Non-starch polysaccharide-degrading enzymes alter the microbial community and the fermentation patterns of barley cultivars and wheat products in an *in vitro* model of the porcine gastrointestinal tract

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Running title: NSP-degrading enzymes alter intestinal fermentation patterns

This is an Accepted Article that has been peer-reviewed and approved for publication in the *FEMS Microbiology Ecology*, but has yet to undergo copy-editing and proof correction. Please cite this article as an "Accepted Article"; doi: 10.1111/j.1574-6941.2011.01074.x

Keywords: pig, NSP, enzymes, *in vitro* fermentation, microbial ecology

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Abstract

An *in vitro* experiment was carried out to assess how non-starch polysaccharide (NSP)-degrading enzymes influence fermentation of dietary fibre in the pig large intestine. Seven wheat and barley products and cultivars with differing carbohydrate fractions (CHO) were hydrolyzed using pepsin and pancreatin in the presence or not of NSP-degrading enzymes (xylanase and β-glucanase) and the filter retentate subsequently fermented with sow fecal bacteria for 72 h. Dry matter, starch, crude protein and β-glucan digestibilities during hydrolysis were measured. Fermentation kinetics of the hydrolyzed ingredients were modelled. Short-chain fatty acids (SCFA) production and molar ratio were compared after 12, 24 and 72 h. Microbial communities were analyzed after 72 h of fermentation using terminal restriction fragment length polymorphism (TRFLP). Besides the expected influence of the cereal type or cultivar, the results showed an increase of *in vitro* nutrient digestibility ($P<0.001$), whereas fermentability as well as SCFA production decreased ($P<0.001$) with addition of the enzyme. SCFA and bacterial community profiles indicated also a shift from propionate to acetate and an increase in cellulolytic *Ruminoccocus*- and xylanolytic *Clostridium*-like

bacteria. This is explained by the increased proportion of slowly fermentable insoluble CHO and the lower proportion of rapidly fermentable β-glucan and starch in the retentate when grains were incubated with NSP-degrading enzymes. Shifts induced by the enzymes were also different for the 4 barley varieties investigated, showing that the efficiency of the enzymes to influence digestion and intestinal fermentation depends on the structure of the CHO fractions in cereal products and cultivars.

Introduction

Indigestible carbohydrates (CHO) such as non-starch polysaccharides (NSP) and resistant starch are considered as antinutritional factors for pigs since their presence negatively affects digestion and absorption processes and animal performance. NSP-degrading enzymes are often supplemented to the diets of monogastric animals to reduce the negative effects of NSP, especially arabinoxylans and β-glucan, and to improve animal performance. The basis for the mode of action of the enzymes is the partial degradation of soluble NSP in the upper digestive tract, which directly decreases digesta viscosity and increases passage rate (Bartelt et al., 2002; Vahjen et al., 2007). Nutrients that were initially unavailable to digestion processes, due to lower access of endogenous enzymes, become available and thereby increase the nutrient supply to the animal (Simon, 1998). The degradation of complex β-glucan and arabinoxylan to lower molecular weight compounds not only decreases viscosity and increases nutrient digestibility but may also facilitate the access of bacteria in the distal small intestine as well as the large intestine to fermentable substrate. In cereals, NSP and resistant starch content are highly variable from one grain species and type to the other, but also between cultivars of the same grain species and type (Izydorczyk & Dexter, 2008). Indigestible CHO induce changes in the composition and metabolic activity of the gut microbiota (Louis et al., 2007; Topping, 2007). The efficiency of digestive processes and the intestinal microbial ecophysiology of monogastric animal such as pigs can therefore be affected by the choice of cereal cultivars with specific CHO composition in the basal diet. For example, using the variability in β-glucan content within barley and oat cultivars, it is possible to manipulate the composition of the intestinal microbial communities and their activity (Pieper et al., 2008; Jha et al., 2010). Short-chain fatty acids (SCFA), mainly acetate, propionate and butyrate, produced during the fermentation of indigestible CHO in the intestines might favour the intestinal health. For example, butyrate, as major energy source for the epithelial cells, has been shown to improve digestive and absorptive capacities of the small intestine in pigs (Bindelle et al., 2008).

Indigestible CHO fermentation also increases bacterial growth of health-promoting bacteria such as lactobacilli and bifidobacteria and reduces protein fermentation in the large intestine (Bindelle et al., 2008). This would reduce the concentration of bacterial metabolites with known negative impact on animal health, such as ammonia, hydrogen sulfide, phenolic compounds or amines (Heo et al., 2009).

The addition of NSP-degrading enzymes to the diets of growing pigs affect the concentration of short-chain fatty acids (SCFA) in the small intestine (Diebold et al., 2004), indicating increased bacterial activity (Haberer et al., 1999). Enhanced bacterial growth in the small intestinal contents of piglets after addition of a multi-enzyme preparation containing xylanases, arabinoxylanases and β-glucanases was also reported (Osswald et al., 2006). Another study by Hirsch et al. (2006) revealed the enhancing effect of xylanase addition to diets for weaned pigs on jejunal total bacteria and lactobacilli 16S rRNA gene abundances and the enhancement of specific *Lactobacillus* spp. These observations suggest that addition of NSP-ases enzymes in the diet disrupts the fibre matrix embedding digestible CHO, i.e. starch, increasing accessibility for the digestive enzymes of the pig with positive outcomes in terms of diet conversion indexes. Simultaneously, the access to digestible and indigestible CHO for fermentation by intestinal bacteria is increased. Even if depolymerisation of the polymers is not the limiting step in the fermentation reaction chains of arabinoxylans and β-glucans in the intestines of pigs (Williams et al. in press), this increased access can favour small intestinal growth of lactobacilli. Conversely, Rosin et al. (2007) observed reduced *E. coli* abundance in ceacums of chicken fed corn and wheat based-diets supplemented with NSP-ases. Improved fermentation of substrates in the upper intestinal tract would furthermore be expected to change the flow of fermentable substrates to the large intestine, thereby changing microbial ecophysiology. However, information detailing how the addition of enzymes to cereals differing in CHO composition would affect substrate availability for fermentation in the gut, microbial communities' composition in the large intestine and fermentation metabolites is scarce.

