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Effect of dietary supplementation with butyrate and probiotic on the survival of Pacific white shrimp after challenge with *Vibrio alginolyticus*

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ABSTRACT - This study evaluated the performance, immunology, and survival of the Pacific white shrimp *Litopenaeus vannamei* to experimental challenge to *Vibrio alginolyticus* based on the use of the probiotic *Lactobacillus plantarum* and the combined use of probiotic and butyrate. Four different diets resulted from the addition of additives: butyrate, probiotic, butyrate + probiotic, and control (no additives). The attractiveness of the diets was assessed by the percentage of positive choices and rejections, using a dual-choice Y-maze format aquarium. The shrimps were fed during four weeks and performance parameters, intestinal microbiota, and immunological parameters were all evaluated. Subsequently, the shrimps were challenged with *V. alginolyticus* and after 48 h, survival and immunological parameters were evaluated. The results showed increased attractiveness and intake, but only with diets supplemented with sodium butyrate. However, other diets were not rejected. No difference in performance or immunological parameters was observed among the different diets. Also, among the treatments, no difference in *Vibrio* spp., or total heterotrophic bacteria counts, was found in the intestinal tract. However, the lactic acid bacteria count was higher in the intestinal tract of shrimps fed diets supplemented with probiotic. After bacterial challenge, shrimp fed all diets had a greater survival when compared with the control group. *Lactobacillus plantarum* and sodium butyrate increase the resistance of shrimp to infection with *V. alginolyticus*, but do so without affecting performance, immunological parameters, or *Vibrio* spp., and total heterotrophic bacteria counts in the intestinal tract.

Key Words: animal production, lactic acid, organic acids

Introduction

The Pacific white shrimp *Litopenaeus vannamei* is the most cultivated species at 73.2% of the total volume produced (FAO, 2012). However, shrimp production has been drastically reduced in some countries as a consequence of the emergence of several diseases associated with aquaculture (Lightner, 2011), including early mortality syndrome (EMS) caused by a strain of *Vibrio parahaemolyticus*, mostly in Asia and Latin America (Lightner et al., 2012; Tran et al., 2013). Infections caused by *Vibrio* are considered an important problem in shrimp farming, causing symptoms such as anorexia, inactivity, low growth rate, muscular necrosis and, consequently, high mortality (Chiu et al., 2007; Lafferty et al., 2015).

As treatment against vibriosis, antibiotics have been commonly used; however, inappropriate or excessive use in aquaculture has led to the selection of resistant bacteria (Deifordt et al., 2011). In addition, the use of antibiotics in aquaculture can affect human health and the environment as a result of residue contamination (Costanzo et al., 2005). Thus, for a sustainable development of the aquaculture sector, it is necessary to take steps towards an alternative to replace the use of antibiotics.

As such alternative measures, probiotics and organic acids, as well as their salts, have been widely implemented in aquaculture to decrease outbreaks of bacterial diseases. Probiotics may be defined as "a live microbial feed supplement which beneficially affects the host animal by improving its intestinal microbial balance" (Gram et al., 1999). Lactic acid bacteria (LAB) are widely used as probiotics by their rapid reproduction, production of antimicrobial compounds, such as bacteriocins, hydrogen peroxide, organic acids, and lactic acid and the capacity to stimulate host immune response (Gatesoupe, 2008).

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Organic acids can also inhibit bacteria, mostly Gram-negative, reducing the pH environment and forming chelate complexes with such essential minerals as iron, thus limiting the growth of other microorganisms (Jones, 1998; Lückstädt, 2008). Many organic acids are also available in sodium, potassium, and calcium salts, having the advantages of being odorless and less corrosive. They are also easy to manipulate as a feed additive by their solidity and lack of volatility (Partanen and Mroz, 1999).

This study evaluated the performance, immunology, and survival of the Pacific white shrimp *Litopenaeus vannamei* to experimental challenge to *Vibrio alginolyticus* based on the use of the probiotic *L. plantarum* and the combined use of probiotic and butyrate.

Material and Methods

Lactobacillus plantarum was the bacterial strain used as the probiotic. It was isolated from adult shrimps of *L. vannamei* (Vieira et al., 2007) and maintained in the collection of microorganisms in Florianópolis, SC, Brazil (27.5949° S, 48.5482° W). Sodium butyrate (C_3H_7COONa) was chosen as the organic salt by having presented the best results of *in vitro* inhibition against *V. alginolyticus* among eight different salts in previous tests.

