Duodenum has the greatest potential to absorb soluble non-ammonia nitrogen in the nonmesenteric gastrointestinal tissues of dairy cows

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Abstract: In cattle, dietary protein is gradually degraded into peptide-bound amino acids (PBAA), free amino acids (FAA), and ultimately into ammonia by the rumen microbes. Both PBAA and FAA are milk protein precursors, and the rumen and small intestines are the main sites where such precursors are produced and absorbed. This work was designed to investigate the expression of the peptide transporter PepT1 and the AA transporters ASCT2, y+LAT1, and ATB0,+, and the concentrations of PBAA, FAA, and soluble protein in the rumen, omasum, and duodenum of dairy cows. Tissues and digesta were collected from six healthy Chinese Holstein dairy cows immediately after the animals were slaughtered. The expression of transporters was analyzed by real-time quantitative polymerase chain reaction (PCR). The FAA concentration was assessed using an amino acid (AA) analyzer, PBAA concentration by quantification of AA before and after acid-hydrolysis by 6 mol/L HCl, and soluble protein concentration by quantification of the bicinchoninic acid content. The results showed that the relative abundance of mRNA of the transporters and the soluble non-ammonia nitrogen (SNAN) concentration of each fraction were greater in the duodenum than in the rumen or omasum. These results indicate that the duodenum is the predominant location within the nonmesenteric digestive tract for producing milk protein precursors. In addition, PBAA was the largest component of SNAN in the digesta from the rumen, omasum, and duodenum. In conclusion, the duodenum has the greatest concentrations of SNAN and PBAA, and the greatest potential for absorption of SNAN in the form of PBAA in the nonmesenteric gastrointestinal tissues of dairy cows.

Key words: Soluble non-ammonia nitrogen, Nonmesenteric gastrointestinal tissues, Transporters, Dairy cows

1 Introduction

Protein, as one of the most important nutrients for ruminants, is gradually degraded by microbes into peptide-bound amino acids (PBAA), free amino acids (FAA), and ultimately ammonia after entering the rumen. Then, the PBAA, FAA, and ammonia are used for the synthesis of microbial protein by rumen microbes. Some dietary soluble protein can escape ruminal degradation and increase the supply of amino acid (AA) for milk production (Choi et al., 2002c). Soluble non-ammonia nitrogen (SNAN) in omasal digesta (OD) indicates the presence of the terminal products of rumen degradation and partly constitutes the liquid phase nitrogen available for entering the intestine (Oh et al., 2008). Previous reports have found that a significant amount of nitrogen (N) can escape degradation in the rumen in the form of peptides and supply AAs for the intestine, especially in the proximal part (Volden et al., 2002; Rémond et al., 2009). PBAA and FAA constitute milk protein precursors (Tagari et al., 2008), and the rumen and small
intestine may be the main parts of the gastrointestinal where such precursors are produced and absorbed (Zhao et al., 2012). Peptides are hydrolyzed inside enterocytes and AAs are released together with those absorbed by AA transporters. Through the blood circulation, AAs are delivered mainly to the mammary gland where they serve as building blocks for milk protein synthesis. The main site of peptide adsorption is nonmesenteric-drained viscera, and nonmesenteric drainage coming from the rumen, reticulum, omasum, abomasum, duodenum and spleen (Webb et al., 1993). The duodenum is an important component of the digestive tract, forming part of the nonmesenteric system and small intestine. Many studies concerning the rumen and omasum have been published, but few have investigated the concentration and proportion of peptides and other forms of SNAN in the rumen, omasum, and duodenum.

