

Effect of prodigiosin on the alleviation of the intestinal inflammation of weaned rats based on ¹H-NMR spectroscopy study and biochemistry indexes

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Weaning results in intestinal dysfunction, mucosal atrophy, transient anorexia, and intestinal barrier defects. In this study, the effect of prodigiosin (PG) on the intestinal inflammation of weaned rats was investigated by using ¹H-NMR spectroscopy and biochemistry indexes to regulate the intestinal metabolism. After administration for 14 days, the body mass of the PG group was increased by 1.29- and 1.26-fold compared with those of the control and alcohol groups, respectively, using a dose of 200 µg PG·kg⁻¹ body weight per day. PG increased organic acid content and decreased moisture, pH values, and free ammonia in feces. In addition, PG alleviated the intestinal inflammation of weaned rats. The analysis of ¹H-NMR signal peak attribution and the model validation of metabolic data of feces contents showed that PG significantly affected the metabolism of small molecular compounds in the intestinal tract of weaned rats. This study presents the promising alternative of using PG to alleviate intestinal inflammation effectively in the intestinal tract of weaned rats.

Keywords: Prodigiosin/pharmacokinetics. Proton Magnetic Resonance Spectroscopy/method. Intestinal Absorption/drug effects. Metabolomics/ methods. Rats/weaned. Metabolism/drug effects.

INTRODUCTION

Mammals generally suffer from biological inflammations due to physiological challenges and environmental changes in the wake of weaning (Moesser, Pohl, Rajput, 2017; Smith *et al.*, 2010). These biological inflammations involved in animal metabolic disorders, gastrointestinal dysfunction, damages to the intestinal mucosal barrier function, and disruption in the intestinal microbial function (Smith *et al.*, 2010). These adverse symptoms could cause intestinal inflammation and dampen the growth and health of the animal (Pohl *et al.*, 2017).

Several adverse events associated with weaning include diet change, maternal separation, and transportation (Campbell, Crenshaw, Polo, 2013). The underlying mechanisms of external regulation factors to minimize the

unhealthy effects of weaning are extremely complicated (Moesser, Pohl, Rajput, 2017). To circumvent the adverse consequences, the nutrition components of L-glutamate and L-aspartate supplementation were capable of attenuating the intestinal inflammation in weaned animals (Duan *et al.*, 2016). In addition, other supplements such as cromolyn (Mereu *et al.*, 2015), glutamine (Wang *et al.*, 2015), photoperiod (Niekamp *et al.*, 2007, probiotics (Hayakawa *et al.*, 2016), and bile acid (Ipharraguerre *et al.*, 2013; Diego-Caberol *et al.*, 2015) were used to relieve the inflammation by weaning (Maia *et al.*, 2014; Trckova *et al.*, 2017). However, these nutritional components had no effective action on the alleviation of adverse events in the intestinal tract by weaning. The development of an effective component has a crucial role in alleviating the adverse effect of weaning.

As a secondary metabolite produced by *Serratia marcescens*, prodigiosin (PG) is a red pigment with a molecular formula of C₂₀H₂₅N₃O (Stankovic *et al.*, 2014). PG has many bioactivities, including anti-malaria, anti-oxidation, immune regulation, and anti-cancer (Nisha,

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Kumar, Kumar, 2015). However, the effect of PG administration on the intestinal inflammations of weaned mammals has not been reported. In this study, weaned rats were fed with PG to determine whether PG alleviated intestinal inflammation of weaned rats.

METHODS AND MATERIAL

Rats, PG, and main instruments

PG produced by *S. marcescens* was isolated by ethanol extraction and purified using column chromatography. The 19-day male Sprague Dawley (SD) rats of 39–57 g weight were from Changzhou Carvins Co., Ltd., under the license number SCxk Su, 2014-0007. This study was approved by the Institutional Animal Care and Use Committee of Hefei University of Technology. Animal care and handling were strictly performed in compliance with US National Institutes of Health Publication 85-23 “Guide for the Care and Use of Laboratory Animals.”

