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Degradation of Monosaccharides, Disaccharides, and Fructans in the Stomach of Horses Adapted to a Prebiotic Dose of Fructooligosaccharides and Inulin

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ABSTRACT

For a period of 20 days, 12 horses either received a prebiotic supplementation with fructooligosaccharides and inulin via Jerusalem artichoke meal (JAM) or corncob meal without grains (CMG) as placebo. The horses were euthanized 1 hour postprandial, gastric digesta was sampled from pars nonglandularis (PNG) and pars glandularis (PG), and concentrations of starch, mono- and disaccharides, fructans, D- and L-lactic acid, and short chain fatty acids were analyzed. Concentrations of starch and simple sugars were widely the same in JAM supplemented and not supplemented meals. However, fructans were less than half as much without supplementation as with supplementation of JAM. Glucose, fructose, sucrose, and fructans disappeared to a larger extent with prebiotic supplementation than without (106.6% vs. 86.7% glucose, 73.1% vs. 66.8% fructose, 91.5% vs. 14.7% sucrose, and 68.3% vs. 35.4% fructans remained in PNG; 81.9% vs. 38.3% glucose, 52.2% vs. 53.4% fructose, 47.1% vs. 0% sucrose, and 48.5% vs. 31.7% fructans remained in PG with CMG vs. JAM feeding). Disappearance of simple sugars and fructans was primarily associated with appearance of n-butyric acid (r = -0.21 – r = -0.33).

1. Introduction

Prebiotics selectively promote growth and/or activity of hindgut microorganisms that influence the health of the host [1]. In horses, inulin-type fructans with different degrees of polymerization (DP), which display the number of monomeric units in the molecule, are common prebiotics with the cecum and colon as primary targets [2]. The DP of a prebiotic substance determines its fermentability in the digestive tract. The average DP is about 4 for short-chain fructooligosaccharides (FOS), 12 for inulin, and 25 for long-chain FOS (high molecular weight inulin) [3]. Due to limited data availability, the recommended daily prebiotic dose for horses, which is 0.2 g/kg body weight, is given as the sum of prebiotic substances regardless of their DP [4]. This dose should avoid any risk of laminitis, which may follow an overload of the hindgut with rapidly fermentable carbohydrates [4,5]. Horses might benefit from prebiotics especially under stressful situations such as sudden diet changes [6], starch overload [7], transport [8], and antimicrobial therapy [2]. However, water-soluble carbohydrates including inulin-type fructans and phlein-type fructans are probably fermented by microbes or degraded by acid hydrolysis and plant enzymes in the stomach [9–12]. As a result, the DP of FOS might be reduced [10,13] and low molecular weight sugars, especially fructose, released. The changed substrate availability affects the microbial community and leads to increased concentrations of organic acids (especially of n-butyrate) in the stomach [13,14] with gastric ulceration as a potential detrimental outcome for horses [15,16].

Another issue with potential health concern is the clear postprandial insulinemic response following fructan intake, which was observed both in horses predisposed to laminitis [17] and in horses that were not predisposed [18]. The concomitant blood glucose concentration was not affected [17,18]. In monogastric animals, it is

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unlikely that short chain fatty acids (SCFA) of gastric origin trigger insulinemic response [19]. In this context, fragments from breakdown of fructans in the lower gut are more suspicious. Thus, fructose might stimulate secretion of glucagon-like peptide-1 (GLP-1) and therefore insulin [20,21]. The remainder of fructans probably passes in oligomeric forms into the hindgut, where it is subjected to microbial fermentation.

On the basis of a previous study on effects of applying FOS and inulin as prebiotics on gastrointestinal functions in horses [12,13,14,16,22], a re-analysis of the data was performed. The objective of this re-analysis was to test in more detail how adaptation of horses to repeatedly applied prebiotic doses of FOS and inulin by JAM affects disappearance of monomeric and dimeric carbohydrates and fructans in the stomach compared to feeding a basal diet supplemented with CMG as placebo.

