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Beneficial Impact and Molecular Mechanism of *Bacillus Coagulans* on Piglets Intestine

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Abstract: This research was to investigate beneficial impact and molecular mechanism of *B. coagulans* on piglets intestine. Twenty-four 21 days old weaned piglets were allotted to three treatments: control group (basal diet), B6 group (basal diet + 2×10^6 CFU/g *B. coagulans*), B7 group (basal diet + 2×10^7 CFU/g *B. coagulans*). The results showed that compared with control group, B6 and B7 group significantly decreased diarrhea rate and the concent of CHOL, GGT and DAO in plasma; decreased villus height and increase crypt depth in jejunum and ileum; increased the activities of SOD and CAT and decreased the concent of MDA and H_2O_2 in intestine. These data suggested that supplementing *B. coagulans* had beneficial impacts on promoting nutrients metabolism, maintaining intestinal integrity and alleviating oxidative stress and diarrhea. Futher research of molecular mechanisms showed that, these beneficial impacts were regulated by changing expression levels of related proteins (including HSP70, Caspase-3, Bax, Villin and Occludin), and genes (including *RPL4*, *IFN-α*, *IFN-β*, *IFN-γ*, *MX1*, *MX2*, *OAS1*, *IL-1β*, *IL-4*, *CXCL-9*, *CCL-2*, *AQP3*, *SGLT-1*, *LPL*, *INSR* and *$b^{0,+}AT$*), and altering community composition of gut microbiota (particularly family *Clostridiaceae*, *Enterobacteriaceae*, and *Veillonellaceae* and genus *Prevotella*, *Turicibacter*, and *Lactobacillus*).

Keywords: *Bacillus coagulans*; Intestinal function; Gut microbiota; Weaned piglet

1. Introduction

In the modern intensive pig production process, piglet feeding has become one of the most important aspects [1]. Weaning of piglets involves complex events, including environmental and dietary stresses that interfere with gut development and adaptation [2], which is one of the most critical developmental stages of the digestive tract when food is changed from maternal milk to a solid diet [3]. This is a period of starvation associated with the absence of the dam, impairment of energy status, and thermoregulation, as shown by

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behavioral and biochemical changes [2-4]. After early weaning, the piglets were prone to physiological and nutritional stress reactions and the immature development of their own organs, which resulted in the decrease of feed intake, slow weight growth, poor mental state and severe diarrhea [5,6]. The long time occurrence of these symptoms will lead to high morbidity and mortality of piglets [1]. This not only causes economical losses in pig production, but also contributes to public health risk from pathogenic bacteria-infected pork products, which has been perplexing pig breeding industry for a long time [7], especially in post-antibiotic era.

The intestine is not only the terminal organ for digestion and absorption of dietary nutrients, but is also crucial for preventing the entry of exogenous pathogens into the systemic circulation [8]. Thus, intestinal integrity is vital to survival, growth, and health of both animals and humans. Extensive studies have demonstrated that piglets early-weaning can result in gut mucosal injury and dysfunction [9,10]. Neonates are prone to various stresses, particularly early-weaning, resulting in intestinal mucosal injury and absorptive dysfunction [8-11]. The consequences are the occurrence of diarrhea, reduced growth, and even deaths, leading to a great deal of economic loss [11]. Therefore, it is urgent to find and explore high quality and safe antibiotic replacement products to improve a series of adverse reactions caused by weaning piglet syndrome, and to promote intestinal health.

The concept of probiotics, which are considered beneficial to the gastrointestinal tract as an alternative to antibiotics, attracts increasing interests of animal nutritionists and livestock producers [12]. It is defined as a live microbial feed supplement that is beneficial to health [13]. The beneficial effects of probiotics in recent reports is mainly reflected in these aspects: 1) produce a large number of active enzymes in the metabolism process, promote the absorption of nutrients, thereby improving the conversion efficiency of feed; 2) promote the synthesis and metabolism of protein and vitamins; 3) inhibit the reproduction of harmful bacteria in the intestinal tract, promote the growth of beneficial bacteria, and maintain the dynamic balance of gut microbiota; 4) regulate the immune function of animal body to a certain extent; 5) reduce the irritating gas in feces, thereby purify the air environment and reduce the pollution [14-17].

As one kind of gram positive bacteria, *Bacillus coagulans* (*B. coagulans*) is a lactic acid producing bacterial species, which is catalase positive, spore forming, motile, and facultative anaerobe [18]. Spores of *B. coagulans* have strong resistance, resurrection and stability, can be activated in the acidic environment of the stomach and begin germinating and proliferating in the intestine [19,20]. Spores can adapt to the low oxygen environment in the intestinal tract and reach the gastrointestinal tract smoothly, and then can play the effect of lactic acid bacteria in the gut [20]. Because of this characteristics, *B. coagulans* is often used in veterinary applications, especially as a probiotic in cattle, poultry and shrimp, and many studies of its beneficial effects have been continuously reported [21].

Nevertheless, there is still limited evidence suggesting whether or how *B. coagulans* could affect molecular function, promote gut health and maintain intestinal homeostasis in piglets. Therefore, this research was to investigate beneficial impact and molecular mechanism of *B. coagulans* on piglets intestine, by means of supplementing two levels of *B. coagulans* to the basal diet, and analyzing of molecular biology indexes. Moreover, this study might ulteriorly reveal the principle how *B. coagulans* benefit the intestine via the maintenance of homeostasis and the regulation of biomolecular.

2. Result and Discussion

2.1 Effects on Growth Performance and Nutrient Metabolism

During the experimental period, average daily gain (ADG), average daily feed intake (ADFI) and diarrhea rate was observed and calculated (Table 1). There was no significant difference in ADG and ADFI

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among three groups, whereas the difference of diarrhea rate was remarkable. Compared with the control group, B6 group significantly reduced the diarrhea rate between day 0 and 10, day 10 and 21 as well as day 0 and 21 ($P < 0.05$), B7 group significantly reduced the diarrhea rate between day 0 and 10 as well as day 0 and 21 ($P < 0.05$). In addition, the diarrhea rate between day 10 and 21 in B6 group was also exceedingly lower than that in B7 group ($P < 0.05$).

Diarrhea is one of the most challenging problems in weaned piglets breeding. In the metabolic process, *B. coagulans* secretes the antibacterial peptide substance coagulin as well as lactic acid, which inhibits *Listeria*, *Micrococcus*, *Leuconostoc* and *Enterococcus* etc [22]. There is evidence from animal research suggests that *B. coagulans* is effective in both treating as well as preventing recurrence of *Clostridium difficile* associated diarrhea [23]. In this study, we found the diarrhea rate was significantly reduced by the supplementation of *B. coagulans*, and 2×10^6 CFU/g *B. coagulans* had better effect in the second half of the trial.

Table 1. Effects of *B. coagulans* on growth performance of weaned piglets

Item	Control	B6	B7
Day 0-10			
ADG /g	295.35±70.08	334.07±51.97	344.68±65.62
ADFI /g	239.40±84.79	263.20±62.74	267.60±77.96
F/G	1.28±0.21	1.28±0.12	1.29±0.04
Diarrhea rate /%	27.0^{b}	9.0^{a}	5.0^{a}
Day 10-21			
ADG /g	592.02±122.77	642.62±99.67	635.90±96.64
ADFI /g	403.27±119.01	432.27±119.55	409.82±166.78
F/G	1.48±0.06	1.53±0.22	1.56±0.11
Diarrhea rate /%	11.8^{b}	0.9^{a}	5.5^{b}
Day 0-21			
ADG /g	450.72±96.11	497.22±77.65	495.68±72.39
ADFI /g	325.24±97.15	342.10±81.62	354.95±81.05
F/G	1.40±0.10	1.46±0.06	1.41±0.09
Diarrhea rate /%	19.5^{b}	5.2^{a}	5.7^{a}

Plasma biochemical indicators were dedected to reflect metabolic function of piglets, the results were shown in Table 2. Compared with the control group, B7 group obviously decreased total cholesterol (CHOL) and gamma glutamyl transpeptidase (GGT) and increased triglyceride (TG) in plasma ($P < 0.05$).

Table 2. Effects of *B. coagulans* on plasma biochemical indicators of weaned piglets

Item	Control	B6	B7
TP (g/L)	49.68±2.70	48.86±4.17	48.16±3.31
CHOL (mmol/L)	1.77±0.29^{b}	1.71±0.19^{ab}	1.51±0.23^{a}
TG (mmol/L)	0.37±0.02^{a}	0.41±0.09^{ab}	0.46±0.08^{b}
GLU (mmol/L)	5.54±0.84	5.48±0.16	5.51±0.85
GGT (mmol/L)	36.23±8.94^{b}	32.48±4.08^{ab}	28.67±5.25^{a}

Cholesterol levels in the blood are one of the routine blood testing indicators, that is associated with many cardiovascular diseases [24]. The cholesterol in the blood is the main cause of atherosclerosis, either high or low level of cholesterol can lead to the damage of animal health [25]. GGT is mainly derived from the

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secretion of liver mitochondria, which is an essential enzyme for the metabolic process of amino acids and proteins [26]. GGT is also an indicator of oxidative stress that reflects the damage of oxygen free radicals to a variety of cells, the elevation of GGT indicates the damage of liver and bile duct epithelium [27]. The results of this study showed that, supplementing *B. coagulans* has the effect of lowering blood cholesterol, regulating metabolism of amino acids and proteins and decreasing stress reactive in liver. Of note, although triglyceride in plasma had a significant difference in this test, the content was still in the normal range.

In summary, the experiments of effects on growth performance and nutrient metabolism indicated that after weaning, supplementing *B. coagulans* could alleviate piglets diarrhea and regulate nutrients metabolism. For this, the analysis of gut microbiota and expression levels of relative proteins and genes was carried out next to reveal this regulation mechanism futher.

