Amino Acids (2014) 46:2489–2501
DOI 10.1007/s00726-014-1807-y

ORIGINAL ARTICLE

Important impacts of intestinal bacteria on utilization of dietary amino acids in pigs

Yu-Xiang Yang · Zhao-Lai Dai · Wei-Yun Zhu

Received: 26 May 2014 / Accepted: 5 July 2014 / Published online: 26 July 2014

Abstract Bacteria in pig intestine can actively metabolize amino acids (AA). However, little research has focused on the variation in AA metabolism by bacteria from different niches. This study compared the metabolism of AA by microorganisms derived from the lumen and epithelial wall of the pig small intestine, aiming to test the hypothesis that the metabolic profile of AA by gut microbes was niche specific. Samples from the digesta, gut wall washes and gut wall of the jejunum and ileum were used as inocula. Anaerobic media containing single AA were used and cultured for 24 h. The 24-h culture served as inocula for the subsequent 30 times of subcultures. Results showed that for the luminal bacteria, all AA concentrations except phenylalanine in the ileum decreased during the 24-h in vitro incubation with a increase of ammonia concentration, while 4 AA (glutamate, glutamine, arginine and lysine) in the jejunum decreased, with the disappearance rate at 60–95 %. For tightly attached bacteria, all AA concentrations were generally increased during the first 12 h and then decreased coupled with first a decrease and then an increase of ammonia concentration, suggesting a synthesis first and then a catabolism pattern. Among them, glutamate in both segments, histidine in the jejunum and lysine in the ileum increased significantly during the first 12 h and then decreased at 24 h. The concentrations of glutamine and arginine did not change during the first 12 h, but significantly decreased at 24 h. Jejunal lysine and ileal threonine were increased for the first 6 or 12 h. For the loosely attached bacteria, there was no clear pattern for the entire AA metabolism. However, glutamate, methionine and lysine in the jejunum decreased after 24 h of cultivation, while glutamine and threonine in the jejunum and glutamine and lysine in the ileum increased in the first 12 h. During subculture, AA metabolism, either utilization or synthesis, was generally decreased with disappearance rate around 20–40 % for most of AA and negligible for branch chained AA (BCAA). However, the disappearance rate of lysine in each group was around 90 % throughout the subculture, suggesting a high utilization of lysine by bacteria from all three compartments. Analysis of the microbial community during the 24-h in vitro cultivation revealed that bacteria composition in most AA cultures varied between different niches (lumen and wall-adherent fractions) in the jejunum, while being relatively similar in the ileum. However, for isoleucine and leucine cultures, bacteria diversity was similar between the luminal fraction and tightly attached fraction, but significantly higher than in the loosely attached fraction. For glutamine and valine cultures, bacteria diversity was similar between the luminal and loosely attached fractions, but lower than that of tightly attached bacteria. After 30 subcultures, bacteria diversity in arginine, valine, glutamine, and leucine cultures varied between niches in the jejunum while being relatively stable in the ileum, consistent with those in the 24-h in vitro cultures. The findings may suggest that luminal bacteria tended to utilize free AA, while tightly attached adherent bacteria seemed in favor of AA synthesis, and that small intestinal microbes contributed little to BCAA metabolism.

Keywords Amino acids · Luminal bacteria · Tightly attached adherent bacteria · Loosely attached adherent bacteria · Small intestine · Pig

Y.-X. Yang · W.-Y. Zhu (✉)
Laboratory of Gastrointestinal Microbiology, Nanjing Agricultural University, Nanjing 210095, China
e-mail: zhuweiyun@njau.edu.cn

Z.-L. Dai
College of Animal Science and Technology, China Agricultural University, Beijing 100193, China

Abbreviations

AA	Amino acids
EAA	Essential amino acids
BCAA	Branched-chain amino acids
DGGE	Denaturing gradient gel electrophoresis

Introduction

Recent studies have revealed that amino acids (AA) are not only nutrients, but also critical regulatory factors of metabolic pathways (Chamorro et al. 2010; Xi et al. 2011). Many AA could play a critical functional role in the intestine as reviewed by Wu (2009, 2010) and Dai et al. (2011). Arginine and its product ornithine are essential for the proliferation, differentiation and repair of intestinal epithelial cells (Wu and Morris 1998), thus improving intestinal barrier function (Wang et al. 2009). Dietary supplementation of 1 % glutamine to weanling piglets prevents jejunal atrophy during the first week post-weaning and improves growth performance (Wu et al. 1996), increases jejunal villus height (Wang et al. 2008), maintains gut health and prevents intestinal dysfunction (Wu et al. 2011). Although AA is critical to the integrity and development of the intestines, researches have revealed that 30–60 % of dietary AA disappears through the first-pass intestinal metabolism (Stoll et al. 1998), especially glutamine, glutamate and aspartate with a disappearance rate above 90 % (Wu 2009). However using jejunal mucosal cells, Chen et al. (2007, 2009) reported that pig intestinal mucosal cells could degrade branched-chain amino acids (BCAA) substantially, but not for other essential amino acids (EAA). Our previous in vitro studies with microbes derived from the lumen of pig small intestines suggested that small intestinal microbes could extensively metabolize lysine, arginine, threonine, glutamate, leucine, isoleucine, valine and histidine, with a disappearance rate higher than 50 % (Dai et al. 2010, 2012). Thus, as suggested in reviews (Fuller and Reeds 1998; Torrallardona et al. 2003; Wu 2009), luminal microbes in the small intestine play an important part in AA metabolism, especially that of EAA.

Luminal microbes in the intestine are a combination of food-derived bacteria and bacteria shed off from the gut mucosa. Thus, luminal bacteria cannot represent the host-commensal microbe. According to the niche microhabitat, the intestinal bacteria are generally divided into luminal bacteria and adherent bacteria (Eckburg et al. 2005), and the adherent bacteria are subdivided into those tightly and those loosely attached (Van den Abbeele et al. 2011). The unique niches of adherent bacteria enable a closer contact with the host, both at the local and system level (O'Toole et al. 2000; Savage 1972) than luminal bacteria. Many researches have revealed that the composition of mucosal adherent bacteria is different from luminal free bacteria, while few studies have focused on their different roles in nutrient metabolism. For example, *Eubacterium*, *Lactobacillus* and *Clostridium* always colonize in the lumen, while *Fusobacterium*, *Spirochaetes* and *Helicobacter* prefer to stay on mucosa (Probert and Gibson 2002). It has also been suggested that mucosal-associated bacteria were better adapted to utilize long-chain endogenous substrates, while luminal bacteria were better equipped to ferment the shorter-chain exogenous dietary substrates (Libao-Mercado et al. 2009; Macfarlane and Macfarlane 1997). Thus, different niche environments contribute to the difference of microbial composition in lumen and mucosa. However, information on the role of adherent microbes in the intestine of humans or monogastric animals is limited. In the rumen, wall-associated bacteria are different from microbes in solid and liquid phase both in diversity and distribution (Sadet et al. 2007). Although accounting for only about 1 % of the total microbial population in the rumen, the role of wall-associated bacteria is strategically important. They can effectively recycle protein of sloughed epithelial cells and produce urease to convert urea into ammonia which is necessary for nitrogen metabolism (Stewart 1997). For the monogastric animals, therefore, we assume that similar compartmentation of the gut microbes may also exist and microbes in different compartments may exert their respective unique functions in the intestine.

To our knowledge, no study has focused on the AA metabolism by bacteria from gut wall and mucosa, especially in the small intestine of monogastric animals. No information is available on AA metabolism profiles of microbes from different niche compartments. Therefore, the current study tested the hypothesis that AA metabolism by bacteria varied in different compartments of the small intestine by combining in vitro incubation and molecular fingerprint technique to compare the AA metabolic profiles and bacteria composition in different compartments of pig small intestine. The findings would provide further insight into the characterization of AA metabolic profiles by bacterial community in different gut compartments.

