM response, which was a bit lower than the response of the broilers, but these chickens generated an IgG response, which was higher and lasted longer than the response in broilers. Other research groups also found a correlation between lower body weight and higher antibody titers to non-replicating antigen in chickens, although they only investigated different boiler lines (191, 259). In a recent study in our laboratory comparing different broiler lines, it was also found that old-fashioned chicken lines (old Dutch breeds with high body weights, the ancestors of the present broiler lines) had higher antibody titers than the heavier genetically improved broiler lines (Kramer *et al*., personal communication). These differences between broilers and layers may be caused by differences in the structure and function of lymphoid organs, such as the spleen, due to genetic selection. After selection of layer-type chickens for high and low responders to SRBC, the spleen of the high responders contained larger B cell areas (159).

Obviously layer-type and broiler chickens differ enormously in body weight at 6 weeks of age. Our data indicate, although we only compared one type of layer-type chickens (White Leghorn) and broilers (Ross), that the observations concerning a negative relationship between bodyweight and antibody titer also seem to be relevant for chickens of these different types. Although in our experiments the IgG response in layer-type chickens was higher than in broilers, the IgM responses were

similar in both types. From an evolutionary point of view the primary IgM response may be more important than the more sophisticated secondary response, and therefore has not been a major factor in genetic selection.

It was found that broilers and layer-type chickens differ considerably in the humoral response after immunization. Subsequently, we have investigated whether these types of chickens also differ in cellular responses. T cells from broiler- and layer-type chickens, isolated from blood and spleen, proliferated after stimulation with ConA. The height of this proliferation is much lower in broilers than in layer-type chickens. Since ConA is used to measure the maximum functional capacity of the T cells, this means that the overall T cell immune response in broilers is lower than in layer-type chickens.

The specific T cell response against TNP-KLH is detectable in spleen, but not in peripheral blood. This is surprising, because peripheral blood is supposed to reflect the events occurring in lymphoid organs, such as the spleen. The difference in proliferation may therefore, at least to a large extent, be related to the proportions of responding lymphocytes in spleen and blood. Otherwise differences may exist in the functional status of the antigen-presenting cells present in the two organs that are necessary during *in vitro* restimulation. In view of the results after ConA stimulation, the last explanation seems more relevant. In spleen, both specific and non-specific T cell responses were detectable: the cellular response in layer-type chickens is considerably higher than the response in broilers. The *in vitro* restimulation showed an optimum at a TNP-KLH concentration of 15 µg/ml in layer-type chickens. In contrast, restimulation of spleen cells of broilers was only detectable at the highest concentration TNP-KLH. In conclusion, the cellular reaction in broilers occurred at a lower level and a higher concentration of antigen or mitogen is needed in order to detect this (re)stimulation.

In summary, we found that layer-type chickens show two types of immune responses: antigen-specific IgM and IgG humoral responses and antigen-specific cellular response. In broilers, part of the immune responses seems to dysfunction due to the genetic selection on bodyweight and feed conversion. Nevertheless, broilers seem to be able to survive with this immune response in the present husbandry systems. However, the moment that the pressure on the immune system will be higher, for example by withdrawal of antibiotics from the feed, health problems may occur. The fact that broilers have some remaining IgG response and T cell response, however, indicates that application of immunomodulating substances, like probiotics, may be feasible in poultry husbandry.

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5

Development and validation of a new *in vitro* assay for selection of probiotic bacteria that express immune stimulating properties in chickens *in vivo*

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Abstract

Oral administration of immunoprobiotic bacteria may support animal health. Species specificity of such microorganisms required appropriate selection. An *in vitro* assay for the selection of immunoprobiotic lactic acid bacteria (LAB) was developed in chicken. The assay allowed testing of large numbers of individual strains. Immune stimulation *in vitro* correlated well with the *in vivo* situation in two experiments and no false negative results occurred. Therefore this assay is an appropriate selection tool for immunomodulating properties of LAB in chicken.

Introduction

Probiotics were defined as 'a live microbial food supplement that beneficially affects the host by improving its intestinal balance' (87). Numerous studies reported a wide variety of health-promoting properties influencing the host intestinal balance, competitive exclusion, lactose intolerance, diarrhea, mucosal immune response, blood cholesterol and cancer. Since *in vivo* evaluations of probiotic properties are time-consuming, labor-intensive and require large numbers of animals for selecting candidate probiotic LAB strains many *in vitro* assays have been developed (193, 287). These included selection for gut and stomach conditions such as acid- and bile tolerance and adhesion to gut mucus or intestinal cell lines (Caco-2 and HT-29) (30, 52). Also selection methods were described for probiotics that express antimicrobial activity (1, 29, 181), growth inhibition of unwanted flora elements (132) and competitive exclusion (288). Also an *in vitro* gut model (171, 291) has been described to evaluate gut microbial ecology.

After oral or parenteral administration of LAB in humans and mice T cells and macrophages may be stimulated (55, 59, 178). Based on such properties we developed an *in vitro* system for rapid pre-selection of LAB with immunomodulating properties. For T cell proliferation following mitogenic stimulation the activation of accessory cells is required. The pre-selection assay was based on a Concanavalin A (ConA) mitogen induced lymphocyte proliferation assay in which enhancement or inhibition of the response was the result of the immunomodulating properties of LAB for which either T cells or accessory cells may be sensitive. Since chickens do not have lymph nodes, spleen cell suspensions were used that include all relevant cell types. Spleen cells were incubated with LAB and a suboptimal concentration of Con A to allow positive or negative modulation of the responses. To validate this assay typical LAB strains selected in the *in vitro* assay were evaluated in two *in vivo* experiments.

Materials and Methods

Chickens

Eggs of layer type (LSL White Leghorn, Charles River) chickens were bred and raised under routine specific pathogen free (SPF) conditions (no vaccinations, antibiotics or coccidiostatics). For all experiments chickens were fed a starter diet for pullets (Arkervaart, Leusden, the Netherlands) and water ad libitum. Stable conditions: temperature 25 °C, lightcycle 16 hours light and 8 hours dark and humidity 55%. Young chicks had extra heating in order to prevent cold stress. At the start of the *in vivo* experiments animals were equally divided over the groups with respect to weight and gender. The animals were numbered individually and housed per group receiving the same treatment. The pens were closed with plates at the sides and between two pens in use one pen was kept empty to avoid contact and

prevent cross-contamination. All animal experiments were in compliance with Dutch regulations on animal experimentation.

Bacteria

LAB strains used in *in vivo* experiments were identified using fatty acid and sodium dodecyl sulfate-polyacrylamide gel electrophoresis analyses and 3 strains by 16S-rDNA-sequence analysis (BCCM/LMS, Gent, Belgium) The LAB were cultured overnight (18 hours) in MRS broth (de Man, Rogosa and Sharpe, Oxoid Ltd.) after inoculation with 1% of a fresh full-grown culture. The LAB were cultured at 30°C or 37°C dependent on the optimal culture temperature of the strain. To accurately count the bacteria, appropriate dilutions were plated in double-layered MRS agar plates and incubated 2 days at 30°C or 37°C.

Antibody determination by ELISA

Serum antibodies to TNP were determined by a direct enzyme-linked immunosorbent assay (ELISA) as described previously (148). Briefly, TNP-bovine serum albumin (BSA, Sigma, St. Louis USA) was coated and sera were serially diluted in twofold. Responses were determined with mouse monoclonal antibodies CVI-ChIgM-59.7 and CVI-ChIgG-47.3, specific for chicken IgM and IgG (23, 135). Detection was performed with rabbit-anti-mouse-HRPO (DAKO A/S, Glostrup, Denmark) and the substrate tetramethylbenzidine (0.1 mg/ml) and H_2O_2 (0.005 % v/v). Extinction was measured at 450 nm. Antibody titers were calculated as the dilution of the sample giving an extinction value of 1 above the background. Geometric mean titers (GMT) of individual 2-log titers, S.E.M. and antilog (2^{GMT}) values were calculated.

Lymphocyte proliferation assay (LPA) for selection of immunoprobiotics

Spleens of 6-week-old chicks were removed aseptically. Single cell suspensions were prepared and mononuclear cells were enriched over a Ficoll-Paque gradient (Amersham Pharmacia Biotech, Uppsala Sweden). Cells were washed three times in phosphate-buffered saline (PBS, Gibco, Life Technologies) and diluted in culture medium: RPMI 1640 Dutch modification (Gibco, Life Technologies) containing 1% normal chicken serum (Gibco, Life Technologies), 5×10^{-5} M ß-mercapto-ethanol, 2 mM L-glutamin and a mix of antibiotics 5% for sterility and to prevent overgrowth by LAB (penicillin 10^6U/l, streptomycin 1.0 g/l, amphotericin B 60 mg/l, polymyxin B 0.50 g/l and kanamycin 20x con). Triplicate cultures with 10^6 viable cells in 200 µl per well were incubated in flat bottom 96-well plates (Costar, Corning Inc., Corning NY, USA) in a humidified incubator at 41°C with 5% CO_2. Tests included cultures with medium + cells and medium + cells + LAB. Cultures with medium alone, medium + LAB served as controls. The proportion of LAB amounted to 10% of the number of cells. The cultures were incubated without and with 1, 4, or 10 µg/ml

ConA. After 68 h of incubation 0.4 µCi per well ^{3}[H]-thymidine (Amersham Pharmacia Biotech, United Kingdom) was added. Four hours later the plates were harvested onto fiberglass filters and counted (cpm: counts per minute) in a liquid scintillation counter (Betaplate, Wallac Oy, Turku, Finland). The stimulation index (SI) was calculated by dividing the proliferation induced by ConA plus the bacteria corrected for ^{3}[H]-thymidine built in by LAB, by the proliferation induced by ConA only and multiplied by 100.

$$SI = \frac{\text{cpm [cells + ConA + LAB]} - \text{cpm [cells + LAB]}}{\text{cpm [cells + ConA]}} * 100$$

SI = 100 means no stimulation with the bacterial strain tested. To allow comparison between chickens and between experiments, an internal reference strain (*L. paracasei* LW122) was included in every assay. For inter- an intra-assay comparison the SI of this strain was set to 100 and the SI induced by the other strains tested with cells from the same spleen in the same assay was normalized to the SI of the reference strain. This normalized SI is indicated as NSI.

Antigen-specific responses in cell proliferation and serology

For immunization and antigen-specific proliferation 2,4,6-trinitrophenyl (Eastman Kodak, Rochester NY, USA) conjugated to keyhole limpet hemocyanin (KLH; Sigma) was used as described previously (49).

***In vivo* experiment 1: The effect of feeding of LAB on the humoral immune response after priming**

To induce immunomodulation LAB were orally administered daily to 6 groups of 13 chickens aged 3 weeks. Groups 1-6 daily received respectively 10^{9} *L. paracasei* LW122 (group 1), *L. murinus-animalis* LW121 (group 2), *L. buchneri* LW 50 (group 3), *L. buchneri* LW 83 (group 4), *S. alactolyticus* LW 89 (group 5) in PBS or PBS alone (group 6) for five consecutive days. The fifth day all chickens were intravenously immunized with a suboptimal amount of antigen: 20 µg TNP-KLH in 0.2 ml 0.9% NaCl. On days 7, 10 and 14 after immunization serum samples were taken.

Statistical analysis was performed in the statistical package GenStat (90). To exclude confounding plate effects, a random plate effect was included in the analysis of variance (ANOVA) model. Linear mixed models for the peak IgM and IgG responses (day 10 after immunization) were analyzed by residual maximum likelihood methods (REML).

***In vivo* experiment 2: the effect of feeding of LAB on the specific humoral and cellular responses after priming and booster**

The set up was similar as for experiment 1. Now 11 groups of 17 layer type chickens orally received 10^9 LAB for 5 consecutive days: *L. paracasei* LW122 (group 1), *L. reuteri* LW 81 (group 2), *L. brevis* LW5 (group 3), *L. brevis* LW 40 (group 4), *L. brevis* LW 167 (group 5), *L. plantarum* LW 143 (group 6), *L. paracasei* LW120 (group 7), *L. murinus-animalis* LW121 (group 8), *L. buchneri* LW 50 (group 9), *L. paracasei* LW 122 (group 10) in PBS or PBS alone (group 11). Four weeks after priming LAB administration was repeated. Serum samples were taken on days 5, 7 and 10 after priming and days 5, 7, 10 and 14 after booster. One week after primary and secondary immunization we performed LPA on spleen cells of six chickens per group. These spleen cells were incubated with medium, (re)stimulated with 15 µg/ml TNP-KLH, 1, 4 and 10 µg/ml ConA, *L. plantarum* LW 143 and *L. paracasei* LW122 (LAB strains made up 10% of the number of cells in a well together with suboptimal ConA concentrations).

Statistical analyses were performed in GenStat. Treatments were assigned differently to test plates than in experiment I to avoid confounding. ANOVA comprised treatments as the only experimental factor. Treatments were grouped into 3 clusters according to the results in the *in vitro* test. The differences between the clusters and the homogeneity within the clusters were analyzed with respect to the peak of the serological responses; that is, the IgM peaked on day 5 after booster immunization but for the IgG responses, the kinetics were different dependent on the strain of LAB that was used (day 5-7 after booster immunization).

Results

Set up of the *in vitro* assay for selection of immunomodulating LAB

The modulation of *in vitro* lymphocyte proliferation was used to pre-select immunomodulating LAB. Assay conditions were developed in various stages.

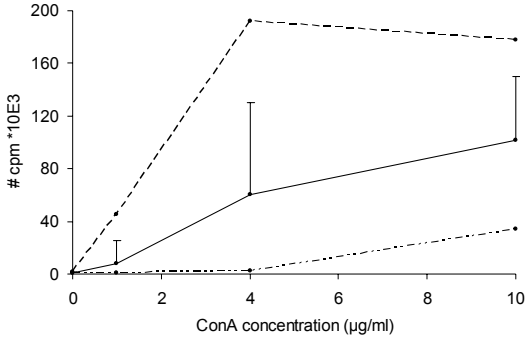


Figure 1. Dose-response curves for ConA induced chicken spleen cell proliferation for selection of the true suboptimal ConA concentration for threshold stimulation. Indicated is the mean dose-response curve with standard deviation (n= 33). To indicate individual variation width the dotted line indicates the dose-response for the individual with the lowest response, the dashed line indicates the highest responder. A useful common suboptimal dose could not be selected. Solid line=average of 33 chicken spleens + S.D.

Antibiotics were added to the culture medium to prevent the LAB strains from growing and thereby interfering with the proliferation in the assay. All strains tested died within 24-36 hours after incubation, which ensured that differences in sensitivity to antibiotics between LAB strains were excluded. LAB alone did not induce lymphocyte proliferation *in vitro* to such extent that it could be used for selection (not shown). Therefore we developed a co-stimulation assay for LAB with suboptimal mitogen (ConA) concentrations to allow the selection for enhancement of the ConA induced response. The proliferative spleen cell response in control chickens showed an almost linear relation between the ConA concentration and incorporation of ^{3}[H]-thymidine (figure 1). However, individual dose-response curves showed large variation. This may be concluded from the lowest and highest dose-responses that were observed (figure 1). The proliferation of cells with medium only was constantly low for all chickens (30 - 500 cpm; figure 1). Proliferation following incubation with 10 µg/ml ConA varied from 40 000 – 180 000 cpm. The steepness of the dose-response curve also showed large individual variation. Therefore we decided to use both 1 and 4 µg/ml ConA for suboptimal stimulation in all further assays and used the results of a true suboptimal concentration for the calculations (table 1).

Table 1. Selection of true suboptimal ConA concentration in spleen cell proliferation assay[a,b]

Spleen	ConA Concentration (µg/ml)			
	0	1	4	10
1	120	138	**5991**	51375
2	146	256	**11641**	61318
3	921	**63058**	93108	103028
4	158	141	**12425**	68796
5	101	**1368**	60754	81428

[a] *Cultures were incubated with three concentrations of ConA and a medium control was included.*

[b] *Data were expressed as cpm for each spleen. For calculation of SI/NSI the results in bold were used.*

Table 2. In vitro selection of LAB based on immunomodulation of sub-optimal stimulated chicken spleen cells[a].

Exp.	*LAB Strain*	Spleen/Animal					NSI		Select	Responder
		1	2	3	4	5	Mean	S.D.		Chickens
1	*L. paracasei LW 122*	100 (232)	100 (299)	100 (117)	100 (229)	100 (705)	100	0	+	5/5
	L. buchneri LW 50	37 (85)	39 (117)	90 (105)	77 (177)	55 (399)	60	23	-	1/5
	L. buchneri LW 83	32 (74)	41 (124)	78 (92)	56 (129)	34 (237)	48	19	-	0/5
	L. plantarum LW 143	34 (78)	34 (100)	85 (100)	45 (103)	12 (86)	42	27	-	0/5
	L. murinus-animalis LW 121	100 (232)	65 (196)	107 (125)	78 (179)	42 (295)	78	26	+	3/5
	L. paracasei LW 120	92 (214)	81 (242)	98 (115)	62 (141)	37 (262)	74	25	+	3/5
	S. alactolyticus LW 89	5 (12)	8 (23)	16 (19)	8 (18)	26 (184)	13	9	-	0/5
2	*L. paracasei LW 122*	100	100	100	100	100	100	0	+	5/5
	L. buchneri LW 50	67	71	37	48	22	49	20	-	0/5
	L. buchneri LW 83	72	70	34	64	56	59	15	-	0/5
	L. plantarum LW 143	38	45	N.D.	66	31	45	15	-	0/4
	L. murinus-animalis LW 121	64	65	111	74	52	73	23	+	2/5
	L. paracasei LW 120	60	53	103	99	87	80	23	+	3/5
	S. alactolyticus LW 89	1	2	6	0	4	3	2	-	0/5
3	*L. paracasei LW 122*	100	100	100	100	100	100	0	+	5/5
	L. brevis LW 5	305	96	129	114	496	228	172	+	5/5
	L. brevis LW 7	511	112	90	85	945	349	379	+	4/5
4	*L. paracasei LW 122*	100	100	100	100	100	100	0	+	5/5
	L. brevis LW 5	210	115	183	320	269	219	79	+	5/5
	L. brevis LW 7	316	191	252	323	229	262	57	+	5/5
5	*L. paracasei LW 122*	100	100	100	100	100	100	0	+	5/5
	L. brevis LW 40	49	20	33	103	170	75	62	+	2/5
	L. reuteri LW 81	133	173	198	131	128	152	31	+	5/5
6	*L. paracasei LW 122*	100	*	100	100	100	100	0	+	4/4
	L. brevis LW 40	96	*	47	92	65	75	23	+	2/4
	L. reuteri LW 81	77	*	378	100	277	208	144	+	3 /4
7	*L. paracasei LW 122*	*	100	100	100	100	100	0	+	4/4
	L. brevis LW 167	*	47	26	78	36	47	22	-	0/4
8	*L. paracasei LW 122*	100	*	100	100	100	100	0	+	4/4
	L. brevis LW 167	18	*	78	27	77	50	32	-	0/4
9	*L. paracasei LW 122*	100	100	100	100	100	100	0	+	5/5
	L. brevis LW 5	121	210	142	92	66	126	55	+	4/5
	L. brevis LW 7	189	212	323	77	70	174	105	+	3/5
	L. reuteri LW 81	138	278	253	34	94	159	104	+	4/5

[a] Nine experiments are shown that were normalized for the results obtained with an internal reference strain. This illustrates reproducibility, sensitivity of individuals and the result of application of selection criteria
NSIs were calculated with reference to strain L. paracasei LW122 set to 100. The average of NSI > 70% was used as cut-off value for positive selection (+/-).
** No correct/useful suboptimal ConA concentration observed.*
For experiment 1 also non-normalized SIs are shown between brackets.

In cultures with ConA only the response varied between 10% and 60% of the positive control (cells with 10 µg/ml ConA). Enhancement of the responses induced by LAB was calculated from the set of cultures with true suboptimal responses to ConA alone and those with suboptimal ConA + LAB. The SI was calculated as the ratio of the proliferation induced by the LAB + ConA and by ConA only (SI range 10–900). For experiment 1 these SIs are shown in parentheses in table 2. The results were normalized with respect to the reference strain (*L. paracasei* LW122) set to 100. For normalized SI (NSI) > 70 the strain was considered to have an immuno-enhancing effect. This cut-off value was calculated in retrospect from the relation between the *in vivo* and *in vitro* experiments and builds-in a safety-margin to prevent exclusion of valuable strains.

A typical experiment (experiment 1, table 2) shows that a broad range in response was observed. The results of 9 different *in vitro* experiments were presented in table 2. The normalization with respect to an internal reference (*L. paracasei* LW122) allowed comparing different experiments based on the use of NSI values. For experiment 1 also the non-normalized SI values are given between brackets. This table illustrated that a LAB strain did not have exactly the same value in every chicken, but that its positive or negative effect is reproducibly detected. Apart from *L. paracasei* LW 122 (the reference strain), also *L. reuteri* LW81 and *L. brevis* LW5 and LW7 were found to have a positive stimulating effect *in vitro*. As is clear from table 2 not all chickens within a breeding line react in the same way to stimulation with immunoprobiotics. Therefore the frequency of chickens reactive in a positive manner was included as a selection criterion. LAB strains showing the highest induction potential also had the broadest working pattern (table 2).

Correlation of *in vitro* immunomodulating probiotic activity with *in vivo* experiments

Several *in vitro* selected strains (positive, intermediate and negative) were tested *in vivo* for validation of the assay and to establish the predictive value for immune stimulation of LAB *in vivo*. In experiment 1 five strains were tested. Immune stimulation was evaluated as enhancement of serological responses to priming with KLH-TNP *in vivo*. *L. paracasei* LW122 had a significant enhancing effect on the specific humoral immune response (P<0.001). *L. murinus-animalis* LW121 showed

an enhancing effect that was not significant (P <0.20). The other strains evaluated did not have a response enhancing effect (figure 2).

These results showed that significant immune stimulation could be induced in a strain dependent manner. The LAB strains that enhanced ConA-induced cell proliferation *in vitro* also induced immune stimulation after oral application of these LAB *in vivo*.

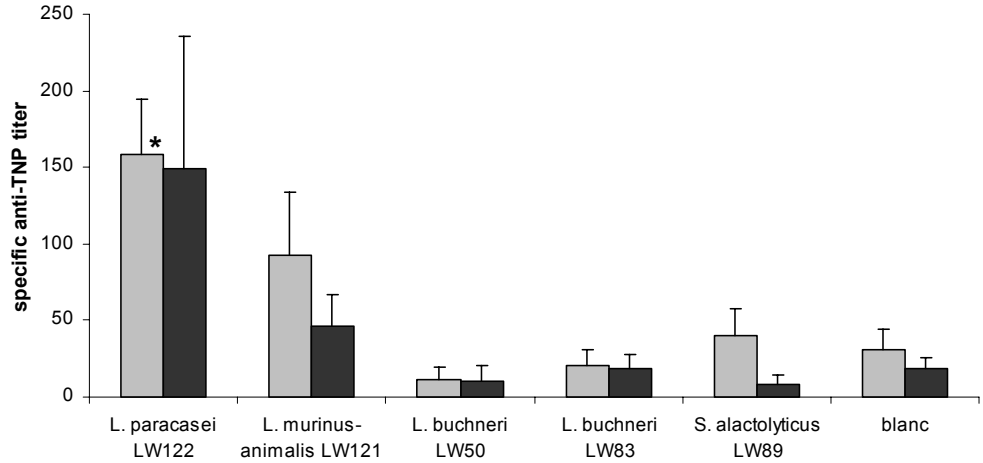


Figure 2. Specific IgM and IgG anti-TNP titers (+S.E.M.) in layer type chickens 10 days after primary intravenous immunization with TNP-KLH in chickens that were fed LAB for 5 consecutive days preceding the antigen injection. L. paracasei LW122 had a positive effect on the specific IgM anti TNP-KLH response (P<0.05). Grey bars: specific IgM anti TNP titer; black bars: specific IgG anti-TNP titer.

Nine LAB strains were tested for their effect on the secondary immune response in *in vivo* experiment 2. As expected, oral administration of *L. paracasei* LW 122 did not generate an IgM or IgG antibody titer to the antigen in the group that was not immunized with TNP-KLH. Three clusters of serological response patterns were observed. The group that was immunized but did not receive LAB (cluster I) generated the lowest IgM response (figure 3a). The groups that received LAB strains that were not enhancing the *in vitro* proliferative response generated a similar low IgM titer as the group that did not receive LAB (cluster II: *L. buchneri* LW 50, *L. plantarum* LW 143, *L. brevis* 167; p = 0.695). No false negatives were observed. Cluster III strains had an intermediate or positive effect on the ConA-induced proliferation *in vitro* and also enhanced the IgM titers *in vivo*. Responses of LAB in cluster III: *L. paracasei* LW122, *L. paracasei* LW120, *L. brevis* LW40, *L. murinus-animalis* LW121, *L. reuteri* LW81, *L. brevis* LW5) were statistically different from responses induced by LAB from cluster I (P = 0.004). Also individual strains

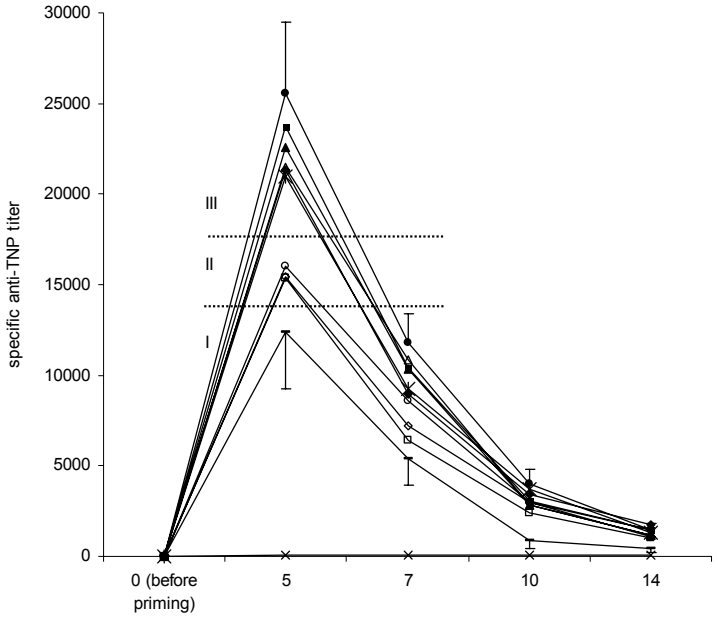
i.e. *L. paracasei* LW122 (P = 0.019), *L. murinus-animalis* LW121 (P = 0.033) and *L. paracasei* LW120 (P = 0.024) responses differed significantly from the response of the group without lactobacilli. For IgG the picture was not as clear (figure 3b). All strains except *L. reuteri* LW81 strains did not have an effect on the IgG response on day 5 after secondary immunization. However, it appeared that the IgG titer of the groups receiving the LAB strains *L. murinus-animalis* LW 121, *L. paracasei* LW120, *L. plantarum* LW143, *L. brevis* LW40 and LW 167 and *L. buchneri* LW50 showed a slower decline than was observed for the titers in the chickens that did not receive LAB. The responses of clusters 2 and 3 were significantly different (P = 0.031). Experiment 2 demonstrated that the results of the *in vitro* assay correlated well with the immunostimulation during primary and secondary IgM response but less well with the IgG response.

Discussion

Selected LAB have been shown in various systems to express immune-enhancing properties (33, 130, 185, 215, 223). In order to select in a relatively simple manner LAB that express immune stimulating properties *in vivo* in chickens an *in vitro* assay was set up. The assay was based on enhancing proliferation of suboptimally stimulated lymphocytes by probiotic bacteria. To verify whether selected LAB inducing immune stimulation *in vivo* could be selected in simple procedures *in vitro*, the enhancement of proliferation in response to suboptimal ConA was thought to provide a suitable model. ConA proliferation of T cells is dependent on the presence and activation of accessory cells. It was hypothesized that those LAB that were able to enhance T cell proliferation *in vitro* by activation of either accessory cells or T cells might do the same *in vivo*. This assumption was based on observation of enhanced secretion of IFN-γ, phagocytosis and expression of complement receptors on phagocytes (28, 165, 214, 308). But in the *in vitro* assay LAB and spleen cells were co-cultured, which is quite different from the potential interactions that at first sight can be expected after oral administration of LAB *in vivo*. However, LAB may induce expression of various cytokines at the level of GI-tract-associated lymphoid cells (169). In addition, gut dendritic cells (DCs) may penetrate the epithelial cell layer without disrupting the barrier function and directly sample gut-associated bacteria (237). Via this route also LAB may differentially affect DC maturation (48) and stimulate the local T cells. Subsequently, the circulating pool of mucosa-homing T-lymphocytes may exert an immune outcome at distant (mucosal) sites (50). Such an effect might be measured as immune stimulation or adjuvant activity since activated T-helper cells could also move to the spleen and stimulate the Th2 cells in e.g. antibody production.

