University of Glasgow

Garber, A., Hastie, P.M., Handel, I. and Murray, J.M.D. (2017) In vitro fermentation of different ratios of alfalfa and starch or inulin incubated with an equine faecal inoculum. *Livestock Science*, (doi:10.1016/j.livsci.2017.05.002)

This is the author's final accepted version.

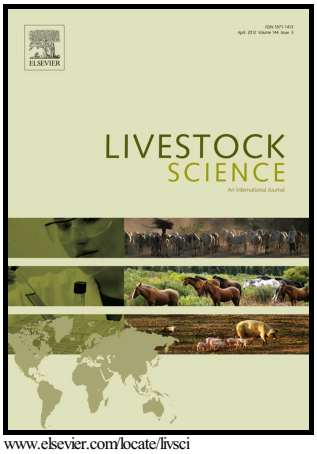
There may be differences between this version and the published version. You are advised to consult the publisher's version if you wish to cite from it.

http://eprints.gla.ac.uk/143569/

Deposited on: 04 July 2017

Enlighten – Research publications by members of the University of Glasgow
http://eprints.gla.ac.uk33640

Author's Accepted Manuscript



www.elsevier.com/locate/livsci

In vitro fermentation of different ratios of alfalfa and starch or inulin incubated with an equine faecal inoculum

A. Garber, P.M. Hastie, I. Handel, M.D. Murray

PII: S1871-1413(17)30132-4
DOI: http://dx.doi.org/10.1016/j.livsci.2017.05.002
Reference: LIVSCI3210

To appear in: *Livestock Science*

Received date: 31 October 2016
Revised date: 28 April 2017
Accepted date: 2 May 2017

Cite this article as: A. Garber, P.M. Hastie, I. Handel and M.D. Murray, *In vitro* fermentation of different ratios of alfalfa and starch or inulin incubated with an equine faecal inoculum, *Livestock Science* http://dx.doi.org/10.1016/j.livsci.2017.05.002

This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting galley proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

ACCEPTED MANUSCRIPT

In vitro fermentation of different ratios of alfalfa and starch or inulin incubated with an equine faecal inoculum

A. Garber[a*], P.M. Hastie[a], I. Handel[b], .M.D. Murray[a]

[a]University of Glasgow, College of Medical, Veterinary and Life Sciences, School of Veterinary Medicine, Bearsden Road, Glasgow, G61 1QH, UK

[b]Division of Veterinary Clinical Sciences, Royal (Dick) School of Veterinary Studies, University of Edinburgh, Easter Bush, Roslin, Midlothian, EH25 9RG, UK

[*]Corresponding author. Anna Garber, Tel.: +44 141 3305746, +44 7557 119149, a.garber.1@research.gla.ac.uk

Accepted manuscript

Abstract

The aim of this work was to assess the impact of substituting starch (S) or inulin (I) with high-temperature dried alfalfa (HTDA) as substrates for *in vitro* fermentation with an equine faecal inoculum. A series of experiments were conducted to assess the fermentation kinetics of HTDA (chopped [CA] or ground [GA]) and either S or I mixed in the following ratios; 100:0, 80:20, 60:40, 40:60 and 20:80 S/I: CA/GA, respectively. For each experiment, a further set of bottles containing identical ratios of S/I:CA/GA were also prepared, with the exception that the alfalfa received a simulated foregut digestion treatment (SFD) as prior to incubation. Total gas production increased ($P<0.05$) as the ratio of S/I to alfalfa increased. Total gas production was lower in bottles containing SFD-treated alfalfa ($P<0.001$). Dry matter loss decreased proportionately with increasing level of alfalfa substitution of S/I ($P<0.001$). Values for pH were lower in bottles containing S or I, with pH values in bottles

ACCEPTED MANUSCRIPT

containing S alone falling to almost 6 and those with I dropping to pH 5 and under. However, the substitution of S or I with 40 percent alfalfa produced pH values above 6.7, which is within physiological levels encountered in the large intestine of the horse. Consequently, there appears to be considerable potential to buffer the deleterious effects of high-starch/fructan diets with the substitution of these substrates with high-temperature dried alfalfa.

Keywords: alfalfa, starch, inulin, equine, faeces, *in vitro* gas production

1. Introduction

Diets high in fibre are required for normal gastrointestinal function in horses (Harris et al., 2016). Meanwhile, diets high in non-structural carbohydrates (NSC), such as starch and fructan, are known to elicit microbial disturbances often leading to clinical disorders like laminitis and colic (Hudson et al., 2001; Bailey et al., 2004).

In diet-induced laminitis lactic acidosis has been described as a consistent characteristic (Garner et al., 1977). Diet has been linked to laminitis for a long time; in fact diet-induced laminitis was first described around 350 BCE as barley disease in reference to the development of laminitis after the consumption of excessive amounts of cereal grain (starch overload). High-starch diets have been associated with hindgut acidosis in horses, and grain overload is often implicated in the onset of this debilitating condition (Garner et al., 1975). In pasture-induced laminitis it is proposed that it is the ingestion of high levels of grass fructan that elicits the onset of laminitis in this instance.

The increase in orchard grass hay intake (from 0.5 to 1.0 kg hay/100 kg BW/day) was shown to cause a dose-dependent elevation of the faecal pH when horses were fed a constant amount of crushed oats (1kg/100kg BW/day) (Zeyner et al., 2004). However, another study

Accepted manuscript

ACCEPTED MANUSCRIPT

suggested that the pH of the gastric juice was significantly higher in samples obtained 2 through 5 hours after feeding when horses were fed alfalfa hay-grain diet, compared with values when horses were fed bromegrass hay (Nadeau et al., 2000), indicating that forage type is also important. Alfalfa is a legume known to have a high buffering capacity due to its high organic acid and protein levels (Merry et al., 2000). Moreover, alfalfa has been reported to negate the deleterious effects that high NSC diets can have on gastric pH due to protein's buffering capacity which neutralises the ability of the short chain fatty acids (SCFA) to cause acid injury and ulceration. (Nadeau et al., 1998). However, little work has been done to assess the effect of alfalfa on the hindgut of horses fed diets high in NSC. This high-buffering capacity of alfalfa may be exploited to counter the acidotic effects associated with high NSC intakes on the large intestinal environment of the horse; however, no information currently exists to support this premise.

The aim of this study was to assess the effect of substituting starch or inulin with increasing ratios of chopped or ground HTDA used as substrates for *in vitro* fermentation with an equine faecal inoculum.

Accepted manuscript

2. Materials and Methods

Experiment 1: Ground alfalfa and starch or inulin

Three identical series of 160 ml serum bottles were used to assess the fermentation characteristics of ground (to pass through a 1mm dry mesh screen) high-temperature dried alfalfa (GA) and starch (S: Fisher Scientific, Loughborough, UK: s/7960/50) or inulin (I: Fisher Scientific, Loughborough, UK: 9005-80-5: from Dahlia tubers) added to each bottle in the following ratios; 100:0 , 80:20 , 60:40 , 40:60 and 20:80 to give a total sample mass of 1 g (± 0.5%) per bottle.

ACCEPTED MANUSCRIPT

A further set of bottles containing identical ratios of GA: starch or inulin was also prepared, with the exception that the alfalfa received a simulated foregut digestion treatment (SFD) prior to incubation with the starch. The SFD treatment was adapted from the method of Furuya et al. (1979), whereby of 20 ml of pepsin HCl (0.075 mol l^{-1} HCl; 2 mg pepsin ml^{-1} [Fisher Scientific UK, Loughborough, UK]) was added per gram of substrate. Samples were then thoroughly mixed and incubated for 1 h at 38^{o}C. Following incubation, samples were neutralised by the addition of 0.2 M NaOH. An aqueous solution of porcine pancreatin (1 ml) was then added to each tube, samples were then mixed and incubated at 38^{o}C for 2 h. The pancreatin (α - amylase, lipase and protease) solution was prepared by mixing the contents of one Pancrex V capsules (approximately 9000 BP units α - amylase per capsule, Paines & Byrne Ltd., Greenford, UK) with 9ml of distilled water. The mixture was then thoroughly mixed and centrifuged at 1500 x *g* for 10 min and the supernatant used as the pancreatic enzyme solution. Following incubation, samples were filtered through a funnel fitted with porosity 3 filter paper under reduced pressure. The residue was then washed with two volumes (50 ml) of distilled water and then dried at 40^{o}C for 48 h.