Materials and methods

Medium and stock solutions

Unless otherwise indicated, deionized and distilled water was used to prepare all solutions. An anaerobic medium was prepared as described by Dai et al. (2010). Trace mineral solution consisted of 25 mg $MnCl_2{\cdot}4H_2O$, 20 mg $FeSO_4{\cdot}7H_2O$, 25 mg $ZnCl_2$, 25 mg $CuCl_2{\cdot}2H_2O$, 50 mg $CoCl_2{\cdot}6H_2O$, 50 mg SeO_2, 250 mg $NiCl_2{\cdot}6H_2O$, 250 mg $Na_2MoO_4{\cdot}2H_2O$, 31.4 mg $NaVO_4$ and 250 mg H_3BO_3 in

1 L water. Fatty acids solution consisted of 6.85 mL acetic acid, 3 mL propionic acid, 1.84 mL butyric acid and 0.55 mL valeric acid in 1 L water. Vitamin solution consisted of 27.35 g KH_2PO_4, 10.2 mg biotin, 10.25 mg folic acid, 82 mg calcium D-pantothenate, 82 mg nicotinamide, 82 mg riboflavin, 82 mg thiamin–HCl, 82 mg pyridoxine–HCl, 10.2 mg para-amino benzoic acid and 10.25 mg vitamin B_{12} in 500 mL of water. Haemin solution was prepared by dissolving 0.05 g haemin in 0.05 mol/L NaOH, followed by adding boiled water to make a final volume of 1 L under continuous CO_2 flush. Reducing agent solution was prepared by dissolving 20.5 g $Na_2S{\cdot}9H_2O$ in 1 L boiled water, which was also processed under the environment of CO_2 flushing. Bicarbonate solution was prepared freshly on the day of the experiment by dissolving 8.2 g Na_2CO_3 in 100 mL of boiled water (continuously gassed with CO_2). Phosphate-buffered saline consisted of 4.0 g NaCl, 0.1 g KCl, 0.72 g $Na_2HPO_4{\cdot}2H_2O$ and 0.12 g KH_2PO_4 in 500 mL of water and was used after autoclaving. Tween–phosphate saline shares the same ingredient with phosphate-buffered saline besides 250 μL Tween-80. Resazurin solution consisted of 100 mg resazurin in 100 mL of water. AA stock solutions were separately prepared by dissolving glutamate, glutamine, histidine, arginine, threonine, proline, methionine, valine, isoleucine, leucine, phenylalanine and lysine, respectively, in boiled water under continuous CO_2 flush to reach a final concentration of 0.09 mol/L. Each AA stock solution was autoclaved at 121 °C for 15 min after fully dissolving glutamine. The vitamin solution, reducing agent solution and glutamine stock solution were filter-sterilized into pre-gassed autoclaved serum bottle through a 0.22 μm filter.

Preparation of inocula

Intestinal samples used in this study were derived from four healthy finisher Duroc × Landrace × Yorkshire pigs fed a corn- and soybean meal-based diet. The small intestine, mainly the jejunum and ileum (50 cm long each from distal part), were dissected and segmented into pre-gassed sterilized ice-bagged flask with sterile threads. The segments were taken back to the laboratory within 30 min for the preparation of luminal, loosely attached or tightly attached bacteria. The inocula from each compartment were a mixture of equal portions of samples from four pigs.

Luminal bacterial inocula were prepared as described previously (Dai et al. 2010). Briefly, the digesta was gently squeezed out into a sterile flask while continuous gassing with CO_2. 300 mL of pre-warmed anaerobic phosphate buffer was then injected into the segment with a sterile syringe. After several times of gentle flush, the washing buffer was collected into the flask which contained the digesta. The flask was then sealed with a sterile rubber stopper, followed by vigorous shaking. The solution was filtered through six layers of sterile cheesecloth into pre-gassed serum bottles sealed with a butyl rubber stopper and an aluminum crimp cap. Bottles were then placed into a 37 °C water bath and served as inocula. Five milliliter of inoclua were taken from each bottle and mixed with the same volume of 40 % anaerobic glycerol. The mixture was stored at −25 °C until use for the extraction of DNA and further analyses.

Loosely attached adherent bacterial inocula were prepared as described by Gong with modifications (Gong et al. 2002). Briefly, the remaining segments were dissected vertically and washed in a Petri dish. The washed gut was transferred into the sterile flask containing 0.1 % Tween–phosphate buffer and agitated vigorously. The filtered washing solution was collected, followed by 27,000×*g* centrifuging. The bacteria were re-suspended in 300 mL anaerobic phosphate buffer and transferred into sterile serum bottles sealed with butyl rubber stoppers and aluminum crimp caps. The bottles were then placed in a 37 °C water bath and served as inocula. The same volumes of inocula were prepared as luminal bacteria for further analyses.

Tightly attached adherent bacterial inocula were made by washing the remaining segment in anaerobic phosphate buffer three times, followed by dissecting the segment into small pieces with sterile scissors under constant CO_2 flush. The dissected gut pieces were inoculated into anaerobic medium and incubated at 37 °C for 3 h to allow the release of tightly attached bacteria. The gut walls were removed from the bottle before AA stock solution was added.

In vitro incubation

The medium was prepared as described by Dai et al. (2010). The basal medium contained the following chemicals per L: 0.6 g KCl, 0.6 g NaCl, 0.2 g $CaCl_2{\cdot}2H_2O$, 0.5 g $MgSO_4{\cdot}7H_2O$, 4 g glucose, 0.5 g NH_4Cl, 1.46 g KH_2PO_4, 3.55 g Na_2HPO_4 1 mL resazurin solution, 10 mL trace mineral solution, 10 mL haemin solution, 10 mL fatty acid solution and 50 mL bicarbonate solution. An aliquot of 74 mL basal medium was added to the serum bottle fitted with a butyl rubber stopper and an aluminum crimp seal. After autoclaving, one milliliter filter-sterilized vitamin solution and autoclaved reducing agent solution were injected into each bottle. To mimic contribution of bacteria to the AA metabolism in each gut segment, four milliliter of inocula from digesta, luminal flush and gut wall was simply added into the medium without normalization of the bacteria density. The control groups contained inocula, but without AA. Three replicate bottles were used for each culture. After adding 10 mL of one of the AA stock solution into each corresponding bottle, the bottles were incubated at 37 °C and the experiment began (0 h). Samples (1 mL)

were taken from each bottle at 0, 6, 12 and 24 h and stored at −25 °C for the determination of AA concentration. Additional 2-mL samples were obtained from the culture after a 24-h period of incubation and mixed with the same volume of 40 % sterile anaerobic glycerol. The mixture was stored at −25 °C until use for the extraction of DNA.

Subculture experiment

A subculture of the small intestinal bacteria was carried out to confirm that the disappearance or the release of AA in the culture was due to bacteria rather than the host enzyme. The medium of subculture was the same as described above. Eight milliliter basal medium and 1 mL AA stock solution were added into Hungate tube and then autoclaved. Before inoculation, 0.1 mL of filter-sterilized vitamin solution and autoclaved reducing agent solution was injected into each tube. Aliquots (1 mL each) from the previous 24-h culture medium were transferred to pre-warmed tubes and incubated at 37 °C for 24 h. The subcultures were used as inocula for the next 24 h of subculture, such that 30 subcultures were obtained. One milliliter sample was taken from the culture medium at the beginning and the end of 24 h of inoculation of the 10th, 20th and 30th subcultures. All samples were stored at −25 °C for the determination of AA concentration. Additional 2-mL samples were obtained after a 24-h period of incubation and mixed with the same volume of 40 % sterile anaerobic glycerol. The mixture was stored at −25 °C until use.

Amino acids analyses

The derivation of the sample was determined according to Smith and Macfarlane (1997) with modifications. Internal standard norleucine was added to the sample and then mixed with 300 μL of trichloroacetic acid. The solution was kept at 4 °C for 1 h followed by 13,000×*g* centrifugation for 15 min. The supernatant was mixed with 1 mol/L triethlyamine and 0.1 mol/L phenylisothiocyanate (PITC). After heating at 25 °C in a water bath for 1 h, 200 μL n-hexane was added to the solution. The lower layer was diluted and 20 μL was analyzed using the Hitachi HPLC system (Hitachi, Japan) fitted with a 4.6-mm × 250-mm Venusil-AA HPLC column (40 °C, Agela Technologies, Newark, DE, USA) and a UV detector (254 nm). Concentrations of AA were calculated using authentic AA standards (Wako, Chuo-ku, Osaka, Japan).

DNA extraction

Culture media at 0, 6, 12 and 24 h during in vitro cultivation and at 0 and 24 h during the 30th time of transfer were used for microbial DNA isolation, as well as the inocula from each compartment before the experiment. The samples were centrifuged at 9,000×*g* for 5 min at 4 °C. The supernatant fluid was discarded and the bacteria pellet suspended with sterile CTAB buffer (CTAB 4 g, NaCl 16.4 g, 1 mol/L Tris–HCl 20 mL (pH 8.0), 0.5 mol/L EDTA 8 mL, dissolved in 200 mL water). Total genomic DNA was extracted using the bead-beating and phenol–chloroform extraction method as previously described (Dai et al. 2010).

