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Evaluation of an in vitro system to simulate equine foregut digestion and the influence of acidity on protein and fructan degradation in the horse´s stomach

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1 Summary

Evaluation of an in vitro system to simulate equine foregut digestion and the influence of acidity on protein and fructan degradation in the horse´s stomach

The aim of the present study was to improve an in vitro system in order to collect data on the digestion of different forages in the horse´s upper gastro-intestinal tract. Therefore, in vitro foregut digestion of several forages (grass mixture for horses, grass mixture for cows (GMC), tall fescue, English perennial ryegrass (ER), white clover, lucerne) was performed in two phases with pepsin and amylase, whereas microbial fermentation was neglected. The results are consistent with current data from in vivo studies, including a degradation of crude protein and monosaccharides as well as a relative increase of fibres. Interestingly, a loss of fructan was measured in two feedstuffs (ER/GMC).
As acid hydrolysis was suspected to be responsible for this, the effect of different pH-values (2, 3 and 4) on the fructan degradation of ER and GMC was tested subsequently. As expected, the highest degradation of protein was shown at the lowest pH (protein in ER/GMC at pH 2: 6.1/8.2 % DM and at pH 4: 7.7/10.6 % DM), whereas fructan degradation was highest at pH 4 (fructan in ER/GMC at pH 2: 1.63/1.95 % DM and at pH 4: 1.31/0.91 % DM). We presume that not only acidic hydrolysis but also plant enzymes cause the loss of fructans in an acidic environment.

Keywords: acidic hydrolysis, polymerization, pepsin, pankreatin, predigestion

2 Zusammenfassung

Etablierung eines in vitro Systems, das die präzäkalen Verdauungsprozesse des Pferdes simuliert und der Einfluss des Säuregrades im Magen auf den Abbau von Proteinen und Fruktanen

Das Ziel der Studie war die Optimierung eines in-vitro Systems um bestmögliche Daten zur Verdauung verschiedener Futtermittel im vorderen Verdauungstrakt des Pferdes zu erhalten. Hierfür wurden verschiedene Grünfutter (Grasmischung für Pferde, Grasmischung für Rinder (GMC), Rohrschwingel, Englisches Raygras (ER), Weissklee, Luzerne) in einem in vitro System mit Pepsin und Pankreatin verdaut, wobei mikrobielle Abbauprozesse ausgeschlossen wurden. Die Ergebnisse der Analysen entsprechen Daten aus in vivo Studien, in Hinsicht auf einen Abbau der Proteine und Monosaccharide, sowie einer relativen Zunahme der Fasern. Wiedererwartend wurde auch eine Abnahme des Fruktangehaltes in zwei Futtermitteln (GMC, ER) gemessen.
Da vermutet wurde, dass eine saure Hydrolyse für den Abbau verantwortlich war, wurde der Effekt verschiedener pH-Werte (2,3 und 4) auf den Fruktanabbau untersucht. Wie erwartet wurde Protein am stärksten bei dem niedrigsten pH-Wert abgebaut (Protein in ER/GMC bei pH 2: 6.1/8.2 % TS und bei pH 4: 7.7/10.6 % TS), wohingegen der Fruktanabbau bei pH 4 am grössten war (Fruktan in ER/GMC bei pH 2: 1.63/1.95 % TS und bei pH 4: 1.31/0.91 % TS). Wir vermuten, dass neben der sauren Hydrolyse auch Pflanzenenzyme für den präzäkalen Fruktanabbau beim Pferd verantwortlich sind.

Schlüsselwörter: saure Hydrolyse, Polymerisation, Pepsin, Pankreatin, Vorverdauung

3 Manuskript

Evaluation of an in vitro system to simulate equine foregut digestion and the influence of acidity on protein and fructan degradation in the horse´s stomach

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3.1 Summary

The aim of the present study was to improve an in vitro system in order to gather optimized information on the digestion of different forages in the horse´s upper gastro-intestinal tract. Therefore, in vitro foregut digestion of several forages was performed (*Part 1*). The effect of different pH-values on the fructan degradation of two selected grasses (*Part 2*) was tested subsequently.