For selection in the *in vitro* assay the definition of suboptimal stimulation was critical. Chickens are produced in breeding-lines and even though the number of breeding fathers was limited, chicken cells in the *in vitro* assay showed wide

variation in ConA induced proliferation. Suboptimal rather than optimal ConA concentrations induced relatively large standard deviations. However, using multiple



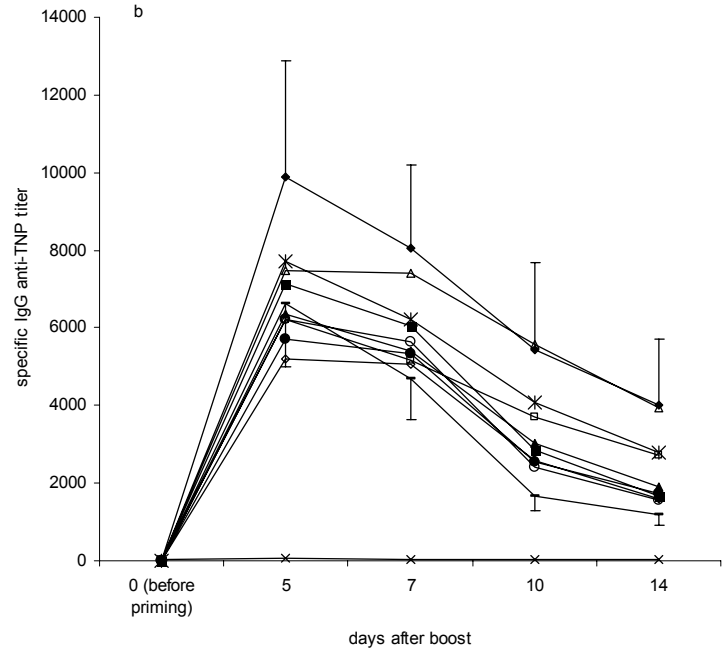


Figure 3. Specific anti-TNP titers (+S.E.M.; panel a represents IgM and panel b represents IgG) in layer type chickens on days 5, 7, 10 and 14 after secondary intravenous immunization with TNP-KLH.
For clarity only the highest and the lowest line have S.E.M. bars. S.E.M. for other data were of similar proportions. The dashed lines represent the separation in clusters: Cluster I received no oral LAB; Cluster II received oral administration of LAB that had no effect in vitro and Cluster III that included evaluation of LAB strains that showed intermediate/positive effects in vitro.
L. paracasei LW122 (closed circle); L. paracasei LW 120 (closed square); L. brevis LW 40 (triangle); L. reuteri LW 81(closed diamond); L. brevis LW 5(star); L. plantarum LW 143 (open circle); L. buchneri LW 50 (open square); L. murinus-animalis LW121 (open triangle); L. plantarum LW 167 (open diamond); no LAB (striped); L. paracasei LW 122, in non immunized chicks (cross).

suboptimal concentrations allowed to reproducibly determine the relative stimulation at one selected ConA concentration and to compare that value to the control situation (shown in table 1). This, however, reduced the number of strains that could be evaluated using one chicken spleen.

L. paracasei LW 122 in the preliminary experiment had a positive effect upon oral administration on the *in vivo* humoral responses and *ex vivo in vitro* proliferation. This confirmed results obtained with this strain in the mouse system (169). Therefore, *L. paracasei* LW 122 was used as an internal reference in each chicken which allowed intra- and inter-assay comparison. A strain was considered positive when the proliferation in the spleen cells of the same chicken was > 70% of NSI induced by *L. paracasei* LW122. Some LAB strains induced proliferation in spleen cells of every chicken *in vitro* (5/5, see table 2), other strains only in a few chickens. This effect was also seen *in vivo* both in humoral and in proliferative responses. This broad variation in immunomodulation by lactobacilli in different individuals has also been observed in mice and man (166, 225). Strain selection should therefore not only be based on the strength of the effect but also on the proportion of positively reactive chickens.

Results from LAB strains tested both *in vitro* and *in vivo* correlated well between results of *in vitro* proliferation assays and modulation of the induction of an antigen-specific IgM titer *in vivo*. The LAB strains that had a positive effect *in vivo* and *in vitro* were: *L. paracasei* LW 122 and LW 120, *L. brevis* LW 5 and LW 40, *L. reuteri* LW 81 and *L. murinus-animalis* LW 121. The strains *S. alactolyticus*, *L. plantarum* LW143, *L. brevis* 167, *L. buchneri* LW 50 and LW 83 had no effect *in vitro* or *in vivo*. This indicates that false negatives were not observed. In this experiment LAB were fed for only 5 days prior to immunization. This might explain why the IgM titers showed a better correlation to the *in vitro* proliferation results than was observed for the IgG titers that in general increase later than IgM. The delay between LAB feeding and IgG switching may have been too long to induce a significant effect. For

modulating the IgG titers it might be necessary to feed LAB for a longer period or in higher doses.

The strains that were investigated were chosen for the purpose of validating the selection system. Not all strains were expected to be strong immune modulators. The strains differed in origin/source and fermentation pattern (obligate or facultative anaerobe, sugar fermentation). Some strains were known for other purposes or properties in other animals. *L. buchneri* was used as bacterial inoculant for silage (67), *L. plantarum* was selected for production of fermented liquid feed (pH effect) (289) and *S. alactolyticus*, being the most abundant commensal in the pig GI tract (36), was therefore not likely to be a strong immunomodulator.

In *in vivo* experiment 2 different strains from the same species (e.g. the three different *L. brevis* strains) did not have the same effect on the humoral response. This indicated individual strains had different probiotic properties, independent on the effect of their closely related family members, and therefore every individual strain needs to be analyzed.

The *in vitro* assay discriminated between various degrees of immunomodulation. There was no quantitative relation between NSI *in vitro* and *in vivo* immune stimulation. In this *in vitro* assay the LAB and the effector cells (lymphocytes and macrophages) were brought together in direct contact and optimal proportions. In the *in vivo* situation after oral administration the contact situation and proportions will probably be quite different. Moreover, other LAB properties or host related factors such as survival in the GI-tract, acid and bile tolerance, growth rate, and adhesion to the gut will play an important role in *in vivo* health stimulation. This could explain why some strains were promising *in vitro*, but showed little effect *in vivo*.

For this *in vitro* assay spleens were used from SPF chickens not treated with LAB. When LAB strains were administered to chickens *in vivo*, the SI of these strains in the *in vitro* assay remained unchanged for spleen lymphocytes (data not shown). Oral administration did not lead to proliferative anti-LAB responses and the effect of probiotic LAB strains in the *in vitro* assay was not influenced by the lactobacilli present in the gut of the animal. The proliferation to the antigen with which the animals were immunized was altered minimally by previous oral application of lactobacilli. This could be negatively influenced because of the relatively long period after feeding before the proliferation was tested.

The present assay significantly reduced experimental animal use. This *in vitro* assay only uses 10 chickens to test 10-15 strains. *In vivo* testing takes at least 15 animals per LAB strain. In addition, evaluating 10-15 LAB strains *in vitro* takes less than a week whereas an *in vivo* experiment lasts 4-6 weeks. An advantage of this *in vitro* test is that simultaneous analysis of the cytokine profile induced by the by LAB

in spleen cells is possible. This adds to further understanding and selection based on the mechanism of action of immunomodulation by LAB. Differences in cytokine profiles that are induced might explain the major differences in immunomodulation even for closely related LAB strains.

In conclusion, this *in vitro* assay is suitable for pre-selection of LAB on immunomodulating properties *in vivo* in chickens. The pre-selection assay thus far showed no false negative results. Strains that have a positive effect on *in vitro* proliferation of spleen lymphocytes also have a positive influence on specific humoral immune responses *in vivo*. Although this assay was developed for chicken, it possibly applies to other species as well, though it remains to be demonstrated whether the responses to model antigens can be reproduced in modulation of chicken response to pathogens.

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6

Immunomodulation by probiotic lactobacilli in layer- and broiler- type chickens

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Abstract

The aim of the experiments was to evaluate whether selected probiotic *Lactobacillus* strains have different immunomodulating effects in layer- and broiler-type chickens. Humoral and cellular specific and non-specific immune responses were studied by experiments in cellular proliferation, entry and survival of *Salmonella* bacteria in gut and spleen leukocytes, immunoglobulin isotypes and specific immunoglobulin titers. The effect of two different feeding regimes (short and continuous feeding) and doses for application of lactobacilli was studied. The Lactobacillus strains that were evaluated showed modulating effects on the immune system of layer- and broiler-type chickens. In broiler type chickens the lactobacilli had a stimulating effect when the chickens were young (up to 3 weeks) and the dose was relatively high, whereas in layer type chickens a lower effective dose and discontinuous administration was also effective. Immunoprobiotic lactobacilli can have a positive effect on humoral and cellular immune responses in layer- and broiler-type chickens, but the Lactobacillus strain to be used, the age of the animals and effective dose of lactobacilli to be administered need to be optimized.

Introduction

Lactobacilli are normal components of the healthy intestinal microflora. Selected Lactobacillus strains are used as probiotics with supposed health-promoting activities. These probiotic properties come in various categories and include beneficial influence on: intestinal balance (242), gut mucosal barrier (243) and mucosal immune response (214). Some probiotics have competitive exclusion effect for specific pathogens (79)and help reduce duration of diarrhea (176). In addition, beneficial effects have been observed on lactose intolerance (95), blood cholesterol concentrations (58) and cancer (177).

Probiotics are used to develop and maintain a healthy intestinal microflora in young animals, like newly hatched chicks (203). In regular poultry housing chickens are raised under hygienic conditions in absence of the mother hen. As a consequence, the young chicks do not acquire a maternal microflora that matches with the natural antibodies transfused with the yolk, thereby leaving them vulnerable to colonization by pathogenic bacteria.

Chickens have genetically been selected either for improved feed conversion and rapid growth or for production of eggs. This selection for economically important production traits has led to two physiologically quite different types of chickens: broiler- and layer-type chickens. As a consequence these chicken types differ in body weight gain, maximum lifespan and in a negative relation between performance traits of chickens and immunological responses (32, 158, 206, 226, 311). Layer- and broiler-type chickens differ in their immune response to infectious disease and to model antigens such as TNP-KLH (trinitrophenyl-conjugated keyhole limpet hemocyanin) and sheep red blood cell (SRBC) (148, 158). Also in turkeys a direct relation between a lower mitogenic leukocyte response to Concanavalin A and higher antibody response to sheep red blood cells was observed in turkeys selected for high body weight (163, 164). These results indicate that selection for high body weight (as in broiler type chickens) can affect immunological parameters for disease resistance like antibody production or mitogenic responses.

Various effects of probiotic lactic acid bacteria have been described in specific animals or man. These effects are depending on strain, host, dose, timing and viability of the strain (63, 131, 169, 221, 222). Some probiotic strains were selected in rodents or pigs for application in humans (71, 153, 213). This indicates that it is not excluded that probiotics act over various species. Although broiler- and layer-type chickens belong to the same species there are considerable phenotypic differences between these chickens. Next to the enormous difference in growth and life expectancy the development of the immune system is possibly also quite different. Since antibody responses (IgG) and non-specific proliferative responses in broiler type chickens were reduced as compared to that in a layer type chickens

under the same conditions (148) especially broiler type chickens might need stimulation to reach immune responses comparable to layer type chickens.

Therefore, we hypothesized that the interaction of lactobacilli with the chicken gut and immune system and therefore their immunomodulating effect might differ qualitatively and/or quantitatively between chicken types. Recently we selected immunoprobiotics in LSL chickens (149). *L. paracasei* LW 122 had a positive effect on the specific antibody titer and non-specific cell proliferation. *L. plantarum* LW 143 did not have an effect on the antibody titer, but contributed to resistance to *Salmonella* Enteritidis (118). Layer type chickens were used since their life span better accommodated the duration of the experiments necessary for evaluation of immunoprobiotic effects modulating responses to priming and booster immunization.

We investigated whether the immunoprobiotic properties of lactobacilli that were observed in layer type chickens expressed similar effects in broiler type chickens. The immunoprobiotic effect may be dependent on the amount of lactobacilli administered (9, 92, 213) therefore we studied two different lactobacilli feeding regimes. High doses were administered for a long period with fermented liquid feed (118). In other experiments the chickens received lactobacilli only during five days before an immunological challenge. As prototype for layer type chicken we have chosen LSL White Leghorn and as broiler type chicken Ross 208.

Materials and methods

Chickens

Eggs of layer type chicken (LSL White Leghorn, Charles River) were bred and raised under routine specific pathogen free (SPF) conditions (no preventive vaccinations, no antibiotics and no coccidiostatics). One-day old Ross 208 broiler type chickens were obtained from a parent flock with a *Salmonella*-free history. Fluff and paper pads from the hatching cabin were examined on the presence of *Salmonella.* When all samples were negative for *Salmonella*, the chickens were used in the experiments.

Housing

At the start of the *in vivo* experiments the animals were divided equally over the groups for weight and gender (experiment II, in experiment I only female chicks were used). The animals were numbered individually and housed in groups that received the same treatment. The pens were closed with plates at the sides and between two pens a space as big as one pen was kept empty to avoid unwanted spreading of the bacteria. The pens were placed on a concrete floor with about five cm of sawdust bedding. Stable conditions were as follows: temperature 33 °C gradually declining to 18°C and the humidity was 55% throughout the experiments.

In experiment I the light: dark scheme was 23 hours: 1 hour and in experiment II 16 hours: 8 hours.

Experimental design

All animal experiments were in compliance with Dutch regulations on animal experimentation.

Experiment I: Continuous feeding of L. paracasei LW 122 in fermented liquid feed (Fermented liquid feed): The effects of immunoprobiotics selected in layer type chickens on broiler type chickens

One-day old chickens were divided into three groups of 20 animals each. Group 1 received fermented liquid feed fermented with *L. plantarum*, group 2 received fermented liquid feed fermented with *L. plantarum* and *L. paracasei*, and group 3 received dry control feed. The chickens received the feed from day one. On days 5 and 33 the animals were immunized with TNP-KLH (trinitrophenyl-conjugated keyhole limpet hemocyanin). On days 6 and 7 after second immunizations chickens were sacrificed for lymphocyte proliferation assay (LPA) on spleen cells. On days 7, 10 and 14 after both immunizations serum samples were taken from all chickens for total and specific IgM and IgG anti-TNP-titer analysis in ELISA.

Experiment II: Temporary oral administration of L. paracasei LW 122: Comparison of response to immunoprobiotics in layer- and broiler-type chickens

Two groups of 15 LSL chickens (groups 1 and 2) and two groups of 15 Ross chickens (groups 3 and 4) aged three weeks were administered either *L. paracasei* (groups 1 and 3) or buffer (groups 2 and 4). The chickens received lactobacilli for five consecutive days one week before immunization (days 8 to 12). On days 15, 17 and 19, five animals of each group were immunized with TNP-KLH. Seven days after immunization animals were sacrificed and blood, spleen and samples from caecum and ileum were collected.

Lactic acid bacteria

Lactic acid bacteria strains were identified by BCCM/LMS (Ghent, Belgium) using fatty acid and SDS-PAGE analysis and *L. paracasei* LW 122 was also identified by 16S rDNA sequence analysis. The lactic acid bacteria were cultured overnight (18 hours) in MRS broth (de Man, Rogosa and Sharpe, Oxoid Ltd.) after inoculation of MRS with 1% of a fresh full-grown culture. Lactic acid bacteria were cultured at the optimal growth temperature for each strain, 30°C (*L. plantarum*) and 37°C (*L. paracasei*). To accurately count the bacteria, cultures were diluted in Pepton Physiological Salt solution (BioTrading, Mijdrecht the Netherlands) and plated out in

double layered pour-plates with MRS agar. The plates were incubated two days at the same temperature as the growing conditions of the culture (30°C or 37°C).

Feed preparation and administration of lactobacilli to the chickens

In experiment I fermented liquid feed was used. The fermented liquid feed was prepared as described by Heres (118). 200 g dry feed (pelleted feed, sterilized by gamma radiation (0.9 Mrad) (starter diet for pullets, Arkervaart, Leusden the Netherlands) was mixed with 280 g water and 1 ml of a fresh overnight culture of *L. plantarum* LW143 (289)or 1 ml *L. plantarum* + 1 ml *L. paracasei* LW122 (149, 169). The mixes were incubated for two days at 30°C. After fermentation the pH was about 4 (4.0 when fermented with *L. plantarum* 4.0 only and 4.2 with both strains). The fermented liquid feed contained 10^9-10^{10} colony forming units (cfu) *L. plantarum* per gram or about $4*10^9$ cfu *L. plantarum* and about $2*10^9$ *L. paracasei* per gram mixed fermented liquid feed and was stored at 4°C until use (max. two days). Seeds (corn, peeled barley and wheat, all coarsely ground) were added to make the feed more crumbly during the first days (fermented liquid feed: seeds = 3:2). On days 5 and 6 the seeds were added to a level of 10% (w/w) and after day 6 no seeds were added. The same quantities of seeds were added to the dry feed of the control group. Water and feed were available *ad libitum* in both experiments.

In experiment II the lactobacilli were orally administrated using a syringe mounted with a gavage. This allowed administering the lactobacilli in the upper part of the throat. During five consecutive days the groups received 10^9 cfu freshly cultured *L. paracasei* in 0.2 ml Na_2CO_3 buffer and the control group 0.2 ml Na_2CO_3 buffer.

Gut content analysis

On days 3, 7, 14 and 35 five chickens per group were randomly selected for necropsy. The intestines were removed aseptically. The contents of the crop, ileum and caecum were emptied in separate sterile stomacher bags and immediately diluted with cold Buffered Peptone Water (BPW, Oxoid, Haarlem the Netherlands). These samples were macerated during 2 minutes and serial diluted in saline. Enterococci were enumerated on Kanamycine Aesculine Azide (KAA) agar, *Enterobacteriaceae* on Violet Red Bile Glucose (VRBG) agar and lactobacilli on Rogosa-agar (all three agars from Oxoid). The plates and counted after 20-24 hours incubation at 37°C (KAA and VRBG) and after 3 days at 30°C (Rogosa). The pH was measured immediately after gathering of the contents by inserting an electrode (Ingold electrode, Mettler Toledo) into the lumen of crop, ileum and caecum.

Antigen

2,4,6-trinitrophenyl (TNP; Eastman Kodak, Rochester NY, USA) was conjugated to KLH (Sigma, St. Louis, USA) as described previously (49). In animal experiment I

chickens were intravenously immunized with 200 µg TNP-KLH in 0.2 ml 0.9% NaCl and in experiment II intravenously with 50 µg TNP-KLH in 0.5 ml 0.9%NaCl. The lower concentration of TNP-KLH was selected in experiment II because we had found earlier (148) that layer type chickens needed a lower concentration of TNP-KLH than broiler type chickens in order to produce a suboptimal specific IgM response. In experiment II we did not want to immunize with two different antigen concentrations within one experiment and therefore selected a concentration which induces a response in both types of chickens.

ELISA

Total antibody concentrations IgM and IgG were measured in serum using a double antibody sandwich ELISA as previously described (155). An external standard (ITK diagnostics B.V., Uithoorn the Netherlands) with known IgM and IgG concentrations was used to indicate total antibody concentrations of the samples (IgG titer 1000 = 5.6 mg/ml and IgM titer 1000 = 0.1 mg/ml).

Anti-TNP antibody titers were determined by means of a direct ELISA as described previously (148). Briefly, TNP-bovine serum albumin (BSA, Sigma, St. Louis USA) was coated and sera were serially diluted in twofold. Responses were determined using mouse monoclonal antibodies CVI-ChIgM-59.7 and CVI-ChIgG-47.3, specific for chicken IgM and IgG (23, 135). In both ELISAs detection was performed with horseradish-peroxidase conjugated rabbit-anti-mouse (DAKO A/S, Glostrup Denmark) and the substrate tetramethylbenzidine (0.1 mg/ml) and H_2O_2 (0.005 % v/v). Extinction was measured at 450 nm.

Antibody titers were calculated as the dilution of the sample giving an extinction value of 1 above the background. Geometric mean titers of individual 2-log titers, S.E.M. and antilog (2^{GMT}) values were calculated.

Antigen specific restimulation assay *in vitro*

Cellular responses were determined by LPA as described previously (148). In short, spleens were removed aseptically and single cell suspensions were prepared. The mononuclear cells of the spleen were enriched over a Ficoll-Paque gradient (Amersham Pharmacia Biotech, Uppsala Sweden) and washed in phosphate buffered saline (PBS). 10^6 viable cells per well were incubated in 0.2 ml RPMI 1640 Dutch modification containing 1% normal chicken serum, glutamine, ß-mercaptoethanol and antibiotics) for 68 hours in a humidified incubator at 41°C with 5% CO_2. Spleen cells were incubated with the antigen to be tested (10 µg/ml ConA or 15 µg/ml TNP-KLH). After 92 hours 0.4 µCi per well ^{3}[H]-thymidine (Amersham Pharmacia Biotech, United Kingdom) was added and 4 hours later the plates are harvested onto fiberglass filters and counted by liquid scintillation spectroscopy

(Betaplate, Wallac Oy, Turku Finland). All assays were performed in triplicate cultures.

Entry and survival assay

The assay to determine entry and survival of naladixic acid resistant strain of *Salmonella enterica* serotype Enteritidis PT4 (296) in leukocytes was used to measure the probiotic effect on the leukocytes and was slightly modified from the method previously described by Kramer *et al*. (155, 157).

Briefly, the intestine (ileum and caecum) was isolated, opened longitudinally, rinsed thoroughly with PBS, and cut in 0.5–1 cm pieces. All tissue pieces were incubated for 10-15 min in PBS containing 0.145 mg/ml dithiotreithol (DTT) and 0.37 mg/ml EDTA in a shaking water bath (110 strokes/min, 37°C). The pieces of small intestine were rinsed once with RPMI 1640 Dutch modification containing 5% FCS and incubated in RPMI 1640 Dutch modification supplemented with 5% FCS, 0.15 mg/ml collagenase, and 0.1 mg/ml DNase in a shaking water bath (200 strokes/min, 37°C) during 75–90 min. The supernatant and the pieces of intestine were subsequently squeezed through 70 µm nylon gauze (Cell strainer Falcon 2350, Becton Dickinson, Leiden, The Netherlands) using RPMI 1640 Dutch modification containing 5% FCS and 0.1 mg/ml DNase. The spleen was cut in small pieces, incubated in RPMI 1640 Dutch modification with 1 mg/ml collagenase for 10 min at 37°C, and squeezed through a 70 µm nylon gauze.

The single cell suspensions of ileum, caecum or spleen leukocytes were incubated in triplicates with 100 µl of a *S.* Enteritidis suspension. Adding gentamicin 30 minutes later (200 µg/ml in order to the kill extracellular bacteria) finished the entry phase in all cultures. The number of bacteria that entered the cells during 30 minutes was determined by washing the cells and one of the three identical cultures was resuspended in PBS with 1% saponin to lyse the cells. Appropriate dilutions of the *Salmonella*s released from the cells were plated onto Brilliant Green Agar plates enriched with naladixic acid (100 ppm). To determine the survival of *S.* Enteritidis after entering the leukocytes two remaining cell suspensions were washed and resuspended in RPMI 1640 with FCS and gentamicin (20µg/ml). These cultures were incubated for another 14 h or 24 h in a humidified incubator at 37 °C with 5% CO_2. Thereafter the chicken cells were lysed and the frequency of surviving bacteria in leukocytes was determined by plating on Brilliant Green Agar.

Statistics/data analysis

The significance of differences between groups at the same timepoint were analyzed with Student's T-test (two-tailed and two sample with unequal variance).

Results

Two Lactobacillus strains have been tested in layer- and broiler-type chickens in two different application regimes, a temporary and a continuous application model and different doses. The effect of the administration of lactic acid bacteria on pH and microflora in the GI-tract of chickens as well as the effect on non-specific and specific humoral and cellular responses were evaluated.

Effects on pH

After feeding fermented liquid feed (experiment I) the pH in the crop was lower pH than was observed in the control group ($P<0.05$, table 1). This difference was probably due to the fermentation products like lactic and acetic acid. Unexpectedly, feeding fermented liquid feed led to a slightly, non significant, increase of the pH in caecum and jejunum as compared to the pH in the same gut sections as in the chickens that received control feed.

Effects on Microflora

Following fermented liquid feed feeding (experiment I) the number of *Enterococci* was higher in crop, jejunum and caecum of the control group than was determined for chickens in the fermented liquid feed groups, especially in the first week of feeding ($P=0.001$). The control group showed larger day-to-day variation in frequency of lactobacilli in crop, caecum and jejunum. The frequency of lactobacilli was about 10 fold lower in the control group than in the fermented liquid feed groups ($P=0.05$) in all segments of the GI-tract tested. To estimate the relative frequency of the strains of lactobacilli that were fed to the chickens, gut samples were investigated. Some (n = 10) typical colonies were analyzed with the API-CH50-test (BioMérieux) to verify the identity of the colony. All colonies tested had exactly the same API-pattern as the strains in the starter culture. It was concluded that the majority of the lactobacilli in caecum of the fermented liquid feed fed groups consisted of *L. plantarum* or *L. plantarum* + *L. paracasei* from the starter cultures.

Table 1. Effects of probiotics in fermented liquid feed fermented with either L. plantarum or L. plantarum + L. paracasei compared to control feed on the pH, number of Enterococci, Lactobacilli and Enterobacteriaceae in crop, jejunum and caecum[a,b].

	Group	Crop				Jejunum				Caecum			
Day of feeding		3	7	14	35	3	7	14	35	3	7	14	35
PH	Control	5.4 (0.7)	4.8 (0.3)[a]	4.8 (0.3)[a]	5.0 (0.5)[a]	6.9 (0.6)	6.7 (0.5)	6.7 (0.6)	6.4 (0.5)	6.4 (0.5)	6.1 (0.4)	6.1 (0.4)	6.2 (0.4)
	L. plantarum	4.2 (0.1)[g]	4.1 (0.2)[g]	4.0 (0.2)[g]	4.2 (0.1)[g]	7.3 (0.4)	7.2 (0.3)	7.1 (0.3)	6.7 (0.2)	6.5 (0.3)	6.5 (0.4)	6.4 (0.3)	6.6 (0.3)
	L. plantarum + L. paracasei	4.3 (0.2)	4.0 (0.1)[g]	4.0 (0.1)[g]	4.5 (0.1)[e]	7.0 (0.6)	7.1 (0.3)	6.9 (0.1)	7.0 (0.2)	6.5 (0.3)	6.4 (0.4)	6.3 (0.2)	6.7 (0.2)
Enterococci	Control	5.8 (0.5)	5.7 (0.9)	4.4 (0.6)	5.6 (0.9)	7.4 (1.5)	8.3 (0.6)	5.1 (0.2)	6.6 (0.9)	10.0(0.6)	9.3 (0.7)	6.8 (0.9)	7.4 (0.8)
	L. plantarum	4.5 (0.5)	4.2 (1.2)	4.2 (1.2)	5.8 (1.0)	4.7 (0.9)	6.5 (2.0)	5.9 (1.2)	6.7 (1.2)	8.2 (0.9)	7.9 (1.8)	6.6 (0.9)	7.2 (0.6)
	L. plantarum + L. paracasei	4.5 (0.4)	4.7 (1.4)	4.4 (0.6)	6.2 (0.5)	4.8 (0.4)	6.3 (1.5)	5.6 (0.9)	7.1 (0.9)	7.9 (1.0)	7.4 (1.1)	7.2 (0.4)	7.5 (1.0)
Lactobacilli	Control	7.9 (1.3)	8.7 (0.9)	8.3 (0.5)	8.3 (0.8)	7.6 (1.8)	9.0 (0.5)	7.9 (0.4)	7.7 (0.9)	9.3 (0.7)	9.5 (0.6)	8.8 (0.6)	7.6 (1.0)
	L. plantarum	9.2 (0.5)	9.2 (0.7)	9.2 (0.9)	9.5 (0.2)	8.5 (0.7)	8.9 (0.4)	9.1 (0.5)	9.5 (0.5)	9.8 (0.8)	9.7 (0.6)	9.4 (0.9)	9.9 (0.6)
	L. plantarum + L. paracasei	9.3 (0.3)	9.1 (0.9)	9.2 (0.2)	8.7 (0.5)	9.2 (0.2)	9.3 (0.3)	9.1 (0.4)	9.7 (0.3)	10.2(0.3)	10.4(0.3)	9.8 (0.2)	10.1(0.3)
Enterobacteriacea	Control		1.0 (1.8)	2.9 (1.1)	2.9 (2.3)			1.4 (1.7)	5.2 (1.3)		0.5 (2.1)	3.8 (3.6)	4.9 (1.7)
	L. plantarum			0.5 (1.1)	2.8 (1.6)			0.6 (0.9)	2.8 (2.1)			1.3 (2.5)	4.1 (2.2)
	L. plantarum + L. paracasei		1.4 (1.7)	0.6 (0.6)	4.1 (2.2)		1.5 (2.2)	0.9 (1.7)	7.4 (0.8)		3.9 (4.3)	3.7 (4.0)	6.8 (1.1)

[a] *The table shows mean pH (stdev) and 10log cfu's (stdev) on day 3, 7, 14 and 35 of feeding.*

[b] *Different subscripts in the same column indicate significant differences (n [a,b,c] : P<0.05).*

Effect of feeding putative immunoprobiotic bacteria on the humoral immune response

Non-specific immunoglobulin levels (experiment I)

After primary immunization the chickens that received fermented liquid feed fermented with *L. paracasei* + *L. plantarum* had higher total IgM titer than the control group (*P*= 0.02). The group fed with *L. plantarum* fermented liquid feed showed a similar trend after priming. After secondary immunization the group that received with *L. paracasei* + *L. plantarum* had a higher total IgG titer in serum than the control group (*P*= 0.05) and the group that received fermented liquid feed fermented with *L. plantarum* only (*P*= 0.009). This *L. plantarum* group also had a higher total IgM titer than the control group (*P*= 0.005, table 2).

Table 2 Effect of continuous feeding of lactobacilli with fermented liquid feed on total serum IgM and IgG titers in broiler type chickens (n=15)[a,b].

		IgM titer	IgG titer
	Standard sample	5698.2 (69.2)	1079.8 (15.9)
Priming	Control	2320.7 (36.9)[a]	1303.7 (29.4)[a]
	L. paracasei + *L. plantarum*	3671.2 (59.2)[c]	2261.7 (71.3)[b]
	L. plantarum	3692.1 (102.2)[b/c]	1789.7 (63.0)
Boost	Control	3924.3 (62.6)[a]	4394.0 (67.2)[a]
	L. paracasei + *L. plantarum*	5378.2 (83.8)[b]	6800.5 (127.9)[c/d]
	L. plantarum	7899.3 (169.3)[d/b]	3863.0 (34.0)[a]

[a] *Mean titers (S.E.M.) from serum samples on day 7 after priming and booster immunization.*

[b] *Different subscripts in the same column indicate significant differences (n [a,b,c] : P<0.05).*

TNP-specific immunoglobulin levels (experiments I en II)

After priming fermented liquid feed prepared with *L. plantarum* and fermented liquid feed prepared with *L. plantarum* + *L. paracasei* both had a positive effect on the IgM titer (both *P*= 0.07) in the broiler type chickens, but after boost no stimulation of the TNP-specific IgM response was detected (table 3).