The aim of the present study was to evaluate the influence of an NSP-degrading enzymatic mixture on the digestion and fermentation characteristics of the CHO fraction of wheat products and barley cultivars and the consequences on intestinal microbial communities, using an *in vitro* model of the porcine gastro-intestinal tract.

Materials and methods

Ingredients

The Rovabio™ Excel AP enzyme mixture used in this study was provided by Adisseo (Commentry, France). The enzyme is a complex of non-starch polysaccharidases (containing mainly endo-1,4-β-xylanase and endo-1,3/1,4-β-glucanase) and produced from *Penicillium funiculosum*.

Six barley and wheat cultivars and products were chosen according to their NSP composition and content, starch composition and β-glucan, content (Table 1): whole wheat, wheat bran, 2 hulless barleys cultivars with very high content of β-glucan (CDC Fibar and SB 94893) and 2 hulled barley cultivars with higher content of xylans in their hulls (AC Metcalfe and McLeod). A diet used in previous *in vivo* experiments was also included in the set. All ingredients were ground to pass a 1 mm screen using a centrifugal mill (Retsch Mill ZM1, Newtown, PA).

In vitro *enzymatic digestion and fermentation*

The 6 cereal products and the diet underwent an *in vitro* enzymatic hydrolysis that simulates digestion in the upper digestive tract, using porcine pepsin and pancreatin as described elsewhere (Boisen & Fernández, 1997), followed by an *in vitro* fermentation that simulates the fermentation occurring in the hindgut (Bindelle et al., 2007a). Briefly, ingredient samples were hydrolysed with porcine pepsin (pH 2, 39°C, 2h) and porcine pancreatin (pH 6.8, 39°C, 4h). Residues were filtered through a 42 μm Nylon cloth,

washed twice with 96 % ethanol and 99.5 % acetone and dried at 60°C overnight. Dry matter digestibility during hydrolysis (IVDMD) was recorded. For each ingredient, 2 enzymes addition patterns were implemented: hydrolysis was performed without or with Rovabio^{TM} enzyme mixture (0.5 mg/g ingredient) added during the pepsin digestion step. The experimental scheme was as follows: 7 ingredients × 2 enzyme addition patterns × 2 replicates × 4 periods.

The residues were incubated in an inoculum prepared from fresh faeces of 3 sows from the herd of the Prairie Swine Centre Inc. (Saskatoon, SK, Canada) that were fed a diet free of antibiotics, and mixed to a buffer solution composed of salts and minerals (Menke & Steingass, 1988). The fermentation (39 ± 0.5°C) started mixing 200 mg of the hydrolyzed residues and 30 ml of the inoculum into 140 ml glass bottles equipped with a rubber stopper. During fermentation, gases (CO_2, H_2 and CH_4) and short-chain fatty acids (SCFA) are produced by faecal microbes. SCFA are buffered by the carbonate ions of the buffer solution to release CO_2. Therefore, in this model, total gas production reflects the major end products (gases plus SCFA) of microbial fermentation of CHO and is inversely related to substrate disappearance.. The released gas volumes (fermentation and buffered gas) were regularly recorded over 72h of incubation by measuring bottles inner pressure in order to measure how fast the different ingredients were fermented by the microbes (Mauricio et al., 1999). The experimental scheme was as follows: 7 ingredients × 2 enzyme addition patterns × 3 replicates + 3 blanks (containing only inoculum).

After 72h, fermentation broth was centrifuged (12.000 g for 5 min) and the supernatant removed for analysis of short-chain fatty acids (SCFA). The pellet was further used for extraction of genomic DNA. Additionally, at 12 and 24 h of fermentation, 1 ml of the fermentation broth was also sampled for SCFA determination.

Kinetics of gas production

Gas accumulation curves were modelled using the mathematical monophasic model according to Groot et al. (1996):

$$G = \frac{A}{1 + \frac{B^C}{t^C}}$$

if t > 0, where G (ml/gDM) denotes the gas accumulation to time, A (ml/gDM) the maximum gas volume for t=∞, *B* (h) the time to half asymptote when *G*=*A*/2 and *C* a constant determining the slope of the inflexion point of the profile. From the equation, 2 additional parameters are calculated, Rmax, the maximum rate of gas production (ml/gDM×h), when the microbial population no longer limits the fermentation and tmax, the time at which Rmax is reached.

Measurement of SCFA production

For each sample, 100 µl of supernatant was mixed in a 1.7 ml epperdorf tube with 20 µl of 25% meta phosphoric acid solution to adjust pH to approx. 2, with 500 µl of internal standard solution (100 mM trimethyl acetic acid in acetonitrile) and 880 µl acetonitrile. The solution was centrifuged at 14,000 g for 10 min and the supernatant transferred into a GC vial. Chromatographic analysis was carried out on an Agilent 6890 GC system equipped with a flame ionization detector (FID), a 7683 series liquid injector and auto sampler (Agilent, Germany). Samples were run on a fused-silica capillary column (ZB-FFAP, Phenomenex, USA) with 30 m x 320 µm x 0.25 µm nominal using He as carrier gas at a flow rate of 1.9 ml/min. The flow rate of hydrogen and air was 35 and 350 ml/min, respectively. Electronic pneumatics control (EPC) was run in split mode with split ratio 100:1 at 11.10 psi pressure and total flow of

191ml/min. The temperature program was set as follows: initial temperature, 100°C for 1 min, ramp with 8°C/ min and final temperature 200°C for 13.5 min (Agilent, Germany).