Part 1: We hypothesized that our system produces representative results simulating digestive processes in the upper alimentary tract, but neglects microbial fermentation. In vitro digestion of six forages (grass mixture for horses, grass mixture for cows (GMC), tall fescue, English perennial ryegrass (ER), white clover, lucerne) was performed in two phases with pepsin and amylase. The results are consistent with current data from in vivo studies, including a degradation of crude protein and monosaccharides as well as a relative increase of fibres. Interestingly, a loss of fructan was measured in two feedstuffs (ER/GMC: 4.05/4.42% DM fructan before and 0.59/0.00% DM after simulated foregut digestion).

Part 2: As fructans are thought not to be fragmented by digestive enzymes, another hypothesis was developed: acidic hydrolysis leads to a degradation of fructans. To evaluate the influence of gastric pH on the digestion of fructan and protein, different pH -values (2, 3 and 4) were adjusted in a second series of in vitro foregut digestion trials with ER and GMC. As expected, the highest degradation of protein was seen at the lowest pH (protein in ER/GMC at pH 2: 6.11/8.28 % DM and at pH 4: 7.73/10.64 % DM), whereas fructan degradation was highest at pH 4 (fructan in ER/GMC at pH 2: 1.63/1.95 % DM and at pH 4: 1.31/0.91 % DM). We presume that not only acidic hydrolysis but also plant enzymes cause the loss of fructans in an acidic environment.

Keywords: animal welfare, acidic hydrolysis, polymerization, pepsin, pankreatin, predigestion

3.2 Introduction

Diseases caused by poor nutrition are common in horses. Therefore, it is important to investigate the pathogenesis of digestive disorders (Harris et al., 2006; Shirazi-Beechey, 2008; Vervuert, 2008; Al Jassim and Andrews, 2009; Pollitt and Visser, 2010). In vivo experiments were conducted to analyse digestive processes in the equine gastro-intestinal tract (Moore-Colyer et al., 2002; Brøkner et al., 2012). However, these techniques are often work- and cost-intensive and should be avoided to the interest of animal welfare. Therefore, the development and improvement of in vitro experimental designs is extremely important (Russel and Burch, 1959).

When developing in vitro digestion, that simulates the natural digestion processes of horses, it is essential to know about equine physiological processes. Since equine saliva has no digestive enzyme activity (Al Jassim and Andrews, 2009), enzymatic degradation of the food starts in the stomach. The secretion of hydrochloric acid into the stomach induces an acidic gastric environment (pH 2-5) (Coenen, 1992; Husted et al., 2008). The low pH causes both denaturation of proteins and presumably acidic hydrolysis of polysaccharides (Ince et al., 2013). Pepsinogen is the most important enzyme in the stomach. After secretion into the acidic environment of the lumen, pepsinogen is activated to pepsin. Pepsin splits denatured proteins hydrolytically into polypeptides. The highest metabolic activity of pepsin was measured in a potent acidic environment (pH 1 - 4) between at least 37 – 42 °C (Scharrer and Wolffram, 2004; Worthington and Worthington, 2011). The pancreatic enzymes (peptidase, nuclease, amylase, lipase) are secreted into the lumen of the small intestine, where the pH is increased up to pH 7 by pancreatic secretions. Amylase is particularly relevant for the hydrolytic degradation of starch as it splits the unbranched 1.4-α- glycosidic linkages (Kienzle et al., 1994; Santos et al., 2010).