PCR amplification and DGGE

Primers U0968f-GC (5′-CGC CCG GGG CGC GCC CCG GGC GGG GCG GGG GCA CGG GGG GAA CGC GAA GAA CCT TAC- 3′) and L1401r (5′ -CGG TGT GTA CAA GAC CC-3′) (Nübel et al. 1996) were used to amplify the V6–V8 regions of the bacterial 16S rRNA gene. PCR was performed with the Premix EX Taq version 2.0 (Takara Biotechnology, Dalian, China). The PCR mixture (20 μL) contained 10 μL of Premix EX Taq, 0.3 μL of forward and reverse primers (10 pmol) and 1 μL of diluted template DNA. The reactions were amplified in a Biometra® T1 thermocycler (Gőttingen, Germany) using the following program: 7 min at 94 °C and 35 cycles at 94 °C for 30 s, 56 °C for 20 s, 68 °C for 40 s and 68 °C for 7 min. PCR amplicons were separated by DGGE using the DCode™ system (Bio-Rad Laboratories, Hercules, CA, USA). Polyacrylamide gel at an 8 % concentration was prepared using acrylamide/bisacrylamide 37.5:1 in 0.5× Tris–acetate–EDTA buffer (20 mmol/L Tris, 10 mmol/L acetic acid and 0.5 mmol/L EDTA) with a 38–48 % denaturing gradient (100 % denaturant is equivalent to 7 mol/L urea and 40 % deionized formamide). Electrophoresis was initiated by pre-running for 10 min at 200 V and then for 12 h at 85 V in 0.5× TAE at a constant temperature of 60 °C. The gels were stained with $AgNO_3$ and scanned using GS-800 Calibrated Densitometer (Bio-Rad Laboratories, Hercules, CA, USA). To normalize different gels, five clones generated from DGGE gels in previous experiments (Dai et al. 2010) were used as markers for each gel. Images were normalized using GelCompar II version 4.0 packages (Applied Maths, Kortrijk, Belgium). The clustering was done with the unweighted pair-group method using arithmetic averages (UPGMA). DGGE profiles were quantified to obtain the peak surface of each band (ni) and the sum of the peak surfaces of all bands (*N*). The Shannon index was calculated with the formula: $H = -\sum \mathrm{Pi} \cdot \ln(\mathrm{Pi})$, where $\mathrm{Pi} = \mathrm{ni}/N$.

Calculation and statistical analysis

The rate of disappearance of AA was calculated according to Dai et al. (2010) as follows:

$$D_t(\%) = \frac{\{[\mathrm{AA}_0] - [\mathrm{AA}_t]\} - \{[C_0] - [C_t]\}}{[\mathrm{AA}_0]} \times 100\ \%$$

In the formula above, D_t refers to the disappearance rate of AA in culture medium at t h, [AA_0] is the concentration of AA in the treatment groups at 0 h, [AA_t] is the AA concentration in the treatment groups at t h, [C_0] is the concentration of AA in the control group at 0 h and [C_t] is the AA concentration in the control group at t h.

Data were analyzed by one-way ANOVA and the general linear model procedure was used to evaluate the differences in AA disappearance rates among different compartments, gut segments, incubation times and number of transfer. The Shannon index was further analyzed using the general linear model procedure to evaluate the differences in different compartments and segments. Statistical analyses were performed using SAS (SAS Institute, Cary, NC, USA). p values <0.05 were declared significant.

Results

Utilization and production of AA by bacteria from different compartments of the pig small intestine

The AA metabolism during the 24-h in vitro cultivation by bacteria from different compartments is summarized in Fig. 1. For luminal bacteria, AA concentration in the jejunum and ileum was mainly decreased. About 6–9 mM glutamate, glutamine, lysine and arginine in the jejunum disappeared after 24 h of cultivation ($p < 0.01$). However, the disappearance of other jejunal AA was lower compared to the above 4 AA. The concentration of all AA except phenylalanine decreased at the end of incubation ($p < 0.01$), while the phenylalanine level was significantly increased at 12 h after inoculation.

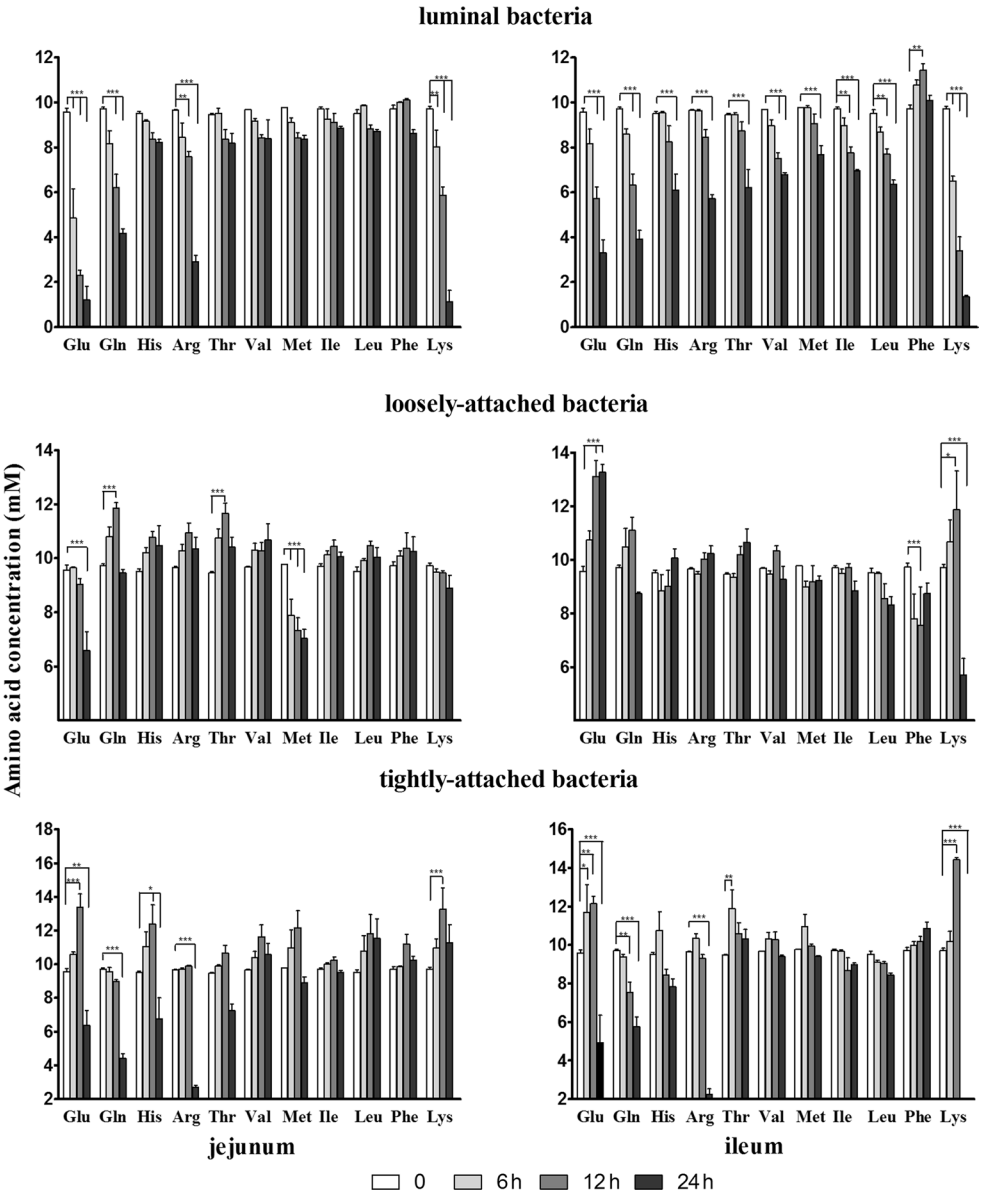


Fig. 1 AA concentration in cultural media at 0, 6, 12 and 24 h during in vitro cultivation. Data are shown as mean ± SEM. The cultivation was done anaerobically for 24 h with three replicates for each bacterial compartment, segment and AA. Significant differences compared to the 0 h are indicated with *asterisks*. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$

