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ORIGINAL ARTICLE

Utilization of amino acids by bacteria from the pig small intestine

Zhao-Lai Dai · Jing Zhang · Guoyao Wu · Wei-Yun Zhu

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Abstract This study determined the utilization of amino acids (AA) by bacteria from the lumen of the pig small intestine. Digesta samples from different segments of the small intestine were inoculated into media containing 10 mmol/L each of select AA (L-lysine, L-threonine, L-arginine, L-glutamate, L-histidine, L-leucine, L-isoleucine, L-valine, L-proline, L-methionine, L-phenylalanine or L-tryptophan) and incubated for 24 h. The previous 24-h culture served as an inoculum for a subsequent 24-h subculture during each of 30 subcultures. Results of the in vitro cultivation experiment indicated that the 24-h disappearance rates for lysine, arginine, threonine, glutamate, leucine, isoleucine, valine or histidine were 50–90% in the duodenum, jejunum or ileum groups. After 30 subcultures, the 24-h disappearance rates for lysine, threonine, arginine or glutamate remained greater than 50%. The denaturing gradient gel electrophoresis analysis showed that *Streptococcus* sp., *Mitsuokella* sp., and *Megasphaera elsdenii*-like bacteria were predominant in subcultures for utilizing lysine, threonine, arginine and glutamate. In contrast, *Klebsiella* sp. was not a major user of arginine or glutamate. Furthermore, analysis of AA composition and the incorporation of AA into polypeptides indicated that protein synthesis was a major pathway for AA metabolism in all the bacteria studied. The current work identified the possible predominant bacterial species responsible for AA metabolism in the pig small intestine. The findings provide a new framework for future studies to characterize the metabolic fate of AA in intestinal microbes and define their nutritional significance for both animals and humans.

Keywords Pig · Small intestine · Metabolism · Bacterial community

Abbreviations

AA	Amino acids
BCAA	Branched-chain amino acids
DGGE	Denaturing gradient gel electrophoresis
PCR	Polymerase chain reaction
PDV	Portal-drained viscera

Introduction

Studies over the last decade indicated extensive first-pass intestinal metabolism of dietary amino acids (AA) in mammals, including the pig (Bergen and Wu 2009; Burrin and Reeds 1997; Elango et al. 2009; Stoll et al. 1998; van der Schoor et al. 2002). By measuring the portal balance, Stoll et al. (1998) found that 30–50% of the dietary essential AA were not available for extra-intestinal tissues and less than 20% of the AA utilized by the small intestine was recovered in mucosal protein. Therefore, it was concluded that most of the extracted AA was catabolized by the gut (Metges et al. 1999; Stoll and Burrin 2006). Intestinal mucosal cells are now known to degrade non-essential AA and branched-chain AA (BCAA) (Chen et al. 2007, 2009; Wu 1998). In contrast, porcine enterocytes cannot degrade lysine, tryptophan, threonine, histidine, phenylalanine or methionine in the presence of physiological AA concentrations due to the lack of key enzymes (Chen et al. 2007, 2009).

Z.-L. Dai · J. Zhang · W.-Y. Zhu (✉)
Laboratory of Gastrointestinal Microbiology,
Nanjing Agricultural University, Nanjing 210095, China
e-mail: zhuweiyun@njau.edu.cn

G. Wu
Department of Animal Science, Texas A&M University,
College Station, TX 77843, USA

There is increasing evidence that microbes in the gastrointestinal tract may play an important role in host protein and AA metabolism (Baker 2009; Blachier et al. 2007, 2010; Kong et al. 2009; Wang et al. 2009a; Yin et al. 2009). Early studies revealed that concentrations of total AA in the lumen of the small intestine (especially in the ileum) were lower in conventional mice, compared with germ-free mice, but no difference was detected for the hindgut (Whitt and DeMoss 1975). Additionally, recent work identified the presence of microbial AA metabolites in urine (Li et al. 2008), feces (Martin et al. 2008, 2009), and plasma (He et al. 2009). Interestingly, 20% of the genes from cDNA-AFLP profiles were related to AA transport and metabolism, particularly in bacteria belonging to the order of Lactobacillales (Booijink 2009).

The objective of this study was to test the hypothesis that gut microbes may extensively utilize AA in a species-dependent manner. We took the approach of combining 24-h in vitro cultivation with 30 subcultures to identify the pattern of AA utilization by microbes derived from different segments of the pig small intestine. The 16S rRNA gene based denaturing gradient gel electrophoresis (DGGE) and sequencing techniques were further employed to assess the bacterial population as related to AA metabolism.

Materials and methods

Medium stock solutions

Unless indicated, deionized and distilled water was used to prepare all solutions. Resazurin solution consisted of 100 mg resazurin in 100 mL water. Trace mineral solution was prepared by sequential addition 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 $NaVO_3$, and 250 mg H_3BO_3 to 20 mL of 0.02 mol/L HCl, followed by addition of water to make up a final volume of 1 L. Haemin solution was prepared by dissolving 100 mg haemin in 5 mL of 0.05 mol/L NaOH, followed by addition of boiled water (continuously gassed with CO_2) to make up a final volume of 1 L. Fatty acid solution consisted of 6.85 mL acetic acid, 3.00 mL propionic acid, 1.84 mL butyric acid, and 0.55 mL valeric acid in 1 L of 0.2 mol/L NaOH. Bicarbonate solution was prepared freshly on the day of experiment by dissolving 8.2 g Na_2CO_3 (anhydrous) in 100 mL boiled water (continuously gassed with CO_2). Phosphate-buffered saline (pH 7.4) consisted of 8.0 g NaCl, 0.2 g KCl, 1.44 g $Na_2HPO_4{\cdot}2H_2O$, and 0.24 g KH_2PO_4 in 1 L water. Sodium phosphate buffer (0.1 mmol/L, pH 7.0) was prepared by mixing 117 mL of 0.2 mol/L NaH_2PO_4 and 183 mL of 0.2 mol/L Na_2HPO_4, followed by addition of water to make up a final volume of 600 mL. Vitamin/phosphate solution consisted of 20.4 mg biotin, 20.5 mg folic acid, 164 mg calcium D-pantothenate, 164 mg nicotinamide, 164 mg riboflavin, 164 mg thiamin-HCl, 164 mg pyridoxine–HCl, 20.4 mg para-amino benzoic acid, and 20.5 mg cyanocobalamin (vitamin B12) in 1 L of solution containing 54.7 g KH_2PO_4. Before use, vitamin/phosphate solution was filter-sterilized (through 0.22 μm filter) into sterile screw-cap tubes. Reducing agent solution was prepared by dissolving 20.5 g $Na_2S{\cdot}9H_2O$ in 1 L boiled water (continuously gassed with CO_2). Except for Bicarbonate solution, all stock solutions were stored at 4°C. Vitamin/phosphate solution was used within 2 weeks after its preparation.

Preparation of inocula

Intestinal samples used in this study were derived from four healthy 5-month-old Duroc × Landrace × Yorkshire pigs fed a corn- and soybean meal-based diet (Chen et al. 2007). Immediately after slaughtering, the small intestine was dissected and segmented with sterile threads (20-cm long for duodenum as well as 50-cm long for jejunum and ileum), placed into vacuum bottles, and flushed with oxygen-free CO_2. The intestine-segments were taken back to the laboratory and processed within 1 h after arrival (Zhang 2009). Briefly, one end of each gut segment was opened with a pair of sterile scissors and digesta was squeezed into a sterile flask that was pre-gassed with oxygen-free CO_2. The weight of digesta was recorded and the opposite end of each gut segment was opened. Proper volume (at the ratio of 9 mL per 1 g digesta) of pre-warmed sterile anaerobic phosphate-buffered saline (pH 7.4) was then injected continuously into the lumen through one end of each gut segment using a sterile syringe. The resultant washing buffer was squeezed through another end of the gut segment into a flask containing digesta. The flask was then sealed with a sterile rubber stopper, followed by vigorous shaking. The solution was filtrated through four layers of sterile cheesecloth into a sterile serum bottle fitted with a butyl rubber stopper and an aluminum crimp seal. Bottles were then placed into a 37°C water bath to provide inocula for the in vitro cultivation experiment. Five milliliter samples were taken from each bottle and mixed with the same volume of sterile 40% anaerobic glycerol. The mixture was stored at −25°C until use for the isolation of DNA and analysis of the bacterial population. All procedures were carried out under a constant stream of CO_2.

In vitro cultivation experiment

For the in vitro cultivation experiment, basal medium was prepared as described by Williams et al. (2005) with

modifications. The basal medium used for this study 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 $MgSO_4{\cdot}7H_2O$, 1 mL "Resazurin Solution", 10 mL "Trace Mineral Solution", 10 mL "Haemin Solution", 10 mL "Fatty Acid Solution", 50 mL "Bicarbonate Solution", 4 g glucose, 0.1 g NH_4Cl, 1.46 g KH_2PO_4, and 3.55 g Na_2HPO_4. Each of select AA (L-lysine, L-threonine, L-arginine, L-glutamate, L-histidine, L-leucine, L-isoleucine, L-valine, L-proline, L-methionine, L-phenylalanine or L-tryptophan) was added separately into their respective bottles at a final concentration of 10 mmol/L (Smith and Macfarlane 1997). An aliquot of 80 mL of the basal medium was then added to a 150-mL serum bottle fitted with a butyl rubber stopper and an aluminum crimp seal. The solution was autoclaved at 115°C for 20 min. Before inoculation, 1 mL of filter-sterilized "Vitamin/Phosphate Solution" and 1 mL of autoclaved reducing agent solution were added into each bottle using a sterile syringe. An aliquot (5 mL) of inocula (derived from the duodenum, jejunum or ileum) was subsequently injected into a pre-warmed medium containing the added AA (10 mmol/L). Negative controls contained all components except AA. Positive controls contained AA but no inoculum. Four bottles were used for each treatment. Bottles were then incubated at 37°C for 24 h. Samples (1 mL) were taken from each bottle immediately after inoculation and at 6, 12 and 24 h after inoculation and then stored at −25°C for the analysis of AA. Additional 2-mL samples were obtained from the culture medium after a 24-h period of incubation and mixed with the same volume of sterile 40% anaerobic glycerol. The mixture was stored at −25°C until use for the isolation of DNA and analysis of the bacterial population. A schematic diagram of the experimental design is shown in Fig. 1.

