

Oral immunoadjuvant activity of *Lactobacillus casei* subsp. *casei* in dextran-fed layer chickens

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We recently reported that synbiotic *Lactobacillus casei* subsp. *casei* together with specific substrate dextran elicited an enhancement in humoral immune response against bovine serum albumin (BSA) as a model antigen in BALB/c mice. The present study was designed to evaluate the oral immunoadjuvant effects of the synbiotic in layer chickens. Using a PCR assay, *L. casei* subsp. *casei* was detected specifically in the intestinal chyme of chickens (10 d of age, Julia strain) fed *ad libitum* on a diet supplemented with 75 mg dextran/kg (dextran-supplemented diet, DSD) and administered orally with 10^7 colony-forming units (CFU) *L. casei* subsp. *casei* in 0.1 ml PBS with the aid of an intubation needle at 1, 2 and 3 d of age. Furthermore, oral administration of 10^7 CFU *L. casei* subsp. *casei* at 1–3 d of age significantly enhanced the production of anti-BSA antibody in DSD-fed chickens (60 d of age) administered orally with 1 mg BSA at 32 and 33 d of age and subcutaneously with 5 µg BSA at 33 d of age. In addition, among bacterial numbers tested, 10^6 CFU *L. casei* subsp. *casei* together with dextran induced an effective increase in humoral immune response to mixed inactivated vaccines against Newcastle disease and avian infectious bronchitis, and the treatment may be advantageous in protecting against these infectious diseases in chickens in actual application. These results suggest that dietary supplementation of *L. casei* subsp. *casei* with dextran leads to immunomodulation of humoral immune responses.

Lactobacillus casei: Dextran: Synbiotic: Chicken

Lactic acid bacteria are contained in a wide variety of fermented food products and are known to be beneficial to the health of man (Ahrne *et al.* 1998). These bacteria are regarded as probiotic and their health-promoting effects, such as improvement of the intestinal microflora and reduction in the incidence of diarrhoea and intestinal infections, have been shown (Fuller, 1989; Rolfe, 2000; Ouwehand *et al.* 2002), while modulation of the immune system has also been reported (Perdigón *et al.* 1986; Matsuzaki & Chin, 2000; Takagi *et al.* 2001).

Prebiotics are non-digestible substances such as oligosaccharides that selectively stimulate beneficial bacterial species in the human colon and have been demonstrated to induce health-promoting effects in the host (Gibson & Roberfroid, 1995). Fructo-oligosaccharides and galacto-oligosaccharides have been demonstrated to facilitate the growth of lactic acid bacteria, while lactulose increased the numbers of *Lactobacillus* species in the intestine of infants (MacGillivray *et al.* 1959; Rowland & Tanaka, 1993; Gibson & Wang, 1994).

A synbiotic is a combination of live bacteria used as a probiotic and their specific substrate as a prebiotic (Gibson & Roberfroid, 1995). We recently demonstrated that the *Lactobacillus casei* subsp. *casei* strains JCM 1134^T and JCM 8129 had specific abilities to utilize dextran (Ogawa *et al.* 2005). Probiotics and prebiotics are becoming recognized as

useful, and are being applied to the feed of livestock and poultry (Bailey *et al.* 1991; Gusils *et al.* 1999; Koenen *et al.* 2002). In the present study, we added a synbiotic to poultry feed and investigated the adjuvant effects of *L. casei* subsp. *casei* in conjunction with dextran in chickens, with bovine serum albumin (BSA) administered as a model antigen, as was a vaccine against Newcastle disease and avian infectious bronchitis.

Materials and methods

Chickens and diets

Unvaccinated male layer chickens (Julia strain) were obtained from GHEN Corporation (Gifu, Japan) and used in all experiments. They were housed in stainless-steel wire cages at a constant temperature of 22°C and fed a commercial diet (PL-1; Oriental Yeast, Tokyo, Japan) as the basal diet (BD). The BD was a commercial layer chicken diet (per 100 g diet: 20.3 g crude protein, 6.5 g crude fat, 6.2 g crude ash, 3.3 g crude fibre, 1477 kJ (353 kcal) digestible energy, 1218 kJ (291 kcal) metabolizable energy) that mainly consisted of maize, white fishmeal, corn oil and vitamin–mineral premix, and satisfied the nutrient demands for layer chickens (Agriculture, Forestry and Fisheries Research Council Secretariat, 1997).

