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Full Length Research Paper

Effects of rough handling on short chain fatty acid production and gastrointestinal pH in broilers and modulatory role of *Lactobacilli*

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The influence of stress due to rough handling (RH) on gastrointestinal tract (GIT) environmental pH, concentration of short chain fatty acids (SCFAs) and modulatory roles of two *Lactobacillus* strains was investigated in broiler chickens. Equal number of chicks was randomly assigned to one of the following treatment groups; (i): no handling + basal diet (control), (ii): RH and + basal diet (RH-BD), (iii): RH + basal diet supplemented with 10^7 CFU of each *Lactobacillus* strain per gram of feed (RH-BDL). Birds fed dietary *Lactobacilli* from day one until the end of the experiment and subjected to RH from day 1 to 21. Digesta from different GIT regions were collected at 14, 28, 35 and 42 days of age and SCFAs and pH were measured. Duodenal, ileal and cecal lactate concentrations together with cecal butyrate level were significantly ($P < 0.05$) decreased in RH-BD birds compared with the control and RH-BDL at 14 and 21 days of age. Acetate concentration was significantly ($P < 0.05$) reduced in the jejunum, ileum and cecum of both RH-BD and RH-BDL birds at 14 days of age. It can be concluded that, stressful condition over the course of the GIT microbial development, negatively affected microbial activity by reduction of the lactate, acetate (as the main substrate for butyric producing bacteria) and ultimately butyrate concentration along the GIT and particularly cecum. This adverse effect was effectively ameliorated by *Lactobacillus* supplementation.

Key words: Gastrointestinal tract (GIT), SCFAs, *Lactobacilli*, stress.

INTRODUCTION

The gastrointestinal tract (GIT) is a multifunctional organ which primarily digests and absorbs nutrients to meet the host demands for the growth and development and simultaneously, protects the host against pathogen bacteria through some factors including saliva, gastric acid, peristalsis, mucus layers, intestinal proteolysis, intestinal microbiota and epithelial cell membranes with intercellular functional complexes (Lai et al., 1976; Tasman-Jones et al., 1987; Wallace, 2008). Intestinal microbiota and their metabolic end products, especially SCFAs, play an important role in maintaining homeostasis in the GIT. Previous studies on rats have demonstrated that, SCFAs affect intestinal motility by the stimulation of nerves and muscles (Cherbut et al., 1998; Ono et al., 2004). Moreover, the intestinal mucus secretion and cell proliferation (Reilly et al., 1995; Shimotoyodome et al., 2000; Gaudier et al., 2004) were affected by luminal

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SCFAs. In addition to the beneficial effects of SCFAs on the structure and function of GIT, it has been shown that, SCFAs also control the whole luminal microbial ecology in broiler chickens (Kubena et al., 2001). Several factors such as the composition of microbiota, diet (Sanderson, 2004) and transit time through the gut has been documented to affect on the SCFAs production in the gut. Thus, the production of SCFAs as a result of the bacterial fermentation can be impaired by altering these factors. It has been reported that, stress disturbed the healthy equilibrium of the intestinal flora, that is, reduced number of *Lactobacilli* and increased number of pathogens (Lan et al., 2004; Selig and Patterson, 2004; Lutgendorff et al., 2008) which may consequently, alter the balance of SCFAs concentration and the GIT pH value. Previous studies on broilers and layers have shown that, rough handling, categorized by psychological stress, was correlated with lower growth rate and eggshell quality, while increasing feed conversion ratio (Hemsworth and Barnett, 1989; Barnett et al., 1992; Jones, 1993). Broiler GIT microbial community is under development during the first three weeks (Snel et al., 2002). Therefore, it can be hypothesized that during this period of time the community of GIT microbiota and subsequently, their metabolism is more susceptible to fearful conditions. Additionally, probiotics appear to counteract fear- induced damage in the GIT. Our previous study demonstrated that the administration of *Lactobacilli* improved intestinal cell proliferation and thus, enhanced the growth performance of chicken exposed to negative physical treatment (Meimandipour et al., 2010).

