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# Beta-glucan from *Agrobacterium sp.* ZX09 improves growth performance and intestinal function in weaned piglets

## Running title

Glucan improves growth and intestinal health

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## Abstract

Beta-glucan is currently under consideration as an alternative to in-feed antibiotics. The aim of the study was to investigate *Agrobacterium sp.* ZX09 beta-glucan on intestinal morphology, cytokine concentration, mucin expression and microbial populations of weaning piglets. Pigs were randomly assigned to one of five dietary treatments supplemented with 0, 25, 50, 100 and 200 mg/kg beta-glucan. Data showed an increase of ADG at the 100 mg/kg group ($P$=0.03). A significant increase in villus height and reduction in crypt depth were fund in ileal tissue at the 100 mg/kg inclusion level ($P$<0.05). Dietary supplementation of 100 mg/kg beta-glucan enhanced IL-10 concentration ($P$=0.04) and gene expression of MUC1 and MUC2 ($P$<0.05) in the jejunum. Dietary supplementation of 100 mg/kg beta-glucan provoked the up-regulation of *Lactobacillus* counts and down-regulation of *Escherichia coli* counts in the cecum ($P$=0.05). Data strongly (Delete) suggested that improved growth performance in response to beta-glucan supplementation at 100 mg/kg in weaned piglets may be explained by the improved intestinal function.

**Keywords:** β-glucan; mucin; cytokine; microbiota; piglet

## 1 Introduction

With widespread prohibition of in-feed antimicrobial compounds, the use of natural supplements, such as beta-glucan, is currently under consideration as an alternative to in-feed antibiotics due to its gut-modulatory function in human and farm animals (Xiong *et al.*, 2015). Beta-glucan resists hydrolysis by mammalian alimentary enzymes, modifies bacterial community, and is easily fermented by the gastrointestinal microbiota to short-chain fatty acids (SCFAs) (Murphy *et al*., 2013). In general, SCFAs play an important role in intestinal health by affecting morphology, mucus production and mucosal gene expression and by protecting against overgrowth

237 with opportunistic pathogens (Yuan *et al*., 2015).

238 Previously, most studies referring the stimulatory properties of glucan presenting in
239 the cell walls of algae, fungi, yeast, cereal grains and a limited number of bacteria
240 have been performed in rats (Belobrajdic *et al.*, 2015), chickens (Tian *et al.*, 2016),
241 fish (Jiang *et al.*, 2016), pigs (Lee *et al.*, 2017) and cattle (Ma *et al.*, 2015). However,
242 no substantial consistent effects of dietary glucan on the intestinal function were
243 found. The divergent results might be caused by the quality of variant glucan used in
244 the study. For instance, the soluble beta-glucan from *L. digitata* may be acting via a
245 different mechanism than the insoluble beta-glucan from *L. byperborea* and *S.*
246 *cerevisiae* (Sweeney *et al.*, 2012). Apart from the solubility, beta-glucan varied in
247 their structure, chemical composition and molecular weight, which may impact their
248 effects on animal performance and gastrointestinal health (Zheng *et al*., 2016).
249 Furthermore, the composition of yeast beta-glucan is complex and not highly pure
250 enough to rule out the complicated effects elicited by beta-glucan (Delete).

251 Efficacy of beta-glucan is likely to link their functional properties, such as purity,
252 structure and molecular weight (Sweeney et al. 2012). In the present study,
253 extracellular beta-glucan with high-molecular-mass was obtained from
254 *Agrobacterium sp.* ZX09. It is a novel high purity and water-soluble polymer, which
255 is composed of a linear chain of glucosyl residues linked through a repeat unit of
256 seven β-(1,3) and two α-(1,3) glucosidic bonds, without any β-(1,6) glucosidic bonds.
257 As a new beta-glucan with specific molecular structure, its safety has been
258 demonstrated in the acute and subchronic experiment (Zhou *et al.*, 2013). The
259 differences between *Agrobacterium sp.* beta-glucan and other sources are shown in
260 table 1 (Zheng *et al*., 2016). As shown in Table 1, compared to beta-glucan in cereal,
261 yeast and fungal formulation, the composition of *Agrobacterium sp.* ZX09 β-glucan is
262 simple and really pure. In addition, the molecular weight of *Agrobacterium sp.* ZX09
263 β-glucan is relatively high in comparision with other sources. The previous studies
264 have shown that beta-glucan preparations in various sources and alteration in
265 molecular structure enhance the growth promotion properties of the molecule by
266 varying degrees, via eliciting gut responses through modulation of immune and

microbial systems (Khan et al. 2016). Thus, we wonder the effects of the new β-glucan source ingestion on the gut-modulating responses including intestinal barrier function, immunity and bacterial populations in piglets. In this study, we hypothesized that β-glucan derived from Agrobacterium sp. ZX09 may aid in gut health of piglets, and that the intestinal modulatory function may depend on the varied dosage of β-glucan in the diets. Hence, the aim of the present study was to assess the intestinal response to the dietary inclusion of *Agrobacterium sp.* ZX09 β-glucans and establish the optimum inclusion level of β-glucan derived from *Agrobacterium sp.* ZX09. However, its intestinal-modulatory activity is uncertain. Hence, the aim of the present study was to establish the optimum inclusion level of beta-glucan derived from *Agrobacterium sp.* ZX09 on growth performance, intestinal morphology, selected bacterial populations, cytokines as well as gene expression of mucins and (Delete) in the weaned piglets. To the best of our knowledge, it is the first in the literature (Delete) to determine the intestinal tissue response to beta-glucan derived from *Agrobacterium sp.* ZX09 (Delete).

## 2 Materials and Methods

All animal procedures described in the present experiment were approved by the Institutional Animal Care and Use Committee of Sichuan Agricultural University.