Determination of microbial composition

DNA was isolated from the fermentation broth using a series of chemical, physical and enzymatic treatments described previously (Hill et al., 2005), with slight modifications. Briefly, 1 ml of fermentation broth was placed in a bead-beating tube (Mo-Bio Laboratories, Solana Beach, CA, USA) and centrifuged. Supernatant was aspirated for SCFA analysis and the remaining pellet was incubated at 37°C for 30 min in 365 µl of lysing buffer A (containing per litre: 200 mg RNAse, 50mM Tris-HCl, 50mM EDTA with 0.5% Tween 20% and 0.5% Triton X- 100, 18.61 g Na_2EDTA and 6.06 g Tris base, pH adjusted to 8.0) added with 7.5 µl lysozyme (100 mg/l) and 20 µl proteinase K (20 mg/l), in order to lyse the bacterial cells. After addition of 135 µl of lysing buffer B (containing per litre: 3M guanidine-HCl and 20% Tween 20), the mixture was incubated for another 30 min at 50°C and subsequently frozen at -70°C for 20 min. After thawing, 700 µl of phenol/chloroform/isoamyl (25:24:1) was added to each tube, and samples were then processed three times in a FastPrep FP 120 instrument (ThermoSavant, Holbrook, NY, USA) at 5 movements s^{-1} for 20 s to release and purify the genomic DNA. After centrifugation at 14,000 g for 15 min, the supernatant was transferred into a new tube and 70 µl of 3M Na acetate and 700 µl isopropanol were added and the precipitated DNA was pelleted for 15 min at 14,000 g. The pellet was washed with 70% ethanol, air dried, re-dissolved in 100 µl MilliQ water and stored at -18°C until further analysis.

For analysis of the microbial communities, a partial fragment of the bacterial 16S rRNA gene was amplified by polymerase chain reaction (PCR) using universal forward primer S-D-Bact-0008-a-S-20 (AGA GTT TGA TCM TGG CTC AG), labelled with 6-carboxyflourescein (6-FAM) and reverse primer R3 (TCT ACG CAT TTC AC) (Dorsch & Stackebrandt, 1992). PCR reactions contained 5µL of 10 x

Incubation Buffer, 1.5 µL of 50 mM $MgCl_2$, 1.5 µL of each primer (10µM), 1.5 µL of each dNTP (10mM) and 0.2 µL of Taq-Polymerase (5 U/µL) and UV-sterilised Millipore water, added until 50 µL. PCR was performed in a Thermolyne Amplitron II temperature cycler (Barnstead/Thermolyne, Dubuque, Iowa) and the program set as follows: 5 min at 95°C, 30 cycles of 95°C for 40 s, 55°C for 40 s and 72°C for 60 s, final extension at 72°C for 10 min. Size and yield of PCR products were checked by electrophoresis in 1.5 % agarose gel after staining with ethidium bromide (0.5µg EtBr/mL agarose). The PCR product was subsequently extracted from the gel using the Qiagen® PCR Purification Kit (Qiagen, Mississauga, ON, Canada) according to manufacturer's protocol and the DNA concentration was measured on a NanoDrop® ND-1000 spectrophotometer (NanoDrop Technologies, Inc., Wilmington, USA).

For terminal restriction fragment length polymorphism (TRFLP) analysis, 100 ng of the PCR product was digested at 37°C for 6 h using 15 U of MspI (Fermentas, Burlington, ON, Canada) in 2µL reaction buffer and UV-sterilized Millipore water, made up to 20 µL. Two µL of the digestion solution were subsequently mixed with 9 µL of formamide and 0.5 µL of an internal size standard (600 LIZ) and denatured at 95°C for 5 min followed by immediately cooling down on ice for 2 min. Fragment sizes were analyzed using an ABI 3130xl Genetic Analyzer in gene scan mode and GeneMapper v3.7 software (Applied Biosystems Inc., Foster City, CA, USA). Fragments that are different in less than + /- 3bp were considered to be identical.

Profiles were normalized and only TRF's with a peak area ratio > 1 % of all TRF's were used for further analyses. TRF's were assigned to bacterial species using the virtual digest tool at the MiCA III Website (http://mica.ibest.uidaho.edu/trflp.php). Diversity of the TRF's profiles was assessed using the Shannon index (Kent & Coker, 1994): $H = -\sum_i P_i \log P_i$, where P_i denotes the relative peak area of the TRF_i.

Chemical analyses

Samples were analyzed for DM (method 967.03; AOAC, 1990) and crude protein contents (CP, method 981.10; AOAC, 1990). Starch and water-soluble β-glucan contents were analyzed colorimetrically after enzymatic hydrolysis according to standard procedures (Megazyme Ltd, Ireland).

The total (t-), soluble (s-) and insoluble (i-) NSP fractions were determined by gas chromatography (Varian Star 3400 GC) equipped with a 30 m fused silica capillary column and a gas flow rate of 36.15 cm/sec after the samples were hydrolyzed with 12 M H_2SO_4 (Englyst & Hudson, 1987). NDF (using heat stable α-amylase), ADF and ADL content of the raw ingredients were performed using F57 filter bag in an Ankom Fiber Analyzer (Ankom Technology Corp., Macedon, NY, USA).