Diets were prepared as shown in Table 1. Ingredients were ground and sieved (500 µm). Subsequently, the micro-ingredients were homogenized in a Y-mixer for 10 min and added to the macro-ingredients for homogenization in a food mixer for 10 min. Soon afterwards, oils and soy lecithin were added, followed by 40% hot water (40 °C). The resulting mixture was pelletized through a meat grinder and dried for approximately 18 h at 40 °C. The organic acid salts were added in the respective quantities to replace kaolin, which was used as a filler. Posteriorly, the probiotic was included in the diet by the inoculation of 10% of the lactic acid bacteria in the diet (Vieira et al., 2008).

Diets were divided as follows: butyrate, probiotic, butyrate + probiotic, and control (diet without additives). Two percent of the organic salt (p/p) and 100 mL of probiotic (1×10^7 CFU mL^{-1}) per kilogram were added to the diet according to the methodology described by Vieira et al. (2008).

The proximate composition of the diets (Table 2) was performed according to the methodology described by the Association of Official Analytical Chemists (AOAC, 2005). Dry matter (DM) was calculated by gravimetric analysis after drying in an oven at 100 °C for 24 h. The ash content was determined gravimetrically by burning in a muffle furnace at 550 °C for 6 h (942.05 in the AOAC, 2005). The method of Kjeldahl (2001.11 in the AOAC, 2005) was used to determine the crude protein content. Crude fiber and ether extract with acid hydrolysis were analyzed by methods 978.10 and 2003.05, respectively (AOAC, 2005).

Feed attractiveness was evaluated using the methodology described by Nunes et al. (2006) through the Y-maze aquarium. A total of 60 shrimps weighing 4.1±1.3 g were fasted for 24 h to stimulate the feeding response before each test. Before every session, the water of each aquarium was changed to avoid the influence of nutrients or the possible presence of remaining feed.

For each observation, two diets were compared. These were offered separately in similar amounts (2 g) and placed individually in the perimeter of one of the Y-maze apparatus arms. Prior to behavioral evaluation, the shrimps were stocked in the acclimation chamber and allowed to acclimate to the Y-maze system for 10 min. For each experimental diet, one different specimen of *L. vannamei* was used at a time. In the event that feed was not detected within a 7-min time limit, the observation was interrupted

Table 1 - Composition of the experimental diets (control and butyrate) used in the growth of *Litopenaeus vannamei* in a clearwater system

Ingredient[1]	Control g.kg^{-1}	Butyrate g.kg^{-1}
Soybean meal	424	424
Fish meal	232	232
Wheat flour	150	150
Kaolin	84	64
Soy lecithin	30	30
Monocalcium phosphate	20	20
Mineral-vitamin premix	15	15
Sodium chloride	12	12
Soybean oil	10	10
Fish oil	10	10
Magnesium sulphate	7.7	7.7
Binder (CMC)	5.0	5.0
Vitamin C	0.6	0.6
Sodium butyrate	0	20

CMC - carboxymethyl cellulose.
[1] Probiotic was added after diet preparation (Vieira et al., 2008).

Table 2 - Proximate composition (dry matter) of the diets used in the growth of *Litopenaeus vannamei* in clearwater system

Nutrient	Control	Butyrate	Probiotic	Butyrate + probiotic
Humidity (%)	10.81	9.79	10.6	10.12
Gross protein (%)	39.51	40.08	41.54	42.01
Ether extract (%)	8.89	8.66	8.51	8.54
Acid detergent fiber (%)	6.25	4.61	4.13	5.45
Ash (%)	21.72	20.64	21.76	20.91

and the animal replaced with another acclimated specimen. The percentages of positive choices for each tested diet relative to all other diets were calculated according to the following expression: positive choices (%) = (total number of choices/total number of comparisons) × 100.

Shrimps (mean weight of 5.28±0.08 g) were stocked in 16 tanks of 800 L (inside a greenhouse) at a density of 40 shrimp m^{-3} in clearwater system with 30% of static water exchange per day. The experiment lasted four weeks between the months of April and May 2015. Animals were fed diets in a quantity equivalent to 6% of biomass divided into four times a day (8:00, 12:00, 14:00, and 17:00 h). The feeding was adjusted according to weight checks conducted weekly. All treatments were performed in quadruplicate.

