Original Research Article

Identification of differential metabolites in liquid diet fermented with *Bacillus subtilis* using gas chromatography time of flight mass spectrometry

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ABSTRACT

Growth and health responses of pigs fed fermented liquid diet are not always consistent and causes for this issue are still not very clear. Metabolites produced at different fermentation time points should be one of the most important contributors. However, currently no literatures about differential metabolites of fermented liquid diet are reported. The aim of this experiment was to explore the difference of metabolites in a fermented liquid diet between different fermentation time intervals. A total of eighteen samples that collected from *Bacillus subtilis* fermented liquid diet on days 7, 21 and 35 respectively were used for the identification of metabolites by gas chromatography time of flight mass spectrometry (GC-TOF-MS). Fifteen differential metabolites including melibiose, sortitol, ribose, cellobiose, maltotriose, sorbose, isomaltose, maltose, fructose, D-glycerol-1-phosphate, 4-aminobutyric acid, beta-alanine, tyrosine, pyruvic acid and pantothenic acid were identified between 7-d samples and 21-d samples. The relative level of melibiose, ribose, maltotriose, D-glycerol-1-phosphate, tyrosine and pyruvic acid in samples collected on day 21 was significantly higher than that in samples collected on day 7 ($P < 0.01$), respectively. Eight differential metabolites including ribose, sorbose, galactinol, cellobiose, pyruvic acid, galactonic acid, pantothenic acid and guanosine were found between 21-d samples and 35-d samples. Samples collected on day 35 had a higher relative level of ribose than that in samples collected on day 21 ($P < 0.01$). In conclusion, many differential metabolites which have important effects on the growth and health of pigs are identified and findings contribute to explain the difference in feeding response of fermented liquid diet.

1. Introduction

Supplementation of probiotics in an adequate amount to human or animals has health-promoting benefits to the host, because many bioactive metabolites including functional oligosaccharides (Sriphannam et al., 2012), organic acids (Gao et al., 2012), antimicrobial peptides (Majumdar and Bose, 1958; Thasana et al., 2010), vitamins (Burgess et al., 2009) and digestive enzymes (Kim et al., 2007; Romero-Garcia et al., 2009) are produced by probiotics during fermentation, and these metabolites together with probiotcs have important roles in terms of rebalance of microbiota and osmotic pressure in intestine (Franks, 2011), improvement of nutrients digestion and absorption (Kim et al., 2007), anti-stress (Mills et al., 2011), and prevention of obesity (Raoult, 2009; Angelakis

et al., 2013), diabetes mellitus (Elliott et al., 2002), hypertension (Ebel et al., 2014) and other intestinal disorders (Caffarelli and Bernasconi, 2007; Weizman, 2010; Fukumoto et al., 2014). However, improper use of probiotics or its fermentation product might result in undesired effects. For example, lactobacillus is often used to prevent animals from diarrhoea, but an experiment reported that oral administration of high-dose *Lactobacillus rhamnosus* to piglets increased the severity of diarrhoea (Li et al., 2012). Bacillus genus is also one of the best probiotics for the controlling of diarrhoea (Kantas et al., 2015), but our feeding experiment showed that feeding of *Bacillus subtilis*-fermented liquid diet to suckling and early weaned piglets caused severe diarrhoea. The specific factors for the diarrhoea caused by the feeding of high dose probiotics or probiotics-fermented diet need to be further clarified.

In this study, gas chromatography time of flight mass spectrometry (GC-TOF-MS) was performed to detect metabolites that produced in *B. subtilis* fermented liquid diet at different fermentation times and to figure out what was the difference in metabolites between different fermentation intervals.

2. Materials and methods

2.1. Fermented liquid diet preparation and sampling

Basal diet was prepared with the ingredients that listed in Table 1. After preparation, 500 g basal diet and 1,100 g tape water were placed into each polypropylene bag with a total of 25 bags, all bags were sealed with heat-sealer and heated in a container with steam at 80°C for 30 min under normal pressure to kill some undesirable microbes, then taken out and placed in a room (indoor temperature:22.5–33.9°C) for fermentation. Six bags of fermented liquid diet were randomly selected and sampled in different times of shelf life (samples collected from day 7 were named as T7; samples collected from day 21 were named as T21; samples collected from day 35 were named as T35). Sample collected from each bag was placed into a 10 mL sterile plastic tube and immediately stored at −80°C for metabolomics study.

