Efficiency of a *Lactobacillus plantarum*-xylanase combination on growth performances, microflora populations, and nutrient digestibilities of broilers infected with *Salmonella* Typhimurium

S. Vandeplas,*[1] R. Dubois Dauphin,† C. Thiry,† Y. Beckers,* G. W. Welling,‡ P. Thonart,† and A. Théwis*

*Animal Science Unit, Gembloux Agricultural University, Passage des Déportés 2, 5030 Gembloux, Belgium; †Bio-Industry Unit, Gembloux Agricultural University, Passage des Déportés 2, 5030 Gembloux, Belgium; and ‡Department of Medical Microbiology, University Medical Center Groningen, 9713 GZ Groningen, the Netherlands

ABSTRACT Three experiments were performed to assess the ability of a *Lactobacillus plantarum* probiotic combined with a xylanase to reduce the effects of *Salmonella* Typhimurium infection in broiler chickens from 1 to 30 or 42 d of age. Chicks were challenged at 3 d of age with 10^8 or 10^5 cfu *Salmonella* Typhimurium/chick. Four diets were studied: a wheat-based diet (C+) supplemented with 0.1 g/kg of xylanase (E) or 10^6 cfu/g of *L. plantarum* (P), or both (PE). Uninfected chicks fed the C diet were used as negative control (C−). Six or 8 chicks were housed per cage with 9 cages/treatment. Growth performance and feed conversion ratio (FCR) were recorded weekly. In experiment 1, bacterial enumeration in ceca was achieved using the fluorescent in situ hybridization technique. *Salmonella* enumeration was realized in excreta by microbiological cultures (experiments 2 and 3). Nutrient digestibilities and AME_n were determined in experiment 3 from d 35 to 39. Infection with *Salmonella* Typhimurium led to a significant decrease in the daily weight gain (DWG) by 23.6 to 32.8%, whereas FCR was increased by 1.0 to 19.7%. Chickens fed the PE diet showed significantly improved performance in comparison with C+ birds (DWG: +12.5% in experiment 1; FCR: −2.1 to 8.6%), and in comparison with the P and E treatments (DWG: +6.3 to 8.3% in experiment 1; FCR: −2.7 to 6.4%). In experiment 3, the FCR was significantly improved by 3% with the PE diet in comparison with C− chickens. The PE combination tended to restore a microflora similar to that of uninfected broilers, whereas the P and E diets had less of an effect on the profile of bacterial communities. At slaughter age, *Salmonella* contamination was reduced by 2.00 and 1.85 log colony-forming units for the E and PE treatment, respectively. The PE diet significantly reduced the crude fat digestibility by 9.2%, in comparison with the C+ chickens. These results suggest that the combination between *L. plantarum* and a xylanase as feed additive could be effective for reduction of the detrimental effect after *Salmonella* Typhimurium infection of broilers.

Key words: probiotic, xylanase, cecal microflora, *Salmonella*, broiler

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INTRODUCTION

In industrial countries, contamination of food from animal origin by human enteric pathogens remains a major public health concern. The major pathogen involved in bacterial foodborne infections worldwide is *Salmonella* spp., with an infection incidence of 38.2 per 100,000 people in 2005 in the European Union (**EU**; EFSA, 2007). Because this microorganism may be carried asymptomatically in the alimentary tract of wild and domesticated birds, consumption of food from poultry origin was identified as the major source of *Salmonella* infection, by spreading via the slaughter process to raw, finished products. In broiler meat, the most commonly reported serovar is *Salmonella* Typhimurium, which represents the predominant serovar associated with salmonellosis in Belgium (National Reference Center for *Salmonella* and *Shigella*, 2007). With the aim of decreasing the incidence of foodborne infections in humans, the EU has progressively established a coherent European legislation [Directive 2003/99/EC and Regulations (EC) no. 2160/2003 and no. 1003/2005], especially for the monitoring and control of *Salmonella* spp. at the primary production, transformation, and distribution levels. From 2010, the European legislation states that poultry meat shall not be placed on the market without any industrial treatment if a 25-g

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[1]Corresponding author: vandeplas.s@fsagx.ac.be

sample is detected *Salmonella*-positive. To match the EU directive, intervention procedures will consequently be progressively implemented by poultry producers to reduce the *Salmonella* colonization in the intestinal tract of poultry and the subsequent potential carcass contamination during slaughter.

Therefore, different strategies have been proposed to prevent *Salmonella* intestinal colonization of chickens at the primary production level, including the use of feed additives (Van Immerseel et al., 2002). The prevention of *Salmonella* infection by competitive exclusion bacteria, which are undefined or partially defined cultures derived from poultry intestinal microflora, have been widely investigated (Schneitz, 2005). To counteract the disadvantages linked to the undefined nature of competitive exclusion products, treatments based on one or some well-identified microbial strains as probiotics have also been studied. Species of *Lactobacillus* have been the most common microorganism studied for their probiotic properties to control *Salmonella* infection (Pascual et al., 1999; Tsai et al., 2005). The positive effects can result from a health effect, with probiotics acting as bioreactors of the intestinal microflora (Netherwood et al., 1999; Mountzouris et al., 2007). By production of antimicrobial substances, stimulation of the immune system, and competition for nutrients and adhesion sites in the gastrointestinal tract (Jin et al., 1997), probiotics may also help to exclude or prevent pathogen colonization in the host.

The use of feed enzymes is also becoming an increasing part of the proposed strategies to mitigate the intestinal colonization of *Salmonella* spp. in poultry. Exogenous enzymes such as xylanase are usually incorporated in poultry wheat-based diets to degrade the antinutritional arabinoxylans (**AX**) of wheat, which may consequently improve the nutrient digestibilities and the growth performance of poultry (Simon, 2000; García et al., 2008). Several studies have showed that such enzymes have also been found to reduce the bacterial colonization in the gut. By improving the digestibility and absorption of nutrients, AX-hydrolyzing enzymes have been hypothesized to leave less residual nutrients to fuel bacterial growth in the small intestine, and more particularly pathogen growth. Vahjen et al. (1998) showed that xylanase supplementation to wheat-based diets for broilers significantly lowered total presumptive enterobacteria and total gram-positive cocci in intestinal samples. Similar results were observed in wheat- or rye-based diets for enterobacteria and gram-positive cocci but also enterococci and total anaerobic microbes (Dänicke et al., 1999; Hübener et al., 2002). According to their effect on enterobacteria, subsequent studies have demonstrated the inhibitory properties of growth-promoting enzymes against *Salmonella* in chickens (Hruby, 2003).

Nevertheless, combining xylanase and probiotic strains in wheat-based diets for chickens, with expected complementary improvement of growth and inhibitory activities against *Salmonella* spp., has not yet been investigated. Hence, the objective of the present work was to study the effectiveness of a feed additive, composed of a *Lactobacillus* strain as probiotic combined with a xylanase, on *Salmonella* Typhimurium colonization in excreta of chickens experimentally infected and fed a wheat-based diet. Moreover, the effects of this combination on the performance and nutrient digestibilities, in relation to modification of the microflora, in infected chickens were evaluated, in comparison with noncontaminated birds.