The aim of the present study was to investigate the effect of early RH over the course of the first three weeks on the major microbial metabolic end product (SCFAs and lactate) and pH in the different locations of broiler GIT. Additionally, the ability of *Lactobacillus* to ameliorate deleterious effects of RH on the amount of luminal SCFAs and pH was also studied.

MATERIALS AND METHODS

Probiotics preparation and supplement feed

Lactobacilli supplementation containing *Lactobacillus salivarius* ssp. *salicinius* JCM 1230 and *Lactobacillus agilis* JCM 1048 was prepared according to the procedure described previously (Lan et al., 2004). The recovery rate of viable bacteria in the freeze dried culture was approximately 3.1×10^9 CFU/g. The freeze dried culture was stored at 4°C and mixed daily into the feed (each 10^7 CFU per gram of feed) to ensure its viability throughout the experimental period. Moreover, the viability of bacterial cells were verified every two weeks interval.

Ethical note

This study was undertaken following the guidelines and approval of the Animal Care Committee of the University Putra Malaysia on animal ethics.

Birds and dietary treatments

A total of 105 day old male broiler chicks were obtained from a local hatchery. Upon arrival, chicks were weighed and randomly divided into 3 equal groups of 35 chicks each in 5 battery cages with wire floors in an environmentally controlled room (2.3 x 9.1 x 3.8 m). Floor space allowed was 923 cm^2 per bird. Ambient temperature on day 1 was set at 32°C and gradually reduced to 23°C by day 21. The relative humidity was between 65 and 75%. All chicks were fed corn and soybean meal based starter (mash form; 21.5% CP and 3000 kcal ME/kg) and finisher (mash form; 19.5% CP and 3100 kcal ME/kg) diets from day 1 to 22 and 22 onwards (Table 1). The diets were formulated to meet or exceed requirements by the NRC (1994) for broiler chickens. From day 1, equal number of chicks was randomly assigned to one of the following treatment groups; (1) no handling + basal diet (control); (2) RH and + basal diet (RH-BD); (3): RH + basal diet supplemented with 10^7 CFU of each *Lactobacillus* strain per gram of feed (RH-BDL). Feed and water were provided *ad libitum* and the birds were under continuous fluorescent lighting.

Rough handling condition

From day 1 to day 21 the chicks were challenged to RH condition, modified from that of Zulkifli and Siti Nor Azah (2004). Briefly, the chicks were caught using both hands, placed in plastic crates and carried to another room. The chicks were suspended by legs in a group and swung gently for 30 s once per day.

Digesta samples from various GIT regions

Five chickens were randomly selected from each treatment group and killed by cervical dislocation at 14, 28, 35 and 42 days of age. The GIT segments were aseptically collected from the loop of the duodenum, midpoint of the jejunum (between the bile duct entry and Meckel's diverticulum), ileum (between the Meckel's diverticulum to the ileo-cecal junction) and cecum. The segments were tied from open sides and placed into an empty sterile plastic bag on ice.

Samples were immediately transferred to the laboratory and intestinal digesta from different parts were homogenized for 2 min with a stomacher (John Morris Scientific Pty. Ltd., Melbourne, Australia). Thereafter, about 0.4 g of homogenized material was resuspended in 2 ml of sterile milli-Q water and the pH of digesta in different parts of GIT was measured. Samples were centrifuged at 18500×*g* for 10 min and the supernatant were stored at -20°C for subsequent SCFAs and lactate analyses.

SCFAs and lactate analyses

SCFAs and lactate were analyzed by HPLC (SPD-M20A, Shimadzu, Japan) using UV detection at 210 nm. The anion exchange column (300×7.8 mm, Aminex HPX-87H; Bio-Rad) was operated at 60°C with 0·009 M-H_2SO_4 (0·6 ml/min) as eluent. Standard solutions of lactic acid, acetic acid, butyric acid and propionic acid of known concentrations were used for column calibration.

Statistical analysis

All analyses were performed using general linear models procedure of SAS (SAS Institute 1994). A one-way analysis of variance (ANOVA) was used to analyse the data and means were separated

Table 1. Composition of the experimental diets (%).