Table 1 Ammonia concentration from bacteria cultures derived from different compartments and segments of the pig small intestine during 24-h in vitro cultivation

mM	Hours after inoculation				SEM	*p* value
	0	6	12	24		
Luminal bacteria						
Jejunum	10.38[a]	14.65[b]	17.12[b]	21.98[c]	0.71	<0.001
Ileum	7.69[a]	8.75[a,b]	9.19[b]	12.77[c]	0.20	<0.001
Loosely attached bacteria						
Jejunum	11.75	11.77	10.84	10.52	0.78	0.725
Ileum	8.23[a]	7.93[a]	5.87[b]	5.20[b]	0.18	<0.001
Tightly attached bacteria						
Jejunum	9.51[a,b]	7.35[b]	5.88[b]	13.77[a]	0.94	<0.01
Ileum	10.36[a,b]	7.08[b]	10.59[a,b]	11.25[a]	0.79	<0.05

SEM standard error of the mean

[a,b,c] Mean values within a row with different superscript letters differ ($n = 3$)

For tightly attached bacteria, all AA concentrations were generally increased during the first 12 h and then decreased, suggesting a synthesis first and then a catabolism pattern. Among them, glutamate in both segments, histidine in the jejunum and lysine in the ileum increased during the first 12 h ($p < 0.05$) and then decreased ($p < 0.05$) at 24 h. The concentrations of glutamine and arginine did not change during the first 12 h, but went through a significant decrease at 24 h ($p < 0.001$) with the final concentration lower than 6 mM. Jejunal lysine was increased to 13.2 mM for the first 12 h ($p < 0.001$), while ileal threonine concentration increased to 11.9 mM at 6 h ($p < 0.01$).

For the loosely attached bacteria, there was no clear pattern for the entire AA metabolism. However, glutamate, methionine and lysine in the jejunum decreased after 24 h of cultivation ($p < 0.001$), while glutamine and threonine in the jejunum, and glutamine and lysine in ileum increased for the first 12 h ($p < 0.001$).

Ammonia concentration during the 24-h in vitro cultivation

Table 1 summarizes the ammonia concentration in each compartment during the 24-h in vitro cultivation. For luminal bacteria, ammonia concentration increased ($p < 0.001$) during the 24-h in vitro incubation, with the value increasing 2.11 and 1.66 times in the jejunum and ileum at the end of cultivation, respectively. This corresponds to the general utilization of AA in luminal bacteria (Fig. 1). For tightly attached bacteria, however, ammonia concentration was generally first decreased and then increased, corresponding to an increase of AA during the first incubation period and then a decrease afterward. Specifically in the jejunum, ammonia level decreased during the first 12 h of incubation but accumulated at 24 h. In the ileum, ammonia concentration decreased during the 6 h of incubation and then increased at 12 and 24 h. For loosely attached bacteria, there was no significant change of ammonia level in the jejunum, while ileal ammonia was significantly decreased at 12 and 24 h after cultivation ($p < 0.05$).

AA metabolism by bacteria derived from different compartments during subcultures

The rates of AA metabolism by subculture bacteria from each compartment are summarized in Fig. 2. After 30 times of transfer, the level of AA metabolism, either utilization or synthesis, was generally decreased as transfer increased. However, the disappearance rate of lysine was around 90 % throughout the subculture ($p < 0.001$), suggesting a high utilization of lysine by bacteria from all three compartments. For glutamine and histidine, the disappearance rate was around 40 % after 30 times of transfer. For arginine, however, while a high disappearance rate was observed in luminal and tightly attached bacteria for the 24-h in vitro cultivation, its disappearance rate in luminal and tightly attached bacteria from both segments sharply decreased during subculture ($p < 0.01$). As for BCAA (leucine, isoleucin and valine), the disappearance rate was negligible in bacteria from each compartment.

Bacteria community change during the 24-h in vitro cultivation and 30 subcultures

Bacteria diversity, as assessed by Shannon indices calculated from the DGGE profiles, is shown in Fig. 3. Analysis of the microbial community during the 24-h in vitro cultivation revealed that bacteria composition in most AA cultures varied between different niches (lumen and wall-adherent fractions) in the jejunum, but relatively similar between niches in the ileum. However, for isoleucine and leucine cultures, bacteria diversity was similar between luminal fraction and tightly attached fraction, but higher than in loosely attached fraction ($p < 0.05$). For glutamine and valine cultures, bacteria diversity was similar between luminal and loosely attached fraction, but lower than that of tightly attached bacteria ($p < 0.01$). After 30 subcultures, bacteria diversity in arginine, valine, glutamine, and leucine cultures varied between niches in the jejunum, but relatively stable in the ileum, consistent with results of the 24-h in vitro cultures.

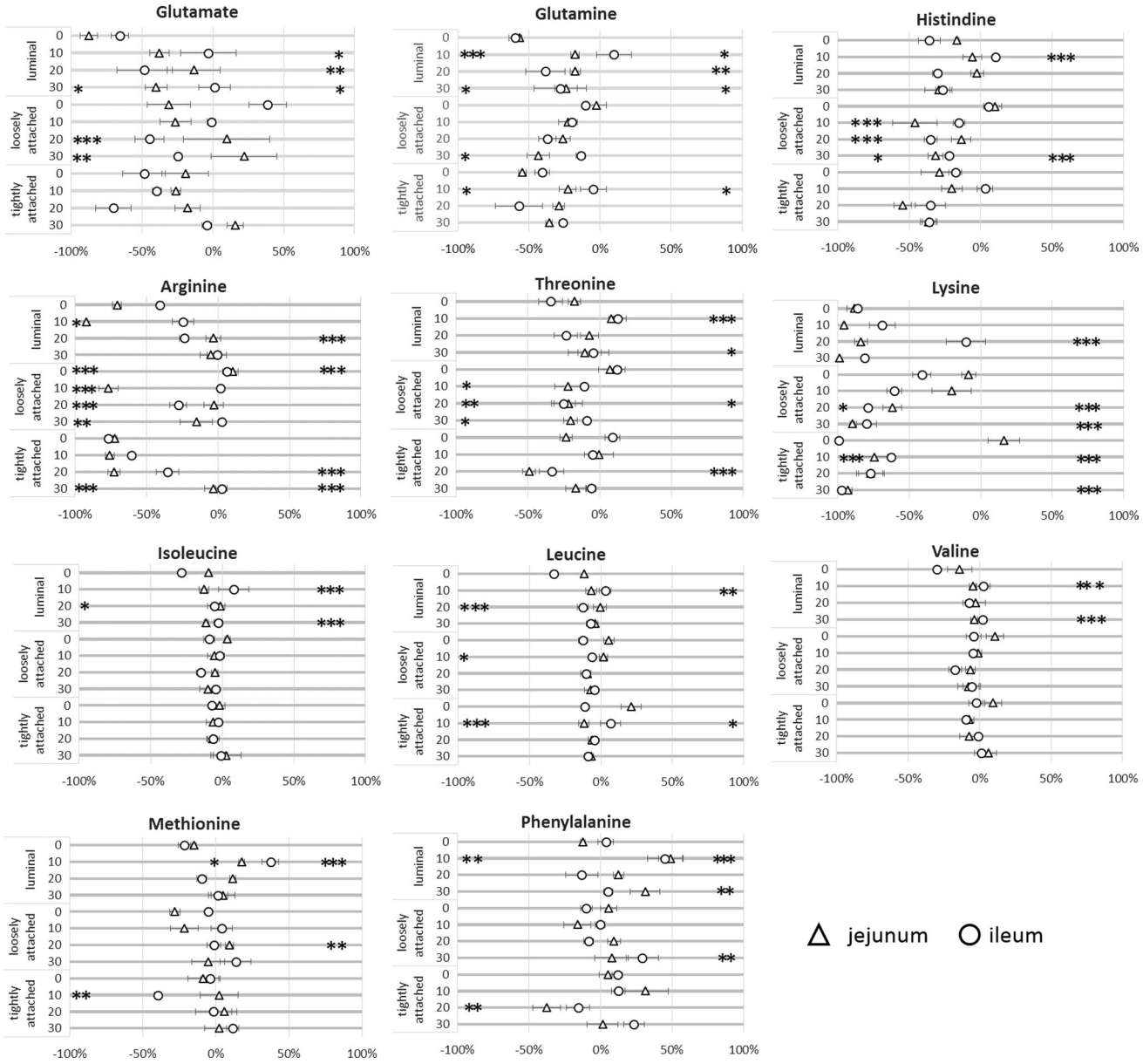


Fig. 2 AA change pattern of jejunum and ileum bacteria from different compartments during subculture. Data are shown as mean ± SEM. Positive value on the *X*-axis means AA release, while negative values represent utilization. Value on the *Y*-axis means times of transfer of bacteria subculture in each compartment. Each time of transfer was cultivated for 24 h with three replicates. Significant differences compared to 24-h (0) cultivation are indicated with *asterisks*. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$. *L* luminal, *LA* loosely attached, *TA* tightly attached