### 2.1 Beta-glucan

The method of beta-glucan preparation was previously described (Zhang et al. 2013). Briefly, *Agrobacterium sp.* ZX09 used in this study was isolated from a soil sample from the ocean coast, and kindly provided by Synlight Bio Co. Ltd of Sichuan, China. Cultures were maintained on HTM agar. A colony of the strain ZX09 was inoculated into a 250 mL flask containing 50 ml medium consisting of 2% sucrose and mineral salt solution. The inoculated preparation was incubated at 28 ℃ on a rotary shaker at 220 rpm for 24 h. A 0.5 mL portion was transferred to a 250 mL flask containing 50 mL fermentation medium. Fermentation was performed on a rotary shaker at 220 rpm for 48 h. The culture broth was diluted more than 3 times with de-ionized water and centrifuged at 12000 g for 30 min to separate cells from the supernatant. The supernatant was added to two volumes of 95% ethanol. Productivity

297 of beta-glucan was expressed in terms of the weight after ethanol precipitation
298 collected by centrifugation at 6000 g for 15 min and dried under reduced pressure.
299 The beta-glucan was further purified according to previously described methods
300 (Chen et al. 2011). The total sugar contents of the fraction were determined by the
301 phenol-sulfuric acid method, using glucose for the standard curve (DuBios *et al.*,
302 1956), and purity of the purified beta-glucan was more than 90%. The mean average
303 molecular weight of purified beta-glucan was estimated from a calibration curve of
304 the standard dextrans obtained by gel filtration on Sepharose CL-4B to be about 2000
305 kDa.

306 **2.2 Experimental Design and Animal Diets**

307 Transition period for feeding lasted for 3 d. After 3 d of acclimatization, a total of
308 180 DLY (Duroc × Landrace × Yorkshire) pigs, weaned at 25 d of age, with an initial
309 body weight of (7.03± 0.03) kg, were blocked on the basis of body weight and litter of
310 origin. Then, the pigs were randomly assigned to one of the five dietary treatments
311 (six pens per treatment and six piglets per pen) as follows: T1, basal diet (T1); T2,
312 basal diet supplemented with 25 mg/kg beta-glucan; T3, basal diet supplemented with
313 50 mg/kg beta-glucan; T4, basal diet supplemented with 100 mg/kg beta-glucan and
314 T5, basal diet supplemented with 200 mg/kg beta-glucan. Experimental feeding
315 period lasted for 28 d ad libitum. Purified beta-glucan from *Agrobacterium sp.* ZX09
316 was extracted according to the precedure described (Zhang *et al.*, 2013), and was
317 kindly provided by Sichuan Synlight Biotech., Ltd.. The total sugar contents of the
318 fraction were determined by the phenol-sulfuric acid method, using glucose for the
319 standard curve (DuBios *et al.*, 1956), and purity of the purified beta-glucan was more
320 than 90%. The mean average molecular weight of purified beta-glucan was estimated
321 from a calibration curve of the standard dextrans obtained by gel filtration on
322 Sepharose CL-4B to be about 2000 kDa（DELETE）. Mash diets based on maize
323 and soybean meal were formulated to approximately meet National Research
324 Council-recommended nutrient requirements for pigs (NRC 2012) (Table 2). The
325 basal diet contained beta-glucan, per se, so it's necessary to measure the beta-glucan
326 contents instead of using theoretical values. Based on the analysis of beta-glucan with

Megazyme kit (Megazyme), each dietary group included 10, 32, 65, 115 and 220 mg/kg beta-glucan, respectively.

**2.3 Animals and Management**

Pigs were housed in pens with six animals confined to each, and had ad libitum access to feed and water. The ambient temperature within the room was approximately 30℃ and decreased by 1℃ each week of the experiment until reaching 24℃. At 08:00 h of d 1, 15 and 29, the body weight and feed intake of all pigs were measured to calculate average daily weight gain (ADG), average daily feed intake (ADFI) and feed conversion. Fecal consistency was scored as follows: 0, normal; 1, pasty; 2, semiliquid; and 3, liquid. The mean cumulative score of diarrhea was calculated as previously described (Zhao *et al.*, 2015) (Delete). Every morning and afternoon during the experiment, the status of anal soft fecal contamination and swelling in each piglet was examined and recorded. These data were then used to calculate the incidence rate of diarrhea, which was taken as a reflection of the incidence of sick weanling pigs. The incidence rate of diarrhea was calculated according to the following formula: [total number of diarrhea pig/(total number of pigs × days of experiment)] ×100%.

**2.4 Sampling and Analyses**

Nutrient fecal apparent digestibility was measured over a 5 d collection period. During collections, total feces weight was recorded daily. At the end of the collection period, fecal samples were pooled and a subsample retained for laboratory analysis. On d 29, following weighing, one piglet in each pen was then anesthetized (with $C_3H_2ClF_5O$) and enthanized by intravenous administration (jugular vein) of 4% sodium pentobarbital solution (40 mg/kg BW). Then, the small intestine was removed, and the jejunum and ileum were quickly isolated, flushed with ice-cold saline. One section (about 2 cm in length ) in each part was placed in 10% phosphate-buffered formalin for histologic analysis, whereas the other section (approximately 18 cm in length) in jejunal tissue was collected as mucosal sample, frozen in liquid nitrogen, and stored at -80℃ until ELISA and RT-PCR analysis. Jejunum is the primary target of nutrient digestion and absorption, while cecum section accounts for microbial

fermentation and metabolite production. The mid-cecum tissues were removed and the digesta sample (approximately 10 g) of each pig was dispensed into two sterilized 5 mL centrifuge tubes for later analysis of microflora.

**2.5 Chemical Analysis for Nutrient Digestibility**

During the experimental period, ash insoluble in hydrochloric acid (AIA) in the diet was used as an inner indigestible marker for the determination of apparent total tract digestibility (ATTD) for nutrients. AIA analysis of the experimental diets and feces were carried out following the AOAC (2012) methods. From d15 to d 19, fecal samples were collected on a pen basis. Concentrations of dry matter (DM), crude protein (CP), ash and gross energy (GE) were determined in feed and feces according to the methods as Smith previously described (Smith et al. 2011). Briefly, following collection, feces were dried at 60°C for 72 h. The feed and dried feces samples were milled through a hammer mill provided with a 1 mm screen (Christy and Norris). The DM was assayed after drying overnight at 103°C. Ash was determined in a muffle furnace (Nabertherm) at 500℃. The nitrogen content of both feed and feces was determined using the LECO FP 528 instrument (UK Limited). The gross energy of the feed and feces was determined using a Parr 1201 oxygen bomb calorimeter (Parr). The beta-glucan content in the diets was determined by a Megazyme kit (Megazyme) (Delete). The digestibility was then calculated using the following formula:

Digestibility (%)=[1-(Nf×Ad)/(Nd×Af)] ×100, in which

Nf = Nutrient concentration in feces (%DM),

Nd = Nutrient concentration in diet (%DM),

Af = AIN concentration in feces (%DM), and

Ad = AIN concentration in diet (%DM).