The apparent digestibility of forages in the foregut of horses was investigated in vivo earlier, using the mobile bag technique. Thus, an apparent crude protein digestibility of 52% was detected for haycubes in the horse´s foregut (Moore-Colyer et al., 2002; Brøkner et al., 2012). Monosaccharides and disaccharides are almost completely absorbed prececally. In addition, a small fraction of cell wall carbohydrates, like hemicellulose and cellulose as well as fructans is degraded in the stomach and small intestine by microbial fermentation (Al Jassim et al., 2005; Coenen et al., 2006; Perkins et al., 2012). Only 5-11% of the NSP, and 8-22% of the NDF, was lost prececally when using the mobile bag technic for analyses by Moore-Colyer et al. (2002) and Brøkner et al. (2012) respectively. Fructans are oligomeric or polymeric polysaccharides and consist of β-linked D-fructofuranosyl units. Inulins are (2-1) linked fructans, levans are (2-6) linked with partial (2-1) linked branches and graminans comprise both types of linkages (Vijn and Smeekens, 1999). Depending on the degree of polymerisation (DP), inulin-type fructans were classified as short chain fructo-oligosaccharides, fructo-oligosaccharides and inulin with mean DP around 3.6, 4 and 12, respectively (Glatter et al. 2016a). This classification is common for inulin-type fructans only. Therefore, a distribution of the chain length is also necessary to classify the other fructans which are of such great importance for digestive processes. Levans and graminans, specific grass fructans, are suspected of causing digestive disorders if they reach the horse´s hindgut in excessive quantities (Longland and Byrd, 2006). The question of how grass fructans are digested prececally is still being discussed. It is assumed that fructans are not degraded by endogenous enzymes (Nilsson et al., 1988). However, Ince et al. (2013) found partial degradation of polymeric fructans to oligomeric fructans when timothy grass was incubated with gastric and small intestine digesta. Glatter et al. (2016b) observed in the stomach of horses post-mortem non-structural carbohydrates including fructans being degraded to a certain degree. In addition the chain-length of inulin-type fructans was shortened when passing from the Pars nonglandularis to the Pars glandularis, where almost complete decomposition of low dose fructans was shown. Microbial fermentation of fructans might be present to some extent, as there is an active microbiome not only in the equine´s hindgut, but also in stomach and small intestines (Mackie and Wilkins, 1988; Coenen et al., 2006). This assumption is supported by results which indicate decomposition of fructans from Jerusalem artichoke meal and a coincident increase of short chain fatty acids with particular emphasis on *n*-butryrate in the horses' stomach (Glatter et al. 2016c). Another probably additional type of prececal fructan degradation could be acidic hydrolysis. Glycosidic linkages are acid-intolerant and could be cleaved in ventral parts of the stomach (Ince et al., 2013). In order to determine the proportion of total degradation caused by acidic hydrolysis and endogenous enzymes only, an in vitro system is required that excludes any fermentation.

The aim of the present study was the adaptation of an in vitro system to simulate equine foregut digestion exclusively by endogenous processes. In addition, we wanted to use this system to study the degradation of proximate nutrients and, in particular, graminan-type fructans of different origins. We hypothesized that i) acidic hydrolysis is responsible for a high degree of fructan degradation in the stomach of horses, and ii) our in vitro system can serve as a model for physiological processes in the equine foregut, that excludes fermentative processes.

3.3 Material and Methods

The following grasses and grass-mixtures were used as feedstuff for the in vitro prececal digestion trials: a grass mixture for horses (GMH; Tab. 1), a grass mixture for cows (GMC; Tab. 1), tall fescue (TF, *Festuca arudinacaea*), English perennial ryegrass (ER, *Lolium perenne* L.), white clover (WC, *Trifolium repens* L.), lucerne (LU, *Medicago sativa* L.). All forages were cultivated as monoculture, harvested on the 20th August 2012 and preserved frozen. Prior to the trial in 2013 the forages were lyophilized (Christ Gefriertrocknungsanlage GAMMA 2-16 LSC, Osterode, Germany) and ground to particles of 2 mm.

Tab. 1: Botanical composition of the seed used for the grass-mixture for horses and grass-mixture for cows (Suter et al., 2004) in g/a (gram per are)

Grass-mixture for horses (SM 485)	[g/a]	Grass-mixture for cows (SM 330)	[g/a]
Tall fescue (*Festuca arundinacea*)	50	Trefoil red clover (*Trifolium pratense*)	20
Timothy (*Phleum pratense*)	30	Timothy (*Phleum pratense*)	25
Early english perennial ryegrass (*Lolium perenne*)	60	English perennial ryegrass (*Lolium perenne*)	70
Late english perennial ryegrass (*Lolium perenne*)	60	Cocksfoot (*Dactylis glomerata*)	55
Smooth meadow grass (*Poa pratensis*)	120	Small-leaved white clover (*Trifolium repens*)	15
Red fescue (*Festuca rubra*)	60	Large-leaved white clover (*Trifolium repens*)	25
Black bent (*Agrostis alba*)	30	Meadow fescue (*Festuca pratensis*)	120
Crested dog's-tail (*Cynosurus cristatus*)	40		

SM 485: Standardmixture 485, SM 330: Standardmixture 330 (Agroscope, Switzerland)

Study protocol

The protocol is based on earlier investigations (Murray et al., 2005; Abdouli and Attia, 2007) but some parameters have been adapted due to the physiological situation in the equine intestinal tract and pre-studies (unpublished data) based on personal communications. The study was performed in two parts:

Part 1 - Preliminary in vitro digestion of all forages

In order to simulate the conditions of the stomach, each forage was stirred in warm water at 38 °C (Baumgartner, 2006) using a magnetic mixer, and the pH-value was adjusted to 3.2 +/- 0.05 (Coenen, 1992; Husted et al., 2008; Glatter et al. 2016c) with 1M HCl-solution. After adding 2.28 g/l Pepsin (Pepsin 2000 FIP-U/g, EC 3.4.23.1, Merck KGaG, Darmstadt, Germany) the mixture was incubated at 38°C (Baumgartner, 2005) for one hour (Van Weyenberg et al., 2006) under constant stirring. In order to simulate the passage into the small intestine, a solution of preheated phosphate buffer (4.4 g/l KH_2PO_4, 4.6 g/l Na_2HPO_4) was then added and the pH-value was adjusted to 6.9 +/- 0.04 by adding 1M NaOH-solution. In addition, 0.25 g/l Pancreatin (Pancreatin, 4 x USP Pancreatin from porcine pancreas, Sigma Aldrich Chemie GmbH, Steinheim, Germany) and thus 7500 U/l Amylase (Kienzle et al., 1994; Richards et al., 2003) was added and the mixture was incubated again at 38°C (Baumgartner, 2006) under constant stirring. After one hour (Van Weyenberg et al., 2005) the pre-digested forage was strained though a layer of four gauzes (ES-Kompressen, Hartmann, EN 14079-VM17, 2355 Wiener Neudorf, Austria); the liquid was poured away and the forage frozen and lyophilized.

Part 2 - Influence of the pH value on gastric degradation of selected forages (ER, GMC)

The simulated foregut digestion of ER and GMC was repeated as described above, however with three different pH-values (2, 3, and 4) during the incubation with Pepsin. Each trail was repeated three times.

Analyses

Part 1: Both the original and the digested materials were analysed for crude nutrients according to the VDLUFA method (Naumann and Bassler, 1997) and for fibre fractions according to the protocol of Van Soest (Naumann and Bassler, 1997) in duplicate. The starch content was determined enzymatically as described by Zeyner et al. (2015). Contents of water-soluble carbohydrates (mono- and dimeric sugars, fructans) were measured by HPLC (Shimazu-Deutschland GmbH, Duisburg, Germany; refraction index; column HPX-87P; Biorad, Hercules, CA, USA) according to a method by Hillegeist and Greef as described by Zeyner et al. (2015).

Part 2: The digested material was analysed for dry matter, crude protein and water-soluble carbohydrates as described above. Characterisation of the structure of fructan isomers in the original material was performed as separation according to its DP using a method by Hillegeist and Greef as modified by Pavis et al. (2001).

Statistical analysis

An analysis of variance (ANOVA) for univariate and multivariate repeated measures was performed with SYSTAT©11 (Systat Software, inc., Point Richmond, CA 94804-2028, USA). The level of significance was pre-set at $p < 0.05$.

3.4 Results

Part 1: The concentrations of crude protein, glucose, fructose and saccharose decreased in the material following simulated foregut digestion, while those of fibre fractions increased simultaneously (Tab. 2). There was a particularly marked loss of fructans in ER and GMC materials (Tab. 3), the only ones with remarkable fructan concentrations in the starting material.

The starch content was analysed in the original material only. The highest values were evaluated for LU (3.68% DM). The GMH (2.29% DM), WC (2.18% DM), ER (2.16% DM), GMC (1.96% DM), TF (1.27% DM) and hay (1.24% DM) contained lower starch contents.

Part 2: Concentrations of fructans did not differ much between the original material of ER and GMC (Tab. 3). After in vitro foregut digestion, the content of fructans in the GMC material was significantly higher ($p < 0.05$) with pH 2 (1.95% DM) than pH 4 (0.91% DM). Significantly more fructans remained in the ER material after in vitro digestion than in GMC (Fig. 1). For both forages the concentration of protein (Fig. 2) was significantly lower ($p < 0.05$) with pH 2 (ER: 6.1% DM; GMC: 8.3% DM) than pH 3 (ER: 7.6% DM; GMC: 10.5% DM) and in the same order between pH 2 and pH 4 (ER: 7.7% DM; GMC: 10.6% DM). Characterisation of the fructan structure identified exclusively oligomeric isomers in GMC, whereas in ER about 70% of the fructan was polymeric (29.5% of the fructan in ER consisted of 1-5 units, 34.5% of 6-10 units, 20.2% of 11-15 units, 3.3% of 16-20 units).