The DGGE patterns of bacterial culture with arginine, glutamine and BCAA (leucine, isoleucin and valine) in each compartment of jejunum and ileum during the 24-h in vitro cultivation and 30 subcultures are shown in Fig. 4. As compared to the original inocula (six lanes on the left side), all AA cultures showed disappearance of some bands, indicating that some bacteria were not able to grow with the specific AA as substrate. Some bands, for example those in clusters A–G, were common to all samples with a specific AA culture, while other bands such as those individual bands specified as "a–n", were only specific to certain AA cultures. For example for arginine, bands a, f, g in the jejunum group and a, e in the ileum group were dominant in the luminal group, but not visible in the tightly attached bacteria. During subculture, bands b, c, d in the jejunal tightly attached bacteria became dominant, while they were not visualized in the lumen. For isoleucine cultures, bands h, i, j were exclusively shown in the luminal group in the

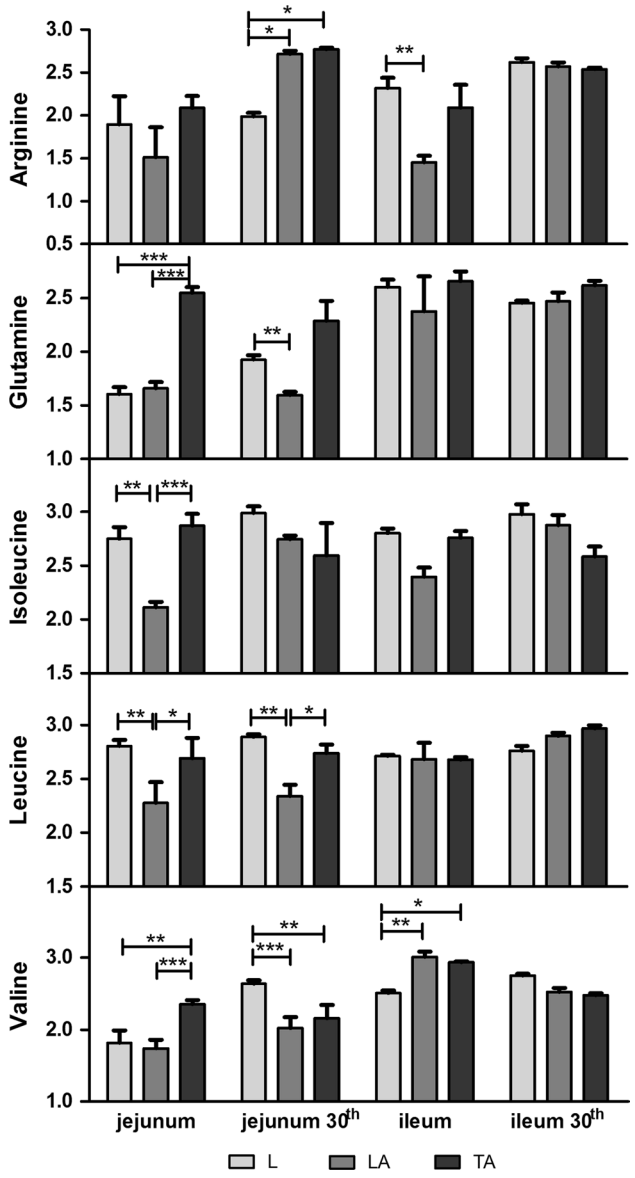


Fig. 3 Biodiversity indices from DGGE fingerprints of the bacterial community in different compartments cultivated with different AA. Data are shown as mean ± SEM. Value on the *Y*-axis is the Shannon index of bacteria in each DGGE gel. Significant differences are indicated with *asterisks*. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. *L* luminal, *LA* loosely attached, *TA* tightly attached

ileum. Bands k, l, m were dominant in the tightly attached bacteria in the jejunum, while band n was shown only in the ileum.

DGGE similarity dendrograms showed overall clusters between luminal bacteria and wall-attached bacteria. For example, arginine, isoleucine and valine cultures had two main clusters. On the other hand, loosely attached bacteria in the jejunum and ileum were segregated in one cluster while luminal and tightly attached bacteria were in another cluster.

Discussion

Small intestinal bacteria are nutritionally important to the host. They provide a vast reservoir of metabolic capabilities that complement the metabolism of the host (El Aidy et al. 2013), especially in AA metabolism (Dai et al. 2010, 2011). It has generally been recognized that luminal and gut wall-associated bacteria communities are different in their composition in the gut of humans (Durbán et al. 2011; Zoetendal et al. 2002), pigs (Su et al. 2008) and chicken (Gong et al. 2002, 2007). Eckburg et al. (2005) suggested that surface-adherent microbial populations may be distinct from luminal bacteria due to the different ecosystem, thus fulfilling different roles in the gut. Indeed, applying PCR-DGGE technique on gastrointestinal microbiota of pigs, studies found that digesta and mucosa samples from the same gut segment had apparently different microbial composition as revealed by DGGE patterns and low similarity index (Simpson et al. 1999; Su et al. 2008). Also, terminal restriction fragment length polymorphism (T-RFLP) analysis revealed that the luminal and mucosal bacteria not only differed in numbers, but also had a completely different microbial composition (Van den Abbeele et al. 2012). However, despite the known compositional variance between luminal and wall-associated bacteria, the metabolic activity of bacteria communities from different compartment niches is unknown. The present study for the first time investigated the role of microbes from different intestinal niches of the pig in AA metabolism.

In 24-h in vitro cultures, concentration of all AA in the ileum except phenylalanine and jejunal glutamate, glutamine, arginine and lysine was significantly decreased in the luminal bacteria group 24 h after inoculation, while ammonia concentration was significantly increased in the culture, indicating that AA, especially glutamate, glutamine, arginine and lysine, was deaminated and utilized by luminal bacteria, which was consistent to our previous study (Dai et al. 2010). These results collectively supported the hypothesis that the extensive in vivo catabolism of EAA by the pig small intestine might result from the action of luminal microbes (Chen et al. 2007, 2009).

A novel finding from the present study is the apparent difference in AA utilization or synthesis between luminal bacteria and tightly attached bacteria. Tightly attached adherent bacteria could produce AA during in vitro cultivation as indicated by the increase of AA concentrations. The concentrations of jejunal glutamate, histidine and lysine and ileal glutamate and lysine were increased for 12 h after inoculation. However, ammonia concentration was decreased during the first 6 or 12 h, indicating that tightly attached bacteria might produce AA through transamination or de novo synthesis. The unique habitat of tightly attached bacteria might contribute to the difference