Statistical analyses

In vitro dry matter digestibility (IVDMD) during pepsin and pancreatin hydrolysis, gas fermentation kinetics and TRF profiles and diversity indexes were analyzed by means of the MIXED procedure of the SAS 9.1 software (SAS Institute, 2004) and comparison of means performed using the LSMEANS statement with a general linear model using 2 criteria of classification (ingredient and Rovabio^{TM} addition) and their interaction. SCFA production and molar ratio were analyzed similarly. However, the model included 3 criteria of classification (ingredient, Rovabio^{TM} addition and sampling time), as well as the 2 and 3 ways interactions. For each grain cultivar, TRF profiles of Rovabio^{TM} treated and untreated grains were compared using a χ^2-test in Minitab 14 (Ryan et al., 2005).

To analyze the complex interactions between cereal types and varieties, carbohydrate fractions and the action of Rovabio^{TM} upon *in vitro* digestibility, fermentation kinetics parameters, SCFA profiles and relative abundance of bacterial groups, a multivariate analysis using the CANOCO statistical package

(version 4.5, Ter Braak & Šmilauer, 2002) was performed. The values for starch, β-glucan, ADF, NDF, cellulose, lignin, tNSP, sNSP and iNSP contents in the cereal varieties as well as the presence of Rovabio^{TM} (0 or 1) were imported as explanatory variables. Square root transformed values for abundance of TRF's, relative SCFA concentration, parameters of fermentation kinetics and IVDMD values were used as response variables. Explanatory and response data were used for direct gradient analysis (redundancy analysis, RDA). RDA is a constrained ordination analysis (canonical ordination), allowing the calculation of variables, describing the interaction of linear combinations of environmental factors and their effect on a set of response variables. Significance of the overall ordination model as well as the importance of explanatory variables in the forward selection procedure during development of the ordination model were tested using Monte Carlo permutation test (n=499).

Results

The addition of the Rovabio^{TM} enzymes increased IVDMD during the pepsin and pancreatin hydrolysis for all ingredients (Table 2, $P<0.001$). However, it did not increased the IVDMD of the diet, which explains the interaction ($P<0.001$) between the effect of Rovabio^{TM} and the ingredient. Similarly, the crude protein, starch and β-glucan digestibilities were increased with Rovabio^{TM} for all ingredients, except for the diet and for starch digestibility in wheat bran.

As a consequence, hydrolyzed ingredients showed different fermentation patterns whether they had been hydrolyzed in the presence of Rovabio^{TM} or not, except for the diet, as shown in Figures 1 and 2 and Table 2. With lower Rmax and higher B and tmax values, ingredients that had been hydrolyzed in the presence of enzymes yielded slower fermentation and less final gas production (A) ($P<0.05$).

SCFA production was generally decreased with Rovabio^{TM} (Table 3) even though in the case of SB94893, SCFA production after 12 h was higher with Rovabio^{TM} than without ($P<0.01$), whereas the trend was inverted after 72 h ($P<0.05$). Rovabio^{TM} increased the molar ratio of acetate and decreased propionate and branched-chain fatty acids (BCFA) of total SCFA, especially in the barley cultivars.

The addition of Rovabio^{TM} influenced the microbial communities after 72 h of fermentation, as indicated by the influence on TRF's 190, 281, 490, 516 and 551 ($P<0.05$) and the interaction between the ingredient and the enzyme on TRF's 218 and 490 ($P<0.05$) (Table 4). Shannon's index of diversity was significantly higher with the two hulless barley cultivars CDC Fibar and SB94893 as compared with the other ingredients except for AC Metcalfe and wheat bran after enzyme addition (Table 4).

Finally, the multivariate analysis yielded a significant ordination model (P = 0.002) showing that the highest variability in *in vitro* digestibility and fermentation parameters as well as in microbial communities was to be ascribed to the cereal type or cultivar. Indeed, the differing composition of these cereal types or cultivars were strongly correlated to the 2 major axes of the ordination model (Figure 3a), especially the β-glucan fraction, which is correlated to the first axis and explains 59.7% of the variability and the insoluble fibre fraction, correlated to the second axis and explaining 15.0% of the variability. A significant part of the variability (5.4%) was taken by the fourth axis, strongly correlated (-0.737) to the effect of Rovabio™ (Figure 3b).

Discussion

The *in vitro* model showed a positive influence of the Rovabio™ NSP-degrading enzymes on pepsin and pancreatin digestibility of DM, CP, starch and β-glucan in barley cultivars and wheat products simulating the gastric and small intestinal digestion in the pig. The addition of the enzymes during the pepsin and pancreatin hydrolysis also influenced the subsequent fermentation profiles when the residues where incubated with pig faeces. However, the positive action of Rovabio™ on nutrient digestibility, as well as on fermentation characteristics and microbial community profiles, seemed to differ according to the cereal source, especially when the two hulless barley cultivars (CDC Fibar and SB94893) were compared. These differences in the effect of the NSP-degrading enzymes are likely due to differences in the complex structure of the fibre matrix that embeds the carbohydrate fraction, including starch. As an example, differences in *in vitro* starch digestibilities between hulless barley cultivars (0.883 and 0.939 for CDC Fibar, without and with Rovabio™,

respectively, and 0.567 and 0.635 for SB94893, without and with Rovabio^{TM}, respectively) originate from different starch structures. More than 90 % of the starch in CDC Fibar is present as amylopectin compared to 46 % amylose found for SB94893 (Pieper et al., 2009). Such difference in starch digestibility according to the structure were also observed in corn by Bird et al. (2007) who recorded a starch digestibility > 96 % at the ileum of pigs that were fed 100 % amylopectin (waxy corn) but only 88 % of digestibility in animals that were fed a high amylose corn. Amylose has a more organised tertiary structure that reduces its solubility and digestibility, compared to amylopectin (Pierce & Stevenson, 2008). Conversely, the similar starch digestibility during pepsin and pancreatin hydrolysis with or without Rovabio^{TM} in wheat bran is probably due to the low starch content of wheat bran *per se* and the high *in vitro* digestibility without Rovabio^{TM} (approx. 98 %), which is consistent with previous observations (Bindelle et al., 2007b).

