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Samenvatting
Het doel van deze studie was na te gaan in welke mate vrije aminozuren gevoelig zijn voor afbraak onder brijvoercondities. Onder de toegepaste condities in dit onderzoek trad er slechts in beperkte mate degradatie van vrije aminozuren in de brijvoeders op.

Summary
The aim of the current study was to determine the degradation of free amino acids in freshly produced liquid feeds. It can be concluded that the level of degradation of free amino acids in liquid feeds might be limited, provided the conditions applied in the current study.

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Wageningen Livestock Research Report 1159

Table of contents

Foreword

The present study has been conducted by Wageningen Livestock Research at the request of the partners of Feed4Foodure, and aimed to evaluate the degradation of free amino acids in liquid feeding conditions.
For the current study, scientists of the mentioned institute worked together with representatives from the various private partners, including Agrifirm, ForFarmers, Trouw Nutrition, De Heus, Cargill, Groep van Zes, DIVA, and Centrico. The authors would like to thank the industry partners of the project team for their worthwhile input.

Dr. Marinus van Krimpen, Study Director

Summary

Providing low-protein diets, that are supplemented with free amino acids to cover the amino acid requirement of pigs, might be helpful in reducing the use of soybean meal of Latin American origin, and thus in reducing the amount of non-EU protein in the diet. In pig farming, liquid feeding is frequently applied. Research from Denmark demonstrated that free amino acids can be highly sensitive for degradation during fermentation of liquid feed. The aim of the current study was to determine the degradation of free amino acids in freshly produced liquid feeds under very mild fermentation conditions.

Two liquid feeds, without and with free amino acids, were compared. On a dry matter basis, feed consisted of 50%, 30%, 10%, and 10% complement diet, liquid wheat starch, potato steam peels, and wheat yeast concentrate, respectively. Free lysine, methionine, threonine, and tryptophan were supplemented to the complement feed with free amino acids. After mixing the liquid feeds, samples of the liquid feed were analysed for pH and amino acid contents after 0, 1, 2, 4 and 8h of incubation. Samples were treated to receive separate fractions with insoluble protein, plant protein, bacterial protein and free amino acids, where amino acid contents were determined in all fractions at each sampling time.

The most important conclusions of this study are summarised below.

- The analysed amino acid contents of the liquid coproducts and the complement feeds were in agreement with the calculated table values. The analysed contribution of the free amino acids to the liquid feed was slightly lower than calculated.
- The sum of analysed amino acids in the distinguished fractions insoluble protein, plant protein, bacterial protein and free amino acids was considerably lower than expected based on the analysed amino acid contents of the liquid coproducts and the complement feeds.
- The pH was 4.2 at the start of the incubation and increased to 4.5-4.6 after 8h of incubation, while pH level was not affected by the free amino acid concentration.
- The liquid feed at the start of the incubation period had a low microbial load and a high concentration of lactic acid.
- The amounts of free Lysine, methionine and threonine that were degraded over the 8h incubation period were estimated to be 9%, 3%, and 0-30%, respectively.

Compared to findings in Danish studies, a relatively low level of degradation of free lysine, methionine, and threonine was observed. These low levels of degradation of free AA might be explained by the limited availability of *Enterobacteriaceae,* as well as by the high concentration of lactic acid at the start of the incubation period. The pH level of the liquid feed remained below 4.6 during the 8h incubation period, indicating no large shift in the presence of volatile fatty acids, while this low pH hampers the development of coliforms, which are able to degrade free amino acids for the production of biogenic amines. It can be concluded that the level of degradation of free amino acids in liquid feeds might be limited, provided the conditions applied in the current study.

1 Introduction

The global demand for animal sourced food in 2050 is expected to increase by 70% compared to the 2000 demand, largely as a result of growth of the world population, increased income levels, and urbanization, mostly in developing regions (FAO, 2009; Alexandratos and Bruinsma, 2012). As a consequence, the world-wide demand for animal feed is expected to increase to 1500 Mton in 2050. From 2005 to 2016, global feed production has increased from 645 to 1070 Mton (Alltech, 2018), with Asia and Africa recording the fastest growth.

Dietary protein for animals originates from a variety of sources including forages, grains, legumes, animal meals and various co-products. Of all the dietary components (e.g. protein, energy, fibre, vitamins, minerals), it is especially the protein component which provides the largest challenge to feed formulation in the future. Major parts of the world are not self-sufficient in terms of protein supply and rely heavily on imported soybean meal (SBM). In the EU, for example, the self-sufficiency rate of feed protein is currently only 42% (EU, 2018).

Providing diets with a reduced crude protein (CP) content, that are supplemented with free amino acids (AA) to cover the AA requirement of pigs, might be helpful in reducing the SBM import from Latin American, and thus in reducing the amount of non-EU protein in the diet. Studies with broilers and growing-finishing pigs, also conducted within the framework of Feed4Foodure, showed that it was possible in low-protein – free AA supplemented diets to reduce 20 to 40% of the original SBM, without compromising animal performance (Van Harn et al., 2017; Pluk and Van Krimpen, 2018).

In European pig production, the use of liquid feed is widely spread (Scholten et al., 1999), and the interest for it has increased during the last years due to the recent total ban of antibiotic growth promoters in the European Union. Fermentation of animal feed could provide protection against pathogens that the raw materials might harbour or that might contaminate the feed during storage (Adams and Nicolaides, 1997; Beal et al., 2002).