MATERIALS AND METHODS

Strains and Cultivation Conditions

The *Salmonella* Typhimurium CWBI-B1199 strain antibiotic penta-resistant (spectinomycin, tetracyclin, ampicillin, chloramphenicol, and nalidixic acid) was used for oral infection challenge assays. Pathogen working culture, from bacterial stock stored at 80°C in 15% glycerol-water, was achieved on plate count agar or tryptone soy broth (Merck, Darmstadt, Germany) for 18 h at 37°C. The *Salmonella* Typhimurium inocula were grown in 15 mL of tryptone soy broth. To determine the final concentration, serial dilutions were made on plate count agar plates. The biomass cells were stored at 4°C and subsequently used in the challenge experiments.

The probiotic strain used to supplement diets was the *Lactobacillus plantarum* CWBI-B659 from the CWBI lactic acid bacteria collection (CWBI, Gembloux, Belgium). The strain was kept frozen at −80°C before being replicated twice for 24 h at 37°C in de Man, Rogosa, and Sharpe (**MRS**) agar (Oxoid Ltd., Basingstoke, UK). The dry powder of the CWBI-B659 culture incorporated in the poultry ration was obtained by bacterial fermentation in 15 L of MRS broth in a 20-L fermentor (Biolafitte & Moritz, Pierre Guerin Technologies, Lyon, France) for 8 to 12 h at 37°C. The fermentation was performed under controlled pH (i.e., 6.0) and with moderate agitation (120 rpm). The culture was then centrifuged at 8,000 × *g* for 30 min at 4°C in a Beckman Avanti J25 I centrifuge (Beckman Instruments, Fullerton, CA). After adding cryoprotective medium containing glycerol 1.5% (wt/vol) and skim milk 20% (wt/vol), the pellet was frozen at −40°C and then lyophilized in a Dura-Top freeze-dryer (FTS Systems Inc., Stone Ridge, NY). The drying conditions were set at 14 Pa of pressure, −25°C, during 18 h. Before use, the freeze-dried culture was analyzed for total viable cell counts. The viable cell count was performed by the standard dilution method on MRS agar after incubation at 37°C for 48 h.

Experimental Diets

Two basal diets, based on wheat at about 600 g/kg from the same batch for all in vivo trials, were formulated to meet Ross broiler nutrient requirements for

grower (1 to 20 d of age) and finisher (21 to 42 d of age) growth periods (Table 1). The chemical compositions of the experimental diets were determined according to the methods of the AOAC (1990). Dry matter was obtained by drying the diet samples for at least 48 h in an oven at 105°C (method 934.01; AOAC, 1990). Nitrogen was determined with the Kjeldahl method (method 988.05; AOAC, 1990). Total protein was obtained by multiplying nitrogen value by 6.25. Fat was measured by Soxhlet extraction with diethyl ether (method 920.39; AOAC, 1990). Measurements of crude fiber were performed according to Weende (method 978.10; AOAC, 1990). The AME was calculated from the gross energy values measured using an adiabatic bomb calorimeter (Parr 1241EF, Parr Instrument Company, Moline, IL).

The enzyme preparation is Belfeed B1100MP (Beldem S.A., Andenne, Belgium), and the producing strain is *Bacillus subtilis* (LMG S-15136). It is a specific pentosanase of xylanase type (105 IU/g) with optimal activity at pH 7 to 8 and added as a dry powder to experimental diets. In the International Union of Biochemistry system, the activity is classified as endo-1.4-β-xylanase (EC 3.2.1.8). It hydrolyzes AX into oligosaccharides and some mono-, di-, and trisaccharides. One international unit is the amount of enzyme that liberates 1 μmol of xylose from birchwood xylan per minute at pH 4.5 and 30°C. The feed additive probiotic was the lactic acid strain *L. plantarum* CWBI-B659, producing lactic acid, able to resist and to hydrolyze bile acids, and able to adhere to gut epithelial tissue (Roblain et al., 2002). Four dietary treatments were produced by addition of the probiotic strain and the xylanase alone or together to the basal diets: central basal diet (**C**), or C basal diet supplemented with 0.1 g/kg of xylanase (**E**), 10^6 cfu/g of *L. plantarum* (**P**), or both (**PE**). The survival of the supplemented bacteria in the diet was assessed after preparation and during storage at room temperature every week. The 4 experimental diets, fed as mash, contained no additional coccidiostats or antibiotics as growth promoters. A preliminary investigation of *Salmonella* presence was performed by culture technique.

Chicken Management and Experimental Design

This study consisted of 3 experiments. Chicks, hatched in the hatchery of the Animal Science Unit (Gembloux Agricultural University, Gembloux, Belgium) and receiving the control wheat-based diet supplemented with 10^6 cfu/g of the probiotic strain *L. plantarum* CWBI-B659 (P) and control wheat-based diet supplemented with the combination of xylanase and probiotic (PE) diets, were sprayed at hatching with the cellular suspension of *L. plantarum* at 10^{10} cfu/mL by a manual vaporizer every hour during 8 h in the

Table 1. Composition of the basal diets

	Grower (0 to 14 d)		Finisher (15 to 42 d)	
Item	Experiment 1	Experiment 2/Experiment 3	Experiment 1	Experiment 2/Experiment 3
Ingredient (g/kg)				
Wheat[1]	592.8	600.0	580.8	600.0
Soybean meal	296.4	283.0	290.4	265.3
Soybean oil	64.5	68.8	87.5	94.0
Vitamin-mineral premix[2]	21.4	20.0	23.0	20.0
L-Lys HCl	2.8	3.5	1.2	1.6
DL-Met	1.4	1.7	0.8	1.3
L-Thr	0.3	0.8	—	0.3
Sodium chloride	1.6	1.3	1.7	0.9
Limestone	—	4.7	—	3.5
Dicalcium phosphate	18.8	16.2	14.5	13.1
Analyzed composition (g/kg of DM)				
DM (g/kg of diet)	882.9	885.9	882.9	884.6
CP (N × 6.25)	26.8	22.1	26.2	22.7
Crude fat	10.6	9.0	11.5	11.8
Crude fiber	4.5	2.7	3.8	2.8
AME[3] (kcal/kg of DM)	3,503	3,546	3,595	3,728
Calculated composition[4] (g/kg of DM)				
Starch	35.2	35.2	35.2	35.2
Met	0.6	0.6	0.5	0.5
Thr	0.7	0.7	0.7	0.6
Lys	1.2	1.2	1.1	1.0

[1]Cultivar Meunier, specific viscosity: 6.2 mL/g.

