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# Influence of differing carbohydrate sources on L-tryptophan metabolism by porcine fecal microbiota studied in vitro

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**Abstract**

Four types of carbohydrate sources, sugar beet pulp (SBP), rye grass hay (RYE), alfalfa hay (ALF) and fructooligosaccharides (FOS) were used to investigate their effects on the metabolism of L-tryptophan to skatole and indole by a mixed bacterial population from the large intestines of pigs. Microbial suspensions were anaerobically incubated at 38 °C, and indole compounds, pH value and volatile fatty acids were analyzed after 24 h fermentation. Effects of carbohydrate sources on the microbial diversity were analyzed using denaturing gradient gel electrophoresis (DGGE) of PCR amplicons of 16S rDNA, cloning and sequencing. Addition of SBP showed decreasing effect on skatole concentration and decreased the relative rate of skatole production significantly, whereas RYE and ALF treatments significantly increased skatole concentration and the relative rate of skatole production. The rate of tryptophan degradation was reduced by addition of ALF remarkably. For the FOS group, skatole concentration and relative rate of skatole production were both significantly reduced. Total and individual VFAs (acetate, propionate, butyrate and valerate) were significantly higher in the four carbohydrate-added groups than in the none-added. Additions of SBP, RYE, ALF and FOS in pig fecal slurries with L-tryptophan markedly decreased the culture medium pH values. The DGGE profiles showed differences between cultures with different carbohydrate substrates, though some bands were common to all cultures. Fermentation with SBP showed a higher bacterial diversity than with others, with three distinct bands appeared. Sequences of the corresponding 16S rRNA of two bands were related to *Eubacterium rectale* (94%) and *Clostridium* sp. (96%), respectively. Three bands were present only in SBP, RYE and ALF samples. Two of these three bands represented 93 and 98% similarity to *Lachnospira pectinoschiza* and *Clostridium disporicum* DSM 5521, respectively. These results suggested that the reduced concentration of skatole observed in the presence of SBP and FOS may be caused by decreased tryptophan degradation to indolic compounds and by shifted microbial metabolism of tryptophan toward indole production at the expense of skatole, resultant from the changed microbial ecosystem and pH value. The bacteria *C. disporicum* DSM 5521 may play a role in the production of skatole.


*Keywords:* Carbohydrate sources; L-tryptophan; In vitro fermentation; Pig fecal bacteria; Skatole

## 1. Introduction

The use of intact male pigs for pork production creates an economic opportunity because they generally have more lean meat and show better feed conversion ratios than castrates. However, it is limited because of the potential of

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boar taint, an off-flavor in meat from some intact male pigs (Babol and Squires, 1995). Moreover, boar taint is not only a unique characteristic of boars, but may be detected at low levels in castrates and gilts as well (Baltic et al., 1997). Skatole (3-methylindole) is one of the two compounds responsible for boar taint and is mainly produced from tryptophan in the gastrointestinal tract of pigs (Jensen et al., 1995a) and humans (Fordtran et al., 1964), and in the rumen of ruminants (Yokoyama and Carlson, 1979). It is widely accepted that anoxic metabolism of L-tryptophan can lead to the production of two alternative volatile lipophilic compounds, indole and skatole. Tryptophan can be degraded to indole directly, or converted to indole-3-acetic acid, and then metabolized to skatole. In pig body, skatole is produced in the large intestine by microbial degradation of L-tryptophan originating from dietary and endogenous protein (Jensen et al., 1995a), and then absorbed into the blood stream. Increased levels of skatole in the fat can give rise to boar taint. Many types of anaerobic bacteria are capable of producing indole from tryptophan, but in contrast, the skatole production is reported only for strains of few genera, *Lactobacillus* bacteria and *Clostridium* bacteria (Jensen et al., 1995b; Jensen and Jensen, 1998).