2.2 Effects on Intestinal Integrity and Redox Status

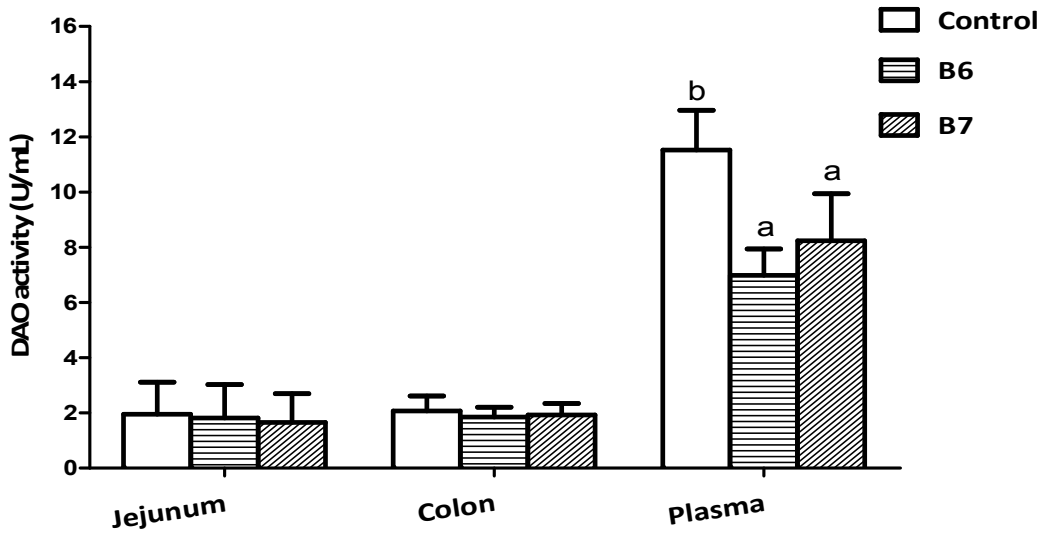

Fig 1. Effects of *B. coagulans* on DAO activity in jejunim, colon and plasma

DAO activity is frequently used as a noninvasive biomarker of alterations in the function and structure of intestinal mucosa [28]. Under certain conditions, cells in intestinal mucosa experience necroses, and slough off into the enteric entocoele, resulting in a decline of DAO levels in intestinal mucosa and an increase of DAO levels in circulation [29]. The data of DAO in this research (Fig 1), which two group relative to control markedly reduced the activity of DAO in plasma ($P < 0.05$), indicated that supplementing *B. coagulans* alleviated intestinal damage caused by weaning stress.

Table 3. Effects of *B. coagulans* on intestinal morphology of weaned piglets

Item	Jejunum			Ileum		
	Control	B6	B7	Control	B6	B7
villus height (μm)	318.7±35.0	350.1±41.4	336.6±41.2	242.1±22.8^{a}	273.3±19.8^{b}	285.2±30.7^{b}
crypt depth (μm)	213.6±18.2^{b}	168.7±15.8^{a}	175.4±20.2^{a}	161.0±11.1	168.0±13.3	173.2±19.5
villus height/crypt depth	1.49±0.11^{a}	2.08±0.17^{c}	1.92±0.15^{b}	1.51±0.09^{a}	1.63±0.12^{b}	1.65±0.08^{b}
villous surface area (cm^{2})	29677±3031	31738±3633	29540±4078	27520±932	28396±3715	28502±2870

The data of intestinal morphology was to reflect intestinal mucosal integrity and injury, that were summarized in Table 3. Compared with control group, both B6 and B7 group significantly decreased the crypt depth and increased the ratio of villus height to crypt depth in the jejunum ($P < 0.05$), significantly increased the villus height as well as the ratio in the ileum ($P < 0.05$). The height of the villus and the depth of

crypt directly reflect the function of the intestinal tract. The atrophy of the villus of small intestine leads to a decrease in the number of mature epithelial cells, then the nutrients will not be fully absorbed by intestine [30]. The depth of crypt reflects the formation rate of villus epithelial cells, and the shallow crypt indicates the rate of cell maturation is increased and the secretory function is enhanced [31]. The data of this sduty showed that supplementing *B. coagulans* strengthen the repair of intestinal damage caused by weaning stress, maintain the integrity of intestinal mucosa.

The intestinal redox status was determined to reflect oxidative stress and antioxidative function of piglets supplemented with *B. coagulans* (Table 4), including the activity of superoxide dismutase (SOD) and catalase (CAT), and the concent of malondialdehyde (MDA) and hydrogen peroxide (H_2O_2). Compared with control group, B6 group remarkably increased the activity of SOD in duodenum and jejunum as well as CAT in colon, decreased the concent of MDA in ileum and colon as well as H_2O_2 in jejunum and colon ($P < 0.05$); B7 group markedly increased the activity of SOD in jejunum as well as CAT in jejunum and colon, decreased the concent of MDA and H_2O_2 in colon ($P < 0.05$). In addition, the difference of SOD, CAT and H_2O_2 in B6 group was bigger than that in B7 group.

Table 4. Effects of *B. coagulans* on intestinal redox status of weaned piglets

Item	Control	B6	B7	Control	B6	B7
	Duodenum			Ileum		
SOD (U/mg)	42.25±3.76^{a}	54.89±4.19^{b}	45.48±3.30^{a}	83.04±5.79	78.95±3.00	79.68±4.52
CAT (U/mg)	17.92±4.41	16.61±2.73	15.59±3.64	9.54±3.08	7.93±1.72	7.92±1.40
MDA (nmol/mg)	4.52±0.77	5.82±1.77	5.69±0.93	6.00±2.40^{b}	3.52±1.11^{a}	4.21±1.40^{ab}
H_2O_2 (nmol/mg)	5.06±1.10^{ab}	4.12±1.01^{a}	5.35±1.21^{b}	8.34±1.80	7.77±3.20	7.49±1.82
	Jejunum			Colon		
SOD (U/mg)	81.55±10.51^{a}	90.53±5.43^{b}	94.21±6.36^{b}	100.54±25.07	95.62±4.92	92.56±12.95
CAT (U/mg)	7.64±1.23^{a}	7.55±1.11^{a}	11.00±2.48^{b}	6.56±1.15^{a}	10.58±2.40^{b}	11.79±2.89^{b}
MDA (nmol/mg)	11.71±4.75	9.61±4.82	13.39±3.96	3.66±1.74^{b}	1.64±0.45^{a}	2.30±0.69^{a}
H_2O_2 (nmol/mg)	30.75±10.31^{b}	19.40±5.24^{a}	23.08±5.72^{ab}	25.25±6.43^{b}	19.61±5.00^{a}	16.80±3.04^{a}

Oxidative stress reflects the unbalance between the systematic phenomenon of reactive oxygen species and the capacity of biosystem to readily detoxicate the reactive intermediaries or to renovate the resulting injury, that frequently occurs after piglets weaning [32,33]. Whereas, cells protect themselves from hydroxyl radicals and other oxygenants by antioxidant enzymes, including SOD and CAT [33,34]. MDA can induce noxious stress in cells and constitute homopolar protein adducts known as advanced lipoxidation end-products (ALEs), which is usually utilized as a marker to evaluate the oxidant stress levels in an biosome [35]. H_2O_2 is the main product of oxidative stress in the body [36]. The results of redox status showed that supplementing *B. coagulans* alleviated oxidative stress and enhanced antioxidative capacity in intestine. Futhermore, supplementing 2×10^6 CFU/g *B. coagulans* had better effect in this test.

In summary, the experiments of effects on intestinal integrity and redox status indicated that after weaning, supplementing *B. coagulans* could alleviate intestinal damage and oxidative stress, maintain intestinal integrity and enhance antioxidative capacity. For this, expression levels of relative proteins and genes was determined next to reveal this regulation and resistant mechanism futher.

2.3 Regulation on Protein Expression

Expression levels of six proteins in jejunum were tested to analyze the regulation of *B. coagulans* on intestinal mucosal stress status and barrier function (Fig 2). Relative to control group, B6 group significantly

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reduced protein expression levels of HSP70, Caspase-3 and Bax, rised expression level of Villin ($P < 0.05$); B7 group significantly reduced protein expression level of Bax, rised expression level of Occludin ($P < 0.05$).

In response to stress, HSP70 is expressed at elevated levels to promote refolding and prevent aggregation of partially-denatured proteins, thereby protecting cells from injury [37]. Caspase-3 is commonly activated by numerous "death" signals to cleave a variety of important cellular proteins, that is responsible for the proteolytic cleavage of many key "death" proteins [38]. Bax resides in the outer mitochondrial membrane, that promotes cell death directly through its putative function as a channel protein versus indirectly by inhibiting cellular regulators of the cell death proteases (caspases) [38,39]. In this research, we found the expression levels of these three proteins were remarkably decreased by *B. coagulans*, implied that *B. coagulans* had beneficial effect on regulating related protein to protect intestine from stresses and injury, which is one of the mechanism of resisting and alleviating weaning and oxidative stress.

Villin is one kind of actin binding protein and a marker of villus cell differentiation, which conduce to prop up the microfilaments of the microvilli of the mucosal villus [40]. Occludin integrate such diverse processes as gene transcription, tumor suppression, and cell proliferation to modulate intestinal mucosal structure and function [41]. After supplementing *B. coagulans*, the expression of these two proteins were regulated and obviously increased, indicated that *B. coagulans* had beneficial effect on maintaining of intestinal barrier function and promoting the growth of villus, which might be exactly one of the key mechanism of alleviating intestinal injury and diarrhea.

