Original Article

Effect of polysaccharides of cassiae seeds on the intestinal microflora of piglets

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The objective of this study was to examine if polysaccharides from Cassiae Seeds (PCS) can be used as prebiotics to improve the intestinal microflora of piglets with an *in vitro* and an *in vivo* trial. The *in vitro* trial was conducted to study the dose-response effect of PCS on the growth of *E. coli 09 and Lactobacillus* with traditional plate count method. The gradient culture mediums, containing 3.2, 1.6, 0.8, 0.4, 0.2, 0.1, 0.05, 0.025 and 0% PCS, were inoculated with *E. coli*09, *Lactobacillus* and cecum content, respectively. PCS had no influence on the growth of *E. coli*$_{O9}$ from rejuvenation fluid, but inhibited the growth of *E. coli*$_{O9}$ from cecum content when the concentration of PCS was higher than 0.1%. *Lactobacillus* counts were significantly increased with 0.1% PCS or higher ($p < 0.05$); and the largest increase was found with 0.8% PCS. With the inoculum of cecum content in the medium, *Lactobacillus* counts increased when the concentration of PCS was 0.4% and 0.8%, whilst *E. coli* 09 counts decreased. The *in vivo* trial was carried out to investigate the effect of PCS on the growth of *E. coli 09 and Lactobacillus* in piglets. Thirty six barrows (average initial BW = 6.5 kg) were randomly divided into 3 groups with 6 each, fed diets supplemented without or with 0.4% or 0.8% PCS. After 14 days, 3 piglets were slaughtered from each group; digesta samples were collected from the ileum, cecum and colon for detection of *E.coli 09* and *Lactobacillus* with plate count method. Samples of the tissue and content of the cecum were taken for detection of caecal microflora profiles with Denaturing Gradient Gel Electrophoresis (DGGE) technique. The dietary inclusion of PCS increased *Lactobacillus* counts, but reduced *E. coli* 09 counts in digesta of ileum, cecum and colon of piglets. The dietary inclusion of 0.8% PCS significantly increased the number of electrophoresis brands of caecal bacterial microflora in mucosa and content of the cecum ($p< 0.05$). These results confirmed the dynamic change in the intestinal microfloa profile with the dietary inclusion of PCS in piglets. Thus, PCS can be used as prebiotics to improve the intestinal microflora.

Key Words: cassiae, polysaccharides, piglet, intestinal microflora, DGGE

Introduction

The intestinal tract is a complex ecosystem, which is inhabited by a dynamic microflora. They are interdependent and competitive with each other. [1-2] Some of them are beneficial, whilst others are harmful to intestinal health. It is generally accepted that the species of microorganisms and their ratio have a major impact on animal health to some extent. Microorganisms consume nutrients, and compete with each other for the limited source of nutrients in the intestinal tract. Studies showed the dietary composition and nutrient concentration had significant influence on the number of microorganisms in the intestinal tract.[3-5] Non-starch polysaccharides (NSP) can not be digested by human being and most of monogastric animals, but can be utilized as nutrients by beneficial bacteria (e.g., Lactobacillus and Bifidobacterium). These polysaccharides, being possible prebiotics, could enhance the proliferation of beneficial bacteria and produce short chain organic acids, which help inhibit the growth of harmful bacteria by reducing the intestine pH and keeping the intestine healthy. [6-8]

There are some limitations for traditional method for analyzing the intestinal microflora considering its inaccuracy and complicated process. However, molecular biology technique improves the accuracy of isolation and identification of bacteria.[9-10] Recently, Denaturing Gradient Gel Electrophoresis (DGGE), based on the technique of 16SrRNA, is well recognized to help analyze the complicated microflora without culturing microorganisms. The objective of this study is to examine whether polysaccharides from Cassiae Seeds (PCS) can be used as prebiotics to improve intestinal microflora. An *in vitro* trial and an *in vivo* trial were carried out to investigate the effect of PCS on the growth of *E. coli 09 and Lactobacillus* with help of the traditional plate cultivation and PCR-DGG technique.

