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Lactobacillus Pentosus Ita23 and L. Acidipiscis Ita44 Enhance Feed Conversion Efficiency and Beneficial Gut Microbiota in Broiler Chickens

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ABSTRACT

Although the use of probiotics especially *Lactobacillus* and *Bifidobacteria* species on growth and feed utilization in poultry production has been extensively studied, the results were inconsistent presumably because the mode of action of probiotic is multi-factorial and each probiotic strain may affect the host in a specific manner. This study investigated the probiotic effect of two strains of *Lactobacillus* (*Lactobacillus pentosus* ITA23 and *Lactobacillus acidophilus* ITA44; 10^9 cells/kg feed) isolated from mulberry (*Morus Alba*) silage on the growth performance, cecal microbial population, and blood cholesterol of broiler chickens. One hundred twenty male broiler chicks (Cobb500) were randomLy allocated to two groups (control and treatment) of six replicates (cages) with 10 chicks per cage. Chicks in the control group received a standard diet and those in the treatment group received the same diet supplemented with 10^9 cells of the above *Lactobacillus* per kg feed. Supplementation of *Lactobacillus* did not affect body weight gain (averaged 1604 g at 35 days old) but feed conversion ratio improved ($p<0.01$) by 6.4% due to reduction in feed intake ($p<0.01$) by birds in the treatment group. Supplementation also increased the population of *Lactobacillus* spp. and reduced pathogens *E. coli* in the cecal samples. Although *Lactobacillus* supplementation tends to reduce serum total cholesterol, low-density lipoprotein (LDL) and triglyceride concentrations, these values were not significantly different from those of the control group. Results of this study showed that *L. pentosus* ITA23 and *L. acidophilus* ITA44 are potential probiotics to be used in poultry diets.

INTRODUCTION

The biggest challenge of present-day commercial poultry production is the availability of quality feed at sustainable and stable price (Lyayi, 2008). Most of of the feed ingredients used for commercial poultry production in many newly developed and developing countries in Asia are imported from different sources depending on their availability and price, and often at the sacrifice of quality. Thus, many approaches, including the use of growth promoter additives (including antibiotics), are used to promote growth and feed conversion efficiency to ensure profitability (Pelicano *et al*., 2002). In view of the severe restriction or total ban on the use of antibiotics as growth promoters in livestock and poultry production, probiotics have been suggested as an alternative to antibiotics (Ahmad, 2006). A probiotic is a live microbial feed supplement that is beneficial to the host animal by inhibiting pathogens to allow the growth of beneficial gut microflora (Jin *et al*, 1998; Ghadban, 2002). The proposed mechanisms of pathogen inhibition by the probiotic microorganisms include competition for nutrients, production of

antimicrobial conditions (e.g. low pH) and compounds (bacteriocins), competition for binding sites on the intestinal epithelium, and stimulation of the immune system (Rolfe, 2000). The diversity of possible modes of action thus suggests that the efficacy of probiotics is strain specific and has to be demonstrated for each strain (Shinde, 2012).

Lactobacillus spp is one of the most extensively studied genera of beneficial bacteria used as probiotics. Another important role of probiotic bacteria is their ability to metabolically influence serum cholesterol and triglyceride levels (Lin *et al*.1989; Taranto *et al.* 1998). The above property of probiotic has been more intensely studied in relation to human. However, there is evidence suggesting that *Lactobacillus* feed supplementation can reduce cholesterol and fatty acid composition of broiler chickens (Kalavathy *et al*., 2006). The purpose of this study was to investigate the probiotic efficacy of two strains of *Lactobacillus*, namely *L. pentosus* ITA23 and *L. acidipiscis* ITA44 which were recently isolated from Mulberry (*Morus Alba*) silage in our laboratory.