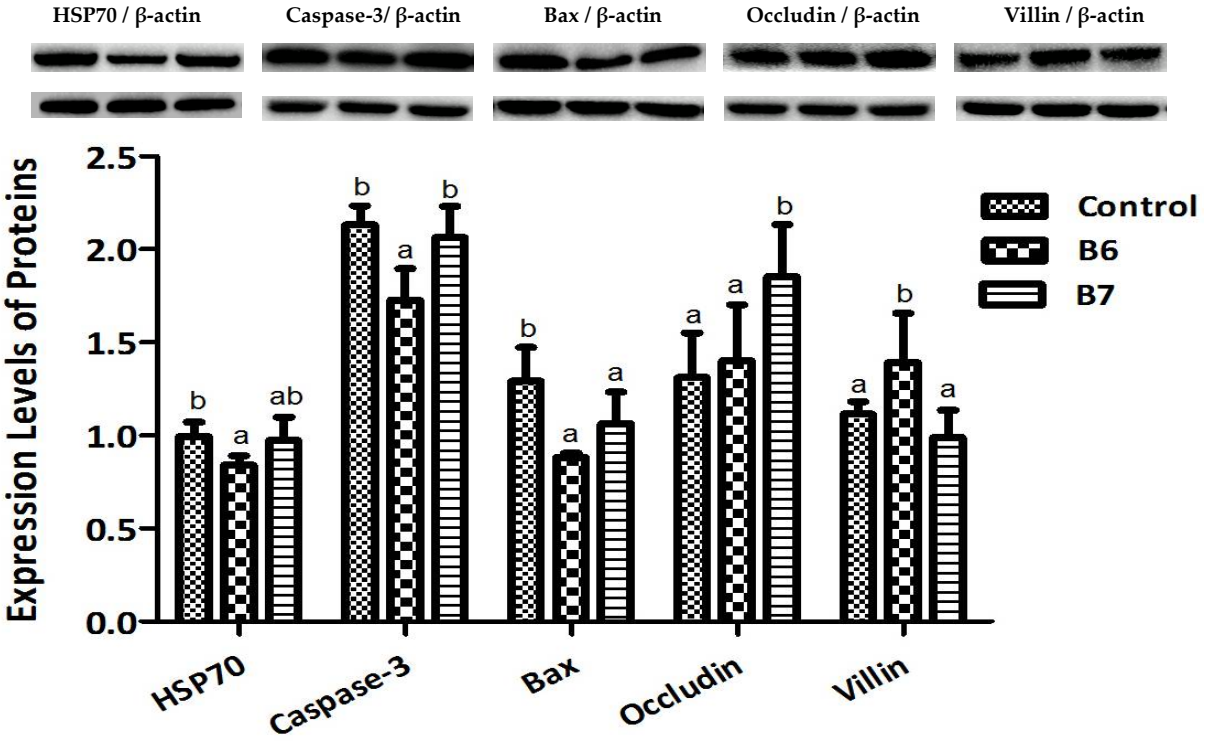

Fig 2. Effects of *B. coagulans* on expression levels of proteins in jejunum

2.4 Regulation on Gene Expression

Expression levels of genes associated with intestinal immune, inflammation, transportation and absorption were tested to analyze the regulation of B. coagulans on relative function. Compared with control group, B6 group obviously increased expression levels of *IFN-α*, *IFN-γ*, *OAS1*, *MX2*, *IL-4*, *CCL-2*, *AQP3* and *LPL* in ileum and *IFN-β*, *SGLT-1* and $b^{0,+}AT$ in colon, decreased expression levels of *IL-4*, *CCL-2* and *IFN-γ* in colon ($P < 0.05$); B7 group remarkably increased expression levels of *IFN-γ*, *OASL*, *OAS1*, *MX2* and *AQP3* in

ileum and *MX1*, *AQP3*, *SGLT-1*, *LPL*, *INSR* and $b^{0,+}AT$ in colon, decreased expression levels of *CXCL-9* in ileum and *CXCL-9*, *IFN-γ* and *IL-1β* in colon ($P < 0.05$).

Table 5. Regulation of *B. coagulans* on gene expression in ileum and colon

Item	Ileum			Colon		
	Control	B6	B7	Control	B6	B7
IFN-α	1.000±0.156^{a}	1.393±0.211^{b}	2.124±0.383^{c}	1.000±0.218	1.041±0.220	1.016±0.323
IFN-β	1.000±0.239^{a}	1.247±0.219^{ab}	1.337±0.313^{b}	1.000±0.141^{a}	2.436±0.483^{b}	0.905±0.205^{a}
IFN-γ	1.000±0.167^{a}	1.276±0.202^{b}	1.191±0.302^{ab}	1.000±0.223^{b}	0.825±0.159^{a}	0.682±0.078^{a}
MX1	1.000±0.135	1.000±0.230	1.196±0.271	1.000±0.172^{a}	0.937±0.159^{a}	1.194±0.188^{b}
MX2	1.000±0.144^{a}	2.015±0.264^{b}	2.649±0.482^{c}	1.000±0.239	0.806±0.203	0.976±0.236
OAS1	1.000±0.148^{a}	1.437±0.313^{b}	1.467±0.354^{b}	1.000±0.130	1.088±0.212	1.001±0.263
IL-1β	1.000±0.214	1.022±0.125	0.846±0.185	1.000±0.257b	0.984±0.113^{b}	0.708±0.130^{a}
IL-4	1.000±0.265^{a}	1.540±0.300^{b}	1.291±0.285^{ab}	1.000±0.168^{b}	0.759±0.166^{a}	0.870±0.229^{ab}
CXCL-9	1.000±0.253^{b}	0.868±0.119^{ab}	0.787±0.158^{a}	1.000±0.204^{b}	1.102±0.269^{b}	0.729±0.186^{a}
CCL-2	1.000±0.205^{a}	1.360±0.325^{b}	1.143±0.275^{ab}	1.000±0.250^{b}	0.646±0.096^{a}	0.862±0.168^{b}
AQP3	1.000±0.217^{a}	2.643±0.708^{b}	2.382±0.602^{b}	1.000±0.233^{a}	0.923±0.230^{a}	1.287±0.265^{b}
SGLT-1	1.000±0.232^{ab}	0.843±0.132^{a}	1.199±0.268^{b}	1.000±0.203^{a}	1.340±0.273^{b}	1.358±0.223^{b}
LPL	1.000±0.203^{a}	1.307±0.276^{b}	1.156±0.216^{ab}	1.000±0.195^{a}	0.857±0.127^{a}	1.250±0.333^{b}
INSR	1.000±0.244	1.104±0.275	1.037±0.206	1.000±0.203^{a}	1.016±0.186^{a}	1.390±0.311^{b}
$b^{0,+}AT$	1.000±0.257	1.017±0.219	1.228±0.211	1.000±0.215^{a}	1.678±0.390^{b}	1.521±0.370^{b}

The cellular response to viral infection includes the induction of genes for type I interferons, *IFN-α* and *IFN-β*, which are produced in most cell types and play a vital role in innate resistance to viral and bacterial infections [42]. *IFN-α/β* can induce the expression of genes encoding for antiviral proteins, particularly myxovirus (MX) and 2′-5′ oligoadenylate synthetases (OAS) [43,44]. *IFNγ*, or type II interferon, is a cytokine that is critical for innate and adaptive immunity against viral, some bacterial and protozoal infections [45]. In this research, we found there were marked alterations about expression levels of these genes after supplementing *B. coagulans*, implied that *B. coagulans* regulated immune-related genes to improve immunity and inhibit pathogens in intestine, which is one of the main mechanism of lowering diarrhea rate.

Oxidative stress is associated with early weaning, and it is suggested that oxidative stress may be one of the main causes of early weaning syndrome [46]. Oxidative stress has been implicated in the development of many chronic inflammatory disorders, such as enteritis, myocarditis and thyroiditis [47]. Antioxidant defense systems may be impaired as a consequence of excessive oxidative stress, and inflammatory responses can be partially mediated by oxidative stress [48]. In this study, some inflammatory cytokines were remarkably changed after supplementing *B. coagulans*, including *IFN-γ*, *IL-1β*, *IL-4*, *CXCL-9* and *CCL-2*, implied that the beneficial impact of *B. coagulans* on alleviating weaning and oxidative stress was regulated by altering expression levels of these inflammatory cytokines.

LPL (lipoprotein lipase) is expressed in heart, muscle, and adipose tissue, which functions as a homodimer, obtains the double functions of triglyceride hydrolase and ligand/bridging factor for receptor-mediated lipoprotein uptake [49]. *INSR* (insulin receptor) is a transmembrane receptor activated by insulin, IGF-I, IGF-II, binding of insulin or other ligands to this receptor activates the insulin signaling pathway, which regulates glucose uptake and release, as well as the synthesis and storage of carbohydrates, lipids and protein [50]. *AQP3* is a selective aquaporin and mainly distributed in intestinal epithelial cells, which can rapidly absorb water in the intestinal cavity into the blood and alter the endocrine environment of

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the intestinal cavity [51]. *SGLT-1* (sodium glucose cotransporters-l) is a high affinity/low capacity transporter of glucose in the mammalian small intestine and kidneys, which is responsible for the entire glucose absorption in small intestine [52]. *$b^{0,+}AT$* ($b^{0,+}$ amino acid transporter) plays a role in the high-affinity and sodium-independent transport of cystine and neutral and dibasic amino acids, and appears to function in the reabsorption of cystine in the kidney tubule [53]. After supplementing *B. coagulans*, expression of genes related to nutrients absorption and transportation were significantly changed, implied that the beneficial impact of *B. coagulans* on promoting nutrients metabolism was regulated by altering expression levels of above genes.