The serum IgM response to TNP-KLH in layer type chickens was slightly lower than was observed in broiler type chickens. In contrast, the IgG response of broiler type chickens was lower than was observed for layers (not significant). In experiment II temporary administration of *L. paracase*i for 5 days prior to immunization with TNP-KLH enhanced the serum IgM and IgG titers in layer type chickens after priming, but not in broiler type chickens under the same conditions (table 4). However,

stimulated IgM titers in layer type chickens reached similar levels as were observed in broiler type chicken controls. For IgG stimulated levels in layer type chickens surpassed IgG level that was observed in broiler type chickens.

Table 3. Effect of continuous feeding with fermented liquid feed on specific serum IgM and IgG anti-TNP titers in broiler type chickens (n=15)[a,b].

		IgM titer	IgG titer
Priming	No TNP-KLH	113.8 (5.3)	7.6 (2.1)
	Control	270.6 (16.3)[a]	13.6 (1.1)
	L. paracasei + *L. plantarum*	694.6 (37.5)[b]	17.1 (1.3)
	L. plantarum	781.4 (51.4)[b]	16.1 (2.2)
Boost	Control	6793.8 (361.2)	117.8 (4.6)[a]
	L. paracasei + *L. plantarum*	4299.6 (224.2)	88.6 (7.3)
	L. plantarum	6427.3 (321.3)	55.3 (3.8)[c]

[a] *Mean titers (S.E.M.) from serum samples on day 7 after priming and booster immunization.*
[b] *Different subscripts in the same column indicate significant differences (n [a,b,c] : P<0.05).*

Table 4. The effect of feeding of 10^9 L. paracasei LW122 for 5 days prior to immunization with TNP-KLH in layer- and broiler-type chickens (n=15) on the specific serum IgM and IgG anti-TNP titers[a].

	IgM titer	IgG titer
Layer	1351.9 (245.1)	716.5 (136.6)
Layer + *L. paracasei*	2227.2 (255.3)	1243.7 (186.8)
Broiler	2019.3 (419.3)	329.6 (112.4)
Broiler + *L. paracasei*	2188.7 (400.4)	242.7 (66.4)
No TNP-KLH	29.7 (0.7)	37.7 (1.3)

[a] *Titers (± S.E.M.) are shown for day 7 after primary immunization.*

Modulation of the cellular immune response
Lymphocyte proliferation (experiment II)

Under identical test conditions the non-specific proliferative response to ConA was higher in spleen cells of layer type chickens than was observed in spleen cells of broiler type chickens, but temporary feeding *L. paracasei* did not have an effect on this response in both types of chickens (table 5). The specific proliferative response to TNP-KLH was enhanced in spleen cells of both layer- and broiler-type chickens that orally received *L. paracasei* as compared to the control groups that did not

receive the lactobacilli. However, the immune enhancing effect was more pronounced in spleen cells of broiler type chickens (*P*= 0.023) than was observed in spleen cells of layer type chickens (not significant).

Table 5. The effect of temporary feeding of L. paracasei LW 122 to layer- and broiler-type chickens (n=15) on the proliferative responses of spleen cells to ConA and TNP-KLH in vitro[a,b].

	KLH-TNP	ConA
Layer	62157 (7048)	27489 (7322)
Layer + *L. paracasei*	73083 (7837)	21828 (6426)
Broiler	56690 (9183)[a]	9069 (4709)
Broiler + *L. paracasei*	93214 (12004)[c]	12423 (4967)

[a] cpm (± S.E.M.) are shown for assays performed on day 7 after primary immunization with TNP-KLH.
[b] Different subscripts in the same column indicate significant differences (n [a,b,c] : P<0.05).

Modulation of the innate immune response: Entry and survival (experiment II)

The effect of temporary feeding of *L. paracasei* was measured as the effect on entry, survival and replication of *S.* Enteritidis in leukocytes of spleen, ileum and caecum one week after the last feeding of *L. paracasei.*
Both entry (measured after 30 minutes) and survival/replication (measured after 14 and 24 hours) of *S.* Enteritidis in spleen and caecum cells was similar in cells of layer- and broiler-type chickens. However, entry and survival of *S.* Enteritidis were significantly higher in ileum derived cells from broiler type chickens than observed in layer type chickens (figure 1).

Feeding of *L. paracasei* LW 122 had an enhancing effect on the entry of *S.* Enteritidis in isolated leukocytes of the caecum of layer type chickens. With *L. paracasei* the entry was 1890 cfu/gram and without 633 cfu/gram (*P*= 0.002). In ileum- and spleen cells of these chickens no effect of feeding probiotic bacteria on uptake of *S.* Enteritidis was observed. Feeding *L. paracasei* did not have any effect on the bacterial entry in the gut and spleen cells of broiler type chickens.
In caecum cells of broiler type chickens fed with *L. paracasei* LW 122 the survival and intracellular replication of *S.* Enteritidis after 14 h incubation was significantly reduced as compared to caecum cells of chickens that did not receive lactic acid bacteria (*P*=0.04). But in caecum cells from layer type chickens the reverse was observed (*P*=0.05). Feeding of *L. paracasei* LW 122 enhanced survival and replication of *S.* Enteritidis in spleen cells of broiler type chickens, but in layer type chickens no effect was observed.

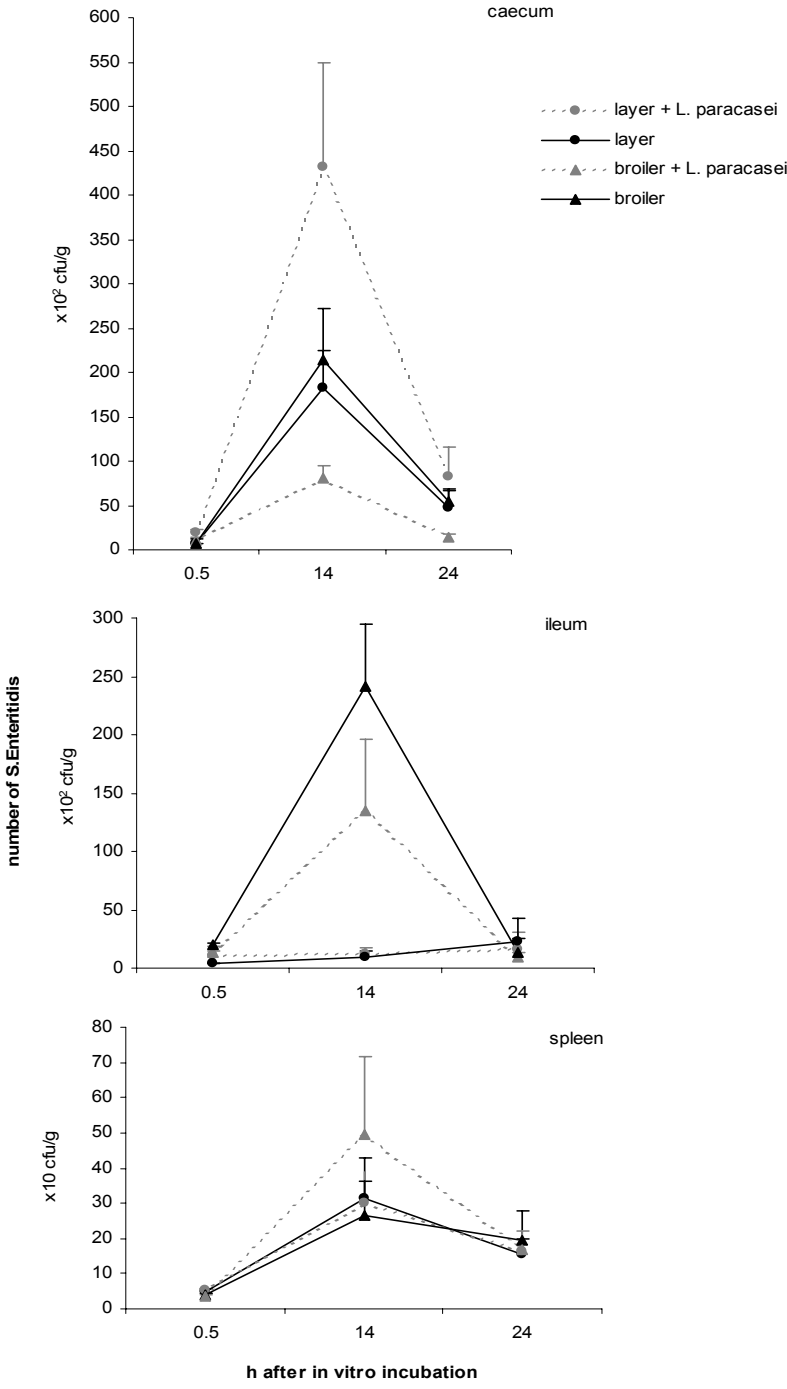
caecum
ileum
spleen
layer + L. paracasei
layer
broiler + L. paracasei
broiler
number of S.Enteritidis
x10² cfu/g
x10 cfu/g
0
50
100
150
200
250
300
350
400
450
500
550
600
10
20
30
40
50
60
70
80
0.5
14
24
h after in vitro incubation

Figure 1. The effect of feeding of L. paracasei LW 122 on the innate immune response. Entry and survival of S. Enteritidis (S.E.) are shown in isolated leukocytes from caecum (a), ileum (b) and ileum (c) of layer- and broiler-type chickens (±S.E.M.) after in vitro exposure at one week after the last feeding of the lactobacilli (n=15) in experiment 2. In order to keep the figures clear the statistical significance is described below:
(a) In caecum L. paracasei had a significant enhancing effect on the entry of S.E. (P=0.002) in the layer type chicken. In broiler type chickens which had L. paracasei the survival of S.E. after 14h was significantly reduced as compared to cells of chickens that did not receive lactic acid bacteria (P=0.04). In caecum cells of layer type chickens the reverse was observed (P=0.05).
(b) For ileal cells the survival and replication of S.E. after 14 h was higher in cells of broiler- than in cells of layer type chickens (P=0.04).
(c) No statistical differences.

After 24 h of incubation of isolated cells with *S.* Enteritidis no differences were found between the two chicken lines with respect to survival in spleen or GI-tract cells with or without prior application of *L. paracasei* to the chickens.

Discussion

This study clearly showed that feeding or temporary oral administration of lactobacilli could modulate physiological aspects of the GI-tract and various aspects of the chicken immune response.

Microflora and fermented liquid feed

Obviously the feeding of fermented liquid feed in broiler type chickens modulated the composition of the microflora in the crop, jejunum and caecum. The frequency of lactobacilli was about 10-fold higher in all three parts of the GI-tract studied. Especially in the first week of fermented liquid feed feeding the frequency of enterococci was reduced. This might be explained by competition for nutrition, space or adherence between lactobacilli and enterococci (249, 250). Lactobacilli are important components for a balanced microflora. The reduced day-to-day variation in frequencies of lactobacilli in crop, ileum and caecum that was observed during fermented liquid feed feeding was an indication for a stabilizing effect of lactobacilli on the intestinal microbiota and was in agreement with earlier findings of Heres *et al*. (117). This stabilizing effect as well as the increased frequency of lactobacilli together with a reduced frequency of *Enterobacteriaceae* was also observed in pig (295). From this we conclude that the selected lactobacilli expressed beneficial effects on the host flora composition.

Daily administration of fermented liquid feed proved to be an effective vehicle to increase levels of live lactobacilli in the GI-tract. However, in contrast to fermented

liquid feed feeding in pigs, a disadvantage of using fermented liquid feed in chicken was the relatively low food intake (117) and therefore a variable intake of lactobacilli (1 gram fermented liquid feed ~ 10^9 cfu lactobacilli). Therefore in the present experiment the number of lactobacilli per day was probably not constant and this might have influenced some of the results. Therefore in the second experiment the chickens were inoculated individually with a fixed number of lactobacilli.

Immune function analysis

The entry of phagocytosis of *S.* Enteritidis in caecum derived cells was enhanced by *L. paracasei* LW122 in layer type chickens. Similarly probiotic lactic acid bacteria enhanced phagocytosis in mice (26, 27, 213, 215, 256). These probiotic properties were dose dependent (92, 213, 215, 256). Though in these latter experiments phagocytosis was studied with inert/passive materials, the observed dose-effect probably did play a role in our experiments with live *S.* Enteritidis. Although the same number of lactobacilli was administered to broiler- and layer-type chickens, the gut of broiler type chickens was relatively heavier and longer and therefore the number of lactobacilli may have differed between the chicken types with respect to the gut surface and volume. Apart from intrinsic differences in immunity this might therefore explain part of the observed differences.

Our results indicated that feeding *L. paracasei* to broiler type chickens enhanced the phagocytic and bacterial activity of the gut cells (caecum, ileum). But in spleen cells the survival of S. Enteritidis was slightly enhanced as compared to the control group. In contrast, feeding of *L. paracasei* did not influence the phagocytic and bacterial activity in ileum and spleen cells of layer type chickens, although in the caecum of layer type chickens survival of *S.* Enteritidis was enhanced. These observations may be better explained by intrinsic differences in the quality of the innate immune system of layer- and broiler-type chickens than by differences in relative doses of lactobacilli only. The enhanced activity at the level of the gut in broiler type chickens may provide an advantage in resistance and control of *S.* Enteritidis or other infections in broiler type chickens.

Simultaneous feeding of *L. paracasei* and *L. plantarum* with fermented liquid feed enhanced total IgM and IgG titers (figure 1). It appeared that both Lactobacillus chickens contributed differentially to the a-specific enhancement of antibody titers. Following priming IgM titers were enhanced both with the mixed fermented liquid feed and with *L. plantarum* only. Especially following booster immunization *L. plantarum* alone had a stronger stimulating effect on the IgM titer than mixed fermented liquid feed. This indicated that no fading of the stimulation of a-specific or polyclonal stimulation was observed. The total IgG level in broiler type chickens was enhanced by mixed fermented liquid feed feeding only following booster immunization which included a longer duration of administration. Fermented liquid feed with *L. plantarum* alone did not enhance polyclonal IgG. However, in

experiment II following a feeding period of only 5 days prior to immunization such an a-specific enhancement was not observed at the timepoint investigated. Not only the difference in doses of lactobacilli but also the delay between the last feeding of lactobacilli and the actual measurement of the response may have biased these results.

Temporary feeding of *L. paracasei* LW 122 (previously called *L. reuteri*-1 on functional characteristics) preceding immunization had an adjuvant-like effect on the specific humoral responses to TNP-KLH in layer type chickens (149, 150). When fermented liquid feed was fed to broiler type chickens (experiment I) an adjuvant-like effect was observed, but only for antigen specific IgM response after priming (figure 2). However, in experiment II, where layer- and broiler-type chickens were compared under the same conditions of temporary feeding with lactobacilli an adjuvant-like effect was not observed in broiler type chickens and only a weak adjuvant effect (IgM and IgG) was measured in layer type chickens (figure 3). The differences in experimental conditions such as doses of lactobacilli and the feeding regimen (continuous or discontinued at the time of immunization) may have resulted in different kinetics for the immunomodulation effects. Also an age effect may play a role. The maximum life-span of broiler type chickens is about 5-6 weeks. In experiment I (fermented liquid feed) the broiler type chickens were 5 weeks old at priming, but in the second experiment they were only 5 days old, creating sufficient time for a secondary immunization. A host-dependent effectiveness of lactobacilli in the gut associated immune system of layer type chickens was observed by Balevi *et al*. (9) based on competitive exclusion. It tentatively might be concluded that in young broiler type chickens the adjuvant effect is absent due to a lower state of immune maturation that rendered the cells less susceptible to the effect of immunoprobiotics. However, this still does not explain the levels of specific anti-TNP Ig production in broiler type chickens in experiment II even without stimulation by lactobacilli was as high as was observed in layer type chickens following such a stimulation. Although both layer- and broiler-type chickens were 5 weeks old in experiment II their immune system still may differ in maturity. As a consequence of the short maximum life span of broiler type chickens the maturation of (part of) their immune system may develop in an accelerated way as compared to the development in layer type chickens. Quantitative data on B cell development may support this hypothesis (J. B. Cornelissen, personal communication).

In broiler type chickens lactobacilli did not influence the non-specific proliferative response of isolated spleen cells, but the specific response to (re)stimulation with antigen *in vitro* was enhanced by *L. paracasei* even following temporary administration prior to the application of the immune stimulus. This indicated that for specific antigens such as e.g. vaccine antigens, immunoprobiotic lactobacilli might enhance disease resistance especially in broiler type chickens.

Interaction strain/gut/immune system

The interaction of gut, immune system and microflora forms a complex interface for mutual interactions between these systems that may be influenced by various management factors. As broiler- and layer-type chickens differ enormously in growth and maximum life span it may be assumed that they also differ in their interactions with the actual bacterial microflora. Administration of *L. paracasei* enhanced T cell proliferation more in broiler- than in layer-type chickens (figure 4). This could be caused by intrinsic differences in the regulation of cellular responses between the chickens. Present experiments confirm our observation, that showed the proliferative responses layer type spleen cells could indeed only become enhanced to a limited extend by lactobacilli (150). The enhancement that was observed in broiler type chickens, but not in layer type chickens of the same age might be caused by differential sensitivity to lactobacilli between the chicken lines due to intrinsic differences in development of the immune system.

As was described by Kramer *et al*. (156) lighter weight broiler type chickens reacted to *Salmonella* bacteria preferably by innate immune mechanisms while a heavier weight chicken fought these bacteria especially via the adaptive immune system. For comparison between layer- and broiler- these differences might even be more extreme since their weight differences are more pronounced than between heavy and light broiler type lines.

Recently Clancy (50) described a putative mechanism for immunomodulation by probiotic bacteria. According to this model probiotic bacteria activate dendritic cells in Peyers' patches which in turn stimulate the mucosa homing circulating pool of T-lymphocytes generated from within the Peyers' patch. In this way these T cells might also exert their immune modulation at distant mucosal sites. A higher number of lactobacilli that reached the Peyers' patches could activate more DC's and indirectly increase the immune response. The present experiments demonstrated that Lactobacillus strains could have differential effects on the microflora and the immune system.

Feeding large amounts of lactobacilli positively influenced the microflora composition of young chickens. The frequency of unwanted bacteria was lower and the frequency of lactobacilli was higher in treated animals. The microflora was stabilized sooner in the treated than in the untreated animals. The age of the chickens and the amount of lactobacilli were important variables in the experiments. These seemed to be responsible for differences in stimulating the proliferation, the entry in leukocytes and humoral responses. The interplay of the Lactobacillus strain, dose of lactobacilli age of the chicken and the interaction of the bacteria with the host gut and immune system and maybe even more make this a subtle but complex way of immunomodulation. This implies that in order to obtain a desired effect, these variables should be investigated and balanced in an optimal way.

The present experiments showed that Lactobacillus strains had different effects on the GI-tract and immune system depending on the type, genetic make-up and age of the chicken. Though the magnitude of the effects per animal is limited, non-specific enhancement of the immune system may support animal health of a flock in an inexpensive way.

Under the chosen conditions both humoral and proliferative responses were enhanced (adjuvant-like effect) and to draw firm conclusions about the modulation of innate responses further investigation is needed. Furthermore the Lactobacillus strain, dose, and duration of application played a role in modulation effects. This implied that in order to obtain a desired effect these variables should be further investigated, optimized and balanced.

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Effects of probiotics on immunological aspects of *Salmonella* Enteritidis infection in chicken

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Submitted for publication

Abstract

Recently selected probiotics i.e. immunomodulating lactobacilli, were orally administered to young chickens prior, during and following *Salmonella* infection. Resistance to the infection was not changed by the lactobacilli. As compared to controls, in chickens treated with probiotic lactobacilli the clearance of the systemic infection from spleen and liver was enhanced. However, cytokine levels in the gut, *Salmonella* specific IgM titers and *Salmonella* specific proliferation of splenic lymphocytes all were decreased as compared to controls. We hypothesize that the probiotic lactobacilli reduced shedding and enhanced clearance of *Salmonella*, thereby reducing the antigenic load, which led to lowered cytokine production, reduced specific IgM titers and *in vitro* proliferation.

Introduction

Chickens harbor a complex gastro-intestinal microflora in which lactobacilli are considered to confer beneficial influences to the host. From probiotic studies with humans and animals it has become clear that health related effects of lactobacilli are strain-dependent (112, 152, 169) and assert a set of vital roles regarding to host resistance (16, 62, 118). E.g., some Lactobacillus strains prevented colonization of pathogens in the GI-tract by competing for epithelial adhesion sites and essential nutrients and/or by production of inhibitory compounds.

In newly hatched chickens, the gut is sterile and both the hygienic measures in commercial breeding and the separation of the young from the adult bird prevent the chick from uptake and ingestion of the adult intestinal microflora. The importance of such a flora was firstly demonstrated by Nurmi and Rantala (204, 234) who treated chickens with cecal flora of adult chickens, which than showed resistance to colonization with *Salmonella*. Lactobacilli also enhanced resistance against *Salmonella* colonization (116, 227). In chickens fed liquid feed fermented with *L. plantarum* the susceptibility for *Salmonella* was decreased (118). However, this effect of fermented liquid feed could not be mimicked with artificially acidified feed. This implied that the lactic acid bacteria played an important role in reducing *Salmonella* colonization apart from their acid production (116). Next to competitive exclusion and acid production, disease resistance could be increased by microbial anti-pathogen substances secreted by probiotics (e.g. bacteriocidins as reuterin, acidophilin, nisin, hydrogen peroxide, etc.) or by immunomodulation of the host by probiotics.

Recently, immunomodulation by specific Lactobacillus strains was described in chicken (150, 152). This immunomodulation was characterized as enhanced specific antibody responses and proliferation of splenocytes. Such immunomodulation might enhance resistance to a pathogenic infection. Augmentation of resistance to *Salmonella* by lactobacilli due to anti-*Salmonellae* protective immunity mediated by the mucosal tissue (antibody titers to *Salmonella* in serum and GI-tract were increased) was described in rodents by (220). Enhanced cell-mediated immunity against pathogenic bacteria mediated by lactic acid bacteria (LAB) contributes to increased protection and host resistance in mice and rat (60, 64, 94, 257).

Therefore, the combination of competitive exclusion and immunomodulation conferred by properly selected probiotic lactobacilli was expected to have a positive effect on the *Salmonella* infection in chicken as well. This was experimentally verified in layer type chickens that were fed with *L. paracasei* or *L. brevis* plus *L. paracasei* prior to and during a *Salmonella* infection. We used *Salmonella* Enteritidis (*S*.E.) phage type 4 infection because this is an important pathogen in food safety and phage type 4 is the most prevalent phage type in the Netherlands (239). The

experiment showed that the probiotics enhanced clearing of liver and spleen from *Salmonella*, however simultaneously local cytokine production, IgM titers and proliferation to *S*.E. were suppressed.

Materials and Methods

Animal experiments

The animal experiments were in compliance with the institutional animal experiment committee and in accordance with the Dutch regulations of animal experimentation.

Chickens

Eggs of layer type chickens (LSL White Leghorn, Charles River), obtained from a parent flock with a *Salmonella*-free history were hatched in our animal facilities. Fluff and paper pads from hatching cabin and transport boxes were examined for presence of *Salmonella*. Before inoculation with *S*.E. the animals were checked on the presence of *Salmonella* (cloaca swabs). Chickens were used in the experiments only if all samples were *Salmonella*-negative. Chickens were raised under routine SPF (specific pathogen free) conditions: no preventive vaccinations, no antibiotics, and no coccidiostatics. Feed (starter diet for pullets, Arkervaart, Leusden, the Netherlands) and water were available ad libitum.

Housing

After inoculation with *Salmonella* chickens were housed individually in pens with wire floors with sawdust bedding to collect droppings. The pens were placed with an interspace of at least 20 cm. Sides, back and roof of each pen were closed with plastic sheets outside. Direct contact between the chickens was prevented. Front sides of the cages were wired and open. To prevent spreading of bacteria between pens and chickens, feed and water equipment were handled with 70% alcohol disinfected gloves.

Animal experiment

During the experiment lactobacilli and *Salmonella* suspensions were orally administrated using a syringe mounted with a gavage, allowing administration of lactobacilli and *Salmonella* in the upper part of the throat. From day 1 of age, 4 groups of 40 layer type chickens daily received orally 10^9 lactic acid bacteria or buffer. On the seventh day of LAB (or buffer) administration, groups 1-3 were orally infected with 10^3 cfu *Salmonella* enteritidis in PBS and group 4 received PBS. This dose was established to infect 90% of 7 day old layer type chickens in a preceding titration experiment (data not shown). From day 7 on the chickens received lactobacilli or buffer for 5 days a week during the rest of the experiment. Group 1 received *Salmonella* Enteritidis only; group 2 received *Salmonella* Enteritidis and *L.*

paracasei LW122; group 3 received *Salmonella* Enteritidis and a mix of *L. paracasei* LW122 and *L. brevis* LW5 and group 4 received buffer only. At the day of *Salmonella* inoculation blood samples were taken from 5 animals per group and during the experiment on days 4, 7, 14 and 21 after *S*.E. inoculation blood samples were taken from al animals. Ten animals per group were sacrificed on day 4, 7, 14 and 21 after *S*.E. inoculation. From these animals spleen and liver and caecum content were removed aseptically and kept on ice. Pieces of jejunum, ileum and caecum were removed of which one half was directly frozen in liquid nitrogen for RNA isolation and the other half was collected in buffered 4% formaldehyde for histological purposes. Caecum, liver, and spleen were used for bacteriological examination and on day 21 after infection half of the spleen was used for immunological research. From day 1 to 11 and on days 14 and 16 after inoculation cloaca swabs were taken to monitor shedding of *S*.E.

Bacterial strains, growth conditions and microbiological determinations
Lactic acid bacteria

LAB strains were identified (BCCM/LMS Ghent Belgium) using fatty acid and SDS-PAGE analysis and 16S rDNA sequence analysis. LAB were cultured overnight (18 hours) in MRS broth (de Man, Rogosa and Sharpe, Oxoid Ltd.) at 37°C, after inoculation of MRS with 1% of a fresh full-grown culture. Cultures were diluted in Pepton Physiological Salt (PPS) solution (BioTrading, Mijdrecht the Netherlands) and plated in double layered pour-plates with MRS agar to count the bacteria. The plates were incubated 2 days at 37°C.

***Salmonella* Enteritidis**

A naladixic acid resistant strain of *Salmonella enterica* serotype Enteritidis PT4 isolated from chicken was used (296). This strain is pathogenic for humans and it may cause clinical disease especially in young or weak chickens. For inoculation a fresh overnight culture of *S.* E. were prepared by growing the bacteria in buffered peptone water (BPW, BioTrading, The Netherlands). The bacterial culture was incubated at 37ºC under vigorous shaking (100 shakes per minute). The viable counts in the inoculum suspensions were determined by diluting and plating on Brilliant Green Agar (with 100µg/ml naladixic acid, BGA-Nal+) for 24 hours at 37 °C.

For detection of *Salmonella* in chickens prior to inoculation with *S*.E., cloaca swabs were incubated in BPW at 37°C for 24 hours. Enriched cultures were plated on Modified Semi-Solid Rappaport Vassiliadis broth (MSRV, BioTrading, the Netherlands) and incubated during 24 hours at 42°C. Suspected cultures were subcultured on BGA-Nal^+ for *Salmonella* determination for 24 hours at 37°C. Cloacaswabs of the chickens were enriched in BPW (24 h at 37 °C) before plating on BGA-Nal+ plates. Positive diagnosis depended on the presence of one or more typical colonies.

To detect *Salmonella* in caecumcontent or liver, 1 g of cecal content or liver was homogenized in 9 ml BPW and serial diluted in BPW. Caecum content was plated onto square BGA-Nal+ plates according to the track dilution method described by Jett (134) and liver on round plates, one dilution per plate. Since the spleens were too small to collect 1g tissue, the whole spleen was weighed, ground in 2 ml BPW, and followed the same procedure as for liver.

Immunological determinations
Specific humoral response

Anti-*Salmonella* antibody titers were determined in a direct ELISA as described previously (296). Briefly, *S*.E. LPS was coated overnight in 96-wells high binding ELISA plates (Greiner, Nürtingen, Germany). Sera were serially diluted in twofold and incubated for one hour at 37°C. The isotype specific responses were determined using mouse monoclonal antibodies CVI-ChIgM-59.7 and CVI-ChIgG-47.3, specific for chicken IgM and IgG (23, 135). Detection was performed with rabbit-anti-mouse-HRPO (DAKO A/S, Glostrup Denmark) and the substrate tetramethylbenzidine (0.1 mg/ml) and H_2O_2 (0.005 % v/v). Extinction was measured at 450 nm. Antibody titers were calculated as the dilution of the sample giving an extinction value of 1 above the background. Geometric mean titers (GMT) of individual 2-log titers, S.E.M. and antilog (2^{GMT}) values were calculated.

Lymphocyte proliferation assay (LPA)

Cellular responses were determined by lymphocyte proliferation assay (LPA) as described previously (148). In short, spleens were removed aseptically and single cell suspensions were prepared. 10^6 viable cells per well were incubated in 0.2 ml RPMI 1640 Dutch modification (containing 1% normal chicken serum, glutamine, ß-mercaptoethanol and antibiotics) for 68 hours in a humidified incubator at 41°C with 5% CO_2. Spleen cells were incubated with the antigen to be tested, 10 µg/ml ConA or 10 µg/ml *S*.E. antigen (prepared by culture of *S*.E. in BPW, concentrated by centrifugation, washed with PBS and inactivated by sonification (four times 30 seconds on ice) and freeze-thawing for four times). For ConA after 68 hours and for *S*.E. antigen after 92 hours 0.4 µCi per well ^{3}[H]-thymidine (Amersham Pharmacia Biotech, United Kingdom) was added and 4 hours later the plates are harvested onto fiberglass filters and counted by liquid scintillation spectroscopy (Betaplate, Wallac, Turku Finland). All assays were performed in triplicate.