Ingredient	Starter diet (0-21 Day)	Grower diet (22-42 Day)
Corn	61.2	64.5
Soy bean meal	28.3	24.5
Palm oil	2	3.5
Fish meal	5	4
Calcium carbonate	1.2	1.2
Dicalcium phosphate	1.3	1.3
Vitamin premix*	0.25	0.25
Mineral premix†	0.25	0.25
Methionine	0.2	0.2
Calculated composition		
Crude protein	21.5	19.5
ME (Kcal/kg)	3000	3100
Lysine	1.16	1.02
Methionine	0.57	0.54
Calcium	1.00	0.96
Non-phytate phosphorous	0.46	0.44
Sodium	0.13	0.13

*Provided per kg of diet: vitamin A, 4500 IU; vitamin D3, 1000 IU; vitamin E, 50 mg; vitamin K, 1.5 mg; vitamin B12, 0.02 mg; vitamin B2, 3 mg; pantothenic acid, 5 mg; niacin, 20 mg; choline chloride, 150 mg; and folic acid, 0.5 mg; †Provided per kg of diet: zinc, 40 mg; iron, 80 mg; iodine, 0.8 mg; manganese, 60 mg; copper, 8 mg; selenium, 0.2; and cobalt, 0.4 mg.

by Duncan's multiple range test. Results were considered statistically significant at $P < 0.05$.

RESULTS

Gastrointestinal tract SCFAs and lactate concentration

Concentration of SCFAs and lactate in different parts of the GIT over the course of the experiment are shown in Tables 2, 3, 4 and 5. At 14 and 21 days of age, broilers of RH-BD treatment had a lower concentration of lactate in the duodenum, ileum and cecum compared with the two other groups (Table 2, $P < 0.05$). Thereafter, from 28 to 35 days of age, RH and *Lactobacillus* supplementation did not affect duodenal lactate concentration. Acetate was not detected in the duodenum during the experimental period. Broilers in RH-BD and RH-BDL treatment groups had a significant lower jejunal acetate concentration at 14 and 21 days of age. At 28 days of age, broilers in RH-BDL had the lowest ($P < 0.05$) jejunal acetate concentration compared with other treatment groups. Acetate concentration in the ileum was significantly ($P < 0.05$) lower in RH-BDL treatment and intermediate in RH-BD group when compared with the control at 14 days of age (Table 3). Moreover, both RH-BD and RH-BDL broilers had significant ($P < 0.05$) lower cecal acetate concentration at 14 days of age. Broilers in RH-BDL treatment had lower cecal acetate concentration at 21 days of age ($P < 0.05$). In the duodenum, jejunum and ileum, propionate was only detected at 14 days of age. On day 14, the amount of propionate was significantly ($P < 0.05$) higher in the duodenum of RH-BDL birds when compared with those of RH-BD and the control groups, while RH-BD was in-between (Table 4). Both RH-BD and RH-BDL treatments had the highest amount of jejunal propionate compared with the control at 14 days of age. Butyrate was mainly found in the cecum during the experimental period (Table 5). Broilers of RH-BD had a significant ($P < 0.05$) lower concentration of cecal butyrate at 14 and 21 days of age as compared with other groups.

The pH of digesta from the GIT

Data on the effects of RH and *Lactobacillus* supplementation on pH values in various segments of the GIT are shown in Table 6. Duodenal and jejunal pH values significantly ($P < 0.05$) increased in broilers of RH-BD treatment compared with the control and RH-BDL groups at 14 and 21 days of ages. *Lactobacilli* supplementation lowered ($P < 0.05$) ileal pH value in broilers of RH-BDL when compared with RH-BD birds at 35 days of age.

DISCUSSION

Influence of RH and *Lactobacillus* supplementation on SCFAs and lactate concentration

It has been reported that, many host and environmental-related factors affect bacterial metabolism and SCFAs formation in the gut, including diet, age, neuroendocrine system activity, stress, pancreatic and other secretions in the digestive tract, mucus production, disease, antibiotics and epithelial cell turnover times (Macfarlane et al., 2008). As shown in Tables 2, 3, 4 and 5, SCFAs production were affected by RH and *Lactobacillus* supplementation in a different manner along the GIT. Decreased concentration of lactate, acetate and butyrate in the GIT- related to RH was probably due to the change of microbiota (Lan et al., 2004; Selig and Patterson, 2004) and/or their metabolic activities in the chicken intestine. In our previous work, broilers of RH-BD and RH-BDL showed higher (37 and 27%, respectively) blood corticosterone concentration at 14 and 28 days of age (Meimandipour et al., 2010) which might negatively affect the bacterial composition and their metabolic activities in the gut. Previous studies illustrated that, stress hormones create the opportunity for pathogens to grow in the GIT by diminishing the growth of protective bacteria (*Lactobacilli* and bifidobacteria), by increasing the

Table 2. Influence of *Lactobacillus* supplemented diet and RH on the concentration of lactate in the different GIT regions of broiler chicken.