Research from Denmark, however, demonstrated that free AA can be highly sensitive for degradation during fermentation of liquid feed, with a disappearance of 34%, 38%, and 42% of free lysine, threonine and methionine, respectively, after 12 hours of fermentation of a liquid feed containing high levels of lactic acid bacteria, *Enterobacteriaceae*, and yeasts. The level of disappearance of the free AA was much higher compared to protein-bound AA.

The extent of degradation of free AA might be dependent of various factors, e.g. fermentation time, pH of the liquid, temperature of the liquid, diet structure (meal vs. pellet), and the presence of *Enterobacteriaceae* (Adams and Nicolaides, 1997; Geary et al., 1999; Beal et al., 2002; Adesogan and Salawu, 2004; Niven et al., 2006).

It was hypothesized that sensitive conditions for high level of AA degradation would be i) pH above 4, ii) relative high liquid temperature (± 37^0C), and iii) use of non-heat-treated feed. The aim of the current study was to determine the degradation of free AA in freshly produced liquid feeds under very mild fermentation conditions.

2 Material and methods

2.1 Dietary treatments

In this *in vitro* study, 2 dietary treatments were compared, a liquid feed without and with free AA. Amino acid degradation was measured in these diets after 0, 1, 2, 4 and 8h of incubation.

2.2 Composition of the liquid feed

A liquid feed was prepared consisting of complement feed, 3 liquid co-products, and water (Table 1).

Table 1 *Dry matter content of ingredients, and diet composition of the liquid feed on as-fed (g) and dry matter (%) basis.*

Ingredient	DM content ingredient	Liquid feed	
Basis	(g/kg)	As-fed basis 25% DM (g)	DM basis 100% DM (%)
Complement diet	874	17.9	50.0
Liquid wheat starch	214	43.8	30.0
Potato steam peal	110	28.4	10.0
Wheat yeast concentrate	250	12.5	10.0
Water	0	22.4	0.0
Total		125.0	100.0

Fresh liquid coproducts were provided by ForFarmers (Lochem, the Netherlands) and stored in a freezer until starting the experiment. The liquid coproducts were thawed and as-fed basis mixed in the ratio mentioned in Table 1. To induce conditions of mild fermentation, this mixture was fermented for 12h at room temperature to stimulate the development of acetic acid, butyric acid, and lactic acid producing bacteria. Thereafter, complement feed, liquid co-products, and water where mixed in the ratio mentioned in Table 1 to receive 125 g of liquid feed with a calculated dry matter (DM) content of 25%. The liquid coproducts were a mixture of 10% back slopping of 12h-fermented coproducts and 90% fresh coproducts. On a dry matter basis, liquid feeds consisted of 50%, 30%, 10%, and 10% complement diet, liquid wheat starch, potato steam peels, and wheat yeast concentrate, respectively. Two complement wheat-SBM diets, with and without supplementation of free AA, were used (Table 2). To maintain the level of bacteria possibly present in the feed ingredients, complement diets were not heat-treated, but produced as mash. Finally, to stimulate mild fermentation during incubation, temperature of the liquid feed was set 37^0C.

Table 2 *Ingredient composition (g/kg) of the complement diets.*

	Without free AA	With free AA
Wheat	627.4	627.4
Soybean meal	300.0	300.0
Limestone	22.9	22.9
Molasses	20.0	20.0
Premix growing-finishing pigs	9.9	9.9
Monocalciumphosphate	9.0	9.0
L-Lysine HCL	0.0	5.0
L-Threonine	0.0	2.5
DL-Methionine	0.0	2.5
L-Tryptophan	0.0	0.8
Diamol	10.8	0.0
Total	1000.0	1000.0

The table values (CVB, 2016) of AA content in the complement feeds without and with free AA, as well as the calculated contribution of the free AA to the AA supplemented feed are provided in Table 3.

Table 3 *Table values (CVB, 2016) of amino acid contents of the complement diets (g/kg as fed basis) without and with addition of free amino acid as well as the contribution of the free amino acids.*

	Without free amino acids	With free amino acids	Contribution free amino acids
Lysine	10.60	14.51	3.91
Methionine	3.08	5.56	2.48
Cysteine	3.63	3.63	0.00
Threonine	7.67	9.93	2.26
Tryptophan	2.65	3.43	0.78

The liquid feed with free AA was optimised to meet the nutrient recommendations of growing pigs (CVB, 2016), with ratios of Met, Thr. and Trp to Lys of 38, 62 and 20%, respectively. In Table 4, the calculated total AA content, the AA content from plant origin, the AA content from free AA origin and the ratio of free AA to total AA are shown. The ratio of free AA to total AA ranged from 15% in case of Trp to 31% in case of Met.

Table 4 *The calculated total AA content (g/kg 88% DM), the AA content from plant origin (g/kg), the AA content from free AA origin (g/kg) and the ratio of free AA to total AA (%) of Lys, Met, Thr, and Trp in the liquid feed.*

	Totaal AA (g/kg)	Plant AA (g/kg)	Free AA (g/kg)	Ratio (%) Free AA/Tot. AA
Lysine	10.62	8.65	1.97	19
Methionine	4.07	2.82	1.25	31
Threonine	7.46	6.23	1.23	17
Tryptophan	2.67	2.27	0.40	15

The table values (CVB, 2016) of AA in the liquid coproducts (g/kg fresh product) are shown in Table 5.

