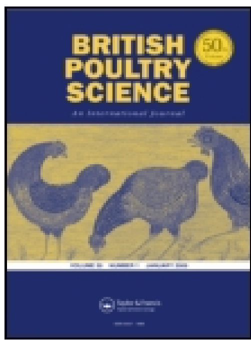

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Immunomodulation by probiotic lactobacilli in layer- and meat-type chickens

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Abstract 1. The aim of the experiments was to evaluate whether selected probiotic lactobacillus strains have different immunomodulating effects in layer- and meat-type strain chickens.
2. Humoral and cellular specific and non-specific immune responses were studied by experiments on cellular proliferation, entry and survival of *Salmonella* bacteria in gut and spleen leukocytes, immunoglobulin isotypes and specific immunoglobulin titres.
3. The effects of two different feeding regimes (short and continuous feeding) and doses for administration of lactobacilli were studied.
4. The lactobacillus strains that were evaluated showed modulating effects on the immune system of layer- and meat-type chickens.
5. In meat-type strain 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.
6. Immunoprobiotic lactobacilli can have a positive effect on humoral and cellular immune responses in layer- and meat-type strain chickens, but the lactobacillus strain to be used, the age of the animals and effective dose of lactobacilli to be administered need to be optimised.

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 (Salminen *et al.*, 1998), gut mucosal barrier (Salminen *et al.*, 1996) and mucosal immune response (Perdigon *et al.*, 1995). Some probiotics have a competitive exclusion effect for specific pathogens (Fedorka-Cray *et al.*, 1999) and help reduce duration of diarrhoea (Marteau *et al.*, 2001). In addition, beneficial effects have been observed on lactose intolerance (Gilliland, 1989), blood cholesterol concentrations (De Roos and Katan, 2000) and cancer (Matsuzaki, 1998).

Probiotics are used to develop and maintain a healthy intestinal microflora in young animals, like newly hatched chicks (Nuotio *et al.*, 1992). In regular poultry housing chickens are raised under hygienic conditions in the absence of the mother hen. As a consequence, the young chicks do not acquire a maternal microflora matched to the natural antibodies transfused with the yolk, thereby leaving them vulnerable to colonisation by pathogenic bacteria.

Chickens have been selected genetically 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: meat- and layer-type strains which differ in body weight gain, maximum lifespan and in a negative relationship between performance traits of chickens and immunological responses (Boa-Amponsem *et al.*, 1991; O'Sullivan *et al.*, 1991; Kreukniet *et al.*, 1994; Praharaj *et al.*, 1995; Yunis *et al.*, 2000). Layer- and meat-type strains 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) (Kreukniet *et al.*, 1994; Koenen *et al.*, 2002*a*). In turkeys a direct relationship between a lower mitogenic leukocyte response to Concanavalin A and higher antibody response to SRBCs was

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observed in turkeys selected for high body weight (Li *et al.*, 1999, 2000). These results indicate that selection for high body weight (as in meat-type strains) can affect immunological variables for disease resistance like antibody production or mitogenic responses.

Various effects of probiotic lactic acid bacteria have been described in animals and man, which depend on strain, host, dose, timing and viability of the strain (Perdigon and Alvarez, 1992; Perdigon *et al.*, 1999; Maassen *et al.*, 2000; Isolauri *et al.*, 2001; De Waard *et al.*, 2003). Some probiotic strains have been selected in rodents or pigs for application in humans (Perdigon *et al.*, 1991; Dunne *et al.*, 2001; Kos *et al.*, 2003). This indicates that it is possible for probiotics to act over several species. Although meat- and layer-type strains belong to the same species there are considerable phenotypic differences between them. As well as the enormous difference in growth and life expectancy, the development of the immune system may also be quite different. Because antibody responses (IgG) and non-specific proliferative responses in meat-type strains are reduced compared to layer-type strains under the same conditions (Koenen *et al.*, 2002*a*), meat-type strains in particular might need stimulation to develop immune responses comparable to layer-type strains. We hypothesised 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 strains. Recently we selected immunoprobiotics in LSL chickens (Koenen *et al.*, 2002*b*, 2003). *L. paracasei* LW 122 had a positive effect on specific antibody titre and non-specific cell proliferation. *L. plantarum* LW 143 had no effect on antibody titre, but contributed to resistance to *Salmonella enteritidis* (Heres *et al.*, 2003). Layer-type strains were used because their lifespan was more appropriate for the duration of the experiments necessary to evaluate the immunoprobiotic effects modulating responses to priming and booster immunisation.

We investigated whether the immunoprobiotic properties of lactobacilli that were observed in layer-type strains expressed similar effects in meat-type strains. The immunoprobiotic effect may depend on the amount of lactobacilli administered (Perdigon *et al.*, 1991; Balevi *et al.*, 2001; Gill and Rutherfurd, 2001), therefore we studied two different lactobacilli feeding regimes. High doses were administered for a long period with fermented liquid feed (Heres, 2003). In other experiments the chickens received lactobacilli for only 5 d before immunological challenge. As exemplar for layer-type strains we have chosen LSL White Leghorn and as a meat-type strain Ross 208.

MATERIALS AND METHODS

Chickens

Eggs of LSL White Leghorn (Charles River) were obtained and hatched under routine specific pathogen free (SPF) conditions (no preventive vaccinations, no antibiotics and no coccidiostatics). One-day-old Ross 208 meat-type chicks were obtained from a parent flock with a *Salmonella*-free history. Fluff and paper pads from the hatching cabin were examined for 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 into groups on the basis of 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 enclosed with plates at the sides and between two pens a space as big as one pen was kept empty to avoid undesired spreading of the bacteria. The pens were placed on a concrete floor with about 5 cm of sawdust bedding. Housing conditions were as follows: temperature 33°C gradually declining to 18°C while humidity was 55% throughout. In experiment I the light:dark periods were 23 h:1 h and in experiment II 16 h:8 h.