Peptides have their own transport systems independent of those responsible for transporting FAA (Webb, 1990). It is generally recognized that the main method of absorption is by intermediate peptide transporters. AAs are transported by different transporter systems. However, in the small intestine, omasum, and rumen of sheep and dairy cows peptides are transported mainly by peptide transporter 1 (PepT1) (Gilbert et al., 2008). Transporter ASCT2 is a neutral AA transporter and \( y^+LAT1 \) is a cationic transporter, and both are expressed in the apical membrane of the small intestine. \( \text{ATB}^{0,+} \) can transport both neutral and cationic AAs and is expressed in the basolateral membrane of the small intestine (Bröer, 2008; Liao et al., 2008; 2009). However, the expression profiles of these transporters in the nonmesenteric gastrointestinal tissues of dairy cows are unknown. Therefore, the purpose of this study was to investigate the SNAN concentrations of each fraction in the rumen, omasum, and duodenum, the proportion of peptides, and the expression of PepT1 and AA transporters on the membrane of the nonmesenteric gastrointestinal.

2 Materials and methods

2.1 Animal management

Six healthy Chinese Holstein dairy cows in late lactation, weighing (643.0±67.4) kg and with milk production of (13.40±2.05) kg/d, were killed by stunning with a captive-bolt pistol, followed by exsanguination. Before slaughter, the cows were separately housed in individual tie-stalls with free access to water. To satisfy basic needs and standardize the rumen condition, a basal diet was fed to the cows twice daily, at 07:00 and 19:00. The proportions of dietary ingredients and the chemical composition of experimental feeds are shown in Table 1. Three hours elapsed between the last feed and the slaughtering of the cows.

<table>
<thead>
<tr>
<th>Dietary ingredient</th>
<th>Proportion (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Silage corn</td>
<td>23</td>
</tr>
<tr>
<td>Chinese wildrye</td>
<td>26</td>
</tr>
<tr>
<td>DDGS</td>
<td>10</td>
</tr>
<tr>
<td>Corn</td>
<td>26</td>
</tr>
<tr>
<td>Bran</td>
<td>6.8</td>
</tr>
<tr>
<td>Bean cake</td>
<td>2</td>
</tr>
<tr>
<td>Cottonseed meal</td>
<td>4</td>
</tr>
<tr>
<td>Additive-mineral and vitamin</td>
<td>0.3</td>
</tr>
<tr>
<td>CaHPO4</td>
<td>1.4</td>
</tr>
<tr>
<td>Salt</td>
<td>0.5</td>
</tr>
<tr>
<td>Composition</td>
<td></td>
</tr>
<tr>
<td>Dry matter</td>
<td>76.9</td>
</tr>
<tr>
<td>Crude protein</td>
<td>11.0</td>
</tr>
<tr>
<td>Ether extract</td>
<td>3.3</td>
</tr>
<tr>
<td>Ash</td>
<td>4.2</td>
</tr>
<tr>
<td>Neutral detergent fiber</td>
<td>31.0</td>
</tr>
<tr>
<td>Acid detergent fiber</td>
<td>19.8</td>
</tr>
<tr>
<td>Intake</td>
<td>15.2 (kg/d)</td>
</tr>
</tbody>
</table>

\(^1\) Distillers dried grains with solubles. * All data are expressed as percentage except for intake (kg/d)

2.2 Real-time quantitative polymerase chain reaction (qPCR) of transporter mRNA

Ruminal, omasal, and duodenal epithelium were taken from the cows immediately after slaughter. Total RNA was extracted using a TRIZol kit (Invitrogen, USA). The purity and quantity of total RNA were measured using a NANODROP 2000 Spectrophotometer (Thermo, USA). RNA of all samples had a ratio of optical density at 260 nm to that at 280 nm between 1.8 and 2.0. Synthesis of cDNA was carried out using PrimeScript RT reagent Kit Perfect Real Time (TaKaRa, Shiga, Japan). The primers for PepT1 and GAPDH were from Xu et al. (2014). The primers of ASCT2, \( y^+LAT1 \), and \( \text{ATB}^{0,+} \), separated by at least
one intron on the corresponding genomic DNA, were designed and synthesized (Biotechnology, China) as in Table 2. The qPCR of reverse-transcription products was performed using an ABI Prism 7500 Sequence Detection System (Applied Biosystems, USA). Components of a 20-μl qPCR reaction were used according to the manufacturer’s protocol (TaKaRa). The PCR conditions were 95 °C for 30 s, 40 cycles of 95 °C for 5 s and 60 °C for 34 s using a continuous fluorescence measurement. Sequencing of the amplified PCR products showed a 100% match to known bovine DNA sequences in GenBank. Each cDNA sample from each cow was analyzed in triplicate. 