Abbreviations: BD, basal diet; BSA, bovine serum albumin; CFU, colony-forming units; DSD, dextran-supplemented diet; IBV, avian infectious bronchitis virus; LCSD, *L. casei* subsp. *casei*-supplemented diet; NDV, Newcastle disease virus.

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The experimental diets were a dextran-supplemented diet (DSD), which was BD supplemented with 75 mg dextran (average molecular weight, 10 000; Meito Sangyo Co. Ltd, Nagoya, Japan) per kg, and an *L. casei* subsp. *casei*-supplemented diet (LCSD), as described later. Water and food were provided *ad libitum*. All the birds were clinically normal throughout the study and there were no significant differences among the treatment groups for average body weight gain and feed conversion ratio (data not shown). The Animal Care and Use Committee of Asahi University approved all procedures.

Bacterial strain

L. casei subsp. *casei* strain JCM 1134^T (type strain), which has an ability to utilize dextran, was aerobically cultured in MRS (Man–Rogosa–Sharpe) broth (Difco Laboratories, Detroit, MI, USA) at 37°C (Ogawa *et al.* 2005). For oral administration of *L. casei* subsp. *casei*, the number of organisms in culture was adjusted to 10⁸ colony-forming units (CFU)/ml spectrometrically at 660 nm. To prepare LCSD, the organisms were centrifuged at 1500 *g* for 10 min and the precipitate was lyophilized. The lyophilized *L. casei* subsp. *casei* was supplemented with 10⁶–10⁸ CFU/kg DSD.

Detection of *L. casei* subsp. *casei* in gastrointestinal tract

For this experiment, forty layer chickens were divided into four groups: BD + Lcc (BD with 10⁷ CFU *L. casei* subsp. *casei*), BD alone, DSD + Lcc (DSD with 10⁷ CFU *L. casei* subsp. *casei*) and DSD alone, with each group consisting of ten chickens. The BD + Lcc and DSD + Lcc groups were orally administered 10⁷ CFU of the organisms in 0.1 ml PBS (Sigma Chemical Co., St. Louis, MO, USA) with the aid of an intubation needle (Natume Co. Ltd, Tokyo, Japan) at 1, 2 and 3 d of age, while the BD alone and DSD alone groups received 0.1 ml PBS at the same times. Water and food were provided *ad libitum*. At 10 d of age, intestinal chyme was taken from the small intestine. DNA was then extracted from 200 mg of each intestinal chyme sample and suspended with *L. casei* subsp. *casei* using a GFXTM genomic blood DNA purification kit (Amersham Biosciences, Uppsala, Sweden). PCR was performed using an *L. casei* subsp. *casei*-specific primer pair that was designed according to the 16S rRNA sequence D16551 of *L. casei* subsp. *casei* strain JCM 1134^T. The primer sequences were 5'-TTG GTC GAT GAA CGG TGC TTG CAC TGA GAT TCG ACT TAA-3' (position 40–84; forward) and 5'-GGC AGT CTT ACT AGA GTG CCC AAC TC-3' (position 1135–1160; reverse). For each reaction, 25 µl of reaction mixture was prepared that consisted of 1 × buffer without MgCl₂, 1.5-mM MgCl₂, 20-µM each dNTP, 0.1-µM primer and 1.5 U *Taq* DNA Polymerase (Takara Bio Inc., Kyoto, Japan). Amplification for the organism-specific 16S rRNA gene was programmed as follows: pre-incubation at 94°C for 2 min, followed by 30 cycles at 94°C for 1 min, then at 70°C for 1 min and at 72°C for 1 min, with a final extension at 72°C for 7 min and cooling to 4°C in an iCycler thermal cycler (Bio-Rad Laboratories, Hercules, CA, USA). For PCR using a ubiquitous primer pair, the annealing temperature was changed to 55°C. The PCR products were electrophoresed in 1.5% (w/v) agarose

gel and then visualized under UV fluorescence after staining with ethidium bromide.