Tab. 2: Analyzed crude nutrients and Van Soest fibres of six lyophilized forages before (orig.) and after (dig.) in vitro digestion, simulating the equine foregut

Forages		DM [g/kg]	CA [g/kg]	CP [g/kg]	CF [g/kg]	NDF [g/kg]	ADF [g/kg]	ADL [g/kg]
Grassm. Horses	orig.	941	107	165	230	478	256	19
	dig.	919	72	94	349	641	386	24
Grassm. Cows	orig.	909	102	196	221	435	259	21
	dig.	928	68	108	350	614	410	34
Tall Fescue	orig.	943	103	177	253	535	286	20
	dig.	931	70	90	351	681	400	27
Engl. Ryegrass	orig.	943	118	165	222	461	248	18
	dig.	922	78	94	328	619	376	22
White Clover	orig.	915	102	191	176	333	248	40
	dig.	931	82	145	304	459	392	62
Lucerne	orig.	935	98	200	224	364	255	52
	dig.	932	73	148	351	526	412	85

DM: dry matter, CA: crude ash, CP: crude protein, CF: crude fibre, ADF: acid detergent fibre, ADL: acid detergent lignin, NDF: neutral detergent fibre, Grassm. Horses: grassmixture for horses, Grassm. Cows: grassmixture for cows, Engl. Ryegrass: English Ryegrass, g/kg: gram per kilogram, orig.: undigested original material, dig.: after simulated foregut-digestion

Tab. 3: Analyzed carbohydrates of six lyophilized forages before (orig.) and after (dig.) in vitro digestion, simulating the equine foregut

Forages	Fructan [%DM]		Sucrose [%DM]		Glucose [%DM]		Fructose [%DM]	
	orig.	dig.	orig.	dig.	orig.	dig.	orig.	dig.
Grassm.Horses	0.96	0.52	0.82	0.13	1.79	0.00	1.46	0.28
Grassm.Cows	4.42	0.00	1.71	0.11	1.65	0.28	1.85	0.17
Tall Fescue	0.73	0.77	1.04	0.09	1.27	0.00	1.48	0.19
Engl. Ryegrass	4.05	0.59	1.87	0.14	1.51	0.00	1.88	0.27
White Clover	0.47	0.62	0.81	0.00	1.93	0.18	0.90	0.10
Lucerne	0.60	0.60	1.41	0.14	0.58	0.00	0.53	0.10

DM: dry matter, orig.: undigested original material, dig.: after simulated foregut-digestion, Grassm. Horses: grassmixture for horses, Grassm. Cows: grassmixture for cows, Engl. Ryegrass: English Ryegrass