2.5 Regulation on Gut Microbiota

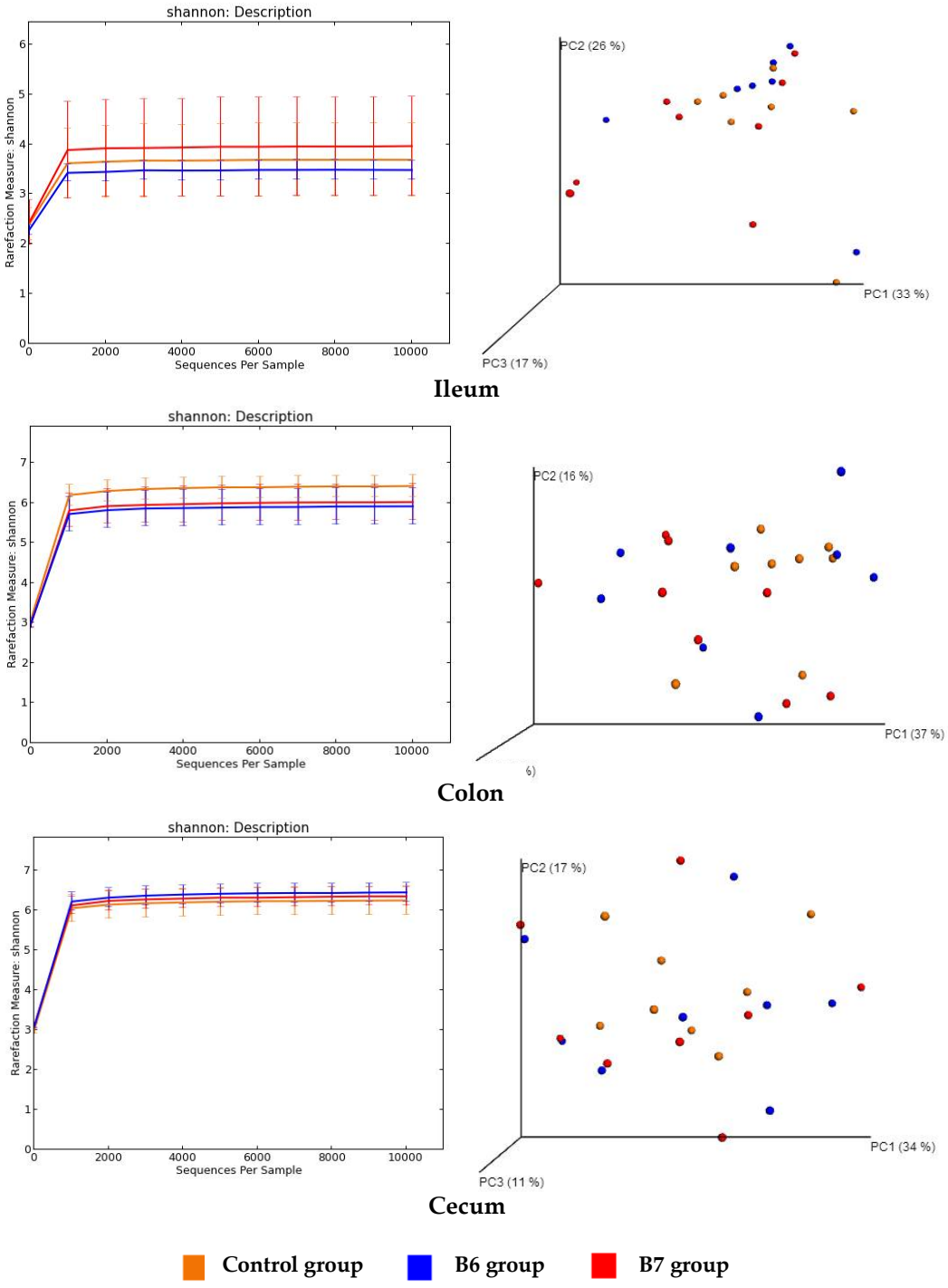

Fig. 3 Shannon α-diversity index and β-diversity

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The diversity analysis of gut microbiota in ileum, colon and cecum was shown in Fig 3. There was significant difference about Shannon α-diversity index between control group (6.43±0.27) and B7 group (5.92±0.45) in colon (P=0.039), but no difference among three groups in ileum and cecum. There was significant difference about β-diversity (unweighted Unifrac) between control and B6 group (P=0.021) as well as B6 and B7 group (P=0.021) in ileum, between control and B7 group in colon (P=0.015), and between control and B6 group (P=0.036) as well as control and B7 group (P=0.001) in cecum.

A total of 1,313,016 reads were obtained from ileum in three groups, 407,289 reads from control group, 558,738 from B6 group, and 346,989 from B7 group. A total of 2,003,105 reads were obtained from colon, 707,573 reads from control group, 650,742 from B6 group, and 651,835 from B7 group. And a total of 3,521,123 reads were obtained from cecum, 902,813 reads from control group, 1,213,060 from B6 group, and 1,405,250 from B7 group.

Legend	Phylum	B6	B7	C
	p__Bacteroidetes	6.3%	0.2%	1.1%
	p__Cyanobacteria	1.2%	0.4%	0.6%
	p__Actinobacteria	0.0%	0.0%	0.0%
	p__Firmicutes	67.5%	80.8%	82.4%
	p__Proteobacteria	24.4%	18.5%	15.7%
	p__Spirochaetes	0.4%	0.0%	0.0%
	p__Tenericutes	0.4%	0.0%	0.0%
	Other	0.1%	0.0%	0.1%

Ileum

Legend	Phylum	B6	B7	C
	p__Actinobacteria	0.5%	0.2%	0.7%
	p__Bacteroidetes	35.5%	42.9%	42.6%
	p__Cyanobacteria	1.5%	0.5%	0.5%
	p__Firmicutes	55.6%	48.8%	48.0%
	p__Proteobacteria	4.9%	3.7%	5.2%
	p__Spirochaetes	1.0%	2.9%	2.0%
	p__TM7	0.4%	0.5%	0.2%
	Other	0.6%	0.3%	0.7%

Colon

Legend	Phylum	B6	B7	C
	p__Actinobacteria	0.3%	0.4%	0.2%
	p__Bacteroidetes	40.8%	39.8%	42.6%
	p__Cyanobacteria	0.8%	1.3%	1.3%
	p__Firmicutes	49.7%	47.9%	49.1%
	p__Proteobacteria	3.2%	4.9%	4.6%
	p__Spirochaetes	4.6%	4.6%	1.3%
	p__TM7	0.2%	0.6%	0.1%
	Other	0.2%	0.4%	0.8%

Cecum

Fig 4.1 The community composition at phylum level

Legend	Class	B6	B7	C
	c__Mollicutes	0.1%	0.0%	0.0%
	c__Bacteroida	6.2%	0.2%	1.1%
	c__4C0d-2	0.1%	0.0%	0.0%
	c__Chloroplast	1.1%	0.4%	0.6%
	c__Bacilli	19.4%	19.0%	35.0%
	c__Clostridia	47.7%	61.8%	47.5%
	c__Erysipelotrichi	0.4%	0.0%	0.0%
	c__Gammaproteobacteria	23.4%	18.4%	15.5%
	c__Spirochaetes	0.4%	0.0%	0.0%
	c__Alphaproteobacteria	0.1%	0.0%	0.0%
	c__Betaproteobacteria	0.4%	0.0%	0.1%
	c__Epsilonproteobacteria	0.5%	0.0%	0.1%
	Other	0.1%	0.0%	0.1%

Ileum

Legend	Class	B6	B7	C
	c__Coriobacteriia	0.5%	0.2%	0.7%
	c__Bacteroida	35.5%	42.9%	42.6%
	c__4C0d-2	1.5%	0.5%	0.5%
	c__Bacilli	1.7%	1.6%	1.5%
	c__Clostridia	53.3%	46.0%	45.7%
	c__Erysipelotrichi	0.6%	1.2%	0.8%
	c__Betaproteobacteria	2.6%	2.0%	1.8%
	c__TM7-3	0.4%	0.5%	0.2%
	c__Deltaproteobacteria	0.8%	0.9%	0.5%
	c__Epsilonproteobacteria	0.2%	0.2%	0.4%
	c__Gammaproteobacteria	1.3%	0.6%	2.4%
	c__Spirochaetes	1.0%	2.9%	2.0%
	Other	0.6%	0.3%	0.7%

Colon

Legend	Class	B6	B7	C
	c__Coriobacteriia	0.3%	0.3%	0.2%
	c__Bacteroida	40.8%	39.8%	42.6%
	c__4C0d-2	0.8%	1.3%	1.3%
	c__Bacilli	1.8%	2.2%	1.1%
	c__Clostridia	46.7%	44.6%	46.6%
	c__Erysipelotrichi	1.3%	1.2%	1.3%
	c__Betaproteobacteria	1.5%	2.6%	1.5%
	c__TM7-3	0.2%	0.6%	0.1%
	c__Deltaproteobacteria	0.1%	0.1%	0.1%
	c__Epsilonproteobacteria	0.2%	0.4%	0.1%
	c__Gammaproteobacteria	1.4%	1.8%	2.9%
	c__Spirochaetes	4.6%	4.6%	1.3%
	Other	0.2%	0.4%	0.8%

Cecum

Fig 4.2 The community composition at class level

The community composition of gut microbiota at five levels were shown in Fig 4.1-4.5. The dominant bacteria at phylum level were *Firmicutes* and *Proteobacteria* in ileum, and *Bacteroidetes* and *Firmicutes* in colon and cecum. The dominant bacteria at calss level were *Bacilli*, *Clostridia* and *Gammaproteobacteria* in ileum, and *Bacteroidia* and *Clostridia* in colon and cecum. The dominant bacteria at order level were *Bacteroidales*,

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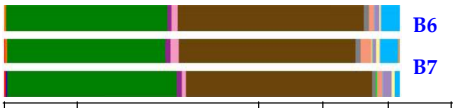
Enterobacteriales, *Clostridiales*, *Lactobacillales* and *Turicibacterales* in ileum, and *Bacteroidales* and *Clostridiales* in colon and cecum. The dominant bacteria at family level were *Turicibacteraceae*, *Lactobacillaceae*, *Clostridiales*, *Clostridiaceae* in ileum, and *Enterobacteriaceae*, *Prevotellaceae*, *Ruminococcaceae*, *Veillonellaceae*, *Paraprevotellaceae*, and *Lachnospiraceae* in colon and cecum. The dominant bacteria at genus level were *Turicibacter*, *Clostridiales*, *Clostridiaceae*, *Enterobacteriaceae*, and *Lactobacillus*, and *Prevotella* in colon and cecum.