2. Materials and Methods

2.1. Animals, Diets, and Experimental Design

Twelve Warmblood horses of both sexes (10 females, 2 males), 14±7.5 years of age, and with a body weight of 534±64.5 kg were used. The horses were kept and cared for by the University of Veterinary Medicine Hannover, Foundation. The experiment was carried out after approval by the Lower Saxony State Office for Consumer Protection and Food Safety in accordance with the German Animal Welfare Act (AZ LAVES 33.4-42502-05-13A385). The horses were kept in single boxes on wood shavings and had free access to tap water and a salt lick consisting of sodium chloride. They received 1.5 kg meadow hay/100 kg body weight/d and crushed oats according to 1.2 g starch/kg body weight/d as fed; offered twice per day to equal parts. Six of the horses received 0.15 g FOS plus inulin/kg body weight/d via JAM (LIVEN GmbH, c/o Lienig Wildfrucht-Verarbeitung, Zossen, Germany). The other 6 horses received an equivalent of CMG as placebo. The horses were randomly allocated to the experimental groups. After 20 days of adaptation, the horses were euthanized approximately 1 hour after they received the morning meal (grain meals plus supplements were consumed completely) by pentobarbital (60 mg/kg body weight). Sedation was precipitated with romifidine (0.12 mg/kg body weight), and anesthesia with diazepam and ketamine (0.05 and 2.2 mg/kg body weight, respectively).

2.2. Sampling and Chemical Analyses

Representative samples of digesta were taken immediately post mortem from the PNG and PG of the stomach and stored at -20°C. Dry feed bulk samples and lyophilized digesta samples were ground to pass 1.0 mm sieve pores. Dry matter (DM) contents were determined according to the Association of German Agricultural Analytic and Research Institutes (VDLUFA; method no. 3.1) [23]. Starch was determined using the amyloglucosidase method (VDLUFA method no. 7.2.5) [23]. Glucose, fructose, sucrose, and fructans were analyzed using high performance liquid chromatography on a KONTRON Instruments unit (Tresser Instruments, Rossdorf, Germany) with refractive index detector (Shodex RI-71; Showa Denko Europe GmbH, Shodex Business, Munich, Germany) and 100 mm × 7.8 mm separation column (Rezex RPM-Monosaccharide Pb+2; Phenomenex Ltd. Deutschland, Aschaffenburg, Germany). The injection volume was 20 μL, and column flow was 0.7 mL/min at constantly 80°C. For the analysis of D-lactic acid, L-lactic acid, and SCFA, 50 g of fresh digesta was mixed with 50 mL distilled water, incubated for 60 minutes, and centrifuged for 10 minutes with 4,000 × *g*. The supernatant was stored at -20°C. The samples were prepared and analyzed for concentrations of D- and L-lactic acid by high performance liquid chromatography tandem mass spectrometry according to Henry et al [24] and Scheijen et al [25] on an API 2000 System (Applied Biosystems, Warrington, UK) with Agilent HPLC 1100 (Agilent Technologies Inc., Santa Clara, CA, USA) and Hypersil ODS separation column (150 mm × 2 mm × 5 µm; Thermo Fisher Scientific Inc., Waltham, MA, USA). The settings were as follows: 2 µL injection volume, 150 μL/min column flow, 25°C constant oven temperature, and 300°C for electrospray ionization. A 99:1 mixture of ammonium formate (1.5 mmol/L water with pH 3.6) and acetonitrile was used as eluent. D- and L-lactic acid were measured using mass transition of 305/89 m/z and the internal standard using 308/89 m/z. The thawed supernatants were priorly centrifuged for 5 minutes at 18,000 × *g*. A portion of 25 μL/sample was mixed with 200 μL of internal standard (DL-lactate-3,3,3-d_3; CDN Isotopes, Quebec, Canada), 825 μL distilled water, and 25 μL 27%-hydrochloric acid, and eluted with 6 mL of 2-methyl-2-butanol plus chloroform (mixed 11:9) on EXtrelut NT 1 columns (Merck KGaA, Darmstadt, Germany). Extraction was carried out using 1 mL of ammonium hydroxide solved in water (0.1 mol/L). After centrifugation for 3 min at 800 × *g*, 100 μL of the liquid phase were transferred to a new sample tube and evaporated under nitrogen at 65°C. The residue was dissolved in 400 μL methanol and centrifuged for 5 minutes at 18,000 × *g*. The supernatant was evaporated under nitrogen at 50°C. The residue was then derivatized with 100 μL of diacetyl-L-tartaric anhydride (50 mg/mL of 4:1 mixed dichloromethane-acetic acid solution) for 30 minutes at 75°C before evaporation under nitrogen at 50°C. The residues were finally dissolved in 100 μL acetonitrile mixed 1:2 with water. SCFA were determined by gas chromatography on a Shimadzu 17A unit (Shimadzu Corp., Kyōto, Japan) with flame ionization detector and 15 m × 0.25 mm × 0.25 μm Nukol separation column (Merck KGaA, Darmstadt, Germany). The injection volume was 1 μL at 25°C, and column flow was 22.1 mL/min (at 100 kPa constant pressure). The oven temperature program was 80°C to 110°C at 10°C/min to 175°C at 15°C/min, then hold at 175°C for 4 minutes. Helium was used as carrier gas and makeup gas. The thawed supernatants were priorly centrifuged for 5 minutes at 2,000 × *g*. Then, 500 μL were mixed with 50 μL of internal standard solution (1.5 g i-caproic acid in 100 mL 80%-formic acid) and centrifuged again for 5 minutes at 2,000 × *g*.