Alcohol was used as the vehicle of PG administration. To compare the effect of alcohol, 90 SD rats were randomly divided into three groups: control group (control, n=30), the rats received normal diet; PG group (n=30), a dose of 200 µg PG·kg⁻¹ body weight per day was administered (containing 5 g of alcohol per 100 g of physiological saline); and alcohol group (n=30), the rats received 5 g of alcohol per 100 g of physiological saline. PG is insoluble in water and soluble in alcohol. In this study, PG was dissolved in alcohol and fed to rats. To eliminate the interference of alcohol, the alcohol group was also tested. All rats freely fed and allowed to drink water. The feeding conditions included an indoor temperature range of 22 °C-25 °C, 12:12 h of day and night cycle from 8:00 am to 8:00 pm, and 50%-70% humidity. Avance DRX-600 high-resolution nuclear magnetic resonance spectrometer and GC-2010 high-efficiency gas chromatography were from Bruker (German) and Shimadzu (Japan), respectively.

The determination of cecal content for ¹H-NMR measurement

After the cecum contents were thawed at 25 °C, 500 mg cecal contents were added into a 2 mL centrifuge tube loaded with 1 mL phosphate buffer solution adjusted to pH 7.4; the solution was prepared by using 1.9 mM Na₂HPO₄, 8.1 mM NaH₂PO₄, and 150 mM NaCl. After whirlpool processing for 1 min, mixing for 10 min, and swirling for 1 min, a centrifugation at 13,000 rpm/min for 1 min was performed to isolate the mixture. A solution

of 400 µL aliquot supernatant was extracted and mixed with 200 µL deuterium oxide (D₂O, containing 0.1% 3-trimethylsilylpropionic acid). After a centrifugation of 13,000 rpm/min at 4 °C for 15 min, a volume of 550 µL supernatant was extracted and added to a 5-mm nuclear tube for further treatment (Romick-Rosendale *et al.*, 2009).

Preparation for ¹H-NMR determination

The ¹H-NMR spectrum of the cecal contents was recorded by the nuclear magnetic resonance spectrometer set at a resonance frequency of 600.58 MHz and a temperature of 294.2 K. One-dimensional hydrogen spectrum of the cecal contents was recorded using a NOESY gppr-1D pulse sequence with a 20 ppm of sampling width, 64 K of sampling point, 3 sec of relaxation delay time, and 32 of cumulative number (Hong *et al.*, 2010). The phases and baselines of ¹H-NMR profiles were corrected using MestReNova-6.1.1 from Mestrelab Research S.L. The catalogs of cecal contents were calibrated using TSP. The integral of each map was an area of δ 0.5-9.0 ppm (removing a residual water peak of δ 4.30–5.10 ppm; an integral interval of 0.01 ppm). The databases for identifying cecal contents were Human Metabolome Database and published literature (Wishart *et al.*, 2007).

Multivariate statistical analysis of ¹H-NMR data

The normalized ¹H-NMR data was introduced into the SIMCA-P 13.0 software package for multivariate statistical data analysis. Principal component analysis (PCA) was used to test the sample grouping in the unsupervised mode and check whether a special sample existed. Partial latent structure discriminant analysis (PLS-DA) was used to evaluate the grouping. Cross validation and permutation tests verified the validity of the model. Orthogonal projection to latent structure-discriminate analysis (OPLS-DA) estimated the sizes of the metabolites to identify the metabolites. Cross validation and analysis of the variance of the cross-validated residual analysis (CV-ANOVA) analyzed the results. The variable importance of the project (VIP) >1.00 represented a significant difference. The difference was significant only when the metabolites met the requirements of both VIP > 1.00 and *P* < 0.05 (Wang *et al.*, 2013).

Measurement of feces moisture, organic acids, and free ammonia

The determination of feces moisture content was

performed as follows. A certain amount of fresh feces was placed onto a glass dish and weighed. The sample was dried at 105 °C until constant mass was achieved. The moisture of feces was calculated in accordance with the following formula: feces moisture content (%) = $100 \times (\text{initial weight} - \text{dry weight}) / \text{initial weight}$. The contents of acetic, propionic, butyric, isobutyric, and total acids were measured by gas chromatography method (Farup, Rudi, Hestad, 2016). The contents of free ammonia were measured as follows. After mixing 0.2 g of feces and 2 g of non-ammonia water, centrifugation was performed at 4000 r/min for 5 min. The supernatant at 1 mL was extracted and mixed with 1 mL of phenol reagent prepared with 0.5 mol/l phenol and 0.001 mol/L of sodium nitroferricyanide. After oscillation, the mixture was mixed with 1 mL of hypochlorite solution (prepared with 0.625 mol/L of sodium hydroxide and 0.03 mol/L of sodium hypochlorite). The absorbance was measured at a wavelength of 625 nm after exposure to a temperature of 60 °C for 5 min.