MATERIALS AND METHODS

Probiotic

The probiotic efficacy of *L. pentosus* ITA23 and *L. acidipiscis* ITA44, isolated from mulberry (*Morus Alba*) silage, was examined in this study. These isolates have been shown to possess high acid and bile tolerance, antioxidant activity, and ability to produce fatty acids (unpublished data). The probiotics were prepared by culturing each inoculum in sterilized MRS broth (Merck, Germany) at 37 °C for 24 h. After incubation, the culture was centrifuged at 6000 rpm for 6 minutes after which the pellet was washed with distilled water by centrifugation at 8000 rpm for 12 minutes. The pellets containing the probiotic cells was freeze dried, grinded, and stored at -80°C until further use. For the feeding trial, the pellets from both *Lactobacillus* (each one containing 10^9 cells/g) were mixed (1:1) before it was added to the feed.

Experimental design

Birds were cared in accordance to the Animal Care and Use Protocol of the Universiti Putra Malaysia Animal Care and Use Committee. One hundred twenty day-old male broiler chicks (Cobb500) from a commercial hatchery in Malaysia were used for the study. The chicks were randomLy allocated to two treatments with six replicates of 10 birds per cage (replicate). Birds in the control group received a standard diet while the second group (probiotic) received similar diet containing 0.1% of a mixture of *L. pentosus* ITA23 and *L. acidophilus* ITA44; 10^9 cells/kg feed. Birds were fed a starter diet for the first 21days and a finisher diet from days 22 to 35 formulated to meet or exceed their nutrient requirements of NRC (1990) (Table 1). Clean drinking water was offered *ad libitum* during the experimental period. Birds were inspected daily to ensure their welfare, and mortality was recorded when it occurred. They were weighed weekly on cage basis throughout the experimental period to determine average body weight (BW) and body weight gain (BWG). Feed intake (FI) per cage was recorded weekly, and weekly feed conversion ratio (FCR) was calculated as FI/BWG.

Table 1 – Ingredient composition and nutrient content of the basal diet

Ingredient (g/kg unless otherwise stated)	Starter (1 to 21 days)	Finisher (22 to 35days)
Ground yellow corn	538.9	603.0
Soybean meal	361.9	305.6
Fish meal	30.0	30.0
Palm oil	37.4	37.4
60% choline chloride	2.5	2.0
Trimix[1]	1.0	1.0
Salt (Na Cl)	2.0	1.0
DL-methionine	1.8	0.4
Limestone	13.0	13.0
Dicalcium phosphate	11.5	6.5
Total	1000.0	1000.0
Calculated composition		
Crude protein	217.0	198.1
Crude fat	63.6	65.6
Crude fibre	38.0	27.8
Calcium	11	9.8
Phosphorus	4.5	3.5
Metabolisable energy (kcal/kg)	3000	3080

[1]Trimix (per kg Trimix): iron 100 g; manganese 110 g; copper 20 g; zinc 100 g; iodine 2 g; selenite 0.2 g; cobalt 0.6 g; santoquin 0.6 g; folic acid 0.33 g; thiamin 0.83 g; pyridoxine 1.33 g; biotin 2 % 0.03 g; riboflavin 2 g; cyanocobalamin 0.03 g; D-calcium pantothenate 3.75 g; niacin 23.3 g; retinol 2000 mg; cholecalciferol 25 mg; α-tocopherol 23,000 mg IU.

On day 35, birds were sacrificed individually by cervical dislocation and blood samples were collected for the determination of serum cholesterol level. The cecal content of two birds per replicate was collected

and stored in liquid nitrogen pending microbial DNA extraction and determination of bacterial population using Quantitative Real-Time PCR.

DNA extraction

DNA was extracted from cecal samples and pure cultures by using the QIAamp DNA Stool Mini Kit (Qiagen Inc., Valencia, CA) according to the manufacturer's protocols. The extracted DNA was stored at -20°C until use. The extracted DNA from pure cultures was used for the production of high concentration of target DNA using normal PCR and preparation of a standard curve. PCR products were purified using the MEGA quick-spin™ (Intron Biotechnology, Inc.), and the purity and concentration of DNA in each sample were measured using a Nanodrop ND-1000 spectrophotometer and number of copies of a template DNA per mL of elution buffer was calculated using the standard formula. Standard curves were constructed using serial dilutions of the PCR products from pure cultures of each bacterial group.