Table 5 *Table values (CVB, 2016) of amino acids in the liquid coproducts (g/kg fresh product).*

	Wheat starch	Potato steam peels	Wheat yeast concentrate
Lysine	0.95	0.84	4.17
Methionine	0.48	0.22	1.45
Cysteine	0.78	0.20	1.28
Threonine	0.83	0.60	2.72
Tryptophan	0.38	0.14	1.02

The table values (CVB, 2016) of AA in the complete liquid feeds, expressed as mg/125 g of liquid feed, are shown in Table 6.

Table 6 *Table values (CVB, 2016) of amino acid contents in the complete liquid feed with and without free amino acids (mg/125 g liquid feed) as well as the calculated contribution of the free amino acids.*

	Without free AA	With free AA	Contribution free AA
Lysine	307	377	70
Methionine	100	145	44
Cysteine	120	120	0
Threonine	221	265	44
Tryptophan	81	95	14

Samples of the liquid feed were analysed after 0, 1, 2, 4 and 8h of incubation. The pH (Escolab, Terschuur, The Netherlands) was measured in the liquid feed at each time sampling point. Average pH values were based on 10, 8, 6, 4, and 2 observations per treatment for 0, 1, 2, 4 and 8 hours of incubation, respectively.

2.3 Analysis of protein fractions

At each time sampling point, 4 different AA fractions were separated and analysed. Fractions were i) soluble free AA, ii) soluble protein-bound AA from feed, iii) protein-bound AA originating from bacterial protein, and iv) insoluble protein-bound AA from feed. It was hypothesised that soluble free and protein-bound AA could be converted to bacterial protein, where insoluble protein-bound AA from feed might become soluble over time. By analysing these fractions, we aimed to get a better understanding of the mode of action of degradation of free AA in liquid feed.
For obtaining the different AA fractions, the method as described by Niven (2006) and De Jonge et al. (2013) were used. Briefly the method included the following steps:

- Step 1: the fraction insoluble protein-bound AA from feed was the residue fraction after centrifuging the liquid feed at 3000 x *g* during 10 min.
- Step 2: the fraction with protein-bound AA from bacteria was the residue fraction after centrifuging the remaining supernatant fraction of step 1 at 20,000 x *g* for 15 min.
- Step 3: the fraction with soluble protein-bound AA from feed was obtained after precipitation of the AA in the remaining supernatant of step 2 by adding trichloroacetic acid (TCA). AA were analysed including a hydrolysis step.
- Step 4: the fraction with free AA was also present in the remaining supernatant of step 2. For this, AA were analysed without hydrolysis step.

Lys, Cys, and Thr contents in the first three fractions were determined after hydrolysis with 6 M HCL during 23 hours. The hydrolysate was adjusted to pH 2.2. Amino acids were separated by ion exchange chromatography and determined by post column reaction with ninhydrin, using photometric detection at 570 nm and at 440 nm for proline (ISO 13903, 2005).
Met was determined after oxidation overnight with performic acid/phenol at 0°C and neutralization with sodium disulphite, followed by hydrolysis with 6 M HCL during 23 hours. The hydrolysate was adjusted to pH 2.2. Amino acids were separated by ion exchange chromatography and determined by post column reaction with ninhydrin, using photometric detection at 570 nm. Met was measured as methionine sulfone (ISO 13903, 2005). Amino acids in the fourth fraction were directly, i.e. without oxidation and hydrolyse, measured in the solution by use the same separation and detection technique as described (ISO 13903, 2005).
Trp was released by an alkaline hydrolysis with LiOH under nitrogen pressure at 120 °C during 16 hours. Trp was separated by reversed phase chromatography with acetate/methanol as eluents and identified and quantified by UV detection at 280 nm (ISO 13904, 2016). The mentioned hydrolysis was not applied to measure Trp in the fourth fraction.

2.4 Microbial populations, fatty acids and toxic amines

At the start of the incubation (t=0h), the liquid feed was analysed on some microbial populations, e.g. counts of *lactic acid* bacteria, *Enterobacterieae,* yeasts and fungi. Besides, in this sample, concentrations of acetic acid, butyric acid, lactic acid and ethanol were determined. Also, contents of some toxic amines, e.g. cadaverine, putrescine, histamine, and spermidine were measured.

3 Results

3.1 Amino acid analysis of ingredients and complement diets

The analysed AA contents (g/kg fresh product) of the liquid coproducts and the AA recovery (%) compared to the table values (CVB, 2016) are shown in Table 7.

Table 7 *Analysed amino acid contents (g/kg fresh product) of the liquid coproducts, and the amino acid recovery (%) compared to the table values (CVB, 2016).*

	Wheat starch		Potato steam peels		Wheat yeast concentrate	
	Analysed	Recovery	Analysed	Recovery	Analysed	Recovery
Lysine	1.17	123.2	0.50	59.5	3.24	77.7
Methionine	0.49	102.1	0.13	59.1	1.16	80.0
Cysteine	0.76	97.4	0.13	65.0	1.36	106.3
Threonine	0.96	115.7	0.36	60.0	2.64	97.1
Tryptophan	0.35	92.1	0.10	71.4	0.80	78.4

In wheat starch, analysed Lys content was 23% higher than the table value, where the analysed values of the other AA were more close to the table values. The analysed AA contents of all AA in the potato steam peels were substantially lower than the table values. The analysed contents of Lys, Met, and Trp in wheat yeast concentrate were lower than the table values, where analysed values of Cys and Thr were close to the table values.