Temperature and dissolved oxygen were measured daily (YSI model Pro 20) and salinity was maintained at 30 ppt. Water pH, ammonia, and nitrite were also measured once a week.

After four weeks, all shrimps were counted and weighted to evaluate weight gain, feed conversion, and survival using the follow equations:

$$\text{Weekly weight gain} = \frac{\text{Final weight (g)} - \text{Initial weight (g)}}{\text{Weeks}}$$

$$\text{Feed conversion} = \frac{\text{Feed intake (g)}}{\text{Final biomass (g)}}$$

$$\text{Survival rate} = \frac{\text{No. survive shrimps}}{\text{Total number stock}} \times 100$$

Five shrimps from each experimental unit were sampled to form a pool per unit for microbiology analyses. The midguts of the shrimps were excised with forceps and scalpel and then homogenized in sterile saline solution in 3% NaCl in a grail. The samples were then serially diluted (1/10) and seeded in Agar Marine culture medium for total heterotrophic marine bacteria and Agar TCBS (Thiosulphate Citrate Bile Sucrose), which is a selective medium for vibrios. Finally, they were incubated at 29 °C for 24 h.

Haemolymph from 10 shrimps per experimental unit was collected to form two pools. The samples were collected with 1 mL sterile syringes with a 21 G needle and cooled at 4 °C. A total of 40 µL hemolymph from each animal were fixed in solution of 4% formaldehyde/MAS (sodium citrate 27 mM, EDTA 9 mM, glucose 115 mM, NaCl 336 mM, pH 7.0) for total haemocyte count (THC). The remaining hemolymph was left to clot at 4 °C. The coagulated haemolymph was frozen at −20 °C and repeatedly centrifuged at 10,000 *g* for 10 min to obtain the serum, which was aliquoted and stored at −20 °C for later use in other immunological analyses.

The number of haemocytes per millilitre of haemolymph was estimated by direct counting in a Neubauer chamber, considering dilution.

To determine serum agglutination titre, serum samples of 50 µL were serially diluted in TBS-1 (50 mM Tris, 150 mM NaCl, 10 mM $CaCl_2$, 5m M $MgCl_2$, pH 7.4) in a flat-bottomed 96-microwell plate. After adding 50 µL of 2% solution from dog erythrocytes in TBS-1 solution, the microplate was incubated for 3 h at 25 °C in a humidified chamber. The control was performed through the substitution of serum by TBS. Serum agglutination titre was defined as the reciprocal of the highest dilution capable of agglutinating erythrocytes.

Phenoloxidase activity (PO) was determined by spectrophotometry (DO490 nm) through the formation of DOPA-chrome pigment after oxidation of the substrate L-dihydroxyphenylalanine (L-DOPA, Sigma Chemical Co., St. Louis, MO, USA), using the methodology described by Söderhäll and Häll (1984). Briefly, serum samples were diluted (1:8) in TBS-2 (10 mM Tris, 336 mM NaCl, 5 mM $CaCl_2$, 10 mM $MgCl_2$, pH 7.6) and 50 µL of this solution were preincubated with an equal volume of the enzyme inducer trypsin (SIGMA, 1 mg mL^{-1}) for 15 min at 20 °C in a flat-bottomed 96-microwell plate. In controls, trypsin and serum were replaced by TBS-2. After incubation, 50 µL of L-DOPA (3 mg mL^{-1}) were added to the wells and the formation of DOPA-chrome was monitored after 0, 5, 10, and 15 min. One unit of the specific activity from PO was equivalent to the variation of 0.001 in the absorbance. min^{-1} mg^{-1} of protein.

The concentration of protein in the haemolymph was estimated by the method of Bradford (1976) through the use of bovine serum-albumin as standard.

At harvest, 10 shrimps (in the intermolt stage) from each tank were transferred to 16 30-L aquaria with aeration (O_2>5 mg L^{-1}) and heating systems (29±1 °C). Each shrimp was injected with 100 µL of *V. alginolyticus* at a concentration of 1×10^7 cfu mL^{-1}, according to the LD_{50} test conducted previously by the authors (data not shown). As a control group, a sampling of shrimps was injected with 100 uL of sterile saline solution. The shrimps were monitored during 48 h and after this period, they were evaluated for survival and immunological parameters. All treatments were performed in quadruplicate.