Experimental design

All animal experiments complied with Dutch regulations on animal experimentation.

Experiment I: continuous feeding of L. paracasei LW 122 in fermented liquid feed: the effects of immunoprobiotics selected in layer-type strains on meat-type strains

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 d 1. On d 5 and 33 the animals were immunised with TNP-KLH. On d 6 and 7 after second immunisations, chickens were killed for lymphocyte proliferation assay (LPA) on spleen cells. At d 7, 10 and 14 after both immunisations, serum samples were taken from all chickens for total and specific IgM and IgG anti-TNP-titre analysis in ELISA.

Experiment II: temporary oral administration of L. paracasei LW 122: comparison of response to immunoprobiotics in layer- and meat-type strains

The 2 × 15 LSL chickens (groups 1 and 2) and 2 × 15 Ross chickens (groups 3 and 4) aged 3 weeks were given either *L. paracasei* (groups 1 and 3) or buffer (groups 2 and 4). The chickens received lactobacilli on 5 consecutive days one week before immunisation (d 8 to 12). On d 15, 17 and 19, 5 animals of each group were immunised with TNP-KLH. Seven days after immunisation animals were killed 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 h) in MRS broth (de Man, Rogosa and Sharpe, Oxoid b.v., Haarlem, the Netherlands) 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 for 2 d at the same temperature as the growing conditions of the culture (30 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 (2003): 200 g dry feed (pelleted feed, sterilised 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* LW 143 (Urlings *et al.*, 1993) or 1 ml *L. plantarum* + 1 ml *L. paracasei* LW 122 (Maassen *et al.*, 2000; Koenen *et al.*, 2003). The mixes were incubated for 2 d at 30°C. After fermentation the pH was about 4 (4·0 when fermented with *L. plantarum* only and 4·2 with both strains). The fermented liquid feed contained 10^9 to 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. 2 d). Seeds (maize, 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 d 5 and 6 the seeds were added to comprise 10% (w/w) and after d 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 administered orally using a syringe mounted with a gavage. This allowed the lactobacilli to be administered in the upper part of the throat. For 5 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 d 3, 7, 14 and 35, 5 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 for 2 min and serially 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 were counted after 20 to 24 h incubation at 37°C (KAA and VRBG) and after 3 d at 30°C (Rogosa). The pH was measured immediately after gathering of the contents by inserting an electrode (Ingold electrode, Mettler Toledo, Tiel, the Netherlands) 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, MO, USA) as described (Claassen *et al.*, 1986). In animal experiment I chickens were intravenously immunised 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 (Koenen *et al.*, 2002*a*) that in layer-type chickens a lower concentration of TNP-KLH induced a suboptimal specific IgM response as compared to meat-type chickens. In experiment II, to avoid two different antigen concentrations in a direct comparison, we selected a TNP-KLH concentration that induced a response in both types of chickens.

Elisa

Total antibody concentrations of IgM and IgG were measured in serum using a double antibody

sandwich ELISA as described by Kramer *et al.* (2001). 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 titre 1000 = 5·6 mg/ml and IgM titre 1000 = 0·1 mg/ml).

Anti-TNP antibody titres were determined by means of a direct ELISA as described (Koenen *et al.*, 2002*a*). TNP-bovine serum albumin (BSA, Sigma) was coated and sera were serially diluted twofold. Responses were determined using mouse monoclonal antibodies CVI-ChIgM-59·7 and CVI-ChIgG-47·3, specific for chicken IgM and IgG (Jeurissen *et al.*, 1988; Bianchi *et al.*, 1990).

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 titres were calculated as the dilution of the sample giving an extinction value of 1 above the background. Geometric mean titres of individual 2-log titres, SEM and antilog ($2^{\text{Geometric mean titre}}$) values were calculated.

Antigen specific restimulation assay *in vitro*

Cellular responses were determined by LPA as described (Koenen *et al.*, 2002*a*). 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). A total of 10^6 viable cells per well were incubated in 0·2 ml RPMI 1640 Dutch modification (developed by Moore *et al.* at Roswell Park Memorial Institute) containing 1% normal chicken serum, glutamin, *β*-mecaptoethanol and antibiotics for 68 h 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 h 0·4 μCi per well ^{3}H-thymidine (Amersham Pharmacia Biotech, Buckinghamshire, UK) was added and 4 h later the plates were harvested on to fibreglass 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 the naladixic acid resistant strain of *Salmonella enterica* serotype *enteritidis* PT4 (*S. enteritidis*, Van Zijderveld *et al.*, 1992) in leukocytes was used to measure the probiotic effect on the leukocytes and was slightly modified from the method described by Kramer *et al.* (2001, 2003*b*). The intestine (ileum and caecum) was isolated, opened longitudinally, rinsed thoroughly with PBS and cut into 0·5 to 1 cm pieces. All tissue pieces were incubated for 10 to 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 to 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 min 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 min 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 on to 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 (20 μg/ml). These cultures were incubated for another 14 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 time point were analysed with the *t*-test (two-tailed and two sample with unequal variance).