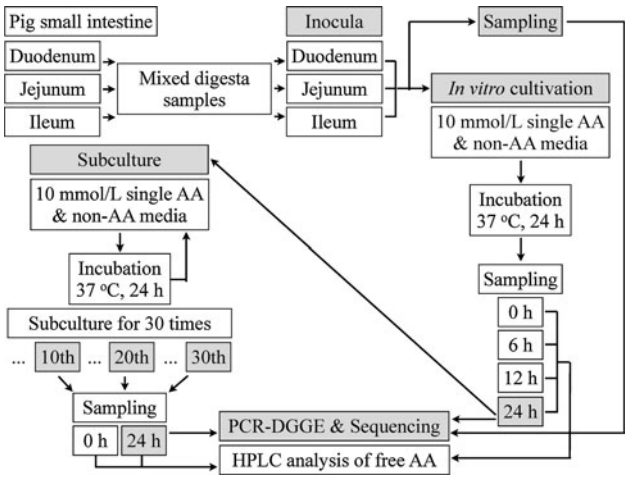


Fig. 1 Schematic diagram of the experimental design of this study

Subculture experiment

The basal medium described above was used for all subculture experiments. Nine milliliter of the basal medium containing 10 mmol/L each of AA (L-lysine, L-threonine, L-arginine, L-glutamate, L-histidine, L-leucine, L-isoleucine or L-valine) were added into each screw-capped tube and then autoclaved. Before inoculation, 100 μL of filter-sterilized "Vitamin/Phosphate Solution" and 100 μL of autoclaved reducing agent solution were injected into tubes using a sterile syringe and incubated at 37°C for 30 min. The previous 24-h culture served as an inoculum for a subsequent 24-h subculture during each of 30 subcultures. In these processes, aliquots (1 mL each) from the 24-h culture medium were transferred to pre-warmed tubes containing an added AA (10 mmol/L) and incubated at 37°C for 24 h. The subcultures were used as inocula for the next 24-h subculture, such that 30 subcultures were obtained. Samples (1 mL) were taken from culture medium at the 10th, 20th and 30th subculture, at the beginning and the end of 24-h inoculation. All samples were stored at −25°C for the analysis of AA. Additional 2-mL samples were obtained from culture medium after 24 h of incubation and mixed with the same volume of sterile 40% anaerobic glycerol. The solution was stored at −25°C until use for the isolation of DNA and analysis of the bacterial population.

Composition of AA in bacteria

Jejunal mixed bacteria and ileal mixed bacteria (obtained from the fifth subcultures of the respective digesta microbiota) as well as pure bacterial species, jejunal *Escherichia coli* (isolated from the jejunum), ileal *Klebsiella* sp. (isolated from the ileum), and ileal *Streptococcus* sp. (isolated from the ileum) were obtained as described by Zhang (2009). To determine total AA (peptide-bound plus free) in bacteria, approximately 20×10^9 cells were washed three times with 5 mL of sterile anaerobic phosphate-buffered saline. Cell pellets were dried, followed by acid or base hydrolysis and AA analysis, as previously described (Li et al. 2009a; Wu et al. 1999). To determine free AA in bacteria, approximately 20×10^9 cells were washed three times with 5 mL of sterile anaerobic phosphate-buffered saline and then lysed with 1 mL of 10% trichloacetic acid. The solution was centrifuged at 10,000×*g* for 5 min, and the supernatant fluid was analyzed for AA analysis (Li et al. 2009a). The content of peptide-bound AA was calculated as a difference between total and free AA in cells.

Incorporation of ^{14}C-labeled AA into protein in bacteria from the pig small intestine

Jejunal mixed bacteria, ileal mixed bacteria, jejunal *E. coli*, ileal *Klebsiella* sp., and ileal *Streptococcus* sp. were

obtained as described above. Bacteria (approximately 20×10^9 cells) were washed three times with 5 mL of sterile anaerobic phosphate-buffered saline. Cells were then cultured at 37°C for 3 h as described above, except that the medium contained one of the following L-[U-^{14}C]-AA plus unlabeled tracers (2 mmol/L): L-arginine, L-glutamine, L-lysine, L-threonine, L-proline, L-leucine, L-methionine, or L-phenylalanine (approximately 250 dpm/nmol). At the end of 3-h culture, cells were washed rapidly three times with 5 mL of sterile anaerobic phosphate-buffered saline, and then lysed with 1 mL of 1.5 mol/L $HClO_4$. The protein pellets were washed three times with 2% trichloacetic acid, followed by solubilization in 0.5 mL of 1 mol/L NaOH. Amounts of ^{14}C radioactivity in protein were determined by a Packard liquid scintillation counter (Meriden, CT), as described by Tan et al. (2009c). The specific radioactivities of intracellular ^{14}C-labeled AA, which were estimated to be 80% of those in culture medium, were used to calculate the rate of incorporation of AA into protein in bacteria.

Analysis of AA in culture medium

Concentrations of AA in culture medium were determined according to the method of Smith and Macfarlane (1997). Briefly, internal standard (10 mmol/L norleucine) was added to samples along with four volumes of 10% trichloroacetic acid. The solution was kept at 4°C for 1 h and then centrifuged at 13,000×*g* for 15 min at 4°C. AA in the diluted supernatant fluid reacted with 0.1 mmol/L phenylisothiocyanate in the presence of 1 mmol/L triethlyamine. After incubation at 25°C for 1 h, the mixture was diluted with water and filtered through a 0.22-μm membrane. An aliquot (20 μL) of the sample was analyzed for AA using the Shimadzu HPLC system (Shimadzu, 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 in samples were calculated using authentic standards (Fluka, St. Louis, MO, USA).

DNA isolation

Thawed liquid samples were vortexed for 2 min and 2 mL of aliquots were transferred into new sterile centrifuge tubes. The samples were centrifuged at 9,000×*g* for 5 min at 4°C. The supernatant fluid was discarded and bacterial cells were washed once with 0.5 mL of ice-cold sterile sodium phosphate buffer (0.1 mmol/L, pH 7.0) and re-suspended with 1 mL of sterile Tris–HCl/EDTA buffer (10 mmol/L Tris–HCl and 1 mmol/L EDTA-disodium; pH 7.6). Total genomic DNA was extracted using the bead-beating and phenol–chloroform extraction method (Zoetendal et al. 2006). After phenol–chloroform extraction, DNA was precipitated with ethanol and re-suspended in 50 μL of Tris–HCl/EDTA buffer. Aliquots (5 μL) were analyzed by electrophoresis on 1.2% agarose gel (w/v) containing Goldview™ (SaiBaiSheng, Shanghai, China) to determine DNA concentrations.

PCR amplification

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 V6–V8 regions of the bacterial 16S rRNA gene. PCR was performed with the *Taq* DNA polymerase kit from Sangon (Shanghai, China). The PCR mixture (50 μL) contained 0.4 μL of *Taq* DNA polymerase (2 U), 5 μL of 10× PCR buffer, 4 μL of 25 mmol/L $MgCl_2$, 1 μL of primers U968-GC and L1401 (10 pmol), 1 μL of 10 mmol/L dNTPs, 1 μL of DNA template. The reactions were amplified in a Biometra® T1 thermocycler (Göttingen, Germany) using the following program: 5 min at 94°C and 35 cycles at 94°C for 10 s, 56°C for 20 s, 68°C for 40 s, and 68°C for 7 min. Aliquots of 5 μL were analyzed by electrophoresis on 1.2% agarose gel (w/v) containing Goldview™ to check the sizes and amounts of the amplicons. Eppendorf® BioPhotometer (Hamburg, Germany) was used for the quantification of DNA.

DGGE analysis of the bacterial population

The amplicons of V6–V8 regions of the bacterial 16S rRNA gene were separated by DGGE using the DCode™ system (Bio-Rad Laboratories, Hercules, CA, USA). Electrophoresis was performed on 8% polyacrylamide gels using a 38–48% denaturing gradient (Konstantinov et al. 2003). Briefly, electrophoresis was initiated by pre-running for 5 min at 200 V and then for 12 h at 85 V in 0.5× Tris–acetate–EDTA buffer (20 mmol/L Tris, 10 mmol/L acetic acid and 0.5 mmol/L EDTA, final concentrations) at a constant temperature of 60°C. The gels were stained with $AgNO_3$ as described by Sanguinetti et al. (1994). Stained gels were scanned using GS-800 Calibrated Densitometer (Bio-Rad Laboratories, Hercules, CA, USA) and the Quantity One® Basic 4.6.6 software for Windows (Bio-Rad).

According to the results of AA utilization by intestinal bacteria, total DNA was extracted from the 24-h cultivation and subculture experiments for PCR-DGGE analysis. Predominant bands (five bands) in pre-run DGGE gels were excised, purified and amplified. Resulting amplicons were mixed with the amplicons of V6–V8 regions of the bacterial 16S rRNA gene from three pure bacteria isolates derived from the subculture experiment (Zhang 2009) and used as markers for all DGGE.