Quantitative Real Time PCR

The following specific primers (16S rRNA region) were used to quantify different bacteria populations according to the method of Navidshad *et al*. (2012):

For total microbes: F-5'-CGG CAA CGA GCG CAA CCC-3' and R-5'-CCA TTG TAG CAC GTG TGT AGC C-3' (Denman & McSweeney, 2006); for *Lactobacillus*: F-5'-CAT CCA GTG CAA ACC TAA GAG-3' and R- 5'-GAT CCG CTT GCC TTC GCA-3' (Wang *et al*., 1996), for E*scherichia coli* F-5'-GTG TGA TAT CTA CCC GCT TCG C-3' and R-5'-AGA ACG CTT TGT GGT TAA TCA GGA-3' (Frahm & Obst, 2003), For E*nterococcus* genus F-5'- CCC TTA TTG TTA GTT GCC ATC ATT-3' and R-5'-ACT CGT TGT ACT TCC CAT TGT-3' (Rinttila *et al*., 2004) and for *Enterobacter* : F- 5'-CAT TGA CGT TAC CCG CAG AAG AAG C-3' and R-5'-CTC TAC GAG ACT CAA GCT TGC-3' (Bartosch *et al*., 2004). Real-time PCR was performed with BioRad CFX96 Touch (BioRad, USA) using optical grade plates. The PCR reaction was performed on a total volume of 25 µL using the iQTMSYBR Green Supermix (BioRad, BioRad, USA). Each reaction included 12.5 µL SYBR Green Supermix, 1 µL of each Primer, 1 µL of DNA samples and 9.5 µL H_2O. The reaction conditions for DNA amplification were 94°C for 5 min, 40 cycles of 94°C for 20 s, 55°C, 58°C, or 60°C for 30 s for total microbes, *Lactobacillus* and other bacteria respectively, and 72°C for 20 s. To confirm the specificity of amplification, melting curve analysis was carried out after the last cycle of each amplification. The expected size of the amplified fragments were 145bp for total microbes, 341 bp for *Lactobacillus* group, 82 bp for E*scherichia coli*, 144 bp for E*nterococcus* genus, and 195 for *Enterobacter*, and were verified on a 2% (wt/vol) Agarose gel for 40 min at 80 V.

To calculate the amount of DNA in digesta samples, first, the calibration standards constructed by amplification of known amounts of target DNA were used to convert the Ct values into amounts of DNA. The estimated values were expressed as log^{10} of each bacteria cell number per gram of digesta.

Statistical analysis

Statistical analyses of the data were performed by comparing birds fed probiotic with the control for each parameter using a two-tailed *t*-test with unequal group variance using SAS Statistical Software (2008). The significance level was set at $p < 0.05$.

RESULTS AND DISCUSSION

Feed is the main component of the total production costs in the poultry industry and therefore, improving the efficiency of feed utilization is essential for profitability. Results of this study show that supplementation of the two *Lactobacillus* spp did not affect BWG, but significantly ($p<0.01$) improved FCR compared with the control feed. The FCR improvement is attributed the lower FI of birds in the *Lactobacillus* supplementation group (Table 2). This result is in agreement with some earlier studies (Kabir *et al*., 2004; Timmerman *et al.*, 2006), whose authors suggested that FCR improved in *Lactobacillus* supplemented birds because probiotic supplementation enhances the activity and release of digestive enzymes thus resulting in better digestion and absorption of nutrients.

Table 2 – Effect of probiotics (*Lactobacillus pentosus* ITA23 and *L. acidipiscis* ITA44) on the feed intake, body weight, weight gain, and feed conversion ratio in broiler chickens between 1 to 35 days of age.