**2.6 Intestinal Morphology Measurements**

Formalin-fixed jejunal and ileal cross-sections were embedded in paraffin wax, and 5 μm slides were cut and stained with hematoxylin and eosin. Villus height (the apex of the villus to the villus-crypt junction) and crypt depth (villus-crypt junction to the base of the crypt) were measured at 40× magnification using an image processing and

analysis system (Leica Imaging Systems Ltd., Cambridge, UK). At least 10 well-oriented intact villi and crypts were examined from each pig.

**2.7 Assessment of Cytokine Concentrations in the Jejunal Mucosa**

Mucosal interleukin-2 (IL-2) was measured using a commercially available swine ELISA kit (BioSource International Inc., Camarillo, CA). IL-1 β , IL-6, IL-10 and TNF-α (tumor necrosis factor-α) in the jejunal mucosa were also measured using commercially available ELISA kits by the manufacturer (R&D Systems Inc., Minneapolis, MN) for pigs, with a within-assay CV of less than 10% for all 3 assays. The assays were analyzed colorimetrically using a BioTek Synergy HT microplate reader (BioTek Instruments, Winooski, VT).

**2.8 RNA Extraction and mRNA Expression of ZO-1, Occludin, Claudin-1 and Mucins in the Jejunal Mucosa**

Total RNA was extracted from snap-frozen jejunal mucosa sample with TRIzol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. The purity of the total RNA was analysed on a NanoDrop spectrophotometer ND1000 (Thermo Scientific, Wilmington, DE, USA). The suitable RNA samples (260:280 ratio ≥ 2.0) were reverse-transcribed using high-capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA) according to the manufacturer's instructions and then amplified by PCR. Primers used for amplification of target ZO-1, Occludin, Claudin-1, MUC1, MUC2 and housekeeping β-actin genes were shown in Table 3. Amplification was carried out in a total of 20 μL, which contained 10 μL Fast SYBR Green Master Mix (Applied Biosystems Inc.), 1.2 μL forward and reverse primer mix (5 μM) and 5 μL cDNA by an Option DNA Engine (Bio-Rad), with PCR programme as follows: 95℃ for 10 s, 40 cycles at 95℃ for 5 s, 60℃ for 25 s, followed by a final single extension step of 72℃ for 5 min. Melt curve analysis was conducted to validate the specificity of the primers. The expression ratio of the target genes relative to the housekeeping gene (β-actin) of each sample was calculated according to the $2^{-\Delta\Delta Ct}$ method (Metzler-Zebeli *et al.*, 2012). All determinations were performed in duplicate.

### 2.9 DNA Extraction and Real-time PCR Analysis of Bacteria in the Caecal Digesta

The genomic DNAs were extracted from 0.1 g of digesta samples using commercially available rapid bacterial genomic DNA isolation kit by the manufacturer (Sangon Bitech, China) for pigs. The copy numbers of total bacteria, *Bifidobacterium*, *Lactobacillus* and *Escherichia coli* in the caecal samples were quantified by real-time PCR on a Bio-Rad CFX96 real-time system (Bio-Rad, USA) using SYBR Green as the fluorescent dye. The primers for each bacterial species were presented in Table 4, and programs of PCR reaction for each bacterial species were as follows: 95℃ for 10 s, 40 cycles at 95℃ for 5 s, 50～60℃ for 25 s, followed by 95℃ for 10 s. A melting curve analysis was generated after each quantitative real-time PCR assay to check and verify the specificity and purity of all PCR products: 40 cycles at 95℃ for 39 s, 55℃ for 1 min, and 95℃ for 1 min. Bacterial counts were presented as $\log_{10}$ copy numbers per gram of dry digesta.

### 2.10 Statistical Analysis

All data from the experiment were analyzed as a complete randomized design using SAS procedure the General Linear Model procedure of the Statistical Analysis Systems Institute (Delete) (SAS Institute, USA). The statistical model used for animal performance, nutrient digestibility, gut morphology, cytokine concentration and microflora (Delete) data analysis included both the linear and quadratic effects of beta-glucan inclusion levels based on regression analysis. The significance between the treatment differences was identified by Duncan's multiple comparisons test in the General Linear Model. Results were expressed as treatment means with their pooled SEM. The probability value, which denotes significance, is $P<0.05$.

## 3 Results

### 3.1 Growth Performance and Nutrient Digestibility

During day 1-14 of the trail, linear increases in food intake were reported, as the level of beta-glucan increased in piglets (Table 5). The pigs fed 100 mg/kg beta-glucan had highly promoted ADFI ($P<0.05$). During day 15-28 of the experiment, dietary beta-glucan inclusion led to an increase in food intake and daily gain (quadratic; $P=0.04$), while a significant effect was observed for daily gain throughout the course of experiment (linear and quadratic; $P=0.03$, respectively). The diarrhea

incidence was alleviated while treated with 100 mg/kg *Agrobacterium sp.* ZX09 beta-glucan compared with control group (liner; $P$=0.04). The 50 mg/kg and 100 mg/kg beta-glucan inclusion levels increased the digestibility coefficients of DM, CP, ash and gross energy ($P$<0.05) (Table 6).

**3.2 Intestinal Morphology**

There was no effect on jejunal morphology with increasing dietary inclusion levels of beta-glucan ($P$>0.05) (Table 7) (Delete). Effects on jejunal morphology with increasing dietary inclusion levels of beta-glucan are shown in Table 7. Increasing the level of beta-glucan from 0 to 200 mg/kg significantly improved the morphology of ileum in piglets. Increased villus height, declined crypt depth and an accelerated villus height: crypt depth ratio were detected following dietary inclusion level of beta-glucan at 100 mg/kg in the diets.