Sequencing of 16S rRNA genes

Representative bands were excised from DGGE gels and DNA was extracted according to the method of Ros et al. (2008) with modifications. Briefly, excised bands were washed twice with 50 μL of Tris–HCl/EDTA buffer and DNA was eluted with 20 μL of Tris–HCl/EDTA buffer overnight at 37°C. The mixture was centrifuged at 8,000×*g* and the supernatant fluid was transferred into a new sterile centrifuge tube. An aliquot (2 μL) of this solution was used for PCR and subsequent DGGE (described above) to check the purity and position by running the original samples in the same gel. PCR products of bands were purified and sequenced commercially (Invitrogen, Carlsbad, CA, USA). PCR products of excised bands and pure bacterial isolates from the subculture experiment (Zhang 2009) were mixed and used as markers in DGGE gels. The sequences were compared to those available in the DNA database from GenBank using the BLAST analysis (Altschul et al. 1990).

Calculation and statistical analysis

The rate of disappearance of AA from culture medium was calculated as follows:

$$D_t = \frac{([AA_0] - [AA_t]) - ([PC_0] - [PC_t]) - ([NC_0] - [NC_t])}{[AA_0]} \times 100\%$$

where D_t is the disappearance rate at t h, $[AA_0]$ is the AA concentration at 0 h in the treatment group, $[AA_t]$ is the AA concentration at t h in the treatment group, $[PC_0]$ is the AA concentration at 0 h in the positive control group, $[PC_t]$ is the AA concentration at t h in the positive control group, $[NC_0]$ is the AA concentration at 0 h in the negative control group, and $[NC_t]$ is the AA concentration at t h in the negative control group.

Data were analyzed by one-way ANOVA and the general linear model procedure to evaluate the differences in AA disappearance rates among different gut segments, different incubation times, and different times of transfer. Statistical analyses were performed using SAS (SAS Institute, Cary, NC). P values ≤ 0.05 were taken to indicate significance.

Results

Utilization of individual AA by cultured bacteria derived from different segments of the pig small intestine

Rates of disappearance of AA from cultured bacteria from each intestinal segment group are summarized in Table 1.

Table 1 Rates of disappearance (%) of AA from bacterial cultures derived from different segments of the pig small intestine

Amino acid	Segments	Hours after inoculation			SEM	*P* value
		6	12	24		
Lysine	Duodenum	20.4^{a}	39.9^{bA}	95.3^{c}	0.95	<0.01
	Jejunum	23.5^{a}	45.1^{bB}	95.6^{c}	1.65	<0.01
	Ileum	20.7^{a}	39.0^{bA}	95.1^{c}	1.12	<0.01
	SEM	1.30	1.78	0.17		
	P value	0.10	<0.05	0.07		
Threonine	Duodenum	7.4^{a}	9.7^{aA}	91.3^{b}	1.90	<0.01
	Jejunum	5.8^{a}	52.4^{bB}	90.7^{c}	2.29	<0.01
	Ileum	8.1^{a}	70.7^{bC}	90.3^{c}	2.49	<0.01
	SEM	3.20	2.11	0.63		
	P value	0.77	<0.01	0.36		
Arginine	Duodenum	20.9^{a}	57.0^{bB}	92.4^{c}	3.07	<0.01
	Jejunum	30.1^{a}	54.5^{bA}	93.1^{c}	3.19	<0.01
	Ileum	20.3^{a}	73.1^{bC}	92.4^{c}	1.67	<0.01
	SEM	3.72	2.89	0.46		
	P value	0.07	<0.01	0.28		
Glutamate	Duodenum	8.1^{a}	48.6^{b}	96.9^{cB}	3.96	<0.01
	Jejunum	13.1^{a}	41.3^{b}	87.5^{cB}	6.39	<0.01
	Ileum	12.2^{a}	38.8^{b}	56.5^{cA}	5.03	<0.01
	SEM	5.27	4.19	6.04		
	P value	0.63	0.13	<0.01		
Histidine	Duodenum	10.9^{a}	12.8^{a}	58.3^{b}	8.57	<0.05
	Jejunum	7.4^{a}	9.9^{a}	50.0^{b}	6.52	<0.05
	Ileum	3.0^{ab}	6.5^{a}	54.1^{b}	2.54	<0.01
	SEM	3.03	2.45	10.35		
	P value	0.10	0.11	0.73		
Leucine	Duodenum	10.2^{a}	18.0^{aA}	85.8^{b}	5.81	<0.01
	Jejunum	10.3^{a}	43.5^{bB}	91.5^{c}	4.07	<0.01
	Ileum	10.5^{a}	46.9^{bB}	92.5^{c}	5.09	<0.01
	SEM	3.36	7.48	3.01		
	P value	0.99	<0.05	0.13		
Isoleucine	Duodenum	9.9^{a}	26.1^{a}	45.5^{bA}	6.69	0.05
	Jejunum	15.1^{a}	32.5^{a}	78.1^{bB}	8.11	<0.05
	Ileum	11.9^{a}	23.5^{b}	80.6^{cB}	3.20	<0.01
	SEM	3.91	9.55	3.78		
	P value	0.46	0.65	<0.01		
Valine	Duodenum	11.0^{a}	21.3^{aA}	67.5^{b}	4.47	<0.01
	Jejunum	11.4^{a}	40.4^{bB}	68.4^{c}	4.60	<0.01
	Ileum	11.1^{a}	39.4^{bB}	75.0^{c}	4.41	<0.01
	SEM	3.45	5.80	3.87		
	P value	0.99	<0.05	0.16		
Proline	Duodenum	ND	11.5	13.9	–	0.75
	Jejunum	ND	16.7	33.5	–	0.08
	Ileum	ND	24.7	35.0	–	0.23
	SEM	–	4.69	8.83		
	P value	–	0.08	0.10		

Table 1 continued

Amino acid	Segments	Hours after inoculation			SEM	*P* value
		6	12	24		
Methionine	Duodenum	ND	1.6^{aA}	7.4^{bA}	–	<0.05
	Jejunum	ND	7.9^{aA}	18.6^{bB}	–	<0.05
	Ileum	ND	19.8^{B}	25.2^{B}	–	0.20
	SEM	–	2.68	3.10		
	P value	–	<0.01	<0.01		
Phenylalanine	Duodenum	ND	8.3^{AB}	8.1	–	0.91
	Jejunum	ND	3.6^{aA}	13.3^{b}	–	<0.05
	Ileum	ND	12.6^{B}	13.2	–	0.88
	SEM	–	2.36	3.10		
	P value	–	<0.05	0.24		
Tryptophan	Duodenum	ND	2.5	3.5	–	0.57
	Jejunum	ND	5.2	12.5	–	0.10
	Ileum	ND	10.0	11.7	–	0.58
	SEM	–	2.39	3.18		
	P value	–	0.05	0.06		

SEM standard error of the mean, *ND* not detected

[a,b,c] Mean values within a row with different superscript letters differ ($n = 4$)

[A,B] Mean values within a column with different superscript letters differ ($n = 4$)

Based on 24-h disappearance rates, AA could be classified into three groups: (a) the high disappearance rate (HDR) group (lysine, arginine, threonine, glutamate and leucine); (b) the medium disappearance rate (MDR) group (isoleucine, valine and histidine); and (c) the low disappearance rate (LDR) group (proline, methionine, phenylalanine and tryptophan). Lysine and arginine were most rapidly utilized, with more than 20% of AA disappearing from the medium after 6 h of cultivation. In the HDR group, approximately 50% of AA disappeared from the medium after 12 h of cultivation and 90% of AA disappeared from the medium after 24 h of cultivation (Table 1). In the MDR group, less than 40% of AA disappeared from the medium after 12 h of cultivation and 50–80% of AA disappeared from the medium after 24 h of cultivation (Table 1). In the LDR group, less than 35% of AA disappeared from the medium after 24 h of cultivation (Table 1).

Differences in bacterial utilization of AA among different segments of the pig small intestine were observed during in vitro cultivation (Table 1). In the HDR group, differences in AA disappearance were observed after 12 h of cultivation for lysine, arginine, threonine and leucine, while a difference was observed for glutamate after 24 h culture (Table 1). The disappearance rate of lysine was higher ($P < 0.05$) with the jejunal inoculum than with duodenal and ileal inocula after 12 h of cultivation (Table 1). For arginine, threonine and leucine, disappearance rates were higher with the ileal inoculum than with duodenal and jejunal inocula ($P < 0.05$ for leucine and $P < 0.01$ for arginine and threonine, Table 1). The 24-h disappearance rate of glutamate was lower ($P < 0.01$) with the ileal inoculum than with jejunal and duodenal inocula (Table 1). In the MDR group, the disappearance rate of valine was lower ($P < 0.05$) with the duodenal inoculum than with jejunal and ileal inocula after 12 h of cultivation. During this period, the disappearance rate of isoleucine was lower ($P < 0.05$) with the duodenal inoculum than with jejunal and ileal inocula (Table 1). In the LDR group, the 12-h disappearance rate of methionine was lower ($P < 0.01$) with duodenal and jejunal inocula than with the ileal inoculum. The disappearance rate of methionine was lower ($P < 0.01$) with the duodenal inoculum after 24 h of cultivation (Table 1). For phenylalanine, a lower rate of disappearance was observed with the jejunal inoculum after 12 h of cultivation ($P < 0.05$).

Utilization of AA by small-intestinal bacteria during subcultures

Rates of the utilization of AA by small-intestinal bacteria during subcultures varied greatly with AA and gut segment (Fig. 2). The 24-h disappearance rate of lysine in cultures derived from jejunal bacteria (the "jejunum group") and ileal bacteria (the "ileum group") remained high during subcultures, with values in a range of 72–95%. However, its disappearance rate in cultures derived from duodenal bacteria (the "duodenum group") decreased ($P < 0.01$) sharply from 95% at transfer 0 to <20% after transfer 20. For threonine, the 24-h disappearance rate decreased ($P < 0.01$) to a lesser extent in all the three intestine groups (e.g., approximately 60–65% for the jejunum group after transfer 20 and the ileum group after transfer 30. The 24-h disappearance rate of arginine remained as high as lysine during subcultures, but a decrease in disappearance rate was observed at transfer 30 for the duodenum group and the jejunum group ($P < 0.01$). For glutamate, the 24-h disappearance rate in the duodenum group and the jejunum group remained high during subcultures (83–97%). Lower rates of disappearance were observed for histidine (e.g., 35–55% for the ileal group; 20–35% for the duodenum group and the jejunum group) and BCAA (e.g., 15–45% for all the intestine groups).