The analysed AA contents of the complement diets (g/kg as fed basis) without and with addition of free amino acid, and the AA recovery (%) compared to the table values are shown in Table 8.

Table 8 *Analysed amino acid contents of the complement diets (g/kg as fed basis) without and with addition of free amino acid, and the amino acid recovery (%) compared to the table values (CVB, 2016).*

	Without free AA		With free AA		Contribution free AA	
	Analysed	Recovery	Analysed	Recovery	Analysed	Recovery
Lysine	11.55	109.0	14.75	101.7	3.20	81.8
Methionine	3.10	100.6	5.17	93.0	2.07	83.5
Cysteine	3.72	102.5	3.86	106.3	0.14	- - -
Threonine	8.34	108.7	10.13	102.0	1.79	79.2
Tryptophan	2.55	96.2	3.17	92.4	0.62	79.5

In the complement feed without free AA, the analysed values of Met, Cys and Trp met the calculated values, where the analysed values of Lys and Thr were higher than calculated. In the diet with free AA the analysed values of Met and Trp were below the calculated values, where the other AA met the calculated values. Based on the analysed differences between the two diets, it was shown that the recoveries of free Lys, Met, and Thr, and Trp in the diets with free AA all were about 80% of the calculated values. Free Cys was not added to the complement feed, and therefore a recovery value was not relevant for this AA.

The recalculated amino acid contents of the liquid feeds with and without free AA, expressed as g/125 g, based on the analysed values of the liquid coproducts and complement feeds, as well as the recovery (%) compared to the table values are shown in Table 9.

Table 9 *Recalculated amino acid contents of the liquid feeds with and without free AA (mg/125 g liquid feed) based on the analysed values of the liquid coproducts and complement feeds, as well as the recovery (%) compared to the table values (CVB, 2016).*

	Without free AA		With free AA		Contribution free AA	
	Analysed	Recovery	Analysed	Recovery	Analysed	Recovery
Lysine	313	102.0	370	98.1	57	81.4
Methionine	95	95.0	132	91.0	37	84.1
Cysteine	121	100.8	123	102.5	3	- - -
Threonine	235	106.3	267	100.8	32	72.7
Tryptophan	74	91.4	85	89.5	11	78.6

In general, the analysed AA values of the recalculated liquid feeds were in the same range as the calculated table values, with differences varying from 0.8% (Thr in feed with free AA) to 8.6% (Trp in feed without free AA). Based on the analysed differences between the two liquid feeds, it was shown that the contribution of free Lys, Met, Thr, and Trp to the diets with free AA was below the calculated table values.

3.2 Development of amino acid concentration over time

Lysine

Table 10 shows the development of the Lys concentration (mg/125 g) over time in the liquid diets without and with free amino acids, subdivided in the fractions i) soluble free AA, ii) soluble protein-bound AA from feed, iii) protein-bound AA originating from bacterial protein, and iv) insoluble protein-bound AA from feed. The expected Lys contents at the start of the incubation were 313 and 370 mg/125 g in the liquid feeds without and with free AA, respectively. At the start of the incubation, the analysed Lys contents were 175 and 221 mg/125 g in the liquid feeds without and with free AA, respectively, indicating a difference of 46 mg/125 g. The expected difference between the two diets was 57 mg/125 g (Table 9), which equals a recovery of added free Lys of 81%. The additional 46 mg free Lys at the start of the incubation resulted in an increase of 20.6 mg (45%) in the insoluble protein-bound AA fraction, 3.1 mg (6.7%) in the soluble protein-bound plant AA fraction and 22.2 mg (48.3%) in the free AA fraction. In the diet without free AA, the reduction in Lys content during the 8h incubation period was 10 mg, indicating a 5.7% reduction in Lys content. In the diet with free AA, the Lys content reduced by 14 mg during the 8h incubation period, indicating a 6.3% reduction in Lys content. During the 8h of incubation, the difference in Lys content between the liquid feed with and without free AA decreased from 46 to 42 mg/125 g, indicating that 9% of the free Lys was degraded over time. This reduction in AA content over the 8h incubation period was entirely caused by a reduction in the insoluble protein-bound AA fraction, where the changes in Lys content in the other fractions were negligible.

Table 10 *Development of lysine concentration (mg/125 g) over time in the liquid diets without and with free amino acids, subdivided in the fractions i) soluble free AA, ii) soluble protein-bound AA from feed, iii) protein-bound AA originating from bacterial protein, and iv) insoluble protein-bound AA from feed.*

	Without free AA					With free AA					Δ AA
Incubation Time (h)	Insol. AA	Bact. AA	Plant AA	Free AA	Total	Insol. AA	Bact. AA	Plant AA	Free AA	Total	
0	168.0	0.2	3.7	2.7	175	188.6	0.3	6.8	24.9	221	46
1	160.6	0.2	5.5	3.6	170	182.4	0.6	5.7	26.0	215	45
2	167.6	0.3	5.6	4.1	178	185.8	0.5	6.7	28.0	221	43
4	154.2	0.8	6.5	4.3	166	177.1	0.6	6.8	26.0	211	45
8	154.2	0.4	6.1	4.4	165	172.3	1.4	6.6	27.0	207	42
Table value (Analysed)		307 (313)		0	307 (313)		307 (313)		70 (57)	377 (370)	