The alfalfa was pre-bloom alfalfa [Medicago sativa: variety: Daisy/Capri mix] that was mown, left to wilt overnight, chopped to 75 mm lengths, dried at 800^{o}C (Van den Broek rotary dryer) for 0.5 min. The alfalfa contained the following: a dry matter (DM) 920 g/kg, crude protein 173 g/kg DM, water soluble carbohydrate 56 g/kg DM, acid detergent fibre 369 g/kg DM and neutral detergent fibre 480 g/kg DM.

Substrate combinations were fermented *in vitro* with an equine faecal inoculum using the *in vitro* gas production (GP) technique of Theodorou et al. (1994). Methods employed for the GP technique were as described by Theodorou et al. (1994); each bottle contained 85ml of media, 4ml of reducing agent and 10ml of microbial inoculum. The faecal inoculum was prepared as described previously (Murray et al., 2005) with the following modifications; ratio

Accepted manuscript

ACCEPTED MANUSCRIPT

of culture media: faeces was 2:1 and homogenized in a blender prior to filtration. The resultant suspension was strained through a triple layer of muslin and collected in a CO_2 filled flask. The faecal inoculum was dispensed immediately after extraction. Freshly voided faeces for the inoculum were collected from one mature horse maintained at pasture and transported in CO_2 pre-filled and pre-warmed flask to the laboratory and processed immediately.

The experiment was a factorial design consisting of five different substrates, two different treatments (+/- SFD) for the HTDA only and three replicate bottles. A total of 34 bottles were included for GP; 30 containing substrate and 4 inoculum blanks (no substrate). Head-space GP readings were taken at 3, 6, 9, 12, 15, 18, 21, 24, 27, 30, 33, 36, 39, 45, 56, 72, 82 and 96 h post-inoculation, with the accumulated gas volume measured using manual pressure transducer and LED digital readout voltmeter (Bailey& Mackey Ltd., Birmingham, UK) (Theodorou et al., 1994) and then released to zero following each reading. After the final reading, bottles were refrigerated at 4^{o}C to arrest fermentation. In addition to GP readings, aliquots (1.2 ml) of culture fluid were removed from each bottle at 0, 6, 12, 18, 24, 30, 36, 45, 56, 72, 82 and 96 h in order to measure the pH of the culture medium. Culture fluid was removed using a 5 ml syringe fitted with a 23 gauge x 1.5 inch needle inserted through the butyl rubber stopper. Previous work by the group (unpublished) showed culture fluid sampling (1.2 ml) at up to 20 time points throughout a 96 hour *in vitro* incubation to have no effect on any *in vitro* fermentation parameters obtained from HTDA. Vessel contents were analysed for DM. Culture fluid was separated from residual plant particles and adherent microbial biomass by vacuum filtration through sintered glass crucibles (porosity 1) and the residue rinsed with two volumes of distilled water. The washed residues were then oven dried to constant weight for the determination of residual DM.

Accepted manuscript

ACCEPTED MANUSCRIPT

Experiment 2: Chopped alfalfa and starch or inulin

Three identical series of 160 ml serum bottles were used to assess the fermentation characteristics of chopped (75 mm length) high-temperature dried alfalfa (CA) and S or I mixed in the following ratios; 100:0 , 80:20 , 60:40 , 40:60 and 20:80 to give a total sample mass of 1 g (± 0.5%) per bottle. A further set of bottles containing identical ratios of CA:S or I were also included using SFD treated alfalfa as described for experiment 1. *In vitro* fermentation procedures, pH and DM measurements were identical to those described in experiment 1.

Data handling and statistical analyses

Gas volume values were calculated for each time point by subtracting the corresponding gas measured in the substrate-negative control bottles and correcting for substrate DM. The mean control profiles for gas produced in inoculated culture bottles in the absence of substrate were subtracted prior to curve fitting analysis. Experimental data were fitted to the multi-phasic model of Groot et al. (1996):

$$Y = A /(1 + (B/t)^{C}) \quad (1)$$

where Y is the gas production (ml/g DM), A is the asymptotic gas production, B is the half time of A, C is the curve shape parameter and t is the time.

The maximal fractional rate of substrate degradation (MFR; h^{-1}) was also calculated:

$$MFR = ((C-1)^{(C-1)/C})/B \quad (2)$$

Accepted manuscript

ACCEPTED MANUSCRIPT

where MFR is the maximal fractional rate of substrate degradation, *B* is the half time of asymptotic gas production and *C* is the curve shape parameter.

Values for the modeled GP parameters and dry matter loss (DML) obtained from each experiment, were analysed for significant differences using two-way ANOVA in GenStat Release 10.1 (Lawes Agricultural Trust, Harpenden, UK). Values for pH measurements from each experiment were analysed by ANOVA using the model: treatment x substrate x time. Comparisons between treatment groups were made by LSD equations.

3. Results

Experiment 1: Ground alfalfa and starch or inulin

Mathematical analysis of gas production curves revealed an interaction ($P<0.001$) between treatment (+/- SFD) and substrate for all of the *in vitro* parameters measured, with the exception DML. GP reduced as the ratio of alfalfa to starch or inulin increased ($P<0.001$: Tables 1 and 2), with values lower overall in bottles containing +SFD alfalfa ($P<0.001$). MFR was also affected by the ratios of alfalfa and starch or inulin as substrates, as well as SFD treatment. MFR values were, in general, higher in bottles containing alfalfa and starch compared to S or I alone ($P<0.001$), with overall MFR values higher in bottles containing – SFD alfalfa compared to alfalfa +SFD. DML decreased proportionately with increasing level of GA substitution of S or I ($P<0.001$). Furthermore, there was no difference in DML between +SFD and – SFD substrates. There was no interaction between substrate and SFD treatment with regards to pH measurements. However, there were differences between SFD treatments, with higher pH values recorded for the –SFD treated material. pH values for starch as the sole substrate did not fall below 6.1 (Figure 1) , which is substantially higher than the value (5.1) recorded for inulin as the sole substrate (Figure 2). The substitution of S

Accepted manuscript

ACCEPTED MANUSCRIPT

with 200 mg of GA produced pH values above 6.7, whereas, a greater amount of alfalfa (400 mg) was required to maintain pH values above 6.7 in bottles containing I.

Experiment 2: Chopped alfalfa and starch or inulin

There was no interaction between treatment (+/- SFD) and substrate for gas production values. GP reduced as the ratio of alfalfa to S or I increased ($P<0.001$) (Table 3 and Table 4). MFR values varied across substrates ($P<0.01$) and between SFD treatments ($P<0.001$). DML decreased with increasing level of CA substitution of S or I ($P<0.001$). Moreover, there was no significant difference in DML between +SFD and – SFD substrates and no interactive effects.

There was no significant interaction between substrate and SFD treatment with regards to pH measurements. However, there were significant differences between SFD treatments, again with higher pH values generally recorded for the –SFD treated material. pH values for starch as the sole substrate did not fall below 6.3 (Figure 3), which again is substantially higher than the values recorded for inulin as the sole substrate (Figure 4) Substitution of S or I with 400 mg of CA was required to maintain pH values above 6.7.