Changes in the composition of the small intestinal bacterial population during in vitro cultivation and subcultures in the presence of different AA

The patterns of DGGE profiles did not change appreciably after 24 h of in vitro cultivation with or without the addition of AA, with predominant bands common in all

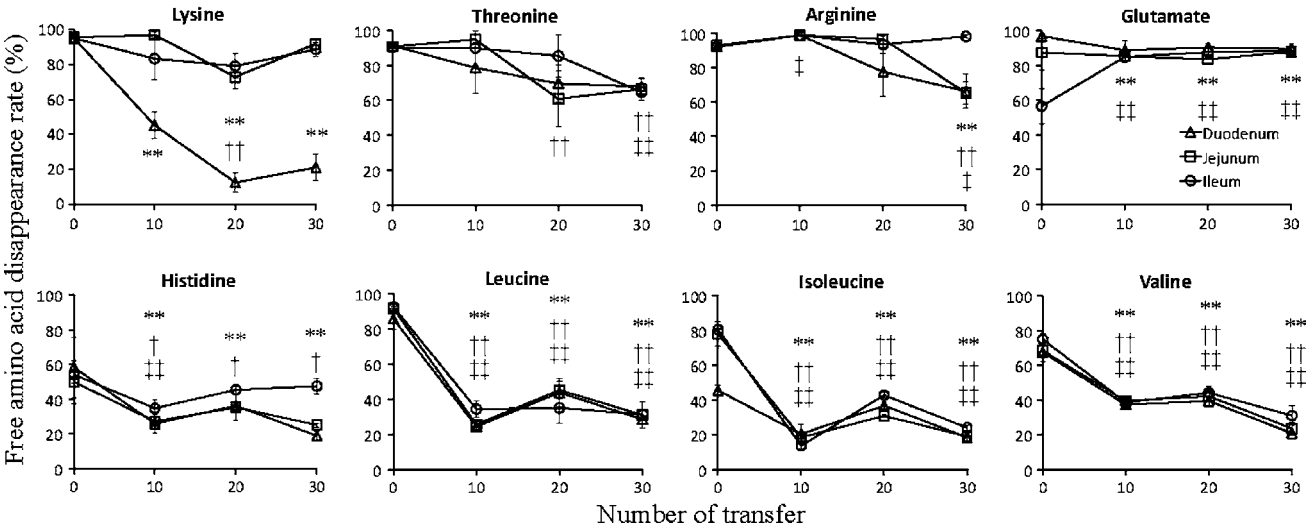


Fig. 2 Disappearance rate of free amino acid after 24 h incubation in subcultures of bacteria derived from the duodenum (*open triangles*), jejunum (*open squares*) and ileum (*open circles*) of pigs. Data are presented as mean ± SD ($n = 4$). Significant difference between transfers and the 24 h in vitro cultivation (transfer 0) are indicated as follows: $^{*}P < 0.05$ (duodenum); $^{**}P < 0.01$ (duodenum); $^{\dagger}P < 0.05$ (jejunum); $^{\dagger\dagger}P < 0.01$ (jejunum); $^{\ddagger}P < 0.05$ (ileum); $^{\ddagger\ddagger}P < 0.01$ (ileum)

samples (Fig. 3). However, the composition of the bacterial population changed in an AA-dependent manner during subcultures. In the non-AA control group, bands at the bottom regions of the DGGE gels disappeared while bands with relative low GC% became predominant and appeared at the upper regions of the DGGE gels. Only bands 3 and 7 remained dominant during subcultures in the DGGE profiles of the control group. Compared to the control group, the DGGE profiles of the arginine and glutamate groups remained constant during subcultures with predominant bands (bands 3–7) at the middle and lower regions in the DGGE gels. For the lysine, histidine and threonine groups, bands at the middle and lower regions of the DGGE gels remained dominant during subcultures (bands 4–7 in the lysine group; bands 4, 6, 8 in the histidine group and bands 4 and 6 in the threonine group), while bands at the upper regions of DGGE gels became predominant during subcultures (band 1 in the lysine group; bands 1 and 2 in the threonine and histidine groups). In comparison with the control group, band 3 disappeared during the whole course of subcultures in the threonine group and in the late period of subcultures in the lysine and histidine groups. For bacterial inocula, the DGGE patterns were similar among different gut segments when treated with the same AA, and bands 3, 4, 6 and 8 became dominant in all cultures after 24 h of in vitro cultivation and were only predominant in the ileum inoculum (especially for band 3). However, with different AA, there were differences in DGGE patterns among different gut segments. For instance, with the addition of lysine to the culture medium, bands at the bottom regions of the lanes of the "duodenum group" remained predominant during subcultures. In the histidine group, bands A and band B became dominant during subcultures in the "duodenum group" and "jejunum group" but not in the "ileum group", whereas band C was only predominant in the "ileum group" after 20 times of subculture (Fig. 3).

Sequencing of the corresponding partial 16S rRNA genes for the predominant bands (including bands derived from pure bacteria isolates) indicated that three pure bacteria isolates were closely related to *Klebsiella* sp. (band 1), *E. coli* (band 3) and *Streptococcus* sp. (band 4) (Table 2). The other five bands were closely related to *Succinivibrio dextrinosolvens* (band 2), *M. elsdenii* (band 5), *Mitsuokella* sp. (band 6), *Anaerovibrio lipolytica* (band 7) and *Acidaminococcus fermentans* (band 8), respectively. The identified bacteria belong to the family of *Veillonellaceae*, *Streptococcaceae* and *Enterobacteriaceae*.

To better illustrate DGGE profiles, Table 3 summarizes the shift of the marker bands identified above during in vitro cultivation and subcultures. In cultures of the non-AA control, the predominant bands were most related to *E. coli*, *Streptococcus* sp., *Mitsuokella* sp. and *A. fermentans* after 24 h of in vitro cultivation. However, some of the bands which were most related to *Streptococcus* sp., *Mitsuokella* sp. and *A. fermentans* disappeared during subcultures and some of the bands which were most related to *Klebsiella* sp., *A. lipolytica* and *S. dextrinosolvens* became dominant. For cultures containing lysine, threonine, arginine, glutamate or histidine, bands which were most related to *E. coli*, *Streptococcus* sp., *Mitsuokella* sp. and *A. fermentans* were predominant in all AA groups after 24 h of in vitro cultivation. Meanwhile, a band which was most related to *M. elsdenii* became predominant in cultures containing threonine or

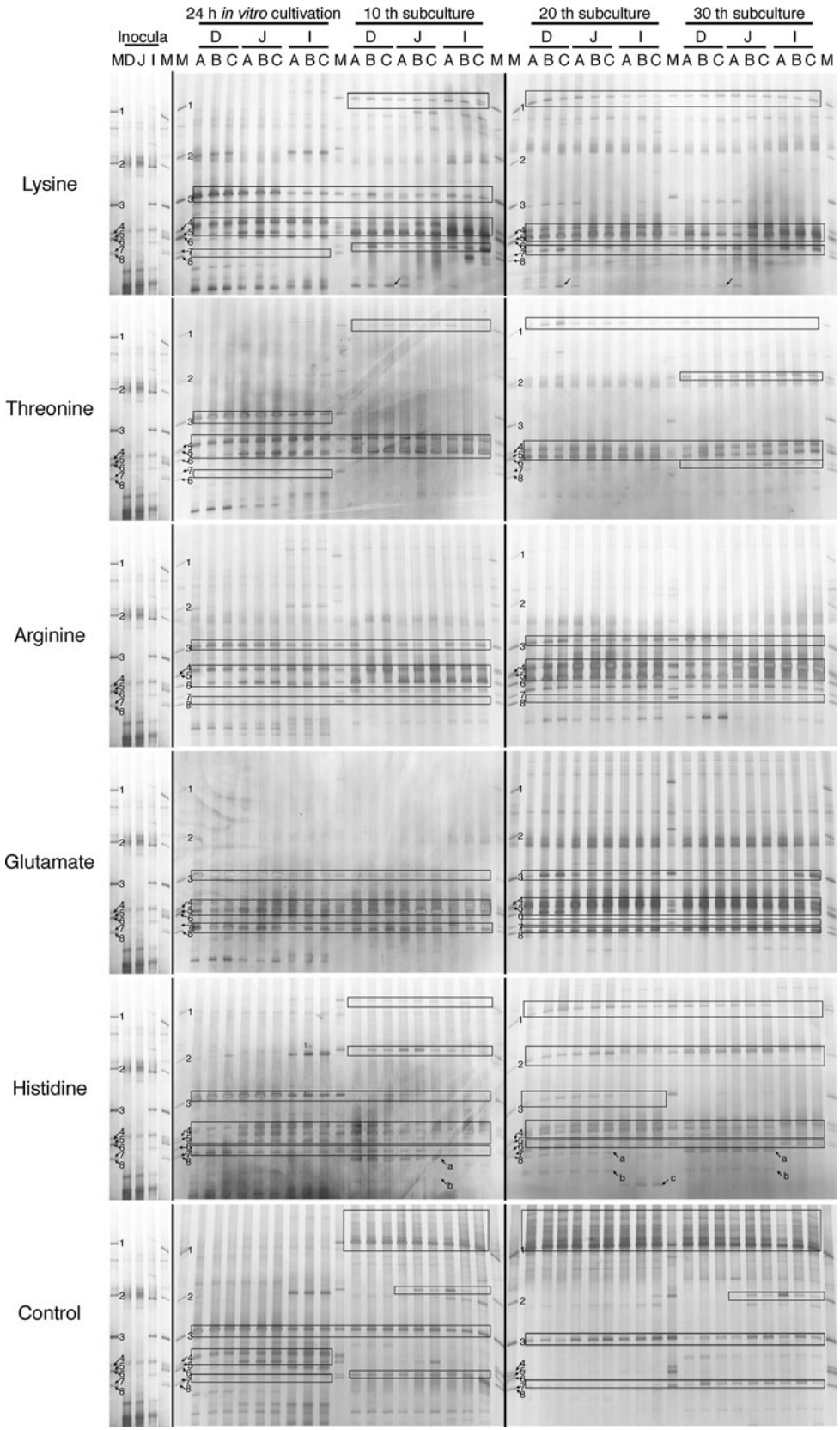
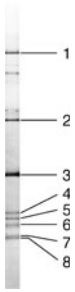