Table 1
Ingredients and nutrient levels of the basal diet (air-dry basis).

Item	Content
Ingredient, %	
Corn	51.0
Wheat bran	7.0
Extruded soybean	30.0
Fishmeal (Peru)	3.0
Lactose	2.0
Sucrose	3.0
Premix[1]	4.0
Total	100.0
Nutrient levels[2], %	
Digestible energy, MJ/kg	13.71
Crude protein	19.67
Calcium	1.05
Total phosphorus	0.66
Lysine	1.32
Methionine + Cystine	0.78

[1] Premix provided per kilogram diet: VA 450,000 IU, VD$_3$ 72,000 IU, VE 2,750 IU, VK$_3$ 100 mg, VB$_1$ 90 mg, VB$_2$ 280 mg, VB$_6$ 190 mg, VB$_{12}$ 0.8 mg, niacin 1,450 mg, pantothenic acid 950 mg, biotin 3 mg, choline chloride 10,500 mg, lysine 40,000 mg, Cu 3,750 mg, Zn 2,750 mg, Fe 2,500 mg, Mn 2,000 mg, I 30 mg, Co 38 mg, Se 10.5 mg, Ca 137,000 mg, P 40,800 mg, NaCl 80,000 mg, and Wole200 (heat-resistant *Bacillus subtilis* HEWD113, effective live bacteria ≥2 × 10^{10} CFU/g) 7,500 mg.
[2] Nutrient levels in the table were analyzed value except digestible energy.

2.2. Metabolites extraction, derivatization and detection

One hundred milligram sample, 0.4 mL methanol-chloroform (vol:vol = 3:1) and 20 μL ribitol (0.2 mg/mL stock in dH$_2$O) were mixed in 2 mL EP tube by vortexing and extracted for 5 min. After that, EP tube was centrifuged for 15 min at 4°C with a speed of 2,410 × g, 0.40 mL supernatant was removed from EP tube and pipetted into a 2 mL glass vial. Glass vial with supernatant was dried in vacuum concentrator at 30°C for 1.5 h, after that, 80 μL methoxymethyl amine salt (dissolved in pyridine, final concentration of 20 mg/mL) was added into the glass vial. Sealed, mixed and incubated glass vial at 37°C for 2 h in an oven, then added 100 μL Bis (trimethylsilyl) trifluoroacetamide (BSTFA, containing 1% tetrachloro-4-methylsulfonyl, vol/vol) into vial, sealed the vial again and incubated it at 70°C for 1 h. Later, added 10 μL fatty acid methyl esters (FAMEs) to the glass vial, and mixed it again for GC-TOF-MS analysis. The GC-TOF-MS analysis was performed using an Agilent 7890 gas chromatograph system coupled with a Pegasus HT time-of-flight mass spectrometer. The system utilized a DB-5MS capillary column coated with 5% diphenyl cross-linked with 95% dimethylpolysiloxane (30 m × 250 μm inner diameter, 0.25 μm film thickness; J&W Scientific, Folsom, CA, USA). Aliquot (1 μL) of the analyte was injected in the splitless mode. Helium was used as the carrier gas, the front inlet purge flow was 3 mL per minute, and the gas flow rate through the column was 20 mL per minute. The initial temperature was kept at 50°C for 1 min, then raised to 330°C at a rate of 10°C per minute, then kept for 5 min at 330°C. The injection, transfer line, and ion source temperatures were 280, 280, and 220°C, respectively. The energy was −70 eV in electron impact mode. The mass spectrometry data were acquired in full-scan mode with the *m/z* range of 85–600 at a rate of 20 spectra per second after a solvent delay of 366 s.

