Interpretive summary

Gamalost, a traditional Norwegian cheese known to have very high angiotensin I-converting enzyme (ACE) inhibitory activity, i.e. antihypertensive effect, was digested by human gastric juice and human duodenal juice at 37°C in an incubator, mimicking conditions in the human body. It was found that its ACE-inhibitory activity was slightly increased after digestion due to the release of many potential ACE-inhibitory peptides from the cheese protein. Norvegia, a Gouda type cheese, had a much lower ACE-inhibitory activity than Gamalost as such, however, during digestion its ACE-inhibitory activity increased almost to the level of Gamalost.

Running head: **ACE INHIBITION IN CHEESES DURING DIGESTION**

Angiotensin I-converting enzyme (ACE) inhibitory activity of the Norwegian autochthonous cheese Gamalost and Norvegia after in vitro human gastrointestinal digestion

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ABSTRACT

The angiotensin I-converting enzyme (**ACE**) inhibitory activity of Gamalost cheese, its pH 4.6 soluble fraction, and Norvegia cheese was monitored before and after digestion with human gastric and duodenal juices. Both Gamalost and Norvegia cheeses showed an increased ACE-inhibitory activity during the gastrointestinal digestion. However, only Norvegia showed pronounced increased activity after duodenal digestion. More peptides were detected in digested Gamalost, compared to digested Norvegia. Most of the peptides in Gamalost were derived from β-CN (casein), some originated from the α_{s1}-CN, while only a very few originated from α_{s2}-CN and κ-CN. In general, the number of peptides increased during gastrointestinal digestion, while some peptides were further degraded and disappeared, however, surprisingly a few peptides remained stable. The aromatic amino acids, such as Tyr, Phe and Trp, the positive charged amino acids (Arg and Lys), and Leu increased after simulated gastrointestinal digestion of Gamalost and Norvegia. After in vitro gastrointestinal digestion, both Gamalost and Norvegia showed high ACE inhibitory activity which may contribute in lowering of mild hypertension.

Key words: Gamalost, ACE-inhibition, human gastrointestinal digestion, peptide composition

INTRODUCTION

Gamalost, an autochthonous mould (*Mucor (M.) mucedo*) ripened Norwegian cheese, is made by acid precipitation of fermented pasteurized skimmed milk and has been shown to have a high angiotensin I-converting enzyme (**ACE**) inhibitory effect (Pripp et al., 2006; Qureshi et al., 2012). Gamalost differ from other mould cheeses by a low fat content (< 0.05% fat) and by the inactivation of the starter bacteria due to intensive cooking of the cheese curd in whey for 1 to 2 h at 90-95°C before the mould is added to the cheese. The development of ACE inhibitory peptides in Gamalost during ripening has previously been investigated (Qureshi et al., 2012), and it was found that the ACE inhibition of the pH 4.6 soluble fraction (SF) differed during ripening due to development of potential bioactive peptides.

A large number of bioactive peptides have been identified in milk, fermented dairy products and cheeses (Meisel, 1998; Clare and Swaisgood, 2000; FitzGerald et al., 2004; Silva and Malcata, 2005; Sieber et al., 2010). These peptides have no bioactivity in the parent protein but are released during fermentation and hydrolysis by native enzymes, rennet enzymes and bacterial enzymes in the cheese and further by digestive enzymes (FitzGerald et al., 2004; Korhonen and Pihlanto, 2006).

For a long time, commercial enzymes like pepsin, trypsin and chymotrypsin from non-human origin have been used in simulated human digestion, and many peptides have been identified as a result of such digestion experiments. Only a few studies have been carried out using human gastrointestinal (**GI**) enzymes under physiological conditions to identify the peptides formed (Mullally et al., 1997; Abubakar et al., 1998; Parrot et al., 2003; Gómez-Ruiz et al., 2004; Hernández-Ledesma et al., 2004; Almaas et al., 2006b; Schmelzer et al., 2007). Different peptide patterns have been observed when using commercial enzymes compared to human enzymes. The in vitro human enzyme digestion results seem to be more consistent with the in vivo digestion studies reported (Chabance et al., 1998; Almaas et al., 2006a; Eriksen et al., 2010).

Human digestion depends on many factors such as composition of the food, gastric pH, buffering capacity of the food, transit time, concentrations and activities of the digestive enzymes and other digestive components (Dressman et al., 1990; Ekmekcioglu, 2002; Moreno, 2007). Gastric juice contains hydrochloric acid, pepsin, mucous, and gastric lipase (only in newborns), whereas pancreatic juice has high contents of bicarbonate, bile salts and digestive enzymes such as trypsin, chymotrypsin, amino and carboxypeptidases, amylase, lipase, and enzymes which aid in digestion of nucleic acids and phospholipids (Ulleberg et al., 2011; Campbell, 2012). Generally, gastric pH ranges from 1 to 3.5 during the fasting period. However, the gastric pH range measured in healthy adults following a meal was 3.9-5.5 depending on the buffering capacity of the food. The duodenal pH is normally around 6.0-7.8 (McCloy et al., 1984; Russell et al., 1993; Ekmekcioglu, 2002; Kalantzi et al., 2006; Campbell, 2012).

Studies on ACE-inhibition after digestion with commercial enzymes have been performed on different milk proteins such as β-lactoglobulin (β-Lg), α-lactalbumin (α-La), whey protein concentrates and cheese. In these studies some antihypertensive peptides of β-casein including the potent Val-Pro-Pro (**VPP**) and Ile-Pro-Pro (**IPP**) peptides have been identified (Mullally et al., 1997; Parrot et al., 2003; Vermeirssen et al., 2003; Gómez-Ruiz et al., 2004; Ohsawa et al., 2008). To our knowledge, no in vitro digestion studies of cheese using human gastric juice (**HGJ**) and human duodenal juice (**HDJ**) evaluating the ACE-inhibitory potential of the cheese digests have been published. Therefore it would be interesting to follow the ACE inhibitory activity of Gamalost, which is known to have a high ACE inhibitory activity (Qureshi et al., 2012), and Norvegia, which is representative for cheese varieties used by many consumers in Norway, during digestion with human gastric and duodenal juices.

The main objective of the present study was to evaluate the ACE-inhibitory activity of two Norwegian cheeses (Gamalost and Norvegia) during digestion with human gastric and duodenal juices and to characterize the peptides and amino acids in the digests.

