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Effects of Adding *Lactobacillus plantarum* I-UL4 Metabolites in Drinking Water of Rats

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Abstract: The objectives of this experiment were to study the effect of adding different levels of *Lactobacillus plantarum* I-UL4 (UL4) metabolite in drinking water on the growth performance, plasma cholesterol concentration, faecal LAB and *Enterobacteriaceae* counts and pH in postweaning rats. A total of 15 female and 15 male post weaning rats were randomly assigned to three groups of drinking water: 100% drinking water, 65% drinking water + 35% UL4 metabolite (35% UL4) and 30% drinking water + 70% UL4 metabolite (70% UL4) for a period of four weeks. The bacteriocin activity for UL4 was 266.67 AU/ml. Daily feed intake, water intake and weekly live weight were measured. Blood plasma from each rat for total cholesterol analysis was obtained at the end of treatment. Faecal samples were taken directly from rectum from each rat and cultured for LAB and *Enterobacteriaceae* a day before commencement of the experiment, and then on days 7, 14, 21, and 28 after feeding the metabolite. The growth performance of control rats was better than the UL4 groups. The 35 and 70% UL4 rats had a lower total plasma cholesterol concentration compared to the control group. The UL4 groups had significantly lower *Enterobacteriaceae* counts on week 3 and significantly (p<0.05) higher LAB counts after 2 weeks of experiment. The 35% UL4 rats had lower faecal pH than other treatment groups. In conclusion, the results showed that the addition of UL4 metabolite in the drinking water reduced the growth rate of rats, especially those treated with 70% UL4, decreased the total cholesterol concentration, increased the faecal LAB counts, reduced *Enterobacteriaceae* counts and faecal pH.

Key words: Rat, *Lactobacillus plantarum* IUL-4 metabolite, *Enterobacteriaceae*

Introduction
Oral administration of some viable bacteria can have advantageous effects in promoting health, and have recently been evaluated as 'probiotics' (Fuller, 1992). Probiotics improve the balance of intestinal flora, which exert beneficial effects by decreasing harmful bacterial metabolites such as amines and indoles (Benno and Mitsuoka, 1992). Recently, some probiotic bacteria have been found to enhance the immunity of the host (Yasui *et al.*, 1992), which may explain the effect of probiotics in prevention of infectious diseases (Gonzalez *et al.*, 1990). Lactic acid bacteria (LAB) as probiotic are known to produce a wide variety of antibacterial substances; as well as inhibitory primary metabolites like acetic acid, lactic acid, propionic acid, ethanol, diacetyl, hydrogen peroxide, bacteriocins and antiobiotic-like substances with activity against Gram-negative bacteria (Earnshaw, 1992). These substances may have bacterial antagonism which has been recognized for over a century but in recent years this phenomena has received more scientific attention. Although there is tremendous interest in the potential health benefits of LAB, however there is currently only limited evidence that LAB metabolites provide beneficial effects to the mammalian host.
In many cases, the precise mechanisms of anti-microbial action cannot be defined because of the complex interaction of several phenomena to produce a combined effect. These interactions are varied depending on the environment in which lactic acid bacteria are located and thus can vary from one to another and indeed between different sites of growth in one particular system (Earnshaw, 1992). Most of the study administered probiotics or LAB cultures or inclusion of LAB fermented product in the diet of animals to alter the pH, *Enterobacteriaceae* and LAB count, cholesterol concentration (Gallaher *et al.*, 1995; Fukushima *et al.*, 1998; Du Toit *et al.*, 1998; Loh *et al.*, 2003). However, there is limited information regarding the effect of direct feeding of metabolites from specific LAB strain to the animals. The objectives of this experiment were to study the effect of adding different levels of *Lactobacillus plantarum* I-UL4 (UL4) metabolites in drinking water on growth performance, plasma cholesterol concentration, faecal *Enterobacteriaceae* and LAB counts and pH in postweaning rats.

Materials and Methods
Culture condition and preparation of LAB metabolite: *Lactobacillus plantarum* I-UL4, isolated from *tapai ubi* was used in this study. These bacteria were obtained from our own collection (Department of Biotechnology, Universiti Putra Malaysia). The LAB strain was kept in

Man Rogosa Sharpe (MRS) broth containing 20% (v/v) glycerol at -20 °C. The LAB strain was revived twice in MRS broth and incubated anaerobically at 30 °C before preparing the metabolite.