Parameter	Treatment		Significant
	Control	Probiotic	
Feed intake (g)	3004.3^{a}±85.2	2829.3^{b}±33.9	**
Body weight (g)	1640.0±25.9	1656.6±29.1	NS
Weight gain (g)	1600.4±25.5	1607.1±28.6	NS
Feed conversion ratio	1.88^{a} ±0.06	1.76^{b}±0.02	**

Data are means ± standard deviation; NS: not significant ($p>0.05$); **: significant at $p<0.01$

In his review, Shinde (2012) reported that the current knowledge on the mode of action of probiotics is only superficial. Several modes of action have been proposed to explain for the beneficial effects of probiotics (Figure 1); however, these effects appear to be multi-factorial and each probiotic strain may affect the host in a specific manner. The positive effects of the *Lactobacillus* strains on FCR in this study could have resulted from a direct nutritional effect of the probiotic alone or in combination with a health effect, with the probiotic acting as a bio-regulator of the intestinal microbiota and reinforcing the host's natural defense systems (Shareef *et al*., 2009). Other authors, such as Anderson *et al* (1999) and Saulnier *et al.*, (2009), mentioned that the beneficial effects of the suppression of pathogenic gut microbiota by probiotics include reduced nutrient utilization by the pathogenic microorganisms and thus more nutrients are made available for the host, as well decreased levels of toxic microbial metabolites that interfere with the host's growth.

Lactobacilli comprise of a large and diverse group of gram positive, non-spore forming, catalase negative-rod bacteria, which are capable of producing lactic acid as the main end-product of carbohydrate fermentation (Pelinescu *et al*., 2009). By producing acids (such as acetic and lactic acids) and other compounds that inhibit the growth of toxin-producing pathogenic bacteria, lactobacilli and other useful bacteria are considered as probiotics (Suskovic *et al.* 2010; Shokryazdan *et al.* 2014). As mentioned above, one of the main features of lactobacilli is their ability to produce lactic acid that reduces the pH of intestinal tract. Low pH environments favor the growth of gram positive bacteria (such as *Bifidobacteria* and *Lactobacillus*) but not of gram negative pathogenic microbes, such as *E-coli* and *Salmonella*, because of the sensitivity of their cell membrane in the acidic condition (Suskovic *et al*., 2010). In addition, by suppressing the production of toxic compounds that may change the morphology of the intestinal wall and reduce the colonization of pathogens on the intestinal wall, probiotics prevent damage to the epithelial cells (Saulnier *et al.*, 2009, Shokryazdan *et al.* 2014) allowing better absorption. The dietary supplementation of *Lactobacillus* in this study did not affect total gut microbial population, but significantly ($p<0.01$) increased the population of lactobacilli and reduced that of *E-Coli* in the cecum samples of broilers (Figure 2). This positive effect on the