MATERIALS AND METHODS

Collection and ripening of cheese

Gamalost cheese, produced as described by Qureshi et al. (2012), was kindly supplied by TINE Meieriet Vik. Five cheeses from each of three separate productions were selected randomly at the dairy. One cheese was frozen (-20°C) at age 0 (after cooking of the cheese in whey but before applying the mould at the surface of the cheese), the remaining four cheeses were frozen after 10 days of ripening. The frozen 10 days old cheeses were thawed and one from each batch was further ripened at the Department of Chemistry, Biotechnology and Food Science (Ås, Norway) at 4°C.

The cheeses were sampled after zero, 10 and 30 days of ripening, grinded and frozen until digestion and further analysis. The sampling of the cheeses was done according to International Dairy Federation (**IDF**) standard 50C (1995) and the cheese was grated with a manual grinder. From each sampling, a pH 4.6 SF was prepared according to the procedure described by Pripp et al. (2006). Three Norvegia cheeses (90 days old) were purchased from a local grocery shop, and used for comparison with Gamalost. The Gamalost cheese, its pH 4.6 SF and Norvegia were further used in simulated digestion experiments.

Aspiration of human gastric and duodenal juices and their activities

Human gastric and duodenal juices were collected from healthy volunteers according to Ulleberg et al. (2011). The juices were collected from 20 fasting volunteers (7 men and 13 women) (average age, 25 ± 5 years). The collected juices were mixed together to make a batch as the juices vary in their activity from individual to individual. The main advantage of making a batch of juices was to reduce the variations in enzyme activity between the individual samples. The collected juices were centrifuged (4500 *g*, 10 min) to remove mucous and cell debris before storing at -20°C or -80°C. The pepsin activity of human gastric juices was measured as described by Sánchez-Chiang et al. (1987), whereas the total proteolytic activity of the human duodenal juices was measured as described by Krogdahl and Holm (1979).

In vitro enzymatic digestion

An in vitro digestion model was performed to simulate human digestion in the stomach (step 1) and the duodenum (step 2) according to Almaas et al. (2006a) with some modifications. The Gamalost (2 g) (containing ~ 50% protein), Norvegia (4 g) (containing ~ 26% protein) and the pH 4.6 SF (150 mg) of Gamalost were dissolved in 10 mL physiological solution (0.9% NaCl). To mimic chewing in the mouth, the sample was incubated (37°C) for 5-7 min in a Stomacher (Seward stomacher 400, West Sussex, UK) with constant shaking. To simulate the gastric phase (step 1), pH was slowly decreased to 2.5 by drop wise addition of 2 M HCl after adding HGJ (15 U/g protein) and the samples were incubated (0.5 h for the pH 4.6 SF and 1 h for the cheeses) at 37°C in a Stomacher. The following duodenal digestion (step 2) was performed by pH adjustment to 7 by 4 M NaOH and then incubated with HDJ (31.2 U/g protein) (1 h for the pH 4.6 SF and 3 h for cheeses) at 37°C. Samples (0.3-0.5 ml) were collected before digestion, after gastric digestion (step 1) and after the subsequent duodenal digestion (step 2). To stop the enzymatic reaction, the samples were immediately transferred to an ice bath and frozen (-20°C). The digestion of each sample was performed in duplicate.

Before further analysis, the samples were thawed and the digestive enzymes were separated from the sample by ultrafiltration through hydrophilic membranes (Amicon Ultra, cut-off MW = 10 kDa, Millipore, Carrigtwohill Corporation, Cork, Ireland) by centrifugation (11148 *g*, 40 min, 4°C). The peptides having molecular weight lower than 10 kDa were collected in the permeate for all the samples.

Chemical analysis of cheese

The dry matter (DM) content of Gamalost was determined according to IDF standard 4/ISO 5534 (2004). The soluble nitrogen (**SN**) content of the 10 kDa permeate of Gamalost cheese and of Norvegia cheese, and the pH 4.6 SF of Gamalost cheese as well as the total nitrogen (**TN**) of Norvegia cheese were determined by the Kjeldahl method (IDF, 1993). A homogeneous sample for TN of Gamalost was difficult to obtain due to formation of precipitates during sample preparation. In addition, due to the presence of denatured whey proteins, foaming occurred during the digestion step in the preparation of the Gamalost sample for TN. Therefore, SN/DM of the Gamalost was used instead of SN/TN.

Identification of peptide sequences

Nano-LC-MS of desalted and concentrated samples was performed according to Qureshi et al. (2012) following the method described by Eriksen et al. (2010) with some modifications. Only peptides with mass above 800 and below 4500 Da were subjected to collision-induced fragmentation and further processing.

Free amino acid (FAA) composition

For the analysis of amino acid composition, the samples were prepared as described by Qureshi et al. (2012) with RP-HPLC using o-phthalaldehyde (OPA) and fluorenylmethyloxycarbonyl chloride (FMOC) derivatisation, following the procedure of Bütikofer and Ardö (1999) with some modifications. One hundred microliter (µl) of the 10 kDa permeate of all the samples was mixed with 100 µL 0.1 M HCl containing 0.4 µmol/mL L-norvalin (Sigma, St. Louis, USA) and 0.4 µmol/mL piperidine-4-carboxylic acid (**PICA**) (Fluka, St.Louis, USA), and used as internal standards.

Preparation of samples for ACE-inhibition assay

The ACE-inhibition assays were performed using the reaction of substrate (hippuryl-histidyl-leucine (Sigma, St. Louis, USA)) and enzyme (extract from rabbit lung acetone powder (Sigma)), with measurement of the liberated hippuric acid (**HA**) by RP-HPLC according to Qureshi et al. (2012) following the method described by Hyun and Shin (2000) with some modifications. Forty µl of the 10 kDa permeate of all samples was used in the assay. Captopril ($C_9H_{15}NO_3S$) (Sigma), a blood pressure lowering pharmaceutical, was used as an inhibitory reference. The ACE-inhibition (%) was calculated by using the formula given below (1):

$$\text{ACE inhibition (\%)} = \frac{\text{HA (control)} - \text{HA (sample)}}{\text{HA (control)}} \times 100 \qquad (1)$$

Where HA (control) denotes the concentration of hippuric acid liberated after reaction of enzyme and substrate (without sample), while HA (sample) represents the hippuric acid released after the reaction of enzyme and substrate in the presence of the sample. The estimated volume of 10 kDa permeates extracted from 1 g of Gamalost was 5 mL whereas the estimated volume of 10 kDa permeates extracted from 1 g of Norvegia was 2.5 mL (as described in the in vitro enzymatic digestion section). The results of the 10 kDa permeates were calculated as the amount of cheese. Hence, the IC_{50} which is the inhibitory concentration of the sample of the freeze dried pH 4.6 SF (in mg/mL) or in mg equivalent cheese per mL required to inhibit 50% of the ACE activity was determined from the linear regression equation by plotting ACE-inhibition (%) versus the inhibitory concentration of each dilution of the sample. The IC_s was calculated using the following formula (2):

$$\mathrm{IC_s} = (\mathrm{C_0} \times \mathrm{V_s} \times \mathrm{L}) \,/\, \mathrm{V} \quad (2)$$