2% (v/v) of overnight culture was inoculated into 1L MRS broth and incubated anaerobically for overnight at 30 °C. The metabolite was collected by separating the bacterial cells with centrifugation at 8000 rpm for 10 min. The metabolite was then kept at 4 °C.

Experimental animals and experimental protocol: The feeding experiment was carried out at Department of Animal Science, Universiti Putra Malaysia. A total of 15 male and 15 female post weaning rats, *Sprague dawley*, 4 week-old with the average body weight of 61g were used in this study. The rats were randomly assigned to 3 treatment groups of 10 each (5 male and 5 female). The treatments were: i) control (100% drinking water), ii) 35% UL4 + 65% drinking water (35% UL4) and iii) 70% UL4 + 30% drinking water (70% UL4). They were housed individually in a 24-hour lit room with well-ventilated air-conditioned environment at 24-26 °C and relative humidity of 60-64%. The rats were given basal feed *ad libitum* for 28 days. The compositions of basal diet are shown in Table 1. All the rats were acclimatized to the respective drinking water for a week before the experiment was started. Daily feed, daily water intake and weekly body weight gain were measured throughout the experiment.

At the end of experiment, the rats were fasted for 12 hours before blood collection. The rats were anaesthetized with dimethyl ether and blood was collected by cardiac puncture into tube containing EDTA (vacutainer®, USA). Plasma was obtained after centrifuging the whole blood at 3000 rpm for 10 min for total cholesterol concentration analysis. Total cholesterol levels were determined through the Enzymatic Endpoint Method using a commercial diagnostic kit (Randox®, UK), as described by Loh *et al.* (2002).

The total plate count was conducted for fresh faecal samples collected directly from the rectum of each rat every week. The pH of the faeces was measured with a pH meter. The faecal (10% w/v) was suspended in sterile peptone water and incubated for an hour before further 10-fold dilutions (v/v) were made with peptone water for *Enterobacteriaceae* and total LAB counts. *Enterobacteriaceae* were plated on EMB-agar (Merck®) and incubated at 37 °C for 24 h, whereas total LAB counts were spread plated on MRS-agar (Merck®) and incubated at 30 °C for 48 h as described by Foo *et al.* (2001). Numbers of colony forming units (CFU) are expressed as log_{10} CFU per gram.

Statistical analyses: Results were expressed as mean ± standard of mean (SEM). The data was analyzed by

Table 1: The compositions of basal diet

Ingredients	Basal Diet
Broken rice	31.70
Corn	30.88
Soybean meal (46% CP)	22.00
Dicalcium Phosphate	1.40
Salt	0.70
Limestone	0.60
DL- methionine	0.50
L- lysine	0.50
Vitamin premix *	2.12
Palm oil	1.60
Fish meal	8.00

*~The vitamin premix provides the following amounts per kilogram of diet: vitamin A, 5200IU; cholecalciferol, 1000IU; vitamin E, 10IU; vitamin K, 1.3mg; riboflavin, 8.0mg; niacin, 25mg; D-calcium pantothenic acid, 10mg; choline chloride, 210mg and vitamin B_{12}, 0.01mg.

two-way analysis of variance (ANOVA). Duncan Multiple Range Test was used to compare the differences of means in CFU of *Enterobacteriaceae* and LAB, faecal pH, plasma total cholesterol concentration and growth performance among treatment groups. Differences of p < 0.05 were considered significant (SAS, 1998).

Results

Growth performance: The growth performance for the different treatment groups is presented in Table 2. The initial live weights were similar (p>0.05) among the treatment groups. Final body weight for 70% UL4 was significantly lower (p<0.05) than the control and 35% UL4. The rats of control had significantly higher (p<0.05) growth rate than those of 70% UL4. However, there was no different (p>0.05) between control and 35% UL4 and between the 35 and 70% UL4 rats. The feed intake for the control rats was significantly higher (p<0.05) than the 35 and 70% UL4. Clear differences (p<0.05) in the water intakes were also observed with the highest water consumption for control rats and the lowest intake for the UL4 rats. The feed conversion ratio from various treatment groups was not significantly different (p>0.05).