Skatole formation can be easily affected by dietary factors (Claus et al., 1994). The type and amount of carbohydrates entering the large intestine have a substantial effect on nitrogen metabolism and may therefore influence the synthesis of indoles (Hawe et al., 1992). Recently studies have shown that inclusions of sugar beet feed (Wood et al., 1993; Whittington et al., 2004), raw potato starch (Claus et al., 2003; Zamaratskaia et al., 2005), chicory (Hansen et al., 2006; Jensen and Hansen, 2006) or whole crop rice (Li et al., 2006) in the diets, decrease skatole contents. Jensen et al. (1995a) observed that an increased content of fermentable carbohydrate in the large intestine resulted in lowered bacterial degradation of both protein and tryptophan and reduced production of skatole. It was hypothesized that these effects of fibre might attribute to the condition that carbohydrate-fermenting bacteria inhibit the proteolytic bacteria. However, there were few studies on the details of related microbial production. Little is known about the effects of carbohydrates sources with different fermentability on skatole production in pigs, although a few studies have been carried out with rapid fermentable source of carbohydrate (Xu et al., 2002a).

Therefore, it was the aim of this study to evaluate the effects of different types of carbohydrates on the metabolism of L-tryptophan to skatole and possible microbial mechanism involved using an in vitro system with pig fecal bacteria. The substrates investigated were selected according to their fermentability: sugar beet pulp (SBP) belonged to the potentially easily fermentative carbohydrates, while rye grass hay (RYE) and alfalfa hay (ALF) were chosen as fermentable fibre as grass fibre and legume family respectively. Besides, fructooligosaccharides (FOS) was used as positive contrast because of its known lowering effect on skatole production in vitro (Xu et al., 2002a; Hansen et al., 2006).

## 2. Materials and methods

### 2.1. Substrates and chemical analyses

The feedstuffs (SBP, RYE and ALF) were obtained from the experimental farm of Zhejiang University, Huajiachi Campus, China. FOS was provided by Friend Living Creature Engineering Co., Ltd. (Henan, China) and the concentration of oligosaccharides was greater than 90% of total mixture. All the air-dried substrates were ground to pass a 1 mm sieve prior to chemical analyses and in vitro fermentation. Dry matter (DM), ash and Kjeldahl-N were determined as described by AOAC (1990). Crude protein (CP) was calculated as Kjeldahl-N × 6.25. Neutral detergent fibre (NDF) and acid detergent fibre (ADF) were determined by the method of Van Soest et al. (1991). Lignin was determined by solubilization of cellulose with sulphuric acid on the ADF residue. Hemicellulose was calculated as NDF–ADF. Chemical composition of the substrates is presented in Table 1.

### 2.2. In vitro fermentation

The in vitro technique was carried out according to the method described by Jensen et al. (1995b) and Xu et al. (2002a). Fresh feces were collected from pigs fed a standard corn–soybean-based diet without added antibiotics or copper and were suspensed in sterile anaerobic mineral salt medium to give a 10% (wt/vol) fecal slurry. The suspension was then filtered through sterile cheesecloth to remove crude particulate material. Ninety-nine mL of this suspension was mixed with 1 mL of L-tryptophan solution (5.1 mg

Table 1
Chemical composition[a] of the carbohydrate sources

| Substrate | Carbohydrate sources[b] | | | |
|---|---|---|---|---|
| | SBP | RYE | ALF | FOS |
| DM (%) | 92.4 | 89.7 | 93.4 | 96.1 |
| CP, % DM | 8.7 | 14.5 | 16.9 | 0.077 |
| NDF, % DM | 55.7 | 37.4 | 37.8 | – |
| ADF, % DM | 38.0 | 31.0 | 19.8 | – |
| Lingin, % DM | 3.0 | 1.0 | 6.6 | – |
| Hemicellulose, % DM | 17.7 | 6.4 | 18.0 | – |
| Ash, % DM | 7.74 | 10.2 | 9.6 | – |