RESULTS

Two lactobacillus strains have been tested in layer- and meat-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 gastrointestinal (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 than was observed in the control group ($P < 0{\cdot}05$, Table 1). This difference was probably attributable to the fermentation products like lactic and acetic acid. Unexpectedly, feeding fermented liquid feed led to a slight, 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 in the chickens in the fermented liquid feed groups, especially in the first week of feeding ($P = 0{\cdot}001$, Table 1). The control group showed larger day-to-day variation in frequency of lactobacilli in crop, caecum and jejunum. The frequency of lactobacilli in the control group was about one-tenth of that in the fermented liquid feed groups ($P = 0{\cdot}05$) 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 analysed 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 the caeca of the fermented liquid feed fed groups consisted of *L. plantarum* or *L. plantarum* + *L. paracasei* from the starter cultures.

Effect of feeding putative immunoprobiotic bacteria on the humoral immune response

Non-specific immunoglobulins (experiment I)

After primary immunisation the chickens that received fermented liquid feed fermented with *L. paracasei* + *L. plantarum* had higher total IgM titres than the control group ($P = 0{\cdot}02$). The group fed with *L. plantarum* fermented liquid feed showed a similar trend after priming. After secondary immunisation the group that received *L. paracasei* + *L. plantarum* had a higher total IgG titre in serum than the control group ($P = 0{\cdot}05$) and the group that received fermented liquid feed fermented with *L. plantarum* only ($P = 0{\cdot}009$). This *L. plantarum* group also had a higher total IgM titre than the control group ($P = 0{\cdot}005$, Table 2).

TNP-specific immunoglobulins (experiments I and 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 titre (both $P = 0{\cdot}07$) in the meat-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 strains was slightly lower than was observed in meat-type strains. In contrast, the IgG response of meat-type strains was lower than was observed for layers (not significant). In experiment II temporary administration of *L. paracasei* for 5 d prior to immunisation with TNP-KLH enhanced the serum IgM and IgG titres in layer-type strains after priming, but not in meat-type strains under the same conditions (Table 4). However, stimulated IgM titres in layer-type strains reached similar levels as observed in meat-type strain controls. For IgG titres in layer-type strains surpassed those in meat-type strains.

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 strains than in spleen cells of meat-type strains, but temporary feeding *L. paracasei* had no 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 meat-type strains that received *L. paracasei* orally as compared to the control groups receiving no lactobacilli. However, the immune-enhancing effect was more pronounced in spleen cells of meat-type strains ($P = 0{\cdot}023$) than was observed in spleen cells of layer-type strains (not significant).

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 min) and survival/replication (measured

Table 1. *Effects of continuous feeding of probiotics in fermented liquid feed on GI tract pH and microflora*

Day of feeding	Group	Crop				Jejunum				Caecum			
		3	7	14	35	3	7	14	35	3	7	14	35
pH	Control	5·4 (0·7)[a]	4·8 (0·3)[a]	4·8 (0·3)[a]	5·0 (0·5)[a]	6·9 (0·6)[a]	6·7 (0·5)	6·7 (0·6)	6·4 (0·5)	6·4 (0·5)	6·1 (0·4)[a]	6·1 (0·4)	6·2 (0·4)
	L. plantarum	4·2 (0·1)[d]	4·1 (0·2)[d]	4·0 (0·2)[d]	4·2 (0·1)[b]	7·3 (0·4)[c]	7·2 (0·3)	7·1 (0·3)	6·7 (0·2)	6·5 (0·3)	6·5 (0·4)[b]	6·4 (0·3)	6·6 (0·3)
	L. plantarum + *L. paracasei*	4·3 (0·2)[d]	4·0 (0·1)[d]	4·0 (0·1)[d]	4·5 (0·1)[b]	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)[a]	5·7 (0·9)[a]	4·4 (0·6)	5·6 (0·9)	7·4 (1·5)[a]	8·3 (0·6)[a]	5·1 (0·2)[a]	6·6 (0·9)	10·0 (0·6)[a]	9·3 (0·7)[a]	6·8 (0·9)	7·4 (0·8)
	L. plantarum	4·5 (0·5)[d]	4·2 (1·2)[c]	4·2 (1·2)	5·8 (1·0)	4·7 (0·9)[d]	6·5 (2·0)[c]	5·9 (1·2)[b]	6·7 (1·2)	8·2 (0·9)[d]	7·9 (1·8)[b]	6·6 (0·9)	7·2 (0·6)
	L. plantarum + *L. paracasei*	4·5 (0·4)[d]	4·7 (1·4)[c]	4·4 (0·6)	6·2 (0·5)	4·8 (0·4)[d]	6·3 (1·5)[c]	5·6 (0·9)[b]	7·1 (0·9)	7·9 (1·0)[d]	7·4 (1·1)[c]	7·2 (0·4)	7·5 (1·0)
Lactobacilli	Control	7·9 (1·3)[a]	8·7 (0·9)[a]	8·3 (0·5)	8·3 (0·8)[a]	7·6 (1·8)[a]	9·0 (0·5)	7·9 (0·4)[a]	7·7 (0·9)[a]	9·3 (0·7)[a]	9·5 (0·6)	8·8 (0·6)	7·6 (1·0)[a]
	L. plantarum	9·2 (0·5)[d]	9·2 (0·7)[d]	9·2 (0·9)	9·5 (0·2)[b]	8·5 (0·7)[b]	8·9 (0·4)	9·1 (0·5)[d]	9·5 (0·5)[b]	9·8 (0·8)[b]	9·7 (0·6)	9·4 (0·9)	9·9 (0·6)[b]
	L. plantarum + *L. paracasei*	9·3 (0·3)[d]	9·1 (0·9)[d]	9·2 (0·2)	8·7 (0·5)	9·2 (0·2)[c]	9·3 (0·3)	9·1 (0·4)[d]	9·7 (0·3)[c]	10·2 (0·3)[c]	10·4 (0·3)	9·8 (0·2)	10·1 (0·3)[c]

The table shows mean pH (SD) and 10 log cfu (SD) at d 3, 7, 14 and 35 of feeding.