4. Discussion

In equids, diets containing high levels of NSC have been associated with the onset of hindgut acidosis, laminitis and colic (Carroll et al., 1987; Clarke et al., 1990; Garner et al., 1977; Rowe et al., 1994). High levels of starch entering the hindgut environments have been seen to elicit detrimental changes to the gastrointestinal tract (Garner et al., 1975) and similar effects have been seen with high levels of fructan (inulin) entering the large intestine of the horse (Pollitt, 2002) high-levels of starch/fructan entering the hindgut can lead to rapid fermentation and the production of volatile fatty acids (VFA). Rapid VFA production has

Accepted manuscript

ACCEPTED MANUSCRIPT

been reported to overload the pH control mechanisms exerted by buffer secretion and absorption of the acids (Wallace et al., 2001), and consequently the hindgut pH decreases, favouring the rapid growth of the gram-positive, lactate-producing *Streptococcus* and *Lactobacillus spp*., which are more acid-tolerant than the gram-negative microbes present in the large intestine (Al Jassim and Rowe, 1999). As a result, lactate production becomes excessive, leading to a further decline in hindgut pH, and a drastic shift in the microbial population from predominantly gram-negative bacteria to primarily gram-positive (Bailey et al., 2004; Milinovich et al., 2006).

A decline in pH was recorded in this present study with pH values in bottles containing inulin rapidly dropping to under pH=6 by 12 hours of incubation. The pH profiles reported here are similar to faecal pH measurements reported by Milinovich et al. (2006) when inulin was administered to horses to experimentally induce laminitis. Maintenance of a healthy gastrointestinal environment requires the pH of the hindgut to be circ. pH 6.7 (Argenzio, 1990) with a drop in pH affecting the microbial populations within the large intestine (de Fombelle et al., 2001; de Fombelle et al., 2003; Julliand et al., 2001) and a pH of 6 representing sub-clinical acidosis, with pH values below 6 representing significant risk to gastrointestinal stability and precipitation of clinical disorders such as colic or laminitis (Al Jassim and Andrews, 2009). The pH in bottles containing starch as the sole substrate also dropped but remained above 6, which concurs with *in vivo* measurements from horses fed high levels of starch (de Fombelle et al., 2001; Julliand et al., 2001).

Diets high in crude protein (14 to 17 %) have been seen to increase rumen buffering capacity (Haaland et al., 1982), indicating that protein in food may act as a buffer against acidity. The macromolecular proteins are thought to be responsible for increasing the protective power of the mucus from penetration of hydrogen ions into the surrounding tissues (Holma and Hegg, 1989). Protein peptides have an amine terminal which can serve as base

Accepted manuscript

ACCEPTED MANUSCRIPT

and a carboxy terminal which can serve as acid. Moreover, proteins have side chains, some of which can be protonated or deprotonated, thus serving as pH buffers (Christensen, 1966) and negating against the drop in hindgut pH as the result of starch overload.

The alfalfa in the study reported here had a crude protein content of 17 percent. In equids it is important to account for protein digestion in the foregut and thus in the present study the +SFD treated alfalfa is more representative of the material entering the hind-gut and therefore it is likely that these values have more physiological relevance. Overall, pH values were higher in bottles containing the –SFD treatment compared to the +SFD materials for all substrate combinations, possibly accounting for the protein removal effect due to SFD and hence reduced buffering capacity of alfalfa on the *in vitro* fermentation. Loss of some alfalfa proteins during the SFD treatment may have reduced the buffering capacity of the +SFD treated alfalfa resulting in lower pH values compared to the –SFD alfalfa.

Nevertheless, the substitution of starch/inulin with 40 % +SFD alfalfa still maintained pH levels within physiological levels. Pre-caecal protein digestibility for alfalfa has been reported to be 28 percent when alfalfa containing 18 percent crude protein was fed to ponies (Gibbs et al., 1988). Therefore, it is possible that the +SFD treated alfalfa still contained considerable levels of crude protein. Alternatively, it may be that the inclusion of a high-fibre substrate negated the deleterious effects of the starch/inulin as it is well known that fibre is required to maintain gastrointestinal health in equids (de Fombelle et al., 2001; Drogoul et al., 2001; Harris et al., 2016; Julliand et al., 2001); therefore, it is possible that other fibrous feedstuffs may have had the same effects seen with the HTDA, an area that warrants further investigation.

Substrate processing also impacts on *in vitro* fermentation (Murray et al., 2010) and in the present study there were notable differences in the rate of fermentation between bottles containing ground and chopped alfalfa. These differences are likely attributable to

Accepted manuscript

ACCEPTED MANUSCRIPT

differences in particle size with a greater surface area available for microbial attachment (Bowman and Firkins, 1993; Emanuele and Staples, 1988) in the ground alfalfa and hence an increase in the rate of degradation. However, pH values were similar in bottles containing ground and chopped alfalfa, which concurs with other equine *in vitro* (Murray et al., 2010) and *in vivo* studies (Drogoul et al., 2000). Furthermore, the gas production technique appears to be a valuable tool for evaluating feedstuff combinations for equids *in vitro*, allowing the kinetics of degradation to be studied as opposed to end-point data and the effect of substrate on pH over time to be measured. Previous work has also shown donor animal variability to be minimal in terms of effects on total gas production and culture fluid pH when high-fibre and high-starch substrates have been investigated *in vitro* (Murray et al., 2006).

Finally, as the ratio of high-temperature dried alfalfa and starch/inulin increased, GP decreased with slower rates of gas production observed. These differences in the extent and rates at which alfalfa and starch/inulin are degraded are attributable to the chemical characteristics of these substrates, with alfalfa containing substantial cell-wall material (Longland et al., 1994) that is fermented less extensively and slower than NSC, such as starch and inulin (McLean et al., 2000; Milinovich et al., 2006). These findings further support the existing literature on the rapid degradation of NSC in the large intestinal environment of the horse and the slower, less extensive degradation of feedstuffs comprising structural cell wall material.

Accepted manuscript

5. Conclusion

The substitution of inulin/starch with HTDA *in vitro* appeared to buffer the deleterious effects of the S/I on *in vitro* fermentation; the substitution of starch/inulin with 40 percent HTDA maintained pH values within physiological levels encountered in the large intestine of the healthy horse. However, the pH measurements in this *in vitro* system may not

ACCEPTED MANUSCRIPT

entirely reflect the *in vivo* situation since the culture medium used in the gas production method is heavily buffered (Theodorou et al., 1994) and due to intestinal absorption of fermentative end-products; hence, further work is required to examine these potential effects *in vivo*.

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

References

Al Jassim, R. A. M., Andrews, F. M., 2009. The Bacterial Community of the Horse Gastrointestinal Tract and Its Relation to Fermentative Acidosis, Laminitis, Colic, and Stomach Ulcers. Veterinary Clinics of North America: Equine Practice 25, 199-215.

Al Jassim, R. A. M., Rowe, J. B., 1999. A better understanding of acidosis and its control. Recent Advances in Animal Nutrition in Austrailia 12, 91-96.

Argenzio, R. A., 1990. Physiology of digestive, secretory and absorptive processes. In: N. A. White (ed.) Equine Acute Abdomen. Lea and Feiber, Philadelphia, pp. 25-35

Bailey, S. R., Marr, C. M., Elliott, J., 2004. Current research and theories on the pathogenesis of acute laminitis in the horse. Equine Vet. J. 167, 129-142.

Bowman, J. G. P., Firkins, J. L., 1993. Effects of forage species and particle size on bacterial cellulolytic activity and colonisation in situ. J. Anim. Sci. 71, 1623-1633.

Carroll, C. L., Hazard, G., Coloe, P. J., Hooper, P. T., 1987. Laminitis and possible enterotoxaemia associated with carbohydrate overload in mares. Equine Vet. J. 19, 344-346.

Christensen, H. N. 1966. Proteins as buffers. Ann. N. Y. Acad. Sci. 133, 34-40.