[2]Premix Leg 2% (Provimi B.V., Rotterdam, the Netherlands) provides (per kg of diet): vitamin A (retinyl acetate), 12,188 IU; cholecalciferol, 2,438 IU; vitamin E (DL-α-tocopheryl acetate), 18.3 IU; pantothenic acid, 42.6 mg; vitamin B_1, 1.2 mg; vitamin B_2, 7.3 mg; vitamin B_3, 9.7 mg; vitamin B_6, 1.2 mg; vitamin B_{12}, 0.024 mg; vitamin K_2, 1.2 mg; folic acid, 0.62 mg; choline chloride, 622.2 mg; calcium, 8,784 mg; phosphorus, 3,660 mg; sodium, 366 mg; magnesium, 36.6 mg; iodine, 0.59 mg; cobalt, 0.59 mg; copper, 2.42 mg; iron, 45.75 mg; manganese, 97.36 mg; zinc, 85.39 mg; selenium, 0.11 mg; methionine, 1,647 mg.

[3]Apparent metabolizable energy values were determined on the basis of gross energy, measured using an adiabatic bomb calorimeter (Parr 1241EF, Parr Instrument Company, Moline, IL).

[4]DSM, Deinze, Belgium.

hatchery environment. The chicks were wing-sexed, wing-banded, and housed in two 3-tier batteries, with each of the batteries containing a total of 18 cages. The combination of the 2 tiers at the same level on each battery represented 1 block. Chickens were placed in 39 × 98 × 62 cm (height × length × width) cages fitted out with a 5-cm square wire mesh bottom and plates underneath to collect excreta. All batteries were placed in a windowless house provided with forced ventilation. The temperature was controlled and gradually reduced from 33 to 20°C until 44 d of age. Chickens had 23 h of light per day during the whole experimental period to ensure frequent feed intake evenly distributed over 24 h. Water and experimental diets were offered for ad libitum consumption. Nine similar cages located in a separated room housed the noninfected chickens fed the C basal diets (C; negative control, **C−**) in identical rearing conditions. Detection of *Salmonella* according to the ISO 6579 standard (Anonymous, 2002) was performed in the house before the start of the experiment. The 3 in vivo trials were performed successively. The experimental protocols complied with the guidelines of the Animal Care and Use Committee (protocol FUSAGx03/03) of Gembloux Agricultural University.

Experiment 1. A total of 270 one-day-old male broiler chickens (Ross) were used. The experiment was carried out from 7 to 28 d of age. From 1 to 6 d of age, chicks were fed the starter basal diet. Confirmation of *Salmonella* absence according to the ISO 6579 standard (Anonymous, 2002) was performed in excreta at 2 d of age. Chicks were infected at 3 d of age by oral gavage with 10^8 cfu of *Salmonella* Typhimurium CWBI-B1199 per chick. A heat stress was used to intensify stress conditions for the birds and favor the intestinal tract colonization by the pathogen. Therefore, the rearing room temperature was reduced by 5°C during the day and restored during the night. This temperature program was applied the day before infection, the day of infection, and the day postinfection. At 7 d of age, 36 groups of 6 chicks with homogeneous weight were obtained by picking up a chick in each of 5-g weight classes, and the groups were randomly housed in batteries. The C, P, E, and PE experimental diets were randomly assigned to 3 cages per block (9 cages per dietary treatment), each cage representing 1 replicate. The C basal diet was distributed to 54 *Salmonella*-free chicks housed in 9 cages of the separated house with 6 chicks/cage. The chickens were weighed individually at 7 and 28 d of age. Feed intake per cage was recorded at the same time and feed conversion ratio (**FCR**) was calculated on a cage weight basis. To measure bacterial populations from the gut, 1 cage per block was randomly selected from each dietary treatment. At 19 d of age, 2 chickens per cage were killed by an intracardiac dose of Nembutal (Abbott Laboratories, Abbott Park, IL) at 2 mL/kg of live weight to collect aseptically 1 cecum per chicken.

Experiment 2. Experiment 2 was similar to experiment 1 with regard to the sex and number of chickens, the experimental design, the infection protocol with *Salmonella* Typhimurium, and the growth performance recording. To quantify *Salmonella* in excreta by conventional microbiological methods, excreta were collected aseptically from 4 cages selected randomly per treatment at 4, 9, 16, 23, and 30 d of age.

Experiment 3. Two hundred eighty-eight 1-d-old broiler chickens (Ross) were fed the starter basal diet from 1 to 6 d of age. At 7 d of age, they were randomly divided into 36 groups of 8 chicks each (4 males + 4 females) with homogeneous weight. Each group was assigned to a cage of the 3-tier batteries. The C, P, E, and PE experimental diets were randomly assigned to 3 cages per block (9 cages per treatment), each cage representing 1 replicate. The C basal diet was distributed to 72 *Salmonella*-free chicks housed in 9 cages of the separated house with 8 chicks (4 males + 4 females) per cage. The experiment was carried out from 7 to 42 d of age. Confirmation of *Salmonella* absence according to the ISO 6579 standard (Anonymous, 2002) was performed in excreta at 2 d of age. At 3 d of age, all of the chicks were orally inoculated with 1 mL of *Salmonella* Typhimurium CWBI-B1199 at 10^5 cfu/mL, with a heat stress as described above. The chickens were weighed individually at the beginning (7 d) and then weekly until the end (42 d) of the experiment. Feed intake by cage was recorded at the same time, and FCR was calculated on a cage weight basis. *Salmonella* quantification was performed by conventional microbiological methods in excreta collected aseptically from all of the 45 cages (9 cages × 5 diets) at 4, 11, 18, 25, 32, and 44 d of age. From 35 to 38 d of age, 6 of the 9 cages per treatment (2 cages per block, 6 × 8 chicks per treatment) were used to perform a balance trial. To determine the nutrient digestibilities and the AME of experimental diets, total collection procedure and ad libitum feeding were performed according to Bourdillon et al. (1990) with some modifications. The digestion balance trial started on d 29 with a 6-d adaptation period. During this period, birds were fed their respective experimental diets as during the performance trial. On d 35, after an 18-h fast, chicks were fed the experimental diets for 4 d, followed by an 18-h fast. Fresh diets and noningested food were dried for minimum of 24 h in an oven at 105°C. Excreta were quantitatively collected twice daily, pooled per cage for the whole experimental period, and stored at −20°C, before being freeze-dried.

Measurements

Analysis of Cecal Microbial Communities by Fluorescent In Situ Hybridization. Samples of the fresh cecal contents were homogenized and diluted 10-fold by weight in PBS (pH 7.5). To remove particulate material, the samples were centrifuged (700 × *g* for 3 min) and 1 mL of supernatant was treated with freshly prepared 4% paraformaldehyde to fix the cells. The samples were incubated overnight at 4°C, transported to the University of Groningen (Groningen, the Nether-

lands) on dry ice, and then stored at −80°C until they were analyzed.