[a–c]Different letters in the same column stand for significant differences within the column (one letter difference $P<0{\cdot}1$; two letters difference $P<0{\cdot}05$; three letters difference $P<0{\cdot}01$; four letters difference $P<0{\cdot}001$).

Table 2. *Total serum IgM and IgG titres (SEM) in meat-type strains (n = 15) at d 7 after priming and booster immunisation with TNP-KLH and continuous feeding of fermented liquid feed containing probiotics*

		IgM titre	IgG titre
	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 (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 (34·0)[a]

[a–c]Different letters in the same column stand for significant differences within the column (one letter difference $P<0·1$; two letters difference $P<0·05$; three letters difference $P<0·01$; four letters difference $P<0·001$).

Table 3. *Specific serum IgM and IgG anti-TNP titres (SEM) in meat-type strains (n = 15) at d 7 after priming and boost immunisation with TNP-KLH during continuous feeding with fermented liquid feed containing probiotics*

		IgM titre	IgG titre
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–c]Different letters in the same column stand for significant differences within the column (1 letter difference $P<0·1$; 2 letters difference $P<0·05$; 3 letters difference $P<0·01$).

Table 4. *Specific serum IgM and IgG anti-TNP titres (SEM) in layer- and meat-type strains (n = 15) at d 7 after primary immunisation with TNP-KLH and feeding 10^9* L. paracasei *LW 122 for 5 d prior to immunisation with TNP-KLH*

	IgM titre	IgG titre
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)

Table 5. *The effect of temporary feeding of* L. paracasei *LW 122 to layer- and meat-type strains (n = 15) on the proliferative responses of spleen cells to ConA and TNP-KLH* in vitro

	TNP-KLH	ConA
Layer	62 157 (7048)	27 489 (7322)
Layer + *L. paracasei*	73 083 (7837)	21 828 (6426)
Broiler	56 690 (9183)[a]	9069 (4709)
Broiler + *L. paracasei*	93 214 (12 004)[c]	12 423 (4967)

CPM (±SEM) are shown for assays performed at d 7 after primary immunisation with TNP-KLH.
[a–c]Different letters in the same column stand for significant differences within the column (one letter difference $P<0·1$; two letters difference $P<0·05$; three letters difference $P<0·01$).

after 14 and 24 h) of *S. enteritidis* in spleen and caecal cells was similar in cells of layer- and meat-type strains. However, entry and survival of *S. enteritidis* were significantly higher in ileum derived cells from meat-type strains than observed in layer-type strains (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 strains. With *L. paracasei* the entry was 1890 cfu/g and without 633 cfu/g ($P = 0·002$). In ileum and spleen cells of these chickens no effect of feeding probiotic bacteria on uptake of *S. enteritidis* was observed. Feeding *L. paracasei* had no effect on bacterial entry in the gut and spleen cells of meat-type strains.

In caecal cells of meat-type strains fed with *L. paracasei* LW 122 the survival and intracellular replication of *S. enteritidis* after 14 h incubation was reduced as compared to caecum cells of chickens that did not receive lactic acid bacteria ($P = 0·04$). But in caecal cells from layer-type strains the reverse was seen ($P = 0·05$).

For ileal cells the survival and intracellular replication of *S. enteritidis* after 14 h incubation was higher in cells from meat- than layer-type strains ($P = 0·04$). Feeding of *L. paracasei* LW 122 reduced the survival and replication in ileal cells of meat-type strains but had no effect on *S. enteritidis* in ileal cells of layer-type strains.

Feeding of *L. paracasei* LW 122 enhanced survival and replication of *S. enteritidis* in spleen cells of meat-type strains, but in layer-type strains no effect was observed. 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*.