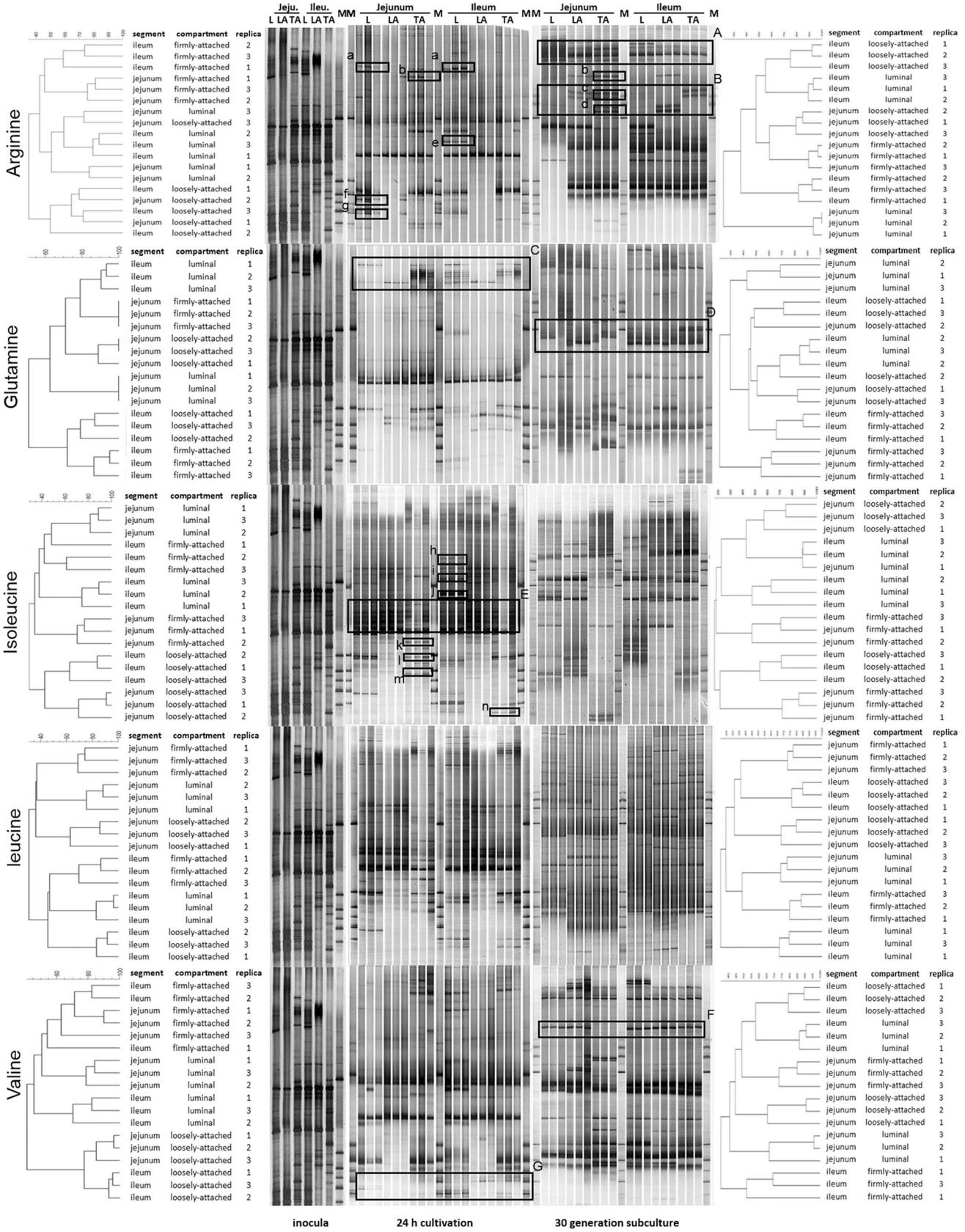
between luminal bacteria on AA metabolism. For healthy animals under physiological state, the epithelial surface bacteria and inner adherent bacteria cannot easily contact with digesta due to the protection of mucus gel that is secreted by goblet cells in the gut epithelium (Pullan et al. 1994), which indicates that luminal digesta may not be an easily available source for tightly attached adherent bacteria. On the other hand, Metges (2000) explained that the ammonia derived from endogenous urea hydrolysis by microbial urease was directly absorbed by epithelial cells. Our finding that the elevation of AA concentration and decrease of ammonia concentration in tightly attached adherent group may support the speculation that ammonia was the preferred source for AA biosynthesis by bacteria on the epithelium (Metges 2000).

Unlike the tightly attached bacteria, the loosely attached adherent bacteria in the present study showed no clear pattern of AA metabolism during the 24-h in vitro cultivation (Fig. 1). As loosely attached adherent bacteria in the present study might contain villous- and crypts-attached microbe (Stoll and Burrin 2006), AA concentration at 24 h was the outcome of villous and crypts bacteria. This could be one of the reasons why AA metabolism by loosely attached adherent bacteria varies among different AA and segments. Further, the acquisition method of mucosal-attached bacteria could cause discrepancy in AA metabolism (Croucher et al. 1983). Loosely attached bacteria could also be contaminated by the shed bacteria from the tightly attached group (Probert and Gibson 2002) and it was unlikely to obtain pure loosely attached bacteria (Van den Abbeele et al. 2011). Thus, the AA metabolic pattern of the loosely attached bacteria varied with each other.

The difference in bacteria composition in different compartments might contribute to the varying AA metabolism. Previous studies have revealed that gut bacteria have their preferred niches. For example, *Lactobacillus sobrius* was abundant in the ileum digesta of sulking piglets, while *Streptococcus suis* was not detectable in mucosa (Su et al. 2008). 16S rRNA gene sequence and metagenomic analyses of bacterial membership also suggested that mucosa-associated ileal microbiota harbored greater bacteria diversity than the lumen (Looft et al. 2014). However, exactly what types of bacteria species or community could lead to varying AA metabolism remains unclear. The difference in AA metabolism by bacteria from each compartment might have important biological function to the host. Studies with germ-free and conventionalized mice suggested that gut bacteria might alter the distribution of free AA in both small and large intestines (Whitt and DeMoss 1975). Traditionally, AA utilization by bacteria in the digestive tract represents the nutritional waste in monogastric animals. However, a number of studies suggested that the gut bacteria play an important role in not only nutrient absorption, but also host health (Dai et al. 2012, 2013; Klose et al. 2010). Thus, the flourishing lumen bacteria, although utilizing nutrients such as AA, might be functionally important to health. Moreover, the synthesizing ability of AA by tightly attached bacteria might counterbalance the utilization of AA by luminal bacteria to some extent and contribute to the host AA requirement.

In the 24-h cultivation, the inocula included gut tissue residues, microbes and their cell materials; thus it was difficult to elucidate the contribution of microbes to AA metabolism. The subculture approach can eliminate the influence of host enzyme and other substances from gut tissue debris through 30 times of transfer, which could attribute all the effects to the microbes. Results showed that the disappearance rate of lysine in each culture remained around 90 % after 30 times of transfer (Fig. 2), suggesting lysine-metabolizing bacteria could be dominant in each compartment and that intestinal bacteria could make a substantial contribution to lysine metabolism. For arginine, however, the disappearance rate in each culture after 30 times of transfer was significantly decreased. This was in accordance with a previous study showing that a significant decrease in the disappearance rate was observed for arginine at the 30th time of transfer in the jejunum group (Dai et al. 2010). The disappearance rate of BCAA was negligible after 30 subcultures, while the disappearance rate of glutamine, histidine and lysine groups was around 40 %, suggesting that pig small intestinal bacteria may preferred to utilize dietary lysine, histidine and glutamine. This was also in agreement with our previous study (Dai et al. 2010). Our result, together with previous reports, support the hypotheses that dietary essential and nonessential AA degraded in the small intestine may result from gut bacteria (Chen et al. 2007, 2009).

Another novel finding from the present study is the apparent difference in AA-metabolizing bacteria diversity between luminal and wall-adherent fractions. Although bacteria diversity was similar between luminal and tightly attached bacteria (Fig. 3), there were apparently different bands present in different niches (Fig. 4). Previous studies had shown that luminal bacteria and gut wall-adherent bacteria were different in their community compositions (Van den Abbeele et al. 2012; Durbán et al. 2011). The present study for the first time found that the luminal and tightly attached bacteria derived from pig small intestine also differed in AA-metabolizing bacteria populations as revealed by DGGE (Fig. 4) and its diversity index (Fig. 3). For example for arginine, some bands were specific to luminal fractions (bands a and e in Fig. 4) and others specific to wall-attached fractions (band b in Fig. 4), while for isoleucine, bands h, i and j were specific to ileum lumen and bands k, l and m were specific to wall-attached fraction in the jejunum. This suggested the difference in AA metabolism specificity of bacteria from different niches.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inocula
24 h cultivation
30 generation subculture

◀**Fig. 4** DGGE of PCR products of V6–V8 regions of 16S rRNA gene of samples from the bacterial community in different compartments of inocula (*left*), 24-h cultivation (*middle*) and 30th (*right*) subculture. PCR products of triplicate samples from each group were run in the same DGGE gel. Marker (*M*) includes different PCR products of five bands excised from a previous experiment. The UPGMA (unweighted pair group method using arithmetic averages) dendrogram was generated from DGGE profiles. *A–G* means a cluster of bands, *a–n* means individual dominant bands. *L* luminal, *LA* loosely attached, *TA* tightly attached

The difference in AA metabolism profiles between luminal bacteria and gut wall-attached bacteria may well be linked with different substrate source in their specific ecological environments and consequently different function in AA metabolism. A number of studies had shown that dietary protein including protein source and AA composition can greatly influence the gut microbial community (Fuller and Reeds 1998; Libao-Mercado et al. 2009). It had been suggested that the luminal bacteria were mostly able to degrade dietary protein and consequently utilize degradation products such as AA (Dai et al. 2011), while mucosa-associated microbes used more AA derived from endogenous products (Libao-Mercado et al. 2009). The different source and AA composition of dietary and endogenous protein may partly explain our finding that AA-metabolizing bacteria communities were different in luminal and tightly attached fractions.