For fluorescent in situ hybridization (**FISH**) analysis, samples from the ceca were assessed with general and group-specific 16S ribosomal RNA targeted oligonucleotide probes. Seven probes were used to enumerate bacterial groups: Eub338 for total bacteria (Amann et al., 1990); Chis150/Clit135 for the *Clostridium histolyticum* group and the *Clostridium lituseburense* group, which comprise *Clostridium perfringens* and *Clostridium difficile,* respectively (Franks et al., 1998); Lab158 for lactobacilli/enterococcci (Harmsen et al., 1999); Enfl3/Enfm2 for *Enterococcus excretais/Enterococcus faecium* (Waar et al., 2005); and Bif164 for *Bifidobacterium* spp. (Langendijk et al., 1995). The FISH method used was described previously by Harmsen et al. (2002). Briefly, 10 μL of diluted cell suspensions was applied to gelatin-coated slides and hybridized with 10 μL of each oligonucleotide probe (50 μg/mL of stock solution) in 110 μL of hybridization buffer overnight. The total number of cells was determined by staining with 4′,6-diamidino-2-phenylindole. Fluorescent cells were counted with an epifluorescence microscope (Leica DMRXA, Leica Camera AG, Solms, Germany; Jansen et al. 1999). Twenty-five fields were counted in duplicate for each sample.

Excreta Quantification of Salmonella Typhimurium. The counting method (cfu) of *Salmonella* in chicken excreta was based on direct plating on brilliant green agar (Biokar Diagnostics, Beauvais, France) without an enrichment step. Ten grams of fresh excreta sample was diluted 10-fold with buffered peptone water and mixed thoroughly. The samples were serially diluted and plated on brilliant green agar. The plates were incubated aerobically at 37°C for 18 to 22 h.

Balance Trial. Diets and excreta samples were ground through a 1-mm sieve and stored at 4°C until analysis. Apparent digestibilities of nitrogen, crude fat, crude fiber, DM, and the AME_n of the diets were estimated from the analyses of excreta and of distributed and noningested feeds, according to the methods of the AOAC (1990) as described above. For excreta, DM was obtained by freeze-drying the samples. Nitrogen in the excreta was calculated as total nitrogen minus nitrogen in the uric acid. Uric acid was analyzed by the method of Terpstra and de Hart (1974). For fat analysis by Soxhlet extraction, excreta samples were first hydrolyzed with 3 *M* HCl. The AME_n values were determined as described by Bourdillon et al. (1990).

Statistical Analysis

To test the effect of the experimental diets on the bird performance, the ANOVA was conducted with the GLM procedure of SAS (SAS Institute Inc., Cary, NC). A randomized complete block design was used, a single cage representing the experimental unit (replicate) for FCR, daily weight gain (**DWG**), and digestive parameters. Experimental performance data were analyzed by 3-way ANOVA, including the effect of the experimental diet (n = 5), the block (n = 3), and any interactions when appropriate. The initial weight (d 7) of the broiler chicks in the performance experiments was used as covariate in the model. A 2-way ANOVA (diet × block) was applied for results of the balance trial. The results were presented as means and SEM calculated by standard procedures. Means were compared using the Newman-Keuls test (Dagnelie, 1996) and considered significant at $P < 0.05$. Microbiological data for *Salmonella* Typhimurium concentration and FISH analysis were performed using the MIXED procedure (SAS Institute Inc.). For data collected over time (*Salmonella* Typhimurium concentration), a repeated-measures-in-time analysis was conducted, and the smallest value of the Akaike's information criteria was used to select the most appropriate covariance structure. Effects in the model included time and treatment as fixed factors, cage as a random factor, and treatment × time interaction. When treatment × time interactions were significant, variables were analyzed within time periods. Differences among treatments were considered significant when $P < 0.05$.

RESULTS

The C, E, P, and PE experimental diets were negative for *Salmonella* for the whole period of the 3 experiments (data not shown). The uninoculated 2-d-old chicks were also *Salmonella*-free before the start of the 3 in vivo trials. No clinical signs were recorded in any of the birds in experiment 1, 2, or 3.

Experiment 1: Growth Performance and Microflora Measurements

The effects of block and of diet × block interaction on growth performance were not significant and only *P*-values for diet effect were reported in Table 2. The infection pattern with *Salmonella* Typhimurium CWBI-B1199 led to a significant decrease of the DWG of control infected birds by 32.8% (43.2 vs. 64.3 g/bird per day), in comparison with uninfected birds. In the same way, the FCR of infected chickens fed the control basal diet was increased by 19.7% (1.40 vs. 1.17) after infection.

The *L. plantarum* strain and the xylanase preparation, added separately to the C basal diet fed to infected chickens (positive control; **C+**), significantly increased the DWG of infected birds from 43.2 g/bird per day to 45.9 and 46.8 g/bird per day, with similar mean improvement for the 2 additives. The FCR was significantly improved by probiotic or xylanase supplementation, with a mean reduction of FCR values by 6.4% (1.31 vs. 1.40) in comparison with infected birds fed the C+ control diet. Combination of *L. plantarum* CWBI-B659 strain and xylanase Belfeed B1100MP significantly increased growth rate by 12.5% (48.6 vs. 43.2

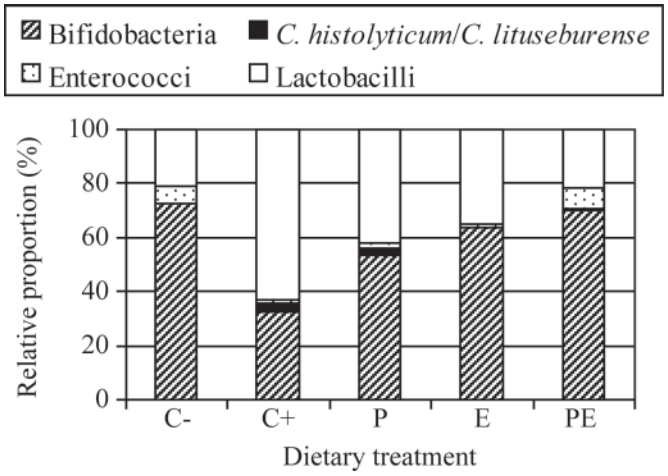

Figure 1. Relative proportions in regard to total hybridized cells of microbial communities from ceca of 19-d-old chicken, according to the dietary treatment (n = 4). C− = control wheat-based diet fed to noninfected chickens; C+ = control wheat-based diet fed to infected chickens; E = control wheat-based diet supplemented with 0.1 g/kg of the xylanase Belfeed B1100MP (Beldem S.A., Andenne, Belgium); P = control wheat-based diet supplemented with 10^6 cfu/g of the probiotic strain *Lactobacillus plantarum* CWBI-B659; PE = control wheat-based diet supplemented with the combination of xylanase and probiotic. Composition *P*-values for the treatment effect were as follows: bifidobacteria, $P = 0.1034$; *Clostridium histolyticum*/*Clostridium lituseburense*, $P < 0.0001$; enterococci, $P = 0.0004$; lactobacilli, $P = 0.0221$.

g/bird per day) and decreased FCR by 8.6% (1.28 vs. 1.40), in comparison with nontreated infected birds.