Histopathological analysis of jejunum

The investigated rats fasted for 12 h and then anesthetized by intraperitoneal injection with pentobarbital sodium at 45 mg kg⁻¹ body weight. The rats were sacrificed, the cecum was longitudinally cut, and the cecum contents were collected. A 1–4 cm jejunum was fixed in a solution of 4% paraformaldehyde (v/v). After washing with water, ethanol gradient dehydration, xylene treatment, and conventional paraffin embedded treatment, the jejunum tissue was cut into 5 µm slices. After de-waxing, rehydration, HE staining, dehydration, transparent, and sealing, the images were acquired using a digital image acquisition system (Van Beers-Schreurs *et al.*, 1998).

RESULTS

Body weight, feces moisture, and pH

The body mass of the PG group was 145.7 g, which was 1.29- and 1.26-fold of the control and alcohol groups, respectively, after successive administration for 14 days (Figure 1). The moisture content of feces from the PG group significantly decreased compared with those from the control and alcohol groups ($P < 0.05$) (Figure 2). In addition, the pH values of feces from the control, alcohol, and PG groups were 7.2, 7.3, and 6.8, respectively. Therefore, PG administration resulted in the increase of body mass and the decrease of feces moisture and pH value.

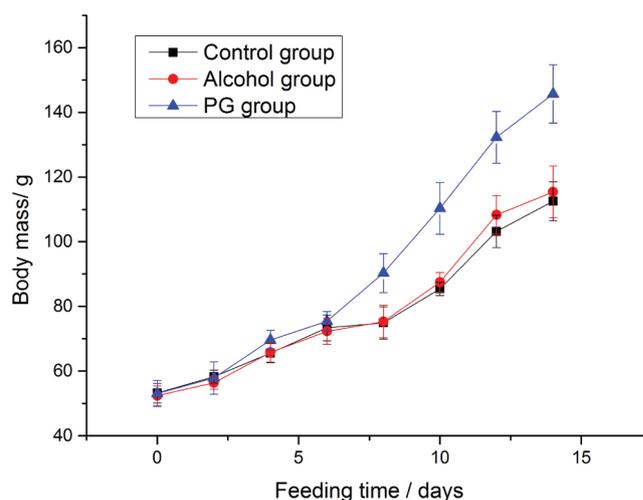


FIGURE 1 - Effect of PG on the body mass.

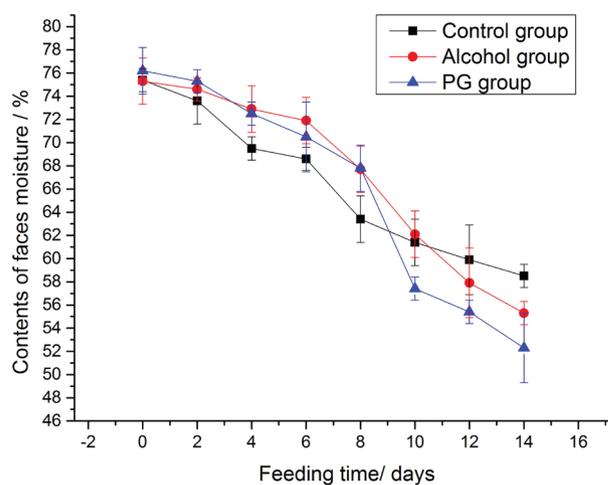


FIGURE 2 - Effect of PG on the contents of feces moisture.

¹H-NMR signal peak attribution of the cecal contents

The typical 600 MHz ¹H-NMR original spectra of 43 metabolites were identified, and the ¹H-NMR spectra of the feces metabolites from the control and PG groups were also recorded (Figure 3). Table I lists the specific metabolites represented by each peak. The detected specific materials included bile acids, short chain fatty acids, amino acids, ketone, choline, and its metabolites, such as glucose, tricarboxylic acids, taurine, uracil, and hypoxanthine. Various small molecule metabolites associated with nutritional metabolism were detected.