2.3 Sampling and chemical analysis

Ruminal digesta (RD), omasal digesta (OD) and duodenal digesta (DD) were taken from the cows immediately after slaughter. The digesta were collected in 50-ml centrifuge tubes and immediately frozen (−80 °C). Before the analysis, RD and DD were kept in an ice bath and then filtered through 8 layers of gauze. A total of 50 ml of filtrate was saved for later experiments. Five grams of ODs were mixed with 30 ml of McDougall’s buffer in a ratio of 1:6 (w/v) (McDougall, 1948), and kept in an ice bath for 24 h. The digesta was made according to the procedure proposed by Choi et al. (2002b). All the filtrates were centrifuged at 20,000×g for 15 min at 4 °C to eliminate small particles and rumen protozoa and bacteria. Once completed, the supernatant was divided into two portions: one was assigned to the assessment of SNAN concentration (FAA-N), and the other was hydrolyzed by trichloroacetic acid. The trichloroacetic acid was substituted by 25% perchloric acid, and the supernatant was divided into three parts to measure the concentrations of FAA, peptides, and soluble protein. Concentrations of soluble FAA in the supernatant without hydrolysis were determined using an AA analyzer (Biochrom 20, Pharmacia Biotech Ltd., Cambridge, UK). The peptide concentration was estimated as N from hydrolysed supernatant minus N present as FAA. Soluble protein was estimated from the hydrolysis of bicinchoninic acid and quantified by its optical density at 562 nm using a microplate reader (Molecular Devices, USA), based on the standard curve of bovine serum albumin. The tissues of the rumen, omasum, and duodenum were immediately taken from the cows after slaughter. The duodenal mucosa was scraped from the proximal part of the small intestine. The rumen and omasal papillae were sheared from rumen and omasal tissues. The epithelium was scraped from tissues and frozen in 1.5-ml centrifuge tubes at −80 °C.

2.4 Calculations and statistical analysis

To quantify the expression of PepT1 and AA transporters, the relative quantification method proposed by Schmittgen and Livak (2008) was used. In this study, the SNAN concentration of each fraction was calculated for the 50 ml of fluid or digesta of the gastrointestinal, as described by Choi et al. (2002b; 2002c): 

\[ C_{\text{FAA-N}} = \frac{C_{\text{FAA}}}{6.25} \]

\[ C_{\text{PBAA-N}} = \frac{(C_{\text{TAA}} - C_{\text{FAA}})}{6.25} \]

\[ C_{\text{soluble protein N}} = \frac{C_{\text{psp}}}{6.25} \]

where \( C_{\text{FAA}} \) is the concentration of FAA in the supernatant, \( C_{\text{TAA}} \) is the concentration of FAA after hydrolyzing by 6 mol/L HCl, and \( C_{\text{psp}} \) is the concentration of protein quantified by bicinchoninic acid.

The rumen was used as calibrator and GAPDH was used as an internal control: 

\[ -\Delta\Delta C_T = (C_{T, \text{gene of interest}} - C_{T, \text{internal control}, A}) - (C_{T, \text{gene of interest}} - C_{T, \text{internal control}, B}) \]

where sample B was the calibrator and sample A was a sample from one of the other two parts.