Where C_0 is the initial sample concentration (mg/mL), V_s is the sample volume (40 µL), L denotes the dilutions used (0.5, 0.25, 0.125) and V is the total reaction volume (340 µL). From the IC_{50} values of the freeze dried pH 4.6 SF, the IC_{50} values of the pH 4.6 SF were also calculated as amount of Gamalost by using the weight (g) of freeze dried pH 4.6 SF extracted from 1 g of cheese. The ACE-inhibitory potential (**IP**) per unit cheese weight (mg captopril equivalents per kg cheese) was calculated by the formula given below (3):

$$\mathrm{ACE\ (IP)} = \mathrm{IC_{50}\ (captopril)} \times \mathrm{pH\ 4.6\ SF\ or\ cheese\ amount} / \mathrm{IC_{50}\ (pH\ 4.6\ SF)} \quad (3)$$

Where IC_{50} (captopril) and IC_{50} (pH 4.6 SF) are the concentrations (mg/mL) of captopril and the freeze dried pH 4.6 SF (mg/mL), respectively whereas pH 4.6 SF and cheese amount represent the amount (mg) of freeze dried powder from 1 g cheese and amount of cheese (mg) present in 1 mL of the physiological solution (0.9% NaCl) respectively.

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Statistical analysis

Statistical analysis was performed by Minitab statistical software version 15 (Minitab Inc., State College, PA, USA). The Shapiro-Wilk test was used for testing the assumption of normal distribution of the data, which were satisfied for all variables. Individual cheeses from the same batch were assumed to be independent. Two way analysis of variance (**ANOVA**) was performed with replicate block (random variable) and age (fixed variable) of Gamalost cheese to test H_0; that the ACE-inhibitory activity in the Gamalost and its pH 4.6 SF did not change during ripening. Similarly, two way ANOVA was carried out with replicate block (random variable) and digestion steps (fixed variable) (including undigested samples) and Norvegia to test H_0 that the ACE-inhibitory activity did not change during digestion of Gamalost and its pH 4.6 SF at each ripening stage and Norvegia at 90 days. Tukey's test for pair wise comparison was used to test the differences between means. For all comparisons, the level of significance was set to $P < 0.05$.

RESULTS

Soluble nitrogen (SN) of Gamalost, its pH 4.6 SF and Norvegia cheese

The SN/DM (%) of the 10 kDa permeates of Gamalost and its pH 4.6 SF and SN/TN (%) of the 10 kDa permeates of Norvegia is presented in table 1. The SN/DM (%) of Gamalost and its pH 4.6 SF increased during ripening as well as during subsequent digestion. A difference between the different batches of Gamalost was found during ripening on the content of SN/DM (%), whereas in the digested Gamalost, an effect of productions was found on the SN/DM (%) after 30 days of ripening (results not shown). The SN/TN (%) content of Norvegia cheese (90 days) also increased significantly during both the gastric and duodenal digestion, and the SN/TN (%) of Norvegia reached its peak after duodenal digestion.

ACE-inhibition during ripening and after gastrointestinal digestion

The results of the ACE-inhibition (%) measurements and the IC_{50} values of Gamalost (10-30 days), its pH 4.6 SF and of Norvegia (90 days) are presented in Figure 1 and 2 respectively. Optimum ACE-inhibitory activity (%) and the lowest IC_{50} values of the Gamalost and its pH 4.6 SF was observed after 10 days of ripening. Digestion affected the ACE-inhibitory activity of Gamalost and its pH 4.6 SF, as well as Norvegia. The highest ACE-inhibitory activity of Gamalost was observed after gastric digestion, and further duodenal digestion did not seem to affect the ACE-inhibitory activity further. The IC_{50} values showed similar results after gastric and duodenal digestion of Gamalost (Figure 2). By recalculation of the IC_{50} of the pH 4.6 SF to the corresponding amount of the original Gamalost (10 and 30 days), the trend was similar to what was found for the pH 4.6 SF. The IC_{50} values were higher in the Gamalost cheese before digestion (2.36 and 1.85 in Gamalost and the recalculated pH 4.6 SF, respectively, at 10 days) and lower in Gamalost after gastric digestion (0.82 and 1.65 in Gamalost and the recalcultated pH 4.6 SF, respectively, at 10 days) as well as duodenal digestion (0.99 and 2.22 in Gamalost and the recalcultated pH 4.6 SF, respectively, at 10 days). Unlike, Gamalost, Norvegia showed a gradual increase in the ACE-inhibitory activity with a drastic decrease in IC_{50} values after the successive gastric and duodenal digestion. However, Gamalost differed from Norvegia by showing much lower IC_{50} values at all digestion steps. The IC_{50} value of captopril measured in the assay was 4.1×10^{-6} (mg/mL) $\pm$ 1.3×10^{-7}. There was an influence of different productions on the IC_{50} values of Gamalost during ripening whereas no influence of productions was found on the ACE-inhibition (%) of Gamalost cheese during ripening. There was an effect of different productions on the ACE-inhibition (%) of pH 4.6 SF at 30 days Gamalost during GI digestion. An effect of different productions was also found on the IC_{50} values in 30 days Gamalost during GI digestion (results not shown).

On the basis of the results of the IC_{50} values of Gamalost, its pH 4.6 SF and of Norvegia, the ACE-IP per unit cheese weight was calculated (Figure 3). Gamalost cheese as well as its respective pH 4.6 SF showed high ACE-IP after gastric digestion but after duodenal digestion the IP-values were somewhat reduced. Norvegia, however, showed an increasing trend after subsequent digestion, but its ACE-IP remained at lower levels as compared to Gamalost.

Peptides generated during ripening and after gastrointestinal digestion

Many peptides were identified during ripening and GI digestion. A summary table (table 2) shows the number of peptides present before and after digestion of Gamalost, its pH 4.6 SF and of Norvegia. Most peptides derived from β-CN, whereas some were released from α_{s1}-CN and only a few derived from α_{s2}-CN and κ-CN. In short, Gamalost cheese contained almost twice as many peptides as detected in its pH 4.6 SF and Norvegia.