**3.3 Gene Expression Related to Mucosal Barrier Function**

In the study, we studied the impact of beta-glucan on mRNA expressions of tight junction proteins from jejunal mucosa (Figure 1). There was no statistical significant difference in ZO-1 (Figure 1A) and Claudin-1 (Figure 1B) expressions while comparing between dietary groups (P>0.05). Dietary supplementation with beta-glucan at 200 mg/kg significantly down-regulated the expression level of Occludin in the jejunum ($P$<0.05) (Figure 1C). To our surprise, 100 mg/kg beta-glucan induced the up-regulation of MUC1 (Figure 2A) and MUC2 (Figure 2B) expressions in the jejunal mucosa of piglets, whereas, the transcripts were inhibited when pigs fed with 200 mg/kg beta-glucan compared with the control animals ($P$<0.05).

**3.4 Cytokine Concentrations in Jejunal Mucosa**

In the present study, linear increased concentration of IL-10 (linear; $P$=0.04) and reduced concentration of TNF-α (linear; $P$=0.05) in the jejunal mucosa of weaned piglets were observed with increasing treatment of beta-glucan (Table 8). An increase in IL-10 and the decline in IL-2 and TNF-α were detected in the jejunum of pigs supplemented with 100 mg/kg beta-glucan but not at the higher dietary inclusion level.

### 3.5 Selected Bacterial Populations in Cecum

This study indicated that beta-glucan diet changed the selected bacterial populations in the cecum of piglets (Table 9). Increasing the level of beta-glucan from 0 to 200 mg/kg had no effect on the total bacteria in the cecum of piglets. There was no linear or quadratic effect on *Bifidobacterium* with increasing levels of beta-glucan (linear, $P$=0.1; quadratic, $P$=0.44), while the values at 25 mg/kg and 50 mg/kg dosage were significantly higher than the control group ($P$<0.05). In addition, 100 mg/kg beta-glucan further improved the number of *Lactobacillus* and decreased the copy of *Escherichia coli* ($P$<0.05).

## 4 Discussion

A novel high purity; water-soluble extracellular beta-glucan of specific molecular weight was used in the present study. To the best of our knowledge, it is the first in literature (Delete) It is confirmed that *Agrobacterium* sp. ZX09 beta-glucan could improve growth performance in weaned piglets and the efficacy was dependent on their inclusion levels. A complex interaction exists between growth performance and disease susceptibility in pigs fed beta-glucan (Vetvicka *et al.*, 2014). Piglets get stress and subsequently had gut misfunction in the post weaning challenge for a period of one month. Based on the improved growth performance of piglets fed by beta-glucan in our previous research (data not shown) and other study (Lee *et al.*, 2017), feeding for 28 days after weaning was considered reliable time to assess the impact of beta-glucan. The reason for differences in means of ADFI, but with no linear or quadratic effects is based on two statistical analyses used in the experiment. Linear or quadratic effects were obtained from regression analysis, while the significance difference between the means of treatment was drawn from the general linear model. No necessary connections exist between such two kinds of statistical analyses. Many studies reported that beta-glucan supplementation enhanced growth performance in pigs (Sweeney *et al.*, 2012; Lee *et al.*, 2017). But the current results are inconsistent with no effects on non-immunochallenged piglets (Hester *et al.*, 2012). An alternative explanation for this discrepancy involves in the differences of optimum concentration, purity, molecular weight, conformation, chemical modification and solubility of

beta-glucan in the diet formulation. Current report strongly demonstrates that optimal dosage of beta-glucan derived from *Agrobacterium* sp. ZX09 was 100 mg per kg of diet for weaned piglets, while the optimal dose of other tested beta-glucans were variant and their sources were mainly obtained from cell wall of *Saccha-romycetes cerevisiae* (Shao *et al.*, 2016; Tian *et al.*, 2016) and subtaintially purified from oat (Suchecka *et al.*, 2016; 2017). Beta-glucan content obtained from *Agrobacterium* sp. ZX09 is higher (>90%) than that in *Saccha-romycetes cerevisiae* and oat (60-80%), thereby existing more sensitive biological activities. Besides, the subjects test with beta-glucan are not the same.

In the present study, pigs fed beta-glucan had higher digestibility of DM, CP, Ash and GE. And the promoted nutrient digestibility contributes to the improved growth performance following beta-glucan ingestion. The dietary inclusion of *Agrobacterium* sp. ZX09 beta-glucan at 100 mg/kg could benefit the gut morphology, mucosal barrier function and caecal microflora, resulting in the increased digestion of nutrients during the passage through the small intestine of pigs and the decreased diarrhea incidence. The up-regulations of MUC1 and MUC2 expression are consistent with the protective role of mucins in the formation of a gut barrier after mucosal stimulation by dietary fiber (Enns *et al.*, 1994). In other studies, mixed-linked beta-glucan, supplemented either in the form of cereals or as a concentrate, was readily fermented, reduced the intestinal number of enterobacteria and increased intestinal butyrate concentrations in growing pigs (Metzler-Zebeli *et al.,* 2010). Of particular interest, the 200 mg/kg beta-glucan inclusion level inhibited the stimulation of Occludin, MUC1 and MUC2 production, which is likely to link with the relatively high diarrhea occurrence when pigs were fed with 200 mg/kg beta-glucan, which was interpreted as being deleterious for piglets, though no reduction in growth performance was detected throughout the course of the study.

The morphology is important to maintain the normal intestinal function, especially digestive and absorptive properties (Suthongsa *et al.*, 2017). In the present study, beta-glucan modified gut morphology followed by changes in an increase of villus height and a decrease of crypt depth in the ileum of weaned pigs. It is hypothesized

537 that dietary supplementation with beta-glucan increased the digestion and absorption
538 of nutrients during the passage through the small intestine of pigs, which may clarify
539 the underlined mechanisms that *Agrobacterium sp.* ZX09 derived beta-glucan at
540 dosage of 100 mg/kg in the diets improved the growth performance and reduced the
541 diarrhea (Delete).