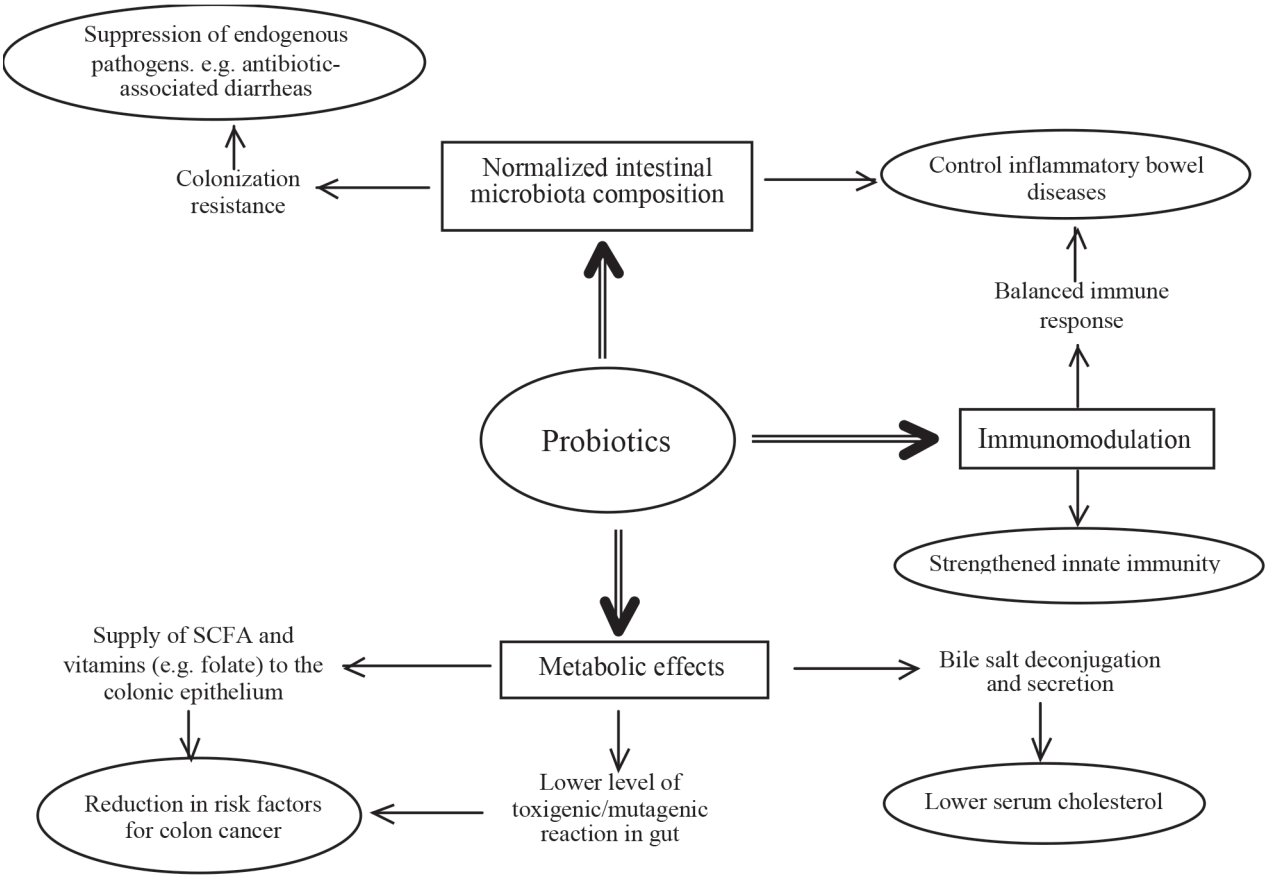

Figure 1 – Mode of action of probiotics (modified from Shinde, 2012)

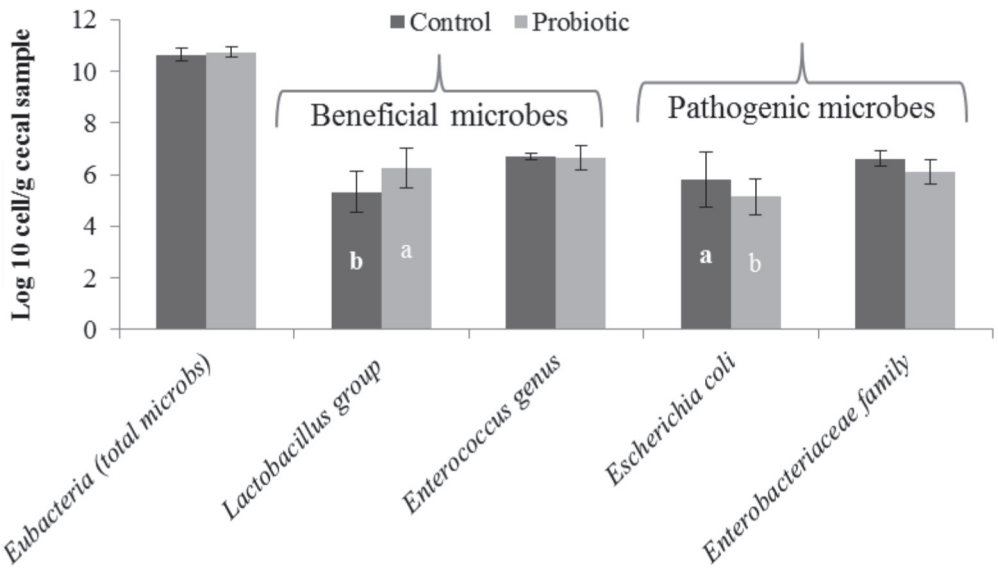

Figure 2 – Effect of probiotics (*Lactobacillus pentosus* ITA23 and *L. acidipiscis* ITA44) on the cecal bacteria of 35-day-old broiler chickens

gut microbiota may have resulted in a more efficient nutrient digestion and absorption in the *Lactobacillus* treatment group, thereby improving their FCR.