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Materials and methods

Preparation of PCS

PCS was prepared in the Sino-Germany Center of Food Science and Engineering of Nanchang University, obtained from Semen Cassiae seeds by isolation and extraction technique with boiling water, followed by precipitation and dialysis technique with ethanol (80%, v/v). The PCS is hazel powder of 86.5% polysaccharides, whose molecular weights range from 160,000 to 210,000 Da. These polysaccharides consist of D-mannose and D-galactose with molar ratio of 6:1, connected as follows: [-Man (β1-4)-Man-(β1-4) Man (β1-4)-Man (β1-4)-Man (β1-4)-Man (β1-6 Gal)-]n[11]

The effect of PCS on the growth of E. coli 09 and Lactobacillus in vitro

3.2% PCS was diluted to different gradients (3.2, 1.6, 0.8, 0.4, 0.2, 0.1, 0.05 and 0.025%) with TSB culture (Shanghai Medical Reagent Co Ltd.), which was also used as the blank. 100 mL of liquid culture medium for each gradient was transferred to the 150 mL conical flask, sterilized at 121℃ for 15 min, then cooled for use. The culture medium was inoculated with *E. coli* 09, *Lactobacillus* and cecum content, respectively. Three replicates of experiments were conducted for each combination of culture medium and inoculation. 100 μL of Serotype *E. coli* 09 rejuvenation fluid (The Institute of Microorganism, Chinese Academy of sciences), containing approximately 10 organisms, was added to the culture medium for *E. coli 09* inoculation; 2 mL of *Lactobacillus* rejuvenation fluid (The Institute of Microorganism, Chinese Academy of sciences), containing 200 organisms, was added to the culture medium for *Lactobacillus* inoculation. Cecum content was detected with 2×10^7 *Lactobacillus* and 6×10^6 *E. coli* 09 per gram. It was diluted with sterile water until the dilution contains 6 to10 *E. coli* 09 and 200 *Lactobacillus* per 100 μL in order to inoculate the similar number of organisms from cecum content as from the rejuvenation fluid in the culture medium. 100μL of the dilution was added to the culture medium for *E. coli 09* and *Lactobacillus* inoculation. The culture medium was incubated at 37℃ for 24 h in an oscillator incubator set at 1200 rpm.

After incubation, the enumeration of microorganisms in each bottle was conducted with the Plate Count Method. The *E. coli* 09 was detected in MacConkay culture (Shanghai Medical Reagent Co Ltd.), and the *Lactobacillus* was detected in the improved tomato juice agar culture (Shanghai Medical Reagent Co Ltd.).

The effect of PCS on the growth of E. coli 09 and Lactobacillus in piglets in vivo

Eighteen barrows (Duroc × Landrace × Yorkshine), with an average initial body weight of 6.5 ± 0.52kg, were individually housed. The animals were randomly divided into three groups of 6 barrows per group and fed diets supplemented without or with 0.4 or 0.8% PCS, named as the control, low PCS and high PCS treatment, respectively. Water was freely available.

For each group, 3 piglets were slaughtered on day 14 of the trial. Ileum, cecum and colon were freshly obtained. Approximately 50 mg of intestinal content of each segment was collected in the 1.5 mL sterilized centrifuge tube, diluted 10 times with sterilized water, and mixed thoroughly. Then the liquid mixture was further diluted to 10^{-6}. 100 μL of 10^{-4}, 10^{-5} and 10^{-6} dilution were added to EMB and LBS, coated uniformly, then incubated at 37℃ for 24h and 72h, respectively. Three replicates of experiments were conducted for each dilution. After incubation, the colony count was determined.

DGGE of mucosa and content of the cecum

Cecum content was pressed into a sterilized coffee pot containing sterilized physiological saline of 4℃ and mixed thoroughly by twitching the stopcock for a few minutes. The liquid mixture was transferred into the sterilized centrifuge tube. The above procedure was repeated and another portion of liquid mixture was obtained and added to the same centrifuge tube. The combined liquid mixture was centrifuged at 4000 rpm for 10 min; the supernatant was transferred at another sterilized centrifuge tube, then centrifuged at 1300 rpm for 15 min. Removing the supernatant, 50 mg of the precipitate, the microorganism talus of cecum content, was weighed into 2mL centrifuge tube. Total DNA of the microorganism talus of cecum content was extracted with QIAamp®DNA Stool Mini kit. The same procedure was followed to obtain the microorganism talus and total DNA of cecum tissue.

Total DNA extracted from samples was used as templates to amplify fragments including 339-GC-f and AG-3'-539-r with help of primer, which is specific for V3 region of 6SrRNA of most bacteria, with Eppendorf Gene Amplifier.