Fig. 3 DGGE of PCR products of V6–V8 regions of 16S rRNA gene of samples from the inocula (*D* Duodenum, *J* Jejunum, *I* Ileum), the 24-h samples of in vitro cultivation experiment and the 24-h samples of subculture experiment (10th subculture, 20th subculture and 30th subculture). PCR products of triplicate samples were run in the same DGGE gel and indicated as A, B and C. Marker (M) includes different PCR products of 5 bands excised from pre-run DGGE gels of mixed bacterial cultures and 3 bands from pure bacterial cultures, as indicated by 1–8 (see details in Table 2). Bands that were either predominant or changed during subcultures were marked with *rectangles*. *Arrows* (a–c) indicate bands whose changes were gut segment-specific

Table 2 Details of the sequences generated in this study and their similarity to the known sequences of cultured bacteria in GenBank

Marker	Band no.	Origin[a]	Length (bp)	Accession number	Cultured bacteria	Similarity (%)
	1	Pure culture	1,425	FJ655784	*Klebsiella* sp.	99
	2	DGGE band	394	FJ821508	*Succinivibrio dextrinosolvens*	98
	3	Pure culture	1,439	FJ949576	*Escherichia coli*	99
	4	Pure culture	1,442	GQ139522	*Streptococcus* sp.	99
	5	DGGE band	409	FJ821510	*Megasphaera elsdenii*	90
	6	DGGE band	393	FJ821509	*Mitsuokella* sp.	99
	7	DGGE band	395	FJ821511	*Anaerovibrio lipolytica*	93
	8	DGGE band	393	FJ821512	*Acidaminococcus fermentans*	100

[a] Sequences of the PCR product of genomic DNA from pure bacteria isolates derived from the subculture experiment (Zhang 2009; primers: 8f and 1510r, Favier et al. 2002) or DNA derived from bands excised from DGGE gels (shown as the markers in Fig. 3)

Table 3 Changes in the composition of identified bacteria and bands during in vitro cultivation and subculture in response to addition of lysine, threonine, arginine, glutamate, or histidine

Band no.	Closest cultured relative	Lysine				Threonine				Arginine				Glutamate				Histidine				Control[c]			
		0[a]	10	20	30	0	10	20	30	0	10	20	30	0	10	20	30	0	10	20	30	0	10	20	30
1	*Klebsiella* sp.	−[b]	+	+	+	−	+	+	+	−	−	−	−	−	−	−	−	−	+	+	+	−	+	+	+
2	*Succinivibrio dextrinosolvens*	−	−	−	−	−	−	−	+	−	−	−	−	−	−	−	−	−	+	+	+	−	+	−	+
3	*Escherichia coli*	+	+	−	−	+	−	−	−	+	+	+	+	+	+	+	+	+	+	+	−	+	+	+	+
4	*Streptococcus* sp.	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−	−	−
5	*Megasphaera elsdenii*	−	+	+	+	+	+	−	−	+	+	+	+	−	+	+	+	−	−	−	+	−	−	−	−
6	*Mitsuokella* sp.	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−	+	+	+	+	+	−	−	−
7	*Anaerovibrio lipolytica*	−	+	+	+	−	−	+	+	−	−	−	−	−	−	+	+	−	−	−	−	−	+	+	+
8	*Acidaminococcus fermentans*	+	−	−	−	+	−	−	−	+	+	+	+	+	+	+	+	+	+	+	+	+	−	−	−

[a] Times of subculture: 0, in vitro cultivation; 10, 10th subculture; 20, 20th subculture; 30, 30th subculture. Samples were all derived from the 24-h culture of in vitro cultivation and subculture experiment

[b] Indicate that the band was predominant (+) or not predominant (−) in the 24-h samples from in vitro cultivation and subculture experiment according to the DGGE profiles in Fig. 3

[c] No AA was added to the control medium

arginine after 24 h of in vitro cultivation, but not in other AA groups or the control culture. During subcultures, bands which were most related to *Streptococcus* sp. and *Mitsuokella* sp. were predominant in all five AA groups. Bands which were most related to *E. coli* and *A. fermentans* remained dominant in subcultures containing arginine, glutamate or histidine but disappeared in subcultures containing lysine or threonine. A band which was most related to *M. elsdenii* remained dominant during subcultures with arginine and became dominant in subcultures containing lysine or glutamate and disappeared in subcultures containing threonine. Compared to the 24-h in vitro cultivation, a band which was most related to *Klebsiella* sp. became dominant in subcultures containing lysine, threonine or histidine, but was undetectable in subcultures containing arginine or glutamate. A band, which was most related to *S. dextrinosolvens* and was predominant in 24-h in vitro cultivation, became dominant in subcultures containing histidine. At the same time, a band which was most related to *A. lipolytica* was absent in all samples from the 24-h in vitro cultivation and remained absent in subcultures containing arginine or histidine but became dominant in subcultures containing lysine, threonine or glutamate.

Composition of AA in bacteria and incorporation of AA into bacterial proteins

Free glutamate plus glutamine represented 1.8, 1.7, <0.1, <0.1, and 2.3% of total AA (peptide-bound and free), respectively, in jejunal mixed bacteria, ileal mixed bacteria, jejunal *E. coli*, ileal *Klebsiella* sp., and ileal *Streptococcus* sp. Free aspartate plus asparagine represented 0.74,

Table 4 Composition of peptide (protein)-bound amino acids in bacteria from the pig small intestine

Amino acid	Jejunal bacteria[A]	Ileal bacteria[B]	*Escherichia coli*[C]	*Klebsiella* sp.[D]	*Streptococcus* sp.[E]
nmol/10^8 cells					
Aspartate[F]	33.9 ± 2.27[a]	50.5 ± 0.26[b]	50.4 ± 3.31[b]	38.3 ± 1.28[a]	23.8 ± 0.18[c]
Glutamate[G]	40.3 ± 1.65[a]	46.4 ± 0.23[ab]	54.0 ± 2.67[b]	42.4 ± 0.87[a]	27.2 ± 0.11[c]
Serine	13.6 ± 0.21[a]	19.8 ± 0.19[b]	22.7 ± 0.26[c]	19.3 ± 0.25[b]	9.84 ± 0.05[d]
Histidine	3.34 ± 0.16[a]	4.88 ± 0.13[b]	6.86 ± 0.29[c]	5.93 ± 0.07[d]	2.13 ± 0.02[e]
Glycine	39.8 ± 0.65[a]	39.8 ± 0.47[a]	53.3 ± 1.55[b]	48.4 ± 1.36[b]	20.3 ± 0.54[c]
Threonine	15.2 ± 0.93[ab]	18.0 ± 0.17[b]	23.6 ± 0.65[c]	13.3 ± 0.28[a]	13.0 ± 0.75[a]
Arginine	16.5 ± 1.03[a]	27.7 ± 0.90[b]	21.9 ± 0.27[c]	15.7 ± 0.32[a]	16.9 ± 0.34[a]
Alanine	38.7 ± 0.90[a]	30.9 ± 0.76[b]	46.0 ± 0.63[c]	41.7 ± 1.02[c]	17.2 ± 0.10[d]
Tyrosine	13.4 ± 0.98[a]	18.3 ± 0.11[b]	24.1 ± 0.82[c]	16.1 ± 0.17[ab]	13.3 ± 0.07[a]
Tryptophan	2.79 ± 0.02[a]	3.31 ± 0.05[b]	8.86 ± 0.17[c]	5.60 ± 0.12[d]	1.88 ± 0.02[e]
Methionine	5.74 ± 0.33[a]	4.28 ± 0.03[b]	6.57 ± 0.56[c]	6.80 ± 0.13[c]	2.04 ± 0.14[d]
Valine	19.0 ± 0.72[a]	27.9 ± 0.35[bc]	34.4 ± 2.60[b]	25.5 ± 0.52[c]	14.1 ± 0.26[a]
Phenylalanine	8.98 ± 0.51[a]	11.8 ± 0.14[b]	18.4 ± 1.22[c]	12.0 ± 0.19[b]	6.74 ± 0.25[d]
Isoleucine	16.0 ± 0.56[a]	22.1 ± 0.13[b]	24.6 ± 0.97[b]	20.7 ± 0.22[b]	11.2 ± 0.23[c]
Leucine	17.9 ± 0.45[a]	23.1 ± 0.33[b]	33.1 ± 0.84[c]	28.7 ± 0.23[d]	12.5 ± 0.12[e]
Lysine	33.5 ± 0.86[a]	21.2 ± 0.27[b]	26.6 ± 0.79[c]	29.6 ± 0.61[c]	10.7 ± 0.10[d]
Proline	14.8 ± 0.50[a]	17.6 ± 0.12[a]	53.0 ± 1.37[b]	40.0 ± 0.38[c]	16.8 ± 0.24[a]
Cystine	0.42 ± 0.00[a]	0.29 ± 0.00[b]	3.57 ± 0.01[c]	1.33 ± 0.01[d]	0.23 ± 0.00[e]

Values are mean ± SEM, $n = 4$

[A] Jejunal mixed bacteria

[B] Ileal mixed bacteria

[C] Jejunal *Escherichia coli*

[D] Ileal *Klebsiella* sp.