From 4 to 19 d of age, FISH enumeration in cecal samples, reported as relative proportion of the total hybridized cells in Figure 1, showed differences between the treatments for clostridia ($P < 0.0001$), lactobacilli ($P = 0.0221$), enterococci ($P = 0.0004$), and bifidobacteria ($P = 0.1034$). Until 19 d of age, *Salmonella* infection, in comparison with uninfected chickens, increased the relative proportion of lactobacilli and clostridia genera by 43.8% ($P = 0.0412$) and 2.31% ($P = 0.0529$), respectively, in relation to total hybridized cells. In contrast, the relative proportion of bifidobacteria population decreased by 37.2% ($P = 0.0785$) after *Salmonella* Typhimurium infection. Otherwise, *Salmonella* reduced the relative proportion of *E. faecium*/*Enterococcus faecalis* by 0.07% ($P = 0.0003$) only, in comparison with uninfected broilers. The probiotic and enzyme treatments, alone or combined, reversed nearly completely the effect of contamination. Bacteria relative proportions observed for the PE treatment were not significantly different from the uninfected birds. In contrast, the P and E treatments showed significant differences in comparison with C− birds while not being significantly different from C+ birds. The combination of xylanase and *L. plantarum* increased the relative proportion of bifidobacteria by 36.7% and decreased the relative proportion of lactobacilli by 42.9% in comparison with C+ treatment. Furthermore, the relative proportions of clostridia and *E. faecium*/*E. faecalis* were also similar to those in the healthy broilers, with an increase of 6.1% at 19 d of age of relative proportion of enterococci in relation to total hybridized cells by xylanase-*L. plantarum* supplementation, as compared with infected C+ chickens.

Experiment 2: Growth Performance and Mean Concentration of Salmonella Typhimurium in Excreta

The effects of block and of diet × block interaction on growth performance were not significant and only *P*-values for diet effect were reported in Table 3. Infection of 3-d-old chicks with *Salmonella* Typhimurium CWBI-B1199 had a significant detrimental effect on growth performance of infected birds, with a 23.6% decrease (46.4 vs. 60.7 g/bird per day) of DWG and a 2.4% increase (1.45 vs. 1.36) of FCR in comparison with uninfected animals.

The *L. plantarum* strain and the xylanase preparation, added separately to the C+ basal diet, had no

Table 2. Growth performance from 7 to 28 d of age of male broiler chickens infected with 10^8 cfu *Salmonella* Typhimurium CWBI-B1199 according to the dietary treatment in experiment 1

	Dietary treatment[1]						
Item	C−	C+	E	P	PE	SEM	*P*-value
BW[2]							
Initial weight (g/bird), 7 d	134.7[b]	138.7[a]	143.3[a]	130.3[b]	133.6[b]	2.08	0.0009
Final BW (g/bird), 28 d	1,437[a]	986[c]	1,065[b]	1,083[b]	1,145[b]	27.3	<0.0001
DWG[3] (g/bird per day)	64.3[a]	43.2[d]	46.8[bc]	45.9[c]	48.6[b]	1.62	<0.0001
Feed consumption[2]							
Feed intake (g of DM/bird per day)	78.9[a]	60.9[b]	60.7[b]	61.1[b]	65.8[b]	1.55	<0.0001
FCR[4] (feed intake/DWG)	1.17[c]	1.40[a]	1.31[b]	1.31[b]	1.28[b]	0.017	<0.0001

[a–d]Means within a row lacking a common superscript are significantly different ($P < 0.05$).

[1]C− = control wheat-based diet fed to noninfected chickens; C+ = control wheat-based diet fed to infected chickens; E = control wheat-based diet supplemented with 0.1 g/kg of the xylanase Belfeed B1100MP (Beldem S.A., Andenne, Belgium); P = control wheat-based diet supplemented with 10^6 cfu/g of the probiotic strain *Lactobacillus plantarum* CWBI-B659; PE = control wheat-based diet supplemented with the combination of xylanase and probiotic. Composition of the control wheat-based diet is given in Table 1. From 1 to 6 d of age, chickens were fed the starter control diet.

[2]Each mean represents 9 cages with 6 birds each.

[3]DWG = daily weight gain.

[4]FCR = feed conversion ratio.

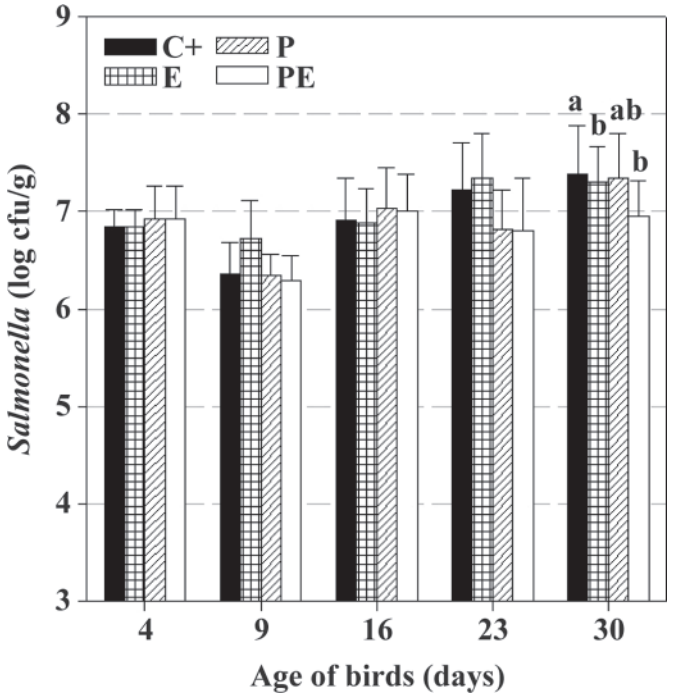

Figure 2. *Salmonella* Typhimurium concentration in excreta at different times of male broiler chickens infected with 10^8 cfu *Salmonella* Typhimurium CWBI-B1199 (experiment 2). C+ = control wheat-based diet fed to infected chickens; E = control wheat-based diet supplemented with 0.1 g/kg of the xylanase Belfeed B1100MP (Beldem S.A., Andenne, Belgium); P = control wheat-based diet supplemented with 10^6 cfu/g of the probiotic strain *Lactobacillus plantarum* CWBI-B659; PE = control wheat-based diet supplemented with the combination of xylanase and probiotic. Bars represent mean log colony-forming units per gram of excreta content, and error bars indicate the SD. For a given time, the values marked with a letter above the bars are significantly different from the control group ($P < 0.05$).

significant effect on DWG of infected birds. The FCR was significantly improved from 1.45 to 1.42 with xylanase or xylanase combined with probiotic supplementation, in comparison with infected birds fed the C+ control diet. The *L. plantarum* CWBI-B659 strain had no significant effect on the FCR, in comparison with nontreated infected birds.