[a] DM = Dry Matter; CP = Crude Protein; NDF = Neutral Detergent Fibre; ADF = Acid Detergent Fibre.
[b] SBP = sugar beet pulp; RYE = rye grass hay; ALF = alfalfa hay; FOS = fructooligosaccharides.

of tryptophan in 1 mL of $H_2O$) in the fermentation bottles, and 1 g of substrate (SBP, RYE, ALF and FOS) was then added. Fermentations of L-tryptophan without any substrates added were designated as Control. Samples were incubated in triplicate at 38 °C for 24 h. All procedures were completed under a constant flow of carbon dioxide ($CO_2$). At the end of fermentation, 1 mL of each culture media sample was taken for analysis of indolic compounds, volatile fatty acids (VFAs), and microbial populations.

Considering the difference in the contents of protein between the substrates, the determinations were also made for each treatment containing all components except for the L-tryptophan. The skatole concentrations in tryptophan-added groups are at least 2 times than that in tryptophan-absent groups for Control, SBP, RYE, ALF and FOS, respectively. Likewise, the indole concentrations in tryptophan-added groups are at least 3 times than that in tryptophan-absent groups for Control, SBP, RYE, ALF and FOS, respectively. Therefore, tryptophan was added in excess relative to substrate crude protein content. Indolic compounds were corrected by the blanks.

A separate gas production (GP) test was performed using the method developed by Theodorou et al. (1994) as modified by Mauricio et al. (1999). Cumulative gas volumes were estimated from a pressure transducer, interfaced with a computer allowing the accumulated head-space gas pressure values to be directly entered into the computer. These pressure measurements were then used to estimate the generated gas volumes (Mauricio et al., 1999).

### 2.3. Analytical methods

Skatole and indole were assayed by HPLC using fluorescence detection as described by Jensen et al. (1995b). Briefly, 1 mL of medium was added to 2 mL of HPLC grade methanol in a centrifuge. The mixture was vortex-mixed, placed at −20 °C for 30 min to accelerate precipitation of the particulate materials and then centrifuged at 2700 ×*g* for 10 min. One mL of the supernatant was transferred to an Eppendorf tube and centrifuged at 15,000 ×*g* for 30 min and 20 μL of the following supernatant was injected into the HPLC. The chromatographic conditions were described elsewhere (Li et al., 2008). The lower limit of sensitivity was 1.47 and 0.25 μmol/L for skatole and indole respectively.

The VFAs were analysed by a GC method as outlined by Hu et al. (2005). Analysis was performed with Shimadzu GC-2100, equipped with a Flame Ionization detector (FID) and 30 m × 0.25 mm × 0.25 μm column (HP-INNOWAX, 19091N-133). One mL of fermentation fluid was immediately mixed with 0.2 mL 25% metaphosphoric acid, and then centrifuged at 20,000 ×*g* for 20 min. The supernatant was injected with a syringe, and the temperature of the injector/detector and the column was 260 °C and 220 °C, respectively. Nitrogen was used as a carrier. As an indicator of the protein fermentation, branched chain proportion (BCP) was calculated. This is the ratio of the sum of isobutyrate and isovalerate to the total VFAs (Awati et al., 2005).

The pH of the fermentation medium was measured with a precise pH meter (Model PB-20, Sartorius).