Adjuvant effects of *L. casei* subsp. *casei* on humoral immune responses following oral and subcutaneous immunization with bovine serum albumin in chickens

For this experiment, forty chickens were divided into four groups as in the previous experiment. The BD + Lcc and DSD + Lcc groups were orally administered 10⁷ CFU of the organisms in 0.1 ml PBS with the aid of an intubation needle at 1, 2 and 3 d of age. The BD alone and DSD alone groups received 0.1 ml PBS at the same times. Then, all chickens in the four groups were orally immunized with 1 mg BSA (Sigma Chemical Co.) in 0.2 ml PBS at 32 and 33 d of age, or immunized subcutaneously with 5 µg BSA incorporated into 0.1 ml Freund incomplete adjuvant (Difco Laboratories) at 32 d of age. Serum specimens were collected at 60 d of age. Anti-BSA IgG titres were measured by ELISA as described previously (Ogawa *et al.* 2005), with serum specimens obtained from non-immunized BD-fed chickens used as a control.

Effect of oral administration of *L. casei* subsp. *casei* on humoral immune response following vaccination against Newcastle disease and avian infectious bronchitis

Twenty layer chickens received BD *ad libitum* until 7 d of age and were then divided into two groups of ten birds each. After 8 d of age, the groups were given either BD or LCSD containing 10⁶–10⁸ CFU/kg DSD *ad libitum*; then at 40 d of age, chickens in both groups were subcutaneously immunized with a combined inactivated vaccine, New Bronz[®] MG (GHEN Corporation), against Newcastle disease and avian infectious bronchitis; after which serum specimens were collected at 68 d of age. The concentrations of IgG specific for Newcastle disease virus (NDV) and avian infectious bronchitis virus (IBV) in sera were determined using Newcastle disease and avian infectious bronchitis ELISA kits (GHEN Corporation), respectively, which were performed according to the manufacturer's instructions. The IgG concentrations against NDV and IBV were obtained by calculating the sample to positive (S:P) ratio as follows (Wang *et al.* 2002): (absorbance value of the sample serum–absorbance value of the negative control serum)/(absorbance value of the positive control serum–absorbance value of the negative control serum).

Statistical analyses

The experiment is a typical repeated measures experiment, and chickens were assigned to the treatment groups according to completely randomized design in the experiment. Comparisons of IgG concentrations between the groups immunized with BSA, or NDV and IBV were assessed using one-way ANOVA, with Scheffe's test. Statistical significance was set at $P < 0.01$ and $P < 0.05$. All analyses were done using Microsoft[®] Excel Version 9.0 (Microsoft-Japan, Tokyo, Japan) and Statcel (OMS, Tokorozawa, Japan) software packages. The results are presented as means with their standard errors.

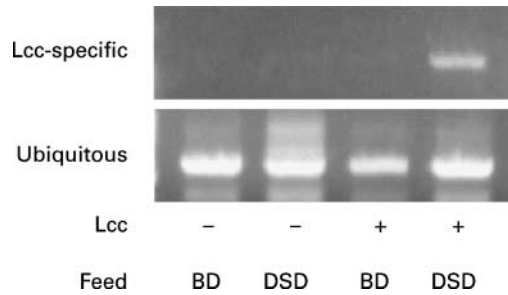


Fig. 1. Detection of *Lactobacillus casei* subsp. *casei* (Lcc) in intestinal chyme of chickens. Chickens fed a basal diet (BD) or a dextran-supplemented diet (DSD) were administered PBS orally with (+) or without (-) Lcc. PCR amplification of DNA samples prepared from the intestinal chyme specimens was performed using Lcc-specific and -ubiquitous primers. All experiments were performed at least three times and representative results are presented.

Results and discussion

We recently reported that only two strains of *L. casei* subsp. *casei* among the various lactic acid bacteria we tested had an ability to utilize dextran as a prebiotic (Ogawa *et al.* 2005). In the present study, we attempted to perform specific detection of *L. casei* subsp. *casei* in the gastrointestinal tract of chickens using a PCR method. In the DSD-fed groups, oral administration of *L. casei* subsp. *casei* resulted in its definite detection in intestinal chyme samples (Fig. 1). On the other hand, it was not detected in intestinal chyme from the BD-fed groups regardless of administration. These findings indicate that dextran in food was able to maintain the growth of *L. casei* subsp. *casei* in the intestines of chickens. In addition, PCR products were also detected in all of the groups using a ubiquitous primer set. Prebiotics have been generally defined as supplements for the growth of lactic acid bacteria, such as *Lactobacillus* and *Bifidobacterium* species (Matsuzaki *et al.* 1988). The present results indicate that dextran has the ability to function as an effective prebiotic in chickens.