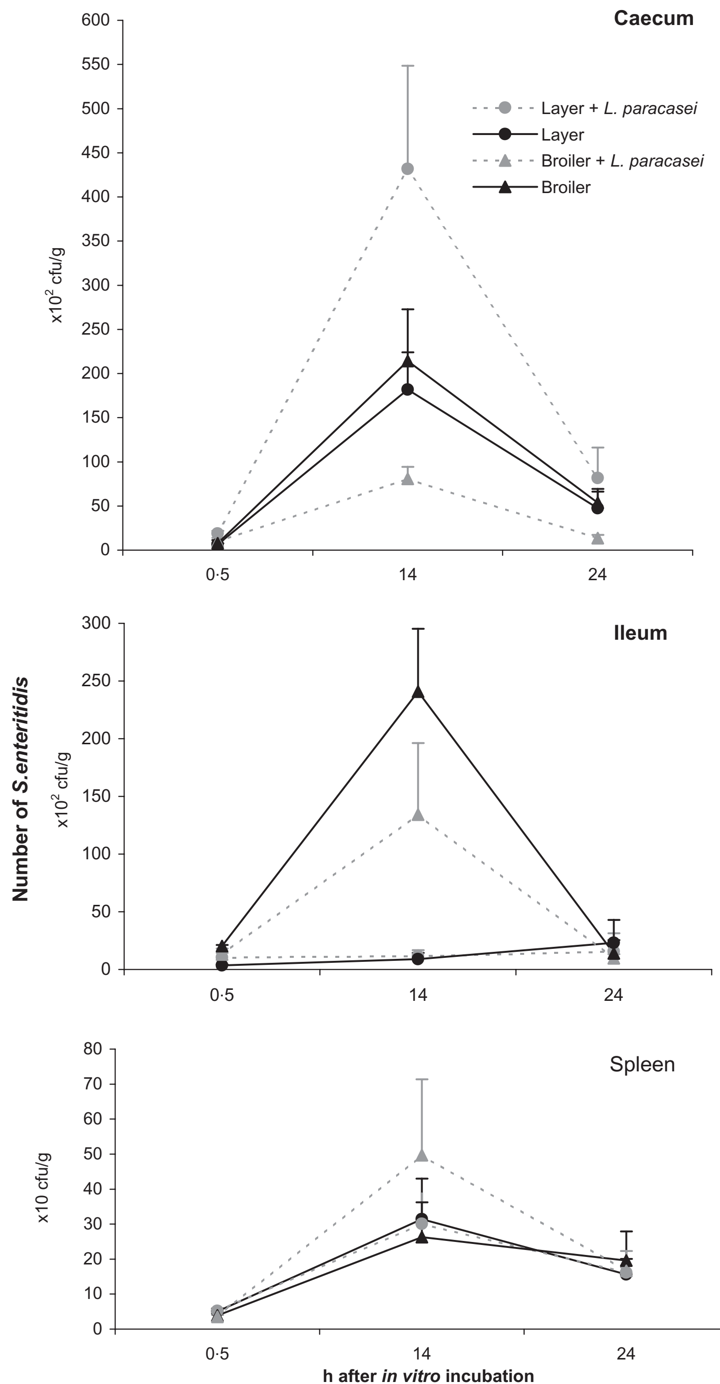

Figure 1. *The modulation of the innate immune response by feeding* L. paracasei *LW 122 in layer- and meat-type strains. Entry and survival of* S. enteritidis *are shown in isolated leukocytes from caecum (a), ileum (b) and spleen (c) of layer- and meat-type strains (±SEM) after in vitro exposure at one week after the last feeding of the lactobacilli (n = 15) in experiment II. (a) In caecum* L. paracasei *had a significant enhancing effect on the entry of* S. enteritidis *(P = 0·002) in the layer-type strain. In meat-type strains which had* L. paracasei *the survival of* S. enteritidis *after 14 h was significantly reduced as compared to cells of chickens not receiving* L. paracasei *(P = 0·04). In caecum cells of layer-type strains the reverse was observed (P = 0·05). (b) For ileal cells the survival and replication of* S. enteritidis *after 14 h was higher in cells of meat- than in cells of layer-type strains (P = 0·04). (c) No statistical differences.*

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

The feeding of fermented liquid feed in meat-type strains 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.

In the first week in particular 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 (Schiemann and Olson, 1984; Savage, 1987). Lactobacilli are important components for a balanced microflora. The reduced day-to-day variation in frequencies of lactobacilli in crop, ileum and caecum observed during fermented liquid feed feeding was an indication of a stabilising effect of lactobacilli on the intestinal microbiota and consistent with earlier findings of Heres *et al.* (2003). This stabilising effect as well as the increased frequency of lactobacilli together with a reduced frequency of *Enterobacteriaceae* was also observed in pigs (Van Winsen *et al.*, 2001). From this we conclude that the selected lactobacilli had beneficial effects on the host flora composition.

Daily administration of fermented liquid feed proved to be an effective vehicle in increasing live lactobacilli in the GI tract. However, in contrast to fermented liquid feed feeding in pigs, a disadvantage of using it in chicken was the relatively low food intake (Heres, 2003) and therefore a variable intake of lactobacilli (1 g fermented liquid feed $\sim 10^9$ 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. So, in the second experiment the chickens were inoculated individually with a fixed number of lactobacilli.

Immune function analysis

The phagocytosis of *S. enteritidis* in caecum derived cells was enhanced by *L. paracasei* LW 122 in layer-type strains. Similarly probiotic lactic acid bacteria enhanced phagocytosis in mice (Bloksma *et al.*, 1981, 1983; Perdigon *et al.*, 1986, 1991; Shu and Gill, 2002). These probiotic properties were dose dependent (Gill and Rutherfurd, 2001). Though in their experiments phagocytosis was studied with inert/passive materials, dose effects probably did play a role in our experiments with live *S. enteritidis*. Although the same number of lactobacilli was administered to meat- and layer-type strains, the gut of meat-type strains was relatively heavier and longer and therefore the number of lactobacilli may have differed between the chicken strains with respect to the gut surface and volume. In addition to intrinsic differences in immunity this might explain part of the observed differences.

Our results indicated that feeding *L. paracasei* to meat-type strains enhanced the phagocytic and bacterial activity of the gut cells (caecum, ileum). But in spleen cells the survival of *S. enteritidis* was slightly enhanced in comparison to the control group. In contrast, feeding of *L. paracasei* did not influence the phagocytic and bacterial activity in ileum and spleen cells of the layer-type strain, although in the caecum of the layer-type strain 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 meat-type strains than by differences in relative doses of lactobacilli. The enhanced activity in the gut of meat-type strains may provide an advantage in resistance and control of *S. enteritidis* or other infections.