2.3. Data analysis

Chroma TOF 4.3X software of LECO Corporation and LECO-Fiehn Rtx5 database were used for raw peaks exacting, the data baselines filtering and calibration of the baseline, peak alignment, deconvolution analysis, peak identification and integration of the peak area (Kind et al., 2009). SIMCA-P+ software (V13.0,Umetrics AB, Umea, Sweden) was run for principal component analysis (PCA) and orthogonal partial least squares discriminant analysis (OPLS-DA), peaks with similarity greater than 700, variable importance projection (VIP) exceeding 1.0 and *P* < 0.05 by T-test were selected as the reliable different metabolites.

3. Results

3.1. Metabolites detection and identification

Total ion chromatograms (TIC) of fermented liquid diet samples collected on days 7, 21 and 35 were shown in Fig. 1. A total of 476 raw peaks were detected by GC-TOF-MS and identified with LECO-Fiehn Rtx5 database in 18 samples. After processed by numerical simulation, noise filtering and data standardization, and 439 valid peaks were used for later metabolomics analysis.

3.2. Results of PCA and OPLS-DA

All samples were within the 95% Hotelling T2 ellipse, there was a good separation in peak clusters between T7 and T21 samples and the 2 principal components explained 40.7% of the total variances (Fig. 2A). No clear partition in peak clusters between T21 and T35 samples and the first and second principal components explained 45.6% of the total variances (Fig. 2B). Peak clusters between T7 and
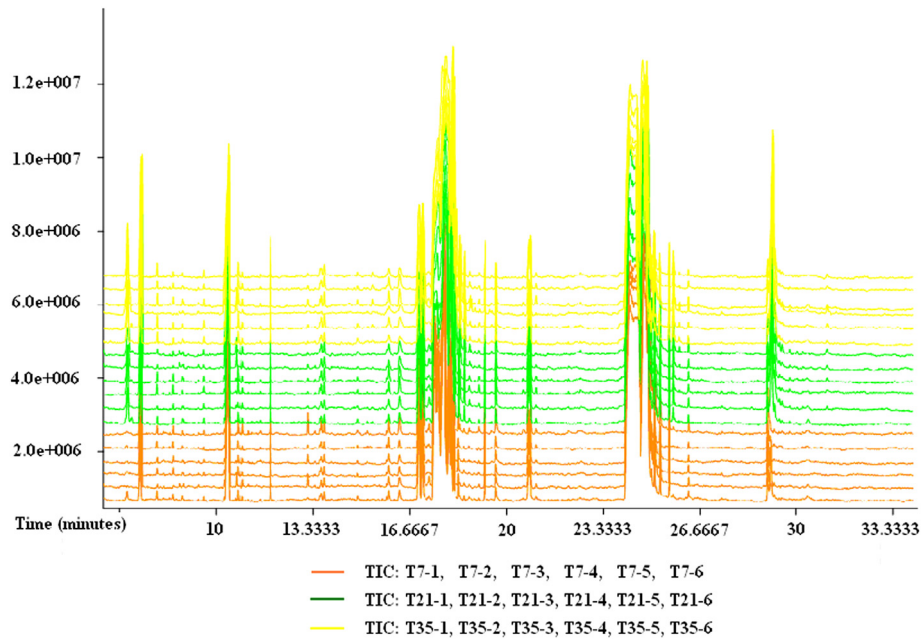
Fig. 1. TIC chromatograms of GC-TOF-MS for fermented liquid diet at different fermentation durations. TIC: total ion chromatogram; T7: samples of fermented liquid diet collected at day 7; T7-1 to T7-6: numbers of samples collected at day 7; T21: samples of fermented liquid diet collected at day 21; T21-1 to T21-6: numbers of samples collected at day 21; T35: samples of fermented liquid diet collected at day 35; T35-1 to T35-6: numbers of samples collected at day 35.
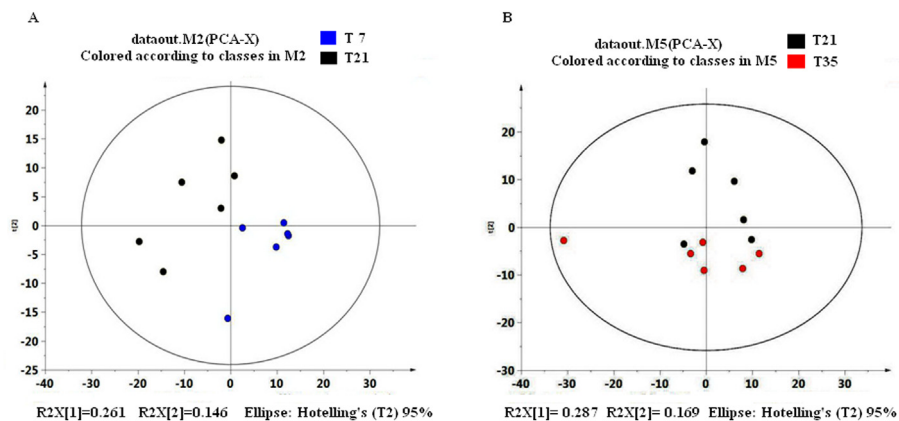