Methionine

Table 11 shows the development of the Met concentration (mg/125 g) over time in the liquid diets without and with free amino acids, subdivided over the different fractions. The expected Met contents at the start of the incubation were 95 and 132 mg/125 g in the liquid feeds without and with free AA, respectively. At the start of the incubation, the analysed Met contents were 73 and 104 mg/125 g in the liquid feeds without and with free AA, respectively, indicating a difference of 31 mg/125 g. The expected difference between the two diets was 37 mg/125 g (Table 9), which equals a recovery of added free Met of 84%. The additional 31 mg free Met at the start of the incubation resulted in an increase of 16.3 mg (52.6%) in the insoluble protein-bound AA fraction, 1.4 mg (4.5%) in the soluble protein-bound plant AA fraction and 13.4 mg (43.2%) in the free AA fraction. In the diet without free AA, the Met content reduced by 4 mg after 8h of incubation, indicating a 5.5% reduction in Met content. In the diet with free AA, the Met content reduced by 5 mg after 8h of incubation, indicating a 4.8% reduction in Met content. After 4h of incubation, the difference in Met content between the liquid feed with and without free AA was similar to the difference at the start of the incubation period, assuming that no free Met was degraded over time. During the 8h of incubation, the difference in Met content between the liquid feed with and without free AA decreased from 31 to 30 mg/125 g, indicating that 3% of the free Met was degraded over time. The reduction in AA content over the 8h incubation period was entirely caused by a reduction in the insoluble AA fraction, where the changes in Met content in the other fractions were negligible.

Table 11 *Development of methionine concentration (mg/125 g) over time in the liquid diets without and with free amino acids, subdivided in the fractions i) soluble free AA, ii) soluble protein-bound AA from feed, iii) protein-bound AA originating from bacterial protein, and iv) insoluble protein-bound AA from feed.*

	Without free AA					With free AA					Δ AA
Incubation Time (h)	Insol. AA	Bact. AA	Plant AA	Free AA	Total	Insol. AA	Bact. AA	Plant AA	Free AA	Total	
0	70.1	0.1	1.8	0.8	73	86.4	0.2	3.2	14.2	104	31
1	66.9	0.1	2.7	1.0	71	85.9	0.4	2.7	15.5	105	34
2	69.4	0.2	2.6	1.2	73	85.1	0.3	2.9	16.1	104	31
4	65.4	0.4	2.8	1.2	70	83.6	0.4	2.8	14.5	101	31
8	64.8	0.2	2.8	1.2	69	81.8	0.7	2.7	14.2	99	30
Calculated (Analysed)		100 (95)		0	100 (95)		100 (95)		44 (37)	145 (132)	

Threonine

Table 12 shows the development of the Thr concentration (mg/125 g) over time in the liquid diets without and with free amino acids, subdivided in the different fractions. Based on the analysed AA values of the liquid coproducts and the complement feeds, the expected Thr contents at the start of the incubation were 235 and 267 mg/125 g in the liquid feeds without and with free AA, respectively. At the start of the incubation, the analysed Thr contents were 213 and 253 mg/125 g in the liquid feeds without and with free AA, respectively, indicating a difference of 40 mg/125 g. The expected difference between the two diets was 32 mg/125 g, which equals a recovery of added free Thr of 125%. The additional 40 mg free Thr at the start of the incubation resulted in an increase of 19.6 mg (49.0%) in the insoluble protein-bound AA fraction, 2.9 mg (7.3%) in the soluble protein-bound plant AA fraction and 17.3 mg (43.3%) in the free AA fraction. In the diet without free AA, the Thr content remained more or less constant over the 8h incubation period. In the diet with free AA, the Thr content reduced by 9 mg after 8h of incubation, indicating a 3.6% reduction in Thr content. After 8h of incubation, the difference in Thr content between the liquid feed with and without free AA was 28 mg/125, assuming that about 30% of the free Thr was degraded over time. The reduction in AA content over the 8h incubation period was entirely caused by a reduction in the insoluble protein-bound AA fraction, where the changes in Thr content in the other fractions were negligible.

Table 12 *Development of threonine concentration (mg/125 g) over time in the liquid diets without and with free amino acids, subdivided in the fractions i) soluble free AA, ii) soluble protein-bound AA from feed, iii) protein-bound AA originating from bacterial protein, and iv) insoluble protein-bound AA from feed.*

	Without free AA					**With free AA**					**Δ AA**
Incubation Time (h)	Insol. AA	Bact. AA	Plant AA	Free AA	Total	Insol. AA	Bact. AA	Plant AA	Free AA	Total	
0	200.0	0.3	3.7	9.1	213	219.6	0.4	6.6	26.4	253	40
1	191.2	0.2	5.5	12.1	209	215.1	0.7	5.6	28.2	250	41
2	196.8	0.4	5.6	13.3	216	215.8	0.6	6.3	28.5	251	35
4	191.9	1.0	6.2	12.8	212	208.9	0.7	6.3	26.7	243	31
8	197.8	0.4	5.9	12.1	216	208.7	1.5	6.0	28.0	244	28
Calculated (Analysed)		221 (235)		0	221 (235)		221 (235)		44 (32)	265 (267)	

3.3 Development of pH over time

The development of pH in the liquid feed with and without free AA during the 8h incubation period is shown in Figure 1.

