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Effects of fermented products on performance, faecal pH, *Enterobacteriaceae* and lactic acid bacteria counts and interrelationships, and plasma cholesterol concentration in rats

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ABSTRACT

Fermented products (FP), a mixed product of raw fish, sea weed, rice bran, sugar cane juice, were obtained in a process of fermentation using combination of cultures of lactic acid bacteria (LAB). The objectives of this study were to assess the effects of diets with different levels of FP on performance, faecal pH, *Enterobacteriaceae* and LAB counts and their relationships, and plasma cholesterol concentration in rats. A total of 24 Sprague Dawley (10 weeks of age) female rats were assigned individually into three groups of 8 rats per treatment. The three dietary treatments were: 1. control diet (basal diet), 2. basal diet + 10% FP and 3. basal diet + 20% FP. The final liveweight, growth rate, total feed intake and feed conversion ratio were not significantly different for all the treatment groups. Addition of FP to the diets reduced the *Enterobacteriaceae* population in faeces of the rats and significantly ($P<0.05$) increased numbers of LAB as compared with control rats. The faecal pH in rats fed with FP was more acidic than in rats fed with basal diet. The correlation analyses between LAB counts and *Enterobacteriaceae* counts, LAB counts and faecal pH and *Enterobacteriaceae* counts and faecal pH, suggest that FP provides an acidic environment, which encourages the growth of LAB and then leads to the inhibition of *Enterobacteriaceae* growth. The plasma cholesterol concentrations for rats fed with FP were significantly lower than that of control rats.

KEY WORDS: fermented products, growth performance, *Enterobacteriaceae*, lactic acid bacteria, cholesterol, rats

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INTRODUCTION

The extensive use of antibiotics as growth promoters in animal husbandry may have public health consequences as the resistance develops in a number of pathogenic bacterial species in the exposed animals. Therefore, there is a need to reduce the usage of antibiotic growth promoters and alternative methods need to be explored. Recently, lactic acid fermented feeds are suggested as alternatives. Lactic acid fermented feeds have made up a significant part of nutrition in animals (Demecková et al., 2002; Van Winsen et al*.,* 2002). Some strains of lactic acid bacteria (LAB) administered orally during first few days of life have shown beneficial effects on the growth of rats (Hargrove and Alford, 1978) and pigs (Underdahl et al., 1982).

Fermented feed contains high numbers of lactic acid bacteria, high number of yeast, a low pH and high concentration of lactic acid (Brooks et al., 2001). In general, the desirable effect of microbial activity in fermented feed may be caused by its biochemical activity. Microbial enzymes break down carbohydrates, lipids, proteins, and other feed components. This can improve food digestion in the animal's gastrointestinal tract, and thus increase nutrient uptake (Nout, 2001).

Fermentation can also increase the safety of feeds by removing their natural toxic components, or by preventing the growth of disease-causing microbes (Nout, 2001). It was observed that fermented feed reduced the number of *Enterobacteriaceae* (Urlings et al., 1993) and *Escherichia coli* in the gastrointestinal tract of pigs (Van Schie, 1987). Epidemiological studies on pig farms showed that the *Salmonella* prevalence on "fermented feed" farm was significantly lower than on "dry feed" farms (Van der Wolf et al., 1999). The reduced *Enterobacteriaceae* numbers in fermented feeds was accompanied by the increased number of LAB. These effects were related to low faecal pH (Urlings et al., 1993; Demecková et al., 2002; Van Winsen et al., 2002), but the correlation relationships were not determined. Various fermented diets can be prepared by either direct microbial inoculation in raw food materials or milk based source (Hólund, 1993). However, the effects of fermented fish (FP) with mixture of lactobacilli cultures as additive in the diet of animals have not been studied. There also has been considerable interest in the hypocholesterolaemia effects of fermented products containing LAB (Du Toit et al., 1998). However, the findings of those studies are inconsistent (Fletcher, 1995). Thus, the objective of this present study was to evaluate the effects of FP as additive in the diet of rats on performance, pH, *Enterobacteriaceae* and LAB counts in the faeces and their relationships, and plasma cholesterol concentration.