Model validation of metabolic data of cecal contents

PCA, PLS-DA, and OPLS-DA multivariate statistical analyses were used to determine the possible

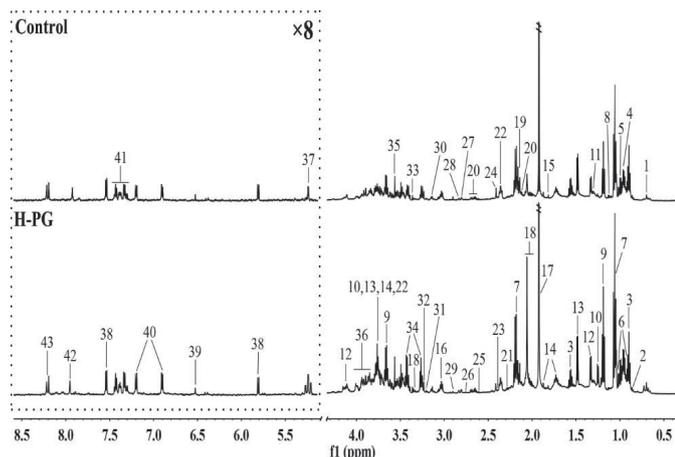


FIGURE 3 - $^1\text{H-NMR}$ spectra of the feces metabolites from the control group and PG group.

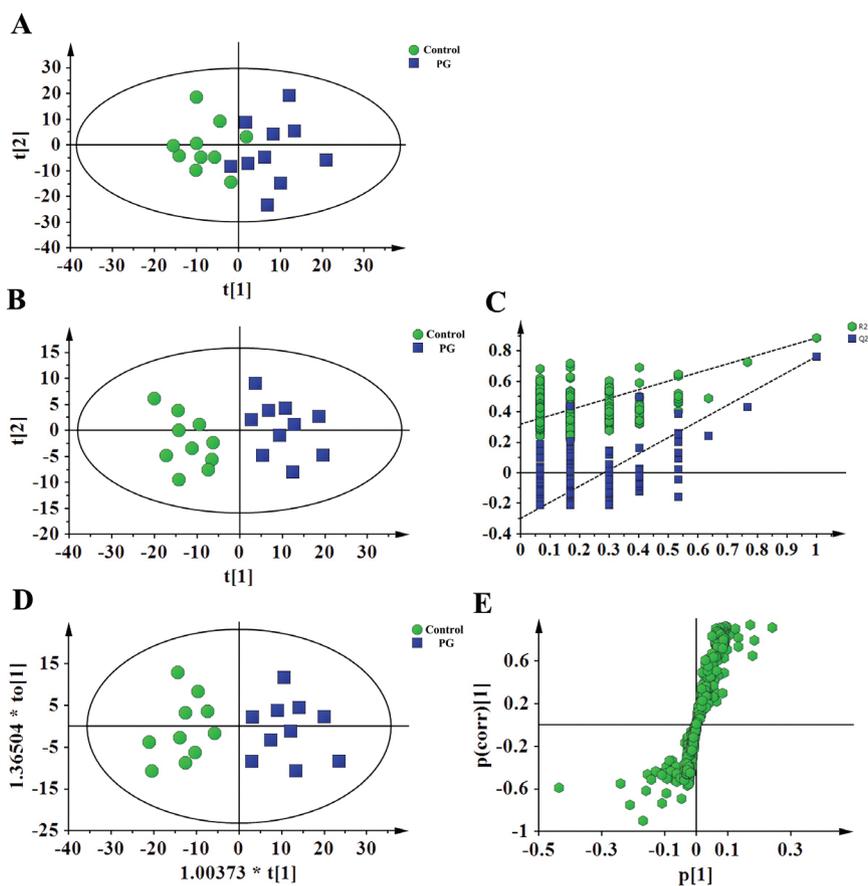
effects of PG on the metabolomics of the cecal contents (Figure 4). The PCA scores were $R^2\text{X} = 0.825$ and $Q^2 = 0.649$, which indicated that the control and PG groups were discernible. No sample point fell outside the oval of the PCA score (Figure 4A). The PLS-DA model scores were $R^2\text{X} = 0.708$, $R^2\text{Y} = 0.900$, and $Q^2 = 0.686$. Figure 4B shows the distributions of the sample point. The PG and control groups had a distinct difference shown in Figure 4C on the basis of the sample profiles of arrangement and verification. Figures 4D and 4E illustrate the score and load diagram of the OPLS-DA model, respectively. Therefore, the model validation of metabolic data of cecal contents showed that PG significantly affected the metabolism of small molecular compounds in the intestinal tract of weaned rats.