Item (day)	Group			*P*-value
	Control	RH-BD	RH-BDL	
	Duodenum (µmol/g of content) content)			
14	29.69±1.76[a]	23.07±1.31[b]	27.85±1.41[a]	0.001
21	35.20±2.29[a]	31.42±1.38[b]	36.23±1.02[a]	0.001
28	35.59±2.79	34.45±3.09	37.55±2.21	0.313
35	39.53±2.93	36.07±3.58	37.84±2.00	0.293
	Jejunum (µmol/g of content)			
14	36.45±3.15	32.58±2.32	35.44±3.24	0.208
21	47.12±3.03	46.94±3.42	48.07±2.67	0.856
28	38.29±2.05	37.81±3.30	39.73±3.76	0.674
35	36.93±3.91	36.19±2.77	36.81±2.03	0.951
	Ileum (µmol/g of content)			
14	48.17±4.24[a]	37.84±2.31[b]	46.16±4.59[a]	0.022
21	66.50±3.81[a]	49.20±3.92[c]	56.97±3.64[b]	0.001
28	37.72±2.59	38.33±3.34	41.82±2.42	0.138
35	57.95±2.22	56.74±3.18	54.81±3.57	0.380
	Cecum (µmol/g of content)			
14	37.73±1.84[a]	31.72±1.67[b]	36.91±2.28[a]	0.027
21	7.66±0.94[ab]	6.14±1.25[b]	8.95±1.28[a]	0.031
28	7.77±0.36	7.69±0.82	7.99±0.54	0.800
35	6.75±0.27	6.54±0.51	6.64±0.35	0.181

Means within a row with no common superscript differed significantly ($P < 0.05$). Control, birds subjected to the normal human contact and fed basal diet; RH-BD, birds subjected to rough handling from day 1 to day 21 and fed basal diet; RH-BDL, birds subjected to rough handling from day 1 to day 21 and fed basal diet containing *Lactobacilli*.

Table 3. Influence of *Lactobacillus* supplemented diet and RH on the concentration of acetate* in the ileum and cecum of broiler chicken.

Item (day)	Group			*P*-value
	Control	RH-BD	RH-BDL	
	Jejunum (µmol/g of content)			
14	0.65±0.12[a]	0.32±0.20[b]	0.20±0.15[b]	0.001
21	0.79±0.15[a]	0.20±0.25[b]	0.19±0.01[b]	0.001
28	1.23±0.19[a]	1.19±0.20[a]	0.67±0.20[b]	0.014
35	1.26±0.21	1.23±0.26	1.03±0.29	0.276
	Ileum (µmol/g of content)			
14	2.93±0.14[a]	1.44±0.18[b]	0.70±0.11[c]	0.001
21	6.39±0.48	6.89±0.89	5.98±0.56	0.235
28	5.63±0.52	5.03±0.77	4.65±0.41	0.109
35	2.58±0.24	2.68±0.49	2.25±0.07	0.199
	Cecum (µmol/g of content)			
14	28.69±2.35[a]	16.90±2.21[b]	16.68±1.35[b]	0.001
21	52.11±2.52[a]	48.52±3.41[a]	35.09±3.77[b]	0.001
28	50.72±2.96	48.82±3.06	47.53±3.71	0.695
35	48.99±3.95	46.82±3.91	44.55±2.17	0.529

*Acetate was not found in the duodenum. Means within a row with no common superscript differed significantly ($P < 0.05$). Control, birds subjected to the normal human contact and fed basal diet; RH-BD, birds subjected to rough handling from day 1 to day 21 and fed basal diet; RH-BDL, birds subjected to rough handling from day 1 to day 21 and fed basal diet containing *Lactobacilli*.