MATERIAL AND METHODS

Experimental animals

The protocol of this experiment was approved by the Research Committee of the University Putra Malaysia (Malaysia). A total of 24 female rats, Sprague Dawley, 10 week-old with the average body weight 152 g were housed in individual cages in a 24-h light room with well-ventilated air-conditioned environment at 24-26°C and relative humidity of 60-64%. Daily feed intake and weekly body weight gain were measured throughout the experiment. Feed and drinking water were provided *ad libitum*. The experiment was carried out for four weeks.

Diets and preparation of fermented products

The diet (antibiotic-free) was formulated to meet the nutrient requirements of the rats according to the National Research Council (1995) recommendations. The compositions of basal diet are shown in Table 1. The rats were randomly assigned to three treatments. Each treatment groups consisted of eight rats. The three dietary treatments were: 1. basal diet (as control), 2. basal diet + 10% FP and 3. basal diet + 20% FP. All the rats were adapted to the respective diets for a week before the experiment was started.

TABLE 1

The composition of basal diet, %

Ingredients	Basal diet
Broken rice	20.00
Maize	30.88
Soyabean 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[1]	2.12
Palm oil	1.60
Fish meal	8.00

Composition of fatty acids, %

	Basal diet	Basal diet + 10% FP	Basal diet + 20% FP
Total saturated fatty acids	27.8	26.31	24.96
Total PUFA n-3[2]	0.34	0.37	0.48
Total PUFA n-6[2]	43.10	41.60	39.32

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

[2] PUFA = polyunsaturated fatty acid

The fermented product (FP) consisted of (%): raw fish (*Rastrelliger kanagurta*) 40, Chinese seaweed 8, rice bran 40 and sugar cane juice 12. The raw fish was crushed and mixed thoroughly with rice bran. The mixture was then mixed with sugar cane juice and combination cultures of LAB (3% from total amount) (obtained from Jia Yi Nutrition Technologies Sdn. Bhd., Malaysia) in a closed 20 l solid fermenter. The product was stirred hourly and air dried every two days. The mixture was fermented for 7 days at 80°C. The pH of the final product was 5.4 and contained 10^6 CFU of LAB/g of FP. The final product was in mash form and brownish in colour.

Faecal sampling and bacteriological analysis

Faecal samples were collected directly from the rectum of each rat every week. The pH of the faeces was directly measured with a pH meter. The faecal matter (10% w/v) was suspended in sterile peptone water and incubated for one h 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.

Total cholesterol concentration

At the end of experiment, the rats were fasted for 12 h before blood collection. The rats were anaesthetized with diethyl 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).

Statistical analysis

Results were expressed as mean ± standard error of mean (SEM). One-way analysis of variance (ANOVA) followed by the Duncan Multiple Range Test was used to compare the differences in CFU of *Enterobacteriaceae* and LAB, faecal pH, plasma total cholesterol concentration and growth performance due to treatment/time effects. Relationships between the weekly faecal pH, weekly *Enterobacteriaceae* counts and weekly LAB counts were investigated using the Spearman's Rank Correlation. All statistics were carried out using the SPSS 8.0 software (Chicago, IL; USA) (SPSS, 1998).

RESULTS

The growth performance for the different treatment groups is presented in Table 2. The initial and final liveweight, growth rate, total feed intake and feed conversion ratio were similar (P>0.05) for all the treatment groups.

TABLE 2

Effect of fermented products on growth performance of rats

Indices	Control	Fermented products (FP)	
		10%	20%
Initial body weight, g	151.7 ± 9.08	151.7 ± 7.50	152.2 ± 8.09
Final body weight, g	197.2 ± 9.25	200.8 ± 5.92	206.3 ± 6.99
Growth rate, g/day	1.62 ± 0.14	1.75 ± 0.16	1.93 ± 0.26
Total feed intake, g	396.7 ± 17.55	405.9 ± 15.39	391.2 ± 15.18
Feed conversion ratio	9.16 ± 0.84	8.59 ± 0.57	7.82 ± 0.71

the results are presented as mean values ± SEM. All values were not significantly different among each other (P>0.05)