Quantitative measurement of cytokine mRNA in jejunum
RNA extraction and cDNA preparation

Total RNA was extracted from 50-100 mg tissue of jejunum of five animals per group (not pooled) and homogenized with liquid nitrogen using mortar and pestle. The homogenized tissue samples were dissolved in 1 ml of TRizol Reagent

(Invitrogen, Breda, The Netherlands) per 50-100 mg of tissue using a syringe and 21-G needle passing the lysate for 10 times. The cleared homogenate was used for phase separation with chloroform as described by the manufacturer. The isolated RNA was quantified by E260 nm. RNA (200 ng) was incubated at 70°C for 10 minutes with random hexamers (0.5µg, Promega Benelux BV, Leiden, The Netherlands), then reverse transcribed in a final reaction volume of 20 µl containing Superscript RNase H-reverse transcriptase (200 Units, Invitrogen, Breda, The Netherlands), RNAsin (40 U, Promega Benelux BV, Leiden, The Netherlands), dNTP (2 mM Promega Benelux BV, Leiden, The Netherlands), 5x First Strand Buffer (Invitrogen, Breda, The Netherlands) and 0.1M DTT (Invitrogen, Breda, The Netherlands), for 50 minutes at 42°C. The reaction was inactivated by heating at 70°C for 10 minutes. cDNA was stored at -20°C until use.

Real time quantitative PCR

Real-time PCR was performed with online detection of the PCR reaction based on fluorescence monitoring (LightCycler, Roche Diagnostics, Mannheim, Germany). Primer and probe sequence were selected with MolBiol software (Berlin, Germany). Primers or probe combinations were designed such that the sequence of interest of the relevant genes was located across an exon-intron boundary (table 1).

Hybridization probes (TIB MolBiol, Berlin, Germany) were used to monitor the amount of specific target sequence product. Quantitative results were determined from the cycle threshold value (signal rises above background level). For quantification standard curves of the cytokine and 28S product were used. Products for PCR optimization and the standard curve were prepared with a plasmid construct containing the region of interest. PCR reactions were performed according to the LightCycler kit instructions (FastStart DNA Master Hybridization Probes, Roche Diagnostics, Mannheim, Germany). Real-time PCR was performed in 20 µl containing 1µM of each primer, 0.2 µM of each probe, $MgCl_2$, 2 µl template and 2 µl Fast Start mix. The reaction mix and samples were loaded into glass capillary tubes. Pre-incubation and denaturation of the template DNA occurred for 10 minutes at 95°C, whereas the PCR cycling was done 10 sec at 95°C, 10 sec ± 56°C (table 1) and 25 sec at 72°C for 45 cycles. Temperature transition rates were set at 20°C/s. A LightCycler instrument (Roche Diagnostics) was used for amplification and detection. To avoid contamination, filter pipette tips were used and reagents were mixed in rooms separate from rooms where DNA was present. A negative control, containing reagents only, and standard 10-fold dilution series were included in each run. A standard curve was generated from the threshold cycles (Ct) of the standard dilution series by LightCycler software version 3.5. The slope of the standard curve was around -2.97, meaning that the overall reaction efficiency for the standard curve was 1.0 - 1.3.

Table 1 Primers and annealing temperatures

Cytokine	primer forward sequence 5'-3' primer reverse sequence 5'-3'	probe FL sequence 5'-3' probe LC red 640 sequence 5'-3'	$MgCl_2$	Annealing temperature
28S	CAAGTCCTTCTGATCGAG TCAACTTTCCCTTACGGTAC	GCGGGTGGTAAACTCCATCT X GGCTAAATACCGGCACGAGACCG p	4 mM	56 °C
IFN-γ	TTCGATGTACTTGGAAATGC TTGCATCTCCTCTGAGACTG	GAAAGATATCATGGACCTGGCCAAGC X CCCGATGAACGACTTGAGAATCCAG p	4 mM	55 °C
IL-2	CAGTGTTACCTGGGAGAAGTG GCAGATATCTCACAAAGTTGGTC	CTTGTTGTTAGCTTCACAGATCTTGCATTCACT X CGGTGTGATTTAGACCCGTAAGACTCTTGAG p	3 mM	58 °C
IL-6	AGGACGAGATGTGCAAGAAG TGCTGTAGCACAGAGACTCG	CGAACAGGCCGCTGGAGAGC X CGTCAGGCATTTCTCCTCGTCGAAG p	3 mM	59 °C
IL-8	ATTCAAGATGTGAAGCTGAC AGGATCTGCAATTAACATGAGG	CATCCGAAGAAGGCATCATGAAGC X TTCCATCTTCCACCTTCCACATCG p	4 mM	56 °C
IL-18	CGTCCAGGTAGAAGATAAGAG AGGAGTCTTCTTCCTCAAAG	CTCCTTCCCTAAATCGAACAACCAT X TCCCATGCTCTTTCTCACAACACAT p	4 mM	56 °C

Calculation of mRNA concentration
The PCR reaction can be described as Nn=No*En (198), where N is the concentration of a sample (identified by a given cycle number), No is the starting concentration of the sample, E is the overall reaction efficiency and n is the baseline crossing point in number of cycles. To calculate the concentration cytokine mRNA the concentration of cytokine mRNA of each individual chicken was divided by the concentration of the 28S mRNA of the same chicken. At each time point the mean of the cytokine [mRNA]/28S [mRNA] of five chickens was taken.

Crypt/villi ratios in jejunum, ileum and caecum

Intestinal tissue sections, 5 µm thick, were cut and stained with haematoxylin and eosin. Five chickens per group were examined at each time point. The crypt-depth and villus-length were measured from the sections using morphometric software (Image Pro Plus 4.1, Media cybernetics).

Statistics/data analysis

Statistics were performed on the data of the chickens that were excreting *Salmonella* Enteritidis in groups 1, 2 and 3 (80-90% of the chickens) and all chickens of the control group. Student's *t*-test was performed at every time point between all groups. The significance of differences between groups at the same time point were analyzed with Student's *t*-test (two-tailed and two sample with unequal variance).

Results
Microbiological determinations

S.E. was detected in cloaca swabs from day 2 after inoculation and on day 4 after inoculation in spleen, liver and caecum content of the *S*.E. infected groups. The highest frequency of *S*.E. cfu's in the liver was detected on day 14 in the group with *S*.E. only, while the groups that received had this peak earlier: the group with *L. paracasei* on day 7 and the group with *L. paracasei* + *L. brevis* on day 4. On day 7, where the group with *L. paracasei* and *S*.E. had the peak frequency of cfu's in the liver and the group with *L. paracasei* and *L. brevis* had started clearance already, the difference in enhancement rate between the two groups with lactobacilli was significant (*P*=0.07). In spleen the Lactobacillus strains had a decreasing effect on the frequency of *S*.E. cfu's on day 7 in both groups with lactobacilli (both *P*=0.04). In caecum no effect of the Lactobacillus strains was found. The number of *S*.E. was $3*10^7$ cfu per gram caecum content on day 4 after infection and remained at this level during the experiment (table 2).

Table 2 Salmonella Enteritidis cfu (^{10}log) per g liver, per spleen and per g caecum content on days 4, 7, 14 and 21 after infection (average of 5 animals (S.E.M.)[a,b,c].

Sample	Treatment		Days after infection			
	Lactobacillus strain	*S*.E.	4	7	14	21
Liver	-	+	2.9(0.4)[a]	3.1(0.3)[a]	3.2(0.4)[a]	nd
	L. paracasei	+	3.0(0.3)[a]	3.6(0.1)[a]	3.1(0.5)[a]	2.2(0.2)
	L. paracasei + *L. brevis*	+	3.4(0.4)[a]	2.8(0.4)[a/b]	2.0(0.3)[a/c]	1.9(0.2)
	-	-	nd[b]	nd[c]	nd[b]	nd
Spleen	-	+	3.1(0.4)[a]	4.3(0.1)[a]	3.5(0.4)[a]	nd
	L. paracasei	+	3.4(0.2)[a]	3.7(0.2)[b]	3.0(0.4)[a]	2.2(0.3)
	L. paracasei + *L. brevis*	+	3.7(0.3)[a]	3.2(0.4)[b]	2.8(0.5)[a/b]	nd
	-	-	nd[b]	nd[c]	nd[b]	nd
Caecum	-	+	7.7(7.3)[a]	7.7(7.3)[a]	7.4(6.6)[a]	7.7(7.1)[a]
Content	*L. paracasei*	+	7.0(6.7)[a]	7.8(7.7)[a]	7.4(7.0)[a]	8.0(7.6)[a]
	L. paracasei + *L. brevis*	+	7.4(7.1)[a]	7.1(6.7)[a]	7.9(7.6)[a]	8.0(7.6)[a]
	-	-	nd[b]	nd[b]	nd[b]	nd[b]

[a] nd = not detectable, below detection limit.
[b] For spleen and liver the highest concentration measured was dilution 10^{-2}, and the estimated value was 1.7 (<50 colonies in spleen or 1g liver and ^{10}log of 50 = 1.7). For caecum content the highest concentration tested was dilution 10^{-3}. Only the samples from the non-infected group were below the detection limit.
[c] Different subscripts in the same column indicate significant differences ($n^{a,b,c}$: P<0.05).

Immunological determinations
Specific humoral response

The *S*.E. specific antibody responses increased after infection. From day 7 the anti *S*.E. titers were significantly higher than in the group without *S*.E. for both IgM and IgG. On day 21 the highest antibody titers were found. The IgM-anti *S*.E. titer in the *L. paracasei* group was decreased compared to the group with *S*.E. only (*P*=0.02, figure 1). This effect was not found in the group with the combination of Lactobacillus strains. On day 21 after infection the IgG titers were still low.

Lymphocyte proliferation assay

On day 21 spleen leukocytes of the group with *S*.E. only proliferated strong to the *S*.E. antigen (figure 2). The proliferation of the spleen leukocytes in the

groups with *S*.E. and lactobacilli was significantly reduced compared to the group with *S*.E. only (*P*=0.01 *L. paracasei* and *P*=0.003 *L. paracasei* + *L. brevis*).

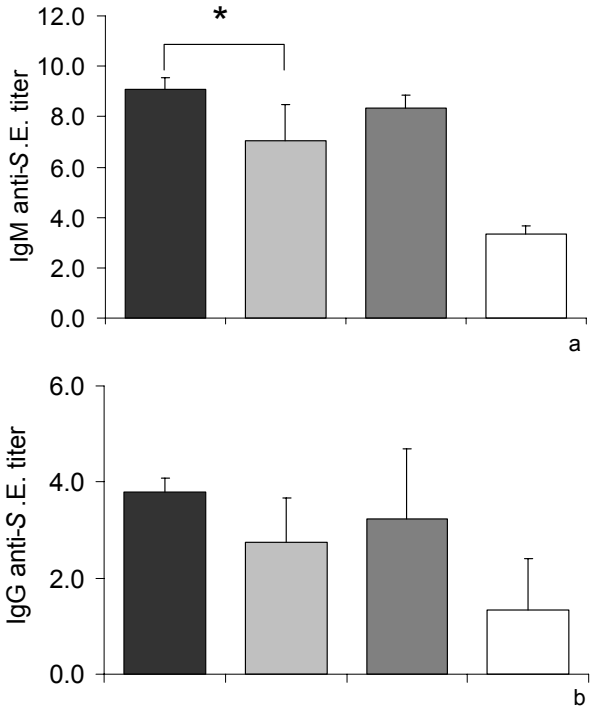

Figure 1. Effect of probiotics on the S.E. specific IgM and IgG titer in serum. Serum anti Salmonella titers IgM (a) and IgG (b) on day 21 after SE inoculation. Following probiotic treatment with L. paracasei the IgM-anti-S.E. titers were decreased (P=0.02). Bars: Black = S.E. infection and no lactobacilli; light grey = S.E. infection and L. paracasei; dark grey = S.E. infection and L. paracasei + L. brevis; white = no S.E. infection and no lactobacilli.

Morphometeric measurements: crypt/villi ratios in caecum, jejunum and ileum

Crypt depth and villus-length were measured on day 4 (early reaction) and day 14 (late reaction) to *Salmonella* infection and application of lactobacilli. No differences were found in crypt depth. In caecum the crypt/villus ratio was reduced after *S*.E. infection. Feeding of Lactobacillus strains seemed to decrease this reduction. The cecal crypt/villus ratio increased with *L. paracasei* (*P*=0.05 compared to the group with *S*.E. only). In jejunum the villus length in chickens of the three *S*.E. infected groups was statistically longer than in the non-infected group (*P*<0.001, not shown).

The crypt/villus ratio in jejunum was not changed by the lactobacilli. In ileum the lactobacilli had a similar effect as was observed in caecum. Feeding of the combination of *L. paracasei* and *L. brevis* protected from a decrease of the crypt/villus ratio (*P*=0.01 compared to the *S*.E. infected group). Statistically the crypt/villus ratio in the group with both lactobacilli was not different from the ratio in the group without *S*.E. infection (*P*=0.931). The early and late reaction were comparable for the proportions between the groups, although on day 4 the differences between the groups were more pronounced, therefore these results are shown in figure 3.

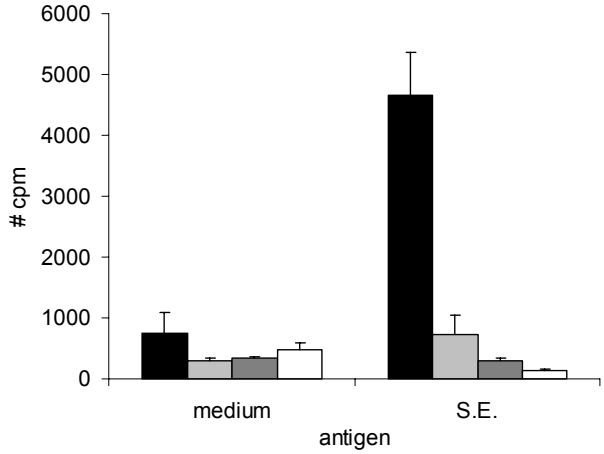

Figure 2. Effect of probiotics on the S.E. specific proliferation of spleen leukocytes. The proliferation of spleen leukocytes to Salmonella antigen on day 21 after inoculation as compared to controls was significantly reduced in the chickens with S.E. + L. paracasei (P=0.01) and for chickens that received S.E. + L. paracasei + L. brevis (P=0.003). For legend to bars, see figure 1.

Discussion

Our hypothesis was that that properly selected probiotics might have a positive effect on disease resistance, e.g. colonization resistance to the pathogen, prevention from/reduced systemic infection, faster recovery from the infection and/or clearance of the infection, and stay in the gut. In our experiment, the effect of feeding the Lactobacillus strains *L. paracasei* LW 122 and *L. brevis* LW 5 on a mild *Salmonella enterica* Enteritidis phage type 4 infection in young layer type chickens was analyzed. The time needed for clearance of the infection was reduced by the Lactobacillus strains, but unexpectedly also a reduction of the anti-*Salmonella* immune response was observed both locally, at level of interleukins in the gut, and systemically at the humoral as well as at the cellular level.

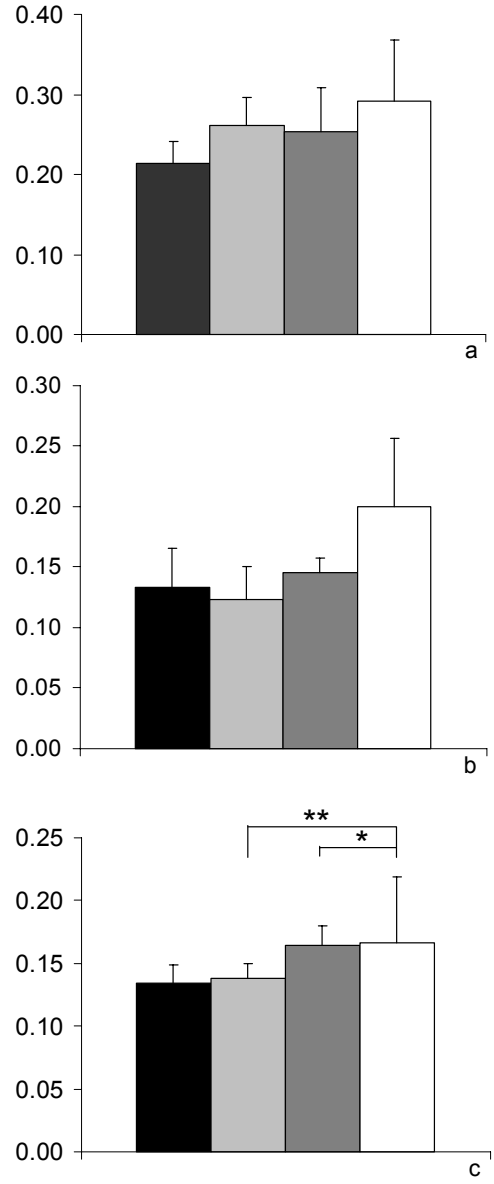

Figure 3. Effect of probiotics on crypt depth and villus-length during S.E. infection. Crypt/villus ratios in caecum (a), ileum (b) and jejunum (c) on day 4 after S.E. infection. As compared to the S.E.-only infected group in caecum the crypt/villus ratio was increased following treatment with L. paracasei (P=0.05). Also in ileum of chickens that received L. paracasei + L. brevis the crypt/villus ratio was increased (P=0.01), but was not statistically different from the group without S.E. infection (P=0.931)). For legend to bars, see figure 1.

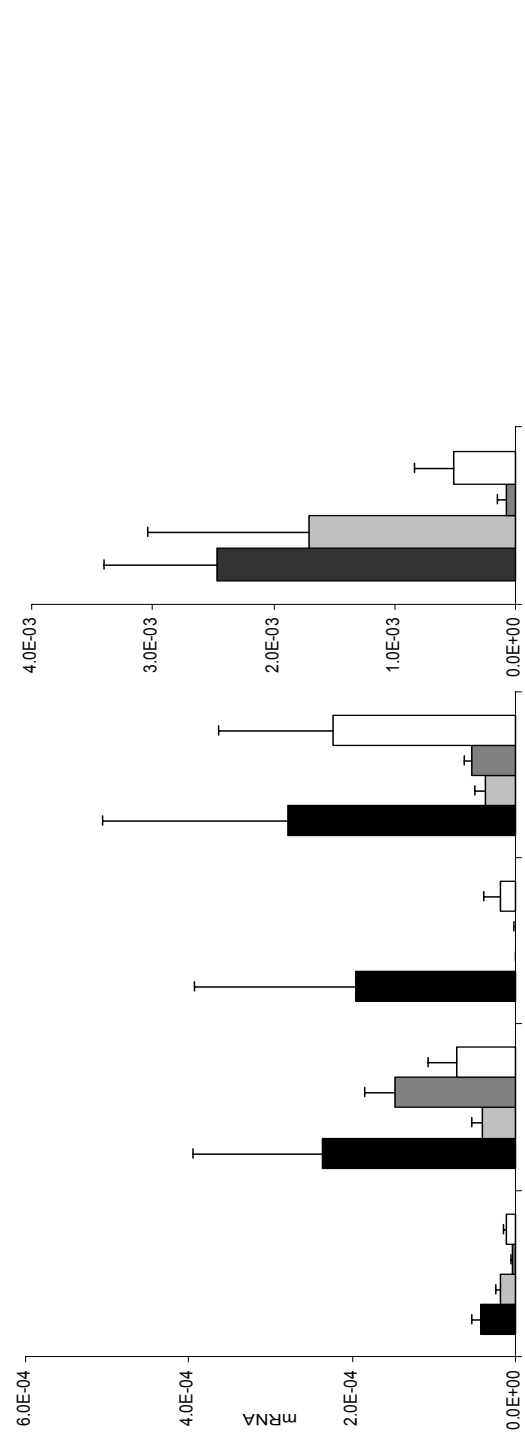

Figure 4. Effect of probiotics and S.E. infection on cytokine mRNA expression in jejunum. On day 7 after S.E. inoculation cytokine mRNA expression levels in jejunum of interferon-γ, Il-2, Il-6, Il-8 and Il-18 were normalized to the individual levels for 28S RNA. The IFN-γ levels were significantly lower in chickens that apart from S.E. received lactobacilli than in chickens that received only S.E. (P=0.03 for both groups that received lactobacilli). Levels of IL-2 in jejunum were reduced in chickens following treatment with L. paracasei as compared to chickens that received S.E. only, while in chickens that received the combination of two strains of lactobacilli had a less strong IL-2 reducing effect (P=0.04 between both Lactobacillus groups). For IL-18 the combination of lactobacilli had a reducing effect (P=0.06) while L. paracasei alone had a less strong reductive effect, as compared to the group with S.E. only. For legend to bars, see figure 1.

Feeding lactobacilli did not prevent the infection from becoming systemic. Since all groups started to shed *S*.E. from day 2 after inoculation, we concluded that susceptibility for *Salmonella* was not altered. The effect of lactobacilli seemed to be restricted to differences in infection-kinetics. Livers and spleens of chickens receiving lactobacilli were cleared sooner from *S*.E. than occurred in chicks that were inoculated with *S*.E. only. Feeding lactobacilli accelerated the *S*.E. infection and clearance but reduced the maximum level of infection in liver and spleen. The combination of two strains of lactobacilli had a stronger effect on the clearance than the *L. paracasei* only. This could be an additive dosis effect because of the administration of two strains, or the supplementary result of two different interactions.

However, both lactobacilli strains were selected for the same immunostimulating properties (150).Feeding lactobacilli did not have an effect on the frequency of *S*.E. in the caecum or the shedding of *Salmonella* in feces. This might be explained by enhanced phagocytosis of *Salmonella* by the lactobacilli (93, 216, 253). By this enhanced phagocytosis the frequency of *Salmonella* bacteria could reach the reduced maximum level sooner after infection (up to 10 days earlier in liver) and enhanced clearance by activated phagocytic cells.

After clearing the infection the *S*.E. bacteria remained present in the gut and the frequency of bacteria in caecum remained stable during the whole experiment. We concluded that *Salmonella* bacteria after a while had become part of the commensal chicken flora. This was confirmed by the fact that the chickens did not show signs of pathology and inoculated chickens kept shedding the bacteria up to 12 weeks (34, 68). Since *S*.E. became part of the commensal flora this is an indication that the immunological elimination of *Salmonella* from the chicken gut probably is not feasible and even probiotic immunomodulating bacteria cannot alter this host-bacteria relationship.

Heres (118) showed that susceptibility of chickens for *Salmonella* was decreased by liquid feed, fermented with *L. plantarum*. This effect could not be mimicked by acidifying dry feed and therefore it was concluded that the LAB might play an intrinsic role in decreasing the susceptibility to *Salmonella* (116). These *L. plantarum* were not immunostimulating (150) and we hypothesized that immuno-enhancing effect together with an acidifying effect of lactobacilli might even further enhance protection against *S*.E. In contrast, the immunological effect of lactobacilli on a *Salmonella* infection was mainly characterized by suppression of different responses. The systemic specific humoral IgM and systemic cellular specific responses were reduced. This was in contrast to the criteria for which these lactobacilli strains were selected (150, 152). As *Salmonella* is an intracellular bacterium the faster clearance was expected to be the result of a Th1-type response (73, 210, 298), and therefore an enhanced production of IFN-γ and its inducing factor IL-18 (188) was expected.

But upon feeding of lactobacilli in the gut the production was of mRNA for several Th1 skewing cytokines (IFN-γ, IL-2, IL-8 and IL-18) was reduced as compared to cytokines in the gut of chicks that received *S.*E. only. Though the systemic immune reactivity was weak, the containment of *Salmonella* in the gut suggested a local immune response or involvement of natural antibodies (IgA) that control commensal bacteria.

The relatively weak immune response could be the result of the relatively low doses of *Salmonella* to which the system was exposed (10^3 cfu). In addition, the accelerated infection kinetics and clearance of the bacteria further reduced the systemic 'antigenic load' which might have resulted in a reduced immune response in chicks fed with lactobacilli as compared to the chickens that received *S.*E. only. In addition, reduction of exposure to *Salmonella* by competitive exclusion using a *Salmonella* vaccine strain resulted in a considerable reduced antibody response after inoculation (187). Not only the dose of *S.*E. but also the route of infection that was used to infect the chickens influenced the quality of the antibody responses (13, 44). The moderate IgM and even weaker IgG response to the oral dose of 10^3 cfu that we applied, equals the response observed by Chart (44) after infection with 10^5 cfu by aerosol where part of the inoculation may enter via the lungs and another part orally. Therefore, the weaker IgG response we found is probably not low because of immunomodulation by the LAB but a result of the low infectious dose. This might also explain the relatively low IgM titer that we observed. In general with *Salmonella* infections both natural and experimental serum IgG titers were detected from one week after infection, that peaked between 2 and 3 weeks after infection and thereafter remained high for at least several months (14, 44, 45). In our experiment, the IgG response was still low at week 3 after infection. However, in all three groups that were infected with *Salmonella* about the same serum IgG response level was observed.

Perdigon (220) described positive effect of lactobacilli on *Salmonella* infection in rodent models. In accordance with our findings, combinations of two Lactobacillus strains had a stronger effect than the single strains. In these experiments, the resistance to the *Salmonella*e based on protective immunity was mainly mediated by the mucosal tissues. The activation of immunocompetent cells in GALT by LAB slightly increased the local inflammatory events although similar as in our experiment no differences were observed in anti-*Salmonella* serum levels between the LAB treated and non-treated groups (212). However, we did not find an indication for increased inflammatory events in the gut. The inflammatory cytokines IL-6, IL-8 in the gut all were reduced. This difference might be host related mouse versus chicken but may also be the result of differences in intrinsic properties of the Lactobacillus strains and infectious agent that was involved (220). The morphology of the caecum and ileum was influenced by the *Salmonella* infection as well as by the lactobacilli. *Salmonella* infection induced shortening of the villi, as in our experiment in caecum and ileum. Similar observations were described by Isaacson

and Stephen *et al*. (127, 275). Probiotic L. reuteri can enhance villus length and crypt depth in ileum (74). Here *L. paracasei* and *L. brevis* diminished or even prevented reduction of the crypt/villus ratio's in ileum and caecum that were the result of *S.*E. infection. Therefore it was concluded, that probiotic lactobacilli can reduce the damage in the gut caused by a *Salmonella* infection. This may result in a more rapid recover from a *Salmonella* infection as it is beneficial to the uptake of nutrients. In conclusion, in the present experiment the combination of Lactobacillus strains that we applied *L. paracasei* LW 122 and *L. brevis* LW 5 in the case of inoculation with *Salmonella enterica* Enteritidis phage type 4 in layer type chickens led to a mild infection that was reduced by the Lactobacillus strains. The dose and route of infection may have contributed to the relatively low immune response at the level of local interleukin expression in the gut and systemic humoral and cellular responses.

Our data do not exclude that a combination of Lactobacillus strains in the case of an infection that does not result in a commensal host relationship could synergize support of different aspects of disease resistance, immune responses and may help reduce physiological damage in the gut.

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8

General discussion

When investigating if probiotics could be an alternative for some properties of antibiotics in chicken, several aspects should be addressed. Firstly, the host in which the probiotics are going to be applied. Chickens have developed in different ways, as in layer- and broiler-type chickens, and their immune system is also differently developed. Secondly, the probiotic strain should be selected for the application in the host. This selection concerns several aspects of the strain (dose, timing), the probiotic properties of this strain (immunomodulating), the host (age, health, environment, immune system) and the combination of the strain with the host. Because the research was focused on immunomodulating lactobacilli in chicken, we selected models (*in vivo* and *in vitro*) to investigate immunomodulating properties of lactobacilli in chicken. Eventually the most promising strains were investigated in an infection model.

In the current chapter the results from the preceding chapters are discussed. This discussion is focused on immunomodulation by probiotic lactobacilli in chicken and followed by a summary of the main conclusions from the research described in this thesis.

Chicken immune system

The opinion is that broiler type chickens are more vulnerable to pathogens than layer type chickens. Heavier broiler type chickens and turkeys showed lower immune responses than less heavy broiler types and turkeys (156, 163, 164). When this relation is broadened to layer and broiler type chickens, the difference in immune response to antigens might be more extreme. To investigate if broiler and layer type chickens respond differently to an antigen qualitatively and/or quantitatively, both types were compared under the same circumstances in chapter 3. The kinetics of the specific IgM anti-TNP response upon TNP-KLH immunization was similar in layer- and broiler-type chickens. Broiler type chickens seemed to generate a good specific IgM response, but a poor specific IgG response. The layer type chickens generated a specific IgM response, which was a bit lower than the response of the broiler type chickens. On the other hand, these layer type chickens generated an IgG response that was higher and lasted longer than the response in broiler type chickens. From an evolutionary point of view, the primary IgM response might be more important for the shorter living broiler type chickens than the more sophisticated secondary response, and therefore has not been a major factor in genetic selection. The short life span of broiler type chickens could also be responsible for a faster development of the immune system in broilers, which could lead to a higher IgM response at the same age as layer type chickens. This hypothesis does not explain the low IgG response in broilers though.

The *in vitro* T cell response (blood and spleen) by broiler type chickens was lower than in layer type chickens. A higher concentration of antigen or mitogen was needed in order to detect (re)stimulation in broiler type chickens (chapter 3, (148)) and the response occurred at a lower level. We conclude that the results of immunological studies can differ between the different chicken types. The poor IgG and cellular responses in broilers might be enhanced to increase disease resistance when removing antibiotics from the chicken feed.

Replacement of antibiotics by probiotics

Replacement of antibiotics by probiotics in feed, fulfilling the same functions, though initially expected, never was our purpose. We knew that an important feature of antibiotics, growth stimulation, was not detected in chickens. The type of Lactobacillus strain, the dose of lactobacilli, the duration of the application period and the host all modulate the properties of lactobacilli (62, 111, 152).