### 2.4. Microbiological analysis

A molecular approach based on denaturing gradient gel electrophoresis (DGGE) analysis and gene sequencing was adopted to study the fecal microbial community after fermentation of the substrates. Total genomic DNA of the samples was isolated using bead-beating method and used as templates to amplify fragments of the 16S rRNA gene (Zoetendal et al., 1998). Agarose gel (1.2%) containing ethidium bromide was performed using 5 μL of the resultant DNA solution to estimate the amount and integrity of the DNA products. PCR products of the V6–V8 regions were amplified as previously described (Wang et al., 2007). The amplicons were separated using C.B.S DGGE system (C.B.S DGGE 2401, C.B.S-SCIENTIFIC COMPANY. INC, USA). Electrophoresis was performed in an 8% polyacrylamide gel of 37.5: 1 acrylamide–bisacrylamide using 38–48% denaturing gradient in 0.5 × TAE buffer. The electrophoresis was initiated by prerunning for 10 min at a voltage of 200 V, and subsequently run at a fixed voltage of 85 V for 15 h at 60 °C and stained with $AgNO_3$ after completion. DGGE analysis of all samples was repeated twice. The Bionumerics software version 4.6 (Applied Maths, Belgium) was used to analyze the DGGE gels.

In order to study phylogenetic diversity of the predominant bacteria, the 16 S rDNA from fermentation sample of SBP group was amplified, cloned and sequenced according to the procedures described by Wang et al. (2007). Finally, homology searches of the Genbank database were performed with BLAST Search (Altschul et al., 1990).

### 2.5. Statistical analysis

One-way analysis of variance was performed using the General Linear Model (GLM) Procedure of SAS (1996). The differences among means for treatments were tested using Duncan's new multiple range test (SAS, 1996).

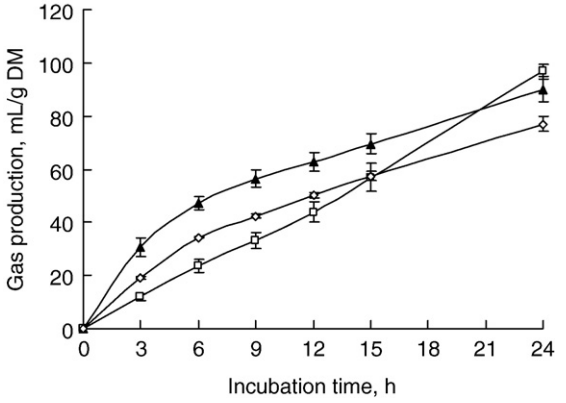


Fig. 1. In vitro cumulative gas production curves of sugar beet pulp (SBP, □), rye grass hay (RYE, ▲), and alfalfa hay (ALF, ◇), using porcine feces as inoculum. Data are means ± SEM.

## 3. Results

### 3.1. In vitro cumulative gas production

The cumulative GP curves were presented in Fig. 1 for SBP, RYE and ALF, using porcine feces as inoculum, respectively. The GP of RYE and ALF were higher than that of SBP at early incubations ($p<0.05$). However, the GP values of RYE and ALF were decreased after 24 h incubation, with the highest GP value in SBP ($p<0.05$).

### 3.2. Metabolism of L-tryptophan by pig fecal slurries

Table 2 shows the in vitro skatole and indole production in pig fecal slurries with L-tryptophan added with different carbohydrate sources. Compared with the Control, a decreased trend in skatole was observed for SBP group, although statistical differences was not significant, whereas RYE and ALF treatments significantly increased skatole concentration ($p<0.05$). Besides, addition of SBP significantly reduced the relative rate of skatole production, whereas RYE and ALF treatments significantly increased it ($p<0.05$). In contrast, the relative rate of indole production was significantly increased by SBP and decreased by RYE and ALF ($p<0.05$). Moreover, rate of tryptophan degradation was reduced by addition of ALF remarkably ($p<0.05$). Overall, skatole and indole concentrations showed opposite trend. For the FOS contrast, skatole concentration and relative rate of skatole production were both significantly reduced ($p<0.05$), and relative rate of indole production elevated ($p<0.05$).