The faecal *Enterobacteriaceae* counts of rats fed control, 10 and 20% FP are shown in Figure 1. At the beginning of the experiment, the *Enterobacteriaceae* counts in the rats were the same. After three weeks of experiment, higher *Enterobacteriaceae* counts were found in faeces obtained from rats fed with control feed compared to those rats fed FP; the faecal *Enterobacteriaceae* counts of rats fed 10 and 20% FP were not significantly different (P>0.05) from each other. At the final week of experiment, the rats fed 20% FP had the lowest (P<0.05) *Enterobacteriaceae* counts.

The faecal LAB counts were not significantly different (P>0.05) between the control feed group and the FP groups at the beginning of the experiment and in the first week of treatment (Figure 2). After two weeks of experiment, faeces excreted from rats fed FP had significantly (P<0.01) higher numbers of LAB compared with faeces from rats fed control diet. At the end of experiment, the rats fed 20% FP had the highest faecal LAB counts.

Figure 3 shows the faecal pH in rats fed control, 10 and 20% FP diets. The pH of the faeces was similar in all groups at the beginning of the experiment and decreased significantly (P<0.05) in rats fed with FP diet after two weeks of experiment. The faecal pH of 10 and 20% FP groups declined with time, whereas those of the control group remained the same.

The plasma cholesterol concentrations for different treatment groups are shown in Figure 4. The lowest (P<0.05) in plasma total cholesterol concentration was found in the 20% FP group, followed by the 10% FP group and control group.

The Spearman's correlation was carried out to investigate the probable relationships between the weekly faecal pH, weekly faecal *Enterobacteriaceae* counts and

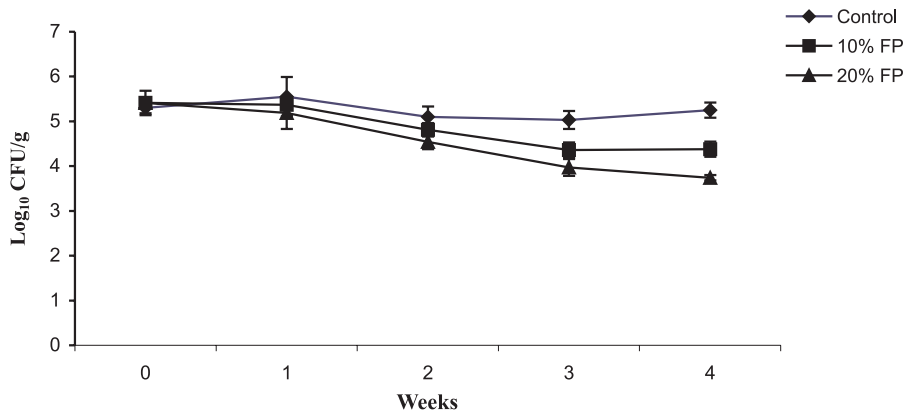

Figure 1. Faecal counts of *Enterobacteriaceae* in rats fed control, 10 and 20% FP diets for 4 weeks

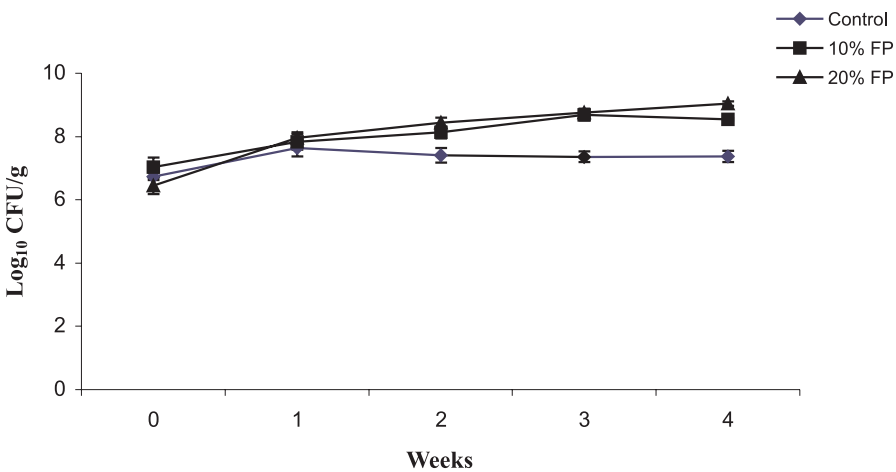

Figure 2. Faecal counts of LAB in rats fed control, 10 and 20% FP diets for 4 weeks