<table>
<thead>
<tr>
<th>Gene</th>
<th>GenBank ID</th>
<th>Sense/antisense primer (5'→3')</th>
<th>Product size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PepT1</td>
<td>NM_001099378</td>
<td>TGGCTGGGGAGAATTCAGAAC/TCCGGCCCTTCTTCAA</td>
<td>239</td>
</tr>
<tr>
<td>ASCT2</td>
<td>NM_00173601.2</td>
<td>CTTGATCTTGGGCGGTCGACT/AGTGGCTCTCGTCGCGA</td>
<td>119</td>
</tr>
<tr>
<td>yTLAT1</td>
<td>NM_001075151.1</td>
<td>AGGGTGTTGCTCTGGTGGTT/CTGGAGGGAATTCATTGACT</td>
<td>138</td>
</tr>
<tr>
<td>ATB6+</td>
<td>NM_001098461.1</td>
<td>GCTGGTGTTATGATTACTTACTGGTC/CCCCAGATGTTTTTCCAGA</td>
<td>150</td>
</tr>
<tr>
<td>GAPDH</td>
<td>AJ000039</td>
<td>GCCAAGAGGGTACTCATCTTC/GGTCAAGGAGGCCATCAGA</td>
<td>197</td>
</tr>
</tbody>
</table>

Table 2 Primers used for real-time quantitative PCR
of the digestive tract. The sample with the lowest $\Delta C_T$ was used as calibrator within each comparison. For the calculation of $PepT1$ expression, the rumen was chosen as calibrator, and for AA transporters, the omasum was chosen. The relative change in the $PepT1$ mRNA was calculated using the $2^{-\Delta\Delta C_T}$ method (Livak and Schmittgen, 2001).

The experimental data were statistically analyzed by analysis of variance (ANOVA) and Duncan’s multiple range tests using SAS method Version 9 (SAS Institute Inc., Cary, NC, USA). The data are presented as the mean-standard error (SE). A level of $P<0.05$ was accepted as being statistically significant.

3 Results

3.1 mRNA expression of $PepT1$ and AA transporters in the rumen, omasum, and duodenum

Expression of $PepT1$, $ATB^{0,+}$, $ASCT2$, and $y^+LAT1$ in the duodenum was significantly higher than that in the rumen and omasum (Fig. 1) ($P<0.05$). $PepT1$ expression in the duodenum was 20.1-fold greater than that in the rumen and 14.3-fold greater than that in the omasum. $ATB^{0,+}$ expression in the duodenum was 33.7-fold greater than that in the rumen and 310.6-fold greater than that in the omasum. The expression of $ASCT2$ in the duodenum was 1.65-fold greater than that in the rumen and 3.1-fold greater than that in the omasum. $y^+LAT1$ expression in the duodenum was 11.0-fold greater than that in the rumen and 55.7-fold greater than that in the omasum.

3.2 Concentrations of FAA-N and PBAA-N in ruminal, omasal, and duodenal digesta

The forage-to-concentrate ratio was 50:50. Mean concentrations of FAA-N (N in the form of FAA), PBAA-N, and soluble protein N are shown in Table 3. The SNAN concentrations of each fraction of the duodenum were 3.8 to 5 times higher than those of the omasum ($P<0.01$), and 7 to 11 times higher than those of the rumen. The ruminal SNAN concentrations of each fraction were 1.8 to 2.2 times lower than those in the omasum. PBAA constituted the largest proportion of SNAN, namely 44.4% to 51.6%, from the duodenum to the rumen. All FAA-N concentrations except Pro in the duodenum were significantly higher than those in the rumen and omasum (Table 4) ($P<0.01$). Most

![Fig. 1 Relative expression of $PepT1$ and AA transporters in the rumen, omasum and duodenum](image)

The mRNA expression of $PepT1$, $ATB^{0,+}$, $ASCT2$, and $y^+LAT1$ in the duodenum was higher than that in the rumen and omasum ($2^{-\Delta\Delta C_T}$ value). For the calculation of $PepT1$ expression, the rumen was chosen as calibrator (a), and for AA transporters, the omasum was chosen as calibrator (b, c, d). Values with different letters (A and B) are considered to be significantly different ($P<0.05$). Data are expressed as mean±SE ($n=6$).
PBAA-N concentrations in the duodenum were significantly higher than those in the rumen and omasum ($P < 0.01$). In addition, cysteine and cystine were oxidatively degraded, due to the use of HCl in the pretreatment.