In general, most of the peptides which were present in Gamalost matched with the peptides present in the pH 4.6 SF, however only a few peptides were common between Norvegia and Gamalost cheese or its pH 4.6 SF (Figures 4-8). The peptide pattern of Norvegia differed from that of Gamalost by a much lower content of peptides. The peptide pattern of Gamalost (10 and 30 days) differed between undigested, gastric and duodenal digested samples, and digestion affected the peptide profile in different ways. Figure 4 and 5 show in more detail the peptide pattern derived from β-CN during gastric and duodenal digestion of Gamalost (10 and 30 days).

Many new peptides were released after gastric and further duodenal digestion. The peptides that were stable during the GI digestion of the 10 days ripened Gamalost were β-CN (126-139), β-CN (129-139), β-CN (144-160), β-CN (192-207), β-CN (193-207) and a few peptides in the 30 days ripened Gamalost (β-CN (78-91), β-CN (129-139), β-CN (144-160), β-CN (193-207)) were also stable through GI digestion. In the pH 4.6 SF (10-30 days) of Gamalost, many peptides derived from β-CN were detected in the extract before digestion. Almost all of the peptides present in the undigested pH 4.6 SF of Gamalost were also detected in the undigested Gamalost cheese. Some peptides derived from β-CN were common between the gastric digested and duodenal digested pH 4.6 SF (results not shown) and Gamalost. In Norvegia cheese, some peptides (β-CN (193-206), β-CN (194-209), β-CN (195-206)) remained stable during GI digestion, however, some new peptides were observed during digestion (Figure 6).

A lower number of peptides were derived from α_{s1}-CN, than from β-CN during GI digestion of Gamalost cheese (Figure 7). In Gamalost cheeses (10 and 30 days), some new peptides derived from α_{s1}-CN were generated during GI digestion, and only one peptide (α_{s1}-CN (180-194)) in 30 days Gamalost remained stable during GI digestion (Figure 7 a and b). The pH 4.6 SF from fresh cheese (0 days) contained a higher number of peptides derived from α_{s1}-CN compared to the pH 4.6 SF of 10 and 30 days ripened cheese (results not shown). Fig. 8 a and b shows the peptides derived from α_{s2}-CN in Gamalost. Only one peptide (α_{s2}-CN (99-115)) remained stable in the 10 days old Gamalost cheese during GI digestion. In addition, Gamalost contained two peptides, β-Lg (124-136) and β-Lg (124-139), derived from β-Lg (table 2). In undigested Norvegia cheese, a few peptides such as α_{s1}-CN (1-13), α_{s1}-CN (1-14), α_{s1}-CN (1-16), α_{s1}-CN (10-23), α_{s1}-CN (15-23) and α_{s1}-CN (26-34) were derived from α_{s1}-CN. Only one peptide (α_{s2}-CN (151-162)) from undigested samples and one (α_{s2}-CN (100-114)) from the HDJ digestion, derived from α_{s2}-CN, were present in Norvegia cheese (results not shown). From κ-CN only a few peptides were observed in Gamalost (30 days), pH 4.6 SF (0 day) and Norvegia (table 3). During GI digestion, only one peptide (κ-CN (155-169)) derived from κ-CN in the pH 4.6 SF from 0 day Gamalost, remained stable.

Most of the peptides identified in Gamalost, its pH 4.6 SF and in Norvegia, had hydrophobic amino acids, such as Ala, Ile, Leu, Met, Phe, Trp and Val (**A**, **I**, **L**, **M**, **F**, **W** and **V**), as well as Pro at any of the three C-terminal positions of peptides. In addition, positive (+) charged amino acids such as Arg (**R**) and Lys (**K**) were detected at any of the three C-terminal positions of a few peptides. Moreover, most peptides in Gamalost, its respective pH 4.6 SF and in Norvegia were generated from internal as well as from the C-terminal sequences of β-, α_{s1}-, α_{s2}- and κ-CN.

Free amino acid contents before and after gastrointestinal digestion

The FAA content (mmol/kg) of Gamalost (10 and 30 days) and of Norvegia before and during digestion are presented in Figure 9 a, b and c. The amino acids Cit and GABA were detected in negligible concentrations in Gamalost and Norvegia and were therefore omitted from Figure 9. Digestion affected the generation of FAA; the content of Pro decreased significantly during gastric digestion of Gamalost whereas the content of Arg, Tyr, Phe, Leu and Lys were not influenced considerably by the gastric digestion. However, after duodenal digestion the content of these amino acids increased significantly. Norvegia (Figure 9 c) had very low concentrations of all amino acids compared to Gamalost, however the amino acids Arg, Tyr, Phe, Trp and Lys increased significantly after duodenal digestion of Norvegia.

DISCUSSION

In Gamalost, the increased ACE-inhibition after gastric digestion indicates a possible further release of potent peptides during digestion. After digestion of Gamalost with HGJ and HDJ, the decreased IC_{50} values might be due to the generation of new peptides which may be more active as compared to the peptides present in the undigested cheese. The considerable decreasing trend of IC_{50} of Norvegia after GI digestion revealed that the released peptides might have a very high ACE-inhibitory effect. The increased content of SN/TN (%) of Norvegia after duodenal digestion is consistent with the results of Parrot et al. (2003), who found that the SN/TN content increased drastically to almost 50% in Emmental cheese water soluble extract (**WSE**) by the action of pepsin and trypsin. A considerable increase of Trp, Phe and Tyr in the digests of Gamalost and Norvegia by human GI digestion is concurrent with the findings of Parrot et al. (2003) and Adt et al. (2011).

The WSE of Asiago d'allevo cheese with peptides having molecular mass less than 3 kDa were reported to have a higher ACE-inhibitory activity than the WSEs containing peptides larger than 3 kDa (Lignitto et al., 2010). Most of the peptides observed in Gamalost, its pH 4.6 SF and in Norvegia had molecular masses lower than 3 kDa. López-Fandiño et al. (2006), reported that peptides with less than 27 amino acids had appreciable ACE-inhibitory activity. There was a slight difference in the IC_{50} values of the non-ultrafiltered pH 4.6 SF (0 and 10 days) in our previous study (Qureshi et al., 2012) and the ultrafiltered pH 4.6 SF of the present study, as some of the peptides most probably were lost during ultrafiltration of the samples in the present study. The difference in the IC_{50} values between the pH 4.6 SF of Gamalost (10 and 30 days) and Norvegia observed in this study compared to the previously mentioned study (Qureshi et al., 2012), might be due to differences in the number of active peptides between different batches of cheese.