Fig. 2. Principal component analysis of *Bacillus subtilis*-fermented liquid diet at different fermentation times. PCA: principal component analysis; T7: samples of fermented liquid diet collected at day 7; T21: samples of fermented liquid diet collected at day 21; T35: samples of fermented liquid diet collected at day 35; M: map; R2X: explanatory variables of the model.

T21 samples or between T21 and T35 samples were clearly discriminated when processed them with OPLS-DA model (Fig. 3A, B), the R2Y for T7-T21 group and T21-T35 group was 0.986 and 0.909, respectively.

3.3. Screening of reliable differential metabolites

Fifteen reliable differential metabolites were screened out between T7 group and T21 group (Table 2) including 10 carbohydrates and carbohydrate conjugates, 3 amino acids and analogues, 1 organic acid and derivative and 1 aliphatic acyclic compound. Compared with T7 group, the relative level of these differential metabolites in T21 group increased ($P < 0.05$ or $P < 0.01$) with the exception of cellobiose, maltose, fructose and beta-alanine. Eight reliable differential metabolites were identified including 5 carbohydrates and carbohydrate conjugates, 1 organic acid and derivative, 1 aliphatic acyclic compound and 1 nucleotide and

analogue when compared fermented liquid diet in T21 group with fermented liquid diet in T35 group (Table 3), the relative level of sorbose, galactinol, cellobiose, pyruvic acid, galactonic acid and pantothenic acid decreased ($P < 0.05$ or $P < 0.01$) and the relative level of ribose and guanosine increased ($P < 0.01$). The reliable differential metabolites shared by T7, T21 and T35 groups were ribose, cellobiose, sorbose, pyruvic acid and pantothenic acid, the relative level of ribose increased ($P < 0.01$) but the relative level of cellobiose decreased ($P < 0.05$) when fermented liquid diet from days 7—35. The relative level of reliable differential metabolites in fermented liquid diet ranked as follows, T7: 4-aminobutyric acid > maltose > fructose > tyrosine > D-glycerol-1-phosphate > cellobiose > ribose > sorbitol > beta-alanine > melibiose > pantothenic acid > pyruvic acid > maltotriose = isomaltose = sorbose, T21: 4-aminobutyric acid > tyrosine > maltose > D-glycerol -1-phosphate > melibiose > ribose > cellobiose > sorbose > sorbitol > pyruvic
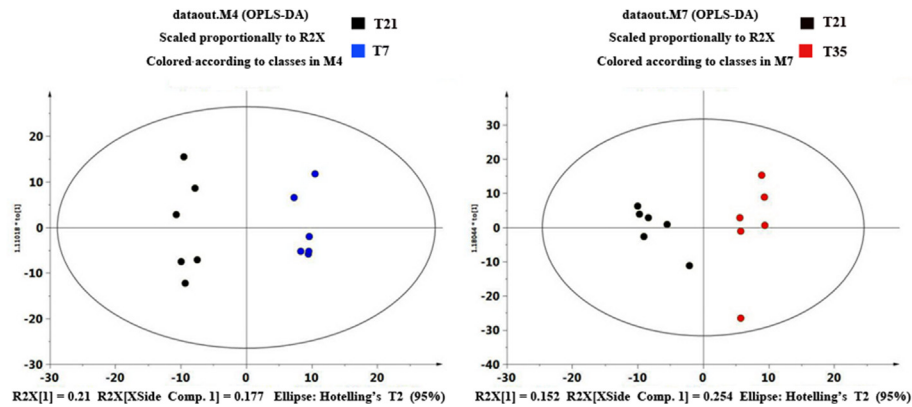
Fig. 3. OPLS-DA score plots of samples collected from different fermentation time points. OPLS-DA: orthogonal partial least squares discriminant analysis; T7: samples of fermented liquid diet collected at day 7; T21: samples of fermented liquid diet collected at day 21; T35: samples of fermented liquid diet collected at day 35; M: map; R2X: explanatory variables of the model.