Simultaneous feeding of *L. paracasei* and *L. plantarum* with fermented liquid feed enhanced total IgM and IgG titres (Figure). It appeared that the lactobacillus strains contributed differentially to the non-specific enhancement of antibody titres. After priming, IgM titres were enhanced by both the mixed fermented liquid feed and with *L. plantarum* only. Following booster immunisation *L. plantarum* alone had a stronger stimulating effect on the IgM titre than mixed fermented liquid feed. This indicated that no fading of the stimulation of non-specific or polyclonal stimulation had occurred. Total IgG titre in meat-type strains was enhanced by mixed fermented liquid feed feeding only after booster immunisation, which included a longer duration of administration. Fermented liquid feed with *L. plantarum* alone did not enhance polyclonal IgG. However, in experiment II after a feeding period of only 5 d prior to immunisation a non-specific enhancement was not observed. Both dose differences and also the interval between the last feeding of lactobacilli and the 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 immunisation had an adjuvant-like effect on the specific humoral responses to TNP-KLH in layer-type strains (Koenen *et al.*, 2002*b*, 2003). When fermented liquid feed was fed to meat-type strains (experiment I) an adjuvant-like effect was observed, but only for antigen-specific IgM response after priming (Tables 2 and 3). However, in experiment II, where layer- and meat-type strains were compared under the same conditions an adjuvant-like effect was not seen in meat-type strains and only a weak adjuvant effect (IgM and IgG) in layer-type strains (Table 4). Differences in experimental conditions may have resulted in different kinetics for the immunomodulation effects. Also, an age effect may play a role. The normal lifespan of meat-type strains is about 5 to 6 weeks. In experiment I (fermented liquid feed) the meat-type strains were 5 weeks old at priming but in the second experiment they were only

5 d old, creating sufficient time for a secondary immunisation. Host-dependent effectiveness of lactobacilli in the gut-associated immune system of layer-type strains was observed by Balevi *et al.* (2001) based on competitive exclusion. One might tentatively conclude that in very young meat-type strains the adjuvant effect is absent due to a lower state of immune maturation that makes cells less susceptible to the effect of immunoprobiotics. However, this still does not explain the titres of specific anti-TNP Ig production in meat-type strains in experiment II which even without stimulation by lactobacilli were as high as in layer-type strains after stimulation. Although both layer- and meat-type strains were 5 weeks old in experiment II their immune systems may still differ in maturity. As a consequence of the short maximum lifespan of meat-type strains their immune system may develop (in part) faster than in layer-type strains. Quantitative data on B-cell development may support this hypothesis (J.B. Cornelissen, personal communication).

In meat-type strains 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*. Thus for specific antigens such as vaccines, immunoprobiotic lactobacilli might enhance disease resistance especially in meat-type strains.

Interaction strain/gut/immune system

The interaction of gut, immune system and microflora may be influenced by various management factors. As meat- and layer-type strains differ enormously in growth and lifespan they probably also differ in their interactions with the actual bacterial microflora. *L. paracasei* enhanced T-cell proliferation more in meat- than in layer-type strains (Table 5), which could be caused by intrinsic differences in the regulation of cellular responses. Our experiments confirm that the proliferative responses of layer-type spleen cells could only be enhanced to a limited extent by lactobacilli (Koenen *et al.*, 2003).

As described by Kramer *et al.* (2003*a*) lighter meat-type strains reacted to *Salmonella* bacteria by innate immune mechanisms, whereas a heavier strain responded to them via the adaptive immune system. Layer and broiler differences in this respect might be greater because of their more pronounced weight differences.

Recently Clancy (2003) 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 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. As more lactobacilli reached the Peyers' patches they would activate more DCs and indirectly increase the immune response.

Feeding large amounts of lactobacilli influenced the microflora composition of young chickens in a beneficial way. The number of unwanted bacteria was lower and the number of lactobacilli was higher in treated animals. The microflora stabilised sooner in the treated than in the untreated animals. The age of the chickens and the dose of lactobacilli were important variables in the experiments. These seemed to be responsible for differences in stimulating proliferation, entry into leukocytes and humoral responses. The interplay of the lactobacillus strain, dose of lactobacilli, age of the chicken, interaction of the bacteria with the host gut and immune system and other factors 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.

Our 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 a very inexpensive way. Under the chosen conditions both humoral and proliferative responses were enhanced (adjuvant-like effect). Furthermore, the lactobacillus strain, dose and duration of application played a role in modulation effects. To draw firm conclusions further investigation is needed.

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REFERENCES

Balevi, T., Ucan, U.S., Coskun, B., Kurtoglu, V. & Cetingul, I.S. (2001) Effect of dietary probiotic on performance and humoral immune response in layer hens. *British Poultry Science*, **42**: 456–461.

Bianchi, A.T., Scholten, J.W., Jongenelen, I.M. & Koch, G. (1990) The use of monoclonal antibodies in an enzyme immunospot assay to detect isotype-specific

antibody-secreting cells in pigs and chickens. *Veterinary Immunology and Immunopathology*, **24**: 125–134.

BLOKSMA, N., DE REUVER, M.J. & WILLERS, J. (1983) Impaired macrophage function as a possible basis of immunomodification by microbial agents, tilorone and dimethyldioctadecylammoniumbromide. *Antonie van Leeuwenhoek*, **49**: 13–22.

BLOKSMA, N., ETTEKOVEN, H., HOFHUIS, F.M., VAN NOORLE-JANSEN, L., DE REUVER, M.J., KREEFTENBERG, J.G. & WILLERS, J. (1981) Effects of lactobacilli on parameters of non-specific resistance of mice. *Medical Microbiology and Immunology*, **170**: 45–53.

BOA-AMPONSEM, K., O'SULLIVAN, N.P., GROSS, W.B., DUNNINGTON, E.A. & SIEGEL, P.B. (1991) Genotype, feeding regimen, and diet interactions in meat chickens. 3. General fitness. *Poultry Science*, **70**: 697–701.