[E] Ileal *Streptococcus* sp.

[F] Aspartate plus asparagine

[G] Glutamate plus glutamine

[a–e] Mean values within a row with different superscript letters differ ($P < 0.05$)

0.59, <0.1, <0.1, and 1.4% of total AA (peptide-bound and free), respectively, in jejunal mixed bacteria, ileal mixed bacteria, jejunal *E. coli*, ileal *Klebsiella* sp., and ileal *Streptococcus* sp. All other free AA represented <0.1% of total AA (peptide-bound and free) in jejunal mixed bacteria, ileal mixed bacteria, jejunal *E. coli*, ileal *Klebsiella* sp., and ileal *Streptococcus* sp. The content of peptide (protein)-bound AA in these bacteria is summarized in Table 4. Aspartate plus asparagine and glutamate plus glutamine were the most abundant AA in proteins of ileal mixed bacteria and ileal *Streptococcus* sp., whereas glycine was the most abundant AA in ileal *Klebsiella* sp. Interestingly, proline was as equally abundant as glutamate plus glutamine in proteins of jejunal *E. coli* and ileal *Klebsiella* sp. (Table 4), whereas lysine was as equally abundant as aspartate plus asparagine in the proteins of jejunal mixed bacteria. The content of glutamate plus glutamine, glycine, and alanine was similar in the proteins of jejunal mixed bacteria. In all of these bacteria, the concentration of cystine was the lowest (Table 4).

Rates of the incorporation of AA into proteins varied greatly with the type of bacteria (Table 5). The rate of incorporation of leucine into proteins was greatest ($P < 0.05$) in ileal mixed bacteria, jejunal *E. coli*, ileal *Klebsiella* sp., and ileal *Streptococcus* sp., whereas the rate of incorporation of glutamine into proteins was greatest ($P < 0.05$) in jejunal mixed bacteria. The rates of incorporation of all the AA into proteins were higher ($P < 0.05$) in jejunal *E. coli* and ileal *Klebsiella* sp. than in jejunal mixed bacteria, ileal mixed bacteria, and jejunal mixed bacteria.

Discussion

Amino acids play important roles in nutrition and physiology (Li et al. 2009b; Rhoads and Wu 2009; Tan et al. 2009a, b). Previous studies of AA metabolism in human faecal microbes demonstrated that lysine, arginine, threonine, histidine, glutamate and aspartate were rapidly fermented

Table 5 Incorporation of ^{14}C-labeled L-amino acids into proteins in bacteria from the pig small intestine

Amino acid	Jejunal bacteria[A]	Ileal bacteria[B]	*Escherichia coli*[C]	*Klebsiella* sp.[D]	*Streptococcus* sp.[E]
nmol/10^8 cells per 3 h					
Arginine	3.69 ± 0.15[a]	3.48 ± 0.11[a]	24.1 ± 0.73[b]	20.9 ± 0.30[b]	7.39 ± 0.52[c]
Glutamine	7.30 ± 0.27[a]	6.01 ± 0.20[b]	24.9 ± 0.35[c]	22.8 ± 0.44[c]	6.80 ± 0.26[ab]
Lysine	5.49 ± 0.12[a]	4.48 ± 0.07[b]	24.2 ± 0.46[c]	22.0 ± 0.50[c]	7.61 ± 0.29[d]
Threonine	5.13 ± 0.20[a]	3.94 ± 0.23[b]	28.4 ± 0.55[c]	17.5 ± 0.23[d]	10.5 ± 0.38[e]
Proline	3.04 ± 0.16[a]	1.08 ± 0.06[b]	19.6 ± 0.63[c]	16.8 ± 0.18[c]	5.78 ± 0.16[d]
Leucine	6.56 ± 0.19[a]	9.26 ± 0.30[b]	36.5 ± 0.49[c]	35.1 ± 0.57[c]	16.9 ± 0.22[d]
Methionine	1.38 ± 0.06[a]	1.24 ± 0.07[a]	9.12 ± 0.57[b]	8.56 ± 0.66[b]	2.41 ± 0.13[c]
Phenylalanine	4.15 ± 0.13[a]	4.54 ± 0.18[a]	16.2 ± 0.38[b]	16.8 ± 0.47[b]	6.09 ± 0.21[c]

Values are mean ± SEM, $n = 4$

[A] Jejunal mixed bacteria

[B] Ileal mixed bacteria

[C] Jejunal *Escherichia coli*

[D] Ileal *Klebsiella* sp

[E] Ileal *Streptococcus* sp

[a–e] Mean values within a row with different superscript letters differ ($P < 0.05$). Culture medium contained 2 mmol/L each of the indicated amino acids plus the respective L-[U-^{14}C]amino acid

during in vitro cultivation (Smith and Macfarlane 1997). The addition of fermentable carbohydrates to the cultivation system reduced AA decarboxylation and the formation of amines by bacteria (Smith and Macfarlane 1996). *Clostridium* and *Bacteriodes* and *Peptostreptococcus* are the predominant AA-fermenting bacteria in the human large intestine (Smith and Macfarlane 1998). Studies with cannulated pigs revealed that lysine, phenylalanine and BCAA synthesized by luminal bacteria can be absorbed by the small and large intestine (Metges 2000; Torrallardona et al. 2003a, b). However, these results cannot explain the net high rate of AA utilization by the gut (van Goudoever et al. 2000). At present, the metabolic fate of nutritionally essential AA in the lumen of the intestine is largely unknown (Wu 2009).

Results of the current study indicated high rates of the utilization of basic (lysine, arginine, and histidine), acidic (glutamate), and neutral (threonine, valine, leucine and isoleucine) AA by intestinal bacteria (Table 1). This supports the previous report that, in young pigs fed a milk protein diet, about 50% of dietary lysine and BCAA were extracted in the first-pass small intestinal metabolism of AA and more than 60% of dietary threonine and 90% of dietary glutamate cannot enter into the portal circulation (Stoll et al. 1998; van der Schoor et al. 2002). Notably, we found that arginine was rapidly taken up by bacteria from culture medium (e.g., more than 20% of the disappearance occurring within 6 h of cultivation). Because enterocytes was not present in the in vitro cultivation, the high rate of arginine utilization must be accounted for by only intestinal microbes. Interestingly, although intestinal bacteria exhibit net use of arginine (Table 3), there is a net output of this AA by the small intestine of postabsorptive pigs (Stoll et al. 1998; Wu et al. 1994). Thus, the gut-derived arginine must be synthesized from mucosal enterocytes of growing pigs (Wu et al. 2009).

Lysine was rapidly utilized with more than 20% disappearing from culture medium after 6 h of cultivation. Since lysine is the first nutritionally limiting AA for pigs (Wu 2009), rapid degradation of luminal lysine by intestinal bacteria may outcompete with the absorption of lysine by enterocytes, thus lowering the entry of dietary lysine into the systemic circulation (van Goudoever et al. 2000). However, the lysine used by the portal-drained viscera (PDV) for protein synthesis is derived from both the arterial circulation and the intestinal lumen (van Goudoever et al. 2000). Thus, luminal utilization of lysine plays an important role in the compartmentalization of lysine metabolism in PDV.

About half of histidine disappeared after 24 h of in vitro cultivation (Table 3). Interestingly, utilization of histidine by the small intestine was not determined by Stoll et al. (1998). Dietary histidine might be extracted during the first-pass metabolism. Indeed, a small amount of dietary histidine was extracted by the ovine small intestine (MacRae et al. 1997). Although, it is still unclear whether dietary or circulating histidine is preferred for utilization by the small intestine (MacRae et al. 1997), the importance of intestinal metabolism (Smith and Macfarlane 1996, 1998) on the immunomodulatory effect of histidine and its metabolites in the gut (Dy and Schneider 2004; Li et al. 2007) should not be neglected.

The gut lumen may be considered as an important "compartment" that harbors exogenous enzymes from both the plant-based diet and gut bacteria. These enzymes could be responsible for one or more steps in AA metabolism. Thus, it should be recognized that we used 5-month-old pigs fed a corn- and soybean meal-based diet in the present study, whereas the previous in vivo work involved either milk formula-fed or sow-reared young pigs (Stoll et al. 1998; Stoll and Burrin 2006). It is possible that the type of diet and the age of pigs may shape the intestinal microbiota and predominance of bacterial strains, therefore influencing AA metabolism and homeostasis.

Another novel finding of this investigation is the gut segment-specific AA utilization. For example, results of the in vitro cultivation (Table 1) indicated that the duodenum could be a significant site for luminal metabolism of dietary glutamate and histidine, whereas lysine, valine, threonine, arginine, leucine and isoleucine might be mainly metabolized in the lumen of the jejunum and ileum. Because the jejunum is the main site for AA absorption (Wu 1998), higher rates of lysine and arginine catabolism in the lumen of the jejunum could outcompete with their transport into enterocytes. Additionally, over a 24-h period of in vitro cultivation, there were more dynamic changes in the utilization of glutamate by duodenal and jejunal mixed bacteria compared with ileal mixed bacteria (Table 1). Notably, glutamate is used for the synthesis of citrulline, arginine and glutamine by luminal bacteria and mucosal enterocytes (Wu et al. 2009; Eklou-Lawson et al. 2009). Considering the length of the pig small intestine (Haynes et al. 2009; Wang et al. 2009b), the compartmentalization of AA metabolism in the lumen of the small intestine could substantially affect the bioavailability of dietary AA to extraintestinal tissues (Fuller and Reeds 1998; Wu 2009).