The concentration results of *Salmonella* in excreta of chickens challenged with *Salmonella* Typhimurium CWBI-B1199 (Figure 2) showed that the combination of xylanase and *L. plantarum* CWBI-B659 was the most effective to significantly reduce the concentrations of *Salmonella*. At 30 d of age, the PE treatment reduced ($P = 0.0116$) the *Salmonella* concentration from 7.39 log colony-forming units per gram to 6.90 log colony-forming units per gram in comparison with the control C+.

Experiment 3: Growth Performance, Mean Concentration of Salmonella Typhimurium in Excreta, and Nutrient Digestibilities

The effects of block and of diet × block interaction on growth performance were not significant and only *P*-values for diet effect were reported (Table 4). In comparison with uninfected birds fed the C− basal diet, infection of 3-d-old chicks with 10^5 cfu *Salmonella* Typhimurium/chick induced, at 42 d, a significant decrease of the DWG by 29.8% (43.9 vs. 62.5 g/bird per day) and a significant increase of the FCR by 1.0% (2.01 vs. 1.99) of infected chickens fed the control basal diet.

The *L. plantarum* strain and the xylanase preparation, added separately to the C+ basal diet, did not significantly improve the growth performance, in comparison with birds fed the C+ diet. In contrast, the *L. plantarum* CWBI-B659 strain combined with the xylanase Belfeed B1100MP significantly improved the FCR by an average of 4.0% (1.93 vs. 2.01) in comparison with birds fed the C+ diet. The FCR observed with the PE diet was also significantly decreased by 4.4% (1.31 vs. 1.37) and 3.0% (1.93 vs. 1.99), at 28 and 42 d of age, respectively, in comparison with FCR values of noninfected chickens given the C− basal diet.

Table 3. Growth performance from 7 to 28 d of age of male broiler chickens infected with 10^8 cfu *Salmonella* Typhimurium CWBI-B1199 according to the dietary treatment in experiment 2

	Dietary treatment[1]						
Item	C−	C+	E	P	PE	SEM	*P*-value
BW[2]							
Initial weight (g/bird), 7 d	145.5^b	156.8^a	157.3^a	143.5^b	144^b	1.2	<0.0001
Final BW (g/bird), 28 d	1,417^a	1,103^b	1,085^b	1,046^b	1,092^b	25.9	<0.0001
DWG[3] (g/bird per day)	60.7^a	46.4^b	45.7^b	44.6^b	46.3^b	1.04	<0.0001
Feed consumption[2]							
Feed intake (g of DM/bird per day)	84.6^a	66.8^b	63.9^b	64.2^b	64.5^b	1.76	<0.0001
FCR[4] (feed intake/DWG)	1.36^c	1.45^a	1.42^b	1.46^a	1.42^b	0.008	<0.0001

$^{a–c}$Means within a row lacking a common superscript are significantly different ($P < 0.05$).

[1]C− = control wheat-based diet fed to noninfected chickens; C+ = control wheat-based diet fed to infected chickens; E = control wheat-based diet supplemented with 0.1 g/kg of the xylanase Belfeed B1100MP (Beldem S.A., Andenne, Belgium); P = control wheat-based diet supplemented with 10^6 cfu/g of the probiotic strain *Lactobacillus plantarum* CWBI-B659; PE = control wheat-based diet supplemented with the combination of xylanase and probiotic. Composition of the control wheat-based diet is given in Table 1. From 1 to 6 d of age, chickens were fed the starter control diet.

[2]Each mean represents 9 cages with 6 birds each.

[3]DWG = daily weight gain.

[4]FCR = feed conversion ratio.

Table 4. Growth performance from 7 to 42 d of age and nutrient digestibilities of a mixed population of broiler chickens infected with 10^5 cfu *Salmonella* Typhimurium CWBI-B1199 according to the dietary treatment in experiment 3

	Dietary treatment[1]						
Item	C−	C+	E	P	PE	SEM	*P*-value
BW[2]							
Initial weight (g/bird), 7 d	112.6[a]	89.1[d]	99.7[b]	96.2[c]	97.0[c]	0.48	<0.0001
Final BW (g/bird), 42 d	2,350[a]	1,572[b]	1,648[b]	1,672[b]	1,605[b]	49.2	<0.0001
DWG[3] (g/bird per day)							
28 d	48.9[a]	33.5[b]	33.6[b]	33.9[b]	34.4[b]	0.51	<0.0001
42 d	62.5[a]	43.9[b]	44.3[b]	44.8[b]	45.2[b]	0.64	<0.0001
Feed consumption[2]							
Feed intake (g of DM/bird per day)							
28 d	68.1[a]	46.4[c]	45.6[d]	47.9[b]	46.2[c]	0.13	<0.0001
42 d	123.7[a]	87.9[b]	87.3[b]	90.3[b]	87.2[b]	0.71	<0.0001
FCR[4] (feed intake/DWG)							
28 d	1.37[b]	1.37[b]	1.36[b]	1.40[a]	1.31[c]	0.006	<0.0001
42 d	1.99[b]	2.01[a]	1.97[b]	2.02[a]	1.93[c]	0.006	<0.0001
Digestibility[5] (%)							
Crude fiber	15.49[c]	20.77[abc]	26.22[a]	17.03[bc]	22.61[ab]	1.996	0.012
Crude fat	89.01[a]	86.92[a]	79.57[b]	78.34[b]	78.95[b]	0.863	<0.0001
DM	80.69	75.49	80.51	79.53	80.28	1.939	0.3893
Protein	85.12	59.93	74.17	66.37	78.56	5.940	0.0638
AME_n (kcal/kg of DM)	3,906	3,630	3,890	3,815	3,855	81.04	0.2837

[a–d]Means within a row lacking a common superscript are significantly different ($P < 0.05$).

[1]C− = control wheat-based diet fed to noninfected chickens; C+ = control wheat-based diet fed to infected chickens; E = control wheat-based diet supplemented with 0.1 g/kg of the xylanase Belfeed B1100MP (Beldem S.A., Andenne, Belgium); P = control wheat-based diet supplemented with 10^6 cfu/g of the probiotic strain *Lactobacillus plantarum* CWBI-B659; PE = control wheat-based diet supplemented with the combination of xylanase and probiotic. Composition of the control wheat-based diet is given in Table 1. From 1 to 6 d of age, chickens were fed the starter control diet.

[2]Each mean represents 9 cages with 8 birds (4 males + 4 females) each.

[3]DWG = daily weight gain.

[4]FCR = feed conversion ratio.