The use of living organisms as probiotics is recommended as an alternative to antibiotics for use as preventive, therapeutic and growth-promoting materials (Sun, 1984; Fuller,

1989). *Lactobacillus* species are beneficial bacteria found in the indigenous microbial flora of the intestinal tract of man and animals, and are frequently used as probiotic organisms (Ahrne *et al.* 1998). To examine the oral immunoadjuvant effect of *L. casei* subsp. *casei* with or without dextran-feeding, we measured the anti-BSA IgG concentrations in serum samples from chickens immunized orally or subcutaneously with BSA (Fig. 2(a,b)). Both types of immunization resulted in a significant increase in anti-BSA IgG concentrations in DSD-fed chickens administered with *L. casei* subsp. *casei*. However, the administration of *L. casei* subsp. *casei* or dextran-feeding alone resulted in no increase in concentrations of anti-BSA IgG. These findings indicate that *L. casei* subsp. *casei* together with dextran resulted in an increase in humoral immune responses against BSA in layer chickens as well as BALB/c mice (Fig. 2; Ogawa *et al.* 2005).

Gibson & Roberfroid (1995) have advocated use of a synbiotic approach for microflora management, in which a probiotic and prebiotic are used in combination, and demonstrated that combinations of *Bifidobacterium* species with a fructo-oligosaccharide and *Lactobacillus* species with lactitol improved survival of the probiotic bacteria available for prebiotic fermentation, resulting in advantages to the host. Recently, administration of antibiotics to livestock and poultry has been limited or prohibited in Europe because it can promote drug resistance of pathogens (van Den Bogaard *et al.* 2000; World Health Organization, 2003). Since the 1950s, it has been reported that antibiotic-fed mice and guinea-pigs were very sensitive to *Salmonella*, *Shigella* and *Vibrio* infections (Freter, 1956; Miller, 1959). Accordingly, a specific synbiotic protocol of *L. casei* subsp. *casei* and dextran in combination is a reasonable substitute and a promising candidate for antibiotic replacement.

NDV, IBV, Infectious laryngotracheitis virus, avian influenza virus and pneumovirus are pathogens that often affect the respiratory tract of chickens (Villegas, 1998). Among the complications that result from infection, Newcastle disease is a fatal disease seen in poultry in many parts of the world, while these pathogens also increase susceptibility to a wide variety of other infections and diseases (Alexander, 1997). Infectious

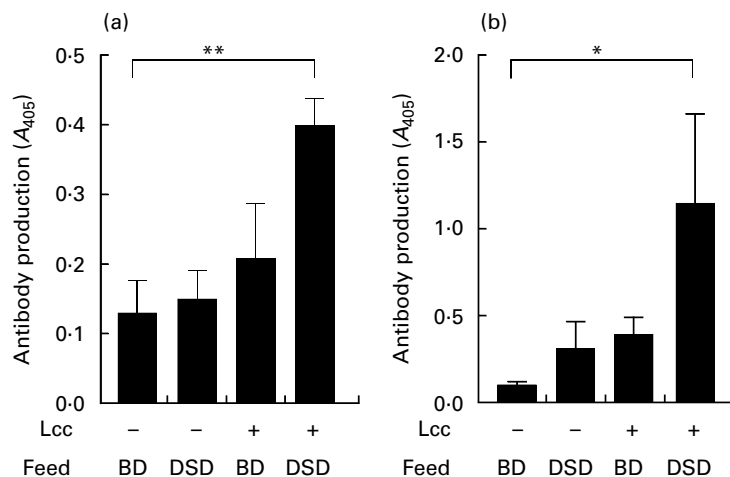


Fig. 2. Bovine serum albumin (BSA)-specific IgG responses of chickens fed a basal diet (BD) or a dextran-supplemented diet (DSD) and administered PBS orally with (+) or without (-) *Lactobacillus casei* subsp. *casei* (Lcc). BSA was given orally (a) or subcutaneously (b). Values are means with their standard errors shown by vertical bars for ten chickens per group. Mean values were significantly different: * $P < 0.05$, ** $P < 0.01$.