Specific primer design

GC hairpin is rich in GC bases, which pair up with each other, and usually stable and difficult to be split. Attaching GC hairpin to one end of DNA prevents DNA from splitting into two single strands. To investigate the diversity of intestinal microflora with PCR-DGGE technique, the positive direction primer 5' was linked to GC hairpin. The amplified PCR complex was difficult to split in the running gel containing denaturants, whilst it was easy to split in DGGE. PCR complex, containing no GC hairpin, could split into two single strands at some gradient of running gel.

Single-stranded DNA migrates from negative to positive potential in DGGE, depending on the size of DNA instead of the base sequence of DNA. All of PCR complexes with the same length could split into single-stranded DNA of the same length. So the PCR complexes with the same length have similar electrophoresis behavior and can not be completely separated in DGGE.

Fingerprint of 200bp from 339-539

40 bp GC hairpin was added to 5'. Primer sequence was as follows.

339-GC-f: 5'-
CGCCCGGGGCGCGCCCCGGGCGGGGCGGGGGCA
CGGGGGG-ACTCCTACGGGAGGCAGC
AG-3' 539-r: 5'-GTATTACCGCGGCTGCTGGCA-3'

PCR reaction system as follows (total system: 25μL):

10×PCR buffer:	2.5μL
10mM dNTPs	0.5μL

primer 1 (forward,10 pmol/μL): 1.25μL
primer 2 (reverse,10 pmol/μL) 1.25μL
Dimethylsulfoxide (DMSO; 4%) (V/V): 1.0μL
Taq DNA polymerase (5 units/μL): 0.25μL
DNA (×100 diluted, 5μL in 495μL water): 0.5μL
Sterile deionized water (use for PCR only): 17.75μL

Procedure of PCR:

PCR for DNA			
Pre-heat	94℃	4min	
Denature	94℃	30sec	29 cycles
Anneal	56℃	30sec	
Extension	72℃	2min	
Final extension	72℃	10min	
Hold	4℃		

As indicated in Table 1, two denaturized gel solutions were prepared with low and high concentration, and poured into a mold to form the linear gradient gel with gradient gel tools. The gel solidified after standing for 1h. 5μL sample was spotted in gel; and electrophoresis was conducted at 100V for 16-18h, allowing genes of different bacteria to be separated from each other [12]. After separation was completed, the fractions of DNA fragments of different length were visualized using an Ag^+ dye specific for DNA. DGGE profiles were analyzed for similarities between the samples by a specialized gel software (Molecular Analyst version 2.15, Bio-Rad, California, USA).

Statistical analysis

All data were represented as means ± standard deviation (SD). A one-way analysis of variance was used to analyze all data. Differences were regarded significant at $p \leq 0.05$.

Results

The influence of PCS on the growth of E. coli 09 and Lactobacillus in vitro

The dose-response effect of PCS on the growth of *E. coli 09* and *Lactobacillus* was presented in Table 2. There were no significant differences ($p>0.05$) in counts of *E. coli09* among treatments with different concentrations of PCS in the single *E. coli 09* culture. Compared with the control, there was a significant increase ($p<0.05$) at counts of *Lactobacillus* in the single *Lactobacillus* culture when the concentration of PCS was higher than 0.1%; and the largest increase was found when the concentraion of PCS was 0.8%. There was no difference ($p>0.05$) in counts of *Lactobacillus* between treatemnts with 0.05% and 0.025% PCS. With the inoculum of cecum content in the culture, compared with the control, Lactobacillus counts increased, whilst E. coli 09 counts decreased when the concentration of PCS was 0.4% and 0.8%. But it is noted that PCS inhibited the growth of Lactobacillus when the concentration of PCS was up to 1.6% and 3.2%, very probably because PCS had great water holding capacity and reduced water activity in vitro.

The effect of dietary inclusion of PCS on the growth of E. coliO9 and Lactobacillus in vivo

Table 3 showed the effect of the inclusion of PCS on the growth of E. coli 09 and Lactobacillus in piglets. The counts of *Lactobacillus* increased in digesta of ileum, cecum and colon of piglets fed the diet with the high inclusion of PCS, while the counts of *E. coli* 09 decreased.