Total cholesterol concentration: Fig. 1 shows the plasma total cholesterol concentration for the control, 35% UL4 and 70% UL4. Total plasma cholesterol concentration of the control rats was significantly higher (p<0.05) than those of UL4 rats

Faecal LAB counts: The faecal LAB counts for the control, 35% UL4 and 70% UL4 is shown in Fig. 2. The faecal LAB counts were not significantly different (p>0.05) between the control feed group and treated groups at the beginning and the first week of the treatment. However, the faecal LAB counts decreased progressively for the control rats after one week of

Table 2: Effects of different levels of *Lactobacillus plantarum* I-UL4 (UL4) on growth performance in postweaning rats

Treatments	Control	35% UL4	70% UL4
Initial body weight, g	60.67±1.48	61.67±2.36	62.76±0.18
Final body weight, g	222.5±21.58[a]	215.67±20.52[a]	207.67±19.31[b]
Growth rate, g/day	5.78±0.77[a]	5.48±0.69[ab]	5.12±0.64[b]
Total feed intake, g	432.17±35.1[a]	398.0±29.50[b]	391.0±36.80[b]
Total water intake, ml	742.83±39.80[a]	597.33±27.80[b]	576.17±34.20[b]
Feed conversion ratio	2.77±0.19	2.76±0.18	2.9±0.19

The results are presented as mean values ± SEM. Values with different superscripts within row differ significantly at p=0.05.

treatment. Faecal LAB counts for 35% UL4 were significantly lower (p<0.05) than the other treatment groups on day 14. However, there was no significant different (p>0.05) for faecal LAB counts between control and 70% UL4. On day 21, the control had a lower (p<0.05) counts than the 70% UL4. However, there were no significant different (p>0.05) between control and 35% UL4 and among the UL4 groups. The faecal LAB counts for the control rats were the lowest (p<0.05) as compared with rats treated with metabolites on the last week of experiment.

Faecal *Enterobacteriaceae* counts: The faecal *Enterobacteriaceae* counts for the control, 35% UL4 and 70% UL4 is shown in Fig. 3. The *Enterobacteriaceae* counts for the control and 35% UL4 rats increased consistently throughout the experiment but decreased in week 4. In contrast, the *Enterobacteriaceae* counts of rats fed 70% UL4 decreased at the beginning and increased after one week of the experiment. The control rats had the highest faecal *Enterobacteriaceae* count (p<0.05) than the other treatment groups on day 21.

Faecal pH: Fig. 4 shows the faecal pH for the control, 35% UL4 and 70% UL4. The faecal pH was found to be significantly different (p<0.05) among the treatment groups after one week of experiment. The rats treated with 35% UL4 had the lowest faecal pH (p<0.05) than other treatment groups on day 14. However, no differences were observed (p>0.05) between control and 70% UL4. On day 21, there were no differences between UL4 and control groups. However, the faecal pH for 35% UL4 was significantly lower (p<0.05) than those other treatment groups at the end of experiment.

Discussion
The results showed that the final body weight, growth rate, feed intake and water intake between treatments appeared to be significantly different. The lesser total water consumption observed in rats treated with UL4 might be associated with poorer taste of the drinking water than the control water. The undesirable taste of UL4 might affect the appetite of rats. This explanation could be supported by the results of lower feed intake. The lower water and feed consumption in rats treated

with UL4 thereby affect their growth rate and final body weight, particularly those treated with higher concentration of metabolite in drinking water. These findings could not be supported by other studies because up to date there is no similar study being carried out. However, other studies included probiotics, LAB fermented food or organic acids in the diets of animals (Underhal *et al.*, 1982; Watkins and Kratzer, 1984; Eckel *et al.*, 1992; Loh *et al.*, 2003). *Lb. plantarum* I-UL4 used in present study is a facultative heterofermentative lactobacilli (Pot *et al.*, 1994). Metabolites produced are characterized by the accumulation of organic acids, primarily lactic and acetic acids, with a concomitant reduction in pH (Lindgren and Dobrogosz, 1990). Eckel *et al.* (1992) reported that addition of 6, 12 and 18 g/kg formic acid to the diet of weaned piglets resulted in better growth performance by 23, 31 and 29%, respectively. Trezona (2001) also claimed that additions of the organic acids such as acetic acid, citric acid, fumaric acid, lactic acid and formic acid produced by probiotics showed a positive effect on growth in pigs.