Interestingly, there was a high variability in β-glucan digestibility between the 4 barley varieties used. Mixed linked β-glucan is mainly located in the starchy endosperm of the grain where it makes up to 85 % of the cell wall polysaccharides (Izydorczyk & Dexter, 2008). Therefore, solubilisation of β-glucan during pepsin and pancreatin hydrolysis and the increase in digestibility due to the action of Rovabio^{TM} are likely linked (Fig 3a). High amylose grains such as SB94893 showed very limited β-glucan digestibility as compared to waxy, high amylopectin starches (CDC Fibar), whereas grains with a normal starch structure (approx. 75 % amylopectin and 25 % amylose) showed an intermediate β-glucan digestibility, which supports this hypothesis. The slower pancreatin hydrolysis of amylose thus could reduce the accessibility and/or efficiency of β-glucan hydrolysis by the exogenous NSP-degrading enzymes. Furthermore, the decreased fermentability of hydrolyzed grains

with addition of Rovabio^{TM} (Figure 1 & 2) can be ascribed to lower contents in highly fermentable substrates such as starch (average of 160 g/kgDM with enzymes vs. 191 without) and possibly β-glucan (average of 6.1 g/kgDM with enzymes vs. 6.9 without) in the hydrolyzed grains, as well as to the increase in insoluble-NSP.

The major influence of carbohydrate composition on fermentation profiles of barley and oat cultivars was already observed (Pieper et al., 2009). Except for the diet and SB94893, the enrichment in insoluble fiber induced slower (B and Rmax) and less extended (A) fermentation. Consistently with Diebold et al. (2004) using another xylanase source with wheat-base diets, more acetate and less propionate were produced in Rovabio^{TM} hydrolyzed grains and fibrolytic species were favoured by the enzymes as indicated by the increase in the profile of TRF 281 (Figure 3a). This fragment length was assigned to *Ruminococcus flavefasciens*-like or *Clostridium xylanolyticum*-like phylotypes, previously described to possess capabilities to degrade cellulose and xylans (Flint et al., 2008; Rogers & Baecker, 1991). The grain types also played a significant role in the changes in bacterial populations observed after the addition of Rovabio^{TM}. The influence of the NSP-ases was particularly high in CDC Fibar since the diversity in the composition of the bacteria population was significantly reduced by the enzymes, as measured through the Shannon index. The whole TRF profile for CDC Fibar tended (P<0.09) to change subsequently after Rovabio^{TM} addition, as indicated by the χ^2-test. Wheat fermenting microbial populations showed an increase in *Eubacterium halii*- and *E. limosum*-like phylotypes (TRF 218) and a strong decrease in *Clostridium*-like phylotypes, members of the *Clostridium* cluster I (TRF 516, Collins et al., 1994). This might be an interesting prospect for health concerns in terms of interaction between the prebiotic potential of wheat NSP and the addition of NSP-ases enzymes as this bacterial cluster also

contains several pathogenic clostridia. As revealed by the multivariate analysis (Figure 3a), TRF 516 was associated with soluble-NSP and β-glucan, CHO fractions that were decreased after Rovabio^{TM} addition, whereas TRF 218 was strongly influenced by the insoluble fiber content of the grain.

Consistent with the improved rates of substrate fermentation as depicted by gas production rates (Rmax), SB94893 with Rovabio^{TM} showed an unusual increase in SCFA production after 12 h of fermentation (333 mg/gDM without enzymes vs. 351 with enzymes, $P<0.01$), as compared to the other grain samples. This is a possible consequence of an increased accessibility to the amylose and β-glucan matrix of the endosperm, following the action of NSP-degrading enzymes during pepsin and pancreatin hydrolysis. After 24 h and 72 h, SB94893 behaved like the other grains, i.e. NSP-ases treated grains yielding less SCFA than untreated grains. Thus, for similar ingredients the transposition of the *in vitro* results *in vivo* is not obvious as the response to the enzymes will depend on the actual transit time in the upper gut. The loss of indigestible but fermentable carbohydrates during *in vitro* hydrolysis prior to fermentation through solubilisation could also appear as a limitation of the *in vitro* method. Nevertheless, these substrates are likely be fermented by the microbial population at very early stage *in vivo*, in the small intestine, without reaching the hindgut. Therefore, the filtration step after pepsin and pancreatin hydrolysis will simulate the fact that sugar moieties and soluble oligosaccharides will not be available for the fermentation in the large intestine. The consistency between *in vitro* Pieper et al (2009) and *in vivo* Bindelle et al (2010) in the ranking of barley varieties according to the SCFA profiles and their efficiency to prevent *Salmonella* colonization in the intestines of pigs support this assumption.

In addition to changes in carbohydrate structure, indigestible protein content decreased in all grain types and cultivars with the addition of Rovabio^{TM}. This increase in CP digestibility consecutive to enzyme addition was higher in barley compared to wheat and wheat bran. As a consequence, reduced protein fermentation metabolites (BCFA) were observed in the Rovabio^{TM} treated barleys.