Before being subjected to statistical analysis, the bacteriological count data were transformed into log10 (x + 1) and the serum agglutination data were transformed into log2 (x + 1). Data homoscedasticity was assessed by the test of Bartlett. After verifying the assumptions of normality

and homoscedasticity, data were subjected to unifactorial analysis of variance supplemented by the Tukey test of separation of averages, both at the significance level of 5%. Attractiveness was analyzed by using the non-parametric chi-square. Differences in survival levels between treatments were analyzed by Kaplan-Meier log-rank χ^2 tests.

Results

Diet supplemented with butyrate had higher attractiveness, but only when compared with control diet (Table 3). The other diets showed no significant differences when compared among themselves.

Water quality parameters remained within acceptable standards for marine shrimp (Boyd and Gautier, 2000) without great temperature (29.56±0.30), oxygen (6.07±0.05), pH (8.37±0.08), ammonia (0.55±0.19), or nitrite (0.13±0.11) variations. After four weeks, shrimps of the different treatments showed no significant differences in performance parameters (Table 4).

Vibrio spp. and total heterotrophic bacteria counts in the midgut revealed no differences among treatments (Table 5) when compared with the control group, while the lactic acid bacteria count was higher in diets supplemented with the probiotic (P<0.05).

At the end of the experiment and before challenge, serum agglutination titre did not differ among the treatments (Table 6). Total haemocyte count was, however, higher in the butyrate treatment when compared with probiotic and butyrate + probiotic treatment, but did not differ with the control group. Phenoloxidase activity was significantly higher in the probiotic treatments compared with the other treatments.

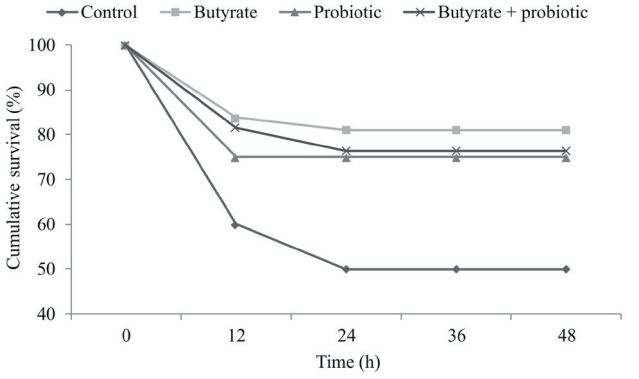
Table 3 - Frequency of positive choices (%) of shrimp *Litopenaeus vannamei* kept in the Y-maze and fed different diets

Diet	Control	Butyrate	Probiotic	Butyrate + probiotic
Control	-	80*	60	40
Butyrate	20	-	40	50
Probiotic	40	60	-	50
Butyrate + probiotic	60	50	50	-

* Difference in chi-square test (P<0.05).

After challenge, THC decreased significantly (P<0.003), but only in the butyrate treatment, compared with THC before infection. Also, the butyrate treatment had a lower THC when compared with the butyrate + probiotic treatment, but without showing differences with the other treatments. The control group had no differences in number of haemocytes when compared with the other treatments. Additionally, while the agglutination titre increased significantly in all treatments after infection (P<0.0001), the control group presented no differences compared with other treatments. There were differences only between the probiotic and butyrate + probiotic treatment, with a higher agglutination titre in the probiotic group. No significant differences were noted in PO activity among the treatments; however, enzymatic activity was numerically lower in the control group.

Fourteen hours after challenge, shrimps started to present symptoms of lethargy and necrosis in the tissues, followed by the first deaths. After 48 h, survival was significantly higher in the treatments compared with the control group (Figure 1). However, the results obtained from the use of probiotic combined with organic salt did not significantly differ from the use in isolation.



Treatments (butyrate, probiotic, and butyrate + probiotic) with significant difference (P<0.05) to control group by Kaplan–Meyer log-rank χ2 test.

Figure 1 - Cumulative survival of *L. vannamei* fed different diets (butyrate 2%, probiotic, butyrate 2% + probiotic, and control) after challenge with *V. alginolyticus*.