Accepted manuscript

ACCEPTED MANUSCRIPT

Clarke, L. L., Roberts, M. C., Argenzio, R. A., 1990. Feeding and digestive problems in horses: Physiologic responses to a concentrate meal. Vet. Clin. North Am. Equine Prac. 6, 433-451.

de Fombelle, A., Julliand, V., Drogoul, C., Jacotot, E., 2001. Feeding and microbial disorders in horses: 1 - Effects of an abrupt incorporation of two levels of barley in a hay diet on microbial profile and activities. J. Equine Vet. Sci. 21, 439-445.

de Fombelle, A., Varloud, M., Goachet, A.G., Jacotot, E., Philippeau, C., Drogoul, C., Julliand, V., 2003. Characterisation of the microbial and biochemical profile of different segments of the digestive tract in horses given two distinct diets. J. Anim. Sci. 77, 293-304.

Drogoul, C., de Fombelle, A., Julliand, V., 2001. Feeding and microbial disorders in horses: 2: Effect of three hay : grain ratios on digesta passage rate and digestibility in ponies. J. Equine Vet. Sci. 21, 487-491.

Drogoul, C., Tisserand, J. L., Poncet, C., 2000. Feeding ground and pelleted hay rather than chopped hay to ponies - 2. Consequences on fibre degradation in the cecum and the colon. Anim. Feed Sci. Tech. 87, 131-145.

Emanuele, S. M., Staples, C. R., 1988. Effect of forage particle size on in situ digestion kinetics. J. Dairy Sci. 71, 1947-1954.

Furuya, S., Sakamoto, K., Takahashi, S., 1979. A new in vitro method for estimation of digestibility using the intestinal fluid of the pig. Br. J. Nutr. 41, 511-520.

Garner, H. E., Coffman, J. R., Hahn, A. W., Salem, C., 1975. Equine laminitis of alimentary origin: an experimental model. Am. J. Vet. Res. 36, 441-445.

Garner, H. E., Hutcheson, D. P., Coffman, J. R., Hahn, A. W., 1977. Lactic acidosis: A factor associated with equine laminitis. J. Anim. Sci. 45, 1037-1041.

Accepted manuscript

ACCEPTED MANUSCRIPT

Gibbs, P. G., Potter, G. D., Schelling, G. T., Kreider, J. L., Boyd, C. L., 1988. Digestion of hay protein in different segments of the equine digestive tract. J. Anim. Sci. 66, 400-406.

Groot, J. C. J., Cone, J. W., Williams, B. A., Derbersaques, F. M. A., Lantinga, E. A., 1996. Multiphasic analysis of gas production kinetics for *in vitro* fermentation of ruminant feeds. Anim. Feed Sci. Tech. 64, 77-89.

Haaland, G. L., Tyrrell H, F., Moe, P. W., Wheeler, W. E., 1982. Effect of crude protein level and limestone buffer in diets fed at two levels of intake on rumen pH, ammonia-nitrogen, buffering capacity and volatile fatty acid concentration of cattle. J. Anim. Sci. 55, 943-950.

Harris, P. A., Ellis, A. D., Fradinho, M. J., Jansson, A., Julliand, V., Luthersson, N., Santos, A. S., Vervuert, I., 2016. Review: Feeding conserved forage to horses: recent advances and recommendations. animal: 1-10.

Holma, B., Hegg, P. O., 1989. pH- and protein-dependent buffer capacity and viscosity of respiratory mucus. Their interrelationships and influence of health. Sci. Total Environ. 84, 71-82.

Hudson, J. M., Cohen, N. D., Gibbs, P. G., Thompson, J. A., 2001. Feeding practices associated with colic in horses. J. Am. Vet. Med. Assoc. 219, 1419-1425.

Julliand, V., de Fombelle, A., Drogoul, C., Jacotot, E., 2001. Feeding and microbial disorders in horses: Part 3 - Effects of three hay : grain ratios on microbial profile and activities. J. Equine Vet. Sci. 21, 543-546.

Longland, A. C., Carruthers, J., Low, A. G., 1994. The ability of piglets 4 to 8 weeks old to digest and perform on diets containing two contrasting sources of non-starch polysaccharides. Anim. Prod. 58, 405-410.

Accepted manuscript

ACCEPTED MANUSCRIPT

McLean, B. M. L., Hyslop, J. J., Longland, A. C., Cuddeford, D., Hollands, T., 2000. Physical processing of barley and its effects on intra-caecal fermentation parameters in ponies. Anim. Feed Sci. Tech. 85, 79-87.

Merry, R. J., Jones, R., Theodorou, M., 2000. The conservation of grass. In: A. Hopkins (ed.) Grass, Its Production and Utilisation. Blackwell Science, Oxford, pp. 196-224.

Milinovich, G. J., Trott, D.J., Burrell, A.W., Thoefner, M.B., Blackall, L. L., Al Jassim, R.A.M., Morton, J.M. 2006. Changes in equine hindgut bacterial popultation during oligofructose-induced laminitis. Appl. Environ. Microb. 8: 885-898.

Murray, J. M. D., Bice, R., Moore-Colyer, M., 2010. The effect of particle size on the in vitro fermentation of different ratios of high-temperature dried lucerne and sugar beet pulp incubated with equine faecal inocula. Anim. Feed Sci. 162(1-2),47-57.

Murray, J. M. D., Longland, A., Moore-Colyer, M., Dunnett, C., 2006. The effect of feeding a low- or a high-starch diet on the in vitro fermentative capacity of equine faecal inocula. Anim. Sci. 82(05), 627-635.

Murray, J. M. D., Longland, A. C., Moore-Colyer, M. J. S., Dunnett, C., 2005. The effect of enzyme treatment on the *in vitro* fermentation of lucerne incubated with equine faecal inocula. Br. J. Nutr. 94, 771-782.

Nadeau, J. A., Andrews, F. M., Mathew, A. G., Argenzio, R. A., Blackford, J. T., 1998. The effect of diet on severity of gastric ulcers in horses. Gastroenterol. 114, A238.

Nadeau, J. A., Andrews, F. M., Mathew, A. G., Argenzio, R. A., Blackford, J. T., Sohtell, M., Saxton, A. M., 2000. Evaluation of diet as a cause of gastric ulcers in horses. Am. J. Vet. Res. 61, 784-790.

Pollitt, C. C. 2002. Equine laminitis: a new induction model based on alimentary overload with fructan. Proceedings of the 6th Colic Research Symposium, Manchester, UK.

Accepted manuscript

ACCEPTED MANUSCRIPT

Rowe, J. B., M. J. Lees, and D. W. Pethick. 1994. Prevention of acidosis and laminitis associated with grain feeding in horses. J. Nutr. 124: 2742S-2744S.

Theodorou, M. K., Williams, B. A., Dhanoa, M. S., McAllen, A. B., France, J., 1994. A simple gas production method using a pressure transducer to determine the fermentation kinetics of ruminant feeds. Anim. Feed Sci. Tech. 48, 185-197.

Wallace, R. J., Wallace, S. J. A., McKain, N., Nsereko, V. L., Hartnell, G. F., 2001. Influence of supplementary fibrolytic enzymes on the fermentation of corn and grass silages by mixed ruminal microorganisms *in vitro*. J. Anim. Sci. 79, 1905-1916.

Zeyner, A., Geibler, C., Dittruch, A., 2004. Effects of hay intake and feeding sequence on variables in faeces and faecal water (dry matter, pH value, organic acids, ammonia, buffering capacity) of horses. J.Anim. Physiol. Anim. Nutr. 88, 7-19.

Fig 1: pH profiles for five ground alfalfa (GA):starch (S) combinations; 100:0 S:GA (S), 80:20 S:GA (SGA1), 60:40 S:GA (SGA2), 40:60 S:GA (SGA3) and 20:80 S:GA (SGA4) incubated with an equine faecal inoculum with (+SFD) or without (-SFD) a simulated foregut digestion treatment prior to *in vitro* fermentation (n=3).

Fig 2: pH profiles for five ground alfalfa (GA):inulin (I) combinations; 100:0 I:GA (I), 80:20 I:GA (IGA1), 60:40 I:GA (IGA2), 40:60 I:GA (IGA3) and 20:80 I:GA (IGA4) incubated with an equine faecal inoculum with (+SFD) or without (-SFD) a simulated foregut digestion treatment prior to *in vitro* fermentation (n=3).