[5]Each mean represents 6 cages with 8 birds (4 males + 4 females) each.

Analysis of the digestibility data revealed that chickens infected with *Salmonella* Typhimurium (treatment C+) had lower digestibilities of DM (75.5 vs. 80.7%), crude fat (86.9 vs. 89%, $P < 0.05$), and a reduced AME_n (3,860 vs. 4,098 kcal/kg of DM) of the diet compared with noninfected chickens (treatment C−; Table 4). Protein digestibility was also reduced, but in spite of a very large difference between digestibility values of infected and uninfected birds (59.9 vs. 85.1%), there was no significant difference between the treatments ($P = 0.0638$). Otherwise, infected chickens showed a higher digestibility of crude fiber by 34.1% (20.77 vs. 15.49%), in comparison with healthy birds.

Chickens fed the xylanase-supplemented diet improved digestibilities of crude fiber, DM, protein, and the AME_n by 26.2% (26.22 vs. 20.77%), 6.7% (80.51 vs.75.49%), 23.8% (74.17 vs. 59.93%), and 6.5% (3,890 vs. 3,630 kcal/kg of DM), respectively, as compared with the control birds fed the C+ diet. The probiotic strain *L. plantarum* CWBI-B659 added to the basal diet did not significantly affect the digestibilities of DM, protein, crude fiber, and AME_n. The PE diet did not significantly improve the digestibilities of crude fiber, DM, and AME_n, but significantly increased protein digestibility by 31.1% (78.56 vs. 59.93%), in comparison with the C+ treatment. All of the dietary treatments led to a significantly decreased digestibility of crude fat by 8.5 to 9.9% (78.34 to 79.57 vs. 86.92%), in comparison with the birds receiving the C+ diet.

The measurement of *Salmonella* Typhimurium CWBI-B1199 concentration in excreta by microbiological methods showed variable results from 4 to 32 d of age. Moreover, the *Salmonella* concentration in excreta from 32 to 44 d of age increased by more than 1 log colony-forming unit per gram, whatever the dietary treatment. At 44 d of age, *Salmonella* concentration in excreta was 6.91 log colony-forming units per gram for the control and decreased to 5.06 log colony-forming units per gram, for the chickens fed the PE diet, which was similar for the P treatment, whereas the xylanase supplemented alone to the wheat-based diet showed a negligible reduction, in comparison with control chickens (Figure 3).

DISCUSSION

Colonization of the chicken gastrointestinal tract by *Salmonella* strains produces a strong inflammatory response (Kaiser et al., 2000), associated with harmful effects to the integrity of the intestinal epithelium (Desmidt et al., 1998), that might potentially induce detrimental outcomes on nutrient digestibilities and growth performance. The effect of *Salmonella* colonization on performance was reported by few authors (Nakamura et al., 2002; Gupta et al., 2005) and was confirmed in this study. Experimental infection of broiler 3-d-old chicks with *Salmonella* Typhimurium induced a very pronounced reduction of performance by 20 to

30% for DWG and 2 to 20% for the FCR, which is seldom observed in industrial production. However, challenge models described in literature are generally carried out with high infection doses to study live bird interventions, and that may explain the marked effect observed in this study. Moreover, reported differences in infection effects on growth performance may be due to various infection doses and virulence of the pathogen strain used. Reduced growth performance is related to the decreased feed intake by infected chickens, as observed in this study. Because bacterial pathogens like *Salmonella* have been postulated to cause mucosal damage by different mechanisms (Finlay and Cossart, 1997; Desmidt et al., 1998), *Salmonella* colonization of the intestinal gut at high infection dose might consequently impede nutrient absorption and digestion.

The negative effect of *Salmonella* infection on chicken performance may be related to a reduced diet absorption after pathogen invasion. Regarding the diet digestion, the results of this study pointed out a modification of nutrient digestibilities after *Salmonella* contamination. The competition by *Salmonella* with the host, combined to the inflammatory response and the activation of the immune system, has an energy cost that may lead to reduced energy for bird maintenance and growth at similar AME values of the diets. Moreover, until 19 d of age, *Salmonella* infection reduced the relative proportions of bifidobacteria and enterococci in comparison with normal microflora of uninfected chickens. Modification in the enteric population balance, like the increase of *C. perfringens* numbers, was shown to be directly related to the reduction of growth performance in chickens (Fuller, 1984; Bomba et al., 2006).

Exogenous xylanases are commonly added to wheat-based broiler diets to reduce the antinutritional effects of such rations due to the high content of AX in wheat. The antinutritional effect of wheat AX has been shown to be correlated mainly to the entrapment of nutrients in the polysaccharides structure, the so-called cage effect (Simon, 2000), and to the increased viscosity of the intestinal content in the lumen, in relation with increased bacterial populations of the gut (Mathlouthi et al., 2002; Choct et al., 2004).

By disrupting AX polymers, endoxylanases such as the Belfeed B1100MP may consequently make the nutrients more accessible for endogenous digestive enzymes, increasing the efficiency of diet digestion as observed by García et al. (2008) and Gutierrez del Alamo et al. (2008). However, these experiments were carried out with uninfected birds, whereas *Salmonella* infection may reduce the apparent digestibilities and AME_n so that the effect of exogenous enzyme in our study appeared to be more important than in these studies. Xylanase supplementation appeared particularly to exert a significant effect on the digestibility of crude fiber, which includes the AX fraction of the diet, in comparison with the uninfected birds. In contrast, the enzyme as well as the probiotic exerted a significant detrimental effect on fat digestibility, in comparison with the control diet fed to contaminated chickens. Because Belfeed B1100MP was shown to improve fat digestibility in uninfected birds (our unpublished data), decreased digestibility with the feed additives may be related to interaction with *Salmonella* contamination and increased hydrolysis activity of bile acids, as shown for the *L. plantarum* strain (Roblain et al., 2002).

Moreover, xylanases may exert part of their response from changes in relative microbiota populations. By reducing the digesta viscosity, exogenous enzyme may lower total bacterial counts in intestinal contents, as already reported by several authors (Hübener et al., 2002; Mathlouthi et al., 2002), and especially gram-positive cocci and enterobacteria (Vahjen et al., 1998; Hübener et al., 2002) or populations of undesirable organisms such as *C. perfringens* (Hübener et al., 2002), which was observed in this study with the xylanase alone or in combination with the *Lactobacillus* strain. *Clostridium perfringens* infection of broiler chickens may cause subclinical or clinical disease associated with necrotic enteritis (Johansson, 2006) and thickening of the intestinal epithelium that may induce reduction of nutrient absorption (Mead, 2000). At the same time, degradation of AX by xylanase activity releases mono- and oligosaccharides in the intestinal lumen that may be fermented in the ceca by specific bacterial species.