The difference in AA-metabolizing bacteria community between luminal and tightly attached fractions may also be caused by endogenous urea. In ruminants, Pei found that epithelium fraction had more unknown species and higher density of methanogens and suggested that the more oxygenated and higher urea concentration around the epithelium might be the possible reason for the discrepancy of the AA metabolism in the lumen and tightly attached bacteria (Pei et al. 2010). In monogastric animals, however, little information was available on the bacteria diversity in different niches of the same intestinal segment. The mechanism of urea release from the small intestine tissue may also exist in monogastric animal. It has already been established that there is considerable urea and other nitrogen source cycling via gut lumen and wall in humans, with nitrogen reappearing in the gut by both secretion and diffusion as endogenous nitrogen (Metges et al. 1999). Van den Abbeele et al. (2012) also demonstrated that bacteria living in the human gut epithelium are confronted with an oxygen gradient, as oxygen is continuously released from the blood toward the mucous layer. The urea secretion will inevitably stimulate the urea-hydrolyzing bacteria to flourish and the oxygenated environment will enable more facultative anaerobe to colonize (Metges and Loh 2003). These facts may partly explain our finding that the diversity of luminal bacteria was lower than that of tightly attached bacteria in the jejunum (Fig. 3). Further, AA-metabolizing microbial colonization in humans was strongly influenced by gut nitrogen secretions (Macfarlane and Macfarlane 1997). In the present study, it was also interesting to note that bacteria diversity was higher in tightly attached bacteria than luminal content in the jejunum, while in the ileum, luminal bacteria had a higher diversity than tightly attached bacteria (Fig. 3). This may be due to the difference of endogenous secretions. Fuller and Reeds (1998) suggested that endogenous secretions, such as urea, appeared to be higher in the proximal small bowel (such as jejunum) than in the ileum of pig. Nyachoti et al. (1997) also reviewed that the amounts of endogenous nitrogen entering the gut of growing pigs were higher in the proximal part of the small intestine than in the distal part. The lower urea and nitrogenous compound secretion in the ileum may lead to the lower diversity in adherent microbial community in the ileum, as shown in the present study. Obviously, further research is needed to illustrate the urea or nitrogenous compound secretion in the segments of the gut and their influences on gut microbiota.

The community composition of AA-metabolizing bacteria changed after 30 subcultures (Figs. 3, 4). Many bands were present in the 24-h in vitro cultivation but disappeared in the 30th subculture, while others became enriched. For example, bands in cluster E with cultures of isoleucine and bands in cluster G in the valine culture disappeared in the 30th culture. On the other hand, with cultures of arginine, bands in region A and B were not shown in the 24-h culture, but became predominant in subcultures. This suggested that the bacteria in the 30th culture were able to utilize these specific AA.

Unlike other AA, glutamine was rapidly utilized by both luminal and tightly attached bacteria, while glutamine synthesis was only found in loosely attached bacteria (Fig. 1). Since glutamine was the primary product of ammonium assimilation and served as nitrogen donor in various biosynthetic reactions, its concentration must be maintained either by synthesis in the bacteria cell or taking up from the environment (Merrick and Edwards 1995; Reitzer 2003). Dai et al. (2011) suggested that the decrease of glutamine and increase of glutamate constituted a universal pathway in bacteria in the small intestine. This was in agreement with the present study that glutamine was greatly utilized by bacteria in the small intestine, regardless of whether they were luminal or tightly attached. The high utilization of glutamine by tightly attached bacteria may suggest that glutamine was the major fuel not only for the small intestinal mucosal cells (Burrin and Davis 2004), but also its resident microbial community. Subculture results indicated that bacteria could still utilize glutamine after 30 times of transfer (Fig. 2). This suggested that glutamine-utilizing bacteria could be dominant in luminal and tightly attached

bacteria and thus glutamine could be critical for microbial growth.

Although there were dominant bands in the 30th subculture (Fig. 4), the utilization of BCAA by bacteria from all three compartments was nearly negligible (Fig. 2). This may suggest that on one hand the bacteria could not utilize a great amount of BCAA and on the other hand a little amount would be sufficient to support the growth of these bacteria. Research found that in milk protein-fed piglets, 40 % of leucine, 30 % of isoleucine and 40 % of valine in the diet were extracted by the portal-drained viscera in first pass (Stoll et al. 1998). The current results suggested that these BCAA extracted in the first-pass metabolism were not utilized by bacteria in the lumen or those attached to the wall in pig small intestine. Interestingly, other studies found a high activity of BCAA transaminase, but not those for other EAA in intestinal mucosal cells (Chen et al. 2007, 2009). Thus, these collective findings may suggest that extensive catabolism of other EAA may result from bacteria, while the extraction of BCAA was mostly transaminated by mucosal cells. This may also imply that the intestinal microbes and the enterocytes may coordinate with each other in AA metabolism.

The findings from the current study suggested that gut bacteria could significantly impact AA metabolism in pigs. Luminal bacteria in the small intestine can quickly utilize dietary AA and thus decrease AA supply to the epithelial cells, which might be a nutritional waste to the host. However, the tightly attached bacteria could utilize the recycled ammonia for AA synthesis (Fuller and Reeds 1998), which might provide extra AA to the host to meet the requirement. Moreover, given that small intestinal cells can only metabolize BCAA (Chen et al. 2007, 2009) while the intestinal bacteria has the ability to degrade all the EAA (the present study), it is possible that small intestinal bacteria might coordinate with the epithelial cells in AA metabolism in the small intestine.

In conclusion, bacterial metabolism of AA varied in each compartment of small intestinal bacteria. Luminal bacteria tended to utilize free AA, while tightly attached adherent bacteria were in favor of AA synthesis for 6–12 h. The metabolism of AA by loosely attached adherent bacteria included both anabolism and catabolism. Lysine metabolism remained high after the 30th subculture, while others decreased to various extents during the subculture. AA-metabolizing bacteria composition varied between different niches in the jejunum, while being relatively similar in the ileum. These findings for the first time demonstrated that metabolism of AA by bacteria in the small intestine of pig was compartmentalized between the luminal and wall-adherent fractions. The results may provide further insights toward our understanding of the utilization and function of dietary AA in the intestine of both humans and monogastric animals. Further studies concerning the AA requirement of pig should not only consider the AA utilization of luminal bacteria, but also the AA-synthesizing ability of tightly attached bacteria.

Acknowledgments This work was supported by the National Key Basic Research Program of China (2013CB127300). The author thanks the PhD student Jingfei Zhang for assistance with the experiments.

Conflict of interest The authors declare that they have no conflict of interest.

References

Burrin DG, Davis TA (2004) Proteins and amino acids in enteral nutrition. Curr Opin Clin Nutr Metab Care 7(1):79

Chamorro S, De Blas C, Grant G, Badiola I, Menoyo D, Carabano R (2010) Effect of dietary supplementation with glutamine and a combination of glutamine–arginine on intestinal health in twenty-five-day-old weaned rabbits. J Anim Sci 88(1):170–180

Chen L, Yin YL, Jobgen WS, Jobgen SC, Knabe DA, Hu WX, Wu G (2007) In vitro oxidation of essential amino acids by jejunal mucosal cells of growing pigs. Livest sci 109(1–3):19–23

Chen L, Li P, Wang J, Li X, Gao H, Yin Y, Hou Y, Wu G (2009) Catabolism of nutritionally essential amino acids in developing porcine enterocytes. Amino Acids 37(1):143–152

Croucher SC, Houston AP, Bayliss CE, Turner R (1983) Bacterial populations associated with different regions of the human colon wall. Appl Environ Microbiol 45(3):1025–1033

Dai ZL, Zhang J, Wu G, Zhu WY (2010) Utilization of amino acids by bacteria from the pig small intestine. Amino Acids 39(5):1201–1215

Dai ZL, Wu G, Zhu WY (2011) Amino acid metabolism in intestinal bacteria: links between gut ecology and host health. Front Biosci 16:1768–1786

Dai ZL, Li XL, Xi PB, Zhang J, Wu G, Zhu WY (2012) Metabolism of select amino acids in bacteria from the pig small intestine. Amino Acids 42(5):1597–1608

Dai ZL, Li XL, Xi PB, Zhang J, Wu GY, Zhu WY (2013) L-Glutamine regulates amino acid utilization by intestinal bacteria. Amino Acids 45(3):501–512

Durbán A, Abellán JJ, Jiménez-Hernández N, Ponce M, Ponce J, Sala T, D'Auria G, Latorre A, Moya A (2011) Assessing gut microbial diversity from feces and rectal mucosa. Microb Ecol 61(1):123–133