DGGE of mucosa and content of the cecum

Fig 1 and 2 demonstrated DGGE profiles of mucosa and content of the cecum, respectively. The effect of dietary inclusion of PCS on the number of electrophoresis bands of mucosa and content in the cecum was presented in Table 4. There were different electrophoresis brands, between control and PCS. More bands such as d and e were shown except the common as a, b and c in PCS. There were significant differences ($p<0.05$) in fingerprints of the

Table 1. Composition of the gradient gel

	Low concentration gel (35%)	High concentration gel (65%)
50X TAE buffer	300 μL	300μL
Acrylamide/biacrylamide	3.75 mL	3.75 mL
Deionized formamide	2.1 mL	3.9 mL
Urea	2.205 g	4.095 g
Dcode dye	0μL	150μL
10% (w/v) ammonium persulfate	150μL	150μL
Putrescine (TEMED)	15μL	15μL
Sterile water	To 15 mL	To 15 mL

Table 2. Effect of PCS on the growth of *Ecoli*$_{09}$ and *Lactobacillus in vitro*

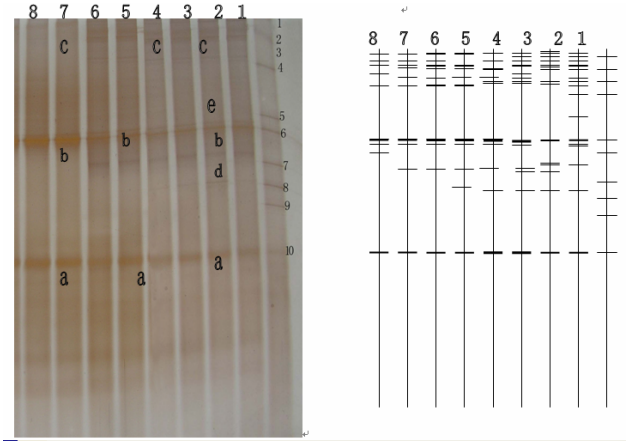
Microbe \ PSC gradient		1	2	3	4	5	6	7	8	9
		3.2%	1.6%	0.8%	0.4%	0.2%	0.1%	0.05%	0.025%	0
Ecoli09 log (cfu/mL)		3.33± 0.05^{a}	3.45± 0.04^{a}	3.44± 0.08^{a}	3.44± 0.10^{a}	3.46± 0.10^{a}	3.39± 0.07^{a}	3.43± 0.08^{a}	3.42± 0.10^{a}	3.45± 0.12^{a}
Lactobacillus log (cfu/mL)		4.90± 0.13^{ab}	5.17± 0.10^{a}	5.43± 0.08^{a}	5.26± 0.07^{a}	5.31± 0.05^{a}	5.21±0.07^{a}	4.81± 0.05^{ab}	4.79± 0.18^{b}	4.72± 0.11^{b}
Content piglets cecum	*Ecoli09* Log (cfu/mL)	2.91± 0.09^{ab}	2.84± 0.04^{ab}	2.71± 0.13^{a}	2.39± 0.12^{a}	2.77± 0.17^{ab}	2.83± 0.13^{ab}	3.07± 0.12^{b}	3.07± 0.09^{b}	3.12± 0.12^{b}
	Lactobacillus log (cfu/mL)	4.50± 0.03^{ab}	4.58± 0.05^{ab}	4.70± 0.07^{a}	4.74± 0.03^{a}	4.62± 0.08^{ab}	4.67± 0.06^{ab}	4.31± 0.05^{b}	4.37± 0.05^{b}	4.33± 0.11^{b}

Note: a,bMeans within same rows without a common superscript have different significantly ($p < 0.05$)

Table 3. Effect of PCS on intestinal microorganisms in vivo ((Lgcfu)/g)

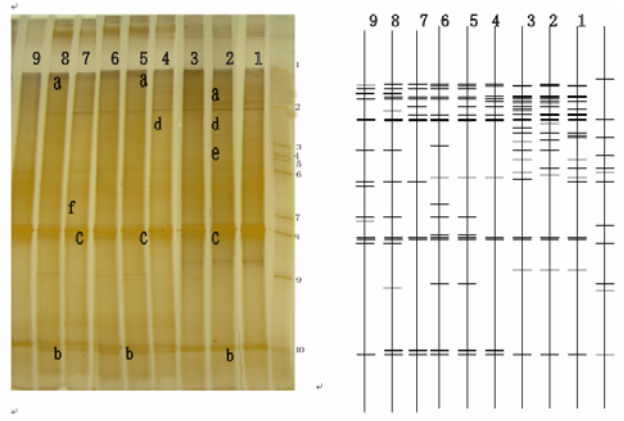
		Control group	0.4% PCS (low dose)	0.8% PCS (high dose)
Lactobacillus	Ileum	7.93±0.12^{b}	8.22±0.27^{ab}	8.68±0.21^{a}
	cecum	7.65±0.39^{b}	8.53±0.21^{a}	8.84±0.11^{a}
	colon	8.39±0.08^{b}	8.67±0.17^{ab}	8.96±0.14^{a}
Ecoli$_{09}$	Ileum	4.76±0.13^{a}	4.43±0.13^{a}	4.35±0.25^{a}
	cecum	4.93±0.26^{b}	4.51±0.11^{ab}	4.41±0.12^{a}
	colon	4.80±0.14^{b}	4.47±0.18^{ab}	3.98±0.09^{a}