Table 2
In vitro skatole and indole production in pig fecal slurries with L-tryptophan added with different carbohydrate sources ($n=3$)

| Items | Carbohydrate sources[1] | | | | | SEM |
|---|---|---|---|---|---|---|
| | Control | SBP | RYE | ALF | FOS | |
| Skatole concentration (μmol/L) | 33.8$^{c}$ | 29.5$^{c}$ | 81.1$^{b}$ | 94.4$^{a}$ | 18.9$^{d}$ | 2.535 |
| Indole concentration (μmol/L) | 174.1$^{b}$ | 203.4$^{a}$ | 156.0$^{c}$ | 78.0$^{d}$ | 179.9$^{b}$ | 3.330 |
| Rate of tryptophan degradation (%)[2] | 83.2$^{b}$ | 93.2$^{a}$ | 94.8$^{a}$ | 68.9$^{c}$ | 79.5$^{b}$ | 2.059 |
| Relative rate of skatole production (%)[3] | 16.3$^{c}$ | 12.7$^{d}$ | 34.2$^{b}$ | 54.9$^{a}$ | 9.5$^{e}$ | 0.889 |
| Relative rate of indole production (%)[4] | 83.8$^{c}$ | 87.4$^{b}$ | 65.8$^{d}$ | 45.2$^{e}$ | 90.5$^{a}$ | 0.889 |

[1]Control, SBP, RYE, ALF and FOS represent fermentation of L-tryptophan without and with sugar beet pulp, rye grass hay, alfalfa hay and fructooligosaccharides, respectively.
[2]Rate of tryptophan degradation = (skatole production + indole production) / added tryptophan.
[3]Relative rate of skatole production = skatole production / (skatole production + indole production).
[4]Relative rate of indole production = indole production / (skatole production + indole production).
[a–e]Values within the same row with different superscripts are significantly different ($p<0.05$).

Table 3
Gas production and fermentation parameters in the end-products of fermentation in pig fecal slurries with L-tryptophan added with different carbohydrate sources ($n=3$)

| Items | Carbohydrate sources[1] | | | | | SEM |
|---|---|---|---|---|---|---|
| | Control | SBP | RYE | ALF | FOS | |
| pH | 6.69$^{a}$ | 6.36$^{c}$ | 6.37$^{c}$ | 6.50$^{b}$ | 5.31$^{d}$ | 0.012 |
| VFA[2], mmol/L | | | | | | |
| Acetate | 13.66$^{d}$ | 34.54$^{c}$ | 36.09$^{bc}$ | 36.83$^{b}$ | 41.97$^{a}$ | 0.631 |
| Propionate | 3.60$^{d}$ | 10.27$^{c}$ | 14.44$^{b}$ | 10.86$^{c}$ | 29.27$^{a}$ | 0.335 |
| Butyrate | 0.79$^{e}$ | 1.24$^{d}$ | 1.62$^{b}$ | 1.43$^{c}$ | 3.02$^{a}$ | 0.046 |
| Isobutyrate | 1.42$^{c}$ | 1.50$^{c}$ | 1.73$^{b}$ | 2.69$^{a}$ | 0.96$^{d}$ | 0.051 |
| Valerate | 0.36$^{d}$ | 0.51$^{c}$ | 0.67$^{b}$ | 1.21$^{a}$ | 1.21$^{a}$ | 0.027 |
| Isovalerate | 0.81$^{b}$ | 0.72$^{c}$ | 0.85$^{b}$ | 1.20$^{a}$ | 0.43$^{d}$ | 0.018 |
| Total | 20.6$^{d}$ | 48.8$^{c}$ | 55.4$^{b}$ | 54.2$^{b}$ | 76.9$^{a}$ | 0.920 |
| BCP[3] | 9.010$^{a}$ | 3.789$^{c}$ | 3.881$^{c}$ | 5.979$^{b}$ | 1.504$^{d}$ | 0.0994 |

[1]Control, SBP, RYE, ALF and FOS represent fermentation of L-tryptophan without and with sugar beet pulp, rye grass hay, alfalfa hay and fructooligosaccharides, respectively.
[2]VFA = volatile fatty acid.
[3]BCP = branched chain proportion, representing the ratio of the sum of isobutyrate and isovalerate to the total VFAs.
[a–e]Values within the same row with different superscripts are significantly different ($p<0.05$).