### 4 Discussion

Macromolecular proteins, DNA, and RNA can be precipitated by 25% perchloric acid. Peptides (of less than fifty AAs) can be hydrolyzed to FAAs by HCl. Peptides greater than six AAs are generally thought to be cleaved to fewer than six AAs by pancreatic-released proteases/peptidases. The smaller size peptides are then degraded into di- or tri-peptides and FAAs by a variety of peptidases in the apical membranes of intestinal epithelial cells (Clark et al., 1992). PBAAs entering the duodenum from the abomasum have two sources: microbial metabolism and digestion of microbial protein by gastric acid and pepsin. In this study, more of the duodenal PBAA may have come from microbial protein due to the low lactation yield of the dairy cows, the requirements of which can be almost met by the microbial protein alone (Zhu et al., 2013). In addition, digesta represent serial events resulting in concentrations that are not proportional to absorption events. For example, omasal digesta is concentrated relative to rumen and duodenal digesta and, at least in vitro, omasal tissue appears to have a greater capacity for dipeptide absorption than does ruminal tissue (Matthews and Webb, 1995; Matthews et al., 1996; McCollum et al., 2000). Therefore, we investigated the expression of peptide and AA transporters and the concentrations of PBAAs, FAA and soluble protein in the rumen, omasum, and duodenum of dairy cows to find out which organ has the greatest potential to absorb SNAN in nonmesenteric gastrointestinal tissues.

### Table 3  SNAN concentrations of each fraction in the ruminal, omasal, and duodenal digesta ($n=6$)

<table>
<thead>
<tr>
<th>Composition</th>
<th>Concentration (mg N/100 ml)</th>
<th>SE</th>
<th>$P$-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>FAA-N</td>
<td>22.42$^{A}$ 49.87$^{B}$ 189.77$^{A}$</td>
<td>24.504</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>PBAA-N</td>
<td>40.01$^{B}$ 73.30$^{B}$ 285.46A</td>
<td>35.798</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Soluble protein N</td>
<td>15.10$^{B}$ 34.57$^{B}$ 168.41A</td>
<td>16.394</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Total N</td>
<td>77.53$^{B}$ 157.73$^{B}$ 643.64A</td>
<td>65.757</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>PBAA-N/Total N</td>
<td>51.6% 46.5% 44.4%</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

SE: standard error of means. Values in the same row with different letters (A and B) are considered to be significantly different ($P<0.01$)
The expression of AA transporters in the duodenum was significantly higher than that in the rumen and omasum (Fig. 1). ASC2T is a high-affinity neutral AA transporter at the apical membrane and can interact with anionic AAs at the low pH of the intestine, especially glutamate (Bröer, 2008). ATB0,++ is a high-affinity neutral and cationic AA transporter expressed at the apical membrane. Liao et al. (2009) found ATB0,++ and y+LAT1 mRNA expression in the duodenum, jejunum, and ileum of cattle (growing steers), and mRNA expression by duodenal epithelia was greater than that by the jejunum or ileum. The trend of expression of y+LAT1 and ATB0,++ mRNA was relevant to our current findings. Briefly, their expression was highest in the duodenum. This result indicates that the duodenum may have a greater capability for the absorption of neutral and cationic AAs than the rumen and omasum. In addition, the concentration of FAA-N in the duodenum was greater than those in the rumen and omasum (Tables 3 and 4). This result agrees with previous studies reporting that AAs escape from the rumen due to the degradation of protein by rumen microbes and enter the duodenum, thus differing from those in the feed consumed (Piepenbrink and Schingoethe, 1998; Taghizadeh et al., 2005). Incubation of the intestine also has a considerable effect on AA profiles for all feedstuffs. For most feedstuffs, this can be explained by the fact that secretions of trypsin, chymotrypsin, elastase, and carboxypeptidases A and B from the pancreas show specificity for PBAA. Their maximal activity occurs in the proximal segments of the small intestine (Chen et al., 1987; Taghizadeh et al., 2005). In the present study, concentrations of PB-Thr, PB-Tyr, PB-Phe, and PB-Arg in the omasum were different from those in the duodenum, confirming the activity of pancreatic enzymes. Although both AA concentrations and AA transporter mRNA expression were greater, the magnitude of the AA concentration was much less than that of mRNA expression. The concentration of AAs and the expression of AA transporters in the duodenum were significantly higher than those in the rumen and omasum.