542 It is generally recognized that IL-1β, IL-2, IL-6 and TNF-α are regarded as
543 pro-inflammatory cytokines, which modulate immunity, regulate nutrient utilization,
544 and depress growth performance of postnatal animals. The cardinal anti-inflammatory
545 cytokine IL-10 can inhibit T-cell proliferation, development and function as well as
546 the secretion of Th1- and Th2-type cytokines, furthermore, can also suppress the
547 activity of the signal transduction of nuclear transcription factor kB, which is a major
548 transcription factor of pro-inflammatory cytokines (Zhang *et al.*, 2019). In this study,
549 IL-2, IL-10 and TNF-α as three representative cytokines, had significant differences
550 among groups. The activation of dectin-1 pathway by beta-gluan of high molecular
551 weight, coupled with the activation of TLRs, would explain the enhanced IL-2 and
552 TNF-α response. If feeding *Agrobacterium sp.* ZX09 beta-glucan decreases secretion
553 of pro-inflammatory cytokines and promotes secretion of anti-flammatory cytokines,
554 hence, less activation of the mucosal immune system would be achieved. These
555 results concur with research groups (Wang *et al.*, 2008; Li *et al*., 2006) who reported
556 that dietary beta-glucan from *Saccharomyces cerevisiae* partially suppressed increase
557 in TNF-α and IL-6 production and enhanced increase in IL-10 production following
558 beta-glucan digestion in an LPS challenge model. On the contrary, yeast product (YP,
559 a mixture of yeast culture, cell wall hydrolysates and yeast extacts) supplementation
560 in the diet of weaned piglets appears to increase the incidence of diarrhea and has
561 adverse effects on intestinal immune function (Yang *et al.*, 2016). Thus, we concluded
562 that depending on the strain, species and preparation process, pure beta-glucan from
563 *Agrobacterium sp.* ZX09 appears to be strong modulators of intestinal inflammation
564 in the piglets, while some beta-glucan fractions from yeasts are not.

565 Structure and metabolites of the gut microbial community are closely related to
566 metagenomic function and nutrient metabolism (Zhang *et al.*, 2017; Luo *et al.* 2018).

In this study, beta-glucan had the ability to increase *Lactobacillus* and *Bifidobacterium* counts, and to reduce *Escherichia coli* number. A similar effect was demonstrated on the beneficial microbiota in pigs fed mulberry (*Morus alba* L.) leaf polysaccharides (Zhao *et al.*, 2015). However, these data are in contrast to previous studies using 0.1 g/kg beta-glucan from *Saccharomyces cerevisae* or 300 mg/kg laminarin derived from *Laminaria digitata* (Murphy *et al.*, 2013; Zhou *et al.*, 2013), which all resulted in decreased number of *Escherichia coli* with no statistically significant effects on *Lactobacillus* and *Bifidobacterium* communities in their faecal microbiota. We hypothesize that the discrepancy may be attributed to the polymerization of beta-glucan. With a higher molecular weight, beta-glucan from *Agrobacterium* sp. ZX09 potentially remains intact for longer time period as a substrate for different bacteria phynotype, therefore exerting a prebiotic promotion of beneficial bacteria while suppression of deleterious bacteria to a greater extent than *Saccharomyces cerevisae and Laminaria digitata* examined in the cecum. Additionally, alteration in microflora may also influence mucin synthesis and secretion, as adherence of beneficial bacteria to mucosal epithelia stimulates up-regulation of MUC1 and MUC2 expression in vivo and in vitro (Capaldo *et al.*, 2017). Hence, beta-glucan supplementation at 100 mg/kg may have increased MUC1 and MUC2 expression indirectly by acting as a substrate for the resident microbiota, which, in turn, upregulated mucin production.

Production of mucins and tight junction proteins in mucosa has played important roles in formation and integrity of gut barrier. In the study, dietary supplementation with *Agrobacterium* sp. ZX09 beta-glucan at 100 mg/kg decreased the diarrhea occurrence, possibly by inducing gene up-regulation of MUC1 and MUC2 in the jejunal mucosa of weaned piglets. This up-regulation is consistent with the protective role of mucins in the formation of a gut barrier after mucosal stimulation by dietary fiber (Enns *et al.*, 1994). Additionally, alteration in microflora may also influence mucin synthesis and secretion, as adherence of beneficial bacteria to mucosal epithelia stimulates up-regulation of MUC1 and MUC2 expression in vivo and in vitro (Capaldo *et al.*, 2017). Hence, beta-glucan supplementation at 100 mg/kg may have

increased MUC1 and MUC2 expression indirectly by acting as a substrate for the resident microbiota, which, in turn, upregulated mucin production. Of particular interest, the 200 mg/kg beta-glucan inclusion level inhibited the stimulation of Occludin, MUC1 and MUC2 production, which is likely to link with the relatively high diarrhea occurrence when pigs were fed with 200 mg/kg beta-glucan, as compared to the control. The decrease of mucin gene expression in the 200 mg/kg beta-glucan treatment was interpreted as being deleterious for piglets, though no reduction in growth performance was detected throughout the course of the study (Delete).

## 5 **Conclusions**

Beta-glucan derived from *Agrobacterium* sp. ZX09, at dietary inclusion level of 100 mg/kg, improved growth performance in weaned piglets through modulation of the intestinal health. Supplementation with 100 mg/kg beta-glucan positively changed the morphology in the ileum, increased IL-10 concentration, also decreased IL-2 and TNF-α concentration in the jejunal mucosa of piglets. Moreover, the dietary inclusion of 100 mg/kg beta-glucan provoked the emergence of a more improved barrier function in the jejunum, meanwhile it had the most profound effect on microbial community in the cecum of piglets. However, higher inclusion level of beta-glucan at 200 mg/kg exerted the deleterious effects on gene expression of MUC1 and MUC2 in the jejunum of weaned piglets. Therefore, we concluded that 100 mg/kg beta-glucan extracted from *Agrobacterium* sp. ZX09 was considered the dietary optimum dose for the weaned piglets in the present study.

## **Conflict of interest**

There are not any conflicts of interest.

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627 farm.