The presented results are consistent with previous reports regarding the structure-activity relationship between ACE-inhibition and the available peptides (López-Fandiño et al., 2006; Haque and Chand, 2008). The presence of hydrophobic (Tyr, Phe, Trp, Ala, Ile, Leu, Val, Met) or positive charged (+) amino acids such as Arg and Lys as well as Pro at any of the three C-terminal positions of the peptides show good binding of ACE (López-Fandiño et al., 2006; Haque and Chand, 2008; He et al., 2011). It was observed that Gamalost also contained some of the peptides in which the two potent tripeptides, IPP and VPP, were present in an encrypted form within their sequences. However, the pH 4.6 SF of Gamalost and Norvegia contained very few of those peptides. The generation of a few peptides from the hydrophobic para-κ-CN in 30 days old Gamalost and in Norvegia might explain the susceptibility of para-κ-CN (residues 1-105) towards hydrolysis by HGJ and HDJ. Gamalost is an acid coagulated cheese, and the glycomacropeptide (**GMP**) is retained on the κ-casein. However, the peptides κ-CN (149-169) and κ-CN (155-169) derived from the hydrophilic GMP were found in the undigested cheese in the pH 4.6 SF from the unripened Gamalost (day 0) and further degradation occured during digestion. These peptides were not found after 30 days of ripening which indicated that the GMP was completely degraded. The presence of two peptides, derived from β-Lg following duodenal digestion revealed the presence of some whey proteins in Gamalost which is reasonable as the cheese was cooked in whey during manufacturing and denatured whey proteins were therefore retained in the cheese matrix.

Chymotrypsin, trypsin and pepsin have specific amino acid targets during hydrolysis of proteins. It has been shown in many studies that trypsin attacks on the carboxyl side of positively charged amino acids such as Arg and Lys of the peptide sequences, as well as cleaving before Pro (Neurath, 1957; Custódio et al., 2005; Rodriguez et al., 2008). It has also been reported that chymotrypsin has a broader specificity spectrum than trypsin, and therefore attacks on the carboxyl-side of non-polar, hydrophobic amino acids or aromatic amino acids (Tyr, Phe and Trp) (Neurath, 1957). Pepsin has been shown to hydrolyse the amino side of the Leu residues and, like chymotrypsin, it also attacks on the carboxyl side of the aromatic amino acids (Neurath, 1957; Auffret and Ryle, 1979). Schmelzer et al. (2007) concluded, by an in vitro peptic digestion of β-CN, that pepsin cleaves the C-terminal region that is rich in hydrophobic residues. Our findings are mostly consistent with the aforementioned reports regarding cleavage site specificities due to the activity of enzymes present in HGJ and HDJ. During HGJ digestion of 10 or 30 days ripened Gamalost, some peptides such as β-CN (59-93), β-CN (126-140), β-CN (129-141), β-CN (193-209) and β-CN (193-209) were generated, which have also been detected in the peptic digests of β-CN during in vitro digestion by pepsin (Schmelzer et al., 2007). Some of the peptides, β-CN (125-140), β-CN (126-141), β-CN (126-142), β-CN (129-140), β-CN (143-163), β-CN (190-209), β-CN (191-209), β-CN (192-209), present in undigested Gamalost in the present study, were also detected by Schmelzer et al. (2007) in pepsin digested β-CN. The *M. mucedo* might have a broader spectrum of cleavage specificities than pepsin as it was observed in undigested Gamalost cheese that hydrophobic amino acids were present at the C-terminal position of the peptides. The presence of Lys and Arg at the C-terminal end of the peptides may be attributed to the action of plasmin (Upadhyay et al., 2004). However, as minor activity of plasmin (due to denaturation) was expected in Gamalost after cooking of the cheese curd in whey for 1 to 2 h at 90-95°C, the presence of Lys and Arg at the C-terminal position of some of the peptides of Gamalost most probably were due to the activity of *M. mucedo*.

The appearance of some common peptides either after gastric digestion or duodenal digestion among pH 4.6 SF of Gamalost of different ripening times (0-30), as well as between 10 and 30 days old Gamalost, might also indicate the common cleavage sites of peptides cleaved by HGJ and HDJ. Most reports on identification of peptides after in vitro digestion of cheese have used commercial enzymes of non human origin (Abubakar et al., 1998; Gómez-Ruiz et al., 2004; Contreras et al., 2009). When comparing peptides released by commercial enzymes with peptides generated with human enzymes, very few peptides matched with previously reported ACE-inhibitory peptides (Schmelzer et al., 2007). Pepsin plays an important role in the primary partial digestion of protein (10 to 15%) resulting in production of long peptides whereas secondary degradation of peptides was done by trypsin and chymotrypsin resulting in oligopeptides (Goodman, 2010). More peptides were formed during gastric digestion compared to duodenal digestion which is manifested from the number of peptides shown in table 2.

In the present study, some peptides were not degraded and remained stable after GI digestion. If the peptides reach the cardiovascular system in an active form, they may exert a physiological, i.e. antihypertensive effect (Segura-Campos et al., 2011). Therefore, presumable absorption of active peptides from cheese through the intestinal tract might result in a mild lowering of blood pressure. To affirm the results of our in vitro study and to clarify the bioavailabilty of the peptides, an epidemiological study on the effect of consumption of Gamalost on blood pressure is in progress.

CONCLUSIONS

Digestion of Gamalost with human GI enzymes increased the ACE-inhibition. Due to the presence of a higher amount of protein as well as higher number of peptides (derived from β-,

α_{s1}-, α_{s2}-, κ-CN and β-Lg), Gamalost showed lower IC_{50} than Norvegia cheese even though Norvegia showed an enormous decrease in the IC_{50} value during gastric and duodenal digestion. Thus, both Gamalost and Norvegia might contribute to a lowering of mild hypertension as some of the peptides remained intact during digestion and may be absorbed through the intestine.

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Figure captions

Figure 1. ACE-inhibition (%) (Mean ± SD) of the < 10 kDa permeates of Gamalost (10 and 30 days), its pH 4.6 SF (0-30 days) and of Norvegia before (undigested; black bars) and after gastrointestinal digestion by human gastric juice (HGJ; white bars) and human duodenal juice (HDJ; line pattern bars). Small letters over the bars (except for Norvegia) represent significant difference ($P < 0.05$) between age of cheese at the same digestion step while capital letters show significant differences ($P < 0.05$) during digestion of the same sample at the same ripening stage.