Probiotics and gut flora

Probiotic lactobacilli might be able to help develop and stabilize the microflora of newly hatched chickens. In chapter 5 we showed that lactobacilli did stabilize the gut flora in crop, jejunum and caecum. When lactobacilli were fed to chickens via

liquid fermented feed, the flora in the gut was stabilized sooner compared to dry feed (152). This stabilization was especially found in the reduced numbers of enterococci in the groups that received fermented feed compared to the dry feed group, together with 10-fold higher numbers of lactobacilli in crop, jejunum and caecum. By stabilizing the gut flora, these chickens should be less susceptible for pathogens that enter the body via the gut. This principle has been proven for Salmonella in broiler type chickens (118) and, according to the same principle, probably also holds for the susceptibility to other pathogens.

Probiotics and immunomodulation

Modulation of the immune response by probiotics has been described since a long time. Such effects were observed by several groups but were hard to reproduce when the bacteria strains and the host to which they were administered were not the same. The seemingly contradictory results that were found in different studies are lined up in review articles. In order to compare effects of probiotics, different aspects of the strain (type, dose, timing, growth phase, duration of application and the host (genetics, environment, health status and age) should be considered. We investigated the different aspects of the strain in chapters 2, 5 and 6, and the role of the host in chapters 6 and 7.

Other investigators have performed experiments and human clinical trials, that were carried out with well-characterized strains (e.g. commercially available and patented or identified by ATCC number), which is an important progress. Another recent improvement is the setup of the experiments, where double blind groups are used. Although such experiments led to verification of immune modulation effects, it is still not known how immunomodulation is induced by probiotics. Currently, the hypotheses are focused on cell-mediated effector functions or DCs, which can be stimulated directly by probiotics. Research concerning which part of probiotic bacteria induces immunomodulation, focuses on cell wall structures as sugars and polymers.

Probiotics may interfere with or enhance immune reactivity at various levels of the immune response process as antigen uptake, antigen processing and presentation in the intestinal mucosal immune system. Investigation of these stages of the immune response offers a grasp on how probiotics or fragments thereof might be delivered in to the systemic immune system and exerts health-stimulating activities. Nevertheless, other routes of entry or stimulation pathways cannot be ruled out. The mechanism(s) by which probiotics modulate immunity are not fully understood. Most published studies focused on *in vitro* or *ex vivo* immune responses, of which the results are difficult to translate to the *in vivo* situation in humans or animals. Although *in vitro* studies might be helpful to unravel the mechanism(s) by which probiotics modulate the immune response, *in vivo* models are more relevant to study the effects of probiotics on immunomodulation (62).

Evaluation of probiotic properties in *in vivo* models is time-consuming, labor-intensive and requires large numbers of animals. Because of the complexity of the interactions of health stimulating probiotics with the host, evaluating probiotics in *in vivo* models is almost comparable to a black box. These interactions vary from surviving the stomach, interaction with the host gut epithelium, interaction with the host gut flora to interaction with the host immune system. Several *in vitro* assays have been set up to be able to evaluate single aspects of probiotics influencing the host health. These assays cover the areas of acid- and bile tolerance and adhesion to gut mucus or intestinal cell lines (Caco-2 and HT-29) (30, 193). Besides selection methods were described for probiotics that express antimicrobial activity (1, 29, 181), growth inhibition of unwanted flora elements (132), competitive exclusion (288), and also an *in vitro* gut model (171, 291) has been described to evaluate gut microbial ecology. Not all assays are validated in the *in vivo* situation, but even if they are, it is important to realize that an *in vitro* assay in general is focusing on one single selected property important for probiotic health stimulation. The results *in vivo* so far may be less promising than the *in vitro* data since immunomodulation by probiotic bacteria is depending on many more factors besides the immune system. If enough probiotic bacteria survive the acid and bile and expand in the gut, the immunomodulating capacity of a strain can have its maximal effect. Besides survival and expansion the bacteria should come into close contact with epithelial cells (adhere to these cells). These aspects of conditions in which probiotic effects are observed are just few examples of all the conditions a strain has to stand or the properties it has to own. A combination of *in vitro* assays, each covering a different aspect of the functioning of probiotics, could be helpful to pre-select promising strains.

In the *in vitro* assay for the selection of immunomodulating capacities of lactic acid bacteria that we developed, immunomodulation was assessed as the modulation of non-specific proliferation of splenocytes. The splenocytes were stimulated with a suboptimal concentration of ConA and the lactic acid bacteria were studied for their immunomodulating capacities of this suboptimal proliferation. The stimulation of proliferation of splenocytes correlated with enhancement of specific IgM responses in layer type chickens (chapter 5). In the *in vitro* assay the interaction of the LAB strain with T/B cells and APCs (macrophages) resulted in extra proliferation, induced by Lactobacillus strains on top of the suboptimal proliferation induced by ConA. ConA proliferation of T cells is dependent on the presence and activation of accessory cells. We hypothesized that those LAB that were able to enhance T cell proliferation *in vitro* either by activation of accessory cells or T cells may do the same *in vivo,* based on their enhancing effect on the secretion of IFN-γ, phagocytosis and expression of complement receptors on phagocytes. Sampling of LAB by DCs from the GI tract may differentially affect DC maturation (48) and stimulate the local T cells. Subsequently, the circulating pool of mucosa-homing T-lymphocytes may exert an immune outcome at distant (mucosal) sites. Such an effect might be measured as immune stimulation or adjuvant activity since activated

T-helper cells could also move to the spleen and modulate the activity of T cell subsets involved in antibody production.

Because of the direct interaction of the bacteria with the T and B cells and APCs cells, the *in vitro* assay can probably also be used to measure the effect of the bacteria on the induction of cytokine production by these cells. The cytokine profiles could help to unravel part of the mechanism of immunomodulation by probiotic bacteria, both enhanced proliferation and decreased proliferation. A similar approach is expected to be successful in other animals.

Stimulation of cell-mediated immune effector functions

Ingestion of probiotic bacteria or fermented milk products triggers spontaneous (108)and enhanced mitogen-induced (306, 307) production of interferon-γ by blood leukocytes. The cells responsible for the interferon production are likely to be NK cells and/or T cells (106, 107, 119). Ingestion of probiotic bacteria enhances the phagocytic capacity of blood polymorphonuclear leukocytes (5, 66, 251), triggers respiratory burst (66, 306) and increases their expression of receptors involved in phagocytosis, especially complement factor 3 (CR3) (111, 211). Blood monocytes are similarly affected, although to a lesser extent (211, 251).

This stimulation of cell-mediated effector functions (IFN-γ production and enhanced phagocyte function) probably results from the production of immune stimulating cytokines and other mediators when probiotic bacteria interact with monocytes/macrophages in the Peyer's patches, intestinal mucosa or other sites. Lactobacilli, as well as other Gram-positive bacteria, are efficient in inducing the production of IL-12 (119, 120). IL-12 is the major stimulator of cell-mediated immune effector functions. IL-12 stimulates IFN-γ production in T cells and NK cells and increases their cytotoxic potential (286). Many other cytokines and mediators will be triggered when probiotic bacteria interact with macrophages and other cells of the innate immune system, some of which may be involved in the enhancement of phagocytic function after intake of probiotics (308). In contrast to IL-12, the macrophage derived cytokine IL-10 (e.g. induced by *L. rhamnosus*) (120) downregulates IFN-γ production and thus opposes the effect of IL-12 on T cells and NK cells (56). Different bacterium strains can induce different cytokines, possibly leading to opposite effects on the immune system.

The induction of cytokines could lead to improved macrophage activity in chicken. In chapter 6 we evaluated the effect of *L. paracasei* on the phagocytosis of Salmonella Enteritidis (*S*.E.) bacteria in gut cells and spleen. In healthy broiler type chickens the phagocytosis and bacterial activity of gut cells (caecum, ileum) was enhanced by *L. paracasei*. In the spleen this bacterial activity was lower. In layer type chickens the probiotics did not have an effect on phagocytic and bacterial activity of the ileum and spleen cells (152). Since we used the same Lactobacillus strain in both chicken

types, the differences on the phagocytic and bacterial activity might be explained by intrinsic differences in the quality of the innate immune system of layer- and broiler type chickens, which were discussed in chapter 4 (148).

In layer type chickens with a *S*.E. infection, the clearance of *S*.E. from the liver and spleen was accelerated in the groups receiving *L. paracasei* or *L. paracasei* + *L. brevis* (chapter 6), probably by enhanced activity of macrophages. In chapter 6 we found that in spleen cells of healthy layer type chickens the macrophage activity was not enhanced by *L. paracasei*. In combination with a *S*.E. infection, the accelerated clearance by liver and spleen indicates that the macrophage activity was enhanced by the lactobacilli, as described by others (93, 216, 253). The phagocytosis in the two experiments was measured differently. In the healthy chickens (chapter 6) the phagocytosis was measured by the number of bacteria entered in spleen and gut cells. In the experiment with the Salmonella infected chickens (chapter 7) the number of bacteria per gram liver or per spleen was calculated at different times, resulting in infection kinetics in the liver and spleen. In a spleen *in vivo*, bacteria can be presented to activated T and B cells, which help clearing the infection (apart from macrophages), while *in vitro* the T cells were not activated by a *S*.E. infection. The combination of *L. plantarum* + *L. paracasei* had a stronger effect on the clearance than the *L. paracasei* only. This could either be a dose effect because of the administration of two strains, or the supplementary or cooperative effect of two different interactions. As Salmonella is an intracellular bacterium, the faster clearance was expected to be the result of a Th1-type response and therefore an enhanced production of IFN-γ and its inducing factor IL-18 was expected. Upon feeding of lactobacilli the production of mRNA for several Th1 skewing cytokines (IFN-γ, IL-2, IL-8 and IL-18) in the gut was reduced as compared to mRNA of cytokines in the gut of chickens that received *S*.E. only. If IL-10 could be measured and would be increased, this would indicate that T and NK cells in the gut were not activated and their cytotoxic potential was not increased as opposed to IL-12 inducing IFN-γ. If *S*.E. was becoming a commensal after infection (as discussed in chapter 7), no increased cytotoxic activity was expected.

Dendritic cells and immunomodulation by lactobacilli.

To clarify the mechanism(s) underlying the immunoregulatory effects of the gut microflora, including probiotic bacteria, recently more attention has been paid to their effect on DCs. DCs occur in most tissues and in particular at sites that interface with external environment, such as the mucosa of the GI-tract, where they reside in the Peyer's patch, lamina propria and draining mesenteric lymph node (145). DCs are the gatekeepers of an immune response and are the principal stimulators of naive Th cells - a property that distinguish them from all other APCs (10). There is increasing evidence that the type/quality of initiated immune response relies on the highly polarized functioning of the DC and that this ultimately depends on DC type and state of activation. The DC function is greatly influenced by locally

present environmental factors (57, 144, 301). After stimulation by the surface of a probiotic bacterium or by degraded parts of this bacterium DCs present all kinds of markers on their surface. These markers affect innate and specific humoral and cellular immunity. Included are variable expression of both antigenic and co-stimulatory signals as well as variations in expression of cytokines, such as IL-10 and IL-12. Since it has been shown that DCs penetrate the epithelium without disrupting the barrier function and directly sample gut-associated bacteria (237), lactobacilli from the gut may differentially affect DC maturation as determined by cytokine and surface marker expression. DC may therefore play a central role in mediating the effects of probiotic bacteria.
The capacity of lactobacilli to variably induce IL-12 and TNF-α, and, to a lesser extent, IL-6 and IL-10, indicates that different strains of Lactobacillus may differentially determine immune responses (57). Besides strain-differences, one strain can dose-dependently induce different cytokine profiles. *L. casei* was observed to induce IL-6, IL-12 and TNF-α when applied in low concentrations, while it also induced IL-10 when applied in high concentration in mice. The strain and the dose of the bacteria affect the interaction with local DCs and thus ultimately dictate whether a Th1, Th2 or Th3 response occurs (48, 57).

IgA responses

Probiotic bacteria induce a specific immune response to themselves, like any other microbes that are taken up from the intestinal lumen. In addition, microbes also stimulate the production of polyreactive antibodies that have other, unknown specificities (42, 229). The function of such non-specific antibodies is unknown, as are mechanism(s) of their induction. However, it is observed that ingestion of probiotics non-specifically stimulates enhanced production of secretory IgA on mucosal surfaces, or serum IgA in the circulation (308).

Several studies have examined the antibody response to peroral (128, 165, 306) or systemic vaccination (151, 152, 306), or to natural infection (142, 143) after and during application of probiotic bacteria. Several strains were used and different results were reported, e.g. increased serum IgA antibody levels against *Salmonella typhi* or rotavirus infection (142, 165), as well as no enhancement of the IgA response was reported (266, 306). It is possible that a particular probiotic strain is more efficient than another in inducing IgA or that the duration of application is an important factor in IgA induction, inducing the differences in results reported. IgA production might be stimulated by IL-6 which is produced by epithelial cells after interaction with probiotic bacteria. From many LAB strains is reported that they enhance intestinal IgA responses and induce IL-6. IL-6 is mainly produced by APCs and promotes terminal differentiation of B cells into plasma cells producing IgA (84, 189).

Adjuvant function

Adjuvants are substances that enhance immune responses to antigens with which they are simultaneously administered. Adjuvants improve antigen presentation to T cells by enhancing the production of T cell stimulating cytokines and enhancing the expression of accessory molecules by APCs. Microbial antigens possess conserved structures that can activate monocytes, macrophages and DCs and therefore are good immunogens. Lactobacilli too possess these conserved structures. In combination with their harmless character Lactobacilli therefore could function as an attractive adjuvant by activating APCs and T cells. In several studies an adjuvant effect was observed after feeding probiotics (150, 165, 169, 219, 228), but this was not always confirmed in other research (99, 112, 179, 283, 308). In chapters 2, 5, and 6 the possible adjuvant effect of probiotic lactobacilli in chickens was evaluated. In our experiments, we found a few strains that had an adjuvant effect on the humoral response to an artificial antigen (TNP-KLH) in both layer and broiler type chickens. The effect was depending on the combination of the strain, host, dose of lactobacilli, and duration of application (150, 152).

The non-specific humoral response was enhanced in broilers while feeding feed fermented with *L. paracasei* and *L. paracasei* + *L. brevis*. The IgM titers were enhanced after priming and booster immunization while the IgG titers were only enhanced after booster immunization while feeding the combination of the two Lactobacillus strains ((152); chapter 6). In experiments where the lactobacilli were only shortly administered (5 days prior to immunization) with a lower dose (10^9 cfu per day), the Lactobacillus strains did not have an adjuvant effect on the non-specific humoral response in layer or broiler type chickens.

Temporary feeding of *L. paracasei* preceding immunization had an adjuvant-like effect on the specific humoral responses in layer type strains ((149, 150); chapters 2 and 5). Continuous feeding with feed fermented with the combination of *L. paracasei* + *L. plantarum* enhanced both specific IgM and IgG titers in broiler type chickens.

The differences in enhancement of the immune response between the different experiments might be the result of differences in dose and feeding regime (temporarily or continuous), which could result in different kinetics of the immunomodulatory effects. The delay between the feeding of lactobacilli and the time point of measuring the humoral responses may influence the results. This delay was even larger for the IgG response, which generally is built up later than the IgM response. The effects on the IgG response were smaller than on the IgM response. The longer period between feeding of the probiotics and the switch to IgG could be too long to induce a significant positive effect. It might be that after feeding the probiotics for a longer period or in higher doses, an adjuvant effect on the IgG response does occur. The differences in stimulation of the IgG response between

broiler and layer type chickens might be related to the lower specific IgG anti TNP-KLH response in broiler type chickens (148).

The age of the chickens might also affect the possibility to modulate the immune response by probiotics, since in young animals the immune system is not as mature as in older animals. In the immature immune system the cells belonging to the immune system might be less susceptible to the effect of probiotics. The adjuvant effect on the specific anti-TNP response was absent in young broiler type chickens after temporary feeding of *L. paracasei*. The combination of the young age and the relatively lower dose of probiotics (in proportion to the body weight) that was administered in broiler type chickens might be responsible for the difference in immunomodulation between the two chicken types within the same experiment ((152); chapter 6).

The probiotic bacteria selected in *in vitro* and *in vivo* experiments did not have an adjuvant effect on the specific humoral immune response in chickens infected with *S*.E. These strains were selected because of their immunomodulating effect. The low dose of *S*.E. led to a mild infection that was reduced by the Lactobacillus strains. The response was not high and was even lower in animals that received the selected probiotic lactobacilli. This reduction in immune response could be the result of the faster clearance of *Salmonella* from the organs. The accelerated infection kinetics and the faster clearance of the bacteria give the immune system less time and opportunity to be in contact with the pathogen and build up an immune response, resulting in a decreased immune response at all parts of the immune response compared to the group with *S*.E. only. Our data do not exclude that a combination of Lactobacillus strains could, in the case of an infection that does not result in a commensal relationship with the host support disease resistance and immune responses.

The adjuvant effect that we observed was never extremely strong and varied between the chickens within a group. We used eggs from a limited number of mother- and father animals in order to reduce the genetic variation between the animals. Within a group of chickens that received the same treatment both responders and non-responders were found. It might be that the relatively low antigen concentration we used for immunizations induced larger within-group variations. The combination of the within-group variation with the small adjuvant effects found made it hard to determine an adjuvant effect. This could also play a role in studies by others and lead to contradicting conclusions.

In layer type chickens the non-specific proliferation of stimulated splenocytes was enhanced by probiotic bacteria when these were added directly to the cell culture. Oral administration of these probiotic bacteria did not have the same enhancing effect on the proliferation of spleen cells *ex vivo* as in the *in vitro* assay. In broiler type chickens the non-specific proliferation was not enhanced. The specific

response to (re)stimulation with antigen *in vitro* was not enhanced in layer type chickens, but was enhanced by *L. paracasei* in broiler type chickens following temporary administration prior to the application of the immune stimulus. This suggests that for specific antigens, such as e.g. vaccine antigens, immunoprobiotic lactobacilli might enhance disease resistance in this particular broiler type chicken.

Strain specificity

A returning aspect in this thesis about research in probiotics is bacterial strain specificity. In the *in vitro* assay, the differences in stimulation of cell proliferation between strains of the same species, but not the same type were clear. Bacterial strain specificity is hypothesized or described in many researches (e.g. 62, 111, 112, 150, 152, 169, 221, 310). In several studies different Lactobacillus strains, or strains from the same species that might not be exactly the same sub-type were compared, leading to conflicting or irreproducible results, as even different strains that are closely related and have a part of their name in common such as *L. brevis* or *L. reuteri* strains do not have the same probiotic properties (150).

Strain specificity could in part be determined by the cell wall of lactic acid bacteria. This cell wall is composed of e.g. peptidoglycans, teichoic acid and polysaccharides which slightly differ from strain to strain. Peptidoglycans may induce adjuvant activity at the mucosal surface (72). Muramyl dipeptide, a lower-molecular weight breakdown product of peptidoglycans may stimulate cytokine production by macrophages, monocytes and lymphocytes. Also teichoic acid may stimulate the production of certain cytokines by monocytes and lymphocytes (113).

Lactic acid bacteria may increase secretory IgA activity in the gastrointestinal tract (114). One specific probiotic strain might induce expression of different surface markers on DCs, APCs and accessory cells than another probiotic strain will, because of the differences in the cell wall. These expressed surface markers can affect the extent of stimulation of T and B cells. Stimulated T cells could increase the T cell reaction or downregulate the existing T cell response. The B cells could be stimulated in production of antibodies (higher antibody titer) or in decreased production of antibodies by deactivation of these cells.

Conclusions

- Lactic acid bacteria can be selected on immunomodulating effect *in vivo* using the *in vitro* assay described in chapter 5.
- Layer and broiler type chickens differ in immune response characteristics, therefore results obtained in one type cannot be interpreted to the other chicken type straight off.
- Probiotic lactobacilli can support in development and stabilization of the gut flora in crop, jejunum and caecum of young chickens and therewith make them less susceptible for pathogens that enter the body via the gut.

- Probiotic lactobacilli can reduce or even prevent the reducing/damaging effect of a Salmonella infection on villus length.
- Because of the broad variation between individual chickens, the strength of an immunomodulating effect as well as the proportion of positive by responding chickens are important to determine if a probiotic strain has a positive effect on the immune response.
- Probiotic lactobacilli can increase macrophage activity and enhance clearance of systemic bacterial infection in liver and spleen.
- Dependent on the strain and dose of the probiotic used, and dependent on the genetics and age of the host and the duration of the application, probiotic lactobacilli can modulate the humoral immune response in chickens.

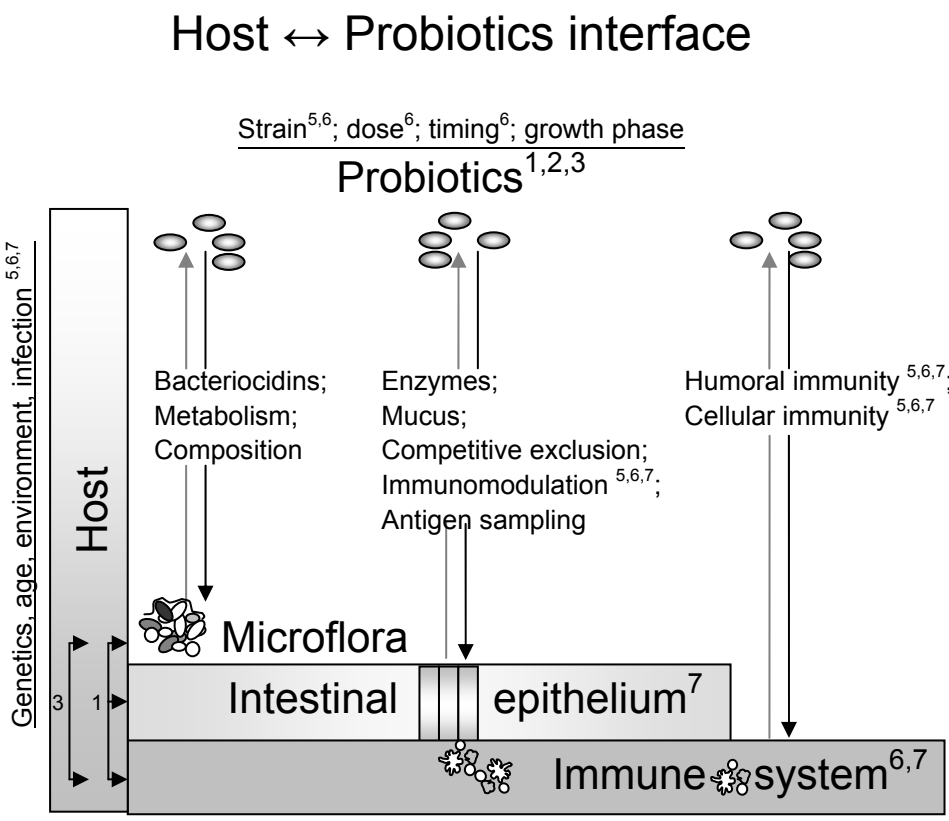

Figure 2: Host-probiotics interface, adapted from (62).Host aspects that can be discriminated are: 1) microflora, 2) intestinal epithelium and 3) immune system. The arrows indicate the interactions or feasible interactions between probiotics and the 3 host aspects or between the aspects themselves. The targets of probiotic activity (probiotic effects) are written in italics. Factors that play a possible role in the probiotic effect are underlined. The superscript numbers refer to corresponding chapters in which the host aspects, probiotic effects or effect related factors are discussed.

In conclusion, probiotic lactobacilli are applicable in chickens for several purposes. These vary from developing and stabilizing the microflora, especially the starterflora of newly hatched animals, decrease or prevent physiological damage in the gut by pathogenic infections, to immunomodulation and enhancing disease resistance or helping to recover faster from an infection.

Not all probiotic lactobacilli are suited for every purpose or all purposes together. The probiotic lactobacilli are no miracle medicine capable of all properties ascribed to them. A probiotic strain can have positive effects on the health, but the strain and the host have to be matched with each other. Improved health does not always imply better or more rapid growth. The combination of the host's age and background genetics with the right bacterium strain and the right dosage and duration of application can have beneficial effects on the gut and the immune system. The observed effects are always limited and do not have the same effect in every single animal. However, when a flock is more resistant to pathogens, the individual animals will be better protected. When all variables important for functioning of probiotics are tuned well to each other, immunomodulation and increased disease resistance by probiotic lactobacilli are possible.

At present it is complex to balance all factors appropriately, but when more knowledge about the mechanism(s) of immunomodulation by probiotic bacteria and the gut microflora and the interaction of these two with the immune system becomes available, it will become easier to select the right strains and their application. This will lead to a more stable chicken health, which is necessary when antibiotics will be removed from the feed and especially when they are held outside where they are exposed to many more pathogens than in the strictly hygienic, more industrial-like farms.

Future research

In order to further investigate the field of probiotic lactobacilli and immunomodulation, more knowledge of the interaction of probiotics with the host is needed. In addition, knowledge of the immunomodulatory mechanism(s) should be developed. With such knowledge probiotic lactobacilli could be rationally selected for different purposes. With help of *in vitro* assays both aspects can be further

investigated. Analysis of cytokine profiles induced by probiotic lactobacilli in NK cells and APCs like macrophages and DCs might help reveal parts of the immunomodulating mechanism(s).

Modern techniques like micro-arrays using DNA-chips allow studies at the gene expression level. Information can be obtained on which genes are upregulated in cells of animals or humans that received a particular probiotic compared to cells of animals or humans which did not receive that particular probiotic treatment. The genes that are up- or down-regulated because of the probiotic might be involved in the immunomodulation observed. The information thus generated could provide more information about the interaction of probiotics with the host and the cell types involved.

There might be a relation between certain glycoproteins and other surface molecules on probiotic bacteria and the direction (Th1, Th2, Th3) and proportion of immunomodulation induced by the strain. When such a relationship does exist, the effect of a probiotic strain could be predicted by the surface molecules present on the surface of the bacteria. These surface molecules could be relatively easy detected in *in vitro* assays.

Probiotics that reduce inflammation could be used for treatment and/or prevention of e.g. inflammatory bowel disease and Crohn's disease, whereas other probiotic strains that stimulate the immune system and stabilize the gut flora could be selected for treatment of viral diarrhea in young children. Probiotics that shift the Th1 –Th2 balance within the immune system in favor of Th1, might be appropriate for application in e.g. allergic responses.

To evaluate the relevance of the immune stimulating lactobacilli that were selected in our TNP-KLH model, it is necessary to evaluate these strains in an infection model. This infection model should induce mild immune responses in order to be able to detect stimulation of the immune response and preferably be a relevant gut pathogen in chicken. The infection by the selected pathogen preferably is to be inhibited by the enhanced immune response in combination with competitive exclusion. Useful infection models should employ Campylobacter, Eimeria, Pseudomonas or Influenza. These pathogens naturally infect the chicken via the gut and cause infections that are relevant for chicken. TNP-KLH, the model-antigen we used in our experiments is a non-replicating antigen to which chicken mount an immune response, but which does not induce an infection. The Salmonella infection we used in our last experiment seemed to fulfill the conditions, but probably because this specific Salmonella bacterium did not induce illness and became a commensal of the chicken flora, the immune response likely is not representative for the immune response to a disease causing microorganism.

The assays to be used to detect the immune responses should be sensitive enough to differentiate between not only enormous differences in stimulation as by adjuvants, but also between subtle differences in immune responses.

When stimulating the immune system, one of the first questions that arise is: is the immune system in healthy, well responding animals not going to be over-stimulated? Such overstimulation might induce adverse effects. Over-stimulation by probiotics, however, has never been reported so far and no clinical evidence about this is available.
Attention should be paid to the safety aspects of bacteria for oral application. Bacteria are capable of exchanging genes with other bacteria in the gut or other environment. For lactobacilli, however, this is not an issue, because of the GRAS-status of the lactobacilli used in food applications.

A continuously important issue in probiotic research is preciseness. The use of well-defined strains and the identification of self-isolated strains are crucial for reproducible experiments. The strain name only is not informative enough, since there are often different strains with the same name, so a molecular biological verification of the strains should be available or be performed. In addition, all factors influencing the results of probiotic application must be well defined and the strain has to be tested in the animal species or in humans beforehand.

References

References

1. Abee, T., T.R. Klaenhammer, and L. Letellier. 1994. Kinetic studies of the action of lactacin F, a bacteriocin produced by Lactobacillus johnsonii that forms poration complexes in the cytoplasmic membrane. Appl Environ Microbiol 60:1006-1013.
2. Adams, M.R., and C.J. Hall. 1988. Growth inhibition of food-borne pathogens by lactic and acetic acids and their mixtures. International J Food Sci Technol 23:287-292.
3. Adler, H.E., and A.J. DaMassa. 1980. Effect of ingested Lactobacilli on *Salmonella infantis* and *Escherichia coli* and on intestinal flora, pasted vents, and chick growth. Avian Dis 24:868-878.
4. Alvarez Olmos, M.I. and R.A. Oberhelman. 2001. Probiotic agents and infectious diseases: A modern perspective on a traditional therapy. Clin Inf Dis 32:1567-1576.
5. Arunachalam, K., H.S. Gill, and R.K. Chandra. 2000. Enhancement of natural immune function by dietary consumption of *Bifidobacterium lactis* (HN019). Eur J Clin Nutr 54:263-267.
6. Asahara, T., K. Nomoto, K. Shimizu, M. Watanuki, and R. Tanaka. 2001. Increased resistance of mice to *Salmonella enterica* serovar Typhimurium infection by synbiotic administration of Bifidobacteria and transgalactosylated oligosaccharides. J Appl Microbiol 91:985-996.
7. Bacha, W.J., and L.M. Bacha. 2000. Digestive system, p. 119-162. *In* D. Balado (ed.), Color atlas of veterinary histology, 2 ed. Lippincott Williams & Wilkins, Baltimore, USA.
8. Bacha, W.J., and L.M. Bacha. 2000. Lymphatic system, p. 69-84. *In* D. Balado (ed.), Color atlas of veterinary histology, 2 ed. Lippincott Williams & Wilkins, Baltimore, USA.
9. Balevi, T., U.S. Ucan, B. Coskun, V. Kurtoglu, and I. S. Cetingul. 2001. Effect of dietary probiotic on performance and humoral immune response in layer hens. Br Poult Sci 42:456-461.
10. Banchereau, J., and R. M. Steinman. 1998. Dendritic cells and the control of immunity. Nature 392:245-252.
11. Barnes, E.M., G.C. Mead, D.A. Barnum, and E.G. Harry. 1972. The intestinal flora of the chicken in the period 2 to 6 weeks of age with particular reference to the anaerobic bacteria. Br Poult Sci 13:311-326.
12. Barrow, P.A. 1992. Probiotics for chickens, p. 571-588. *In* R. Fuller (ed.), Probiotics: the scientific basis, vol. 1. Chapman & Hall, London, UK.
13. Barrow, P.A. 2000. The paratyphoid Salmonellae. Revue Scientifique et Technique De L Office International Des Epizooties 19:351-375.
14. Barrow, P.A., and M.A. Lovell. 1991. Experimental-infection of egg-laying hens with *Salmonella* enteritidis phage type 4. Avian pathology 20:335-348.