Table 4. Influence of *Lactobacillus* supplemented diet and RH on the concentration of propionate* in the different GIT regions of broiler chicken.

Item (day)	Group			*P*-value
	Control	RH-BD	RH-BDL	
	Duodenum(µmol/g of content)			
14	5.05±0.74^{c}	15.66±0.57^{b}	19.29±1.69^{a}	0.001
	Jejunum (µmol/g of content)			
14	5.55±0.80^{b}	19.24±1.19^{a}	19.99±0.93^{a}	0.001
	Ileum (µmol/g of content)			
14	7.78±0.81	8.62±0.76	9.14±0.59	0.072
	Cecum(µmol/g of content)			
14	8.52±1.21	8.13±0.81	9.68±0.56	0.088
21	2.81±0.87	2.26±0.25	3.76±0.96	0.167
28	3.16±0.21	3.57±0.61	4.02±0.74	0.159
35	6.17±1.00	6.17±0.56	6.40±0.67	0.883

*Propionate was not found in the other days (28, 35 and 42) from duodenum to ileum. Means within a row with no common superscript differed significantly (P < 0.05). Control, birds subjected to the normal human contact and fed basal diet; RH-BD, birds subjected to rough handling from day 1 to day 21 and fed basal diet; RH-BDL, birds subjected to rough handling from day 1 to day 21 and fed basal diet containing *Lactobacilli*.

Table 5. Influence of *Lactobacillus* supplemented diet and RH on the concentration of butyrate* in the broiler chicken cecum.

Item day	Group			*P*-value
	Control	RH-BD	RH-BDL	
	Cecum (µmol/g of content)			
14	2.09±0.17^{a}	0.59±0.22^{c}	1.25±0.17^{b}	0.001
21	7.09±1.60^{ab}	5.47±0.78^{b}	8.03±1.06^{a}	0.040
28	5.34±0.66	5.86±0.73	6.33±0.40	0.126
35	7.01±0.64	6.24±0.52	6.57±0.65	0.246

*Butyrate was not found in the other parts of the GIT. Means within a row with no common superscript differed significantly (P < 0.05). Control, birds subjected to the normal human contact and fed basal diet; RH-BD, birds subjected to rough handling from day 1 to day 21 and fed basal diet; RH-BDL, birds subjected to rough handling from day 1 to day 21 and fed basal diet containing *Lactobacilli*.

number of pathogens and through altering the epithelial susceptibility to attachment (Belay and Sonnenfeld, 2002; Bailey et al., 2004). However, it should be taken into account that, changes in SCFAs concentration are not necessarily associated with alteration in the population level of GIT microbiota (Ichikawa et al., 1999; Meimandipour et al., 2010). In fact, carbohydrate degradation to produce SCFAs are interactive processes among various bacteria (Macfarlane and Gibson, 1995), in which only a minor change in the GIT microbiota could profoundly alter its overall metabolism. Besides, in this study, feed intake numerically decreased (3.3%) in RH-BD treatment when compared with the control group in the first week (Meimandipour et al., 2010; Soleimani et al., 2010; Meimandipour et al., 2011). It has been shown that, psychological stress lowered feed intake and net water absorption (Söderholm and Perdue, 2001) and consequently, feed passage rate which all may reduce substrate for the GIT bacteria and ultimately influence SCFAs production (Sakata, 1997).

Broilers in RH-BD and RH-BDL treatment groups showed significantly higher amounts of duodenal and jejunal propionate compared with the control at 14 days of age, which coincided with higher pH value at these sites. Previous *in vitro* study by Belenguer et al. (2007) on human intestinal content fermentation has been shown that, propionate formation was enhanced in higher pH value (6.7) compared with lower one (5.6). Taken

Table 6. Influence of *Lactobacillus* supplemented diet and rough handling on the pH values in the different GIT regions of broiler chicken.