Cholesterol is essential for many body functions because it acts as a precursor of certain hormones and vitamins and it is an important component of cell membranes and nerve cells. It is known that elevated levels of blood cholesterol or other blood lipids are considered risk factors for developing of human coronary heart diseases (Lim *et al*. 2004). Although it may vary from person to person, the lipid component of diet affects serum cholesterol and triglyceride levels. Consumers are increasingly concerned with the consumption of animal products (eggs, meat and milk) because of their high lipid contents. Therefore, a challenge of present-day commercial poultry producers is to produce good quality products (e.g. meat and eggs with lower fat content) at a competitive cost.

Another known mode of action of probiotics is their metabolic effect on the reduction of cholesterol levels through deconjugation of bile salts in the host animal (Figure 1). Some studies reported that probiotics may potentially reduce serum cholesterol levels (Kim *et al*., 2008; Belviso *et al*., 2009). Kalavathy *et al*. (2006) reported that although the supplementation of *Lactobacillus* cultures (consisting of four species) lowered liver cholesterol levels of broiler chickens, this effect was not observed in the muscle. The same authors also observed that the *Lactobacillus* cultures reduced the fat content of the liver, muscle, and carcass of broiler chickens, but showed very little potential to modify fatty acid composition. Our results show that supplementation of the two strains of *Lactobacillus* lowered serum total cholesterol levels, but low density lipoprotein (LDL) and triglyceride levels were not different ($p>0.05$) from the control birds (Figure 3). Since the modes of action of probiotics appear to be multi-factorial and strain specific, the efficacy of probiotics to reduce and modify the fatty acid composition of animal products (meat and eggs) requires further investigations.

Results of this study indicate that supplementation of a mixture of *L. pentosus* ITA23 and *L. acidipiscis* ITA44 improved the FCR of broiler chickens. However, the effect on serum cholesterol and other blood

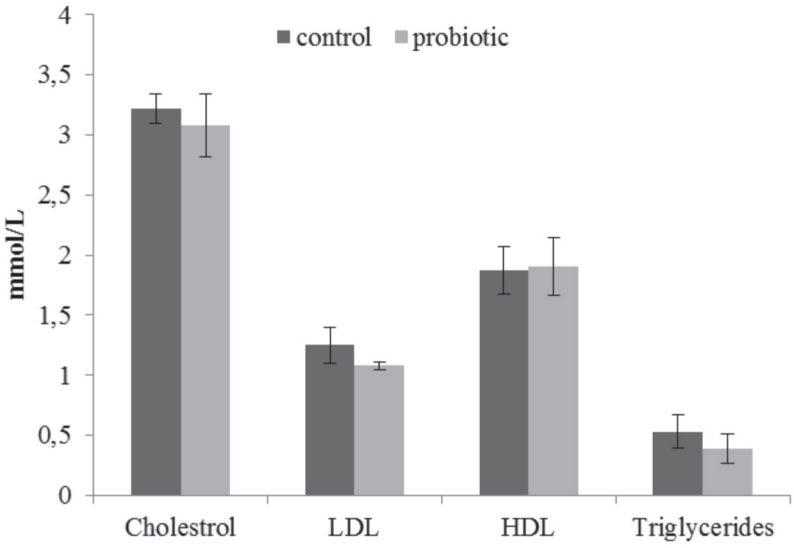

Figure 3 – Effect of probiotics (*Lactobacillus pentosus* ITA23 and *L. acidipiscis* ITA44) on serum cholesterol and triglyceride levels of 35-day-old broiler chickens.

parameters was less evident. Although the actual mechanism by which the supplementation improved FRC was unclear, it is likely that *Lactobacillus* spp act as a probiotic by suppressing pathogenic bacteria, thereby providing a better environment for nutrient digestion and absorption.

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