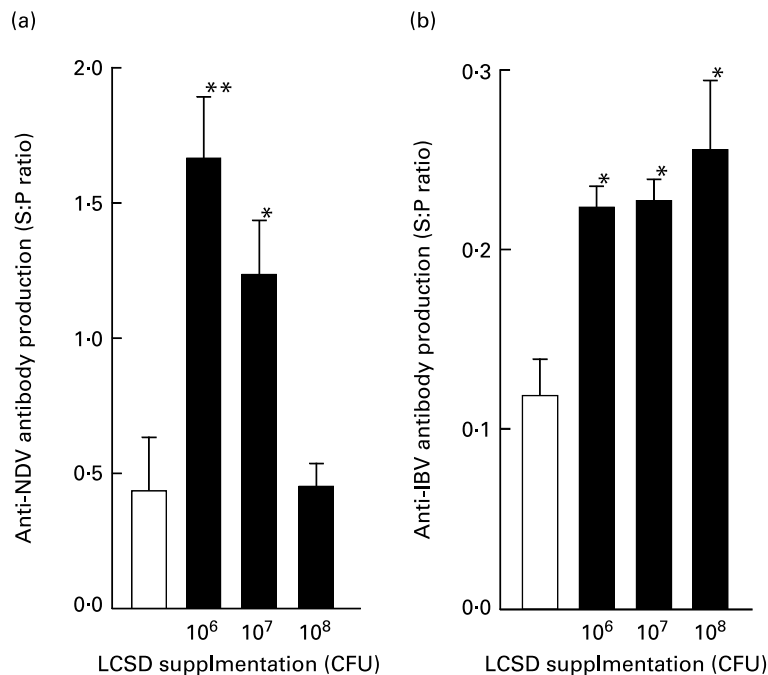


Fig. 3. Newcastle disease virus (NDV)- and avian infectious bronchitis virus (IBV)-specific IgG responses by chickens fed a basal diet (□) or a *Lactobacillus casei* subsp. *casei*-supplemented diet (LCSD(■); 10^6 – 10^8 colony-forming units (CFU) of *L. casei* subsp. *casei*/kg dextran-supplemented diet). The chickens were subcutaneously immunized with inactivated vaccines against NDV and IBV. Concentrations of IgG specific for NDV (a) and IBV (b) in serum specimens were determined by ELISA. The sample to positive (S:P) ratio was determined as described in the Materials and methods section. Values are means with their standard errors shown by vertical bars for ten chickens per group. Mean values were significantly different: * $P < 0.05$, ** $P < 0.01$.

bronchitis is a highly contagious disease caused by coronavirus that affects not only young chickens and broilers, but also birds in lay (Cavanagh & Naqi, 1997). To control those respiratory viruses, live and inactivated vaccines have been developed and used by the poultry industry for many years.

Since administration of *L. casei* subsp. *casei* to DSD-fed chickens markedly increased humoral immune responses specific for BSA as a model antigen (Fig. 2), we next examined the influence of dietary *L. casei* subsp. *casei* supplementation with DSD on IgG production against vaccines for NDV and IBV in chickens with a view to practical application. Oral administration of LCSD containing 10^6 – 10^8 CFU *L. casei* subsp. *casei*/kg DSD resulted in a definite enhancement of humoral immune responses against NDV and IBV in chickens (Fig. 3(a,b)). These findings indicate that 10^6 CFU *L. casei* subsp. *casei* together with dextran exhibited effective oral immunoadjuvant activities in chickens immunized with mixed vaccines against Newcastle disease and avian infectious bronchitis. We previously showed that a variety of derivatives of *N*-acetyl-glucosaminyl- β (1 \rightarrow 4)-*N*-acetylmuramyl-L-alanyl-D-isoglutaminyl-(L)-*meso*-2,6-diaminopimelic acid-(D)-amide, prepared by chemical modification of an enzymatic hydrolysate of *Lactobacillus plantarum* cell-wall peptidoglycan, exerted an effective adjuvant activity when administered orally to mice (Ogawa *et al.* 1986, 1992, 1995). Therefore, these immunoadjuvant effects of lactic acid bacteria containing *L. casei* subsp. *casei* may be attributable to their bacterial surface components and products. The mechanisms by which probiotics exhibit their effects have not been clearly defined. Several studies have characterized the ability of probiotics to modulate the activity and cytokine production of immune

cells in gut-associated lymphoid tissue (Erickson & Hubbard, 2000; Haller *et al.* 2000).

Taken together, our results show that *L. casei* subsp. *casei* exhibits powerful immunoadjuvant activities in dextran-fed layer chickens. They also indicate that dietary supplementation of *L. casei* subsp. *casei* together with dextran is a valuable immunomodulatory protocol for antigen-specific humoral responses towards vaccination.

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