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**Table 1. Structure and content of beta-glcuan preparations**

| Beta-glcuan source | Molecular Weight (kDa) | Content (%) | Structure |
|---|---|---|---|
| Yeast | 5-80 | ≤30% | β-1,3/1,6; β-1,3/1,4 |
| Oat | 5-250 | ≤85% | β-1,3/1,4 |
| Algal | <5 | ≤80% | β-1,3/1,6 |
| *Agrobacterium* sp. ZX09 | 200-3000 | ≥90% | β-1,3 |

**Table 2. The composition and nutrient content of basal diet**

| Ingredients | Composition, g/kg |
|---|---|
| Corn | 306.2 |
| Extruded corn | 277.0 |
| Fish meal | 47.0 |
| Whey powder | 80.0 |
| Soybean meal | 100.0 |
| Extruded full-fat soybean | 60.0 |
| Soy protein concentrate | 70.0 |
| Sucrose | 25.0 |
| L-Lysine·HCL(78%) | 4.3 |
| L-Threonine(98.5%) | 1.0 |
| DL-Methionine(99%) | 1.9 |
| L-Tryptophan | 0.2 |
| Choline chloride | 1.0 |
| Sodium chloride | 2.0 |
| Calcium carbonate | 8.8 |
| Dicalcium phosphate | 3.1 |
| Soybean oil | 10.0 |
| Vitamin premix[1] | 0.5 |
| Mineral premix[2] | 2.0 |
| Nutrient composition, g/kg | |
| Digestible energy[3], MJ/kg | 14.52 |
| Crude protein[4] | 193..9 |
| Total lysine[4] | 13.6 |
| Total methionine and cystine[3] | 7.4 |
| Total tryptophan[3] | 2.2 |
| Total threonine[3] | 8.0 |
| Calcium[3] | 8.2 |
| Phosphorus available[3] | 4.0 |

[1] Provided the following per kg of diet: Vitamin A, 8000 IU; Vitamin $D_3$, 1500 IU; Vitamin E, 25 IU; Vitamin $K_3$, 2.0 mg; Vitamin $B_1$, 2.0 mg; Vitamin $B_2$, 5.0 mg; Vitamin $B_6$, 4.0 mg; Vitamin $B_{12}$, 0.1 mg; Nicotonic, 25 mg; Pantothenic, 12 mg; Folic acid, 0.75 mg; Biotin, 0.2 mg.

[2] Provided the following per kg of diet: Fe ($FeSO_4.7H_2O$),100 mg; Cu ($CuSO_4.5H_2O$), 6 mg; Zn ($ZnSO_4.7H_2O$), 100 mg; Mn ($MnSO_4.H_2O$), 4 mg; Se ($Na_2SeO_3.5H_2O$), 0.35 mg; I (KI), 0.14 mg.

[3] Calculated values: nutrient level in each ingredient × ingredient content in the diet.

[4] Measured values.

**Table 3. Primers used for quantitative RT-PCR of mucosal barrier function**

| Gene | Primers and sequence (5' to 3') | Accession number | Product length |
|---|---|---|---|
| ZO-1 | F: CAGCCCCCGTACATGGAGA<br>R: GCGCAGACGGTGTTCATAGTT | XM_005659811 | 114bp |
| Occludin | F: CTACTCGCTCAACGGGAAAG<br>R: ACGCCTCCAAGTTACCZCTG | NM_001163647.2 | 158bp |
| Claudin-1 | F: TCTTAGTTGCCACAGCATGG<br>R: CCAGTGAAGAGAGCCTGACC | NM001244539 | 106bp |
| MUC1 | F: GTGCCGCTGCCCACAACCTG | XM_001926883.4 | 141bp |

| | | | |
|---|---|---|---|
| | R: AGCCGGGTACCCCAGACCCA | | |
| MUC2 | F:GGTCATGCTGGAGCTGGACAGT<br>R: TGCCTCCTCGGGGTCGTCAC | XM_003122394.1 | 181bp |
| β-actin | F: TCTGGCACCACACCTTCT<br>R: TGATCTGGGTCATCTTCTCAC | DQ178122 | 114bp |

788

789 **Table 4. Primers for real-time PCR of bacteria**

| Items | Primers and sequence (5'-3') | Product length |
|---|---|---|
| *Total bacteria* | F: ACTCCTACGGGAGGCAGCAG<br>R: ATTACCGCGGCTGCTGG | 200 bp |
| *Escherichia coli* | F: CATGCCGCGTGTATGAAGAA<br>R:CGGGTAACGTCAATGAGCAAA | 96 bp |
| *Bifidobacterium* | F: CGCGTCCGGTGTGAAAG<br>R: CTTCCCGATATCTACACATTCCA | 121 bp |
| *Lactobacillus* | F: GAGGCAGCAGTAGGGAATCTTC<br>R: CAACAGTTACTCTGACACCCGTTCTTC | 126 bp |

790

791

792

793 **Table 5. Effects of *Agrobacterium sp.* ZX09 beta-glucan supplementation on**

794 **growth performance of weaned piglets**

| IItem | Beta-glucan inclusion level (mg/kg) | | | | | Significance | | |
|---|---|---|---|---|---|---|---|---|
| | 0 | 25 | 50 | 100 | 200 | SEM | Linear | Quadratic |
| 1-14d | | | | | | | | |
| ADFI (g)[1] | 364.65[b] | 370.40[b] | 377.70[ab] | 416.58[a] | 348.95[b] | 6.44 | 0.05 | 0.42 |
| ADG (g)[2] | 225.60 | 227.18 | 256.83 | 259.21 | 220.83 | 8.77 | 0.49 | 0.22 |
| F/G[3] | 1.61 | 1.62 | 1.48 | 1.60 | 1.59 | 0.06 | 0.56 | 0.29 |
| 15-28d | | | | | | | | |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| ADFI (g) | 615.40$^{ab}$ | 617.93$^{ab}$ | 664.03$^{a}$ | 660.22$^{ab}$ | 609.41$^{b}$ | 8.69 | 0.11 | 0.04 |
| ADG (g) | 336.39 | 348.82 | 368.54 | 366.50 | 336.23 | 5.38 | 0.14 | 0.04 |
| F/G | 1.83 | 1.78 | 1.80 | 1.81 | 1.81 | 0.02 | 0.90 | 0.99 |
| 1-28d | | | | | | | | |
| ADFI (g) | 480.47$^{ab}$ | 494.16$^{ab}$ | 518.89$^{ab}$ | 529.66$^{a}$ | 485.16$^{b}$ | 7.20 | 0.16 | 0.12 |
| ADG (g) | 280.99$^{b}$ | 288.00$^{b}$ | 310.20$^{ab}$ | 325.12$^{a}$ | 278.53$^{b}$ | 5.49 | 0.03 | 0.03 |
| F/G | 1.71 | 1.72 | 1.67 | 1.63 | 1.75 | 0.02 | 0.38 | 0.24 |
| Diarrhea incidence | 6.83$^{ab}$ | 4.17$^{b}$ | 5.00$^{b}$ | 2.67$^{c}$ | 9.83$^{a}$ | 0.72 | 0.04 | 0.08 |

[1]ADFI: Average daily feed intake

[2]ADG: Average daily gain

[3]F/G: Feed intake/daily gain

[a,b,c] Mean values within a row with unlike superscript letters were significantly different (P<0.05).