2.3. Statistical Analysis

Statistical analysis was performed using the SAS 9.4 software package (SAS Institute Inc., Cary, NC, USA). Means of percentages of mono- and dimeric sugars and fructans remaining in stomach digesta were compared between CMG and JAM feeding by pooled t-test. For the analysis of residuals of fructans in PNG, homogeneity of sample variances was not given, and therefore the method of Satterthwaite was used. The studentized residuals were confirmed to have Gaussian distribution by Shapiro-Wilk test. This was not the case for residuals of sucrose in PG, and therefore, the Mann-Whitney U test was used. Differences between CMG and JAM groups with $P < .05$ were considered to be significant. Pearson correlation coefficients were calculated among residuals of simple sugars and fructans and concentrations of D- and L-lactic acid and SCFA in stomach digesta.

3. Results and Discussion

The basic chemical composition of the individual feedstuffs and supplements and the daily diet is given in previous reports [12,14,22]. Digesta of the stomach likely contained nutrients only originating from the morning meal offered approximately 1 hour ante mortem. The proportions of individual feedstuffs in the mixed morning meal were estimated considering the whole offered quantity of oats and the supplements, but only 0.15 kg hay/kg body

Table 1
Concentration of mono- and dimeric sugars and fructans in feed and percentage remaining in stomach digesta approximately 1 hour postprandial.

		(g/kg DM)	Remaining portion (%)	
Item	Treatment	Feed[a]	PNG	PG
Glucose	CMG	15.7	106.6	81.9[b]
	JAM	16.0	86.7	38.3[c]
Fructose	CMG	20.2	73.1	52.2
	JAM	23.3	66.8	53.4
Sucrose	CMG	8.7	91.5	47.1
	JAM	14.9	14.7	0
Mono- and disaccharides	CMG	44.7	87.7	61.0
	JAM	54.5	67.7	31.4
Fructans	CMG	21.1	68.3	48.5
	JAM	48.0	35.4	31.7
Range of SD		0.366 – 4.13	19.6 – 96.3	13.7 – 69.0

CMG, corncob meal without grains; DM, dry matter; JAM, Jerusalem artichoke meal; PG, pars glandularis; PNG, pars non glandularis; SD, standard deviation.
[a] Equals the mean concentration in the morning meal.
[b,c] Different superscripts indicate difference between treatments ($P < .01$).