TABLE I - $^1\text{H-NMR}$ signal assignments for the metabolites in the feces contents

Metabolites	Moieties	δ1H (ppm) and multiplicity
Bile acids	CH_3	0.69(m)
2-Methylbutyrate	CH_3, CH_2	0.86(t), 1.05(d), 1.38(m), 1.50(m), 2.21(m)
Butyrate	CH_3, CH_2	0.89 (t), 1.55 (m), 2.16 (t)
Leucine	$\alpha\text{CH}, \delta\text{CH}_3, \delta\text{CH}_3$	0.91(d), 0.96(d), 3.72(t)
Valine	$\alpha\text{CH}_3, \beta\text{CH}, \gamma\text{CH}_3$	0.99(d), 1.04(d)
Isoleucine	$\gamma\text{CH}_3, \delta\text{CH}_3$	0.94(t), 1.01(d)
Propionate	CH_3, CH_2	1.06 (t), 2.18 (q)
Isobutyrate	CH_3	1.12(d)
Ethanol	CH_3, CH_2	1.19 (t), 3.66 (q)
Methylmalonate	CH_3, CH	1.26(d), 3.76(m)
Threonine	$\alpha\text{CH}, \beta\text{CH}, \gamma\text{CH}_3$	1.32(d), 4.25(m), 3.58(d)
Lactate	$\alpha\text{CH}, \beta\text{CH}_3$	1.33(d), 4.11(q)
Alanine	$\alpha\text{CH}, \beta\text{CH}_3$	1.48(d), 3.77(q)
Lysine	$\alpha\text{CH}, \beta\text{CH}_2, \gamma\text{CH}_2, \delta\text{CH}_2$	3.77(t), 1.89(m), 1.73(m), 1.48(m)
Citrulline	$\gamma\text{CH}_2, \beta\text{CH}_2$	1.56(m), 1.82(m)
Cadaverine	$\text{NH}_2\text{-CH}_2, \text{CH}_2$	1.48(m), 1.73(m), 3.02(t)
Acetate	$\text{CH}_2\text{-C=O}$	1.92(s)
Proline	$\beta\text{CH}_2, \gamma\text{CH}_2, \delta\text{CH}_2$	2.03(m), 2.07(m), 3.35(m), 3.45(m), 4.14(dd)
Glutamine	$\alpha\text{CH}, \beta\text{CH}_2, \gamma\text{CH}_2$	3.68(t), 2.45(m), 2.15(m)
Methionine	$\alpha\text{CH}, \beta\text{CH}_2, \gamma\text{CH}_2, \delta\text{CH}_3$	3.78(m), 2.64(dd), 2.16(m), 2.14(s)
Acetone	CH_3	2.28(s)
Glutamate	$\alpha\text{CH}, \beta\text{CH}_2, \gamma\text{CH}_2$	3.75(m), 2.12(m), 2.35(m)
Pyruvate	CH_3	2.37(s)
Succinate	$\alpha, \beta\text{CH}_2$	2.41(s)
Methylamine	CH_3	2.61(s)
Dimethylamine	CH_3	2.73(s)
Succinimide	CH_2	2.80(s)
Methylguanidine	CH_3	2.84(s)

TABLE I - $^1\text{H-NMR}$ signal assignments for the metabolites in the feces contents (cont.)