Legend	Order	B6	B7	C
	o__Bacteroidales	6.2%	0.2%	1.1%
	o__YS2	0.1%	0.0%	0.0%
	o__Streptophyta	1.1%	0.4%	0.6%
	o__Enterobacteriales	19.9%	13.7%	9.2%
	o__Bacillales	0.1%	0.0%	0.0%
	o__Lactobacillales	9.1%	9.5%	18.5%
	o__Tremblayales	0.3%	0.0%	0.1%
	o__Campylobacterales	0.5%	0.0%	0.1%
	o__Clostridiales	47.7%	61.8%	47.5%
	o__Erysipelotrichales	0.4%	0.0%	0.0%
	o__Spirochaetales	0.4%	0.0%	0.0%
	o__Turicibacterales	10.1%	9.4%	16.5%
	o__Pasteurellales	3.1%	4.5%	6.1%
	o__Pseudomonadales	0.4%	0.1%	0.1%
	o__Spirochaetales	0.9%	2.8%	1.8%
	o__Aeromonadales	0.0%	0.0%	0.1%
	Other	0.1%	0.1%	0.1%

Ileum

Legend	Order	B6	B7	C
	o__Bacteroidales	35.5%	42.9%	42.6%
	o__YS2	1.5%	0.5%	0.5%
	o__Lactobacillales	1.6%	1.2%	1.2%
	o__Turicibacterales	0.1%	0.4%	0.3%
	o__Clostridiales	53.3%	46.0%	45.7%
	o__Erysipelotrichales	0.6%	1.2%	0.8%
	o__Campylobacterales	0.2%	0.2%	0.4%
	o__Aeromonadales	0.8%	0.1%	0.5%
	o__Tremblayales	2.5%	1.9%	1.8%
	o__Desulfovibrionales	0.8%	0.9%	0.4%
	o__Coriobacteriales	0.5%	0.2%	0.7%
	o__Burkholderiales	0.1%	0.1%	0.1%
	o__Enterobacteriales	0.5%	0.4%	0.6%
	o__Pasteurellales	0.1%	0.1%	1.3%
	o__Spirochaetales	0.9%	2.8%	1.8%
	o__CW040	0.4%	0.5%	0.2%
	Other	0.6%	0.3%	0.7%

Colon



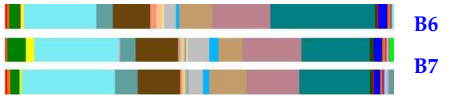
Legend	Order	B6	B7	C
	o__Bacteroidales	40.8%	39.8%	42.6%
	o__YS2	0.8%	1.3%	1.3%
	o__Lactobacillales	1.7%	2.0%	1.0%
	o__Turicibacterales	0.1%	0.1%	0.1%
	o__Clostridiales	46.7%	44.6%	46.6%
	o__Erysipelotrichales	1.3%	1.2%	1.3%
	o__Campylobacterales	0.2%	0.4%	0.1%
	o__Aeromonadales	0.8%	0.7%	1.9%
	o__Tremblayales	1.5%	2.5%	1.4%
	o__Desulfovibrionales	0.1%	0.1%	0.1%
	o__Coriobacteriales	0.3%	0.4%	0.2%
	o__Burkholderiales	0.1%	0.1%	0.1%
	o__Enterobacteriales	0.5%	0.9%	0.8%
	o__Pasteurellales	0.1%	0.2%	0.1%
	o__Sphaerochaetales	0.1%	0.2%	0.0%
	o__Spirochaetales	0.2%	0.6%	0.1%
	Other	0.2%	0.4%	0.8%

Cecum

Fig 4.3 The community composition at order level

Legend	Family	B6	B7	C
	f__Turicibacteraceae	10.1%	9.4%	16.5%
	f__Prevotellaceae	5.1%	0.1%	0.7%
	o__Clostridiales; f	11.1%	13.2%	9.0%
	f__Ruminococcaceae	0.7%	0.0%	0.1%
	f__Clostridiaceae	33.1%	47.3%	36.6%
	f__Peptostreptococcaceae	1.2%	1.0%	1.3%
	o__Streptophyta; f	1.1%	0.4%	0.6%
	f__Streptococcaceae	0.8%	1.5%	1.7%
	f__Paraprevotellaceae	0.9%	0.0%	0.2%
	f__Helicobacteraceae	0.4%	0.0%	0.1%
	f__Lactobacillaceae	8.3%	8.0%	16.8%
	f__Moraxellaceae	0.3%	0.0%	0.1%
	f__Lachnospiraceae	0.2%	0.0%	0.1%
	f__Pasteurellaceae	3.1%	4.5%	6.1%
	f__Veillonellaceae	1.2%	0.1%	0.4%
	f__Erysipelotrichaceae	0.4%	0.0%	0.1%
	f__Enterobacteriaceae	19.9%	13.7%	9.2%
	f__S24-7	0.1%	0.0%	0.1%
	f__Mycoplasmataceae	0.1%	0.0%	0.0%
	f__Spirochaetaceae	0.4%	0.0%	0.0%
	Other	0.7%	0.5%	0.4%

Ileum



Legend	Family	B6	B7	C
	o__Bacteroidales; f	2.7%	4.7%	2.3%
	f__Prevotellaceae	18.3%	21.6%	23.3%
	f__Coriobacteriaceae	0.5%	0.2%	0.7%
	f__Ruminococcaceae	14.5%	14.6%	13.3%
	f__S24-7	4.2%	4.1%	5.8%
	o__YS2; f	1.5%	0.5%	0.5%
	f__Succinivibrionaceae	0.8%	0.1%	0.5%
	f__Lachnospiraceae	8.4%	5.9%	9.5%
	f__Paraprevotellaceae	9.3%	10.5%	10.7%
	o__Clostridiales;f	3.3%	5.7%	3.7%
	f__Lactobacillaceae	1.6%	1.2%	0.9%
	o__Tremblayales;f	2.5%	1.9%	1.8%
	f__Desulfovibrionaceae	0.8%	0.9%	0.4%
	f__Pasteurellaceae	0.1%	0.1%	1.3%
	f__Veillonellaceae	26.3%	17.2%	17.7%
	f__Campylobacteraceae	0.2%	0.2%	0.4%
	f__Enterobacteriaceae	0.5%	0.4%	0.6%
	f__F16	0.4%	0.5%	0.2%
	f__Porphyromonadaceae	0.8%	1.9%	0.5%
	f__Spirochaetaceae	0.9%	2.8%	1.8%
	Other	1.3%	1.7%	1.8%

Colon

Legend	Family	B6	B7	C
	o__Bacteroidales; f	1.7%	2.5%	1.5%
	f__Prevotellaceae	22.2%	21.5%	24.9%
	f__Coriobacteriaceae	0.3%	0.4%	0.2%
	f__Ruminococcaceae	11.6%	12.5%	11.3%
	f__S24-7	2.1%	2.9%	2.7%
	o__YS2; f	0.8%	1.3%	1.3%
	f__Clostridiaceae	1.7%	2.0%	1.6%
	f__Lachnospiraceae	9.0%	7.9%	13.7%
	f__Paraprevotellaceae	12.9%	10.6%	12.2%
	o__Clostridiales;f	3.1%	3.8%	2.9%
	f__Lactobacillaceae	1.7%	2.0%	0.9%
	f__Erysipelotrichaceae	1.3%	1.2%	1.3%
	f__Succinivibrionaceae	0.8%	0.7%	1.9%
	o__Tremblayales;f	1.5%	2.5%	1.4%
	f__Veillonellaceae	21.1%	18.0%	16.8%
	f__Campylobacteraceae	0.2%	0.4%	0.1%
	f__Enterobacteriaceae	0.5%	0.9%	0.8%
	f__F16	0.2%	0.6%	0.1%
	f__Porphyromonadaceae	1.8%	2.2%	1.1%
	f__Spirochaetaceae	4.5%	4.4%	1.3%
	Other	1.1%	1.4%	1.6%

Cecum

Fig 4.4 The community composition at family level

In particular, after supplemented with two levels of *B. coagulans*, the *Firmicutes* percentage had an increase and *Bacteroidetes* percentage had a dropat phylum level in ileum, while it was opposite in colon.

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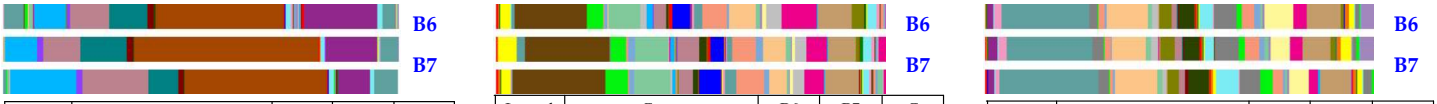
Moreover, there was a slightly but significantly decrease about *Proteobacteria* in ileum and no obvious difference in cecum at phylum level. The main factor in ileum which concretely induced the difference of *Bacteroidetes* was the genus *Prevotella*, inducement of *Firmicutes* were *Clostridiaceae* family and the genus *Turicibacter* and *Lactobacillus*, and inducement of *Proteobacteria* was the *Enterobacteriaceae* family. In addition, the main factor in both colon and cecum which concretely induced the difference was the *Veillonellaceae* family.



Legend	Genus	B6	B7	C
	f__Clostridiaceae; g	32.5%	46.6%	35.8%
	g__Prevotella	5.8%	0.1%	0.9%
	o__Tremblayales; f; g	0.3%	0.1%	0.1%
	o__Streptophyta;f; g	1.1%	0.4%	0.6%
	g__Turicibacter	10.1%	9.4%	16.5%
	f__Peptostreptococcaceae; g	1.2%	1.0%	1.3%
	f__Enterobacteriaceae; g	19.8%	13.6%	9.1%
	g__Actinobacillus	3.0%	4.5%	6.1%
	g__Klebsiella	1.2%	0.4%	0.9%
	g__Helicobacter	0.4%	0.0%	0.1%
	g__Treponema	0.4%	0.0%	0.0%
	f__Veillonellaceae; g	1.1%	0.0%	0.3%
	g__Streptococcus	0.8%	1.5%	1.7%
	g__Clostridium	0.3%	0.2%	0.3%
	f__Ruminococcaceae; g	0.7%	0.0%	0.0%
	o__Clostridiales; f; g	11.1%	13.2%	9.0%
	o__Bacteroidales; f; g	0.4%	0.0%	0.1%
	g__Lactobacillus	8.2%	8.0%	16.8%
	g__Phascolarctobacterium	0.5%	0.0%	0.2%
	Other	1.1%	0.1%	0.1%