weekly faecal LAB counts (Table 3). No correlation ($P>0.05$) was found among these three variables at week 0, 1 and 2 of the experiment. The faecal pH was negatively correlated ($P<0.01$) with faecal LAB counts since the second week of experiment, whereas the other variables were not significantly correlated to each other. Since the third week, the *Enterobacteriaceae* counts were correlated positively ($P<0.05$) with the faecal pH, but correlated negatively with the faecal LAB counts. On the fourth week of experiment, all the four variables were strongly correlated ($P<0.01$) with each other. Faecal LAB counts were negatively correlated with faecal

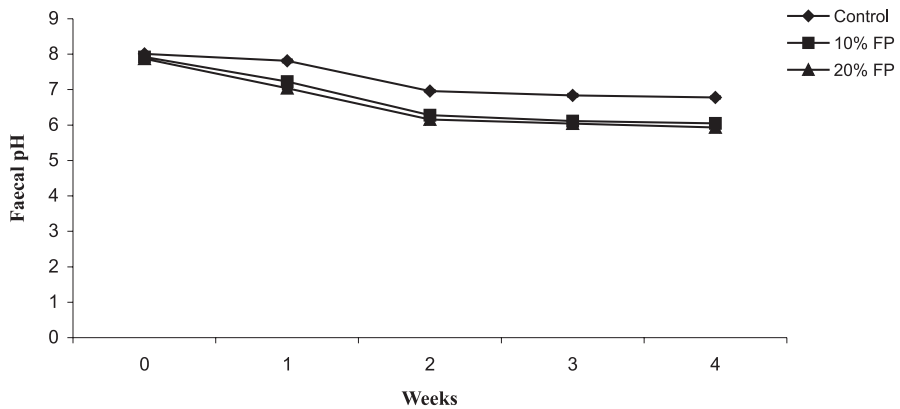

Figure 3. Faecal pH in rats fed control, 10 and 20% FP diets for 4 weeks

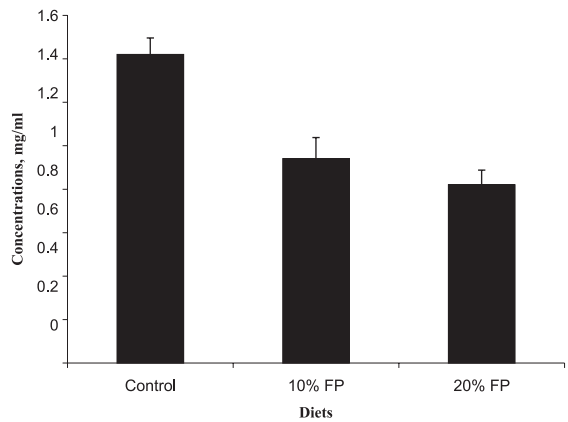

Figure 4. Plasma cholesterol concentration in rats fed control, 10 and 20% FP diets for 4 weeks, bars indicate the standard error of the mean

pH, the faecal *Enterobacteriaceae* counts and plasma cholesterol concentrations (R=-0.64; data was not shown). The faecal *Enterobacteriaceae* counts were positively correlated with faecal pH.