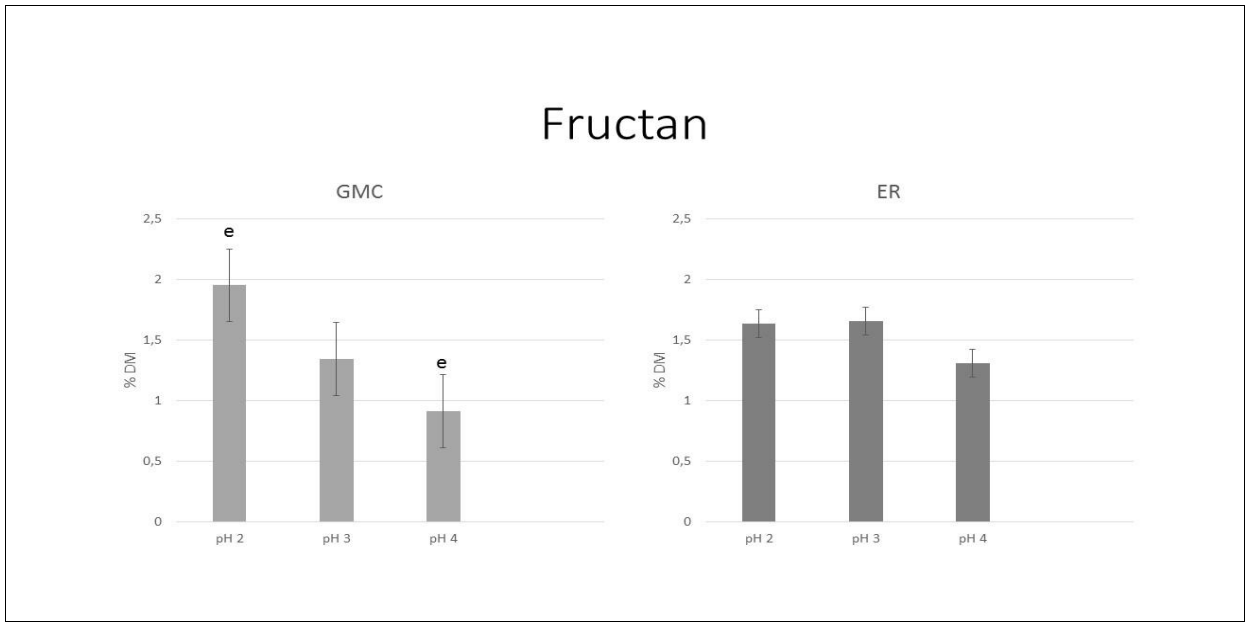

Fig. 1: Average (±standard error) content of fructans in forages (GMC = Grass-mixture for cows; ER = English perennial ryegrass) after in vitro digestion depending on the applied pH. In percent dry matter (% DM), significant differences are marked with small letters

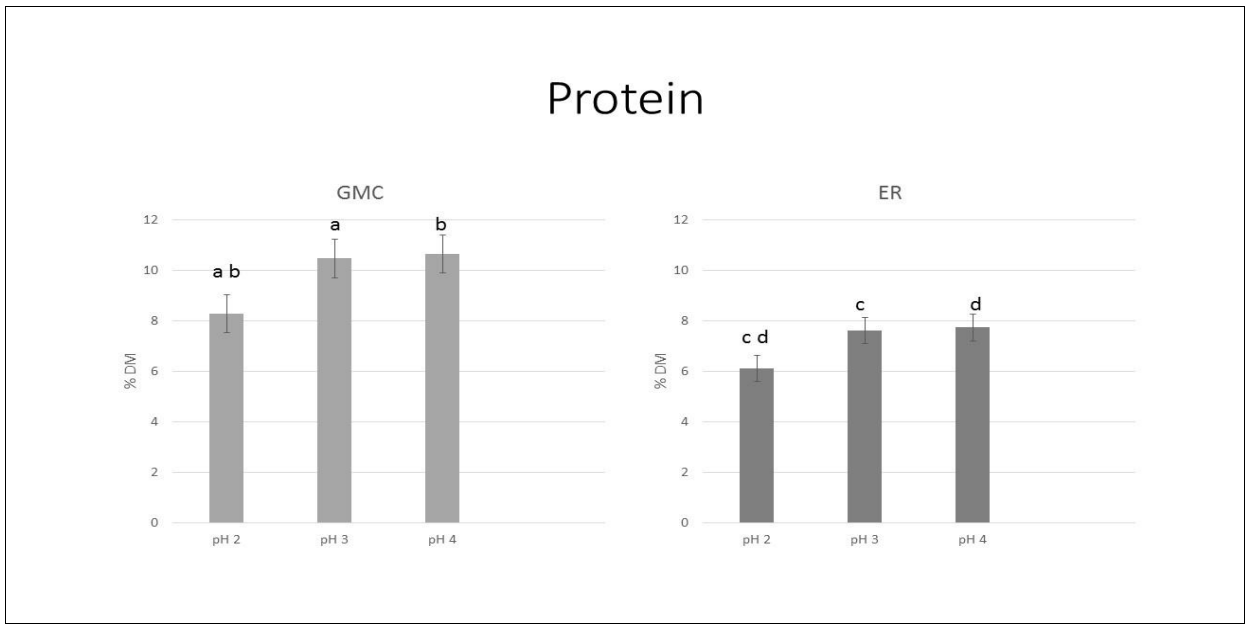

Fig. 2: Average (±standard error) content of protein in forages (GMC = Grass-mixture for cows; ER = English perennial ryegrass) after in vitro digestion depending on the applied pH. In percent dry matter (% DM), significant differences are marked with small letters

3.5 Discussion

In *Part 1*, the preliminary study, six different grasses and grass mixtures were digested in the in vitro simulation of enzymatic foregut digestion. The resulting changes in the content of nutrients correspond in most cases to observations made in vivo (Moore-Colyer et al., 2002; Brøkner et al., 2012). Crude protein was notably degraded in the in vitro simulation. This was expected as a result of the addition of pepsin and is also in agreement with Moore-Colyer et al. (2002). These authors identified an apparent prececal digestibility of 52% for the crude protein of haycobs using the mobile bag technique. The relative increase of fibre fractions measured in the present study is in line with physiological processes, since fibre is not degraded enzymatically (Brøkner et al., 2012). The decrease of mono- and dimeric sugars, such as glucose, fructose and sucrose, which are easily digested and absorbed in the small intestine as monomeric compounds, is also conform with equine physiology (Shirazi-Beechey, 1995; Dyer et al., 2002; Glatter et al., 2016b). These observations confirm the importance of equine prececal digestive processes when simulating equine digestion in vitro. They also verify the implemented protocol for protein and fibre digestion as well as digestion of monosaccharides and disaccharides.