Ileum

Legend	Genus	B6	B7	C
	f__Lachnospiraceae; g	6.7%	5.4%	7.1%
	g__Prevotella	19.3%	22.6%	24.3%
	o__Tremblayales; f; g	2.5%	1.9%	1.8%
	f__Erysipelotrichaceae; g	0.6%	1.1%	0.7%
	g__Roseburia	2.2%	1.2%	2.8%
	g__Succiniclasticum	3.2%	0.6%	0.0%
	f__Enterobacteriaceae; g	0.5%	0.4%	0.6%
	g__Treponema	0.9%	2.8%	1.8%
	g__Succinivibrio	0.7%	0.1%	0.5%
	f__S24-7; g	4.2%	4.1%	5.8%
	g__[Prevotella]	9.2%	9.2%	10.9%
	f__Veillonellaceae; g	1.7%	1.5%	1.2%
	g__CF231	0.9%	1.9%	0.4%
	g__Lachnospira	0.4%	0.3%	0.6%
	f__Ruminococcaceae; g	6.1%	5.9%	6.9%
	o__Clostridiales; f; g	3.2%	5.6%	3.6%
	o__Bacteroidales; f; g	2.7%	4.7%	2.3%
	g__Parabacteroides	0.8%	1.9%	0.3%
	g__Lactobacillus	1.6%	1.2%	0.9%
	f__Clostridiaceae; g	0.8%	2.3%	1.4%
	g__Oscillospira	6.8%	5.9%	4.5%
	g__Anaerovibrio	3.9%	2.7%	2.9%
	g__Phascolarctobacterium	8.6%	7.2%	8.6%
	g__Megasphaera	8.8%	5.2%	4.9%
	g__Ruminococcus	1.1%	1.1%	1.5%
	g__Faecalibacterium	0.5%	1.7%	1.3%
	Other	2.1%	1.5%	2.4%

Colon

Legend	Genus	B6	B7	C
	f__Lachnospiraceae; g	4.2%	4.2%	6.0%
	g__Prevotella	22.2%	22.5%	24.9%
	o__Tremblayales; f; g	1.5%	2.5%	1.4%
	g__p-75-a5	1.0%	0.9%	0.8%
	g__Roseburia	3.5%	2.3%	5.7%
	f__Lachnospiraceae; g	3.9%	3.8%	5.3%
	f__Enterobacteriaceae; g	0.5%	0.9%	0.8%
	g__Treponema	4.5%	4.4%	1.3%
	g__Succinivibrio	0.8%	0.7%	1.9%
	f__F16; g	1.4%	1.6%	1.2%
	g__[Prevotella]	10.6%	9.4%	10.7%
	f__RF16; g	2.1%	2.9%	2.7%
	g__CF231	1.7%	1.4%	0.9%
	g__YRC22	0.5%	1.3%	0.4%
	f__Ruminococcaceae; g	5.6%	6.0%	5.1%
	o__Clostridiales; f; g	3.1%	3.8%	3.9%
	o__Bacteroidales; f; g	1.7%	2.5%	1.5%
	g__Parabacteroides	1.8%	2.2%	1.1%
	g__Lactobacillus	1.7%	2.0%	0.9%
	f__Clostridiaceae; g	1.4%	1.7%	1.4%
	g__Oscillospira	3.2%	3.5%	2.6%
	g__Anaerovibrio	7.4%	4.7%	4.9%
	g__Phascolarctobacterium	8.5%	7.1%	8.0%
	g__Megasphaera	3.3%	3.2%	2.3%
	g__Ruminococcus	1.4%	1.4%	0.6%
	g__Faecalibacterium	1.4%	1.6%	3.0%
	Other	1.1%	1.5%	0.7%

Cecum

Fig 4.5 The community composition at genus level

In gut microbiota of mammals, *Firmicutes* and *Bacteroidetes* were the dominant phyla, followed by *Fusobacteria*, *Proteobacteria*, and *Actinobacteria* [54]. Previous study also presented the similar result that *Firmicutes*, and *Bacteroidetes* were still the main phyla in pigs regardless of growing ages or different intestinal segments [55,56]. *Prevotella* is a genus of Gram- negative bacteria, can cause infections such as abscesses, bacteraemia, wound infection, bite infections, genital tract infections, and periodontitis (Pavillion) [57]. *Veillonellaceae* belongs to the order of *Clostridiales*, which are commonly found in soils and in microbiota of humans and animals, whose wounds and infections are found worldwide [58]. *Enterobacteriaceae* are a large family of Gram-negative bacteria that includes, along with many harmless symbionts, many of the more familiar pathogens, such as *Salmonella*, *Escherichia coli*, *Yersinia pestis*, *Klebsiella*, and *Shigella* [59,60]. After supplemented with two levels of *B. coagulans*, this three kinds of pathogens had different levels of decrease, it might be one of the regulatory mechanisms in intestine that *B. coagulans* could alleviate inflammation, diarrhea and disease. Moreover, the percentage of *Lactobacillus* had sharply rised by twice when supplemented with 2.0×10^{7} CFU/g *B. coagulans*, which is a large group of beneficial bacteria [61], it implied that one ways of *B. coagulans* lower diarrhea promote nutrients absorption and metabolism may be

indirectly via the proliferation of *Lactobacillus*. Additionally, there was an inexplainable result that the percentage of *Clostridiaceae* substantially increased when supplemented with 2.0×10^6 CFU/g *B. coagulans*, which is a general etiological agent of animals. We inferred the rise of it might involve with antibiotic or stress during breeding, and it might be the cause that there was no significant difference of ADG.

3. Material and Methods

3.1 Experimental Design and Sample Collection

The animal-use protocol for this research was approved by the Animal Care and Use Committee of Hubei Province. The 24 healthy crossbred piglets (Duroc × Landrace × Yorkshire) were weaned at 21 d of age and feed with a corn and soybean meal–based diet. Each piglet was individually housed in a 1.20 × 1.10 m^2 steel metabolic cage. After a period of 3 days adaptation, piglets were assigned randomly on the basis of body weight and litter origin to 3 groups: 1) control (C) group which piglets were fed with the basal diet; 2) B6 group which piglets were fed with the basal diet supplemented with 2.0×10^6 CFU/g *B. coagulans*; 3) B7 group which piglets were fed with the basal diet supplemented with 2.0×10^7 CFU/g *B. coagulans*. All diets were isocaloric [62]. On 21th day of the trial, blood, intestine and digesta samples were collected and stored at -80°C until assay as previous study [63].

3.2 Plasma Biochemical Indicators and Intestinal Redox Status

Plasma biochemical indicators were measured with corresponding kits using a Hi-tachi 7060 Automatic Biochemical Analyzer (Hitachi, Japan), the activities of DAO, SOD and CAT and the concent of MDA and H_2O_2 were determined using commercially available kits (Jiancheng Bioengineering Institute, Nanjing, China). Assays were performed in triplicate.

3.3 Intestinal Morphology

To determine intestinal morphology, paraformaldehyde-fixed jejunum and ileum samples were dehydrated and embedded in paraffin. 5-μm sections were cut and then stained with hematoxylin and eosin stain. Intestinal morphology was determined using a light microscope (Leica, Germany) with the Leica Application Suite image analysis software (Leica, Germany). Villus area was quantitated from the perimeter and height of the villi . The ratio of villus height to crypt depth was calculated.

3.4 Expression Levels of Proteins

The expression levels of proteins were proformed by western bloting as described by Hou *et al* [64]. The primary antibodies: HSP70, Caspase-3, Bax, Villin and Occludin (rabbit, 1: 1,000; Cell Signaling Technology, Inc., Danvers, USA), β-actin (mouse 1: 2,000; Sigma-Aldrich Inc., St. Louis, USA). The secondary antibody: anti-rabbit (mouse, 1: 2,000; Zhongshan Golden Bridge Biological Technology Co., Ltd., Beijing, China). Blots were carried out by utilizing a chemiluminescence kit (Amersham Biosciences, Uppsala, Sweden) and image forming system (Alpha Innotech, New York, USA).

3.5 Expression Levels of Genes

The gene expression levels were quantitated by the method of real-time PCR as described by Yi *et al* [65]. The real-time PCR was carried out with primers (Table 6) of these genes as well as reference gene ribosomal protein L 4 (RPL4) and the SYBR® Premix Ex Taq™ (Takara, Dalian, China) on 7,500 Fast Real-Time PCR System (Foster City, USA). Data was analyzed by the $2^{-\Delta}$Ct method as described [65].

3.6 Analysis of Gut Microbiota

Total bacterial DNA was extracted, the gene-specific sequences targeted the 16S V3 and V4 region and amplified with two stage PCR, and then was analyzed by MiSeq sequencing. The result were processed with QIIME as described by Caporaso *et al* [66].

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α-Diversity metrics were calculated using a read depth of 10,000 and a β-diversity distance matrix was calculated based on UniFrac metric, which was used for principal coordinates analysis [67]. The significance of the diet effect on the β-diversity distance matrix was assessed by PERMANOVA analysis [68]. Raw sequence data and detection and removal of chimeras were performed using the software USEARCH and UCHIIME [66,68].

Table 6. Primers for Real-Time PCR analysis

Gene	Forward	Reverse
RPL4	GAGAAACCGTCGCCGAAT	GCCCACCAGGAGCAAGTT
IFN-α	ACTCCATCCTGGCTGTGAGGAAAT	ATCTCATGACTTCTGCCCTGACGA
IFN-β	ATGTCAGAAGCTCCTGGGACAGTT	AGGTCATCCATCTGCCCATCAAGT
IFN-γ	TCTGGGAAACTGAATGACTTCG	GACTTCTCTTCCGCTTTCTTAGGTT
MX1	AGTGCGGCTGTTTACCAAG	TTCACAAACCCTGGCAACTC
MX2	CGCATTCTTTCACTCGCATC	CCTCAACCCACCAACTCACA
OAS1	TGGTGGTGGAGACACACACA	CCAACCAGAGACCCATCCA
IL-1β	CAACGTGCAGTCTATGGAGT	GAGGTGCTGATGTACCAGTTG
IL-4	AGGAGCCACACGTGCTTGA	TTGCCAAGCTGTTGAGATTCC
CXCL-9	CTTGCTTTTGGGTATCATCTTCCT	TCATCCTTTGGCTGGTGTTG
CCL-2	CATAAGCCACCTGGACAAGAAAA	GGGTATTTAGGGCAAGTTAGAAGGA
AQP3	AAGCTGTCCCAAGTAAAGCACAA	GCCCTACTTCCTGTTTCACCAC
SGLT-1	CCCAAATCAGAGCATTCCATTCA	AAGTATGGTGTGGTGGCCGGTT
LPL	AGCCTGAGTTGGACCCATGT	CTCTGTTTTCCCTTCCTCTCTCC
INSR	GGGGCTAAAGAGGAACTATGAGG	AGAGGAAAGCGAAGACAGGAAA
$b^{0,+}AT$	CGAGTACCCGTACCTGATGGA	TGCGTAGAAGGGCGAAGAA

3.7 Statistical Analysis

Data were analyzed using one-way analysis of variance to analysis, expressed as mean values ± SEM. All experimental data was analysed using SPSS (Version 17.0). A *P*-value of < 0.05 was considered statistically significant.