Fig 3: pH profiles for five chopped alfalfa (CA):starch (S) combinations; 100:0 S:CA (S), 80:20 S:CA (SCA1), 60:40 S:CA (SCA2), 40:60 S:CA (SCA3) and 20:80 S:CA (SCA4) incubated with an equine faecal inoculum with (+SFD) or without (-SFD) a simulated foregut digestion treatment prior to *in vitro* fermentation (n=3).

Accepted manuscript

ACCEPTED MANUSCRIPT

Fig 4: pH profiles for five chopped alfalfa (CA):inulin (I) combinations; 100:0 I:CA (I), 80:20 I:CA (ICA1), 60:40 I:CA (ICA2), 40:60 I:CA (ICA3) and 20:80 I:CA (ICA4) incubated with an equine faecal inoculum with (+SFD) or without (-SFD) a simulated foregut digestion treatment prior to *in vitro* fermentation (n=3).

Table 1: Gas production curve fitted parameters [asymptotic gas production (A), maximal fractional rate of substrate degradation (MFR)] and dry matter loss (DML) for five ground alfalfa (GA):starch (S) combinations; 100:0 S:GA (S), 80:20 S:GA (SGA1), 60:40 S:GA (SGA2), 40:60 S:GA (SGA3) and 20:80 S:GA (SGA4) incubated with an equine faecal inoculum with (+SFD) or without (-SFD) a simulated foregut digestion treatment prior to *in vitro* fermentation (n=3). NS: Not significant

	Substrate					
	S	**SGA1**	**SGA2**	**SGA3**	**SGA4**	**Treatment Mean**
A (ml/g DM)						
+SFD	308^{f}	341^{g}	244^{d}	185^{b}	121^{a}	**240**
-SFD		305^{ef}	281^{e}	223^{cd}	209^{bc}	**265**
Substrate Mean	**308**	**323**	**262**	**204**	**165**	
Substrate s.e.d.	9.3 (P<0.001)					
Treatment s.e.d.	5.9 (P<0.001)					
S x T s.e.d.	13.1 (P<0.001)					
MFR						
+SFD	0.062^{ab}	0.068^{b}	0.095^{cd}	0.098^{d}	0.083^{c}	**0.081**
-SFD		0.120^{e}	0.148^{f}	0.114^{e}	0.054^{a}	**0.096**
Substrate Mean	**0.062**	**0.094**	**0.119**	**0.106**	**0.068**	
Substrate s.e.d.	0.0050 (P<0.001)					
Treatment s.e.d.	0.0031 (P<0.001)					
S x T s.e.d.	0.0070 (P<0.001)					
DML (mg/g)						
+SFD	987	940	914	889	868	**918**
-SFD		928	903	870	826	**905**
Substrate Mean	**987**	**934**	**909**	**880**	**847**	
Substrate s.e.d.	10.5 (P<0.001)					
Treatment s.e.d.	6.7 (NS)					
S x T s.e.d.	14.9 (NS)					

Table 2: Gas production curve fitted parameters [asymptotic gas production (A) and maximal fractional rate of substrate degradation (MFR)] for five ground alfalfa (GA):inulin (I) combinations; 100:0 I:GA (I), 80:20 I:GA (IGA1), 60:40 I:GA (IGA2), 40:60 I:GA (IGA3) and 20:80 I:GA (IGA4) incubated with an equine faecal inoculum with (+SFD) or without (-SFD) a simulated foregut digestion treatment prior to *in vitro* fermentation (n=3).NS: Not significant

Accepted manuscript

ACCEPTED MANUSCRIPT

	Substrate					
	I	**IGA1**	**IGA2**	**IGA3**	**IGA4**	**Treatment Mean**
A (ml/g DM)						
+SFD	276d[e]	336[f]	241[cd]	181[b]	123[a]	**231**
-SFD		307[ef]	261[cd]	250[cd]	233[c]	**265**
Substrate Mean	**276**	**321**	**251**	**216**	**178**	
Substrate s.e.d.	13.7 (P<0.001)					
Treatment s.e.d.	8.7 (P<0.001)					
S x T s.e.d.	19.4 (P<0.001)					
MFR						
+SFD	0.094	0.065	0.081	0.096	0.064	**0.080**
-SFD		0.070	0.078	0.068	0.058	**0.074**
Substrate Mean	**0.094[d]**	**0.068[bc]**	**0.080[cd]**	**0.082[cd]**	**0.061[a]**	
Substrate s.e.d.	0.0061 (P<0.001)					
Treatment s.e.d.	0.0038 (NS)					
S x T s.e.d.	0.0086 (NS)					
DML (mg/g)						
+SFD	992	884	809	750	566	800
-SFD		870	748	717	553	776
Substrate Mean	**992[a]**	**878[b]**	**779[c]**	**733[d]**	**560[e]**	
Substrate s.e.d.	22.2 (P<0.001)					
Treatment s.e.d.	14.0 (NS)					
S x T s.e.d.	31.3 (NS)					

Table 3: Gas production curve fitted parameters [asymptotic gas production (A) and maximal fractional rate of substrate degradation (MFR)] for five chopped alfalfa (CA):starch (S) combinations; 100:0 S:CA (S), 80:20 S:CA (SCA1), 60:40 S:CA (SCA2), 40:60 S:CA (SCA3) and 20:80 S:CA (SCA4) incubated with an equine faecal inoculum with (+SFD) or without (-SFD) a simulated foregut digestion treatment prior to *in vitro* fermentation (n=3).NS: Not significant

	Substrate					
	S	**SCA1**	**SCA2**	**SCA3**	**SCA4**	**Treatment Mean**
A (ml/g DM)						
+SFD	341	305	281	208	119	**251**
-SFD		310	258	242	195	**269**
Substrate Mean	**341[d]**	**308[cd]**	**270[c]**	**225[b]**	**157[a]**	
Substrate s.e.d.	19.9 (P<0.001)					
Treatment s.e.d.	12.6 (NS)					
S x T s.e.d.	28.2 (NS)					
MFR						
+SFD	0.069[b]	0.069[b]	0.133[d]	0.157[e]	0.058[a]	**0.097**
-SFD		0.066[b]	0.077[c]	0.066[b]	0.076[c]	**0.071**
Substrate Mean	**0.069**	**0.067**	**0.105**	**0.111**	**0.067**	
Substrate s.e.d.	0.0123 (P<0.01)					
Treatment s.e.d.	0.0078 (P<0.01)					
S x T s.e.d.	0.0175 (P<0.001)					
DML (mg/g)						

Accepted manuscript

ACCEPTED MANUSCRIPT

+SFD	998	949	876	848	841	**903**
-SFD		925	904	865	840	**906**
Substrate Mean	**998^{a}**	**937^{b}**	**890^{c}**	**857^{d}**	**841^{e}**	
Substrate s.e.d.	12.3 (P<0.001)					
Treatment s.e.d.	7.8 (NS)					
S x T s.e.d.	17.5 (NS)					

Table 4: Gas production curve fitted parameters [asymptotic gas production (A) and maximal fractional rate of substrate degradation (MFR)] for five chopped alfalfa (CA):inulin (I) combinations; 100:0 I:CA (I), 80:20 I:CA (ICA1), 60:40 I:CA (ICA2), 40:60 I:CA (ICA3) and 20:80 I:CA (ICA4) incubated with an equine faecal inoculum with (+SFD) or without (-SFD) a simulated foregut digestion treatment prior to *in vitro* fermentation (n=3).NS: Not significant.