Table 2
Results of reliable differential metabolites identified from fermented liquid diet between T7 and T21.

Metabolites	R.T.	Mass	T7[1]	T21[2]	VIP	P-value[3]	Fold change
Melibiose	25.77	160	0.0545	0.3776	1.97	0.00	0.14
Sorbitol	18.21	345	0.0847	0.1043	1.39	0.04	0.81
Ribose	15.37	103	0.1062	0.3578	1.99	0.00	0.30
Cellobiose	24.89	235	0.3077	0.2759	1.53	0.03	1.16
Maltotriose	31.36	204	0.0001	0.0538	2.07	0.00	0.00
Isomaltose	26.05	160	0.0001	0.0059	1.51	0.03	0.02
Maltose	25.29	204	1.5253	0.9369	1.38	0.03	1.63
Fructose	17.66	262	1.3695	0.0001	1.83	0.01	13,695.00
Sorbose	17.56	235	0.0001	0.1401	1.46	0.04	0.00
D-glycerol -1- phosphate	16.32	299	0.6394	0.8895	1.77	0.00	0.72
4-aminobutyric acid	13.71	174	1.6141	1.8736	1.40	0.03	0.86
Beta-alanine	12.42	86	0.0575	0.0310	1.31	0.04	1.85
Tyrosine	18.26	218	0.8436	1.5437	1.95	0.00	0.55
Pyruvic acid	7.22	174	0.0384	0.0781	1.89	0.00	0.49
Pantothenic acid	18.71	291	0.0432	0.0595	1.49	0.03	0.73

R.T. = retention time; VIP = variable importance projection.
[1] Samples of fermented liquid diet collected at day 7.
[2] Samples of fermented liquid diet collected at day 21.
[3] $P < 0.05$ means the difference in concentration of metabolites between T7 and T21 was significant, $P < 0.01$ means the difference in concentration of metabolites between T7 and T21 was extremely significant.

acid > pantothenic acid > maltotriose > beta-alanine > isomaltose > fructose, T35: ribose > guanosine > galactonic acid > pyruvic acid > pantothenic acid > galactinol > cellobiose > sorbose. The pathways concerned were carbohydrate digestion and absorption, protein and amino acid metabolism, vitamin B metabolism, ABC transporters, alkaloids biosynthesis, glycerolipid and glycerophospholipid metabolism, bacterial chemotaxis, insulin secretion and type II diabetes mellitus.

4. Discussion

Health benefits of fermented foods result from the interaction of live *Lactobacillus* or (and) *Bacillus* strains with host and the ingestion of functional metabolites (vitamins, bioactive peptides, organic acids, and fatty acids et al.) produced by probiotics fermentation (Stanton et al., 2005). It was reported that consumption of *Bifidobacterium lactis* LKM512-fermented yogurt increased fecal spermidine levels and significantly reduced the mutagenicity level

Table 3
Results of reliable differential metabolites identified from fermented liquid diet between T21 and T35.

Metabolites	R.T.	Mass	T21[1]	T35[2]	VIP	P-value[3]	Fold change
Ribose	15.37	103	0.3578	1.0126	2.55	0.00	0.35
Sorbose	17.56	235	0.1401	0.0001	1.83	0.04	1,401.00
Galactinol	26.45	204	0.0361	0.0212	1.67	0.04	1.70
Cellobiose	24.85	390	0.2759	0.0052	1.91	0.03	53.06
Pyruvic acid	7.22	174	0.0781	0.0420	1.97	0.01	1.86
Galactonic acid	18.73	292	0.2000	0.1128	1.86	0.02	1.77
Pantothenic acid	18.71	291	0.0595	0.0349	1.82	0.02	1.70
Guanosine	25.17	324	0.1380	0.2387	1.99	0.01	0.58

R.T. = retention time; VIP = variable importance projection.
[1] Samples of fermented liquid diet collected at day 21.
[2] Samples of fermented liquid diet collected at day 35.
[3] $P < 0.05$ means the difference in concentration of metabolites between T21 and T35 was significant, $P < 0.01$ means the difference in concentration of metabolites between T21 and T35 was extremely significant.