4. Conclusion

Supplementing *B. coagulans* in intestine of weaning piglets had beneficial impacts on lowering diarrhea rate, promoting nutrients metabolism, maintaining intestinal integrity and alleviating oxidative stress. Futher research of molecular mechanisms showed that, these beneficial impacts were regulated by changing expression levels of related proteins and genes and altering community composition of gut microbiota.

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Referrence

1. Pluske, J.R.; Hampson, D.J.; Williams, I.H. Factors influencing the structure and function of the small intestine in the weaned pig: a review. *Livest. Prod. Sci.* **1997**, *51, 215-236*.
2. De, G.A.; Resink, J.W.; VanHees, H.M.; Ruuls, L.; Klaassen, G.J.; Rouwers, S.M.; Stockhofe-Zurwieden, N. Supplementation of piglets with nutrient-dense complex milk replacer improves intestinal development and microbial fermentation. *J. Anim. Sci.* **2016**, *94, 1012*.
3. Moeser, A.J.; Pohl, C.S.; Rajput, M. Weaning stress and gastrointestinal barrier development: Implications for lifelong gut health in pig. *Anim. Nutr.* **2017**, *3, 313-321*.
4. Montagne, L.; Boudry, G.; Favier, C.; Le Huërou-Luron, I.; Lallès, J.P.; Sève, B. Main intestinal markers associated with the changes in gut architecture and function in piglets after weaning. *Br. J. Nutr.* **2007**, *97, 45-57*.
5. Moeser, A.J.; Ryan, K.A.; Nighot, P.K.; Blikslager, A.T. Gastrointestinal dysfunction induced by early weaning is attenuated by delayed weaning and mast cell blockade in pigs. *Am. J. Physiol. Gastrointest. Liver. Physiol.* **2007**, *293, G413*.
6. Etheridge, R.D.; Seerley, R.W.; Wyatt, R.D. The effect of diet on performance, digestibility, blood composition and intestinal microflora of weaned pigs. *J. Anim. Sci.* **1984**, *58, 1396-1402*.
7. Hamard, A.; Sève, B.; Le, F.N. Intestinal development and growth performance of early-weaned piglets fed a low-threonine diet. *Animal* **2007**, *1, 1134*.
8. Bergen, W.G.; Wu, G. Intestinal nitrogen recycling and utilization in health and disease. *J. Nutr.* **2009**, *139, 821-825.*
9. Dai, Z.L.; Li, X.L.; Xi, P.B.; Zhang, J.; Wu, G.; Zhu, W.Y. L-Glutamine regulates amino acid utilization by intestinal bacteria. *Amino Acids* **2013**, *45, 501-512.*
10. Brosnan, J.T.; Brosnan, M.E. Glutamate: a truly functional amino acid. *Amino Acids* **2013**, *45, 413-418.*
11. Liu, Y.L.; Huang, J.J.; Hou, Y.Q.; Zhu, H.L.; Zhao, S.J.; Ding, B.Y.; Yin, Y.L.; Yi, G.F.; Shi, J.Y.; Fan, W. Dietary arginine supplementation alleviates intestinal mucosal disruption induced by Escherichia coli lipopolysaccharide in weaned pigs. *Br. J. Nutr.* **2008**, *100, 552-560.*
12. Dai, Z.L.; Wu, G.; Zhu, W.Y. Amino acid metabolism in intestinal bacteria: links between gut ecology and host health. *Front. Biosci.* **2011**, *16, 1768-1786.*
13. Xin, W.; Zheng, R.; Kong, X.F.; Tang, Z.R.; Dong, X.H. Functions of microbiota in monogastric gastrointestinal tract and new practices in animal production. *J. Food. Agri. Environ.* **2010**, *8, 695-702.*
14. Arunachalam, K.D. Role of Bifidobacteria in nutrition, medicine and technology. *Nutr. Res.* **1999**, *19, 1559-1597.*
15. Bon, M.L.; Davies, H.E.; Glynn, C.; Thompson, C.; Madden, M.; Wiseman, J.; Dodd, C.; Hurdidge, L.; Payne, G.; Treut, Y.L.; Craigon, J.; Tötemeyer, S.; Mellits K.H. Influence of probiotics on gut health in the weaned pig. *Livest. Sci.* **2010**, *133, 179-181.*
16. Xuan, Z.N.; Kim, J.D.; Heo, K.N.; Jung, H.J.; Lee, J.H.; Han, Y.K.; Kim, Y.Y.; Han, I.K. Study on the development of a probiotics complex for weaned pigs. *Asian. Austral. J. Anim. Sci.* **2001**, *14, 1425-1428.*
17. Wang, J.Q.; Yin, F.G.; Zhu, C.; Yu, H.; Niven, S.J.; DeLange, C.F.M.; Gong, J. Evaluation of probiotic bacteria for their effects on the growth performance and intestinal microbiota of newly-weaned pigs fed fermented high-moisture maize. *Livest. Sci*. **2012**, *145, 79-86.*
18. Payot, T.; Chemaly, Z.; Fick, M. Lactic acid production by Bacillus coagulans — kinetic studies and optimization of culture medium for batch and continuous fermentations. *Enzyme. Microb. Tech.* **1999**, *24, 191-199.*
19. Zhou, X.; Wang, Y.; Gu, Q.; Li, W. Effect of dietary probiotic, Bacillus coagulans, on growth performance, chemical composition, and meat quality of Guangxi Yellow chicken. *Poul. Sci.* **2010**, 89, 588.
20. Hung, A.T.; Lin, S.Y.; Yang, T.Y.; Chou, C.K.; Liu, H.C.; Lu J.J.; Wang, B.; Chen, S.Y.; Lien, T.F. Effects of Bacillus coagulans ATCC 7050 on growth performance, intestinal morphology, and microflora composition in broiler chickens. *Anim. Prod. Sci.* **2012**, *52, 874.*

21. Ripamonti, B.; Agazzi, A.; Baldi, A.; Balzaretti, C.; Bersani, C.; Pirani, S.; Rebucci, R.; Savoini, G.; Stella, S.; Stenico, A.; Domeneghini, C. Administration of Bacillus coagulans, in calves: recovery from faecal samples and evaluation of functional aspects of spores. *Vet. Res. Commun.* **2009**, *33, 991.*
22. Hyronimus, B.; Marrec, C.L.; Urdaci, M.C. Coagulin, a bacteriocin. *J. Appl. Microbiol.* **1998**, *85, 42-50.*
23. Baron, M. A patented strain of Bacillus coagulans increased immune response to viral challenge. *Postgrad. Med.* **2009**, *121, 114–118.*
24. Kristal-Boneh, E.; Harari, G.; Green, M.S. Circannual Variations in Blood Cholesterol Levels. *Chronobiol. Int.* **1993**, *10, 37-42.*
25. Bae, Y.J.; Choi, M.K.; Kim, M.H. Manganese supplementation reduces the blood cholesterol levels in Ca-deficient ovariectomized rats. *Biol. Trace. Elem.* Res. **2011**, *141, 224-231.*
26. Stole, E.; Smith, T.K.; Manning, J.M.; Meister, A. Interaction of gamma-glutamyl transpeptidase with acivicin. J. Biol. Chem. **1994**, *269, 21435.*
27. Braun, J.P.; Benard, P.; Burgat, V.; Rico, A.G. Gamma Glutamyl Transferase in domestic animals. *Vet. Res. Commun.* **1983**, *6, 77-90.*
28. Agúndez, J.A.; Ayuso, P.; Cornejo-García, J.A.; Blanca, M.; Torres, M.J.; Doña, I.; Salas, M.; Blanca-López, N.; Canto, G.; Rondon, C.; Campo, P.; Laguna, J.J.; Fernández, J.; Martínez, C.; García-Martín, E. The diamine oxidase gene is associated with hypersensitivity response to non-steroidal anti-inflammatory drugs. *Plos. One.* **2012**, *7: e47571.*
29. Kitanaka, J.; Kitanaka, N.; Tsujimura, T.; Terada, N.; Takemura, M. Expression of diamine oxidase (histaminase) in guinea-pig tissues. *Eur. J. Pharmacol.* **2002**, *437, 179–185.*
30. Pluske, J.R.; Williams, I.H.; Aherne, F.X. Maintenance of villous height and crypt depth in piglets by providing continuous nutrition after weaning. *Anim. Sci.* **1996**, *62, 131-144.*
31. Cera, K.R.; Mahan, D.C.; Cross, R.F.; Reinhart, G.A.; Whitmoyer, R.E. Effect of age, weaning and postweaning diet on small intestinal growth and jejunal morphology in young swine. *J. Anim. Sci.* **1988**, *66, 574-584.*
32. Cabello-Verrugio, C.; Ruiz-Ortega, M.; Mosqueira, M.; Simon, F. Oxidative Stress in Disease and Aging: Mechanisms and Therapies. *Oxid. Med. Cell. Longev.* **2015**, *2016, 2.*
33. Doke, N. Involvement of superoxide anion generation in the hypersensitive response of potato tuber tissues to infection with an incompatible race of Phytophthora infestans, and to the hyphal wall components. *Physiol. Plant Pathol.* **1983**, *23, 345–357.*
34. Harris, E.D. Regulation of antioxidant enzymes. *J. Nutr.* **1992**, *122, 625-626.*
35. Del Rio, D.; Stewart, A.J.; Pellegrini, N. A review of recent studies on malondialdehyde as toxic molecule and biological marker of oxidative stress. *Nutr. Metab. Cardiovasc. Dis.* **2005**, *15, 316–328.*
36. Sies, H. Role of metabolic H_2O_2 generation: redox signaling and oxidative stress. J Biol Chem. **2014**, *289, 8735-8741.*
37. Buchmeier, N.A.; Heffron, F. Induction of Salmonella stress proteins upon infection of macrophages. *Science* **1990**, *248, 730.*
38. Snigdha, S.; Smith, E.D.; Prieto, G.A.; Cotman, C.W. Caspase-3 activation as a bifurcation point between plasticity and cell death. *Neurosci. Bull.* **2012**, *28, 14.*
39. Kirkland, R.A.; Saavedra, G.M.; Cummings, B.S.; Franklin, J.L. Bax regulates production of superoxide in both apoptotic and nonapoptotic neurons: role of caspases. *J. Neurosci.* **2010**, *30, 16114.*
40. Friederich, E.; Pringault, E.; Arpin, M.; Louvard, D. From the structure to the function of villin, an actin-binding protein of the brush border. *Bioessays* **1990**, *12, 403-408.*
41. Schneeberger, E.E.; Lynch, R.D. The tight junction: a multifunctional complex. *Am. J. Physiol. Cell. Physiol.* **2004**, *286, 1213-28.*
42. Malcolm, K.C.; Worthen, G.S. Lipopolysaccharide stimulates p38-dependent induction of antiviral genes in neutrophils independently of paracrine factors. *J. Biol. Chem.* **2003**, *278, 15693- 15701.*