Figure 2. IC_{50} values (Mean ± SD) expressed as mg equivalent/mL of Gamalost (10 and 30 days) and Norvegia and pH 4.6 SF (0-30 days) (mg/mL) of Gamalost before (undigested; black bars) and after gastrointestinal digestion by human gastric juice (HGJ; white bars) and human duodenal juice (HDJ; line pattern bars). Small letters over the bars (except for Norvegia) represent significant difference ($P < 0.05$) between age of cheese at the same digestion step while capital letters show significant differences ($P < 0.05$) during digestion of the same sample at the same ripening stage.

Figure 3. ACE-inhibitory potential (IP) (per unit cheese weight, expressed as mg captopril equivalents/kg cheese) (Mean ± SD) of Gamalost (10 and 30 days), its pH 4.6 SF (0-30 days) and of Norvegia before (undigested; black bars) and after gastrointestinal digestion by human gastric juice (HGJ; white bars) and human duodenal juice (HDJ; line pattern bars). Small letters over the bars (except for Norvegia) represent significant difference (P < 0.05) between age of cheese at the same digestion step while capital letters show significant differences (P < 0.05) during digestion of the same sample at the same ripening stage.

Figure 4. The peptides derived from β-CN before (undigested; indicated by U and black lines) and after in vitro gastrointestinal digestion by human gastric juice (HGJ; dark grey lines) and human duodenal juice (HDJ; light grey lines) in the < 10 kDa permeate of 10 days ripened Gamalost.

Figure 5. The peptides derived from β-CN before (undigested; indicated by U and black lines) and after in vitro gastrointestinal digestion by human gastric juice (HGJ; dark grey lines) and human duodenal juice (HDJ; light grey lines) of the < 10 kDa permeate of 30 days ripened Gamalost.

Figure 6. The peptides derived from β-CN before (undigested; indicated by U and black lines) and after in vitro gastrointestinal digestion by human gastric juice (HGJ; dark grey lines) and human duodenal juice (HDJ; light grey lines) of the < 10 kDa permeate of Norvegia.

Figure 7 a and b. The peptides derived from α_{s1}-CN before (undigested; indicated by U and black lines) and after in vitro gastrointestinal digestion by human gastric juice (HGJ; dark grey lines) and human duodenal juice (HDJ; light grey lines) of the < 10 kDa permeate of Gamalost; (a) 10 days ripened Gamalost and (b) 30 days ripened Gamalost.

Figure 8 a and b. The peptides derived from α_{s2}-CN before (undigested; indicated by U and black lines) and after in vitro gastrointestinal digestion by human gastric juice (HGJ; dark grey lines) and human duodenal juice (HDJ; light grey lines) of the < 10 kDa permeate of Gamalost and its pH 4.6 SF; (a) 10 days ripened Gamalost; (b) 30 days ripened Gamalost.

Figure 9 a, b and c. The concentrations (mmol/kg cheese) (Mean ± SD) of the free amino acids (FAA) in the undigested (black bars), HGJ (white bars) and HDJ (line pattern bars) digested Gamalost and Norvegia; (a) Gamalost ripened for 10 days, (b) Gamalost ripened for 30 days and (c) Norvegia ripened for 90 days.

Table 1. Soluble nitrogen (SN) in dry matter (DM) (%) of Gamalost of the 10 kDa permeates of pH 4.6 soluble fraction (SF) of Gamalost (0-30 days) and Gamalost cheese (10 and 30 days) and SN in total nitrogen (TN) (%) of 10 kDa permeates of Norvegia (90 days) before (U) and after gastrointestinal digestion by human gastric juice (HGJ) at pH 2.5 and human duodenal juice (HDJ) at pH 7.0. Means with small superscripts represent difference ($P < 0.05$) between age of cheese within the same column while means with capital superscripts show difference ($P < 0.05$) between digestion steps (including undigested samples) in the pH 4.6 SF or cheese at the same ripening stage within the same rows

Age (days)	Sample type	SN DM^{-1} (%)[1] of pH 4.6 SF			SN DM^{-1} (%)[1] of Gamalost or SN TN^{-1} (%)[2] for Norvegia		
-	-	U	HGJ	HDJ	U	HGJ	HDJ
0	pH 4.6 SF	$0.05^{cB} \pm 0.02$	$0.82^{cA} \pm 0.34$	$0.87^{cA} \pm 0.47$	-	-	-
10	pH 4.6 SF or Gamalost	$4.34^{bC} \pm 0.47$	$8.64^{bB} \pm 0.80$	$11.45^{bA} \pm 0.67$	$6.09^{bC} \pm 0.21$	$6.98^{bB} \pm 0.40$	$10.52^{bA} \pm 0.47$
30	pH 4.6 SF or Gamalost	$6.26^{aB} \pm 0.88$	$12.05^{aA} \pm 2.39$	$12.29^{aA} \pm 1.57$	$7.59^{aC} \pm 0.28$	$8.25^{aB} \pm 0.37$	$12.16^{aA} \pm 0.45$
90	Norvegia	-	-	-	$8.84^{C} \pm 1.02$	$14.13^{B} \pm 0.73$	$51.36^{A} \pm 5.22$

[1]Soluble nitrogen (SN) as a percentage of dry matter (DM) of Gamalost.

[2]Soluble nitrogen (SN) as a percentage of total nitrogen (TN) in Norvegia.

Table 2. Number of peptides in Gamalost (ripened for 10 and 30 days), its pH 4.6 soluble fraction (SF) and Norvegia (90 days) present before (undigested samples (U)) and after gastrointestinal digestion by human gastric juice (HGJ) and human duodenal juice (HDJ)

-					Gamalost cheese								Norvegia			
Protein type	-				10 days				30 days				90 days			
-	-	-	-	-	U	HGJ	HDJ	Total	U	HGJ	HDJ	Total	U	HGJ	HDJ	Total
β-CN	-	-	-	-	32	47	30	72	38	33	19	68	19	14	15	36
α_{s1}-CN	-	-	-	-	12	07	04	20	06	07	05	14	06	00	00	06
α_{s2}-CN	-	-	-	-	01	03	03	05	05	05	01	07	01	00	01	02
κ-CN	-	-	-	-	00	00	00	00	00	02	00	02	01	04	00	05
β-Lg	-	-	-	-	00	00	00	00	00	00	02	02	00	00	00	00
Total					45	57	37	97	51	47	27	93	27	18	16	49
							pH 4.6 SF of Gamalost									
Protein type	0 day				10 days				30 days				-			
-	U	HGJ	HDJ	Total	U	HGJ	HDJ	Total	U	HGJ	HDJ	Total	-	-	-	-
β-CN	06	15	08	22	21	12	08	33	22	15	07	33	-	-	-	-
α_{s1}-CN	05	04	07	10	02	02	02	04	03	01	03	05	-	-	-	-
α_{s2}-CN	00	00	00	00	01	02	02	04	03	03	02	05	-	-	-	-
κ-CN	02	02	02	04	00	00	00	00	00	00	00	00	-	-	-	-
Total	13	21	17	36	24	16	12	41	28	19	12	43	-	-	-	-

Total represents number of peptides (including common once) present before and after digestion in Gamalost, its pH 4.6 SF and Norvegia.