(Matsumoto and Benno, 2004) When fermented soybean with *Bacillus* strains (*Licheniformis KCCM 11053P*, *Licheniformis 58*, and *Amyloliquefaciens CH86-1*). Many beneficial metabolites for health including ribose, alanine, fructose, maltose, melibiose, and sorbitol have been identified, and these metabolites have effects in the improvement of intestinal health and immune response (Baek et al., 2010; Kim et al., 2012).

B. subtilis is a heat-resistant gram positive bacterium and is recognized as a safe microorganism by the Food and Drug Administration (Romero-Garcia et al., 2009). It can secrete enzymes to degrade carbohydrate and protein into mono-and oligo-saccharides, organic acids and amino acids (Valasaki et al., 2008). The GC-TOF-MS results showed that carbohydrates and proteins in the *B. subtilis*-supplemented liquid diet had been partly converted into melibiose, sorbitol, ribose, cellobiose, sorbose, maltose, fructose, alanine, tyrosine, pyruvic acid and pantothenic acid. These metabolites can be used either as substrates for the growth of microbes in the fermented liquid diet or as bioactive materials in host gut to manage animal's health in terms of enteric, cardiovascular and respiratory system.

Extruded soybean or soybean meal generally contains raffinose at a level of 1.0%–2.2% and it often causes monogastric animals to flatulence and diarrhea. In general, the diet for suckling and early weaned piglets consists of high proportion of extruded soybean or soybean meal, when piglets ingest this kind of diet, piglets often suffer from a mild to severe diarrhea. How to decrease the level of raffinose in the diet with high proportion of extruded soyabean or

soyabean meal is very important for the improvement of enteric health of monogastric animals. Raffinose can be converted into melibiose by *B. subtilis B7* and *B15* (Ouoba et al., 2007), data in Table 2 showed that the level of melibiose in the fermented liquid diet increased progressively to a relative constant level as fermentation time advanced, this implied that raffinose in the fermented liquid diet can also be effectively converted into melibiose by *B. subtilis HEWD113* fermentation. The reason for the relative level of melibiose at day 21 was significantly higher than that at day 7 was probably that *B. subtilis HEWD113* grew in its exponential growth phase from day 7 to day 21, so the ability of producing melibiose by *B. subtilis HEWD113* increased progressively. After day 21, *B. subtilis HEWD113* grew in its stationary or death phase and the relative level of melibiose had no significant difference when compared day 21 to day 35. Melibiose is a functional oligosaccharide, it can increase the amount of lactic bacteria in enteric tract and improve stool condition (Boucher et al., 2002).

Some strains of *Zymomonas*, *Candida* and *Lactobacillus* genus can convert lactose, glucose, fructose and maltose into sorbitol (Silveira and Jonas, 2002; Ladero et al., 2007) and sorbitol can be oxidized into sorbose during fermentation (Xu et al., 2014). In the present study, *B. subtilis HEWD113* grew in its exponential growth phase from day 7 to day 21, many kinds of enzymes were secreted by *B. subtilis HEWD113*, therefore, a large number of mono-and di-saccharides were produced and used to produce sorbitol, this resulted in a significantly higher relative level of sorbitol in samples from T21 group when compared with samples from T7 group. During exponential growth phase, the relative level of sorbitol increased and sorbose could be produced by sorbitol oxidization, this should be the reason why the relative level of sorbose increased significantly. After day 21, *B. subtilis HEWD113* grew in its stationary or death phase, insufficient sorbitol and sorbose fermentation in this phase resulted in a very low relative level of sorbose on day 35. Sorbitol was used as an osmotic laxative material for constipation treatment (Di Saverio et al., 2009), but when ingested in large amounts (30–50 g), it led to abdominal pain, bloating problems and mild to severe diarrhoea owing to intestinal malabsorption and increased colonic osmolarity (Islam and Sakaguchi, 2006), especially on an empty stomach, sorbitol sped up transit time and increased stool output (Livesey, 2001). Sorbose is one of the poorly digestible sugars, feeding sorbose to animals decreased body weight, liver and abdominal fat weights by suppressing feed intake (Furuse et al., 1991; Oku et al., 2014). Liquid diet fermented with *B. subtilis* from day 7 to day 21 was high in the levels of sorbitol and sorbose, this should be the contributor to the diarrhhoea of suckling and early weaned piglets.