DISCUSSION

The results showed that there were no significant differences for the body weight, growth rate, feed intake and feed conversion ratio between treatment groups. Bernardeaus et al. (2002) reported that *Lactobacillus acidophilus* supplementation in

TABLE 3
Spearman's Rank Correlation between the faecal pH, *Enterobacteriaceae* counts and LAB counts

Week		Faecal pH	*Enterobacteriaceae* counts
0	Faecal pH	-	-0.065
	LAB counts	0.288	0.276
1	Faecal pH	-	0.098
	LAB counts	0.327	0.220
2	Faecal pH	-	0.254
	LAB counts	-0.595[2]	-0.137
3	Faecal pH	-	0.473[1]
	LAB counts	-0.697[2]	-0.400[1]
4	Faecal pH	-	0.725[2]
	LAB counts	-0.729[2]	-0.725[2]

[1] significantly different at P< 0.05 (2-tailed)
[2] significantly different at P< 0.01 (2-tailed)

drinking water did not affect the weight gain, feed intake and water intake in mice. It has been suggested that the growth stimulating effect of fermented products are more likely to be obtained in situations involving negative stress (Thomke and Elwinger, 1998). However, in the present study, there was no stressor applied to the rats.

The ability of LAB to inhibit the growth of various gram-negative bacteria especially pathogenic *E. coli* is well documented for both *in vitro* (Gopal et al., 2001) and *in vivo* condition (O'Mahony et al., 2001). The results of the present study demonstrated that rats given feeds containing FP had lower faecal *Enterobacteriaceae* counts than those of the control. Similar reduction of faecal *Enterobacteriaceae* have been obtained by the addition of *Lactobacillus plantarum* cultures to the diet of pigs (Van Winsen et al., 2002). Urlings et al. (1993) also reported that provision of the fermented feed to pigs resulted in reduced *Enterobacteriaceae* and *E. coli* counts in gastrointestinal tract.

Incorporation of beneficial microbials in the diet are known to benefit the animals by improving intestinal microflora equilibrium (Fuller, 1989). The results on LAB number in the faeces showed that the addition of FP containing LAB cultures increased the LAB population in the faeces 14 days after feeding. Similar results in increasing faecal LAB numbers have been obtained by the provision of fermented feed to pigs (Du Toit et al., 1998; Demecková et al., 2002; Van Winsen et al., 2002) and by the inclusion of beneficial bacteria in the diet of mouse (Fukushima et al.,

1999). The greater number of faecal LAB in rats with FP could be due to changes of the available substrates passing to the hindgut (Mathers and Goodlad, 1999).

Fermented feed is characterized by high numbers of lactic acid bacteria, a low pH and high concentration of lactic acid (Brooks et al., 2001). A significantly lower pH in faeces of rats fed FP compared with rats fed basal diet was observed. This is in contrast with the results of Urlings et al. (1993), Fransen et al. (1995) and Van Winsen et al. (2002). They found a higher pH of the faeces of pigs fed with fermented feed compared to pigs fed with normal feed. The contradictory results may be attributed to a lower production of VFAs (Urlings et al., 1993; Fransen et al., 1995; Van Winsen et al., 2002), excessive bicarbonate excretion in the colon or a change in metabolic processes in the colon due to a transition of carbohydrate to protein fermentation (Jensen and Jorgensen, 1994). The results of correlation analyses were found significant after two weeks of feeding, suggesting the effects of FP can only be seen after feeding for two weeks.

The results on negative correlations between faecal LAB and the faecal pH and between faecal LAB and faecal *Enterobacteriaceae* further support the theory that the acidic environment enhances the growth of LAB and inhibits the growth of *Enterobacteriaceae* (Van Winsen et al., 2002). We infer that the FP is able to reduce pH in the gastrointestinal tract and thereby enhances the activity of VFA. The increase in VFA concentration may have caused the reduction of *Enterobacteriaceae* in the gastrointestinal tract (Urlings et al., 1993) and therefore reduced *Enterobacteriaceae* shedding. The negative correlation between LAB and *Enterobacteriaceae* can be explained by the fact that high numbers of LAB may have enhanced colonization resistance capability against the *Enterobacteriaceae* (Volaard and Clasener, 1994) as demonstrated in those rats fed with FP. Similarly, Herias et al. (1999) reported that *Lactobacillus* strains inhibited the adherence of *E. coli* to the wall of the intestinal tract. We found a significant positive correlation between *Enterobacteriaceae* and pH. The result agrees with Deng et al. (1998), who reported that higher pH encouraged the growth of *Enterobacteriaceae*.