Table 3. Identified peptides derived from κ-CN in Gamalost (30 days), pH 4.6 soluble fraction (SF) (0 day Gamalost) and Norvegia (90 days) before (undigested samples (U)) and after gastric (G) and duodenal (D) digestion

-	-	-	pH 4.6 SF			Gamalost			Norvegia		
Mr	Fragment	Amino acid sequence[a]	0 day			30 days			90 days		
			U	HGJ	HDJ	U	HGJ	HDJ	U	HGJ	HDJ
1584.83	18-30	F.FSDKIAKYIPIQY.V					+			+	
1796.98	18-32	F.FSDKIAKYIPIQYVL.S								+	
1181.61	56-65	F.LPYPYYAK*P*A.A					+				
2861.53	51-75	L.INNQFLPYPYYAKPAAVRSPAQILQ.W								+	
1536.73	67-79	A.VRSPAQILQWQVL.S							+		
1197.51	96-105	M.ARHPHPHLSF.M								+	
1144.56	116-137	D.KTEIPTINTIASGEPTSTPTTE.A		+							
2196.06	149-169	D.SPEVIESPPEINTVQVTSTAV	+								
1226.59	151-161	P.EVIESPPEINT.V			+						
1541.73	155-169	E.SPPEINTVQVTSTAV	+	+	+						

[a]One letter amino acid codes used.

Dot (.) represents the cleavage site of the peptides.

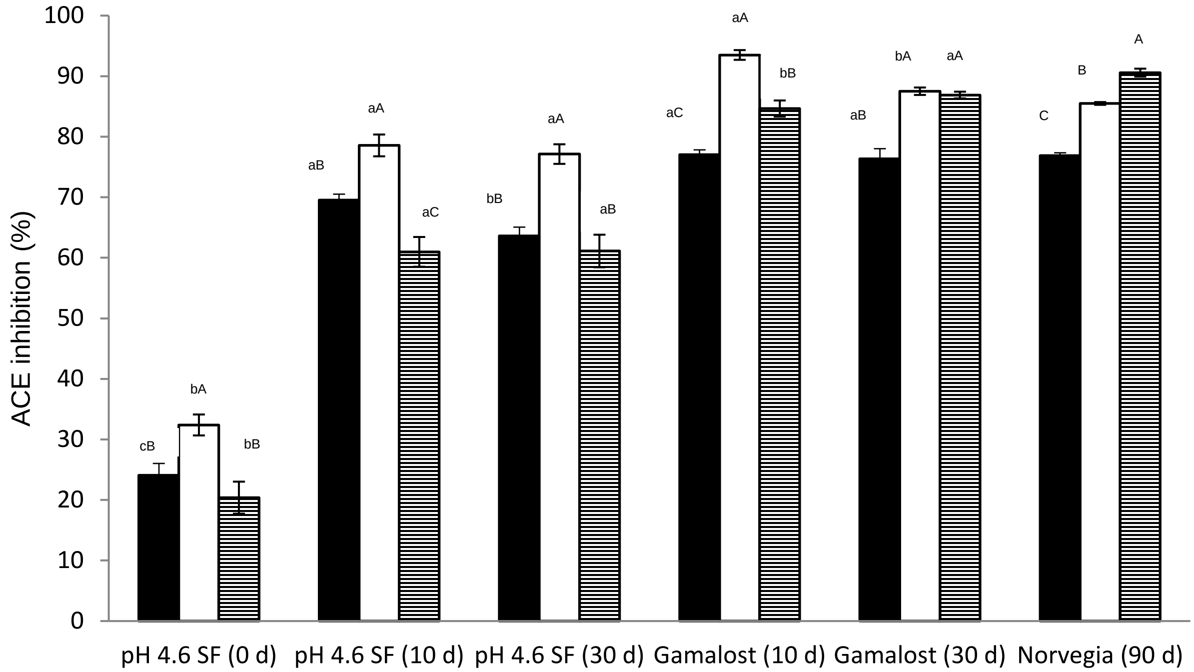

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Figure 1.

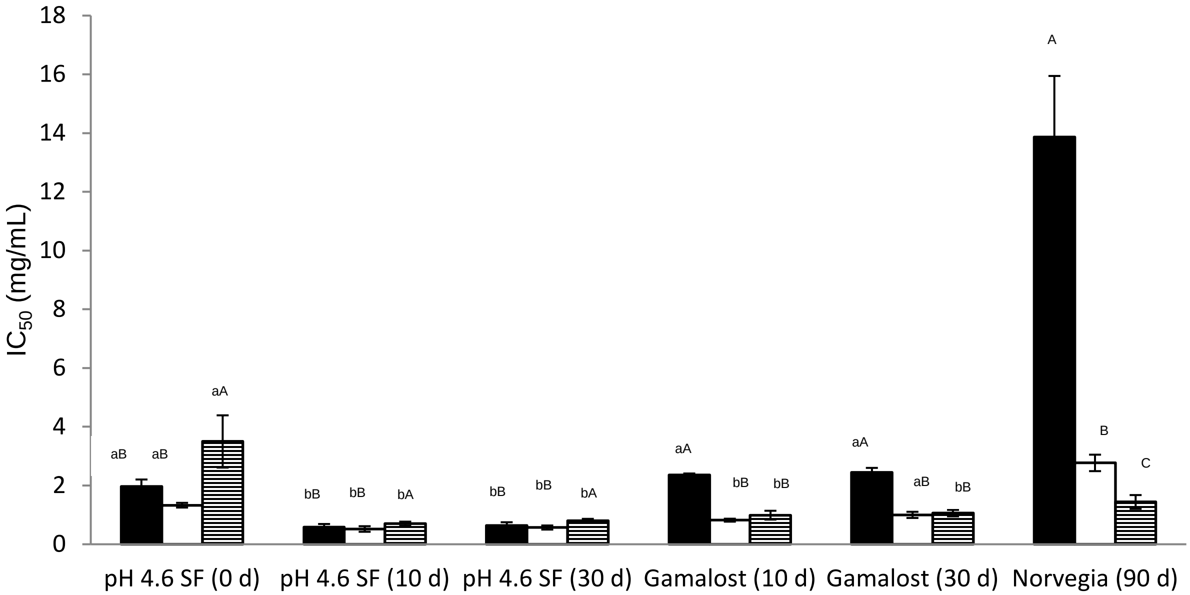

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Figure 2.