The multivariate analysis performed here confirms *in vivo* findings (O'Connell et al., 2005) that in wheat and barley, cereal type had a more dramatic impact on digestibility, SCFA and bacterial population compared to xylanase and β-glucanase addition, even though the enzyme effect was quite consistent. This is evidenced by the fact that the fourth axis of the ordination model was correlated with Rovabio^{TM} (Figure 3b). This axis corroborates the positive influence of the enzymes on *in vitro* digestibility, especially the β-glucan fraction and the negative influence on the fermentation of the hydrolyzed cereals as measured through gas production kinetics (tmax and A) as well as the influence of the enzyme on *Butyrivibrio fibrisolvens* (TRF 32) and uncultured clones (TRF 490).

This study clearly implies that NSP-degrading enzymes can affect nutrient composition entering the large intestine and subsequently alter bacterial composition. However, the extent to which this relationship between the prebiotic potential of NSP in the grains and the addition of Rovabio^{TM} in the diets could result in positive outcomes beyond improved nutrient digestibility depends on grain type and cultivar, due to many interactions between NSP-ases on one hand, and starch, CP and NSP digestibility, as well as bacterial community, on the other hand.

Authors contribution

JB and RP contributed equally to this work.

Acknowledgements

We thank Adisseo (Commentry, France) for funding of the study. R. Pieper was funded by a post-doctoral research grant from the German Academic Exchange Service (DAAD, Germany). J. Bindelle was funded by the Fonds de la Recherche scientifique-FNRS (Brussels, Belgium). We are grateful to the technical staff of the Prairie Swine Centre and the Department of Animal and Poultry Science at the University of Saskatchewan for their assistance.

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Figure 1. Gas production curves during *in vitro* fermentation of the pepsin and pancreatin hydrolyzed complete diet and wheat products in the presence of NSP-degrading enzymes (Rovabio^{TM}) or not.

Figure 2. Gas production curves during *in vitro* fermentation of the pepsin and pancreatin hydrolyzed barley varieties in the presence of NSP-degrading enzymes (Rovabio^{TM}) or not.

Figure 3. RDA [(a) axis 1 & 2; (b) axis 3 & 4] of the influence of the composition of the wheat and barley products and cultivars and the presence of NSP-degrading enzymes (Rovabio^{TM}) during *in vitro* pepsin and pancreatin hydrolysis on *in vitro* digestion and fermentation parameters, SCFA production and molar ratio and dominant bacterial phylotypes (TRF's). Length and angle between arrows indicate the strength of the correlation between variables.

Figure 1.

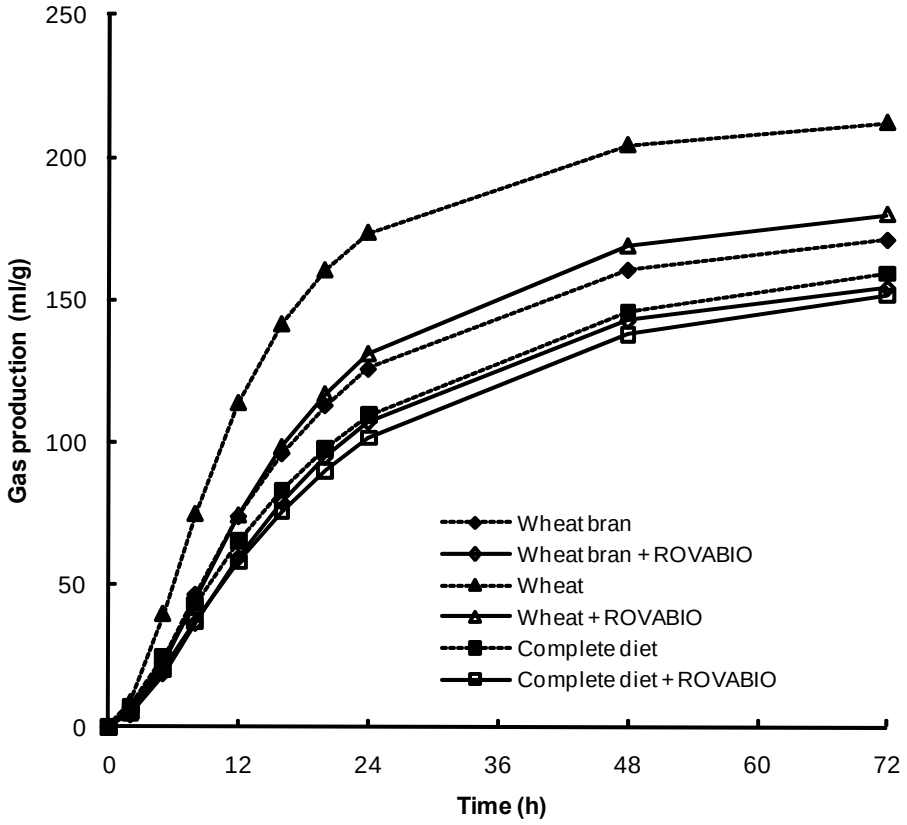
250
200
150
100
50
0
Gas production (ml/g)
0
12
24
36
48
60
72
Time (h)
Wheat bran
Wheat bran + ROVABIO
Wheat
Wheat + ROVABIO
Complete diet
Complete diet + ROVABIO

Figure 2.