CLAASSEN, E., KORS, N. & VAN ROOIJEN, N. (1986) Influence of carriers on the development and localization of anti-trinitrophenyl antibody-forming cells in the murine spleen. *European Journal of Immunology*, **16**: 271–276.

CLANCY, R. (2003) Immunobiotics and the probiotic evolution. *FEMS Immunology and Medical Microbiology*, **38**: 9–12.

DE ROOS, N.M. & KATAN, M.B. (2000) Effects of probiotic bacteria on diarrhea, lipid metabolism, and carcinogenesis: a review of papers published between 1988 and 1998(1). *American Journal of Clinical Nutrition*, **71**: 405–411.

DE WAARD, R., CLAASSEN, E., BOKKEN, G.C., BUITING, B., GARSSEN, J. & VOS, J.G. (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. *Clinical and Diagnostic Laboratory Immunology*, **10**: 59–65.

DUNNE, C., O'MAHONY, L., MURPHY, L., THORNTON, G., MORRISSEY, D., O'HALLORAN, S., FEENEY, M., FLYNN, S., FITZGERALD, G., DALY, C., KIELY, B., O'SULLIVAN, G.C., SHANAHAN, F. & COLLINS, J.K. (2001) *In vitro* selection criteria for probiotic bacteria of human origin: correlation with *in vivo* findings. *American Journal of Clinical Nutrition*, **73**: 386S–392S.

FEDORKA-CRAY, P.J., BAILEY, J.S., STERN, N.J., COX, N.A., LADELY, S.R. & MUSGROVE, M. (1999) Mucosal competitive exclusion to reduce *Salmonella* in swine. *Journal of Food Protection*, **62**: 1376–1380.

GILL, H.S. & RUTHERFURD, K.J. (2001) Viability and dose-response studies on the effects of the immunoenhancing lactic acid bacterium *Lactobacillus rhamnosus* in mice. *British Journal of Nutrition*, **86**: 285–289.

GILLILAND, S.E. (1989) Acidophilus milk products: a review of potential benefits to consumers. *Journal of Dairy Science*, **72**: 2483–2494.

HERES, L. (2003) Epidemiological aspects of *Salmonella* and *Campylobacter* control in broiler chickens: the role of nutritionally induced changes of the gut ecology. *Ph.D. Thesis*, University of Utrecht, the Netherlands.

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. *Poultry Science*, **82**: 603–611.

ISOLAURI, E., SUTAS, Y., KANKAANPAA, P., ARVILOMMI, H. & SALMINEN, S. (2001) Probiotics: effects on immunity. *American Journal of Clinical Nutrition*, **73**: 444S–450S.

JEURISSEN, S.H., JANSE, E.M., EKINO, S., NIEUWENHUIS, P., KOCH, G. & DE BOER, G.F. (1988) Monoclonal antibodies as probes for defining cellular subsets in the bone marrow, thymus, bursa of Fabricius, and spleen of the chicken. *Veterinary Immunology and Immunopathology*, **19**: 225–238.

KOENEN, M.E., BOONSTRA-BLOM, A. & JEURISSEN, S.H.M. (2002*a*) Immunological differences between layer- and broiler-type chickens. *Veterinary Immunology and Immunopathology*, **89**: 47–56.

KOENEN, M.E., HERES, L., CLAASSEN, E. & BOERSMA, W.J.A. (2002*b*) *Lactobacilli* as probiotics in chicken feeds. *Bioscience Microflora*, **21**: 209–216.

KOENEN, M.E., HULST, R. VAN DER, LEERING, M., JEURISSEN, S.H.M. & BOERSMA, W.J.A. (2003) 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**: 119–127.

KOS, B., SUSKOVIC, J., VUKOVIC, S., SIMPRAGA, M., FRECE, J. & MATOSIC, S. (2003) Adhesion and aggregation ability of probiotic strain *Lactobacillus acidophilus* m92. *Journal of Applied Microbiology*, **94**: 981–987.

KRAMER, J., VISSCHER, A.H., WAGENAAR, J.A., BOONSTRA-BLOM, A.G. & JEURISSEN, S.H.M. (2001) Characterization of the innate and adaptive immunity to *Salmonella* Enteritidis pt1 infection in four broiler lines. *Veterinary Immunology and Immunopathology*, **79**: 219–233.

KRAMER, J., VISSCHER, A.H., WAGENAAR, J.A., CORNELISSEN, J.B.J.W. & JEURISSEN, S.H.M. (2003*a*) Comparison of natural resistance in seven genetic groups of meat-type chicken. *British Poultry Science*, **44**: 577–585.

KRAMER, J., VISSCHER, A.H., WAGENAAR, J.A. & JEURISSEN, S.H.M. (2003*b*) Entry and survival of *Salmonella enterica* serotype Enteritidis pt4 in chicken macrophage and lymphocyte cell lines. *Veterinary Microbiology*, **91**: 147–155.

KREUKNIET, M.B., GIANOTTEN, N., NIEUWLAND, M.B. & PARMENTIER, H.K. (1994) *In vitro* T cell activity in two chicken lines divergently selected for antibody response to sheep erythrocytes. *Poultry Science*, **73**: 336–340.

LI, Z., NESTOR, K.E., SAIF, Y.M. & ANDERSON, J.W. (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. *Poultry Science*, **79**: 804–809.