	Substrate					
	I	**ICA1**	**ICA2**	**ICA3**	**ICA4**	**Treatment Mean**
A (ml/g DM)						
+SFD	265	270	262	198	123	**224**
-SFD		344	319	246	215	**278**
Substrate Mean	**265^{bc}**	**307^{c}**	**291^{c}**	**222^{b}**	**169^{a}**	
Substrate s.e.d.	15.8 (P<0.001)					
Treatment s.e.d.	10.0 (P<0.001)					
S x T s.e.d.	22.5 (NS)					
MFR						
+SFD	0.310^{f}	0.170^{de}	0.144^{bcd}	0.151^{cd}	0.202^{e}	**0.196**
-SFD		0.099^{ab}	0.148^{cd}	0.120^{bc}	0.063^{a}	**0.148**
Substrate Mean	**0.310**	**0.135**	**0.146**	**0.135**	**0.133**	
Substrate s.e.d.	0.0165 (P<0.001)					
Treatment s.e.d.	0.0105 (P<0.001)					
S x T s.e.d.	0.0235 (P<0.01)					
DML (mg/g)						
+SFD	992	871	812	734	661	**807**
-SFD		829	769	713	650	**791**
Substrate Mean	**992^{a}**	**850^{b}**	**791^{c}**	**723^{d}**	**656^{e}**	
Substrate s.e.d.	20.0 (P<0.001)					
Treatment s.e.d.	12.7 (NS)					
S x T s.e.d.	28.4 (NS)					

Accepted manuscript

ACCEPTED MANUSCRIPT

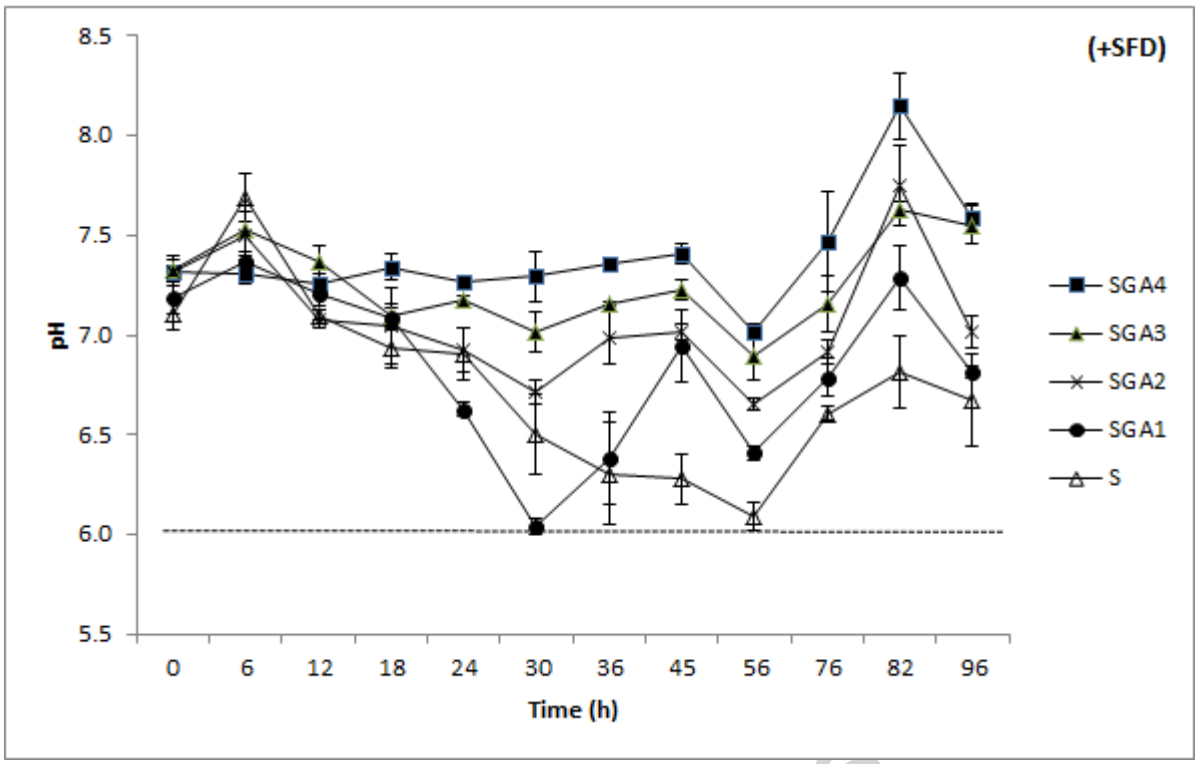
Highlights

- Non-structural carbohydrates (NSC) overload often leads to drop of pH, hindgut microbial disturbances resulting in clinical disorders, which in its turn affect welfare and performance of the horse
- There is a need in developing new nutritional practices to reduce negative effects of the high NSC diets
- Alfalfa is well known for its buffering capacity due to its high protein level
- An *in vitro* study confirmed that there is considerable potential to buffer the deleterious effects of high-starch/fructan diets with the substitution of these substrates with alfalfa

Accepted manuscript

Figure 1

ACCEPTED MANUSCRIPT



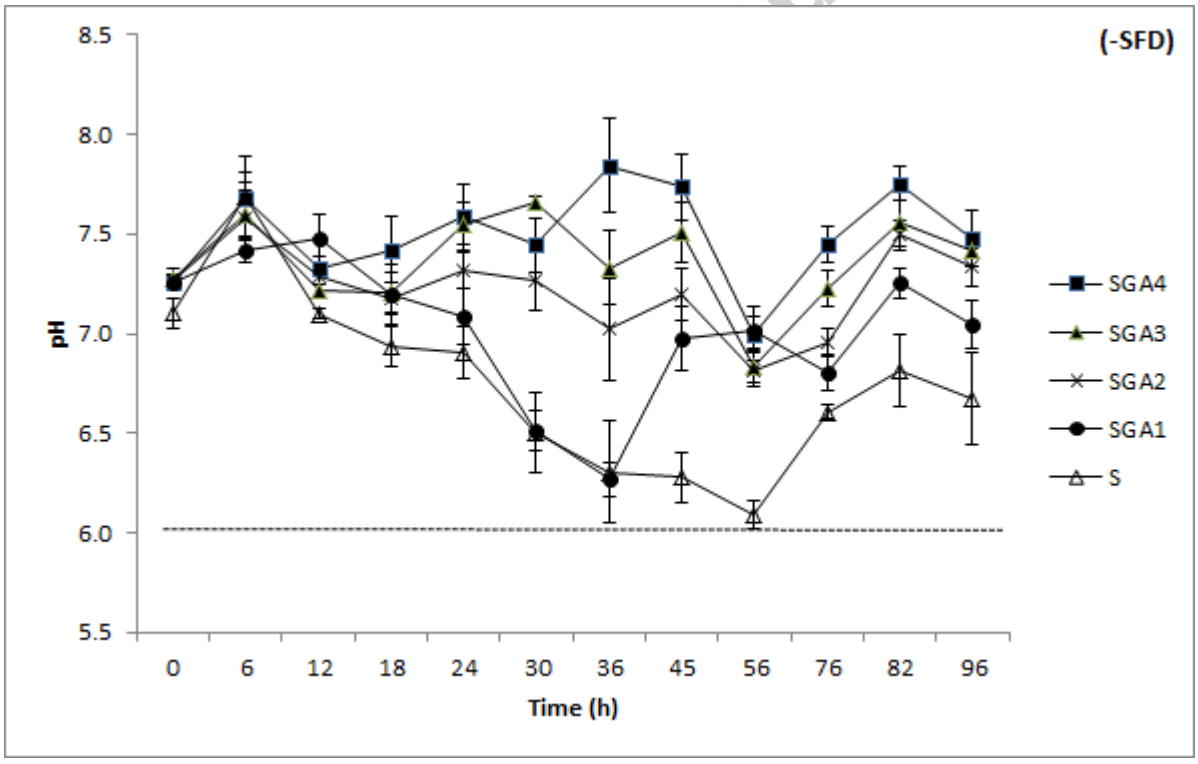
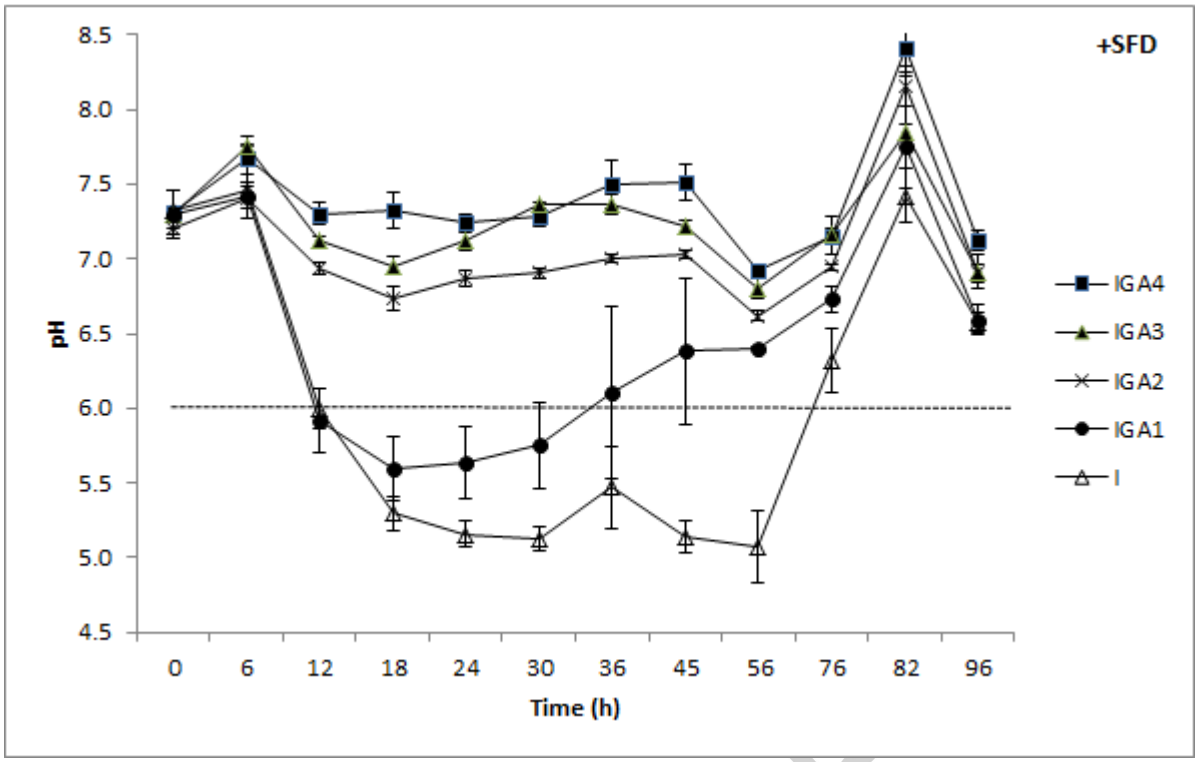