Table 4 - Performance parameters of *Litopenaeus vannamei* cultivated in clearwater system and fed different diets

Diet	Initial weight (g)	Final weight (g)	Weekly weight gain (g)	Feed conversion	Survival (%)
Control	5.34±0.19	12.58±0.31	1.84±0.36	1.9±0.16	96.7±0.8
Butyrate	5.17±0.20	12.11±0.36	1.74±0.29	2.2±0.21	98.3±0.6
Probiotic	5.32±0.21	12.22±0.58	1.73±0.40	2.3±0.28	97.5±0.5
Butyrate + probiotic	5.28±0.22	12.35±0.55	1.77±0.21	2.0±0.09	97.5±1.0

Table 5 - Microbiological count (log) of the intestine of *Litopenaeus vannamei* fed different diets after four weeks of cultivation

Treatment	*Vibrio* sp.	Total heterotrophic bacteria	Lactic acid bacteria
Control	6.56±0.98a	8.54±1.31a	0.79±1.58a
Butyrate	7.59±0.97a	8.54±0.25a	0.67±1.35a
Probiotic	6.97±0.79a	8.33±0.41a	3.54±0.71b
Butyrate + probiotic	7.03±0.51a	8.49±0.42a	3.55±0.65b

Table 6 - Total haemocyte count (THC), phenoloxidase activity (PO), and serum agglutination titre of *Litopenaeus vannamei* shrimp before and after challenge with *Vibrio alginolyticus*

Treatment	THC ($\times 10^6$ mL^{-1})	PO activity (U mg^{-1} min^{-1})	Agglutination titer
Before infection			
Control	44.3±6.3Aab	27.17±8.54Ab	10.83±0.50A
Butyrate	58.57±6.7Ab	17.51±4.45Aab	10.58±0.00A
Probiotic	29.33±7.9Aa	42.38±3.93Ac	11.33±0.50A
Butyrate + probiotic	39.1±8.5Aa	14.20±1.96Aa	11.33±0.50A
After infection			
Control	33.30±7.61Aab	27.17±3.11A	14.08±0.58Bab
Butyrate	26.32±3.92Ba	43.87±6.15B	13.83±0.50Bab
Probiotic	32.21±1.25Aab	33.12±2.48B	14.83±0.50Bb
Butyrate + probiotic	45.05±3.51Ab	48.12±17.60B	13.33±0.50Ba

Uppercase letters indicate significant differences in time; lowercase letters indicate statistical differences among the treatments.

Discussion

The use of additives in diets, including organic acids, or their salts, can change palatability, either decreasing or increasing attractiveness. Silva et al. (2013) found that the attractiveness and the intake of commercial diets for shrimp increased when supplemented with sodium butyrate and propionate. In another study, it was observed that citric and lactic acids also increased attractiveness of diets for tilapia (*Oreochromis niloticus*). However, acetic acid and metacetonic acid had the opposite effect, resulting in the rejection (Xie et al., 2003). In the present study, the only diet presenting differences in attractiveness was the one supplemented with butyrate when compared with the control diet, which was the least attractive diet to shrimps. The other diets showed no differences among them, indicating that the additives did not decrease the attractiveness of feed.

Several studies have reported that organic acids complemented by probiotics can improve performance parameters through the increased activity of digestive enzymes (Wang, 2007; Zhou et al., 2009), improving the digestion of macro and micro nutrients (Lin et al., 2004; Hossain et al., 2007) and the digestibility of protein (Lückstädt, 2008; Buglione-Neto et al., 2013), consequently improving feed conversion and specific growth rate (Kongnum and Hongpattarakere, 2012; Romano et al., 2015). *Litopenaeus vannamei* shrimp fed both organic salts, such as propionate and sodium butyrate, and the probiotic *L. plantarum* had higher final weight, feed efficiency, and survival when compared with the control group (Kongnum and Hongpattarakere, 2012; Silva et al., 2014). In the present study, we did not observe the same behavior, possibly as a result of rearing conditions, since shrimps were not exposed to any stress in our clearwater system with low density and good water quality, providing ideal growth conditions.