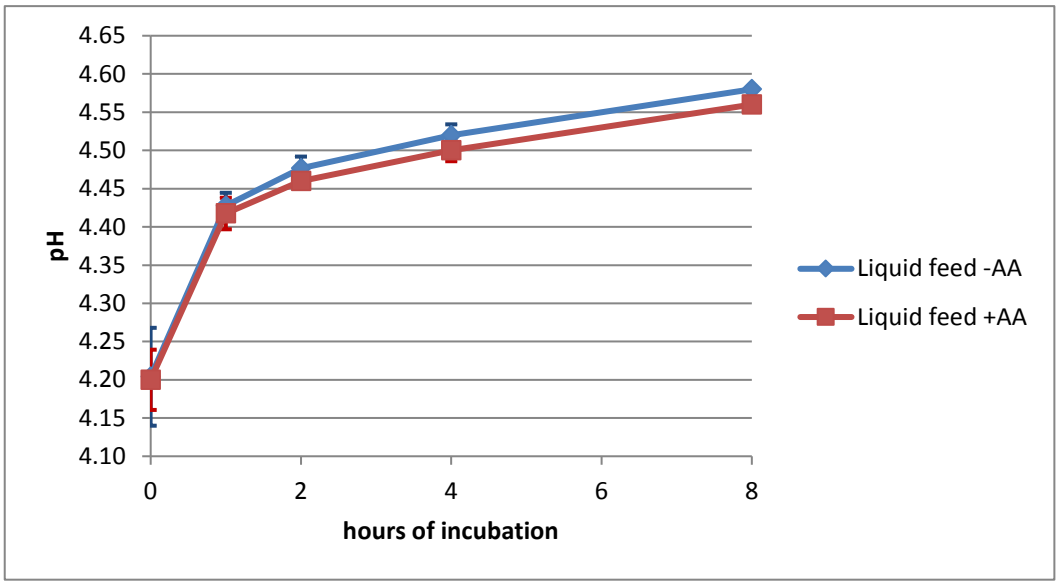

Figure 1 *Development of pH in the liquid feed with and without free AA during the 8h incubation period. Values were based on 10, 8, 6, 4, and 2 observations per treatment for 0, 1, 2, 4 and 8 hours of incubation, respectively.*

At the start of the incubation period, pH was 4.20, and pH increased over time to 4.57 after 8 hours of incubation. The development of pH of the liquid feed over time was not affected by the presence of free AA.

3.4 Microbial populations, fatty acids and ethanol

In Table 13, the counts of some microbial populations (log cfu/g), the concentrations of acetic acid, butyric acid, lactic acid, and ethanol (mmol/kg), and the concentrations of some biogenic amines (mg/kg DM) of the liquid feed at the start of the incubation are presented.

Table 13 *Counts of some microbial populations (log cfu/g), the concentrations of acetic acid, butyric acid, lactic acid, and ethanol (mmol/kg), and the concentrations of some biogenic amines (mg/kg DM) of the liquid feed at the start of the incubation.*

	Unit	Value
Lactic acid bacteria	Log cfu/g	< 2.0
Enterobacteriaceae	Log cfu/g	< 1.0
Yeast	Log cfu/g	< 2.0
Fungi	Log cfu/g	< 3.0
Acetic acid	mmol/kg	18.3
Butyric acid	mmol/kg	5.7
Lactic Acid	mmol/kg	133.3
Ethanol	mmol/kg	8.7
Cadaverine	mg/kg DM	57
Putrescine	mg/kg DM	57
Histamine	mg/kg DM	26
Spermidine	mg/kg DM	23

At the start of the incubation, the counts of lactic acid bacteria, *Enterobacteriaceae*, yeast and fungi all were below the detection limit, indicating a low microbial load of the liquid feed. The lactic acid concentration (133.3 mmol/kg) was the most dominant acid as compared to acetic and butyric acid and ethanol. The concentrations of the determined biogenic amines ranged from 23 (spermidine) to 57 (cadaverine and putrescine) mg/kg DM. These analyses were not conducted at the end of the incubation period.

4 Discussion

4.1 Degradation of free amino acids

The aim of the current study was to determine the degradation of free AA in freshly produced liquid feeds under very mild fermentation conditions. Based on the analysed differences between the liquid feeds without and with free AA during the 8h incubation period, the degradation of free Lys, Met, and Thr were estimated to be 9%, 3% and 30%, respectively. These differences were based on AA analysis in the four separated fractions in the liquid feeds without and without free AA. All variations in AA analysis accumulated in the calculated differences between the two liquid feeds. Taking the differences in AA contents between both feeds over time into account, actually no clear proof for degradation of free Lys and Met could be observed. For Thr, the results more clearly seemed to indicate a substantial degradation of free AA. Contrary to in Lys and Met, the fraction insoluble plant protein-bound Thr remained constant over the 8h incubation period. This was mainly due to the value observed after 8h incubation, which based on the trends in analysis of the other time points, seemed to be an outlier. After excluding this value from the dataset, very similar slopes of the trend lines describing the decrease in insoluble plant protein-bound Thr in both liquid feeds over time were observed (Figure 2).