Metabolites	Moieties	$\delta^1\text{H}$ (ppm) and multiplicity
Trimethylamine	CH_3	2.90(s)
Malonate	CH_2	3.14(s)
Choline	$\text{N-(CH}_3)_3, \alpha\text{CH}_2, \beta\text{CH}_2$	3.20(s), 4.05(t), 3.51(t)
TMAO	CH_3	3.26(s)
Methanol	CH_3	3.36(s)
Taurine	$\text{N-CH}_2, \text{S-CH}_2$	3.27(t), 3.43(t)
Glycine	CH_2	3.56(s)
Serine	$\beta\text{-CH}_2, \alpha\text{-CH}$	3.85(dd), 3.98(dd), 4.00(dd)
α -Glucose	1-CH	5.24(d)
Uracil	CH, CH	5.80(d), 7.54(d)
Fumarate	CH_3	6.52(s)
Tyrosine	$\alpha\text{CH}, \text{CH}_2$	7.19(d), 6.89(d)
Phenylalanine	CH	7.32(m), 7.38(m), 7.43(m)
Unknown	CH	7.96(s)
Hypoxanthine	$\text{N-CH}, \text{CH}$	8.20(s), 8.22(s)

**FIGURE 4** - Multivariate statistical analysis of $^1\text{H-NMR}$ spectra data sets of the feces contents of weaned rats. Each point in A, B, and D represented one sample. A represented the PCA score of the $^1\text{H-NMR}$ spectrum dataset of the cecal contents; B and C respectively represented the PLS-DA model score and the permutation verification; D and E represented the score and load diagram of the OPLS-DA model respectively. The original point in C represented the R^2 value of the model. The square point represented the model Q^2 value. Each point in E represented the chemical shift value with the integral interval.

Effects of PG on the metabolites of cecal content

Table II presents the metabolite profile of PG supplementation in the cecal contents of weaned rats. Bile acid, isobutyrate, propionate, and lactate contents in the PG group were higher than in the control group. PG treatment resulted in the decrease of isoleucine, threonine, alanine, lysine, citrulline, proline, α -glucose, and trimethylamine.

TABLE II - PG affects the small molecule metabolites in the cecal contents

Metabolites	NMR chemical shift (δ)	PG VS the control		
		Fold change	VIP	Change
Bile acids	0.68(m)	2.39	1.71	\uparrow^{**}
Isoleucine	0.94(d)	0.68	1.76	\downarrow^*
Iso-butyrate	1.12(d)	1.43	1.58	\uparrow^*
Threonine	1.32(d)	0.56	1.43	\downarrow^*
Lactate	1.34(d)	1.31	1.34	\uparrow^*
Alanine	1.48(d)	0.77	1.15	\downarrow^*
Lysine	1.73(m)	0.55	1.88	\downarrow^*
Citrulline	1.82(m)	0.69	1.48	\downarrow^*
Trimethylamine	2.91(s)	0.62	1.41	\downarrow^*
TMAO	3.26(s)	0.49	1.97	\downarrow^{**}
α -Glucose	5.24(d)	0.60	1.45	\downarrow^*
Propionate	2.18 (q)	1.38	1.92	\uparrow^*
Proline	3.35(t)	0.61	1.32	\downarrow^*

Organic acids and free ammonia in the cecum of weaned rats

The contents of organic acids in the cecum were measured to investigate the effect of PG on organic acid accumulation (Figure 5). PG administration significantly increased the contents of acetate (9.88 $\mu\text{mol/g}$), propionate (4.25 $\mu\text{mol/g}$), and total acids (17.52 $\mu\text{mol/g}$) compared with the control group ($P < 0.05$). The accumulation amounts of isobutyrate and butyrate were lower than those of acetate and propionate. Alcohol had minimal effect on the content increase of total acids. Therefore, PG was the main factor in the excessive accumulation of total acids in the cecum.