weight. The latter was calculated on the basis of a roughage intake time of approximately 39 min/kg [26]. In the morning meal (i.e., based on the estimated proportions of individual feedstuffs), concentrations of starch were equal in CMG and JAM groups (215 vs. 214 g/kg DM). This was similar with glucose and fructose concentrations; however, more sucrose was offered with JAM than with CMG feeding (Table 1). The CMG group received less than half of fructans offered to the JAM group (Table 1). The percentage of analyzed water-soluble carbohydrates that remained in digesta was gradually reduced from PNG to PG (Table 1). Nothing of them remained in cecal digesta [12]. The disappearance of mono- and disaccharides and fructans might in part have been a result of an outflow of these substances with the liquid phase of the stomach digesta into the small intestines [12] with subsequent absorption and conceivable stimulation of GLP-1 secretion [20,21]. However, fructans and simple sugars might also have been degraded in the stomach by microbial fermentation [13,27,28,29], plant hydrolases [30], and acid hydrolysis [11]. The supplemented fructans including FOS and inulin mainly comprised chains with 16 to 20 monomers (23.3%) and chains with 11 to 15 monomers (21.1%) [13]. By contrast, the analysis of stomach contents revealed that the majority of carbohydrates were present in DP ranges of 1 to 5 or 6 to 10 units, which is a significant shortening of carbohydrate chains [13]. In PNG, more glucose, fructose, sucrose, and fructans disappeared with supplementation of JAM, which was reflected by a change in the composition of the microflora (relative abundance of *Firmicutes* increased and relative abundance of *Bacteroidetes* and *Proteobacteria* decreased) and increase of α-diversity (e.g., the Shannon-Wiener index increased from 0.969 with CMG to 1.089 with JAM; $P < .05$) [13]. This was widely similar within PG digesta, in which substantially less glucose (-43.6%-points on DM basis; $P < .01$) and sucrose (-47.1%-points on DM basis; $P > .05$) remained with JAM. In PG, compositional changes of the bacterial community and increase of α-diversity (e.g., the Shannon-Wiener index increased from 0.934 with CMG to 1.055 with JAM; $P > .05$) were similar to PNG [13]. The pH of the digesta was 4.5 in PNG and 3.4 in PG and did not differ between the experimental groups [14]. The remainder of fructose was equal with CMG and JAM supplementation (Table 1). Inulin contains about 96% fructose [31]. Fructose is also a part of the dimer sucrose. It is likely that microorganisms and gastric acid hydrolyzed fructose as well as other simple sugars. This in turn could have been compensated by fructose originating from inulin degradation and sucrose hydrolysis. Similarly, the high percentages of glucose remaining in the digesta indicate a release of glucose primarily from the breakdown of sucrose. As reported by Glatter et al [14], PNG digesta contained 19.7 versus 23.6 mmol D-lactic acid, 17.2 versus 34.9 mmol L-lactic acid, 22.7 versus 30.9 mmol acetic acid, 1.8 versus 1.4 mmol propionic acid, and 11.5 versus 28.3 mmol n-butyric acid/L; PG digesta contained 8.8 versus 27.7 mmol D-lactic acid, 19.2 versus 24.9 mmol L-lactic acid, 14.7 versus 22.8 mmol acetic acid, 0.5 versus 0.3 mmol propionic acid, and 4.9 versus 8.3 mmol n-butyric acid/L with CMG versus JAM feeding. Appearance or disappearance of fructans and mono- and disaccharides were specifically correlated with the appearance of D- and L-lactic acid and SCFA in gastric digesta (Table 2). This was on rather low level, but provides further evidence for the degradation of fructans and simple sugars in the stomach of horses.

Fructose overload from fructan degradation can cause local epithelial inflammation and acidosis [32]. The disappearance of simple sugars and fructans was correlated with the appearance of n-butyric acid ($r = -0.21$ – $r = -0.33$; $P > .05$; Table 2), which may have the capability to impair the integrity of squamous and glandular mucosa causing acute, multifocal hydropic swelling, and epithelial detachment [15,16]. Butyric acid or applied low doses of salts of butyric acid may also support, for example, gastrointestinal development and absorptive capacity, which was observed in calves [33] and piglets [34–36].