Note: ^{a,b}Means within same rows without a common superscript have different significantly ($p < 0.05$)



Note: high dose group was band 1-3, low dose group was band 4-6, control group was band 7-8

Figure 1. DGGE profiles of microorganisms of cecum content



Note: high dose group was band 1-3, low dose group was band 4-6, control group was band 7-8

Figure 2. DGGE profiles of microorganisms of cecum mucosa

caecal bacterial microflora between piglets receiving different dietary inclusions of PCS. Compared with the control, the dietary inclusion of 0.8% PCS significantly increased the number of electrophoresis brands of the caecal bacterial microflora in mucosa and content of the cecum (p<0.05).

Table 4. The effect of PCS on the number of electrophoresis bands

	Control group	0.4% PCS (low dose)	0.8% PCS (high dose)
Cecum content	10.3±1.0^{a}	11.0±1.0^{ab}	14.0±2.0^{b}
Cecum mucosa	12.7±1.53^{a}	14.3±2.08^{ab}	16.3±1.53^{b}

Note: ^{a,b}Means within same rows without a common superscript have different significantly ($p < 0.05$)

Discussion

The influence of PCS on the growth of E. coli$_{O9}$ and Lactobacillus in vitro

There were no differences in counts of *coliform* bacteria among different concentrations of PCS in the culture inoculated with *E. coli$_{O9}$* only. This finding is in agreement with the results reported by Yan-Chengnong[13], showing no significant influence of PCS on *E. coli$_{O9}$ in vitro*.

Compared with the control, counts of *Lactobacillus* increased when the concentration of PCS was higher than 0.1%, and reached the highest value at 0.8%. However, there was no difference in counts of *Lactobacillus* between treatemnt with 0.025% and 0.05% PCS.

Compared with the control, *Lactobacillus* counts were increased and *E. coli$_{O9}$* counts were reduced in the culture inoculated with cecum content dilution when PCS concentration was 0.1% and higher, and more significantly when PCS concentration was 0.4% and 0.8%. It is obvious that PCS benefits the proliferation of *Lactobacillus* in the culture inoculated with *Lactobacillus* rejuvenation fluid or cecum content. PCS had no influence on the growth of *E.coli$_{O9}$* in the culture inoculated with rejuvenation fluid, but inhibited the growth of *E. coli$_{O9}$* inoculated with cecum content dilution when the concentration of PCS was higher than 0.1%.

In the *in vivo* experiment, *Lactobacillus* counts were increased in digesta of ileum, cecum and colon of piglets fed diets with low and high inclusion of PCS, whilst *E. coli$_{O9}$* counts were reduced, especially with the high

inclusion of 0.8% PCS. It is clear that PCS improved the growth of *Lactobacillus,* but inhibited the growth *E. coli*$_{O9}$ in piglets. Results of the *in vivo* trial suggest PCS could function as prebiotics in the intestinal microflora.[14]

The effect of PCS on the intestinal microflora

Genes of different bacteria can be separated with DNA-DGGE electrophoresis technique. DNA-DGGE profiles of microorganisms of mucosa and content of the cecum showed different numbers of electrophoresis bands can be obtained for each sample after DNA separation was completed. The bands, which can be observed in the same position of all the lanes (e.g., a, b and c), represented the typical strains of bacteria in the intestine. The bands, which showed different mobility and strength (e.g., e and f), indicated there were differences in the species of bacteria between different caecal digesta samples. Compared with control, the number of bands of the caecal bacterial microflora was numerically increased with dietary inclusion of 0.4% PCS (p>0.05), and significantly increased with dietary inclusion of 0.8% PCS (p<0.05). These results confirmed the dynamic change in the intestinal microfloa profile with the dietary inclusion of PCS in piglets. Thus, PCS can be used as prebiotics to improve the intestinal microflora.

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