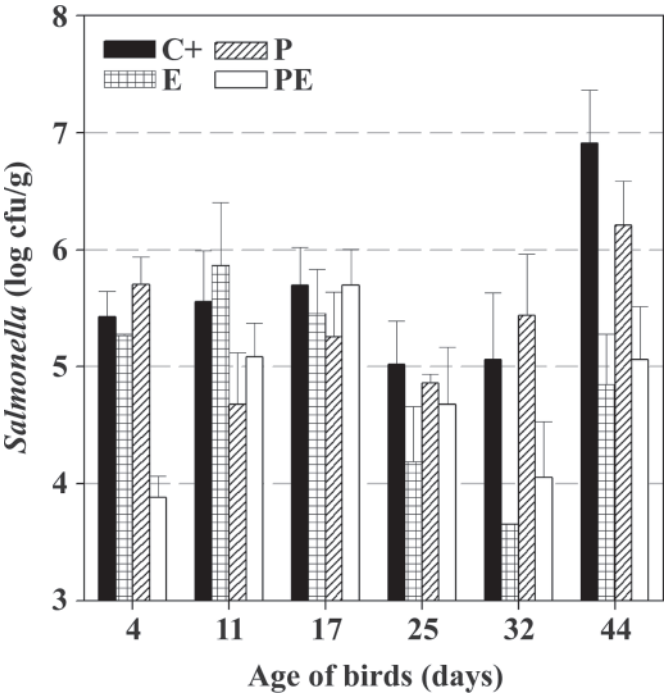

Figure 3. *Salmonella* Typhimurium concentration in excreta at different times of a mixed population of broiler chickens infected with 10^5 cfu *Salmonella* Typhimurium CWBI-B1199 (experiment 3). C+ = control wheat-based diet fed to infected chickens; E = control wheat-based diet supplemented with 0.1 g/kg of the xylanase Belfeed B1100MP (Beldem S.A., Andenne, Belgium); P = control wheat-based diet supplemented with 10^6 cfu/g of the probiotic strain *Lactobacillus plantarum* CWBI-B659; PE = control wheat-based diet supplemented with the combination of xylanase and probiotic. Bars represent mean log colony-forming units per gram of excreta content, and error bars indicate the SD.

The development of these specific AX-fermenting populations (i.e., enterococci; Hübener et al., 2002) may lead to an increase of fatty acid production and a reduction of pH values (Engberg et al., 2004), which may inhibit the growth of gram-negative pathogen bacteria such as *Escherichia coli* (Mathlouthi et al., 2002) or *Salmonella*. In this study, bifidobacteria were shown to be favored by xylanase alone or combined with the *L. plantarum* strain. Courtin et al. (2008) showed that arabinoxylooligosaccharides produced by AX hydrolysis increased the number of bifidobacteria in the ceca of broilers. The increased number of bifidobacteria in the cecum of chicken may be able to decrease the population of *Salmonella*. Indeed, bifidobacteria are known to stimulate butyric acid production in the ceca (Belenguer et al., 2006), which has a detrimental effect on pathogen colonization in young chickens. Because xylanase appeared to be effective on *Salmonella* counts only when the infection dose was low (experiment 3 vs. experiment 2), it is hypothesized that such enzyme-favored specific bacterial communities might only exert efficient competitive inhibition when the pathogen population is limited. The analysis of intestinal bacterial populations by the FISH technique demonstrated that the different dietary additives modified the microflora. However, further investigations have to be carried out with higher numbers of samples and targeted bacteria species to confirm the potential effect of the xylanase alone or with the probiotic. The denaturing gradient gel electrophoresis technique, for example, may help to evaluate the overall intestinal microbial profile (Hume et al., 2003), rather than focusing on a few specific microorganisms.

Among the beneficial effects described for probiotic strains, the most important is the prevention or the reduction of intestinal colonization by bacterial pathogens. Briefly, Mead (2000) proposed 4 mechanisms by which probiotic cultures are able to exclude enteric pathogens: competition for receptor sites, competition for limiting nutrients, production of bacteriocins, and production of volatile fatty acids that may inhibit certain enteric pathogens. Several studies have showed that the growth of Enterobacteriacae like *Salmonella* spp. was inhibited in vitro (Tsai et al., 2005) and in vivo (Higgins et al., 2007; Wolfenden et al., 2007) in the presence of *Lactobacillus* strains. Higgins et al. (2007) showed that oral inoculation of 7.5×10^5 cfu of a culture of a probiotic mixture, based on 11 lactic acid bacteria, induced a 2.4 $\log_{10}$ reduction of *Salmonella* in ceca of 1-d-old chicks inoculated with 4.5×10^3 cfu of *Salmonella* Enteritidis. However, oral gavage is not a practical way of administration in commercial hatcheries or poultry houses. Wolfenden et al. (2007) showed that treating day-of-hatch chicks challenged with 10^4 cfu/chick of *Salmonella* Enteritidis with the same *Lactobacillus*-based probiotic by spray at 10^7 cfu/mL or in the drinking water at 10^6 cfu/mL during 5 d postchallenge induced a significant reduction of *Salmonella* Enteritidis recovery for 15 to 65% in comparison with the controls. In this study, the beneficial action of the *L. plantarum* probiotic on pathological effects induced by *Salmonella* infection was only significant when the strain was combined with the xylanase Belfeed B1100MP in the wheat-based diet. It was hypothesized that the survival and the growth of the *L. plantarum* strain was favored by the xylanase activity, in the same way as other bacterial populations with probiotic potential like bifidobacteria. The enhanced growth of probiotic strains may correspond to increased specific bacterial activities, leading to higher antagonistic effects against *Salmonella* (Jin et al., 1997), to reduction of intestinal pathogen colonization, and to decreased subsequent detrimental effects on growth performance. The upsurge of excreta *Salmonella* Typhimurium concentrations at 44 d of age observed in experiment 3, whatever the dietary treatment applied, may be explained by adaptation of specific strains (Prouty et al., 2004) and by increased pH values in the intestinal chyme, which may partly suppress the mechanisms of *Salmonella* inhibition by *Lactobacillus* strains (Fayol-Messaoudi et al., 2005).

In conclusion, the results presented here revealed a potential complementary effect between a *L. plantarum* probiotic strain and an exogenous xylanase added to a wheat-based diet for broiler chickens experimentally infected at 3 d of age with *Salmonella* Typhimurium. This complementary beneficial effect was partly expressed by FCR improvement and by reduction of *Salmonella* Typhimurium concentrations in excreta, which is more pronounced than with both additives supplemented alone. This complementary action was hypothesized to result mainly as well from reduced overall bacterial populations in the gut, which may decrease nutrient competition with the host, as from changes in composition and metabolic potential of bacterial populations toward specific genera, like enterococci, with notably probiotic properties. To increase the knowledge about the complementary mechanisms of xylanase and *L. plantarum* strain, further research could be conducted by analyzing the ileal microflora and by following AX degradation and environmental conditions in the gastrointestinal tract.

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