The present study demonstrated that rats given UL4 metabolite (35 and 70%) had a lower cholesterol concentration than those of the control rats. The results was in agreement with the study by Rao *et al.* (1981), who reported that metabolites from orotic acid formed during fermentation of dairy products may help to reduce the cholesterol level. According to Jasper *et al.* (1984), uric acid inhibits cholesterol synthesis and orotic acid and hydroxy methyl glutamic acid produced by LAB reduce serum cholesterol. Chikai *et al.* (1987) observed that gnotobiotic rats exhibited an increase in total faecal acids when inoculated with intestinal microflora of human origin able to deconjugate bile acids, resulting in increased cholesterol breakdown. Gilliland *et al.* (1985) showed that *L. acidophilus* itself might take up cholesterol during growth in the small intestine and make it unavailable for absorption into the bloodstream. The rats treated with UL4 had a higher faecal LAB counts than the control rats. The results are therefore an indication that the addition of LAB metabolite will encourage and increase the growth and population of indigenous LAB in the intestine and faeces. Lactobacilli are much better adapted to grow in an acidic
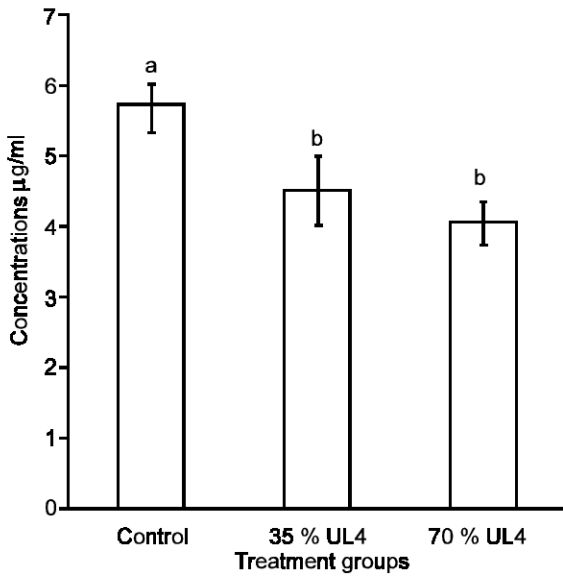
Fig. 1: Effect of different levels of UL4 metabolize on plasma cholesterol concentration in postweaning rat. Error bar indicates standard error of mean
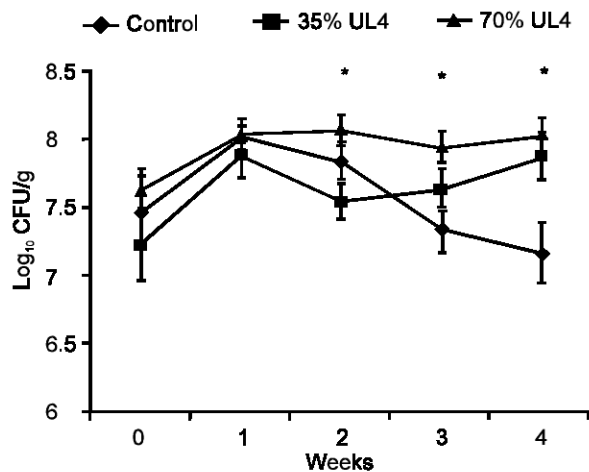Values with different alphabets differ significantly at p=0.05.

Fig. 2: Effect of different levels of UL4 metabolites on faecal LAB counts in postweaning rats. Error bar indicates standard error of mean
*indicates significant difference at p < 0.05.

environment produced by lactic acid bacteria than are pathogens like *Salmonella* (Fuller, 1977). High numbers of LAB may have an enhanced colonization resistance capability against the *Enterobacteriaceae* (Vollaard and Clasener, 1994) as demonstrated in those rats fed with LAB metabolites. Havennaar and Huis Int Veld (1992) showed that one of the most important purposes in using a probiotic is the idea that it influences the
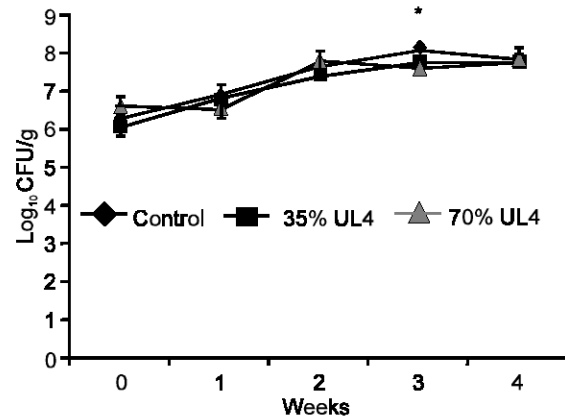