In *Part 2*, the influence of gastric acidity on the degradation of protein and fructan was investigated. Fructans are supposed to reach the horses hindgut undigested (Nilsson et al., 1988). In our study, ER and GMC were the only tested forages with notable fructan contents (g/kg DM: ER 40.5 and GMC 44.2; other forages: < 10). However, fructans of both forages diminished after in vitro digestion. This conforms with observations made by Glatter et al. (2016b) who found mono- and disaccharides, fructans and starch almost completely decomposed in the foregut when they analysed ingesta of euthanized horses, that had been fed a hay-based diet with and without addition of Jerusalem artichoke meal. In their study oligomeric compounds made up the largest group of fructans. In our original material 100% of the GMC fructan was identified as oligomeric, but only 30% of the ER fructan consisted of less than 5 D-fructofuranosyl units. However, there was no significant difference in fructan degradation between ER and GMC after in vitro digestion at any acidity. Ince et al. (2013) used ingesta to supply enzymes as well as the typical microbial flora for in vitro foregut digestion and also observed changes within the fructan polymerization by digestion. They assumed that microbial fermentation or acidic hydrolysis caused degradation of polymeric fructan to oligomeric fructan in stomach and small intestine. Glatter at al. (2016b) described - exclusively for the stomach of horses - differences between the DP of fructans in the Pars nonglandularis and Pars glandularis. These were reversed when Jerusalem artichoke meal was fed instead of fructans from native feedstuffs only. As we used no digesta but only pure enzymes in our experimental set-up, we could exclude microbial degradation of fructan. We hypothesized that acidic hydrolysis causes the decrease in fructan. Therefore, we analysed the influence of different pH values on the degradation of fructan and protein in a subsequent second in vitro digestion trial of GMC and ER. According to the spectrum of acidity in the stomach described by Coenen (1992), the pH values 2, 3 and 4 were adjusted for gastric in vitro digestion. Significantly higher protein degradation was observed at a pH of 2 compared to pH 3 or 4, for both ER and GMC. This may refer to enzymatic degradation by pepsin, pepsin having 90% of the maximal activity at pH 1.5, and 35% of the maximal activity at pH 4.5 (Worthington, 2011). In vivo the H^+-dependent activation of pepsinogen would strengthen the effect. In contrast to these findings, the highest loss of fructans occurred at the lowest acidity (pH 4), which is contrary to the assumption of fructan degradation due to acidic hydrolysis (Fig. 1). Ott et al. (2008) proposed that plant enzymes are responsible for the degradation of fructan. Enzymes in forages might work particularly effectively if the decrease in pH were not as rapid or large. As we do not know if the investigated lyophilized forages still contained any active plant enzymes, the influence of plant enzymes on fructan degradation will have to be the topic of further investigations. Our research covered only a

small range of plant diversity. Therefore, we recommend continuing investigations about different kinds of fructan compositions in forages and their impact on degradation in the horse´s upper gastrointestinal tract. As we were unable to confirm the influence of fructan polymerization, the levan- and graminan-fractions might also be a topic for further research, particularly as most in vivo experiments about fructan degradation in horses are conducted with inulin (Eps and Pollitt, 2006; Milinovich et al., 2010).

3.6 Conclusion

The results suggest that the in vitro system applied in this study provides new possibly comparable results, that simulate foregut digestion by endogenous enzymes in horses. This includes a notable degradation of crude protein and simple sugars as well as a concomitant relative increase of fibres. Contrary to our expectations there was an obvious loss of fructans during in vitro digestion in two forages. We suspected acidic hydrolysis to be responsible for this. Nevertheless, there was no linear increase in the decomposition of fructans with lower pH levels in the simulated stomach content. We consider plant enzymes to contribute to the acidic hydrolysis of fructans, which should be analysed in further investigations.

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