This study is the first to use subcultures to determine AA utilization by intestinal microbes. While the original 24-h in vitro cultivation possibly included gut microbes and enzymes derived from intestinal mucosal cells, the subculture approach exclusively identified the important role for microbes of the gut lumen in AA utilization by the small intestine. Bacteria could only "survive" during subcultures if they could effectively utilize AA in medium for protein synthesis and/or as fuels. Here, we found that the 24-h disappearance rates of lysine, threonine, arginine and glutamate in all samples remained high during 30 subcultures except for lysine in the duodenum group, while the disappearance rates of leucine, isoleucine and valine were greatly reduced during subcultures (Fig. 2). Thus, microbes derived from the pig small intestine may preferentially utilize dietary lysine, threonine, arginine and glutamate for growth. Based on cell growth, AA composition (Table 4), and the incorporation of AA into polypeptides (Table 5), we estimated that protein synthesis represented approximately 40–60% of AA utilized by jejunal mixed bacteria, ileal mixed bacteria, jejunal *E. coli*, ileal *Klebsiella* sp., and ileal *Streptococcus* sp. A transcriptome profiling study of the human ileum microbiota demonstrated that 20% of the sequences retrieved from the cDNA-AFLP profiles functioned in AA transport and metabolism (Booijink 2009). However, further information about which AA could be actively metabolized by the intestinal microbiota is not known. It is possible that some groups of the microbiota in the small intestine utilize dietary AA (e.g., lysine) for their growth while other groups could synthesize the same AA (e.g., lysine) from ammonia and carbon skeletons.

The current work identified the possible predominant bacterial species responsible for extensive AA metabolism in the pig small intestine. In the non-AA control with NH_4Cl as the only nitrogen source in the medium, *Klebsiella* sp., *E. coli*, *A. lipolytica*-like bacteria and, to a lesser extent, *S. dextrinosolvens* were predominant in cultures (Table 3). Compared to the non-AA control, *Streptococcus* sp., *M. elsdenii*-like bacteria and *Mitsuokella* sp. were the predominant bacteria in almost all the five AA treatments except for the histidine group where *M. elsdenii*-like bacteria was not a predominant species (Table 3). In the rumen, *Streptococcus* bacteria could extensively metabolize lysine, threonine, arginine, glutamate, and other AA (Scheifinger et al. 1976). Furthermore, rates of AA transport and utilization were greater than those for peptides in the bacteria (Lin and Armstead 1995; Westlake and Mackie 1990). Other investigators have found that *M. elsdenii* is one of the major AA-fermenting bacteria in the rumen that could extensively metabolize BCAA (Rychlik et al. 2002; Wallace 1986). Our work ("inocula" in Fig. 3) and previous studies (Leser et al. 2002; Konstantinov et al. 2006) showed that species of *Streptococcus* and *M. elsdenii* were abundant in the pig small intestine, as reported for *Streptococcus* in the human ileum (Booijink 2009). Similarly, *Mitsuokella* is present in the stomach, cecum and small intestine of pigs (Mikkelsen et al. 2007; Tsukahara et al. 2002), but functions of the bacterium are unknown. The coexistence of these bacteria in the gut may regulate AA degradation and synthesis (Chassard et al. 2008; Duncan et al. 2004; Flint et al. 2007; Wallace 1996; Williams et al. 2001). It would be important to determine whether microbial AA metabolism differs between the rumen and the small intestine.

Results of this study led to an important discovery that AA could regulate the composition of the intestinal bacterial population. As noted above, species belong to *Streptococcus*, *M. elsdenii* and *Mitsuokella* may be the predominant bacteria community for AA metabolism in the small intestine. This gives rise to the question of whether dietary supplementation with certain AA may regulate the

growth/proportion of the specific bacterial community. Here, we reported that *A. fermentans* became dominant in culture containing arginine, glutamate or histidine, and *S. dextrinosolvens* became dominant in culture containing histidine during subcultures. An early study showed that glutamate was vital for supporting the growth of *A. fermentans* and that bacterial growth decreased when arginine was omitted from the medium (Rogosa 1969). Thus, *A. fermentans* might be one of the major bacteria responsible for glutamate and/or arginine metabolism in the gut. However, the stimulatory effect of histidine on the growth of *A. fermentans* was not found in a previous study (Rogosa 1969). At present, little information is available about AA metabolism in *S. dextrinosolvens*. The preferred substrates for *S. dextrinosolvens* are pectin, dextrin and sugar in the rumen (Russell and Rychlik 2001), suggesting that the bacterium utilizes histidine or its ammonia product for protein synthesis. It is possible that some AA play important roles in prevention and control of gut infectious diseases (Wang et al. 2009a; Schaible and Kaufmann 2005).

Collectively, our results support the proposition that bacteria in the small intestine may be primarily responsible for the degradation of dietary essential and nonessential AA by the small intestine (Chen et al. 2007, 2009). The compartmentalization of AA metabolism in the lumen of the small intestine may exist at three levels: (1) different sites of the small intestine (gut segments, lumen, and mucosa); (2) different cell types (different species of bacteria and their functional redundancy); and (3) different cellular sites (cell membrane, cytoplasm, mitochondria). The regulation of protein and AA metabolism in the lumen of the small intestine at different levels by dietary supplementation of specific AA or other supplements may potentially provide a novel means for regulating microbial composition and activity. Thus, the ecological adaptation of microbes along the gastrointestinal tract may influence metabolism and health in animals and humans, particularly they receive dietary supplementation with prebiotics and probiotics (Bergen and Wu 2009; Booijink et al. 2007; Ley et al. 2006; Martin et al. 2008, 2009).

In conclusion, this study demonstrated high rates of the utilization of lysine, arginine, threonine, glutamate, leucine, isoleucine, valine and histidine by luminal bacteria from the pig small intestine. Using a novel gut-microbe-subculture approach, we found that small intestinal bacteria, particularly *Streptococcus*, *M. elsdenii* and *Mitsuokella,* degraded AA in a species-dependent manner and that protein synthesis was a major pathway for AA metabolism in all the bacteria studied. These results provide a new framework for future studies to characterize the metabolic fate of AA in intestinal bacteria and define their nutritional significance in animals and humans.

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References

Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ (1990) Basic local alignment search tool. J Mol Biol 215:403–410

Baker DH (2009) Advances in protein-amino acid nutrition of poultry. Amino Acids 37:29–41

Bergen WG, Wu G (2009) Intestinal nitrogen recycling and utilization in health and disease. J Nutr 139:821–825

Blachier F, Mariotti F, Huneau JF, Tomé D (2007) Effects of amino acid-derived luminal metabolites on the colonic epithelium and physiopathological consequences. Amino Acids 33:547–562

Blachier F, Lancha AH Jr, Boutry C, Tomé D (2010) Alimentary proteins, amino acids and cholesterolemia. Amino Acids 38:15–22

Booijink CCGM (2009) Analysis of diversity and function of the human small intestinal microbiota. Thesis, Wageningen University Press, The Netherlands

Booijink CCGM, Zoetendal EG, Kleerebezem M, de Vos WM (2007) Microbial community in the human small intestine: coupling diversity to metagenomics. Future Microbiol 2:285–295

Burrin DG, Reeds PJ (1997) Alternative fuels in the gastrointestinal tract. Curr Opin Gastroenterol 13:165–170

Chassard C, Scott KP, Marquet P, Martin JC, Del'homme C, Dapoigny M, Flint HJ, Bernalier-Donadille A (2008) Assessment of metabolic diversity within the intestinal microbiota from healthy humans using combined molecular and cultural approaches. FEMS Microbiol Ecol 66:496–504

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

Chen LX, Li P, Wang JJ, Li XL, Gao HJ, Yin YL, Hou YQ, Wu G (2009) Catabolism of nutritionally essential amino acids in developing porcine enterocytes. Amino Acids 37:143–152

Duncan S, Louis P, Flint HJ (2004) Lactate-utilizing bacteria, isolated from human feces, that produce butyrate as a major fermentation product. Appl Environ Microbiol 70:5810–5817

Dy M, Schneider E (2004) Histamine–cytokine connection in immunity and hematopoiesis. Cytokine Growth Factor Rev 15:393–410

Eklou-Lawson M, Bernard F, Neveux N, Chaumontet C, Bos C, Davila-Gay AM, Tome D, Cynober L, Blachier F (2009) Colonic luminal ammonia and portal blood L-glutamine and L-arginine concentrations: a possible link between colon mucosa and liver ureagenesis. Amino Acids 37:751–760

Elango R, Ball RO, Pencharz PB (2009) Amino acid requirements in humans: with a special emphasis on the metabolic availability of amino acids. Amino Acids 37:19–27

Favier CF, Vaughan EE, De Vos WM, Akkermans AD (2002) Molecular monitoring of succession of bacterial communities in human neonates. Appl Environ Microbiol 68:219–226

Flint HJ, Duncan S, Scott KP, Louis P (2007) Interactions and competition within the microbial community of the human colon: links between diet and health. Environ Microbiol 9:1101–1111

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

Haynes TE, Li P, Li XL, Shimotori K, Sato H, Flynn NE, Wang JJ, Knabe DA, Wu G (2009) L-Glutamine or L-alanyl- L-glutamine prevents oxidant- or endotoxin- induced death of neonatal enterocytes. Amino Acids 37:131–142

He QH, Kong XF, Wu G, Ren PP, Tang HR, Hao FH, Huang RL, Li TJ, Tan BE, Li P, Tang ZR, Yin YL, Wu YN (2009) Metabolomic analysis of the response of growing pigs to dietary L-arginine supplementation. Amino Acids 37:199–208

Kong XF, Yin YL, He QH, Yin FG, Liu HJ, Li TJ, Huang RL, Geng MM, Ruan Z, Deng ZY, Xie MY, Wu G (2009) Dietary supplementation with Chinese herbal powder enhances ileal digestibilities and serum concentrations of amino acids in young pigs. Amino Acids 37:573–582

Konstantinov SR, Zhu WY, Williams BA, Tamminga S, de Vos WM, Akkermans ADL (2003) Effect of fermentable carbohydrates on piglet faecal bacterial communities as revealed by denaturing gradient gel electrophoresis analysis of 16S ribosomal DNA. FEMS Microbiol Ecol 43:225–235