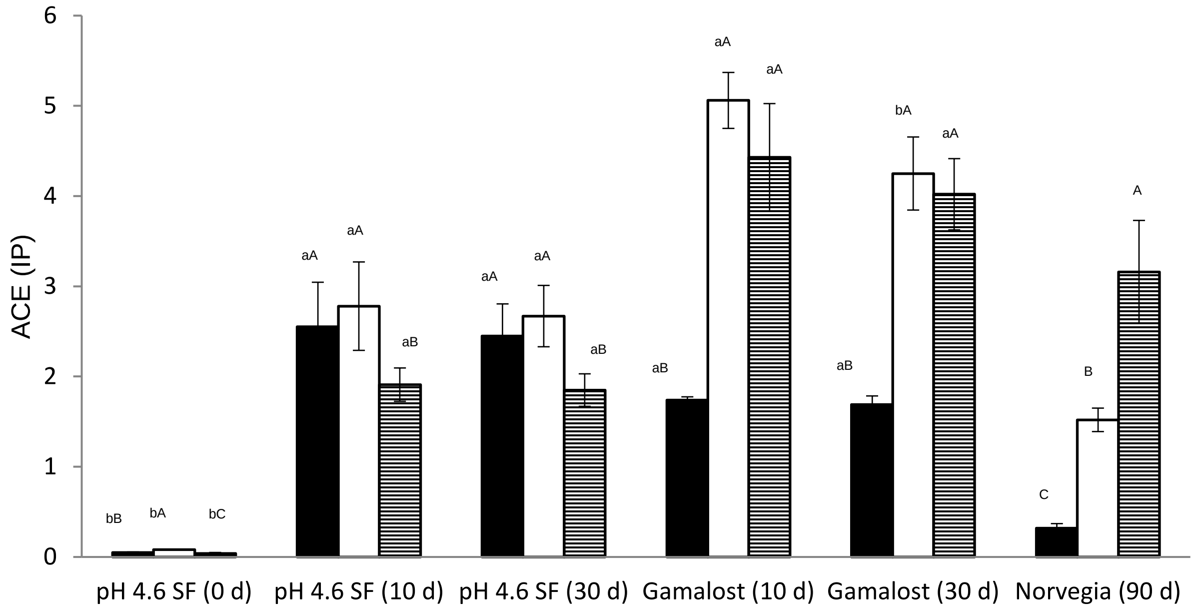

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Figure 3.

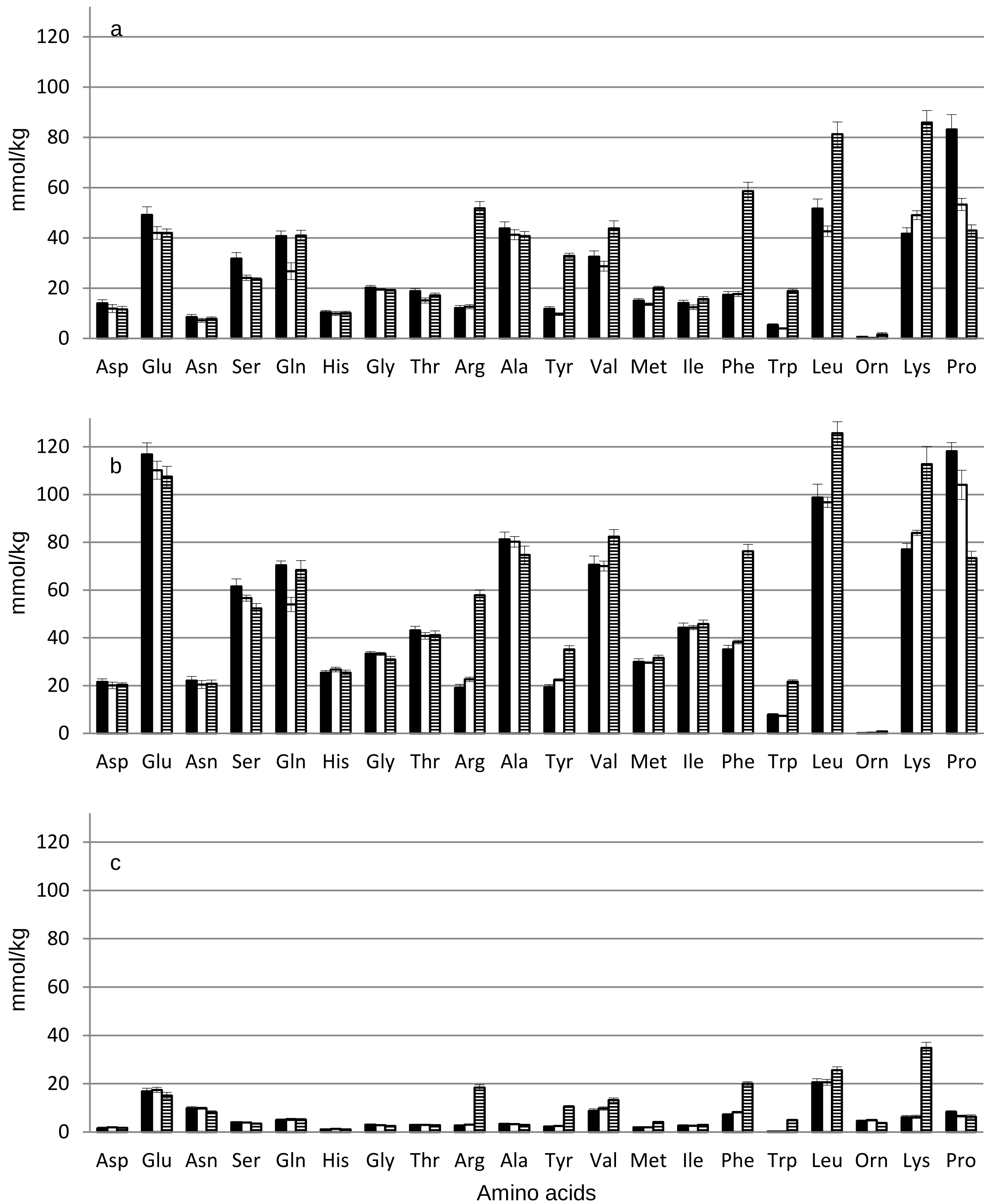

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Figure 9.