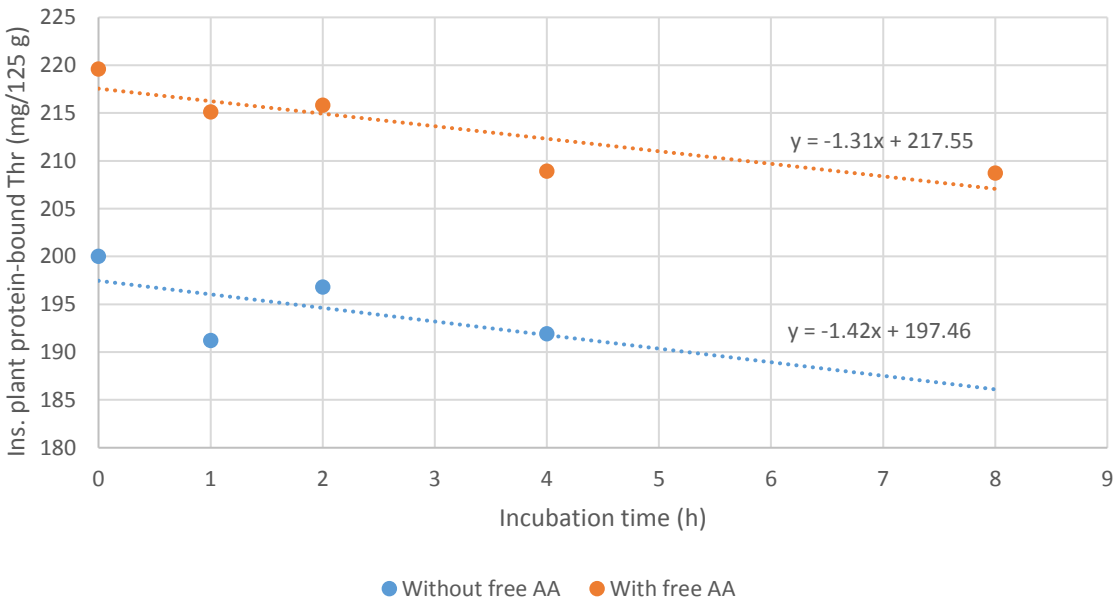

Figure 2 *Trends in development of insoluble plant protein-bound Thr (mg/125 g) over time after excluding the value in the liquid feed without free AA at 8h of incubation from the dataset.*

Based on the trend line in Figure 2, the Thr content in the insoluble plant protein-bound fraction after 8h of incubation has been estimated to be 186.1 mg/125 g, where the analysed value was 197.8 mg/125 g. After summing up this 11.7 mg difference to the current calculated difference between both liquid feeds of 28 mg, it can be concluded that the degradation of free Thr was negligible. If we, however, consider the difference of 11.7 mg less free Thr between the start and the end of the incubation as the complete result of degradation of free AA, then we have to conclude that 30% of free Thr was degraded over the 8h incubation period.

4.2 Impact of microbial load on amino acid degradation

Based on the above mentioned findings, the level of free AA degradation after 8h of incubation in the current study was 9% for Lys, 3% for Met and 0-30% for Thr. In a study with liquid feed conducted by Canibe et al. (2007), the degradation of free Lys, Met, and Thr after 6h of incubation amounted 25%, 31% and 28%, respectively. After 12h of incubation, 34%, 42% and 38% of free Lys, Met, and Thr, respectively, was degraded. In a study with liquid feed of Niven et al. (2006), no free Lys degraded after 4h of incubation, while the level of Lys degradation between treatments after 7h of incubation ranged from 0% to 55%, whereas level of degradation ranged from 0% to 91% after 21h of incubation. It can be concluded that the level of degradation of free AA in the current study were rather low, but that substantial levels of degradation has been reported in literature as well.
The decrease in free AA in liquid feed is the result of the balance between the accumulation of free amino acids caused by proteolysis because of enzymes present in the ration and the degradation of free AA by the available microflora (Canibe et al., 2007). This suggests that the disappearance of free AA is related to the microbial characteristics of the liquid feed. In contrast to the current study, Canibe et al. (2007) applied a specific incubation procedure aiming to prepare a fermented liquid feed with a suboptimal microbial quality, containing rather high levels of *Enterobacteriaceae*. In the study of Canibe et al. (2007) no back slopping was applied from 96h of incubation onwards, representing a steady state of the fermented liquid feed. As shown in Table 14, the microbial load in the samples between 96 and 108h of incubation were much higher as compared to the values in the current study, where contents of butyric and lactic acid were less.

Table 14 *Development of microbial populations (log CFU/g), and concentrations of short-chain fatty acids and ethanol (mmol/kg) during incubation in the study of Canibe et al. (2007), as compared to the values in the current study at T=0.*

Time (h)	Lactic acid bacteria	Entero bacteria ceae	Yeast	Acetic acid	Butyric acid	Lactic acid	Ethanol
Unit	(log CFU/g)			(mmol/kg)			
Canibe, T=0	<3.0	5.0	3.9	4.7	0.1	ND[1]	ND
Canibe, T=6	<3.0	6.0	<3.2	4.9	0.1	ND	1.9
Canibe, T=24	8.1	7.1	3.6	5.9	ND	ND	6.8
Canibe, T=48	9.5	6.7	3.7	20.9	0.2	91.2	15.5
Canibe, T=96 Free AA added	8.8	6.1	4.7	7.7	0.9	11.8	1.0
Canibe, T=102	9.5	6.0	<4.3	19.8	2.1	46.3	3.7
Canibe, T=108	9.5	5.0	5.2	23.9	0.7	70.4	8.7
Current study, T=0	<2.0	<1.0	2.0[2] <3.0[3]	18.3	5.7	133.3	8.7