Eckburg PB, Bik EM, Bernstein CN, Purdom E, Dethlefsen L, Sargent M, Gill SR, Nelson KE, Relman DA (2005) Diversity of the human intestinal microbial flora. Science 308(5728):1635

El Aidy S, Derrien M, Merrifield CA, Levenez F, Dore J, Boekschoten MV, Dekker J, Holmes E, Zoetendal EG, van Baarlen P, Claus SP, Kleerebezem M (2013) Gut bacteria–host metabolic interplay during conventionalisation of the mouse germfree colon. ISME J 7(4):743–755

Fuller MF, Reeds PJ (1998) Nitrogen cycling in the gut. Annu Rev Nutr 18(1):385–411

Gong J, Forster RJ, Yu H, Chambers JR, Sabour PM, Wheatcroft R, Chen S (2002) Diversity and phylogenetic analysis of bacteria in the mucosa of chicken ceca and comparison with bacteria in the cecal lumen. FEMS Microbiol Lett 208(1):1–7

Gong J, Si W, Forster RJ, Huang R, Yu H, Yin Y, Yang C, Han Y (2007) 16S rRNA gene-based analysis of mucosa-associated bacterial community and phylogeny in the chicken gastrointestinal tracts: from crops to ceca. FEMS Microbiol Ecol 59(1):147–157

Klose V, Bayer K, Bruckbeck R, Schatzmayr G, Loibner AP (2010) In vitro antagonistic activities of animal intestinal strains against swine-associated pathogens. Vet Microbiol 144(3–4):515–521

Libao-Mercado AJ, Zhu CL, Cant JP, Lapierre H, Thibault JN, Seve B, Fuller MF, de Lange CF (2009) Dietary and endogenous amino acids are the main contributors to microbial protein in the upper gut of normally nourished pigs. J Nutr 139(6):1088–1094

Looft T, Allen HK, Cantarel BL, Levine UY, Bayles DO, Alt DP, Henrissat B, Stanton TB (2014) Bacteria, phages and pigs: the effects of in-feed antibiotics on the microbiome at different gut locations. ISME J. doi:10.1038/ismej.2014.12

Macfarlane GT, Macfarlane S (1997) Human colonic microbiota: ecology, physiology and metabolic potential of intestinal bacteria. Scand J Gastroenterol 32(222):3–9

Merrick M, Edwards R (1995) Nitrogen control in bacteria. Microbiol Rev 59(4):604–622

Metges CC (2000) Contribution of microbial amino acids to amino acid homeostasis of the host. J Nutr 130(7):1857–1864

Metges CC, Loh G (2003) Intestinal microbial amino acid synthesis and its importance for the amino acid homeostasis of the monogastric host. In: Souffrant WB, Metges CC (eds) Progress in research on energy and protein metabolism. Wageningen Academic Publishers, The Netherlands, pp 579–592

Metges CC, Petzke KJ, El-Khoury AE, Henneman L, Grant I, Bedri S, Regan MM, Fuller MF, Young VR (1999) Incorporation of urea and ammonia nitrogen into ileal and fecal microbial proteins and plasma free amino acids in normal men and ileostomates. Am J Clin Nutr 70(6):1046–1058

Nübel U, Engelen B, Felske A, Snaidr J, Wieshuber A, Amann RI, Ludwig W, Backhaus H (1996) Sequence heterogeneities of genes encoding 16S rRNAs in *Paenibacillus polymyxa* detected by temperature gradient gel electrophoresis. J Bacteriol 178(19):5636–5643

Nyachoti C, De Lange C, McBride B, Schulze H (1997) Significance of endogenous gut nitrogen losses in the nutrition of growing pigs: a review. Can J Anim Sci 77(1):149–163

O'Toole G, Kaplan HB, Kolter R (2000) Biofilm formation as microbial development. Annu Rev Microbiol 54(1):49–79

Pei C-X, Mao S-Y, Cheng Y-F, Zhu W-Y (2010) Diversity, abundance and novel 16S rRNA gene sequences of methanogens in rumen liquid, solid and epithelium fractions of Jinnan cattle. Animal 4(01):20–29

Probert H, Gibson G (2002) Bacterial biofilms in the human gastrointestinal tract. Curr Issues Intest Microbiol 3(2):23–27

Pullan R, Thomas G, Rhodes M, Newcombe R, Williams G, Allen A, Rhodes J (1994) Thickness of adherent mucus gel on colonic mucosa in humans and its relevance to colitis. Gut 35(3):353–359

Reitzer L (2003) Nitrogen assimilation and global regulation in *Escherichia coli*. Annu Rev Microbiol 57(1):155–176

Sadet S, Martin C, Meunier B, Morgavi D (2007) PCR-DGGE analysis reveals a distinct diversity in the bacterial population attached to the rumen epithelium. Animal 1(7):939–944

Savage DC (1972) Associations and physiological interactions of indigenous microorganisms and gastrointestinal epithelia. Am J Clin Nutr 25(12):1372

Simpson JM, McCracken VJ, White BA, Gaskins HR, Mackie RI (1999) Application of denaturant gradient gel electrophoresis for the analysis of the porcine gastrointestinal microbiota. J Microbiol Methods 36(3):167–179

Smith E, MacFarlane G (1997) Dissimilatory amino acid metabolism in human colonic bacteria. Anaerobe 3(5):327–337

Stewart C (1997) The rumen microbial ecosystem. Springer, Germany

Stoll B, Burrin D (2006) Measuring splanchnic amino acid metabolism in vivo using stable isotopic tracers. J Anim Sci 84(13 Electronic Supplement 1):60–72

Stoll B, Henry J, Reeds PJ, Yu H, Jahoor F, Burrin DG (1998) Catabolism dominates the first-pass intestinal metabolism of dietary essential amino acids in milk protein-fed piglets. J Nutr 128(3):606–614

Su Y, Yao W, Perez-Gutierrez ON, Smidt H, Zhu WY (2008) 16S ribosomal RNA-based methods to monitor changes in the hindgut bacterial community of piglets after oral administration of *Lactobacillus sobrius* S1. Anaerobe 14(2):78–86

Torrallardona D, Harris CI, Fuller MF (2003) Pigs' gastrointestinal microflora provide them with essential amino acids. J Nutr 133(4):1127–1131

Van den Abbeele P, Van de Wiele T, Verstraete W, Possemiers S (2011) The host selects mucosal and luminal associations of coevolved gut microorganisms: a novel concept. FEMS Microbiol Rev 35:681–704

Van den Abbeele P, Roos S, Eeckhaut V, MacKenzie DA, Derde M, Verstraete W, Marzorati M, Possemiers S, Vanhoecke B, Van Immerseel F (2012) Incorporating a mucosal environment in a dynamic gut model results in a more representative colonization by lactobacilli. Microb Biotechnol 5(1):106–115

Wang J, Chen L, Li P, Li X, Zhou H, Wang F, Li D, Yin Y, Wu G (2008) Gene expression is altered in piglet small intestine by weaning and dietary glutamine supplementation. J Nutr 138(6):1025–1032

Wang W, Qiao S, Li D (2009) Amino acids and gut function. Amino Acids 37(1):105–110

Whitt DD, Demoss RD (1975) Effect of microflora on the free amino acid distribution in various regions of the mouse gastrointestinal tract. Appl Microbiol 30(4):609–615

Wu G (2009) Amino acids: metabolism, functions, and nutrition. Amino Acids 37(1):1–17

Wu G (2010) Functional amino acids in growth, reproduction, and health. Adv Nutr 1(1):31–37

Wu G, Morris S Jr (1998) Arginine metabolism: nitric oxide and beyond. Biochem J 336(1):1–17

Wu G, Meier SA, Knabe DA (1996) Dietary glutamine supplementation prevents jejunal atrophy in weaned pigs. J Nutr 126(10):2578–2584

Wu G, Bazer F, Johnson G, Knabe D, Burghardt R, Spencer T, Li X, Wang J (2011) Triennial growth symposium: important roles for L-glutamine in swine nutrition and production. J Anim Sci 89(7):2017–2030

Xi P, Jiang Z, Dai Z, Li X, Yao K, Zheng C, Lin Y, Wang J, Wu G (2011) Regulation of protein turnover by L-glutamine in porcine intestinal epithelial cells. J Nutr Biochem 23(8):1012–1017

Zoetendal EG, Von Wright A, Vilpponen-Salmela T, Ben-Amor K, Akkermans ADL, De Vos WM (2002) Mucosa-associated bacteria in the human gastrointestinal tract are uniformly distributed along the colon and differ from the community recovered from feces. Appl Environ Microbiol 68(7):3401–3407