LI, Z., NESTOR, K.E., SAIF, Y.M., BACON, W.L. & ANDERSON, J.W. (1999) Effect of selection for increased body weight on mitogenic responses in turkeys. *Poultry Science*, **78**: 1532–1535.

MAASSEN, C.B., VAN HOLTEN-NEELEN, C., BALK, F., DEN BAK-GLASHOUWER, M.J., LEER, R.J., LAMAN, J.D., BOERSMA, W.J. & CLAASSEN, E. (2000) Strain-dependent induction of cytokine profiles in the gut by orally administered lactobacillus strains. *Vaccine*, **18**: 2613–2623.

MARTEAU, P.R., DE VRESE, M., CELLIER, C.J. & SCHREZENMEIR, J. (2001) Protection from gastrointestinal diseases with the use of probiotics. *American Journal of Clinical Nutrition*, **73**: 430S–436S.

MATSUZAKI, T. (1998) Immunomodulation by treatment with *Lactobacillus casei* strain shirota. *International Journal of Food Microbiology*, **41**: 133–140.

NUOTIO, L., SCHEITZ, C., HALONEN, U. & NURMI, E. (1992) Use of competitive exclusion to protect newly-hatched chicks against intestinal colonisation and invasion by *Salmonella* Enteritidis pt4. *British Poultry Science*, **33**: 775–779.

O'SULLIVAN, N.P., DUNNINGTON, E.A., SMITH, E.J., GROSS, W.B. & SIEGEL, P.B. (1991) Performance of early and late feathering broiler breeder females with different feeding regimens. *British Poultry Science*, **32**: 981–995.

PERDIGON, G. & ALVAREZ, S. (1992) Probiotics and the immune state, in: FULLER, R. (Ed) *Probiotics. The Scientific Basis*, Vol. 1, pp. 145–180 (London, Chapman & Hall).

PERDIGON, G., ALVAREZ, S. & PESCE DE RUIZ HOLGADO, A. (1991) Immunoadjuvant activity of oral *Lactobacillus casei*: influence of dose on the secretory immune response and protective capacity in intestinal infections. *Journal of Dairy Research*, **58**: 485–496.

PERDIGON, G., ALVAREZ, S., RACHID, M., AGUERO, G. & GOBBATO, N. (1995) Immune system stimulation by probiotics. *Journal of Dairy Science*, **78**: 1597–1606.

PERDIGON, G., DE MACIAS, M.E., ALVAREZ, S., OLIVER, G. & DE RUIZ HOLGADO, A.A. (1986) Effect of perorally administered lactobacilli on macrophage activation in mice. *Infection and Immunity*, **53**: 404–410.

PERDIGON, G., VINTINI, E., ALVAREZ, S., MEDINA, M. & MEDICI, M. (1999) Study of the possible mechanisms involved in the mucosal immune system activation by lactic acid bacteria. *Journal of Dairy Science*, **82**: 1108–1114.

PRAHARAJ, N.K., DUNNINGTON, E.A. & SIEGEL, P.B. (1995) Growth, immunoresponsiveness, and disease resistance of diverse stocks of chickens reared under two nutritional regimens. *Poultry Science*, **74**: 1721–1729.

SALMINEN, S., BOULEY, C., BOUTRON-RUAULT, M.C., CUMMINGS, J.H., FRANCK, A., GIBSON, G.R., ISOLAURI, E., MOREAU, M.C., ROBERFROID, M. & ROWLAND, I. (1998) Functional food science and gastrointestinal physiology and function. *British Journal of Nutrition*, **80**: S147–S171.

SALMINEN, S., ISOLAURI, E. & SALMINEN, E. (1996) Clinical uses of probiotics for stabilizing the gut mucosal barrier: successful strains and future challenges. *Antonie Van Leeuwenhoek*, **70**: 347–358.

SAVAGE, D.C. (1987) Factors influencing biocontrol of bacterial pathogens in the intestine. *Food Technology*, **July**: 82–87.

SCHIEMANN, D.A. & OLSON, S.A. (1984) Antagonism by gram-negative bacteria to growth of yersinia enterocolitica in mixed cultures. *Applied Environmental Microbiology*, **48**: 539–544.

SHU, Q. & GILL, H.S. (2002) Immune protection mediated by the probiotic *Lactobacillus rhamnosus* hn001 (dr20) against *Escherichia coli* O157:H7 infection in mice. *FEMS Immunology and Medical Microbiology*, **34**: 59–64.

URLINGS, H.A., BIJKER, P.G. & VAN LOGTESTIJN, J.G. (1993) Fermentation of raw poultry byproducts for animal nutrition. *Journal of Animal Sciences*, **71**: 2420–2426.

VAN WINSEN, R.L., URLINGS, B.A., LIPMAN, L.J., SNIJDERS, J.M., KEUZENKAMP, D., VERHEIJDEN, J.H. & VAN KNAPEN, F. (2001) Effect of fermented feed on the microbial population of the gastrointestinal tracts of pigs. *Applied Environmental Microbiology*, **67**: 3071–3076.

VAN ZIJDERVELD, F.G., VAN ZIJDERVELD-VAN BEMMEL, A.M. & ANAKOTTA, J. (1992) Comparison of four different enzyme-linked immunosorbent assays for serological diagnosis of *Salmonella* Enteritidis infections in experimentally infected chickens. *Journal of Clinical Microbiology*, **30**: 2560–2566.

YUNIS, R., BEN-DAVID, A., HELLER, E.D. & CAHANER, A. (2000) Immunocompetence and viability under commercial conditions of broiler groups differing in growth rate and in antibody response to *Escherichia coli* vaccine. *Poultry Science*, **79**: 810–816.