After PG administration for 14 days, the contents of free ammonia were measured (Figure 6). Free ammonia content decreased and increased in the PG and alcohol groups, respectively, after 14 days of feeding compared with the control group. Therefore, PG

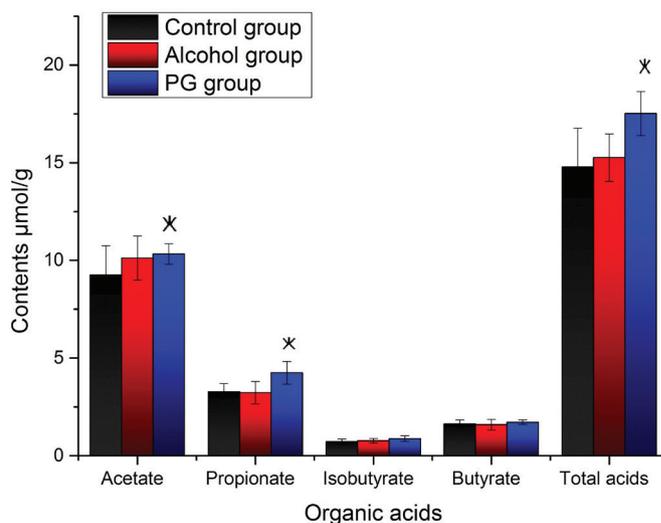


FIGURE 5 - Effect of PG on the accumulation of organic acids in the cecal of weaned rats.

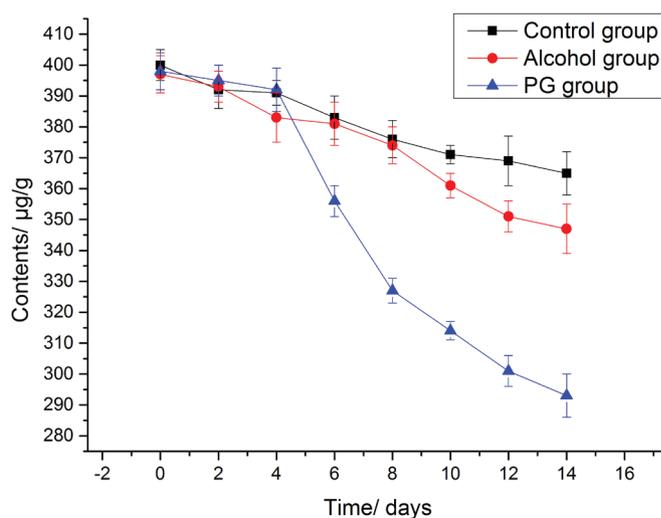


FIGURE 6 - Effect of PG on the contents of free ammonia.

resulted in the decrease of free ammonia in the cecum of weaned rats.

Morphological structure of the jejunum of weaned rats

The effect of PG on the morphological structures of the jejunum was visualized. The control group displayed intact villus, damaged crypt, and incomplete muscular layer, which were shown inside the box and ellipse of Figure 7.A. The structure of the villus, crypt, and muscular layer remained in the state of damage in the alcohol group. However, after PG administration for 14 days, the morphological structures of jejunum showed that the damage of the jejunum had been alleviated (Figure 7.B). By comparing with the morphological structure of the

control group, no inflammation was found in the PG group. The intestinal inflammation was alleviated after feeding PG. Therefore, the administration of PG could minimize the jejunum damage of weaned rats.

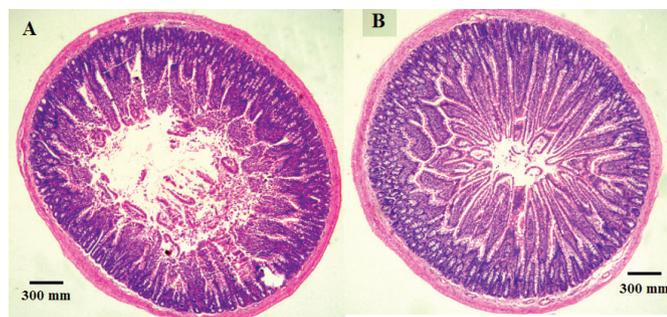


FIGURE 7 - The effect of PG on the jejunum morphology of weaned rats. Note: A: control group; B: PG group. In the Figure 7A, the box and ellipse areas pointed by the arrow were the inflammatory areas. Correspondingly, no inflammatory area was detected in the Figure 7B after feeding PG.