Fig. 3: Effect of different levels of UL4 metabolites on faecal Enterobacteriaceae counts in postweaning rats. Error bar indicates standard error of mean
*indicates significant difference at p < 0.05.
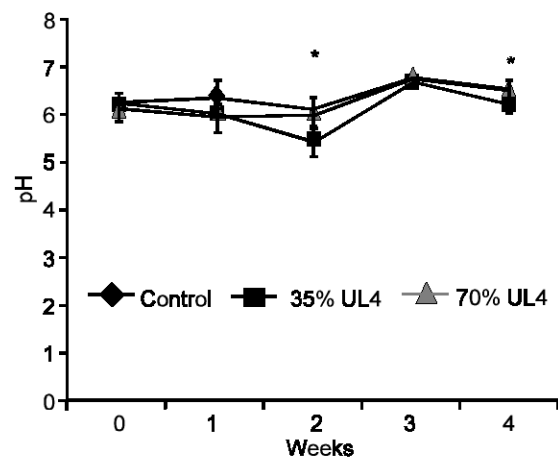

Fig. 4: Effect of different levels of UL4 metabolites on faecal pH in postweaning rats. Error bar indicates standard error of mean
*indicates significant difference at p < 0.05.

indigenous microflora resulting in growth promotion of animals in combination with a higher feed conversion, destruction of anti-nutritional factors, synthesis of vitamins and pre-digestion of proteins.
The effect of UL4 on faecal *Enterobacteriaceae* counts was not very significant in present study, this could be explained by the volume or amount of metabolite consumed by the rats. Nevertheless, the rats given water containing LAB metabolites had a slightly lower faecal *Enterobacteriaceae* counts than those of the control after two weeks of experiment, this could be explained by the present of antibacterial agents (organic acids, peroxides or bacteriocins) that are produced and secreted which have an inhibitory effect on controlling pathogenic microflora (Scheinbach, 1998). Shahani *et al.* (1977)

reported that acidophilic produced by *L. acidophilus* when cultured in milk inhibits a wide range of genera including both Gram-negative (e.g. *Enterobacteriaceae*) and Gram-positive types. Some strains of LAB synthesize substances such as nisin that are inhibitory to other bacteria (Hurst, 1981).

A significantly lower pH in faeces of rats treated with LAB metabolites particularly the 35% UL4 group, compared with the rats treated with normal drinking water was observed at the end of the experiment. Shah (2001) reported that lowering of pH due to lactic acid or acetic acid produced by probiotic bacteria in the gut has a bactericidal or bacteriostatic effect. We infer that the UL4 metabolite was able to reduce pH in the gastrointestinal tract and thereby, enhanced activity of volatile fatty acid (VFA). The volatile fatty acids produced by the indigenous microflora suppressed colonization of harmful bacteria in the gut (Fuller, 1992). The results are corroborated by De Vuyst and Vandamme (1994) who reported that the accumulation of acidic end-products and the concomitant low pH as well as hydrogen peroxide formation results in a wide inhibitory spectrum including both Gram-positive and Gram-negative bacteria (e.g. *Enterobacteriaceae*). The low pH as well as both the dissociation constant (pK_a) and the acid concentration determine the inhibitory activity of both lactic acid and acetic.

Conclusion: The present results demonstrated that the addition of UL4 metabolite in the drinking water of rats reduced the growth performance. This was associated with the lower intake of water and feed. However, the consumption of this metabolite reduced the concentration of plasma cholesterol, increased the faecal LAB population, decreased faecal pH and reduced slightly faecal *Enterobacteriaceae* counts. These results imply that addition of metabolite from *Lb. platarum* in drinking water has an important role in promoting animal's health. Further research will attempt to lower the concentration of metabolite in drinking water with the aim of encouraging the animals to consume more.

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