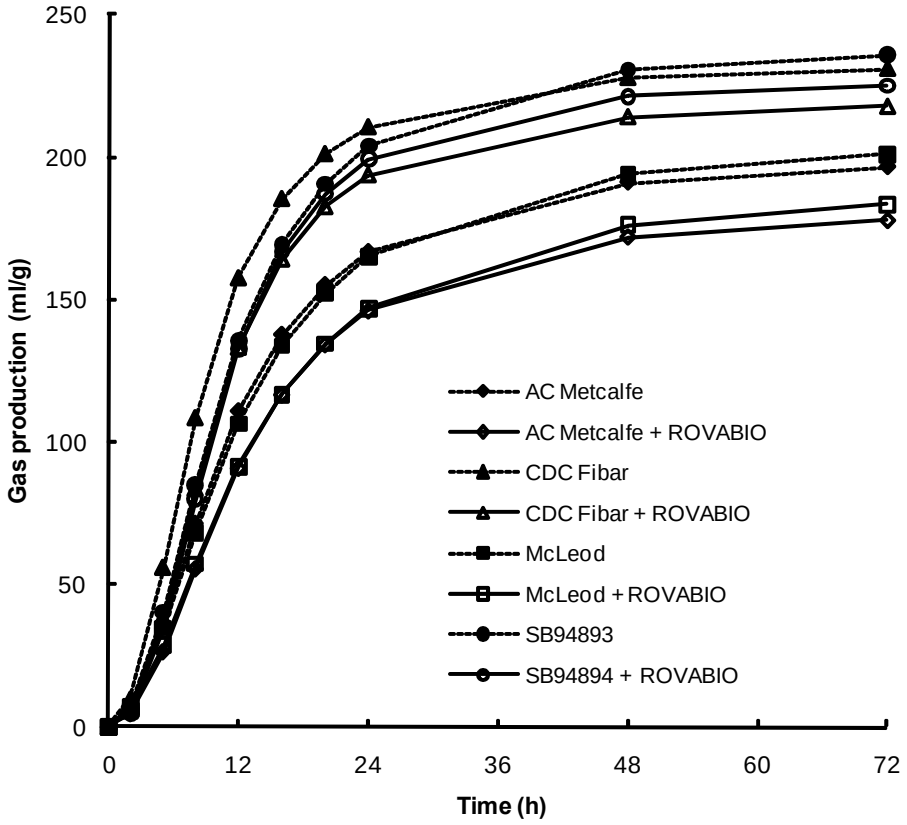
250
200
150
100
50
0
Gas production (ml/g)
0
12
24
36
48
60
72
Time (h)
AC Metcalfe
AC Metcalfe + ROVABIO
CDC Fibar
CDC Fibar + ROVABIO
McLeod
McLeod + ROVABIO
SB94893
SB94894 + ROVABIO

Figure 3a.

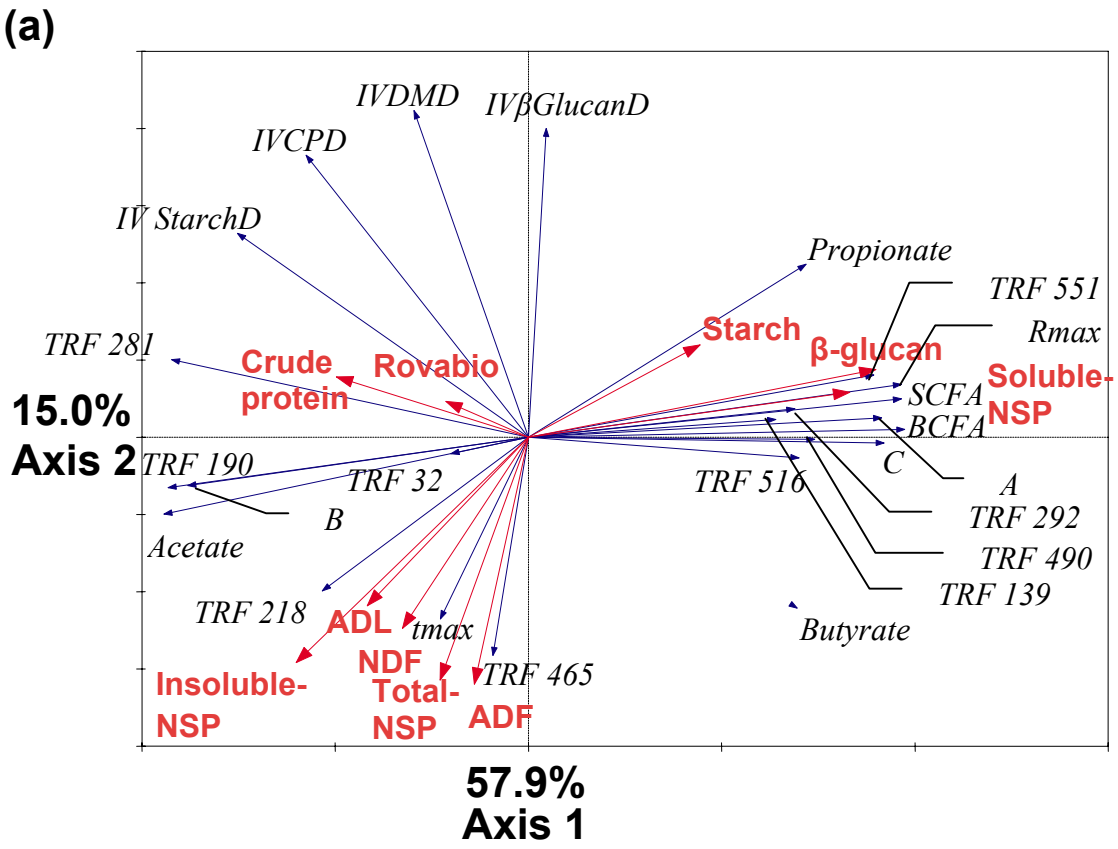
(a)
IVDMD
IVβGlucanD
IVCPD
IV StarchD
Propionate
TRF 551
Rmax
Starch
β-glucan
TRF 281
Crude protein
Rovabio
Soluble-NSP
SCFA
BCFA
15.0%
Axis 2
TRF 190
TRF 32
C
A
B
TRF 516
TRF 292
Acetate
TRF 490
TRF 139
TRF 218
ADL
tmax
Butyrate
NDF
Insoluble-NSP
Total-NSP
TRF 465
ADF
57.9%
Axis 1

Figure 3b.