Item (day)	Group			*P*-value
	Control	UPC-BD	UPC-BDL	
		Duodenum		
14	5.80±0.03[c]	6.13±0.04[a]	6.00±0.03[b]	0.001
21	5.98±0.06[b]	6.12±0.08[a]	5.95±0.08[b]	0.028
28	6.08±0.09	6.14±0.07	6.08±0.07	0.512
35	6.00±0.08	5.99±0.09	5.86±0.07	0.058
		Jejunum		
14	5.92±0.06[c]	6.29±0.07[a]	6.10±0.05[b]	0.001
21	6.11±0.17[b]	6.45±0.16[a]	6.06±0.11[b]	0.014
28	6.67±0.18	6.77±0.15	6.46±0.24	0.121
35	6.26±0.36	6.04±0.42	5.80±0.40	0.319
		Ileum		
14	6.66±0.30	7.17±0.33	7.06±0.44	0.162
21	6.08±0.42	6.79±0.31	6.04±0.66	0.101
28	7.33±0.12[a]	7.46±0.26[a]	6.64±0.40[b]	0.006
35	6.84±0.45	6.53±0.29	6.21±0.85	0.344
		Cecum		
14	6.07±0.09	6.06±0.10	5.96±0.09	0.206
21	5.90±0.40	6.00±0.36	5.95±0.50	0.698
28	6.00±0.31	5.96±0.26	6.05±0.14	0.879
35	6.23±0.16	6.21±0.19	6.14±0.28	0.663

Means within a row with no common superscript differed significantly ($P < 0.05$). Control, birds subjected to the normal human contact and fed basal diet; RH-BD, birds subjected to rough handling from day 1 to day 21 and fed basal diet; RH-BDL, birds subjected to rough handling from day 1 to day 21 and fed basal diet containing *Lactobacilli*.

together, addition of *Lactobacillus* to the diet of RH-BDL broilers did increase the concentration of lactate, propionate and butyrate during the experiment. However, it decreased the amount of jejunal, ileal and cecal acetate in this treatment. This result was similar with those of previous works that demonstrated supplementing the *Lactobacillus* cultures, singly or in a mixture, in the diet of broilers significantly increased the total SCFAs in the ileum and cecum (Jin et al., 1998) and lactate, propionate and butyrate in the GIT of Japanese quail (Strompfova et al., 2005). Furthermore, our previous *in vitro* study (Meimandipour et al., 2009), showed that, *Lactobacillus* supplementation (using the same strains) directly by the production of lactate and propionate and indirectly by cross feeding of butyric producing bacteria increased butyrate concentration. Moreover, the reduction of acetate in broilers of RH-BDL could be associated with the acetate utilization by *Lactobacilli* as an electron acceptor for maintaining their intracellular redox balance during anaerobic fermentation (Takahashi et al., 1995).

Influence of RH and *Lactobacillus* supplementation on pH value

The results showed that, broilers of RH-BD had a significant higher duodenal and jejunal pH at 14 and 21 days of age (Table 6). However, *Lactobacillus* supplementation significantly decreased the pH values in RH-BDL birds when compared with RH-BD treatment. Previous study has been reported that, endogenous corticotrophin releasing hormone (CRF) excreted during stress and exogenously administered CRF stimulate duodenal bicarbonate secretion by release of 3-endorphin from the pituitary, thus, demonstrating a functional hypothalamus-pituitary-gut axis (Lenz, 1989). The enhanced level of bicarbonate ion during the stressful condition may explain the increased pH values

in duodenum and jejunum in 14 and 21 days old RH-BD birds. The higher production of SCFAs and lactate as the metabolic end product of GIT microbial fermentation in RH-BDL birds caused to reduce the duodenal and jejunal pH over the course of stress (Table 6).

In conclusion, the concentration and proportion of SCFAs and lactate changed along the GIT due to psychological stress related to rough handling and *Lactobacilli* administration. Subjecting of broilers to stressful condition over the course of the GIT bacterial development altered environmental pH and bacterial metabolic activities. Increasing of intestinal pH and decrease in the concentration of SCFAs and lactate may result in the reduction of beneficial bacteria in the favour of opportunistic pathogens. The results of this study clearly demonstrated that, *L. salivarius* ssp. *salicinius* JCM 1230 and *L. agilis* JCM 1048 can ameliorate deleterious effects of RH on the GIT from early age by the enhancement of lactate, propionate and butyrate, which subsequently decreased the GIT environmental pH.

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