Konstantinov SR, Awati AA, Williams BA, Miller BG, Jones P, Stokes CR, Akkermans ADL, Smidt H, de Vos WM (2006) Post-natal development of the porcine microbiota composition and activities. Environ Microbiol 8:1191–1199

Leser TD, Amenuvor JZ, Jensen TK, Lindecrona RH, Boye M, Møller K (2002) Culture-independent analysis of gut bacteria: the pig gastrointestinal tract microbiota revisited. Appl Environ Microbiol 68:673–690

Ley RE, Peterson DA, Gordon JI (2006) Ecological and evolutionary forces shaping microbial diversity in the human intestine. Cell 124:837–848

Li P, Yin YL, Li D, Kim SW, Wu G (2007) Amino acids and immune function. Br J Nutr 2007(98):237–252

Li M, Wang B, Zhang M, Rantalainen M, Wang S, Zhou H, Zhang Y, Shen J, Pang X, Zhang M, Wei H, Chen Y, Lu H, Zuo J, Su M, Qiu Y, Jia W, Xiao C, Smith LM, Yang S, Holmes E, Tang H, Zhao G, Nicholson JK, Li L, Zhao L (2008) Symbiotic gut microbes modulate human metabolic phenotypes. Proc Natl Acad Sci USA 105:2117–2122

Li P, Kim SW, Li XL, Datta S, Pond WG, Wu G (2009a) Dietary supplementation with cholesterol and docosahexaenoic acid affects concentrations of amino acids in tissues of young pigs. Amino Acids 37:709–716

Li XL, Bazer FW, Gao H, Jobgen W, Johnson GA, Li P, McKnight JR, Satterfield MC, Spencer TE, Wu G (2009b) Amino acids and gaseous signaling. Amino Acids 37:65–78

Lin JR, Armstead IP (1995) The in vitro uptake and metabolism of peptides and amino acids by five species of rumen bacteria. J Appl Bacteriol 78:116–124

MacRae JC, Bruce LA, Brown DS, Calder AG (1997) Amino acid use by the gastrointestinal tract of sheep given lucerne forage. Am J Physiol Gastrointest Liver Physiol 273:1200–1207

Martin F-PJ, Wang Y, Sprenger N, Yap IKS, Lundstedt T, Lek P, Rezzi S, Ramadan Z, Bladeren PV, Fay LB, Kochhar S, Lindon JC, Holmes E, Nicholson JK (2008) Probiotic modulation of symbiotic gut microbial–host metabolic interactions in a humanized microbiome mouse model. Mol Syst Biol 4:157

Martin F-PJ, Sprenger N, Yap IKS, Wang Y, Bibiloni R, Rochat F, Rezzi S, Cherbut C, Kochhar S, Lindon JC, Holmes E, Nicholson JK (2009) Panorganismal gut microbiome-host metabolic crosstalk. J Proteome Res 8:2090–2105

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

Metges CC, El-Khoury AE, Henneman L, Petzke KJ, Grant I, Bedri S, Pereira PP, Ajami AM, Fuller MF, Young VR (1999) Availability of intestinal microbial lysine for whole body lysine homeostasis in human subjects. Am J Physiol 277:E597–E607

Mikkelsen LL, Højberg O, Jensen BB (2007) Coarse structured feed stimulates members of the genera *Lactobacillus* and *Mitsuokella* as well as propionate and butyrate producers in the pig stomach. Livest Sci 109:153–156

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

Rhoads JM, Wu G (2009) Glutamine, arginine, and leucine signaling in the intestine. Amino Acids 37:111–122

Rogosa M (1969) *Acidaminococcus* gen. n., *Acidaminococcus fermentans* sp. n., anaerobic gram-negative diplococci using amino acids as the sole energy source for growth. J Bacteriol 98:756–766

Ros M, Goberna M, Pascual PA, Klammer S, Insam H (2008) 16S rDNA analysis reveals low microbial diversity in community level physiological profile assays. J Microbiol Methods 72:221–226

Russell JB, Rychlik JL (2001) Factors that alter rumen microbial ecology. Science 292:1119–1122

Rychlik JL, LaVera R, Russell JB (2002) Amino acid deamination by ruminal *Megasphaera elsdenii* strains. Curr Microbiol 45:340–345

Sanguinetti CJ, Dias Neto E, Simpson AJG (1994) Rapid silver staining and recovery of PCR products separated on polyacrylamide gels. BioTechniques 17:915–919

Schaible UE, Kaufmann SHE (2005) A nutritive view on the host–pathogen interplay. Trends Microbiol 13:373–380

Scheifinger C, Russell N, Chalupa W (1976) Degradation of amino acids by pure cultures of rumen bacteria. J Anim Sci 43:821–827

Smith EA, Macfarlane GT (1996) Studies on amine production in the human colon: enumeration of amine-forming bacteria and physiological effects of carbohydrate and pH. Anaerobe 2:285–297

Smith EA, Macfarlane GT (1997) Dissimilatory amino acid metabolism in human colonic bacteria. Anaerobe 3:327–337

Smith EA, Macfarlane GT (1998) Enumeration of amino acid fermenting bacteria in the human large intestine: effects of pH and starch on peptide metabolism and dissimilation of amino acids. FEMS Microbiol Ecol 25:355–368

Stoll B, Burrin DG (2006) Measuring splanchnic amino acid metabolism in vivo using stable isotopic tracers. J Anim Sci 84:E60–E72

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 128:606–614

Tan B, Yin Y, Liu Z, Li X, Xu H, Kong X, Huang R, Tang W, Shinzato I, Smith SB, Wu G (2009a) Dietary L-arginine supplementation increases muscle gain and reduces body fat mass in growing-finishing pigs. Amino Acids 37:169–175

Tan B, Li XG, Kong XF, Huang RL, Ruan Z, Yao K, Deng ZY, Xie MY, Shinzato I, Yin YL, Wu G (2009b) Dietary L-arginine supplementation enhances the immune status in early-weaned piglets. Amino Acids 37:323–331

Tan BE, Yin YL, Kong XF, Li P, Li XL, Gao HJ, Li XG, Huang RL, Wu G (2009c) L-Arginine stimulates proliferation and prevents endotoxin-induced death of intestinal cells. Amino Acids. doi: 10.1007/s00726-009-0334-8

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

Torrallardona D, Harris CI, Fuller MF (2003b) Lysine synthesized by the gastrointestinal microflora of pigs is absorbed, mostly in the small intestine. Am J Physiol Endocrinol Metab 284:E1177–E1180

Tsukahara T, Koyama H, Okada M, Ushida K (2002) Stimulation of butyrate production by gluconic acid in batch culture of pig cecal

digesta and identification of butyrate-producing bacteria. J Nutr 132:2229–2234
van der Schoor SRD, Reeds PJ, Stoll B, Henry JF, Rosenberg JR, Burrin DG, van Goudoever JB (2002) The high metabolic cost of a functional gut. Gastroenterology 123:1931–1940
van Goudoever JB, Stoll B, Henry JF, Burrin DG, Reeds PJ (2000) Adaptive regulation of intestinal lysine metabolism. Proc Natl Acad Sci USA 97:11620–11625
Wallace RJ (1986) Catabolism of amino acids by *Megasphaera elsdenii* LC1. Appl Environ Microbiol 51:1141–1143
Wallace RJ (1996) Ruminal microbial metabolism of peptides and amino acids. J Nutr 126:1326S–1334S
Wang WW, Qiao SY, Li DF (2009a) Amino acids and gut function. Amino Acids 37:105–110
Wang X, Ou D, Yin J, Wu G, Wang J (2009b) Proteomic analysis reveals altered expression of proteins related to glutathione metabolism and apoptosis in the small intestine of zinc oxide-supplemented piglets. Amino Acids 37:209–218
Westlake K, Mackie RI (1990) Peptide and amino acid transport in *Streptococcus bovis*. Appl Microbiol Biotechnol 34:97–102
Whitt DD, DeMoss RD (1975) Effect of microflora on the free amino acid distribution in various regions of the mouse gastrointestinal tract. Appl Microbiol 30:609–615
Williams BA, Verstegen MWA, Tamminga S (2001) Fermentation in the large intestine of single-stomached animals and its relationship to animal health. Nutr Res Rev 14:207–227
Williams BA, Bosch MW, Boer H, Verstegen MWA, Tamminga S (2005) An in vitro batch culture method to assess potential fermentability of feed ingredients for monogastric diets. Anim Feed Sci Technol 123–124:445–462
Wu G (1998) Intestinal mucosal amino acid catabolism. J Nutr 128:1249–1252
Wu G (2009) Amino acids: metabolism, functions, and nutrition. Amino Acids 37:1–17
Wu G, Borbolla AG, Knabe DA (1994) The uptake of glutamine and release of arginine, citrulline and proline by the small intestine of developing pigs. J Nutr 124:2437–2444
Wu G, Ott TL, Knabe DA, Bazer FW (1999) Amino acid composition of the fetal pig. J Nutr 129:1031–1038
Wu G, Bazer FW, Davis TA, Kim SW, Li P, Rhoads JM, Satterfield MC, Smith SB, Spencer TE, Yin YL (2009) Arginine metabolism and nutrition in growth, health and disease. Amino Acids 37:153–168
Yin FG, Liu YL, Yin YL, Kong XF, Huang RL, Li TJ, Wu GY, Hou YQ (2009) Dietary supplementation with Astragalus polysaccharide enhances ileal digestibilities and serum concentrations of amino acids in early weaned piglets. Amino Acids 37:263–270
Zhang J (2009) Isolation and identification of amino acid utilizing bacteria from the porcine small intestine. Thesis, Nanjing Agricultural University, Nanjing, China
Zoetendal EG, Heilig HGHJ, Klaassens ES, Booijink CCGM, Kleerebezem M, Smidt H, de Vos WM (2006) Isolation of DNA from bacterial samples of the human gastrointestinal tract. Nat Protoc 1:870–873