In this study, the mean concentrations of FAA-N were lower than published values (6.30–33.37 mg N/L and 12.20–56.23 mg N/L, respectively) (Choi et al., 2002a; 2002b; 2002c; Choi and Choi, 2003). One reason may be that incomplete acid precipitation of proteins results in overestimated peptide fractions, as discussed by Reynal et al. (2007). The dietary protein proportion (11%) was lower than that found in previous studies (Choi et al., 2002a; 2002b; 2002c; Choi and Choi, 2003). The relatively low dietary protein led to less degradation of protein into SNAN and less use of available SNAN by rumen microbes. Thus, less microbial protein was provided to the duodenum. Another reason may be that different methods to deal with the digesta resulted in different SNAN concentrations left in the liquid phase of the digesta. In addition, small peptides can be absorbed by PepT1 by the epithelial cells of the gastrointestinal tract without further degradation (Xu et al., 2014). The expression of PepT1 increased significantly from the rumen to the duodenum, but not significantly between the rumen and the omasum (Fig. 1). This result indicates that the duodenum may be the major nonmesenteric gastrointestinal for peptide absorption. In addition, the trend of PBAA-N concentrations was similar to the trend of PepT1 expression in the rumen, omasum, and duodenum. This may be because peptides stimulate the expression of PepT1 to transport more peptides across the epithelium. PepT1 increased with the increase in peptide concentration in the nonmesenteric gastrointestinal. In this study, the concentrations of PBAA-N increased from the rumen to the duodenum. This may be because some PBAA-N was used by rumen microbes, and microbial protein was degraded into PBAA by the large number of peptidase enzymes in the duodenum (Clark et al., 1992). These results confirm that the duodenum is the major nonmesenteric gastrointestinal for peptide absorption. In addition, we found that PBAA-N was lower than that found in some previous studies (Choi et al., 2002a; 2002b; 2002c; Choi and Choi, 2003). One reason may be that incomplete acid precipitation of proteins results in overestimated peptide fractions, as discussed by Reynal et al. (2007). Another reason may be that the methods to deal with the digesta may result in different SNAN concentrations left in the liquid phase of the digesta. In this study, a low crude protein diet was used to feed cows with low yield lactation. In China, there are many small and medium dairy farms using Chinese Holstein dairy cows with a...
low milk yield. If a higher crude protein diet was used to feed the cows, the distribution and the proportion of all kinds of SNAN may be similar to those in this study due to the similar gastrointestinal environment.

In conclusion, the mRNA expression of three AA transporters and PepT1 in the duodenum was highest in the nonmesenteric gastrointestinal, as were the concentrations of FAA-N and PBAA-N. This common trend indicates that the duodenum is the major nonmesenteric site for absorbing SNAN. Moreover, PBAA-N concentrations accounted for the largest part of SNAN, suggesting that PBAA has an important role in SNAN absorption and confirming results from previous studies. These results can be used to improve feed regulation and gastrointestinal metabolism to increase milk protein yield and N efficiency.

Compliance with ethics guidelines
Ying-ming XIE, Qing-biao XU, Yue-ming WU, Xin-bei HUANG and Jian-xin LIU declare that they have no conflict of interest.
All institutional and national guidelines for the care and use of laboratory animals were followed.

References