15. Barrow, P.A., J.M. Simpson, and M.A. Lovell. 1988. Intestinal colonisation in the chicken by food-poisoning salmonella serotypes; microbial charcateristics associated with faecal excretion. Av Pathol 17:571-588.
16. Bautista Garfias, C.R., O. Ixta, M. Orduna, F. Martinez, B. Aguilar, and A. Cortes. 1999. Enhancement of resistance in mice treated with *Lactobacillus casei*: Effect on *Trichinella spiralis* infection. Vet Parasitol 80:251-260.
17. Beery, J.T., M.B. Hugdahl, and M.P. Doyle. 1988. Colonization of gastrointestinal tracts of chicks by *Campylobacter jejuni*. Appl Env Micrbiol 54:2365-2370.
18. Befus, A.D., N. Johnston, G.A. Leslie, and J. Bienenstock. 1980. Gut-associated lymphoid tissue in the chicken. I. Morphology, ontogeny, and some functional characteristics of Peyer's patches. J Immunol 125:2626-2632.
19. Bendelac, A., and D.T. Fearon. 2000. Innate immunity - Receptors and effecters of innate immunity - Editorial overview. Curr Op Immunol 12:11-12.
20. Berg, R.D. 1996. The indigenous gastrointestinal microflora. Trends Microbiol 4:430-435.
21. Berg, R.D. 1998. Probiotics, prebiotics or 'conbiotics'? Trends Microbiol 6:89-92.
22. Berg, R.D., and A.W. Garlington. 1979. Translocation of certain indigenous bacteria from the gastrointestinal tract to the mesenteric lymph nodes and other organs in a gnotobiotic mouse model. Infect Immun 23:403-411.
23. Bianchi, A.T., J.W. Scholten, I.M. Jongenelen, and G. Koch. 1990. The use of monoclonal antibodies in an enzyme immunospot assay to detect isotype-specific antibody-secreting cells in pigs and chickens. Vet Immunol Immunopathol 24:125-134.
24. Bjorksten, B. 1999. The intrauterine and postnatal environments. J Allergy Clin Immunol 104:1119-1127.
25. Blaschke, V., K. Reich, S. Blaschke, S. Zipprich, and C. Neumann. 2000. Rapid quantitation of proinflammatory and chemoattractant cytokine expression in small tissue samples and monocyte-derived dendritic cells: validation of a new real-time RT-PCR technology. J Immunol Methods 246:79-90.
26. Bloksma, N., M.J. De Reuver, and J.M. Willers. 1983. Impaired macrophage function as a possible basis of immunomodification by microbial antigens, tilorone and dimethyldioctadecylammoniumbromide. Antonie van Leeuwenhoek 49:13-22.
27. Bloksma, N., H. Ettekoven, F.M. Hofhuis, L. Van Noorle-Jansen, M.J. de Reuver, J.G. Kreeftenberg, and J.M. Willers. 1981. Effects of lactobacilli on parameters of non-specific resistance of mice. Med Microbiol Immunol 170:45-53.
28. Bloksma, N., E. d. Heer, H. v. Dijk, and J.M. Willers. 1979. Systemic augmentation of the immune response in mice by feeding milks with *Lactobacillus casei* and *Lactobacillus acidophilus*. Immunol 37:367-375.

29. Blom, H., and C. Mortvedt. 1991. Anti-microbial substances produced by food associated micro-organisms. Biochem Soc Trans 19:694-698.
30. Blum, S., and R. Reneiro. 2000. Industrial panel statements: adhesion of selected *Lactoabcillus* strains to enterocyte-like Caco-2-cells *in vitro*: a critical evaluation of the reliability of *in vitro* adhesion assays. Proceedings of the 4th Workshop of the PROMEBO-FAIR CT 96-1028 project "Functional Foods for EU health in 2000".
31. Blum, S., and E.J. Schiffrin. 2003. Intestinal microflora and homeostasis of the mucosal immune response: implications for probiotic bacteria? Curr Issues Intest Microbiol 4:53-60.
32. Boa-Amponsem, K., N.P. O'Sullivan, W.B. Gross, E.A. Dunnington, and P.B. Siegel. 1991. Genotype, feeding regimen, and diet interactions in meat chickens. 3. General fitness. Poult Sci 70:697-701.
33. Boersma, W.J.A., M. Shaw, and E. Claassen. 2000. Probiotic bacteria as live oral vaccines. *Lactobacillus* as the versatile delivery vehicle, p. 234-270. *In* R. Fuller and G. Perdigon (eds.), Probiotics, vol. 3. Kluwer Academic Publishers, Dordrecht, the Netherlands.
34. Bolder, N.M., J.A. Wagenaar, F.F. Putirulan, K.T. Veldman, and M. Sommer. 1999. The effect of flavophospholipol (Flavomycin (R)) and salinomycin sodium (Sacox (R)) on the excretion of *Clostridium perfringens*, *Salmonella* enteritidis, and *Campylobacter jejuni* in broilers after experimental infection. Poult Sci 78:1681-1689.
35. Bovee-Oudenhoven, I.M.J. 1998. Dietary modulation of the resistance to intestinal infections. Thesis Agricultural University, Wageningen.
36. Boye, M., T.K. Jensen, K. Moller, T D. Leser, and S.E. Jorsal. 1998. Specific detection of Lawsonia intracellularis in porcine proliferative enteropathy inferred from fluorescent rRNA in situ hybridization. Vet Pathol 35:153-156.
37. Braegger, C.P. 2003. Modulation of intestinal flora with probiotics and prebiotics. Monatssch kinderheilk 151 S1:S21-S23.
38. Branca, F., and L. Rossi. 2002. The role of fermented milk in complementary feeding of young children: lessons from transition countries. Eur J Clin Nutr 56 S4:S16-20.
39. Brandtzaeg, P., I.N. Farstad, F.E. Johansen, H.C. Morton, I.N. Norderhaug, and T. Yamanaka. 1999. The B-cell system of human mucosae and exocrine glands. Immunol Rev 171:45-87.
40. Buyse, J., D.S. Adelsohn, E. Decuypere, and G.C. Scanes. 1993. Diurnal-nocturnal changes in food intake, gut storage of ingesta, food transit time and metabolism in growing broiler chickens: a model for temporal control of energy balance. Br Poult Sci 34:699-709.
41. Cebeci, A., and C. Gurakan. 2003. Properties of potential probiotic *Lactobacillus plantarum* strains. Food Microbiol 20:511-518.
42. Cebra, J.J. 1999. Influences of microbiota on intestinal immune system development. Am J Clin Nutr 69:1046S-1051S.

43. Chan, R.C., G. Reid, R.T. Irvin, A.W. Bruce, and J.W. Costerton. 1985. Competitive exclusion of uropathogens from human uroepithelial cells by Lactobacillus whole cells and cell wall fragments. Infect Immun 47:84-89.
44. Chart, H., A. Baskerville, T.J. Humphrey, and B. Rowe. 1992. Serological responses of chickens experimentally infected with *Salmonella* enteritidis PT4 by different routes. Epidemiol Infect 109:297-302.
45. Chart, H., B. Rowe, A. Baskerville, and T.J. Humphrey. 1990. Serological response of chickens to *Salmonella* enteritidis infection. Epidemiol Infect 104:63-71.
46. Cheema, M.A., M.A. Qureshi, and G.B. Havenstein. 2003. A comparison of the immune response of a 2001 commercial broiler with a 1957 randombred broiler strain when fed representative 1957 and 2001 broiler diets. Poult Sci 82:1519-1529.
47. Chiang, B.L., Y.H. Sheih, L.H. Wang, C.K. Liao, and H.S. Gill. 2000. Enhancing immunity by dietary consumption of a probiotic lactic acid bacterium (*Bifidobacterium lactis* HN019): optimization and definition of cellular immune responses. Eur J Clinical Nut 54:849-855.
48. Christensen, H.R., H. Frøkiær, and J.J. Pestka. 2002. Lactobacilli differentially modulate expression of cytokines and maturation surface markers in murine dendritic cells. J Immunol 168:171-178.
49. Claassen, E., N. Kors, and N. Van Rooijen. 1986. Influence of carriers on the development and localization of anti-trinitrophenyl antibody-forming cells in the murine spleen. Eur J Immunol 16:271-276.
50. Clancy, R. 2003. Immunobiotics and the probiotic evolution. FEMS Immunol Med Microbiol 38:9-12.
51. Coloe, P.,T. Bagust, and L. Ireland. 1984. Development of the normal gastrointestinal microflora of pathogen-specific free-chickens. J Hyg (Lond) 92:79-87.
52. Conway, P.L., S.L. Gorbach, and B.R. Goldin. 1987. Survival of lactic acid bacteria in the human stomach and adhesion to intestinal cells. J Dairy Sci 70:1-12.
53. Corthesy, B., and J.P. Kraehenbuhl. 1999. Antibody-mediated protection of mucosal surfaces. Def Muc Surfaces Pathogenesis Imm Vacc 236:93-111.
54. Cross, M.L. 2002. Microbes versus microbes: immune signals generated by probiotic lactobacilli and their role in protection against microbial pathogens. FEMS Immunol Med Microbiol 34:245-253.
55. Cunningham-Rundles, S., S. Ahrne, S. Bengmark, R. Johann-Liang, F. Marshall, L. Metakis, C. Califano, A. M. Dunn, C. Grassey, G. Hinds, and J. Cervia. 2000. Probiotics and immune response. Am J Gastroenterol 95:S22-S25.
56. D'Andrea, A., M. Aste Amezaga, N.M. Valiante, X. Ma, M. Kubin, and G. Trinchieri. 1993. IL-10 inhibits human lymphocyte interferon gamma-production by suppressing natural killer cell stimulatory factor/IL-12 synthesis in accessory cells. J Exp Med 178:1041-1048.

57. De Jong, E.C., P.L. Vieira, P. Kalinski, J.H. Schuitemaker, Y. Tanaka, E.A. Wierenga, M. Yazdanbakhsh, and M.L. Kapsenberg. 2002. Microbial compounds selectively induce Th1 cell-promoting or Th2 cell-promoting dendritic cells *in vitro* with diverse th cell-polarizing signals. J Immunol 168:1704-1709.
58. De Roos, N.M., and M.B. Katan. 2000. Effects of probiotic bacteria on diarrhea, lipid metabolism, and carcinogenesis: a review of papers published between 1988 and 1998(1). Am J Clin Nutr 71:405-411.
59. De Simone, C.,B. Bianchi Salvadori, R. Negri, M. Ferrazzi, L. Baldinelli, and R. Vesely. 1986. The adjuvant effect of yoghurt on production of gamma-interferon by ConA-stimulated human peripheral blood lymphocytes. Nut Rep Intl 33:419-433.
60. De Simone, C., S. Tzantzoglou, L. Baldinelli, S. Di Fabio, B. Bianchi-Salvadori, E. Jirillo, and R. Vesely. 1988. Enhancement of host resistance against *Salmonella typhimurium* infection by a diet supplemented with yogurt. Immunopharmacol Immunotoxicol 10:399-415.
61. De Simone, C., R. Vesely, R. Negri, B. Bianchi Salvadori, S. Zanzoglu, A. Cilli, and L. Lucci. 1987. Enhancement of immune response of murine Peyer's patches by a diet supplemented with yogurt. Immunopharmacol Immunotoxicol 9:87-100.
62. De Waard, R. 2002. Lactobacilli and host resistance.Thesis Univeristy Utrecht, Utrecht, the Netherlands.
63. De Waard, R., E. Claassen, G.C. Bokken, B. Buiting, J. Garssen, and J. G. Vos. 2003. Enhanced Immunological Memory Responses to Listeria monocytogenes in Rodents, as Measured by Delayed-Type Hypersensitivity (DTH), Adoptive Transfer of DTH, and Protective Immunity, following *Lactobacillus casei* Shirota Ingestion. Clin Diagn Lab Immunol 10:59-65.
64. De Waard, R., J. Garssen, G.C.A.M. Bokken, and J.G. Vos. 2002. Antagonistic activity of *Lactobacillus casei* strain Shirota against gastrointestinal *Listeria monocytogenes* infection in rats. Int J Food Microbiol 73:93-100.
65. De Waard, R.,J. Snel, G.C. Bokken, P.S. Tan, F. Schut, and J.H. Huis in 't Veld. 2002. Comparison of faecal *Lactobacillus* populations in experimental animals from different breeding facilities and possible consequences for probiotic studies. Lett Appl Microbiol 34:105-109.
66. Donnet-Hughes, A., F. Rochat, P. Serrant, J. M. Aeschlimann, and E.J. Schiffrin. 1999. Modulation of nonspecific mechanisms of defense by lactic acid bacteria: effective dose. J Dairy Sci 82:863-869.
67. Driehuis, F., S.J. Elferink, and S.F. Spoelstra. 1999. Anaerobic lactic acid degradation during ensilage of whole crop maize inoculated with *Lactobacillus buchneri* inhibits yeast growth and improves aerobic stability. J Appl Microbiol 87:583-594.
68. Duchet Suchaux, M., M. Mompart, B.F., B.C., L.P., and P. Pardon. 1997. Differences in frequency, level and duration of cecal carriage between four

outbred chicken lines infected orally with *Salmonella* enteritidis. Avian Dis 41:559-567.
69. Dugas, B., A. Mercenier, I. Lenoir-Wijnkoop, C. Arnaud, N. Dugas, and E. Postaire. 1999. Immunity and probiotics. Immunol Today 20:387-390.
70. Dunne, C., L. Murphy, S. Flynn, L. O'Mahony, S. O'Halloran, M. Feeney, D. Morrissey, G. Thornton, G. Fitzgerald, C. Daly, B. Kiely, E. M. Quigley, G. C. O'Sullivan, F. Shanahan, and J. K. Collins. 1999. Probiotics: from myth to reality. Demonstration of functionality in animal models of disease and in human clinical trials. Antonie Van Leeuwenhoek 76:279-292.
71. Dunne, C., L. O'Mahony, L. Murphy, G. Thornton, D. Morrissey, S. O'Halloran, M. Feeney, S. Flynn, G. Fitzgerald, C. Daly, B. Kiely, G. C. O'Sullivan, F. Shanahan, and J. K. Collins. 2001. *In vitro* selection criteria for probiotic bacteria of human origin: correlation with *in vivo* findings. Am J Clin Nutr 73:S386-392.
72. Dziarski, R. 2003. Recognition of bacterial peptidoglycan by the innate immune system. Cell Mol Life Sci 60:1793-1804.
73. Eckmann, L., and M.F. Kagnoff. 2001. Cytokines in host defense against Salmonella. Microbes Infect 3:1191-1200.
74. Edens, F.W., C.R. Parkhurst, I.A. Casas, and W.J. Dobrogosz. 1997. Principles of *ex ovo* competitive exclusion and *in ovo* administration of *Lactobacillus reuteri*. Poult Sci 76:179-196.
75. Egeler, R.M., B.E. Favara, J.D. Laman, and E. Claassen. 2000. Abundant expression of CD40 and CD40-ligand (CD154) in paediatric Langerhans cell histiocytosis lesions. Eur J Cancer 36:2105-2110.
76. Elwinger, K.,C. Schneitz, E. Berndtson, O. Fossum, B. Teglof, and B. Engstom. 1992. Factors affecting the incidence of necrotic enteritis, caecal carriage of *Clostridium perfringens* and bird performance in broiler chicks. Acta Vet Scand 33:369-378.
77. EU. 1999. EU Resolution on antibiotic resistance, p. 1-3, 1999/c 195/01.
78. Famularo, G., S. Moretti, S. Marcellini, and C. De Simone. 1997. Stimulation of immunity by probiotics, p. 133-161. *In* R. Fuller (ed.), Probiotics 2 : applications and practical aspects. London, UK.
79. Fedorka-Cray, P.J., J.S. Bailey, N.J. Stern, N.A. Cox, S.R. Ladely, and M. Musgrove. 1999. Mucosal competitive exclusion to reduce *Salmonella* in swine. J Food Prot 62:1376-1380.
80. Finlay, B.B., and S. Falkow. 1997. Common themes in microbial pathogenicity revisited. Microbiol Mol Biol Rev 61:136-169.
81. Fioramonti, J., V. Theodorou, and L. Bueno. 2003. Probiotics: what are they? What are their effects on gut physiology? Best Pract Res Clin Gastroenterol 17:711-724.
82. Freter, R., E. Stauffer, D. Cleven, L.V. Holdeman, and W.E. Moore. 1983. Continuous-flow cultures as *in vitro* models of the ecology of large intestinal flora. Infect Immun 39:666-675.

83. Fujiwara, S.,H. Hashiba, T. Hirota, and J.F. Forstner. 1997. Proteinaceous factor(s) in culture supernatant fluids of bifidobacteria which prevents the binding of enterotoxigenic *Escherichia coli* to gangliotetraosylceramide. Appl Environ Microbiol. 63:506-512.
84. Fukushima, Y., Y. Kawata, H. Hara, A. Terada, and T. Mitsuoka. 1998. Effect of a probiotic formula on intestinal immunoglobulin A production in healthy children. Int J Food Microbiol 42:39-44.
85. Fuller, R. 1973. Ecological studies on the lactobacillus flora associated with the crop epithelium of the fowl. J. Appl. Bacteriology 36:131-139.
86. Fuller, R. 1977. The importance of Lactobacilli in maintaining normal microbial balance in the crop. Br Poult Sci 18:85-94.
87. Fuller, R. 1989. Probiotics in man and animals. J Appl Bacteriol 66:365-378.
88. Fuller, R. 1992. Probiotics : the scientific basis. 398pp. London, UK.
89. Fuller, R. 1999. Probiotics for farm animals, p. 15-22. *In* G. W. Tannock (ed.), Probiotics: a critical review. Horizon Scientific Press, Wymondham, UK.
90. GenStatCommittee. 2002. GenStat for Windows, 6th ed. Lawes agricultural trust, Rothamsted Experimental Station.
91. Gill, H.S. 2003. Probiotics to enhance anti-infective defences in the gastrointestinal tract. Best Pract Res Clin Gastroenterol 17:755-773.
92. Gill, H.S., and K.J. Rutherfurd. 2001. Viability and dose-response studies on the effects of the immunoenhancing lactic acid bacterium *Lactobacillus rhamnosus* in mice. Br J Nutr 86:285-289.
93. Gill, H. S., K.J. Rutherfurd, J. Prasad, and P.K. Gopal. 2000. Enhancement of natural and acquired immunity by *Lactobacillus rhamnosus* (HN001), *Lactobacillus acidophilus* (HN017) and *Bifidobacterium lactis* (HN019). Br J Nutr 83:167-176.
94. Gill, H.S., Q. Shu, H. Lin, K. J. Rutherfurd, and M.L. Cross. 2001. Protection against translocating *Salmonella* typhimurium infection in mice by feeding the immuno-enhancing probiotic *Lactobacillus rhamnosus* strain HN001. Med Microbiol Immunol 190:97-104.
95. Gilliland, S.E. 1989. Acidophilus milk products: a review of potential benefits to consumers. J Dairy Sci 72:2483-2494.
96. Gilliland, S.E., M.L. Speck, and B.E. March. 1977. Antagonistic action of *Lactobacillus acidophilus* toward intestinal and foodborne pathogens in associative cultures.The host and its microflora: an ecological unit. J Food Prot 40:820-823.
97. Go, Y.Y., I.H. Cho, J.W. Kim, J.I. Lee, H.S. Kim, J.B. Lee, T.J. Kim, C.S. Song, and S.Y. Park. 2003. *Bifidobacterium longum* BL730 confers protective potentials against *Salmonella* infection to mice. Food Sci Biotechnol 12:625-630.
98. Gopal, P.K., J. Prasad, J. Smart, and H.S. Gill. 2001. *In vitro* adherence properties of *Lactobacillus rhamnosus* DR20 and *Bifidobacterium lactis* DR10 strains and their antagonistic activity against an enterotoxigenic *Escherichia coli*. Int J Food Microbiol 67:207-216.

99. Gorbach, S.L. 2002. Probiotics in the third millennium. Dig Liver Dis 34S2:2-7.
100. Gorbach, S.L., and B.R. Goldin. 1990. The intestinal microflora and the colon cancer connection. Rev Infect Dis 12 S2:252-261.
101. Gorden, J., and P.L.C. Small. 1993. Acid resistance in enteric bacteria. Infect Immun 61:364-367.
102. Goren, E., W.A. de Jong, P. Doornenbal, J.P. Koopman, and H.M. Kennis. 1984. Protection of chicks against salmonella infection induced by spray application of intestinal microflora in the hatchery. Vet Q. 6:73-79.
103. Greaves, D.R. 2000. Innate immunity - Antigen recognition - Web alert. Curr Op Immunol 12:9.
104. Guarner, F., and G.J. Schaafsma. 1998. Probiotics. Int J Food Microbiol 39:237-238.
105. Gusils, C., A. Perez Chaia, S. Gonzalez, and G. Oliver. 1999. Lactobacilli isolated from chicken intestines: potential use as probiotics. J Food Prot 62:252-256.
106. Haller, D., S. Blum, C. Bode, W.P. Hammes, and E.J. Schiffrin. 2000. Activation of human peripheral blood mononuclear cells by nonpathogenic bacteria *in vitro*: evidence of NK cells as primary targets. Infect Immun 68:752-759.
107. Haller, D., P. Serrant, D. Granato, E. J. Schiffrin, and S. Blum. 2002. Activation of human NK cells by staphylococci and lactobacilli requires cell contact-dependent costimulation by autologous monocytes. Clin Diagn Lab Immunol 9:649-657.
108. Halpern, G.M., K.G. Vruwink, J. vanderWater, C.L. Keen, and M.E. Gershwin. 1991. Influence of long-term yogurt consumption in young adults. Int J Immunother VII:207-210.
109. Havenstein, G.B., P.R. Ferket, and M.A. Qureshi. 2003. Carcass composition and yield of 1957 versus 2001 broilers when fed representative 1957 and 2001 broiler diets. Poult Sci 82:1509-1518.
110. Havenstein, G.B., P.R. Ferket, and M.A. Qureshi. 2003. Growth, livability, and feed conversion of 1957 versus 2001 broilers when fed representative 1957 and 2001 broiler diets. Poult Sci 82:1500-1508.
111. He, F., H. Morita, A. C. Ouwehand, M. Hosoda, M. Hiramatsu, J. Kurisaki, E. Isolauri, Y. Benno, and S. Salminen. 2002. Stimulation of the secretion of pro-inflammatory cytokines by *Bifidobacterium* strains. Microbiol Immunol 46:781-785.
112. He, F., E.M. Tuomola, H. Arvilommi, and S.J. Salminen. 2000. Modulation of humoral immune response through probiotic intake. FEMS Immunol Med Microbiol 29:47-52.
113. He, Y.W., H. Li, J. Zhang, C. L. Hsu, E. Lin, N. Zhang, J. Guo, K.A. Forbush, and M.J. Bevan. 2004. The extracellular matrix protein mindin is a pattern-recognition molecule for microbial pathogens. Nat Immunol 5:88-97.
114. Health, PDR. 2004. Yogurt. http://www.pdrhealth.com/drug_info/nmdrugprofiles/nutsupdrugs/yog_0307.shtml.

115. Hentges, D.J., W.W. Marsh, B.W. Petschow, M.E. Rahman, and S.H. Dougherty. 1995. Influence of a human milk diet on colonisation resistance mechanisms against *Salmonella typhimurium* in human faecal bacteria-associated mice. Microb Ecol Health Dis 8:139-149.
116. Heres, L. 2003. Effect of acidified feed on susceptibility of broiler chickens for Salmonella and Campylobacter, p.113-128. In: reference nr. 117.
117. Heres, L. 2003. Epidemiological aspects of Salmonella and Campylobacter control in broiler chickens: the role of nutritionally induced chages of the gut ecology. Thesis Utrecht University, Utrecht, the Netherlands.
118. Heres, L., Engel, B., Knapen, F. van, Jong, M.C.M. de, Wagenaar, J.A., Urlings, H.A.P. 2003. Fermented liquid feed reduces susceptibility of broilers for *Salmonella* enteritidis. Poult Sci 82.603-611.
119. Hessle, C., B. Andersson, and A.E. Wold. 2000. Gram-positive bacteria are potent inducers of monocytic interleukin-12 (IL-12) while gram-negative bacteria preferentially stimulate IL-10 production. Infect Immun 68:3581-3586.
120. Hessle, C., L.A. Hanson, and A.E. Wold. 1999. Lactobacilli from human gastrointestinal mucosa are strong stimulators of IL-12 production. Clin Exp Immunol 116:276-282.
121. Holt, P.G., and C.A. Jones. 2000. The development of the immune system during pregnancy and early life. Allergy 55:688-697.
122. Holzapfel, W.H., P. Haberer, J. Snel, U. Schillinger, and J.H. Huis in 't Veld. 1998. Overview of gut flora and probiotics. Int J Food Microbiol 41:85-101.
123. Hooper, L.V., H. Wong Melissa, A. Thelin, L. Hansson, G. Falk Per, and I. Gordon Jeffrey. 2001. Molecular analysis of commensal host-microbial relationships in the intestine. Science Washington DC 291:881-884.
124. Huijsdens, X.W., R.K. Linskens, M. Mak, S.G. Meuwissen, C.M. Vandenbroucke-Grauls, and P.H. Savelkoul. 2002. Quantification of bacteria adherent to gastrointestinal mucosa by real-time PCR. J Clin Microbiol 40:4423-4427.
125. Hume, M.E., L.F. Kubena, T.S. Edrington, C.J. Donskey, R.W. Moore, S.C. Ricke, and D.J. Nisbet. 2003. Poultry digestive microflora biodiversity as indicated by denaturing gradient gel electrophoresis. Poult Sci 82:1100-1107.
126. Hunt, R. 2003. Immunology www.med.sc.edu/micro.
127. Isaacson, R.E. 1998. Enteric bacterial pathogens, villus atrophy and microbial growth. Vet Q 20 S3:S68-72.
128. Isolauri, E. 2003. Probiotics for infectious diarrhoea. Gut 52:436-437.
129. Isolauri, E. 2003. Probiotics in the treatment and prevention of allergies. Monatsschrift kinderheilk 151 S1:27-30.
130. Isolauri, E., J. Joensuu, H. Suomalainen, M. Luomala, and T. Vesikari. 1995. Improved immunogenicity of oral D x RRV reassortant rotavirus vaccine by *Lactobacillus casei* GG. Vaccine 13:310-312.
131. Isolauri, E., Y. Sutas, P. Kankaanpaa, H. Arvilommi, and S. Salminen. 2001. Probiotics: effects on immunity. Am J Clin Nutr 73:444S-450S.

132. Jacobsen, C.N., V. Rosenfeldt Nielsen, A.E. Hayford, P.L. Moller, K.F. Michaelsen, A. Paerregaard, B. Sandstrom, M. Tvede, and M. Jakobsen. 1999. Screening of probiotic activities of forty-seven strains of lactobacillus spp. by *in vitro* techniques and evaluation of the colonization ability of five selected strains in humans. Appl Environ Microbiol 65:4949-4956.
133. Janeway, C.A., Jr., P. Travers, M. Walport, and M.J. Shlomchik (eds). 2001. An introduction to immunobiology and innate immunity. *In* Immunobiology: the immune system in health and disease, 5th ed. Garland Publishing, New York, USA.
134. Jett, B.D., K.L. Hatter, M.M. Huycke, and M.S. Gilmore. 1997. Simplified agar plate method for quantifying viable bacteria. Biotechn 23:648-650.
135. Jeurissen, S.H.M, E.M. Janse, S. Ekino, P. Nieuwenhuis, G. Koch, and G.F. de Boer. 1988. Monoclonal antibodies as probes for defining cellular subsets in the bone marrow, thymus, bursa of fabricius, and spleen of the chicken. Vet Immunol Immunopathol 19:225-238.
136. Jeurissen, S.H.M, E.M. Janse, N. van Rooijen, and E. Claassen. 1998. Inadequate anti-polysaccharide antibody responses in the chicken. Immunobiol 198:385-395.
137. Jeurissen, S.H.M., L. Vervelde, and E.M. Janse. 1993. Structure and function of lymphoid tissues of the chicken. Poult Sci rev. Oxford, England: Elsevier Science Ltd 5:183-207.
138. Jeurissen, S.H.M., F. Wagenaar, and E.M. Janse. 1999. Further characterization of M cells in gut-associated lymphoid tissues of the chicken. Poult Sci 78:965-972.
139. Jin, L.Z., Y.W. Ho, M.A. Ali, N. Abdullah, and S. Jalaludin. 1996. Effect of adherent *Lactobacillus* spp. on *in vitro* adherence of salmonellae to the intestinal epithelial cells of chicken. J Appl Bacteriol 81:201-206.
140. Johnson, I.T. 2001. New food components and gastrointestinal health. Proceedings of the nutrition society 60:481-488.
141. Julian, R.J. 1998. Rapid growth problems: ascites and skeletal deformities in broilers. Poult Sci 77:1773-1780.
142. Kaila, M., E. Isolauri, M. Saxelin, H. Arvilommi, and T. Vesikari. 1995. Viable versus inactivated *Lactobacillus* strain GG in acute rotavirus diarrhoea. Arch Dis Child 72:51-53.
143. Kaila, M., E. Isolauri, E. Soppi, E. Virtanen, S. Laine, and H. Arvilommi. 1992. Enhancement of the circulating antibody secreting cell response in human diarrhea by a human *Lactobacillus* strain. Pediatr Res 32:141-144.
144. Kalinski, P., C.M.U. Hilkens, E.A. Wierenga, and M.L. Kapsenberg. 1999. T-cell priming by type-1 and type-2 polarized dendritic cells: the concept of a third signal. Immunol today 20:561-567.
145. Kelsall, B., and W. Strober. 1999. Gut-associated lymphoid tissue: antigen handling and T-lymphocyte. *In* P.L. Ogra, J. Mestecky, M.E. Lamm, J.R. McGhee, J. Bienenstock, and W. Strober (eds.), Handbook of mucosal immunology, 2nd ed. Academic Press, San Diego, USA.