DISCUSSIONS

The content of organic acids in the intestine increased after feeding PG because of the following reasons. (1) *S. marcescens* PG is an antibacterial agent against Gram-positive and Gram-negative bacteria (Mekhael, Yousif, 2009) and human pathogenic bacteria, namely, *Staphylococcus aureus* and *Klebsiella pneumoniae* (Visalakchi, Muthumary, 2010). (2) PG possesses an anti-inflammatory effect in lipopolysaccharide-stimulated macrophages (Stevenson *et al.*, 2002). (3) The colony number increase is related to acid-producing probiotics. The colony numbers of *Enterococcus* and *Lactobacillus* in the PG group were significantly decreased and increased, respectively, compared with the control group ($P < 0.05$) (Table III). In addition, PG also resulted in the increase in colony number of *Bifidobacterium*. *Lactobacillus* and *Bifidobacterium* can produce acidic products. Therefore, the increase in the number of *Lactobacillus* and *Bifidobacterium* resulted in high accumulation of the content of acidic products, including increase of organic acid content. In addition, the increase of bile acids could protect cecal barriers and inhibit hazardous microbes. The amount decrease of biogenic amines in the cecal contents could sustain the cecal health and minimize the inflammation of amine toxicity. Therefore, multiple factors result in the alleviation of the intestinal inflammation of weaned rats by PG.

Dietary supplementation with bile acid (Diego-Caberol *et al.*, 2015), glutamine (Cruzat *et al.*, 2014), or L-glutamate/L-aspartate could alleviate adverse

TABLE III - The effects of PG on the caecal microflora in weaned rats Ig(CFU/g)

Items	Control group	Prodigiosin group
<i>Lactobacillus</i>	8.81±0.70	9.80±0.94 [#]
<i>Bifidobacterium</i>	9.14±0.94	9.76±0.74
<i>Escherichia coli</i>	9.26±0.66	8.35±0.83 [#]
<i>Enterococcus</i>	7.59±0.54	6.43±0.82 ^{##}

Note: [#] and ^{##} represented the significant difference and the extremely significant difference, respectively.

inflammation caused by weaning (Hayakawa *et al.*, 2016). PG possesses the bioactivity characteristics of anti-malarial, antifungal, immunosuppressant, and antibiotic agents, which could affect the intestinal environment and physical function (Williamson *et al.*, 2006). Weaning dampens the immune system and induces the inflammation in the mammalian body (McLamb *et al.*, 2013); weaning even directly hinders the growth of animals (Moeser, Pohl, Rajput, 2017). Trimethylamine-N-oxide (TMAO) is directly associated with diet (Cho *et al.*, 2016), hinders the animal growth, and leads to renal insufficiency and mortality risk (Hartiala *et al.*, 2014). The content increase of bile acid could stimulate the production of specific metabolites and the gene expression and enhance the protection and barrier function of the intestinal tract (Diego-Caberol *et al.*, 2015). This study showed that PG could significantly decrease the content of TMAO in the cecum of weaned rats. In addition, PG administration directly resulted in the increase of organic acids, decrease of free ammonia, and increase of small molecule acids in the cecal contents. In addition, PG lessened the effect of weaning on intestinal damage, enhanced the intestinal structure, and mitigated the unhealthy effects of the weaning inflammation. Therefore, PG has the potential to overcome the adverse inflammations caused by weaning, control the growth, and diminish weaning-related inflammations. However, the molecular mechanism of PG on the effect of weaned rats remains unclear and needs to be further investigated.

CONCLUSIONS

PG has no genotoxic effect and possesses antimalarial, antibacterial, and anti-cancer activities. However, PG's alleviation of intestinal inflammation has not been reported. In this study, a dose of 200 μg PG kg⁻¹ body per day was used to investigate the effect of PG on the alleviation of the intestinal inflammation of

weaned rats. After PG administration for 14 days, the body mass of the PG group was increased by 1.29- and 1.26-fold compared with the control and alcohol groups, respectively. In addition, PG caused the increase of organic acid content and the decrease of the moisture content, pH value, and free ammonia content in feces. Furthermore, the analysis of ¹H-NMR signal peak attribution and the model validation of metabolic data of feces contents showed that PG significantly affected the metabolites of small molecular compounds in the intestinal tract of weaned rats. PG administration alleviated the intestinal inflammation of weaned rats. PG can be implemented as a nutritional supplementation to alleviate the intestinal inflammation in the weaning of mammals. PG can be used as a drug to alleviate the adverse reactions of mammals to weaning.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest regarding the publication of this paper.

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