43. Takaoka, A.; Yanai, H. Interferon signalling network in innate defence. *Cell. Microbiol.* **2006**, *8, 907-922.*
44. García-Sastre, A.; Biron, C.A. Type 1 interferons and the virus-host relationship: A lesson in Détente. *Science* **2006**, *312, 879.*
45. Matsumiya, T.; Hayakari, R.; Narita, N.; Ito, R.; Kon, T.; Kubota, K.; Sakaki, H.; Yoshida, H.; Imaizumi, T.; Kobayashi, W.; Kimura, H. Role of type I- and type II-interferon in expression of melanoma differentiation-associated gene-5 in HSC-3 oral squamous carcinoma cells. *Biomed. Res.* **2014**, *35, 9-16.*
46. Franco, J.G.; Lisboa, P.C.; Lima, N.S.; Amaral, T.A.; Peixoto-Silva, N.; Resende, A.C.; Oliveira, E.; Passos, M.C.; Moura, E.G. Resveratrol attenuates oxidative stress and prevents steatosis and hypertension in obese rats programmed by early weaning. *J. Nutr. Biochem.* **2013**, *24, 960.*
47. Villehuchet, A.M.; Brack, M.; Dreyfus, G.; Oussar, Y.; Bonnefont-Rousselot, D.; Chapman, M.J.; Kontush, A. A machine-learning approach to the prediction of oxidative stress in chronic inflammatory disease. *Redox. Rep.* **2009**, *14, 23-33.*
48. Wei, Y.H.; Lu, C.Y.; Wei, C.Y.; Ma, Y.S.; Lee, H.C. Oxidative stress in human aging and mitochondrial disease-consequences of defective mitochondrial respiration and impaired antioxidant enzyme system. *Chin. J. Physiol.* **2001**, *44, 1-12.*
49. Jiang, X.C.; Moulin, P.; Quinet, E.; Goldberg, I.J.; Yacoub, L.K.; Agellon, L.B.; Compton, D.; Schnitzer-Polokoff, R.; Tall, A.R. Mammalian adipose tissue and muscle are major sources of lipid transfer protein mRNA. *J. Biol. Chem.* **1991**, *266, 4631.*
50. Sarfstein, R.; Werner, H. Insulin receptor (INSR) and insulin-like growth factor-I receptor (IGF-GR) translocate to nucleus and regulate IGF-GR gene expression in breast cancer cells. *Growth. Horm. Igf. Res.* **2012**, *22, S4*
51. Arnspang, E.C.; Sundbye, S.; Nelson, W.J.; Nejsum, L.N. Aquaporin-3 and aquaporin-4 are sorted differently and separately in the trans-Golgi network. *Plos. One.* **2013**. *8: e73977.*
52. Tavakkolizadeh, A.; Berger, U.V.; Shen, K.R.; Levitsky, L.L.; Zinner, M.J.; Hediger, M.A.; Ashley, S.W.; Whang, E.E.; Rhoads, D.B. Diurnal rhythmicity in intestinal SGLT-1 function, V(max), and mRNA expression topography. *Am. J. Physiol. Gastrointest. Liver. Physiol.* **2001**, *280, G209.*
53. Romeo, E.; Dave, M.H.; Bacic, D.; Ristic, Z.; Camargo, S.M.; Loffing, J.; Wagner, C.A.; Verrey, F. Luminal kidney and intestine SLC6 amino acid transporters of B0AT-cluster and their tissue distribution in Mus musculus. *Am. J. Physiol. Renal. Physiol.* **2006**, *290, F376.*
54. Pedersen, R.; Andersen, A.D.; Molbak, L.; Stagsted, J.; Boye, M, Changes in the gut microbiota of cloned and non-cloned control pigs during development of obesity: gut microbiota during development of obesity in cloned pigs. *BMC Microbiol.* **2013**, *13, 30.*
55. Ramadan, Z.; Xu, H.; Laflamme, D.; Czarnecki-Maulden, G.; Li, Q.J.; Labuda, J.; Bourqui, B. Fecal Microbiota of Cats with Naturally Occurring Chronic Diarrhea Assessed Using 16S rRNAGene 454-Pyrosequencing before and after Dietary Treatment. *J. Vet. Int. Med.* **2014**, *28, 59–65.*
56. Roux, V.; Robert, C.; Raoult, D. Non-contiguous finished genome sequence of Prevotella timonensis, type strain 4401737 T. *Stand. Genomic. Sci.* **2014**, *9, 1344-1351.*
57. Bonatti, H.J.R.; Stauffer, J.; Turan, N.; et al. Leberabszess durch Eikenella corrodens, Prevotella meloninogenica, und Lactobacillus, spp. *Eur. Surg.* **2010**, *42, 58-64.*
58. Sabine, G.; Sabine, W.; Alla, L.; Goegelel H.; Sifri C.D.; Martin, K.; Hinder, R.; Nguyen, J.H. Complete genome sequence of Veillonella parvula type strain (Te3). *Stand. Genomic. Sci.* **2010**, *2, 57-65.*
59. Santiago, A.E.; Ruizperez, F.; Jo, N.Y.; Vijayakumar, V.; Gong, M.Q.; Nataro, J.P. A Large Family of Antivirulence Regulators Modulates the Effects of Transcriptional Activators in Gram-negative Pathogenic Bacteria. *Plos. Pathog.* **2014**, *10, e1004153.*
60. Sassone-Corsi, M.; Nuccio, S.P.; Liu, H.; Hernandez, D.; Vu, C.T.; Takahashi, A.A.; Edwards, R.A.; Raffatellu, M.

Microcins mediate competition among Enterobacteriaceae in the inflamed gut. *Nature* **2016**, *540, 280.*

61. Li, Z.; Hu, W.; Liu, D.; Tian, W.; Yu, G.; Liu, X.; Wang, J.; Feng, E.; Zhang, X.; Chen, B.; Zeng, M.; Wang, H. A Reference Proteomic Database of Lactobacillus plantarum CMCC-P0002. *Plos. One.* **2011**, *6, e25596.*
62. Hou, Y.Q.; Wang, L.; Ding, B.Y. Dietary α-ketoglutarate supplementation ameliorates intestinal injury in lipopolysaccharidechallenged piglets. *Amino. Acids.* **2010**, *39, 555–564.*
63. Yasuda, K.; Dawson, H.D.; Wasmuth, E.V.; Roneker, C.A.; Chen, C.; Urban, J.F.; Welch, R.M.; Miller, D.D.; Lei, X.G. Supplemental dietary inulin influences expression of iron and inflammation related genes in young pigs. *J. Nutr.* **2009**, *139, 2018.*
64. Hou, Y.; Yao, K.; Wang, L.; Ding, B.; Fu, D.; Liu, Y.; Zhu, H.; Liu, J.; Li, Y.; Kang, P.; Yin, Y.; Wu, G. Effects of α-ketoglutarate on energy status in the intestinal mucosa of weaned piglets chronically challenged with lipopolysaccharide. *Br. J. Nutr.* **2011**, *106, 357–363*
65. Yi, D.; Hou, Y.; Wang, L.; Zhao, D.; Ding, B.; Wu, T.; Chen, H.; Liu, Y.; Kang, P.; Wu, G. Gene expression profiles in the intestine of lipopolysaccharide-challenged piglets. *Front. Biosci.* **2016**, *21, 487-501.*
66. Caporaso, J.G.; Kuczynski, J.; Stombaugh, J.; Bittinger, K.; Bushman, F.D.; Costello, E.K.; Fierer, N.; Peña, A.G.; Goodrich, J.K.; Gordon, J.I.; Huttley, G.A.; Kelley, S.T.; Knights, D.; Koenig, J.E.; Ley, R.E.; Lozupone, C.A.; McDonald, D.; Muegge, B.D.; Pirrung, M.; Reeder, J.; Sevinsky, J.R.; Turnbaugh, P.J.; Walters, W.A.; Widmann, J.; Yatsunenko, T.; Zaneveld, J.; Knight, R. QIIME allows analysis of high-throughput community sequencing data. *Nat. Methods.* **2010**, *7, 335–6.*
67. Price, M.N.; Dehal, P.S.; Arkin, A.P. FastTree: computing large minimum evolution trees with profiles instead of a distance matrix. *Mol. Biol. Evol.* **2009**, *26, 1641–50.*
68. Lozupone, C.; Knight, R. UniFrac: a new phylogenetic method for comparing microbial communities. *Appl. Environ. Microbiol.* **2005**, *71, 8228–8235.*