Shrimps receiving the probiotic showed higher LAB colony counts in the intestine compared with groups not receiving the probiotic, proving that *L. plantarum* could remain in the feed and reach the intestine through the ingestion of feed. Similar studies with *L. vannamei* supplemented with *L. plantarum* have also shown that the intestinal microbiota is modified by increasing the concentration of LAB and, consequently, decreasing the concentrations of *Vibrio* spp. (Vieira et al., 2010). A study on *L. stylirostris* reported that the lactic acid bacterium *Pediococcus acidilactici* could also change the microbiota of shrimp and, as a result, decrease the concentrations of *Vibrio* spp. and total heterotrophic bacteria in the intestine (Castex et al., 2008).

Even though differences in *Vibrio* spp. or total heterotrophic bacteria counts were not observed among treatments in a previous study, LAB could still inhibit the growth of different microorganisms by decreasing the pH of their environment, increasing competition for nutrients and sites of adhesion, as well as producing antimicrobial compounds (Gatesoupe, 2008). On the other hand, in addition to decreasing pH, organic acids, or their salts, can produce chelate complexes with minerals, thus inhibiting the growth of bacteria, such as those of the genus *Vibrio* (Whitaker et al., 2010). In *L. vannamei*, studies have shown that organic salts decrease the concentration of *Vibrio* spp. in both hepatopancreas and intestine (Silva et al., 2013, 2014; Romano et al., 2015).

In the experimental challenge with *V. alginolyticus*, shrimps fed diets supplemented with sodium butyrate and/or the probiotic had higher survival than shrimps from the control group ($P<0.019$). *Lactobacillus plantarum* also has proven to be beneficial for *L. vannamei* by increasing humoral and cellular immune response after challenge with *V. alginolyticus* (Chiu et al., 2007) and increasing the survival after infection with *V. harveyi* (Vieira et al. 2007, 2010). Organic salts also increase the survival of shrimps after challenges with *V. harveyi* (Roman et al., 2015) and the survival of tilapia fed 0.5% potassium diformiate

(KDF) after challenge with *Vibrio anguillarum* (Ramli et al., 2005). Diets with 0.3% KDF also help to decrease overall mortality in tilapia challenged with *Streptococcus agalactiae* (Ng et al., 2009). However, we suggest further studies using oral/bath infection, emulating the natural infection route and minimizing possible septicemia caused by direct injection into the animal aiming at obtaining the real benefits of probiotic/organic acid to the gut.

One of the most important determinants of host immunological response in shrimp is PO activity. The results showed that before infection, PO activity was higher in the probiotic treatment when compared with all treatments. However, the supplementation with butyrate seemed to neutralize the effect of the probiotic on the PO activity in the treatment using both feed additives. After infection, even though we did not obtain statistical differences, the numerically higher PO activity in shrimp fed diets supplemented with additives (butyrate, probiotic, and butyrate + probiotic) may have helped to enhance host resistance to *V. alginolyticus* challenge.

Shrimp fed probiotic diets (probiotic and butyrate + probiotic) before infection and shrimp fed butyrate diet after infection had lower THC. However, the TCH in these treatments was close to 30×10^6 cells mL^{-1}, common value for healthy shrimp (Rodríguez and Le Moullac, 2000). In addition, this lower THC did not seem to be prejudicial, once the survival in these treatments (probiotic, butyrate, and probiotic + butyrate) after *V. alginolyticus* challenge was higher than control.

The lower agglutinating titer in shrimps fed diet supplemented with both additives (butyrate + probiotics), although significantly different, does not seem to be biologically important, once the numerical difference is small.

However, in this experiment, the results of the use of probiotic combined with organic salt did not differ significantly from its use in isolation, contrasting with the results obtained in broiler chickens fed organic acids combined with probiotics, presenting better results than individual treatments after infection with *Salmonella enteritidis* (Wolfenden et al., 2007).

Conclusions

Sodium butyrate and *L. plantarum* increase the resistance of *L. vannamei* to challenge with *V. alginolyticus*; however, it does not change the performance or the immunological parameters of shrimp reared in the clearwater system. Adding the probiotic *L. plantarum* to the diet increases LAB counts in the intestine of *L. vannamei*; however, it does not change *Vibrio* spp. or total heterotrophic bacteria counts in the midgut. Finally, the use of *L. plantarum* and sodium butyrate in feed does not alter its attractiveness.

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