Ribose can be biosynthesized by *B. subtilis* with lots of carbon sources (glucose, sorbitol et al.) (Park et al., 2006). Carbon sources such as glucose and sorbitol were abundant in this fermented liquid diet, because corn starch, sucrose and lactose in the fermented liquid diet could be converted constantly into glucose by enzymes that secreted by *B. subtilis HEWD113*, this could increase the level of ribose in fermented liquid diet constantly from day 7 to day 35. Ribose has key roles in energetic metabolism and glycogen synthesis, it can be rapidly metabolized to glucose in the liver via the pentose phosphate to improve adenosine-triphosphate (ATP) production and reduce soreness and fatigue caused by exercise (Peveler et al., 2006).

On the contrary to ribose, the level of cellobiose, maltose and fructose decreased progressively as fermentation advanced, it is probably caused by *B. subtilis* growth, because cellobiose, maltose and fructose are often used as the favorable substrates for the growth of *B. subtilis* (Romero-Garcia et al., 2009; Quigley, 2012). In addition, cellobiose, maltose and fructose can be converted into glucose and products of sugar metabolism, glucose can be used to produce lactic acid or lactate by fermentation to lower the pH of fermented liquid diet, this also resulted in a decrease in level of cellobiose, maltose and fructose from day 7 to day 35. It was reported that cellobiose has effect in reducing serum lipid concentration (Hetzler and Steinbüchel, 2013). Maltose can be taken up by *B. subtilis* through ATP binding cassette (ABC) and serve as sole carbon and energy sources for *B. subtilis* growth (Schonert et al., 2006). Fructose is a palatable monosaccharide and when poorly absorbed, it can cause diarrhoea or bloating (McGuinness and Cherington, 2003), this is also probably the cause for the diarrhoea of suckling and early weaned piglets when fed *B. subtilis* fermented liquid diet to these piglets, because liquid diet fermented with *B. subtilis* from day 7 to day 21 had high levels of fructose.

Pyruvic acid can be produced by lactic acid bacteria using carbohydrates, organic acids or amino acids as substrates (Liu, 2003). The relative level of pyruvic acid increased significantly from day 7 to day 21 and then decreased significantly from day 21 to day 35, this could be caused by the sufficient substrates for pyruvic acid synthesis during its exponential growth phase of *B. subtilis HEWD113*, insufficient materials for the production of pyruvic acid during stationary or death phase and the metabolism of pyruvic acid would lower the relative level of pyruvic acid.

Pantothenic acid could be produced by microbial fermentation (Baigori et al., 1991) and in this study, the relative level of pantothenic acid firstly increased from day 7 to day 21 and then decreased from day 21 to day 35, this also demonstrated that *B. subtilis HEWD113* grew in its exponential growth phase from day 7 to day 21 and then grew in its stationary or death phase from day 21 and day 35.

5. Conclusions

Differential metabolites and their relative levels varied with fermentation duration, and sugar metabolites were the main differential metabolites in the *B. subtilis*-fermented liquid diet and the reliable differential metabolites shared by *B. subtilis*-fermented liquid diet on days 7, 21 and 35 were ribose, cellobiose, sorbose, pyruvic acid and pantothenic acid. Control of fermentation duration is one of the major measures to produce the desired metabolites when ferment carbohydrate-fortified liquid diet with *B. subtilis*, and these findings can help people better understand the difference in feeding response of fermented liquid diet.

Acknowledgement

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