Figure 2

ACCEPTED MANUSCRIPT



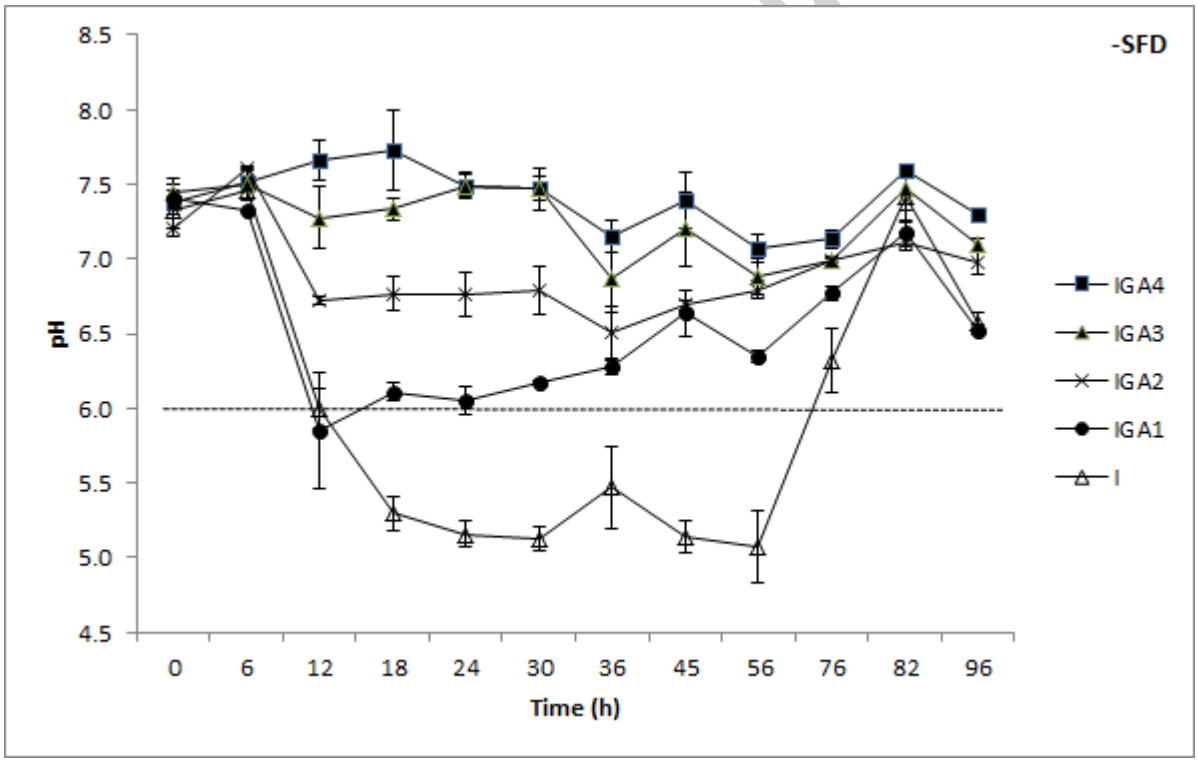
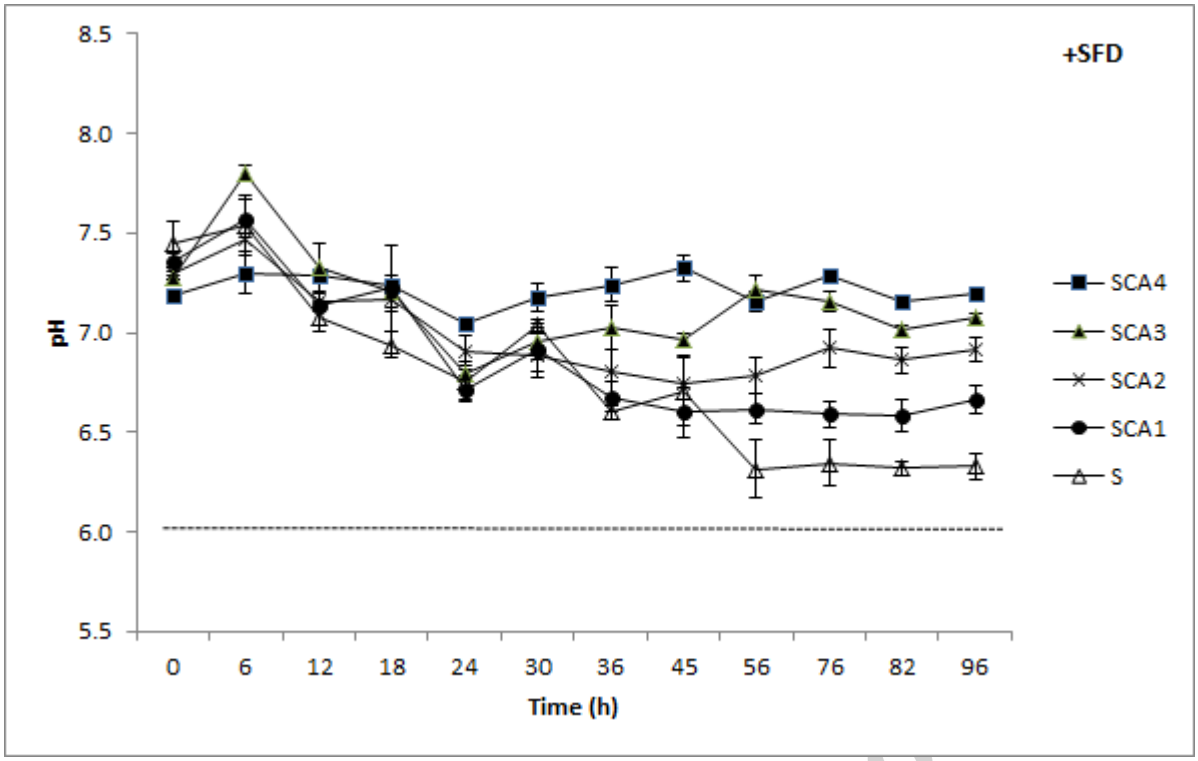