The results of the present study demonstrated that rats given FP had a lower plasma cholesterol concentration than control rats. Similar results in reduction of cholesterol concentration have been obtained by provision of fermented products in the diet of humans (Richelsen et al., 1996) and by the addition of lactic acid bacteria or bifidobacteria in the human diet (Pereira and Gibson, 2002). Fukushima and Nakano (1996) also reported that addition of mixture of *Lactobacillus acidophilus* or *Streptococcus faecalis* in the diet of rats resulted in reduced serum cholesterol concentration. The role of FP in relation to hypocholesterolaemia effect may be inferred from the results obtained from the correlation analysis, which showed that plasma cholesterol concentration was strongly correlated to the LAB counts in the faeces. It is believed that LAB, which are present in higher quantities in FP, plays important role in assimilating and deconjugating bile salts, as bile salts being the

water-soluble excretory end-products of cholesterol. These bile salts may be transformed by enzyme activities of some intestinal bacteria during the enterohepatic cycle (Du Toit et al., 1998). Deconjugated bile salts are excreted in the faeces and this may result in an increased requirement for cholesterol as precursor for the synthesis of new bile salts and thus may reduce the plasma cholesterol concentrations (De Rodas et al., 1996).

CONCLUSIONS

In conclusion, the present results demonstrated that the addition of FP in the diet of rats did not affect significantly the performance, but increased the numbers of faecal LAB counts, while the numbers of faecal *Enterobacteriaceae* and faecal pH were reduced significantly. Concurrently, the concentration of the plasma cholesterol was also significantly reduced in the FP treated animals. The results of correlation analyses show that FP provides low pH environment and high numbers of LAB, this would eliminate many pathogens in the faeces. This study gave further evidence of the importance and benefits of fermented products, indicating that FP has the potential to be a viable alternative to antibiotics as an additive in the diet of animals. This in turn will be reflected in the lower excretion number of *Enterobacteriaceae* and better nutritional quality.

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STRESZCZENIE

Wpływ skarmiania przefermentowanych produktów na rozwój szczurów, pH kału, liczebność *Enterobacteriaceae* i bakterii kwasu mlekowego i ich współzależności oraz stężenie cholesterolu w plazmie krwi

Przefermentowane produkty (FP), stanowiące mieszaninę surowych ryb, wodorostów morskich, otrąb pszennych i soku z trzciny cukrowej, otrzymano w procesie fermentacji stosując kombinację kultur bakterii kwasu mlekowego (LAB). Celem badań było określenie wpływu skarmiania diet zawierających różną ilość FP na rozwój szczurów, pH kału, liczebność *Enterobacteriaceae* i LAB i ich współzależności, oraz na stężenie cholesterolu w plazmie krwi.

Doświadczenie przeprowadzono na 24 samiczkach szczurów Spragne Dawley, w wieku 10 tygodni, podzielonych na 3 grupy po 8 w każdej. Zwierzętom podawano 3 diety doświadczalne: 1. kontrolna (podstawowa), 2. dieta podstawowa +10% FP i 3. dieta podstawowa +20% FP. Końcowa masa ciała, tempo wzrostu, pobranie i wykorzystanie paszy przez szczury nie różniły się istotnie między grupami. Dodatek FP do diet obniżył populację *Enterobacteriaceae* w kale i istotnie ($P<0,05$) zwiększył liczebność LAB w porównaniu z ilością LAB u zwierząt grupy kontrolnej, a pH kału było niższe. Otrzymane współzależności pomiędzy liczebnością LAB i *Enterobacteriaceae*, liczebnością LAB i pH kału, oraz liczebność *Enterobacteriaceae* i pH kału wskazują, że FP powoduje zakwaszenie środowiska, co sprzyja rozwojowi LAB, a w następstwie tego hamuje rozwój *Enterobacteriaceae*.

Stężenie cholesterolu w plazmie krwi szczurów otrzymujących diety z FP było istotnie niższe ($P<0,05$) niż u szczurów kontrolnych.