**Table 6. Effects of *Agrobacterium sp.* ZX09 beta-glucan supplementation on nutrient digestibility**

| Item | Beta-glucan inclusion level (mg/kg) | | | | | SEM | Significance | |
|---|---|---|---|---|---|---|---|---|
| | 0 | 25 | 50 | 100 | 200 | | Linear | Quadratic |
| GE (%)[1] | 79.78$^{bc}$ | 78.72$^{c}$ | 83.65$^{a}$ | 82.45$^{a}$ | 82.16$^{ab}$ | 0.50 | <0.01 | 0.03 |
| Ash (%) | 37.74$^{b}$ | 38.21$^{b}$ | 49.12$^{a}$ | 45.83$^{a}$ | 45.38$^{a}$ | 1.21 | <0.01 | <0.01 |
| DM (%)[2] | 80.15$^{bc}$ | 79.57$^{c}$ | 83.87$^{a}$ | 82.67$^{a}$ | 82.24$^{ab}$ | 0.44 | <0.01 | 0.03 |
| CP (%)[3] | 72.22$^{bc}$ | 70.20$^{c}$ | 75.94$^{a}$ | 76.52$^{a}$ | 75.45$^{ab}$ | 0.70 | <0.01 | 0.02 |

[1]GE: Gross energy

[2]DM: Dry matter

[3]CP: Crude protein

[a,b,c] Mean values within a row with unlike superscript letters were significantly different (P<0.05).

**Table 7. Effects of *Agrobacterium sp.* ZX09 beta-glucan supplementation on intestinal morphology of weaned piglets**

| Item | Beta-glucan inclusion level (mg/kg) | | | | | | Significance | |
|---|---|---|---|---|---|---|---|---|
| | 0 | 25 | 50 | 100 | 200 | SEM | Linear | Quadratic |
| Jejunum | | | | | | | | |
| Villus height (μm) | 406.62 | 394.71 | 455.98 | 466.91 | 408.72 | 17.83 | 0.45 | 0.40 |
| Crypt depth (μm) | 201.29 | 215.50 | 200.22 | 203.11 | 188.18 | 8.25 | 0.90 | 0.55 |
| VH:CD[1] | 2.09 | 1.84 | 2.31 | 2.20 | 2.18 | 0.11 | 0.62 | 0.86 |
| Ileum | | | | | | | | |
| Villus height (μm) | 409.48[b] | 400.23[b] | 426.13[ab] | 502.89[a] | 447.93[ab] | 14.25 | 0.07 | 0.09 |
| Crypt depth (μm) | 258.65[a] | 188.68[b] | 211.29[b] | 184.02[b] | 203.20[b] | 9.02 | 0.03 | 0.05 |
| VH:CD | 1.62[c] | 2.12[bc] | 2.02[bc] | 2.75[a] | 2.22[b] | 0.12 | 0.09 | <0.01 |

[1]VH: CD: Ratio of villus height to crypt depth

[a,b,c] Mean values within a row with unlike superscript letters were significantly different (P<0.05).

**Table 8. Effects of *Agrobacterium sp.* ZX09 beta-glucan supplementation on cytokine concentrations in the jejunum of weaned piglets**

| Item | Beta-glucan inclusion level (mg/kg) | | | | | | Significance | |
|---|---|---|---|---|---|---|---|---|
| | 0 | 25 | 50 | 100 | 200 | SEM | Linear | Quadratic |
| IL-1 β (ng/ mg protein) | 116.72 | 109.25 | 112.67 | 110.93 | 118.49 | 2.12 | 0.19 | 0.56 |
| IL-2 (pg/mg protein) | 79.13[a] | 79.71[a] | 78.98a | 76.81[b] | 77.09ab | 2.04 | 0.07 | 0.21 |
| IL-6 (pg/ mg protein) | 540.33 | 556.39 | 545.98 | 542.19 | 545.04 | 1.96 | 0.13 | 0.98 |
| IL-10 (ng/ mg protein) | 18.66[a] | 20.14[a] | 22.82[ab] | 25.17[b] | 18.58[a] | 1.25 | 0.04 | 0.15 |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| TNF-α (pg/ mg protein) | 150[a] | 140[a] | 110[b] | 110[b] | 130[ab] | 0.03 | 0.05 | 0.36 |

822 [a,b] Mean values within a row with unlike superscript letters were significantly different (P<0.05).

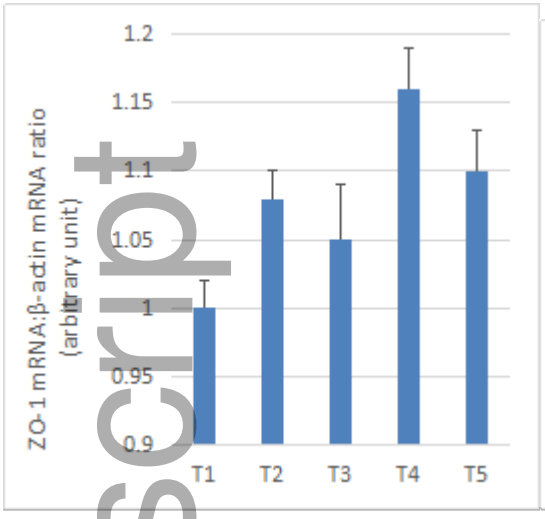
823

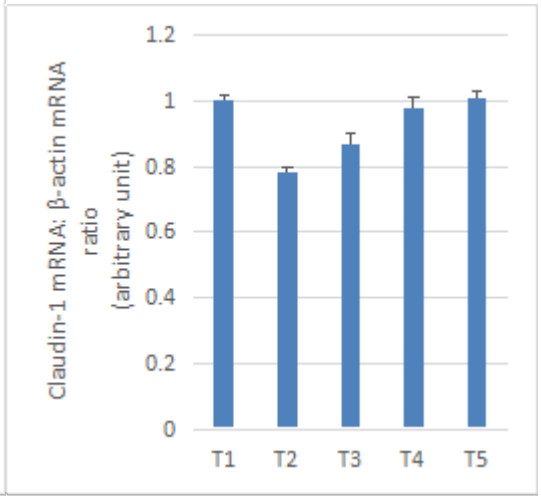
824 **Table 9. Effects of *Agrobacterium sp.* ZX09 beta-glucan supplementation on cecal**