Figure 3

ACCEPTED MANUSCRIPT



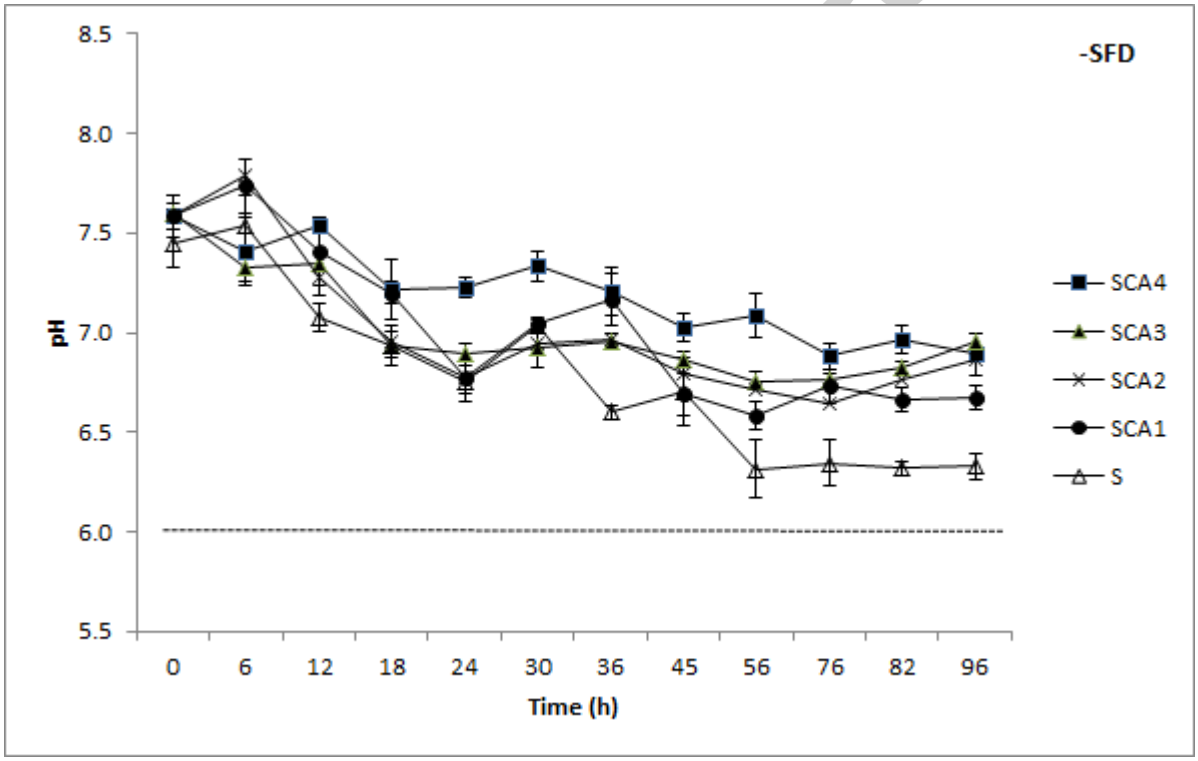
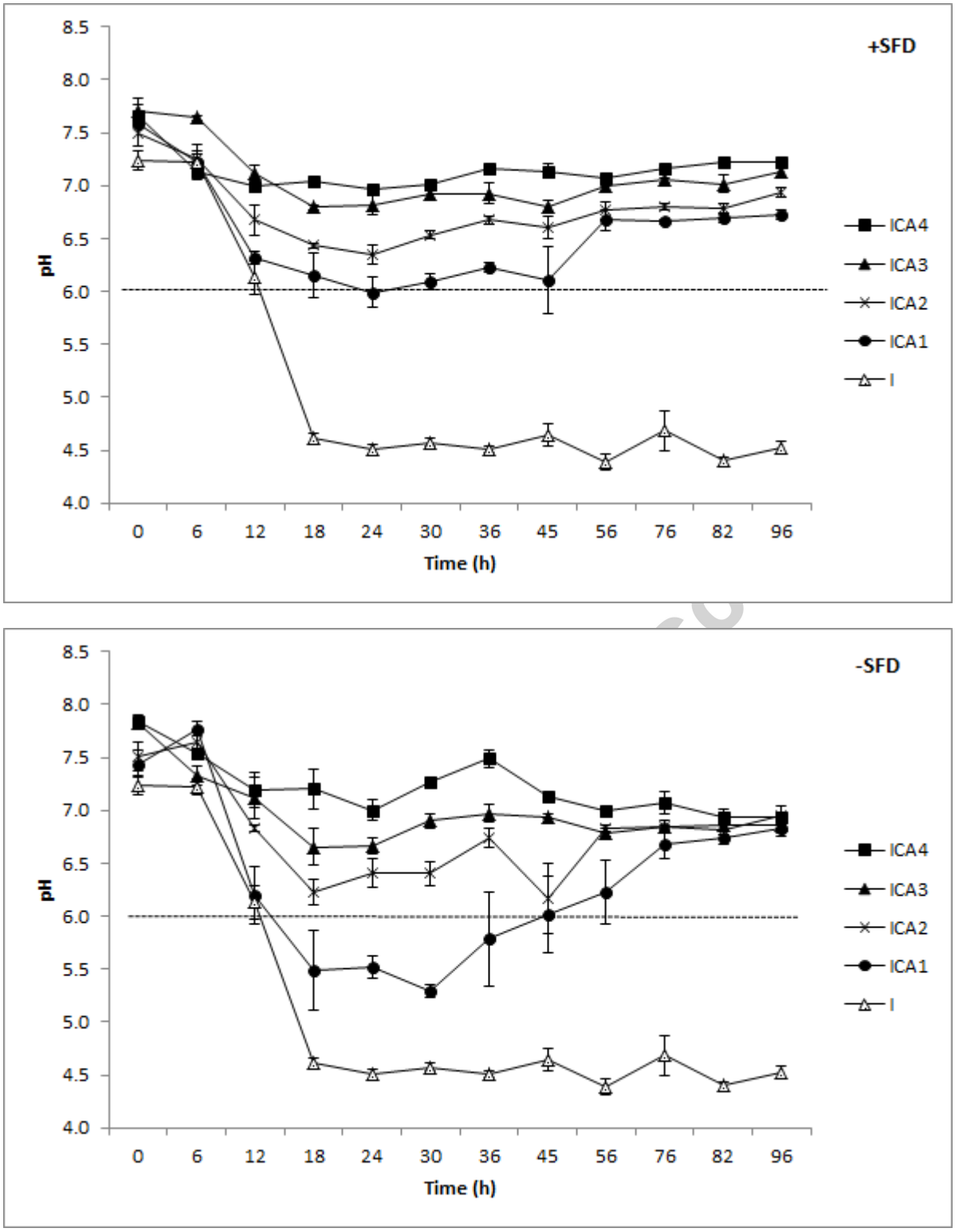


Figure 4

ACCEPTED MANUSCRIPT