### 3.3. Volatile fatty acids and culture medium pH

Production and proportions of VFAs were significantly different between substrates (Table 3). Total and individual VFAs (acetate, propionate, butyrate and valerate) were significantly higher in carbohydrate-added groups than in the Control ($p<0.05$), with the highest contents in FOS-added medium. The FOS group had the lowest isobutyrate and isovalerate. The BCP was significantly reduced for all the four treatments ($p<0.05$), with the lowest value in FOS treatment. For carbohydrate-added groups, BCP tends to decrease in which skatole concentration does so.

Additions of SBP, RYE, ALF and FOS in pig fecal slurries with L-tryptophan markedly decreased the culture medium pH values from 6.69 in Control to 6.36, 6.37, 6.50 and 5.31 ($p<0.05$), respectively, with the lowest pH value in FOS group.

### 3.4. PCR/DGGE analysis

Changes in the DGGE profile were clearly observed between fermentative samples (Fig. 2). Overall, some DGGE bands were common to all samples and there were

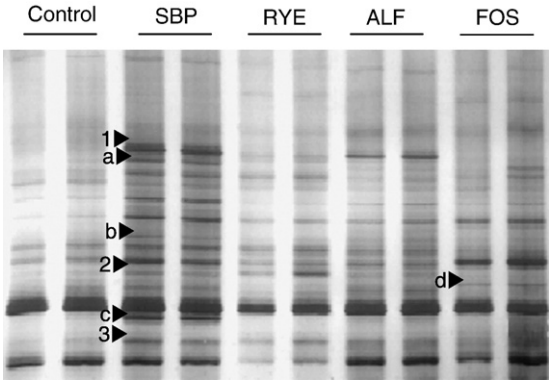


Fig. 2. DGGE of PCR products of V6–V8 regions of 16S rDNA of in vitro fermentation samples. Control, SBP, RYE, ALF and FOS represent fermentation of L-tryptophan without and with sugar beet pulp, rye grass hay, alfalfa hay and fructooligosaccharides, respectively; a, b and c indicate the distinct bands in SBP group; d indicated the distinct bands in FOS group; 1, 2 and 3 indicate the common bands in SBP, RYE and ALF groups.

more bands present in two replicates of carbohydrate-added groups than the Control. The substrate played a major role in determining the fermentative DGGE profiles. The greater number of bands appeared in SBP group, with three distinct bands enriched, as indicated with a, b and c. However, the bands indicated with 1, 2 and 3 were present only in the lanes of SBP, RYE and ALF, but not in FOS. Besides, one distinct band, as indicated with d, appeared only in FOS group.

### 3.5. Identification of cloned 16S rDNA in DGGE patterns

V6–V8 regions in the 16S rDNA from SBP group of cell lysates of 32 transformants were amplified. The mobility of these amplicons after DGGE was compared to those obtained from 16S rDNA of the same fermentation sample, as shown in Fig. 3. Twenty-one clones were assigned to one of the 11 dominant bands in the DGGE profiles, while 10 clones did not match with any of the detectable bands. Based on identical mobility within the gel, bands indicated with 1, 2 and 3 in Fig. 2 matched with clones K22 (accession no. EF640148), clone K19 (accession no. EF640146) and clone K5 (accession no. EF640141), respectively. The 16S rDNA sequences of the three clones showed 93, 97 and 98% similarity to their closest relatives, *Lachnospira pectinoschiza*, uncultured bacterium and *Clostridium disporicum* DSM 5521, respectively. The bands noted with a and c in Fig. 2, matched with clones K10 (accession no. EF640142) and K11 (accession no. EF640143) and were related to *Eubacterium rectale* (94%) and *Clostridium* sp. (96%).