[1]) ND = Not Determined; [2]) Yeast; [3]) fungi

AA are sensitive to microbial transamination, deamination, and decarboxylation (Smith and Macfarlane, 1997; Tavaria et al., 2002), and this microbial catabolism of AA is accompanied by the production of ammonia and toxic amines that are harmful to the gut integrity and gut health (Qaisrani et al., 2015; Apajalahti and Vienola, 2016). Of the determined AA in the current study, only Lys is involved in the production of cadaverine, which is a toxic amine (Halasz et al., 1994).
Niven et al. (2006) studied the metabolism of Lys degradation in liquid feed with and without supplementation of lactic acid bacteria and *E. coli*. They observed that in the control feed and the feed supplemented with E. coli about 90% of free Lys was metabolised after 21h of incubation, where the production of cadaverine accounted for about 50% of the loss of Lys. No Lys was degraded in the liquid feed supplemented with lactic acid bacteria, and the loss of Lys was 20% in the liquid feed supplemented with both lactic acid bacteria and *E. coli.* The rate of Lys degradation was inversely correlated with the lactic acid concentrations. A study of Beal et al. (2002) demonstrated that the concentration of lactic acid in liquid feed needed to be 75-100 mM and the pH below 4.5 to be able to inhibit the growth of coliforms.

Substantial levels of Lys degradation were observed when a non-heat treated mash diet was supplemented to the liquid feed, whereas low levels of Lys degradation occurred in case of using a heat-treated pelleted diet (Canibe and Jensen, 2010). Differences could not be directly related to the presence of microbial populations, e.g. lactic acid bacteria, *Enterobacteriaceae*, and yeasts, although in particular a higher concentration of acetic acid over time was determined in the liquid feed based on mash diet. Although a mash complement feed was used in the current study as well, the rather low levels of degradation of free AA might be explained by the limited availability of *Enterobacteriaceae,* and the high concentration of lactic acid at the start of the incubation period. Moreover, low levels of cadaverine, putrescine, histamine and spermidine were determined in this liquid feed as well. Although in the current study no microbial analysis after 8h of incubation are available, the low pH value after the 8h incubation period indicates that the conditions were still suitable for inhibiting the growth of coliforms, which supports the observed rather low levels of degradation of free AA.

4.3 Amino acid contents of the liquid feed at T=0

Based on the analysed AA contents of the individual components of the liquid feed, e.g. the liquid coproducts and the complement feeds, a good agreement between calculated (CVB, 2016) and analysed amino acid contents was observed, as shown in Table 9. Surprisingly, for Lys and Met, the sum of AA from the four separated fractions in the liquid feed at T=0 was substantially lower than the expected values based on the analysis of the individual components of the liquid feed. The analysed Lys contents in the liquid feed without and with free AA were 44% and 40% below the expected value, respectively, where the analysed Met contents were 33% and 21% below the expected values, respectively. Contrary to this, the analysed Thr contents in the liquid feed without and with free AA were only 4% and 5% below the expected values, respectively, indicating that the applied procedure for determining the different AA fractions worked well for Thr. We hypothesize that the cap in recovery might be explained by the procedure to obtain the soluble plant protein-bound AA. This procedure comprised a hydrolysis step, including treatment of the samples with hot acid for a certain period. Some AA are known to be unstable under these conditions, resulting in racemization or degradation of the AA (Lamp et al., 2018). Met, for instance, can be degraded during acid hydrolysis due to the reaction of sulpher with residual oxygen by the formation of methionine sulfoxide (Lamp et al., 2018). Lys is also very sensible for acid hydrolysis, because it contains a lysyl chain and an α-amino group, making this AA basic. Thr, however, is expected to be less sensible for hydrolysis, because it contains an α-amino group, a carboxyl group, and a side chain containing a hydroxyl group, making it a polar, uncharged amino acid. The high sensitivity of Lys and Met for acid hydrolysis might explain the low recovery of these AA in the liquid feed samples at T= 0. Lamp et al. (2018) published an improved HPLC-method, which enables to correct for the amino losses of unknown sample materials during hydrolysis by including a known protein source in the analysis as well. The hydrolysis kinetics of the known protein source provides information that can be used to correct for AA degradation of the unknown sample. This method, however, was not applied in the current project.

5 Conclusions

The most important conclusions of this study are summarised below.

- The analysed amino acid contents of the liquid coproducts and the complement feeds were in agreement with the calculated table values. The analysed contribution of the free amino acids to the liquid feed was slightly lower than calculated.
- The sum of analysed amino acids in the distinguished fractions insoluble protein, plant protein, bacterial protein and free amino acids was considerably lower than expected based on the analysed amino acid contents of the liquid coproducts and the complement feeds.
- The pH was 4.2 at the start of the incubation and increased to 4.5-4.6 after 8h of incubation, while pH level was not affected by the free amino acid concentration.
- The liquid feed at the start of the incubation period had a low microbial load and a high concentration of lactic acid.
- The amounts of free Lysine, methionine and threonine that were degraded over the 8h incubation period were estimated to be 9%, 3%, and 0-30%, respectively.

Compared to findings in Danish studies, a relatively low level of degradation of free lysine, methionine, and threonine was observed. These low levels of degradation of free AA might be explained by the limited availability of *Enterobacteriaceae,* as well as by the high concentration of lactic acid at the start of the incubation period. The pH level of the liquid feed remained below 4.6 during the 8h incubation period, indicating no large shift in the presence of volatile fatty acids, while this low pH hampers the development of coliforms, which are able to degrade free amino acids for the production of biogenic amines. It can be concluded that the level of degradation of free amino acids in liquid feeds might be limited, provided the conditions applied in the current study.

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