825 **microflora of weaned piglets (Unit: lg copies/gram of dry digests)**

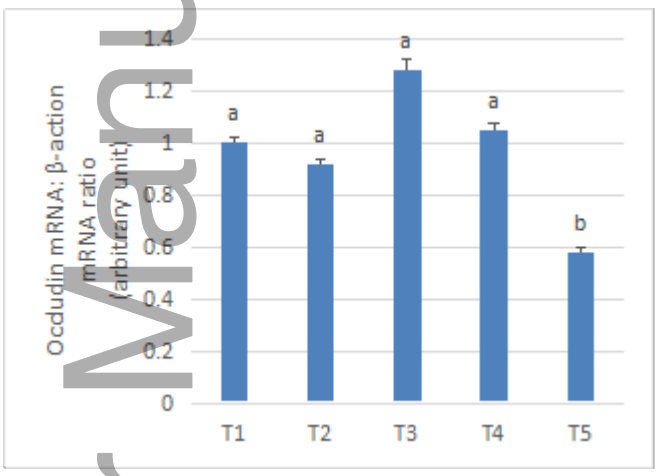
| Item | Beta-glucan inclusion level (mg/kg) | | | | | | Significance | |
|---|---|---|---|---|---|---|---|---|
| | 0 | 25 | 50 | 100 | 200 | SEM | Linear | Quadratic |
| Lactobacillus | 8.20[b] | 8.29[b] | 8.73[ab] | 9.03[a] | 8.77[ab] | 0.11 | 0.08 | 0.49 |
| Bifidobacterium | 8.54[b] | 10.26[a] | 10.41[a] | 9.57[ab] | 9.24[ab] | 0.24 | 0.10 | 0.44 |
| Escherichia coli | 8.80[a] | 7.73[ab] | 7.87[ab] | 7.68[b] | 7.37[b] | 0.24 | 0.05 | 0.41 |
| Total bacteria | 10.51 | 11.49 | 11.58 | 11.46 | 11.53 | 0.21 | 0.50 | 0.209 |

826 [a,b] Mean values within a row with unlike superscript letters were significantly different (P<0.05).



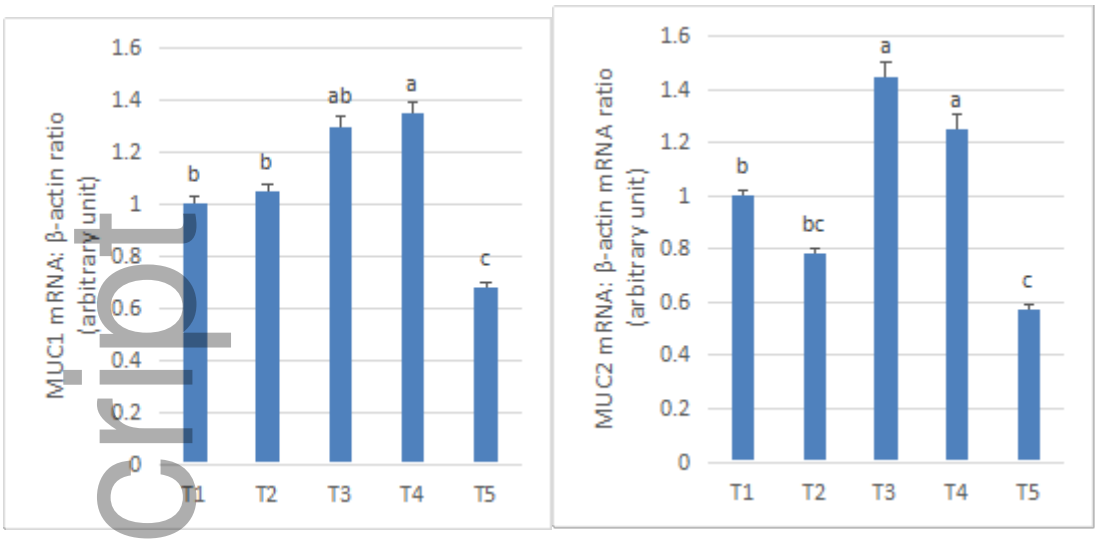




(A) (B)




(c)

Fig.1 mRNA expression level of tight junction proteins in the jejunal mucosa of piglets fed diets with different inclusion levels of beta-glucan. Data are shown as means±SEM. Fig. 1A: ZO-1 mRNA expression level. Fig. 1B: Claudin-1 mRNA expression level. Fig. 1C: Occludin mRNA expression level. Difference letters indicate statistically significant differences between groups ($P$<0.05). T1-control group fed with basal diet; T2-experimental group fed with 25 mg/kg beta-glucan; T3-experimental group fed with 50 mg/kg beta-glucan; T3-experimental group fed with 50 mg/kg beta-glucan; T4-experimental group fed with 100 mg/kg beta-glucan; T5-experimental group fed with 200 mg/kg beta-glucan




(A) (B)

Fig.2 mRNA expression level of MUC1 and MUC2 in the jejunal mucosa of piglets fed diets with different molecular weight beta-glucan. Data are shown as means±SEM. Fig. 2A: MUC1 mRNA expression level. Fig. 2B: MUC2 mRNA expression level. Difference letters indicate statistically significant differences between groups ($P$<0.05). T1-control group fed with basal diet; T2-experimental group fed with 25 mg/kg beta-glucan; T3-experimental group fed with 50 mg/kg beta-glucan; T4-experimental group fed with 100 mg/kg beta-glucan; T5-experimental group fed with 200 mg/kg beta-glucan