Apart from effects on the stomach itself, it was observed in a previous study that a defined dose of inulin on a hay diet induced a clear postprandial insulinemic response in ponies, at least in those individuals that were predisposed to laminitis [17]. Healthy horses also had increased insulin levels after JAM supplementation with a hay and oat diet; however, without increased blood glucose concentration [18]. In the stomach, fructans are partly hydrolyzed to organic acids. Parts of them, however, may escape degradation by acids and microbes in the stomach and flow into the small intestines as oligomeric forms together with monosaccharides and disaccharides released from fructan degradation [10], where they are further decomposed, absorbed, and metabolized. At this, fructose is a major metabolite. The majority of fructose is converted to trioses in the liver [37], but fructose might also be metabolized in the small intestines [38]. In addition to glucose [39–41], fructose might also stimulate GLP-1 secretion, but not the secretion of the glucose-dependent insulinotropic polypeptide, which is followed by a rising level of insulin in the blood [20,21]. Although there are no data on this in horses, it could be a possible explanation for the postprandial insulinemic response to the intake of fructans observed in horses and the lack of an increase of blood glucose concentration at the same time. Frequently or persistently elevated concentrations of circulating insulin in the bloodstream (hyperinsulinemia) have the risk to decrease insulin sensitivity and evoke insulin resistance [42] followed by metabolic dis-

Table 2
Pearson correlation coefficients among percentages of mono- and dimeric sugars and fructans remaining in stomach digesta approximately 1 hour postprandial[a].

	Glucose	Fructose	Sucrose	Fructans	D-Lactic acid	L-Lactic acid	Acetic acid	Propionic acid	n-Butyric acid
Glucose	1	0.86***	0.67***	0.76***	0.17	0.44*	0.18	0.34	-0.29
Fructose		1	0.51*	0.88***	-0.04	0.17	0.28	0.44*	-0.21
Sucrose			1	0.62**	0.58**	0.72***	-0.05	0.37	-0.33
Fructans				1	0.13	0.34	0.11	0.53**	-0.30
D-Lactic acid					1	0.41*	-0.01	0.27	0.11
L-Lactic acid						1	-0.03	-0.05	-0.29
Acetic acid							1	0.06	0.33
Propionic acid								1	0.09
n-Butyric acid									1

[a] Measurements from both stomach compartments and both treatments are included.
* $P < .05$,
** $P < .01$,
*** $P < .001$.

orders such as laminitis [43,44]. Small doses of fructose on top of glucose may increase glucose tolerance in even insulin-resistant individuals [37]. By contrast, high fructose doses within a short time may contribute to hyperinsulinemia especially in obese and otherwise predisposed individuals, because in such, the effect on GLP-1 secretion appears to be intensified [21].

4. Conclusions

Simple sugars and fructans from a meadow hay and oat diet supplemented with FOS and inulin from JAM rapidly disappeared from gastric digesta at the postprandial state. This is probably linked to outflow, microbial fermentation, and acid hydrolysis. The gastric microbial population adapts to recurrent administration of prebiotics. This results in elevated degradation of simple sugars and fructans to lactic acid and SCFA, especially n-butyric acid, which may have gastric and metabolic health impacts. Fructans which are administered as prebiotic dose do not reach the target organ, the large intestine, in their entirety if they are not protected by galenic treatment from breakdown in the stomach.

Author statement

Martin Bachmann: Conceptualization, Methodology, Investigation, Writing – Original draft preparation, Writing – Review and Editing. Maren Glatter: Conceptualization, Methodology, Investigation. Mandy Bochnia: Conceptualization, Methodology, Investigation. Jörg M. Greef: Resources. Gerhard Breves: Conceptualization, Methodology, Resources, Writing – Review and Editing. Annette Zeyner: Conceptualization, Methodology, Resources, Writing – Review and Editing, Supervision, Project administration. All authors approved the submitted version of the article.

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