## 4. Discussion

The present study indicated that addition of FOS to swine fecal slurries resulted in a significant reduction in skatole concentration after fermentation (Table 2), which was in agreement with Xu et al. (2002a). FOS has been shown to enhance the growth of *Bifidobacterium* and *Lactobacillus*, but inhibit *E. coli* and *Clostridium* in several studies (Roberfroid et al., 1998; Gebbink et al., 1999; Xu et al., 2002b). Xu et al. (2002a) observed that addition of FOS to the swine fecal slurries significantly reduced the skatole concentration and the rate of tryptophan degradation, probably because the shifted microbial metabolism of tryptophan to indole production at the expense of skatole, and at least a portion of the


| Clone | Closest relatives | Sequence Similarity (%) | Sequence Length (bp) |
|---|---|---|---|
| K22 | Lachnospira pectinoschiza | 93 | 1517 |
| K10 | Eubacterium rectale | 94 | 1467 |
| K1 | Butyrate-producing bacterium L1-93 | 96 | 1517 |
| K29 | Roseburia faecalis strain M88/1 | 98 | 1608 |
| K17 | Uncultured bacterium clone p-190-05 | 99 | 820 |
| K32 | Unidentified rumen bacterium 5C0d-11 | 92 | 1512 |
| K19 | Uncultured bacterium clone p-190-05 | 97 | 1120 |
| K20 | Clostridium disporicum DSM 5521 | 98 | 1495 |
| K11 | Clostridium sp. 14505 | 96 | 1506 |
| K5 | Clostridium disporicum DSM 5521 | 98 | 1495 |
| K14 | Clostridium butyricum | 94 | 1497 |

Fig. 3. Clones, with percentage of similarity to known sequences in GenBank and sequence length, were retrieved from the fermentation sample with sugar beet pulp.

inhibition of skatole production by FOS occurred at the phase of tryptophan conversion to indole-3-acetic acid. Our PCR/DGGE results confirmed this.

Addition of SBP showed decreasing effect on skatole concentration and relative rate of skatole production (Table 2). However, it was not the case for RYE and ALF treatments. A proposed mechanism is that the presence of fermentable carbohydrates provide a energy source for those intestinal bacteria, and lead to reduced protein catabolism (Konstantinov et al., 2003). Microbial community after fermentation is dependent on the substrate's fermentation characteristics. Potentially highly but slowly fermentable (Gebbink et al., 1999) SBP may provide a continuous presence of substrate for the fibre-degrading bacteria throughout the incubation period (Gebbink et al., 1999), whereas RYE and ALF used in our experiment had relative higher fermentation rate at early incubation, as indicated by higher GP at the first 15 h of fermentation than SBP (Fig. 3). Due to the higher rate of fermentation of RYE and ALF, there might be depletion in the availability of carbohydrate substrate with time, which made microbes to rely on protein as fermentable substrate. Increased protein fermentation led to higher skatole production. Fordtran et al. (1964) observed a delay in tryptophan catabolism in the human large intestine in the presence of fermentable carbohydrate. Moreover, skatole-synthesizing bacteria isolated from the rumen exhibited a reduced production when readily fermentable carbohydrate glucose was incorporated into the incubation medium (Yokoyama and Carlson, 1974). This may apply equally to skatole-producing bacteria in the large intestine of pigs.