146. Kidd, P.M. 2001. Multiple sclerosis, an autoimmune inflammatory disease: prospects for its integrative management. Altern Med Rev 6:540-566.
147. Knarreborg, A., M.A. Simon, R.M. Engberg, B.B. Jensen, and G.W. Tannock. 2002. Effects of dietary fat source and subtherapeutic levels of antibiotic on the bacterial community in the ileum of broiler chickens at various ages. Appl Environ Microbiol 68:5918-5924.
148. Koenen, M.E., A.G. Boonstra-Blom, and S.H.M. Jeurissen. 2002. Immunological differences between layer- and broiler-type chickens. Vet Immunol Immunopathol 89:47-56.
149. Koenen, M.E., Heres, L., Claassen E. and Boersma, W.J.A. 2002. Lactobacilli as probiotics in chicken feeds. Biosci Microflora 21:209-216.
150. Koenen, M.E., R. v.d. Hulst, M. Leering, S.H.M. Jeurissen, and W.J.A. Boersma. 2004. Development and validation of a new *in vitro* assay for selection of probiotic bacteria that express immune-stimulating properties in chickens *in vitro*. FEMS Immunol Med Microbiol 40:119-127.
151. Koenen, M.E., S.H.M. Jeurissen, and W.J.A. Boersma. 2002. Modulation of the immune response by probiotics in chicken. Br J Nutr S1:S120 - 121.
152. Koenen, M.E., J. Kramer, R. v.d. Hulst, L. Heres, S.H.M. Jeurissen, and W.J.A. Boersma. 2004. Immunomodulation by probiotic lactobacilli in layer- and meat type chickens. Br Poult Sci, accepted.
153. Kos, B., J. Suskovic, S. Vukovic, M. Simpraga, J. Frece, and S. Matosic. 2003. Adhesion and aggregation ability of probiotic strain *Lactobacillus acidophilus* M92. J Appl Microbiol 94:981-987.
154. Kraehenbuhl, J.P., and M.R. Neutra. 2000. Epithelial M cells: differentiation and function. Annu Rev Cell Dev Biol 16:301-332.
155. Kramer, J., A.H. Visscher, J.A. Wagenaar, A.G. Boonstra-Blom, and S.H.M. Jeurissen. 2001. Characterization of the innate and adaptive immunity to Salmonella enteritidis PT1 infection in four broiler lines. Vet Immunol Immunopathol 79:219-233.
156. Kramer, J., A.H. Visscher, J.A. Wagenaar, J.B.J.W. Cornelissen, and S.H.M. Jeurissen. 2003. Comparison of natural resistance in seven genetic groups of meat-type chicken. Br Poult Sci 44:577-585.
157. Kramer, J., A.H. Visscher, J.A. Wagenaar, and S.H.M. Jeurissen. 2003. Entry and survival of *Salmonella enterica* serotype Enteritidis PT4 in chicken macrophage and lymphocyte cell lines. Vet Microbiol 91:147-155.
158. Kreukniet, M.B., N. Gianotten, M.B. Nieuwland, and H.K. Parmentier. 1994. *In vitro* T cell activity in two chicken lines divergently selected for antibody response to sheep erythrocytes. Poult Sci 73:336-340.
159. Kreukniet, M.B., S.H.M. Jeurissen, M.G. Nieuwland, N. Gianotten, P. Joling, and H.K. Parmentier. 1996. The B cell compartment of two chicken lines divergently selected for antibody production: differences in structure and function. Vet Immunol Immunopathol 51:157-171.

160. Lanning, D., P. Sethupathi, K.J. Rhee, S.K. Zhai, and K.L. Knight. 2000. Intestinal microflora and diversification of the rabbit antibody repertoire. J Immunol 165:2012-2019.
161. Lee, A. 1985. Neglected niches; the microbial ecology of the gastrointestinal tract. Adv. Microb. Ecol. 8:115-162.
162. Lenoir-Wijnkoop, I. 2003. Host physiology. Factors affecting colonisation. *In* I. Lenoir-Wijnkoop (ed.), The intestinal microflora. Understanding the symbiosis. John Libbey & Company ltd, London, UK.
163. Li, Z., K.E. Nestor, Y.M. Saif, and J.W. Anderson. 2000. Antibody responses to sheep red blood cell and Brucella abortus antigens in a turkey line selected for increased body weight and its randombred control. Poult Sci 79:804-809.
164. Li, Z., K.E. Nestor, Y.M. Saif, W.L. Bacon, and J.W. Anderson. 1999. Effect of selection for increased body weight on mitogenic responses in turkeys. Poult Sci 78:1532-1535.
165. Link-Amster, H., F. Rochat, K.Y. Saudan, O. Mignot, and J.M. Aeschlimann. 1994. Modulation of a specific humoral immune response and changes in intestinal flora mediated through fermented milk intake. FEMS Immunol Med Microbiol 10:55-63.
166. Maassen, C.B., W.J.A. Boersma, J.C.P.A. van Holten, E. Claassen, and J.D. Laman. 1999. Growth phase of orally administered *Lactobacillus* strains differentially affects T helper-cell pathways for soluble antigens: Implications for vaccine development. In:Lactobacilli as antigen delivery system for mucosal tolerance induction in autoimmune disease. Thesis Erasmus University Rotterdam, Rotterdam, the Netherlands.
167. Maassen, C.B.M., W.J.A. Boersma, C. van Holten Neelen, E. Claassen, and J.D. Laman. 2003. Growth phase of orally administered *Lactobacillus* strains differentially affects IgG1/IgG2a ratio for soluble antigens: implications for vaccine development. Vaccine 21:2751-2757.
168. Maassen, C.B.M., J.D. Laman, W.J.A. Boersma, and E. Claassen. 2000. Modulation of cytokine expression by lactobacilli and its possible therapeutic use, p. 176-193. *In* G. Perdigon and R. Fuller (eds.), Immunomodulation by the gut microflora and probiotics, vol. 3. Kluwer Academic.
169. Maassen, C.B.M., C. van Holten-Neelen, F. Balk, J.H. den Bak Glashouwer, R.J. Leer, J.D. Laman, W.J.A. Boersma, and E. Claassen. 2000. Strain-dependent induction of cytokine profiles in the gut by orally administered *Lactobacillus* strains. Vaccine 18:2613-2623.
170. Macfarlane, G.T., and J.H. Cummings. 2002. Probiotics, infection and immunity. Curr Opin Infect Dis 15:501-506.
171. Macfarlane, G.T., S. Macfarlane, and G.R. Gibson. 1998. Validation of a three-stage compound continuous culture system for investigating the effect of retention time on the ecology and metabolism of bacteria in the human colon. Microb Ecol 35:180-187.
172. Male, D., and I. Roitt. 2001. Introduction to the immune system, p. 1-13. *In* I. Roitt, J. Brostoff, and D. Male (eds.), Immunology, 6 ed. Mosby, London, UK

173. Marteau, P., M. Minekus, R. Havenaar, and J.H. Huis in't Veld. 1997. Survival of lactic acid bacteria in a dynamic model of the stomach and small intestine: validation and the effects of bile. J Dairy Sci 80:1031-1037.
174. Marteau, P., and J.C. Rambaud. 1993. Potential of using lactic acid bacteria for therapy and immunomodulation in man. FEMS Microbiol Rev 12:207-220.
175. Marteau, P., J.P. Vaerman, J.P. Dehennin, S. Bord, D. Brassart, P. Pochart, J.F. Desjeux, and J.C. Rambaud. 1997. Effects of intrajejunal perfusion and chronic ingestion of *Lactobacillus johnsonii* strain La1 on serum concentrations and jejunal secretions of immunoglobulins and serum proteins in healthy humans. Gastroenterol Clin Biol 21:293-298.
176. Marteau, P.R., M. de Vrese, C.J. Cellier, and J. Schrezenmeir. 2001. Protection from gastrointestinal diseases with the use of probiotics. Am J Clin Nutr 73:430S-436S.
177. Matsuzaki, T. 1998. Immunomodulation by treatment with *Lactobacillus casei* strain Shirota. Int J Food Microbiol 41:133-140.
178. Matsuzaki, T. 1999. Effects of parenteral adminstration of *L. casei* strain Shirota. Antitumor and immunopotentiating effects, p. 208-239. *In* T. Yokokura (ed.), *Lactobacillus casei* strain Shirota - Intestinal flora and human health. Yakult Honsha Co., Ltd, Tokyo, Japan.
179. Matsuzaki, T., and J. Chin. 2000. Modulating immune responses with probiotic bacteria. Immunol Cell Biol 78:67-73.
180. Matsuzaki, T., Y. Shimizu, and T. Yokokura. 1990. Augmentation of antimetastatic effect on Lewis lung carcinoma (3LL) in C57BL/6 mice by priming with *Lactobacillus casei*. Med Microbiol Immunol 179:161-168.
181. McAuliffe, O., M.P. Ryan, R.P. Ross, C. Hill, P. Breeuwer, and T. Abee. 1998. Lacticin 3147, a broad-spectrum bacteriocin which selectively dissipates the membrane potential. Appl Environ Microbiol 64:439-445.
182. McCracken, V.J., and H.R. Gaskins. 1999. Probiotics and the immune system, p. 85-111. *In* G.W. Tannock (ed.), Probiotics: a critical review. Horizon Scientific Press, Wymondham, UK.
183. Mead, G., and C.S. Impey. 1987. The present status of the Nurmi Concept for reducing carriage of food-poisoning salmonellae and other pathogens in live poultry, p. 57-77, Elimination of pathogenic organisms from meat and poultry. Elsevier Science Publisher B.V.
184. Medzhitov, R., and C. Janeway, Jr. 2000. Innate immunity. N Engl J Med 343:338-344.
185. Mercenier, A. 1999. Lactic acid bacteria as live vaccines, p. 113-128. *In* G. W. Tannock (ed.), Probiotics, A Critical Review. Horizon Scientific Press, Wymondham, UK.
186. Metchnikoff, E. 1907. The prolongation of life. Heinemann, G.P., London.
187. Methner, U., P.A. Barrow, A. Berndt, and G. Steinbach. 1999. Combination of vaccination and competitive exclusion to prevent *Salmonella* colonization in chickens: experimental studies. Int J Food Microbiol 49:35-42.

188. Micallef, M.J., T. Tanimoto, K. Kohno, H. Ikegami, and M. Kurimoto. 2000. Interleukin 18 Induces a Synergistic Enhancement of Interferon Gamma Production in Mixed Murine Spleen Cell-Tumor Cell Cultures: Role of Endogenous Interleukin 12. Cancer Det Prev 24:234-243.
189. Miettinen, M., J. Vuopio-Varkila, and K. Varkila. 1996. Production of human tumor necrosis factor alpha, interleukin-6, and interleukin-10 is induced by lactic acid bacteria. Infect Immun 64:5403-5405.
190. Miles, R.D., A.S. Arafa, R.H. Harms, C.W. Carlson, B.L. Reid, and J.S. Crawford. 1981. Effects of a living nonfreeze-dried *Lactobacillus acidophilus* culture on performance, egg quality, and gut microflora in commercial layers. Poult Sci 60:993-1004.
191. Miller, L L., P B. Siegel, and E A. Dunnington. 1992. Inheritance of antibody response to sheep erythrocytes in lines of chickens divergently selected for fifty-six-day body weight and their crosses. Poult Sci 71:47-52.
192. Molin, G. 2001. Probiotics in foods not containing milk or milk constituents, with special reference to *Lactobacillus plantarum* 299v. Am J Clin Nutr 73:380S-385S.
193. Morelli, L. 2000. *In vitro* selection of probiotic lactobacilli: a critical appraisal. Curr Issues Intest Microbiol 1:59-67.
194. Morelli, L. 2002. Probiotics: clinics and/or nutrition. Dig Liver Dis 34 Suppl 2:S8-11.
195. Morishita, T.Y., P.P. Aye, B.S. Harr, C.W. Cobb, and J.R. Clifford. 1997. Evaluation of an avian-specific probiotic to reduce the colonization and shedding of *Campylobacter jejuni* in broilers. Avian Dis. 41:850-855.
196. Mowat, A.M., and J.L. Viney. 1997. The anatomical basis of intestinal immunity. Immunol Rev 156:145-166.
197. Mukai, T., T. Asasaka, E. Sato, K. Mori, M. Matsumoto, and H. Ohori. 2002. Inhibition of binding of *Helicobacter pylori* to the glycolipid receptors by probiotic *Lactobacillus reuteri*. FEMS Immunol Med Microbiol 32:105-110.
198. Mygind, T., S. Birkelund, E. Falk, and G. Christiansen. 2001. Evaluation of real-time quantitative PCR for identification and quantification of *Chlamydia pneumoniae* by comparison with immunohistochemistry. J Microbiol Methods 46:241-251.
199. Naidu, A.S., W. R. Bidlack, and R.A. Clemens. 1999. Probiotic spectra of lactic acid bacteria (LAB). Crit Rev Food Sci Nutr 39:13-126.
200. Nakamura, T., S. Hoshi, Y. Nagasawa, and S. Ueda. 1995. The effect of route of inoculation on protection by killed vaccines in chickens. Avian Dis 39:507-513.
201. Nisbet, D. 2002. Defined competitive exclusion cultures in the prevention of enteropathogen colonisation in poultry and swine. Antonie van Leeuwenhoek 81:481-486.
202. Nisbet, D.J., D.E. Corrier, C.M. Scanlan, A.G. Hollister, R.C. Beier, and J.R. DeLoach. 1993. Effect of a defined continuous-flow derived bacterial culture

and dietary lactose on Salmonella typhimurium colonization in broiler chickens. Avian Dis. 37:1017-1025.
203. Nuotio, L., C. Schneitz, U. Halonen, and E. Nurmi. 1992. Use of competitive exclusion to protect newly-hatched chicks against intestinal colonisation and invasion by *Salmonella* enteritidis PT4. Br Poult Sci. 33:775-779.
204. Nurmi, E., and M. Rantala. 1973. New aspects of *Salmonella* infection in broiler production. Nature 241:210-211.
205. Opitz, H.M., M. El-begearimi, P. Flegg, and D. Beane. 1993. Effectiveness of five feed additives in chicks infected with *Salmonella* enteritidis phage type 13a. J Appl Poult Res 2:147-153.
206. O'Sullivan, N.P., E.A. Dunnington, E.J. Smith, W.B. Gross, and P.B. Siegel. 1991. Performance of early and late feathering broiler breeder females with different feeding regimens. Br Poult Sci 32:981-995.
207. Ouwehand, A.C., P.V. Kirjavainen, M.M. Gronlund, E. Isolauri, and S.J. Salminen. 1999. Adhesion of probiotic micro-organisms to intestinal mucus. Int Dairy J 9:623-630.
208. Ouwehand, A.C., P.V. Kirjavainen, C. Shortt, and S. Salminen. 1999. Probiotics: mechanisms and established effects. Int Dairy J 9:43-52.
209. Ouwehand, A.C., S. Salminen, and E. Isolauri. 2002. Probiotics: an overview of beneficial effects. Antonie van Leeuwenhoek 82:279-289.
210. Pashine, A., B. John, S. Rath, A. George, and V. Bal. 1999. Th1 dominance in the immune response to live *Salmonella typhimurium* requires bacterial invasiveness but not persistence. Int Immunol. 11:481-489.
211. Pelto, L., E. Isolauri, E.M. Lilius, J. Nuutila, and S. Salminen. 1998. Probiotic bacteria down-regulate the milk-induced inflammatory response in milk-hypersensitive subjects but have an immunostimulatory effect in healthy subjects. Clin Exp Allergy 28:1474-1479.
212. Perdigon, G., S. Alvarez, M.E.N. De Macias, M.E. Roux, and A. Pesce de Ruiz Holgado. 1990. The oral administration of lactic acid bacteria increase the mucosal intestinal immunity in response to enteropathogens. J Food Prot 53:404-410.
213. Perdigon, G., S. Alvarez, and A. Pesce de Ruiz Holgado. 1991. Immunoadjuvant activity of oral *Lactobacillus casei*: influence of dose on the secretory immune response and protective capacity in intestinal infections. J Dairy Res 58:485-496.
214. Perdigon, G., S. Alvarez, M. Rachid, G. Aguero, and N. Gobbato. 1995. Immune system stimulation by probiotics. J Dairy Sci 78:1597-1606.
215. Perdigon, G., M.E. de Macias, S. Alvarez, G. Oliver, and A.A. de Ruiz Holgado. 1986. Effect of perorally administered lactobacilli on macrophage activation in mice. Infect Immun 53:404-410.
216. Perdigon, G., M.E. de Macias, S. Alvarez, G. Oliver, and A.P. de Ruiz Holgado. 1988. Systemic augmentation of the immune response in mice by feeding fermented milks with *Lactobacillus casei* and *Lactobacillus acidophilus*. Immunol 63:17-23.

217. Perdigon, G., R. Fuller, and R. Raya. 2001. Lactic acid bacteria and their effect on the immune system. Curr Issues Intest Microbiol 2:27-42.
218. Perdigon, G., C.M. Galdeano, J.C. Valdez, and M. Medici. 2002. Interaction of lactic acid bacteria with the gut immune system. Eur J Clin Nut 56 S4:S21-26.
219. Perdigon, G., M. Locascio, M. Medici, A.P.D. Holgado, and G. Oliver. 2003. Interaction of bifidobacteria with the gut and their influence in the immune function. Biocell 27:1-9.
220. Perdigon, G., M.E. Nader de Macias, S. Alvarez, G. Oliver, and A.A. Pesce de Ruiz Holgado. 1990. Prevention of gastrointestinal infection using immunobiological methods with milk fermented with *Lactobacillus casei* and *Lactobacillus acidophilus*. J Dairy Res 57:255-264.
221. Perdigon, G., E. Vintini, S. Alvarez, M. Medina, and M. Medici. 1999. Study of the possible mechanisms involved in the mucosal immune system activation by lactic acid bacteria. J Dairy Sci 82:1108-1114.
222. Perdigon, P., and S. Alvarez. 1992. Probiotics and the immune state. *In* R. Fuller (ed.), Probiotics: the scientific basis. p.146-180. Chapman & Hall, London, UK.
223. Pereyra, B.S., and D. Lemonnier. 1993. Induction of human cytokines by bacteria used in dairy foods. Nut Res 113:1127-1140.
224. Pessi, T., Y. Sutas, M. Hurme, and E. Isolauri. 2000. Interleukin-10 generation in atopic children following oral *Lactobacillus rhamnosus* GG. Clin Exp Allergy 30:1804-1808.
225. Pessi, T., Y. Sutas, M. Saxelin, H. Kallioinen, and E. Isolauri. 1999. Antiproliferative effects of homogenates derived from five strains of candidate probiotic bacteria. Appl Environ Microbiol 65:4725-4728.
226. Praharaj, N.K., E.A. Dunnington, and P.B. Siegel. 1995. Growth, immunoresponsiveness, and disease resistance of diverse stocks of chickens reared under two nutritional regimens. Poult Sci 74:1721-1729.
227. Promsopone, B., T.Y. Morishita, P.P. Aye, C.W. Cobb, A. Veldkamp, and J.R. Clifford. 1998. Evaluation of an avian-specific probiotic and *Salmonella typhimurium*- specific antibodies on the colonization of *Salmonella typhimurium* in broilers. J Food Prot 61:176-180.
228. Qiao, H.P., L.C. Duffy, E. Griffiths, D. Dryja, A. Leavens, J. Rossman, G. Rich, M. Riepenhoff Talty, and M. Locniskar. 2002. Immune responses in rhesus rotavirus-challenged balb/c mice treated with bifidobacteria and prebiotic supplements. Ped Res 51:750-755.
229. Quan, C.P., A. Berneman, R. Pires, S. Avrameas, and J.P. Bouvet. 1997. Natural polyreactive secretory immunoglobulin A autoantibodies as a possible barrier to infection in humans. Infect Immun 65:3997-4004.
230. Qureshi, M.A., and G.B. Havenstein. 1994. A comparison of the immune performance of a 1991 commercial broiler with a 1957 randombred strain when fed "typical" 1957 and 1991 broiler diets. Poult Sci 73:1805-1812.

231. Rada, V., and M. Marounek. 1997. Effect of maduramicin and monensin on survival of lactobacillus salivarius 51R administered in hte crop and caeca of young chickens. Arch. Anim. Nutr. 50:25-29.
232. Rada, V., M. Marounek, I. Rychly, D. Santruckova, and K. Vorisek. 1995. Effect of Lactobacillus salivarius administration on microflora in the crop and caeca of broiler chickens. J An Feed Sci 4:161-170.
233. Rafter, J. 2003. Probiotics and colon cancer. Best Pract Res Clin Gastroenterol 17:849-859.
234. Rantala, M., and E. Nurmi. 1973. Prevention of the growth of *Salmonella infantis* in chicks by the flora of the alimentary tract of chickens. Br Poult Sci 14:627-630.
235. Reid, G., A.W. Bruce, J.A. McGroarty, K.J. Cheng, and J.W. Costerton. 1990. Is there a role for lactobacilli in prevention of urogenital and intestinal infections? Clin Microbiol Rev. 3:335-344.
236. Reid, G., and J. Burton. 2002. Use of *Lactobacillus* to prevent infection by pathogenic bacteria. Microb Inf 4:319-324.
237. Rescigno, M., M. Urbano, B. Valzasina, M. Francolini, G. Rotta, R. Bonaiso, F. Granucci, J. P. Kraehenbuhl, and P. Ricciardi-Castagnoli. 2001. Dendritic cells express tight junction proteins and penetrate gut epithelial monolayers to sample bacteria. Nature Immunol 2:361-367.
238. Rhee, K.-J., P. Sethupathi, A. Driks, D.K. Lanning, and K.L. Knight. 2004. Role of commensal bacteria in development of gut-associated lymphoid tissues and preimmune antibody repertoire. J. Immunol. 172:1118-1124.
239. RIVM 2003, 1996-2003 IGZ 4-wekenoverzicht, http://www.infectieziektenbulletin.nl.
240. Rook, G.A., and J.L. Stanford. 1998. Give us this day our daily germs. Immunol Today 19:113-116.
241. Rosenfeldt, V., E. Benfeldt, S.D. Nielsen, K.F. Michaelsen, D.L. Jeppesen, N.H. Valerius, and A. Paerregaard. 2003. Effect of probiotic *Lactobacillus* strains in children with atopic dermatitis. J Allergy Clin Immunol 111:389-395.
242. Salminen, S., C. Bouley, M.C. Boutron-Ruault, J.H. Cummings, A. Franck, G.R. Gibson, E. Isolauri, M.C. Moreau, M. Roberfroid, and I. Rowland. 1998. Functional food science and gastrointestinal physiology and function. Br J Nutr 80 S1:S147-S171.
243. Salminen, S., E. Isolauri, and E. Salminen. 1996. Clinical uses of probiotics for stabilizing the gut mucosal barrier: successful strains and future challenges. Antonie Van Leeuwenhoek 70:347-358.
244. Salminen, S., A. von Wright, L. Morelli, P. Marteau, D. Brassart, W.M. de Vos, R. Fonden, M. Saxelin, K. Collins, G. Mogensen, S.E. Birkeland, and T. Mattila-Sandholm. 1998. Demonstration of safety of probiotics - a review. Int J Food Microbiol 44:93-106.
245. Salminen, S., and A. v. Wright. 1998. Lactic acid bacteria: microbiology and functional aspects. 2nd ed. 617pp. Marcel Dekker (ed.), New York, USA.

246. Salzman, N.H., H.de Jong, Y. Paterson, H.J. Harmsen, G.W. Welling, and N.A. Bos. 2002. Analysis of 16S libraries of mouse gastrointestinal microflora reveals a large new group of mouse intestinal bacteria. Microbiol 148:3651-3660.
247. Sanders, M.E., and T.R. Klaenhammer. 2001. Invited review: the scientific basis of *Lactobacillus acidophilus* NCFM functionality as a probiotic. J Dairy Sci 84:319-331.
248. Savage, D.C. 1981. The microbial flora in the gastrointestinal tract. Prog Clin Biol Res 77:893-908.
249. Savage, D.C. 1987. Factors influencing biocontrol of bacterial pathogens in the intestine. Food Technol. Chicago, Ill.: Institute of Food Technologists:82-87.
250. Schiemann, D.A., and S.A. Olson. 1984. Antagonism by gram-negative bacteria to growth of *Yersinia enterocolitica* in mixed cultures. Appl Environ Microbiol 48:539-544.
251. Schiffrin, E.J., F. Rochat, H. Link-Amster, J.M. Aeschlimann, and A. Donnet-Hughes. 1995. Immunomodulation of human blood cells following the ingestion of lactic acid bacteria. J Dairy Sci 78:491-497.
252. Schneitz, C., T. Kiiskinen, V. Toivonen, and M. Nasi. 1998. Effect of BROILACT on the physicochemical conditions and nutrient digestibility in the gastrointestinal tract of broilers. Poult Sci 77:426-432.
253. Sheih, Y.H., B.L. Chiang, L.H. Wang, C.K. Liao, and H.S. Gill. 2001. Systemic immunity-enhancing effects in healthy subjects following dietary consumption of the lactic acid bacterium *Lactobacillus rhamnosus* HN001. J Am Coll Nutr 20:149-156.
254. Shires, A., J.R. Thompson, B.V. Turner, P.M. Kennedy, and Y.K. Goh. 1987. Rate of passage of corn-canola meal and corn-soybean meal diets through the gastrointestinal tract of broiler and White Leghorn chickens. Poult Sci 66:289-298.
255. Shortt, C. 1999. The probiotic century: historical and current perspectives. Trends Food Sci Technol 10:411-417.
256. Shu, Q., and H.S. Gill. 2002. Immune protection mediated by the probiotic *Lactobacillus rhamnosus* HN001 (DR20) against *Escherichia coli* O157:H7 infection in mice. FEMS Immunol Med Microbiol 34:59-64.
257. Shu, Q., H. Lin, J. Rutherfurd Kay, G. Fenwick Stan, J. Prasad, K. Gopal Pramod, and S. Gill Harsharnjit. 2000. Dietary *Bifidobacterium lactis* (HN019) enhances resistance to oral *Salmonella typhimurium* infection in mice. Microbiol Immunol 44:213-222.
258. Siegel, P.B., W.B. Gross, and J.A. Cherry. 1982. Correlated responses of chickens to selection for production of antibodies to sheep erythrocytes. Anim Blood Groups Biochem Genet 13:291-297.
259. Siegel, P.B., and W.G. Gross. 1980. Production and persistence of antibodies in chickens to sheep erythrocytes. 1. Directional selection. Poult Sci 59:1-5.

260. Snel, J., H.J.M. Harmsen, P.W.J.J. van der Wielen, and B A. Williams. 2002. Dietary strategies to influence the gastro-intestinal microflora of young animal, and its potential to improve intestinal health, p. 37-70. *In* M. C. Blok, H. A. Vahl, I. de Lange, A. van de Braak, G. Hemke, and M. Hessing (eds.), Nutrition and health of the gastro intestinal tract. Wageningen Academic Publishers, Wageningen, the Netherlands.
261. Snel, J., P P. Heinen, H.J. Blok, R.J. Carman, A.J. Duncan, P.C. Allen, and M. D. Collins. 1995. Comparison of 16S rRNA sequences of segmented filamentous bacteria isolated from mice, rats, and chickens and proposal of "Candidatus Arthromitus". Int J Syst Bacteriol. 45:780-782.
262. Soerjadi, A.S., R. Rufner, G.H. Snoeyenbos, and O.M. Weinack. 1982. Adherence of salmonellae and native gut microflora to the gastrointestinal mucosa of chicks. Avian Dis 26:576-584.
263. Soerjadi, A.S., S.M. Stehman, G.H. Snoeyenbos, O.M. Weinack, and C F. Smyser. 1981. The influence of lactobacilli on the competitive exclusion of paratyphoid salmonellae in chickens. Avian Dis. 25:1027-1033.
264. Soerjadi, A.S., S.M. Stehman, G.H. Snoeyenbos, O.M. Weinack, and C.F. Smyser. 1981. Some measure of protection against parathypoid *Salmonella* and *Escherichia coli* by competitive exclusion in chickens. Avian Dis 25:706-712.
265. Soerjadi Liem, A.S.,G.H. Snoeyenbos, and O.M. Weinack. 1984. Comparative studies on competitive exclusion of three isolates of *Campylobacter fetus* subsp. *jejuni* in chickens by native gut microflora. Avian Dis 28:139-146.
266. Spanhaak, S., R. Havenaar, and G. Schaafsma. 1998. The effect of consumption of milk fermented by *Lactobacillus casei* strain Shirota on the intestinal microflora and immune parameters in humans. Eur J Clin Nutr 52:899-907.
267. Speck, M. L. 1972. Control of food-borne pathogens by starter cultures. J Dairy Sci 55:1019-1022.
268. Spencer, R.J., and A. Chesson. 1994. The effect of *Lactobacillus* spp. on the attachment of enterotoxigenic *Escherichia coli* to isolated porcine enterocytes. J Appl Bacteriol 77:215-220.
269. Spriggs, D.R. 1986. Bacteriocins and antagonism: the killing fields. J Infect Dis 153:809-810.
270. Staeheli, P., F. Puehler, K. Schneider, W. Goebel Thomas, and B. Kaspers. 2001. Cytokines of birds: Conserved functions: A largely different look. J Interferon Cytokine Res 21:993-1010.
271. Stappenbeck, T.S., V. Hooper Lora, and I. Gordon Jeffrey. 2002. Developmental regulation of intestinal angiogenesis by indigenous microbes via Paneth cells. Proc Ntl Acad Sci USA 99:15451-15455.
272. Stavric, S. 1992. Defined cultures and prospects. Int J Food Microbiol 15:245-263.
273. Steidler, L. 2001. Microbiological and immunological strategies for treatment of inflammatory bowel disease. Microbes Infect 3:1157-1166.