Branched-chain fatty acids are mainly formed by the metabolism of branched-chain amino acids such as valine, leucine and *iso*-leucine. The BCP was lower with addition of fermentable carbohydrates (Table 3), suggesting that the group without carbohydrate might led to utilization of amino acid present in the medium as a source of energy by microbiota (Macfarlane et al., 1992). For the four carbohydrate-added groups, BCP tends to decrease in which skatole concentration does so, indicating carbohydrate fermentations of SBP, RYE, ALF and FOS could affect the protein fermentations differently and thus had varied influences on skatole production. RYE and ALF differing in soluble carbohydrate, cellulose and hemicellulose contents, will significantly influence fibre fermentation and further affect the protein fermentation. Higher skatole content obtained with wheat bran diet was found in a previous study, and the reason owed to a lack of significant carbohydrate fermentation to prevent protein fermentation because of the relatively high lignin content of the wheat bran (Van Oeckel et al., 1998). Addition of FOS resulted in the highest total VFA (Table 3). This result might partially be responsible for the lowest culture medium pH and further the lowest skatole concentration, because the proteolytic bacteria responsible for the conversion of tryptophan to skatole function optimally at neutral or alkaline pH (Jensen et al., 1995a). However, according to Jensen et al. (1995b), at this pH, the production of skatole is higher than indole, which is opposite to the present study. It is suggested that reduction in skatole production in the incubations with FOS is probably due to factors other than pH. The reduced concentration of skatole in the presence of SBP might also partially due to effects of fermentation on the acidity of the microbial environment.

In the study of Xu et al. (2002a), traditional culture technique was used to examine the microbial ecosystem after fermentation. The culture-dependent methods can only count the easily cultivable organism, which are only a small fraction of the bacterial species present in the gastrointestinal tract. These limitations have led to the development of rRNA-based approaches by which the presence and identification of the bacteria is based on the sequence diversity of the 16S rRNA gene (Amann et al., 1995; Amann and Ludwig, 2000). A combination of PCR and DNA fingerprinting techniques, such as DGGE, have been used successfully to describe the microbial diversity of a variety of ecosystems (Konstantinov et al., 2003; Wang et al., 2007), including in vitro samples (Zhu et al., 2003; Awati et al., 2005).

From the DGGE profiles (Fig. 2), it can be observed that the presence of fermentable carbohydrates, particularly SBP, together with tryptophan resulted in higher diversity of microbial community compared with tryptophan only. Similar results were found in vitro by Awati et al. (2005), and in vivo by Konstantinov et al. (2003). In both studies, addition of fermentable carbohydrates in the diet led to high bacterial diversity and rapid stabilization of the fecal microbial community. As indicated in Fig. 2, three bands marked with 1, 2 and 3 existed in SBP, RYE and ALF samples but not in FOS sample. As we discussed above, carbohydrate-fermenting bacteria may compete with proteolytic bacteria in the culture systems, thus the changed DGGE bands might explain the observed decreasing effect of FOS on skatole concentration. The 16S rDNA sequence of the clone representing band 1 showed 93% similarity to its closest relative, the pectinophilic *L. pectinoschiza* (Fig. 3), which has been derived from normal pig intestines (Cornick et al., 1994) and was concerned with the fermentation of fibrous substrates in the present study. There was no identified sequence of bacteria matching with clone representing band 2, which is likely to be a new, yet unidentified

bacteria. The sequence for the clone representing band 3 had 98% similarity to *C. disporicum* DSM 5521 (Fig. 3 This result was consistent with the known inhibitory effect on *Clostridium* by FOS (Roberfroid et al., 1998; Gebbink et al., 1999; Xu et al., 2002a). The threshold for similarity of 97% is considered as the same species (Stackebrandt and Goebel, 1994). Therefore, the bacteria *C. disporicum* DSM 5521 may be responsible for the degradation of tryptophan to skatole. The sequences for the clones representing bands a and c in SBP sample DGGE lanes showed 94 and 96% similarity to *E. rectale* and *Clostridium* sp., respectively. These two species have both been reported as cellulolytic bacteria (Varel et al., 1995), and are widespread in the guts of mammals. In the present study, four clones related to Clostridium-like amplicons were found on four different positions in DGGE profile, indicating an abundance of species related to Clostridium in the fermentation system.

## 5. Implication

Skatole production could be changed by the shifted microbial composition, which was closely related to different sources of carbohydrate. The SBP could be used as pig feed ingredient to inhibit microbial production of skatole. The results from this study provide a useful implication that bacteria *C. disporicum* DSM 5521 might play a role in the production of skatole.

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