274. Stepankova, R., F. Kovaru, and J. Kruml. 1980. Lymphatic tissue of the intestinal tract of germfree and conventional rabbits. Folia Microbiol (Praha) 25:491-495.
275. Stephen, J., T. S. Wallis, W. G. Starkey, D. C. Candy, M. P. Osborne, and S. Haddon. 1985. Salmonellosis: in retrospect and prospect. Ciba Found Symp 112:175-192.
276. Sudo, N., S. Sawamura, K. Tanaka, Y. Aiba, C. Kubo, and Y. Koga. 1997. The requirement of intestinal bacterial flora for the development of an IgE production system fully susceptible to oral tolerance induction. J Immunol 159:1739-1745.
277. Talham, G.L., H.Q. Jiang, N.A. Bos, and J.J. Cebra. 1999. Segmented filamentous bacteria are potent stimuli of a physiologically normal state of the murine gut mucosal immune system. Infect Immun 67:1992-2000.
278. Tamboli, C.P., C. Caucheteux, A. Cortot, J. F. Colombel, and P. Desreumaux. 2003. Probiotics in inflammatory bowel disease: a critical review. Best Pract Res Clin Gastroenterol 17:805-820.
279. Tannock, G.W. 1990. The microecology of lactobacilli inhabiting the gastrointestinal tract, p. 147-171. *In* K. Marshall (ed.), Advances in microbial ecology, vol. 2. Plenum Press, New York, USA.
280. Tannock, G.W. 1997. Probiotic properties of lactic-acid bacteria: plenty of scope for fundamental R & D. Trends Biotechnol 15:270-274.
281. Tannock, G.W. 2002. Analysis of the intestinal microflora using molecular methods. Eur J Clin Nutr 56:S44-S49.
282. Taranto, M.P., M. Medici, G. Perdigon, A.P.R. Holgado, and G.F. Valdez. 2000. Effect of *Lactobacillus reuteri* on the prevention of hypercholesterolemia in mice. J Dairy Sci 83:401-403.
283. Tejada-Simon, M.V., Z. Ustunol, and J.J. Pestka. 1999. Effects of lactic acid bacteria ingestion of basal cytokine mRNA and immunoglobulin levels in the mouse. J Food Prot 62:287-291.
284. Thatte, J., S. Rath, and V. Bal. 1995. Analysis of immunization route-related variation in the immune response to heat-killed *Salmonella typhimurium* in mice. Infect Immun 63:99-103.
285. Tortuero, F. 1973. Influence of the implantation of *Lactobacillus acidophilus* in chicks on the growth, feed conversion, malabsorption of fats syndrome and intestinal flora. Poult Sci 52:197-203.
286. Trinchieri, G. 1994. Interleukin-12: a cytokine produced by antigen-presenting cells with immunoregulatory functions in the generation of T-helper cells type 1 and cytotoxic lymphocytes. Blood 84:4008-4027.
287. Tuomola, E., R. Crittenden, M. Playne, E. Isolauri, and S. Salminen. 2001. Quality assurance criteria for probiotic bacteria. Am J Clin Nutr 73:393S-398.
288. Tuomola, E.M., A.C. Ouwehand, and S.J. Salminen. 1999. The effect of probiotic bacteria on the adhesion of pathogens to human intestinal mucus. FEMS Immunol Med Microbiol 26:137-142.

289. Urlings, H.A., P.G. Bijker, and J.G. van Logtestijn. 1993. Fermentation of raw poultry byproducts for animal nutrition. J Anim Sci 71:2420-2426.
290. Van der Klis, J.D. and A.J.M. Jansman. 2002. Optimising nutrient digestion, absorption and gut barrier function in monogastrics: reality or illusion? p.1-36. *In* M. C. Blok, H. A. Vahl, I. de Lange , A. van de Braak, G. Hemke, and M. Hessing (eds.), Nutrition and health of the gastro-intestinal tract. Wageningen academic publishers, Wageningen, the Netherlands.
291. Van der Werf, M.J. and K. Venema. 2001. Bifidobacteria: genetic modification and the study of their role in the colon. J Agric Food Chem 49:378-383.
292. Van der Wielen, P.W., S. Biesterveld, S. Notermans, H. Hofstra, B.A. Urlings, and F. Van Knapen. 2000. Role of volatile fatty acids in development of the cecal microflora in broiler chickens during growth. Appl Environ Microbiol 66:2536-2540.
293. Van der Zijpp, A.J. 1983. The effect of genetic origin, source of antigen, and dose of antigen on the immune response of cockerels. Poult Sci 62:205-211.
294. Van der Zijpp, A J., T.R. Scott, and B. Glick. 1986. The effect of different routes of antigen administration on the humoral immune response of the chick. Poult Sci 65:809-811.
295. Van Winsen, R.L., B.A. Urlings, L. J. Lipman, J. M. Snijders, D. Keuzenkamp, J.H. Verheijden, and F. Van Knapen. 2001. Effect of fermented feed on the microbial population of the gastrointestinal tracts of pigs. Appl Environ Microbiol 67:3071-3076.
296. Van Zijderveld, F.G., A.M. van Zijderveld-van Bemmel, and J. Anakotta. 1992. Comparison of four different enzyme-linked immunosorbent assays for serological diagnosis of *Salmonella* enteritidis infections in experimentally infected chickens. J Clin Microbiol 30:2560-2566.
297. Vanbelle, M., E. Teller, and M. Focant. 1990. Probiotics in animal nutrition: a review. Arch Tierernahr. 40:543-567.
298. VanCott, J.L., S.N. Chatfield, M. Roberts, D.M. Hone, E.L. Hohmann, D.W. Pascual, M. Yamamoto, H. Kiyono, and J.R. McGhee. 1998. Regulation of host immune responses by modification of *Salmonella* virulence genes. Nat Med. 4:1247-1252.
299. Vaughan, E.E., and B. Mollet. 1999. Probiotics in the new millennium. Nahr 43:148-153.
300. Vaughan, E.E., B. Mollet, and W.M. deVos. 1999. Functionality of probiotics and intestinal lactobacilli: light in the intestinal tract tunnel. Curr Opin Biotechnol 10:505-510.
301. Vieira, P.L., E.C. de Jong, E.A. Wierenga, M.L. Kapsenberg, and P. Kalinski. 2000. Development of Th1-inducing capacity in myeloid dendritic cells requires environmental instruction. J Immunol 164:4507-4512.
302. Watkins, B.A., and B.F. Miller. 1983. Competitive gut exclusion of avian pathogens by *Lactobacillus acidophilus* in gnotobiotic chicks. Poult Sci 62:1772-1779.

303. Watkins, B.A., B.F. Miller, and D.H. Neil. 1982. *In vivo* inhibitory effects of *Lactobacillus acidophilus* against pathogenic *Escherichia coli* in gnotobiotic chicks. Poult Sci 61:1298-1308.
304. Weinack, O.M., G.H. Snoeyenbos, C.F. Smyser, and A.S. Soerjadi. 1981. Competitive exclusion of intestinal colonization of *Escherichia coli* in chicks. Avian Dis. 25:696-705.
305. Weinack, O.M., G.H. Snoeyenbos, and A.S. Soerjadi Liem. 1985. Further studies on competitive exclusion of *Salmonella typhimurium* by lactobacilli in chickens. Avian Dis 29:1273-1276.
306. Wheeler, J.G., M.L. Bogle, S.J. Shema, M.A. Shirrell, K.C. Stine, A.J. Pittler, A.W. Burks, and R.M. Helm. 1997. Impact of dietary yogurt on immune function. Am J Med Sci 313:120-123.
307. Wheeler, J.G., S.J. Shema, M.L. Bogle, M.A. Shirell, A.W. Burks, A.J. Pittler, and R.M. Helm. 1997. Immune and clinical impact of *Lactobacillus acidophilus* on asthma. Ann All Asthma Immunol 79:229-233.
308. Wold, A.E. 2001. Immune effects of probiotics. Scand J Nutr 45:76-85.
309. Wostmann, B.S., C. Larkin, A. Moriarty, and E. Bruckner Kardoss. 1983. Dietary intake, energy metabolism, and excretory losses of adult male germfree Wistar rats. Lab Anim Sci 33:46-50.
310. Yasui, H., K. Shida, T. Matsuzaki, and T. Yokokura. 1999. Immunomodulatory function of lactic acid bacteria. Antonie van Leeuwenhoek 76:383-389.
311. Yunis, R., A. Ben-David, E.D. Heller, and A. Cahaner. 2000. Immunocompetence and viability under commercial conditions of broiler groups differing in growth rate and in antibody response to *Escherichia coli* vaccine. Poult Sci 79:810-816.
312. Zekarias, B., A.H.M. ter Huurne Agnes, J.M. Landman Wil, J.M.J. Rebel, J.M. A. Pol, and E. Gruys. 2002. Immunological basis of differences in disease resistance in the chicken. Vet Res Paris 33:109-125.
313. Zhou, J.S., P.K. Gopal, and H.S. Gill. 2001. Potential probiotic lactic acid bacteria *Lactobacillus rhamnosus* (HN001), *Lactobacillus acidophilus* (HN017) and *Bifidobacterium lactis* (HN019) do not degrade gastric mucin *in vitro*. Int J Food Microbiol 63:81-90.

Samenvatting

Samenvatting

De darm heeft tegenstrijdige functies. Voedingsstoffen en water moeten de darmwand kunnen passeren, terwijl ziekteverwekkers als bacteriën, virussen, schimmels en parasieten dat juist niet zouden moeten kunnen. Om deze ziekteverwekkers buiten het lichaam te houden wordt de darm beschermd door een slijmlaag en het immuunsysteem. De bacteriën in de darm vormen met elkaar de darmflora. Deze darmflora bestaat uit honderden soorten bacteriën, in totaal vele miljarden bacteriën (geschat wordt 10^{14} in zoogdieren en kippen). Deze bacteriën hebben intensief contact met elkaar, met de gastheer en met het immuunsysteem rond de darm. De bacteriën leven in symbiose met elkaar en beïnvloeden elkaar door synergie, antagonisme en competitie. Daardoor is de darmflora een complex geheel waar veel verschillende soorten bacteriën in een delicate balans met elkaar en met de gastheer leven. Zij dragen bij aan de bescherming van de gastheer tegen ziekteverwekkers doordat de hechte gemeenschap van bacteriën die de flora vormen, indringers niet gemakkelijk toestaat. Maar deze delicate balans kan worden verstoord door drastische veranderingen in voeding, door virulente ziekteverwekkers, antibiotica en stress.

Kippen komen steriel uit het ei; er zijn nog geen bacteriën in hun darm aanwezig. De kuikens krijgen hun darmflora door het contact met de ouderdieren (mest) en van de omgeving door het pikken op de grond. Echter, in de moderne kippenhouderij zijn de hygiënische maatregelen zeer streng en worden de verschillende generaties kippen strikt gescheiden gehouden. Hierdoor krijgen de jonge kuikens nauwelijks de kans om een evenwichtige, gezonde darmflora op te bouwen, terwijl in jonge dieren ook het immuunsysteem nog niet goed ontwikkeld is. De combinatie van de slecht ontwikkelde darmflora, het in ontwikkeling zijnde immuunsysteem, de stressvolle leefomstandigheden heeft tot gevolg dat kippen minder weerstand tegen ziektes hebben.

De huidige kippen zijn in het verleden gefokt op ofwel snelle groei met zo min mogelijk voer (vleesproductie) ofwel de productie van eieren. Door zo te fokken zijn nu twee verschillende soorten kippen ontstaan, de vleeskippen en legkippen. Ze verschillen niet alleen in lichaamsgewicht en eiproductie, maar ook in leeftijdsverwachting en immuunsysteem.
De meeste kippen worden gehouden in industrieachtige boerderijen onder extreem hygiënische omstandigheden, om de infectiedruk te verlagen. Onder minder hygiënische omstandigheden, zoals in biologische dierhouderijen waar de dieren ook naar buiten kunnen lopen, zijn relatief robuustere kippen nodig. Binnenkort wordt het verboden om antibiotica aan het voer toe te voegen. Deze antibiotica hebben een antimicrobieel en groeibevorderend effect. Wanneer geen antibiotica aan het voer worden toegevoegd en steeds meer kippen biologisch gehouden gaan worden, wordt een verbeterde weerstand tegen ziekten en een goed ontwikkeld immuunsysteem nog belangrijker.

De vraagstelling voor dit onderzoek was of immuunstimulerende probiotica als vervanging voor de antibiotica konden worden gebruikt. Probiotica worden meestal omschreven als ‘oraal toegediende levende micro-organismen die een gezondheidsbevorderend effect op de gastheer hebben’. Een aspect van antibiotica wat door probiotica niet kan worden vervangen is het groeibevorderende effect. Wij onderzochten of probiotica het antimicrobiële effect van antibiotica zou kunnen vervangen door stimulatie van het immuunsysteem, om zo de weerstand tegen ziekten te verbeteren.

Melkzuurbacteriën, zoals lactobacillen en bifidobacteriën, zijn vaak geschikte kandidaten voor probiotica. Verschillende melkzuurbacteriestammen kunnen op allerlei manieren de gezondheid bevorderen. Bijvoorbeeld door het moduleren en stimuleren van het immuunsysteem en het versterken van de slijmvliesoppervlakten, zoals de darmwand, waar ziekteverwekkers het lichaam binnen kunnen treden. Een kort overzicht van probiotische effecten is beschreven in hoofdstuk 2 en 3.

Veel onderzoek naar probiotica is gebaseerd op *in vitro* experimenten (niet in de levende gastheer, maar in de reageerbuis). Het werk beschreven in dit proefschrift richt zich op verschillende facetten van probiotica *in vitro* en *in vivo* (in de levende gastheer). De aanpak van het onderzoek beschreven in dit proefschrift was als volgt:

- vergelijken van het immuunsysteem van leg- en vleeskippen
- *in vitro* selectie van immuunmodulerende melkzuurbacteriën; hiertoe werd eerst een model opgezet
- het immuunmodulerende vermogen van oraal toegediende lactobacillen, die *in vitro* geselecteerd waren, in een *in vivo* immunisatie met een model-antigeen onderzoeken (valideren van gemaakte keuzes)
- het immuunmodulerende effect van een stam (*L. paracasei)* in leg- en vleeskippen vergelijken. Omdat deze kippen een verschillend werkend immuunsysteem hebben zou dat invloed kunnen hebben op de werking van probiotica. Daarnaast kunnen ook de dosis van de probiotica, het toedieningsschema van de probiotica en de leeftijd van de gastheer een rol spelen in de immuunmodulatie
- het effect van immuunmodulerende lactobacillus stammen op een veelvoorkomende Salmonella infectie in legkippen onderzoeken in plaats van het immunisatiemodel met een niet ziekteverwekkend antigeen dat in de andere proeven werd gebruikt.

In hoofdstuk 4 wordt het vergelijken van de leg- en vleeskippen beschreven. Het resultaat was dat de vleeskippen een goede korte termijn immuunrespons met antistoffen (humorale respons, IgM) hebben, maar een verlaagde lange termijn humorale respons (IgG) en een verlaagde cellulaire immuunrespons. Legkippen hebben een hogere cellulaire immuunrespons en een hogere IgG respons dan

vleeskippen terwijl de IgM respons wat lager is dan die in de vleeskippen. Deze verschillen in immuunrespons zijn waarschijnlijk door het fokken van de twee verschillende soorten kippen meegeselecteerd en lijken in overeenstemming met de levensverwachting van de kippensoorten: de vleeskippen leven maximaal 6 weken en de legkippen leven enkele jaren.

In hoofdstuk 5 wordt het opzetten van een *in vitro* model voor het selecteren van immuunmodulerende melkzuurbacteriën beschreven. Dit model wordt uitgevoerd op cellen van de milt van de kip. Wanneer deze cellen samen met een stimulerende stof (Concanavaline A) en een immuunmodulerende bacteriestam worden geïncubeerd, gaat een deel van de cellen (de T-cellen) zich vermenigvuldigen. De groei van de cellen is een maat voor de immuunstimulatie door de bacteriestam. De bacteriestam stimuleert deze T-cellen tot vermenigvuldiging doordat er een interactie plaats vindt tussen macrofagen ('grote eters', cellen die ziekteverwekkers opnemen (opeten) en in stukjes aan het immuunsysteem presenteren), de lactobacillus en de T-cel. In de *in vitro* test werden de lactobacillus stam, de T-cellen en de antigeen presenterende cellen samengevoegd. Wanneer een bacteriestam niet immuunstimulerend is, gaan de cellen zich niet vermenigvuldigen. Van één stam, *L. paracasei* werd in de eerste experimenten duidelijk dat deze significante immuunmodulerende capaciteiten heeft *in vivo* (verhoging specifieke IgM en IgG repons tegen TNP-KLH). De vermenigvuldiging van de cellen wordt steeds vergeleken met de vermenigvuldiging die door deze stam, die als referentie-stam werd meegetest in ieder *in vitro* experiment. Het *in vitro* model werd gecontroleerd met twee *in vivo* experimenten en bleek inderdaad een voorspellende waarde te hebben voor de immuunstimulerende eigenschappen van melkzuurbacteriën. Van de meer dan honderd stammen die werden getest bleken slechts vijf een immuunstimulerend effect te hebben dat even groot of iets minder groot was dan dat van de referentie-stam. Op grond van deze experimenten blijkt dat selectie van stammen ook efficiënt *in* vitro plaats kan vinden en door het gebruik van dit model zijn minder proefdieren nodig voor de selectie van immuunstimulerende melkzuurbacteriën.

In hoofdstuk 6 wordt beschreven hoe twee Lactobacillus stammen die *in vitro* geselecteerd waren op grond van immuunstimulerende eigenschappen *in vivo* worden geëvalueerd in leg- en vleeskippen. Omdat in hoofdstuk 4 bleek dat deze dieren immunologisch van elkaar verschillen, zou de werking van immuunstimulerende melkzuurbacteriën kunnen verschillen tussen deze soorten. Het immuunmodulerende effect op de antistofrespons (humorale immuunrespons) en de cellulaire immuunrespons werden bestudeerd in leg- en vleeskippen. In deze experimenten werd duidelijk dat Lactobacillus stammen verschillende effecten op het maagdarmkanaal en immuunsysteem hebben, afhankelijk van het soort kip, zijn genetische achtergrond en de leeftijd van de kip. Hoewel de effecten per dier niet spectaculair zijn, kan niet specifieke stimulatie van het immuunsysteem de gezondheid van een groep dieren op een goedkope manier ondersteunen. Zowel

de humorale als de cellulaire immuunrespons werden versterkt. Ook de invloed van de hoeveelheid lactobacillen per dag en de duur van de toediening van de lactobacillen werden bestudeerd en deze variabelen bleken de immuunstimulatie door de probiotica te beïnvloeden. Om het gewenste effect te verkrijgen moeten al deze variabelen verder onderzocht worden, geoptimaliseerd en gebalanceerd worden.

In hoofdstuk 7 wordt beschreven hoe het effect van twee Lactobacillus stammen, die zowel *in vitro* als *in vivo* een sterk immuunstimulerend effect hebben, in een Salmonella infectie model in legkippen werd bestudeerd. De Salmonella infectie werd sneller uit de lever en de milt verwijderd in de groepen die de Lactobacillus stammen kregen toegediend dan in de groep zonder, maar bleef aanwezig in de darm alsof het een onderdeel van de natuurlijk aanwezige darmflora was (als commensaal). De immuunrespons tegen Salmonella zowel humoraal als cellulair) was verlaagd in de groepen met de probiotische stammen, waarschijnlijk door de kortere aanwezigheid van de Salmonella infectie in het lichaam (milt en lever). De probiotische bacteriën versnelden mogelijk de infectie, waardoor het immuunsysteem minder tijd had om een goede respons op te bouwen. De lage infectiedosis en de infectieroute hebben waarschijnlijk ook bijgedragen aan de lage immuunrespons. Dit betekent niet dat een combinatie van probiotische stammen bij een infectie, die niet uitmondt in een gastheer-commensaal relatie, geen positief effect kunnen hebben op verschillende aspecten van de weerstand, immuunresponsen en fysiologische schade in de darm.

Probiotische lactobacillen kunnen, wanneer ze nauwkeurig geselecteerd worden, de immuunrespons in kippen versterken. Daardoor kunnen probiotica gebruikt worden als voederadditief in kippenvoer om ziekteresistentie te verhogen. Naast het immuunmodulerende effect, kunnen lactobacillen ook helpen bij het opbouwen en stabiliseren van de darmflora in jonge kippen. Vooral in jonge kippen zou deze combinatie een positief effect kunnen hebben.

Curriculum vitae

Op 30 april 1971 werd ik, Marjorie Koenen, geboren in Amsterdam. In 1989 slaagde ik voor mijn gymnasium diploma aan het Coornhert Gymnasium in Gouda. In datzelfde jaar begon ik aan de studie Levensmiddelentechnologie aan de Landbouwuniversiteit Wageningen. Na het behalen van de propedeuse vervolgde ik mijn studie met de nieuwe richting biorocestechnologie. Hiervoor deed ik een afstudeervak moleculaire virologie aan de vakgroep virologie van de universiteit (prof. Just M. Vlak) en een afstudeervak celbiologie en immunologie bij IntroGene, nu Crucell (dr. Ronald Vogels, prof. Dinko Valerio en prof. Willem B. van Muiswinkel). Mijn stage celbiologie en immunologie heb ik in Amerika aan de University of California Davis uitgevoerd (prof. Ronald P. Hedrick, dr. Mark M. Adkison, prof. Willem B. van Muiswinkel). In 1996 studeerde ik af als bioprocestechnoloog met de moleculair-cellulaire specialisatie.
In 1997 trad ik in dienst van het ID-DLO op de afdeling virologie als assistent-onderzoeker bij de varkenspest diagnostiek, waar een screeningslab moest worden opgezet en ik regelmatig de dagelijkse leiding op me nam. Vervolgens heb ik daar gewerkt aan de ontwikkeling van een antigeen-capture ELISA voor de detectie van het varkenspest virus. In 1998 begon ik als aio aan de Erasmus Universiteit te Rotterdam, faculteit der geneeskunde en Gezondheidswetenschappen en was gedetacheerd bij het ID-DLO te Lelystad, afdeling immunologie (nu Animal Sciences Group van Wageningen Universiteit en Researchcentrum, afdeling dier en omgeving, dr. Suzan Jeurissen en dr. Wim Boersma). Hier heb ik gewerkt aan het onderzoek dat is beschreven in dit proefschrift.

Publications

Marjorie E. Koenen, Lourens Heres, Eric Claassen and Wim J.A. Boersma (2002). Lactobacilli as Probiotics in Chicken Feeds, Bioscience Microflora 21(4), 209-216.

Goddeeris B.M, Boersma W.J.A., Cox E., Van der Stede Y., Koenen M.E., Vancaeneghem S., Mast J., and Van den Broek W. (2002).The porcine and avian intestinal immune system and its nutritional modulation. In: Nutrition and health of the gastrointestinal tract, Blok M.C., Vahl H.A., De Lange L., Van de Braak A.E., Hemke G., Hessing M. (eds.), Wageningen Academic Publishers, Wageningen, The Netherlands.

Marjorie E. Koenen, Suzan H.M. Jeurissen and Wim J.A. Boersma (2002). Modulation of the immune response by probiotics in chicken, British Journal of Nutrition 88 Supplement 1, 120-121.

Marjorie E. Koenen, Anneke G. Boonstra-Blom and Suzan H.M. Jeurissen (2002). Immunological differences between layer and broiler type chickens, Veterinary Immunology & Immunopathology 89(1-2), 47-56.

Marjorie E. Koenen, Remy van der Hulst, Mariska Leering, Suzan H.M. Jeurissen and Wim J.A. Boersma (2004). Development and validation of a new *in vitro* assay for selection of probiotic bacteria that express immune stimulating properties in chickens *in vivo*, FEMS Immunology and Medical Microbiology 40(2), 119-127.

Marjorie E. Koenen, Judith Kramer, Remy van der Hulst, Lourens Heres, Suzan H.M. Jeurissen and Wim J.A. Boersma (2004). Immunomodulation by probiotic lactobacilli in layer- and meat type chickens, British Poultry Science, accepted.

Marjorie E. Koenen, Arjan J. Hoekman, Fráncis R.M. Balk and Wim J.A. Boersma. Effects of probiotics on immunological aspects of *Salmonella* Enteritidis infection in chicken, submitted for publication.

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Appendix

Analysis of the Lactobacillus strains frequently used in the research described in this thesis as performed by BCCM/LMS, Ghent, Belgium:

Tests performed:
Recovery and purity check
Cultures were recovered and checked for purity on MRS agar (Oxoid CM361). After anaerobic incubation for 24-48h at 30°C or 37°C as indicated by the client, all cultures showed a homogeneous growth.

Basic microbiological tests
Following microbiological tests were performed: cell morphology, gram stain, oxidase and catalase reaction.

Fatty acid analysis
Cells were grown for 24 h at 28°C on MRS agar (Oxoid CM361); cells of *L. casei* LW 120 were grown for 48 h at 28°C. The cellular fatty acid composition was determined gaschromatographically. The extraction and the analysis were performed conform to the recommendations of the commercial identification system MIDI (Microbial Identification System Inc., Delaware USA). Profiles were compared with the MIDI identification database TSBA40 V.4.10.

1D-protein gelelectrophoresis (SDS-PAGE) and cluster analysis
Cultures were grown in anaerobic conditions on MRS agar (Oxoid CM 361) for 24h at 30°C or at 37°C as depending on the optimal growth temperature. The preparation of the cell extracts and the 1D-protein gelelectrophoresis (SDS-PAGE) were carried out conform with the protocol established by the Research Group of the Laboratory for Microbiology, University Ghent (Pot, B., P. Vandamme and K. Kerstens (1994). Analysis of electrophoretic whole-organism protein fingerprints. Chemical Methods in Prokaryotic Systematics, M. Goodfellow and A.G. O'Donnell (eds.), J. Wiley and sons, Chichester).The normalized and digitized protein patterns were numerically analyzed and clustered with the reference profiles (culture collection strains and recent industrial isolates) in the LAB database as currently available (GelCompar^{TM} 4.2 software, Applied Maths, Belgium).

16S rDNA sequence analysis and phylogenetic study (*L. brevis* LW 5; *L. reuteri* LW 81; *L. paracasei* LW 122)
Genomic DNA of the strains was prepared according to the protocol of Niemann *et al.* (Niemann S., A. Puehler, H.-V. Tichy, R. Simon and W. Selbitschka (1997). Evaluation of the resolving power of three different DNA fingerprinting methods to discriminate among isolates of a natural Rhizobium *meliloti* population, J Appl Microbiol 82: 477-484) or Pitcher *et al.* (Pitcher, D.G., N.A. Saunders and R.J. Owen (1989). Rapid extraction of bacterial genomic DNA with guanidinium thiocyanate, Lett Appl Microbiol 8: 151-156).

16S rRNA genes were amplified by PCR and purified using the QIAquick PCR Purification Kit (Qiagen GmbH), Hilden, Germany).
Complete sequencing was performed using Applied Biosystems, Inc 377 DNA Sequencer and the protocols of the manufacturer (Perkin-Elmer Applied Biosystems Div., Foster City, CA, USA) using the 'ABI PRISM TM BigDye TM Terminator Cycle Sequencing Ready Reaction Kit (with AmpliTaq® DNA Polymerase, Fs)'.The forward and reverse primers resulted in partial overlap of sequences, ensuring highly reliable assembled data.
Sequence assembly was performed by using the program AutoAssembler^{TM} (Perkin-Elmer Applied Biosystems Div., Foster City, CA, USA).
Phylogenetic analysis was performed using the software package BioNumerics (Applied Maths, Belgium) after including the consensus sequence in alignment of small ribosomal subunit sequences collected from the international nucleotide sequence library EMBL. This alignment was pairwise calculated using an open gap penalty of 100% and a unit gap penalty of 0%. A similarity matrix was created by homology calculation with a gap penalty of 0% and after discarding unknown bases. A resulting tree was constructed using the neighbor-joining method.

Results
All strains were positive in the gram staining and negative in the oxidase and catalase reactions.

Strain	Fatty acid analysis	SDS-PAGE	16S rDNA seq. analysis
L. brevis LW 5	Lactobacillus sp.	*L. coryniformis*	>97% sim. with *L. brevis*
L. brevis LW 7	Lactobacillus sp.	*L. brevis*	
L. brevis LW 40	Lactobacillus sp.	*L. brevis*	
L. reuteri LW 46	Lactobacillus sp., possibly *L. reuteri*	*L. reuteri*	
L. buchneri LW 50	Lactobacillus sp., possibly *L. kefiri*	*L. buchneri*	
L. reuteri LW 81	Lactobacillus sp.	*L. reuteri*	>97% sim. with *L. reuteri*
L. buchneri LW 83	Lactobacillus sp., possibly *L. parabuchneri*	*L. buchneri*	
L. paracasei LW 120	Lactobacillus sp., possibly *L. paracasei*	*L. paracasei*	
L. murinus-animalis LW 121	Lactobacillus sp.	*L. murinus-animalis*	
L. paracasei LW 122	Lactobacillus sp., possibly *L. paracasei*	*L. paracasei*	> 97% sim. with *L. paracasei*
L. plantarum LW 143	Lactobacillus sp., possibly *L. parabuchneri*	*L. plantarum*-group	
L. brevis LW 167	Lactobacillus sp., possibly *L. parabuchneri*	*L. plantarum*-group	