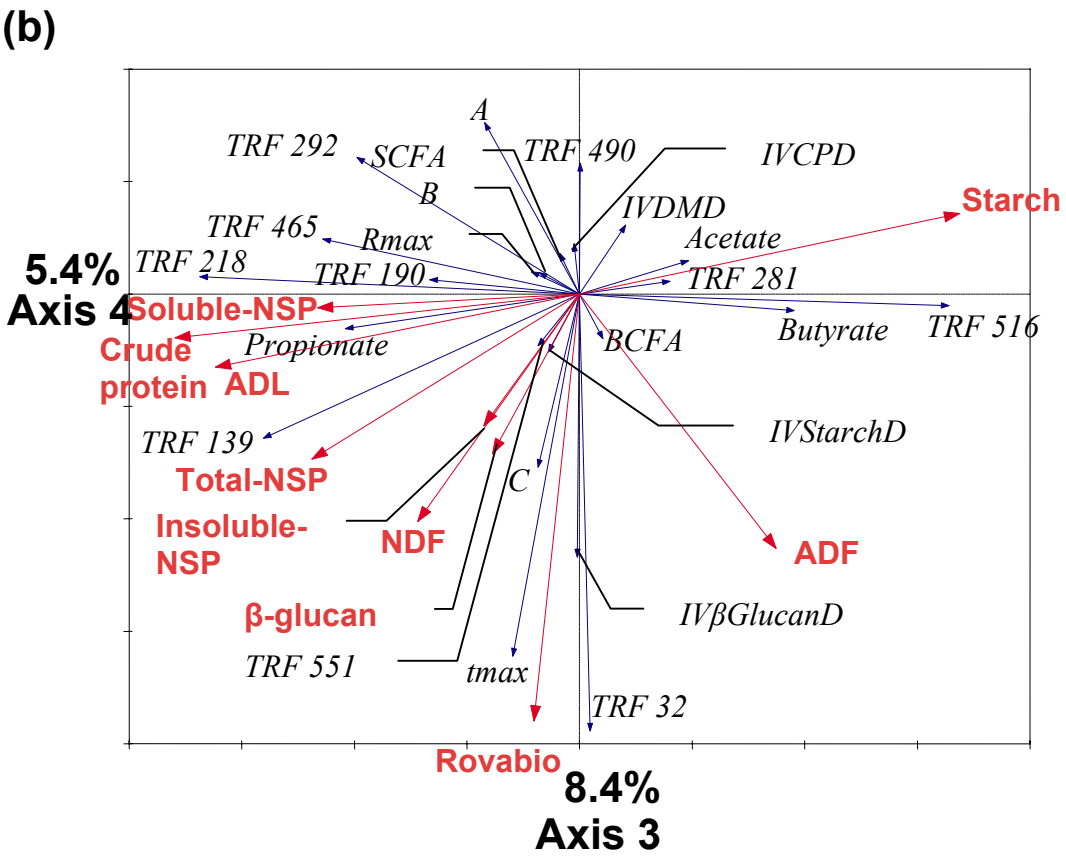
(b)
A
TRF 292
SCFA
TRF 490
IVCPD
B
IVDMD
Starch
TRF 465
Rmax
Acetate
5.4%
Axis 4
TRF 218
TRF 190
TRF 281
Soluble-NSP
Butyrate
TRF 516
Crude protein
Propionate
BCFA
ADL
IVStarchD
TRF 139
Total-NSP
C
Insoluble-NSP
NDF
ADF
β-glucan
IVβGlucanD
TRF 551
tmax
TRF 32
Rovabio
8.4%
Axis 3

Table 1. Chemical composition of the ingredients (g/kg).

Ingredient	**Gross energy**	**Crude protein**	**NDF**	**ADF**	**ADL**	**t-NSP**	**i-NSP**	**s-NSP**	**Starch**	**β-glucan**
	(MJ/kg)									
Diet	17.41	157.6	115.0	42.6	14.0	121.4	106.1	15.3	351.4	8.6
Wheat	16.94	146.1	122.6	32.9	19.0	133.7	91.5	42.2	486.0	6.6
Wheat bran	17.72	201.0	429.6	89.9	34.0	287.4	262.2	25.2	171.7	17.4
Common barley Mc Leod	17.05	115.0	187.0	80.6	11.9	146.1	110.3	35.8	506.6	40.0
Common barley AC Metcalfe	17.20	109.6	182.3	74.5	11.5	132.1	114.3	17.7	527.7	37.2
Hulless barley CDC Fibar	17.29	166.0	152.0	37.0	14.7	136.2	40.5	95.7	401.2	89.2
Hulless barley SB94893	17.60	115.4	153.9	57.6	16.5	177.9	85.0	92.8	424.9	70.3

Table 2. *In vitro* dry matter (IVDMD) (N=8), crude protein (IVCPD), starch (IVStarchD) and β-glucan (IVβGlucanD) digestibilities during pepsin and pancreatin hydrolysis and gas fermentation parameters (A, B, C, Rmax, Tmax) (N=3) modelled according to Groot et al. (1996) of the ingredients when NSP-degrading enzymes were added during pepsin and pancreatin hydrolysis (+) or not (-).

Enzyme	**Ingredient**	**IVDMD**	**IVCPD**	**IV StarchD**	**IVβ GlucanD**	**A** (ml/gDM)	**B** (h)	**C**	**Rmax** (ml/gDM×h)	**tmax** (h)
-	Diet	0.825 c	0.933	0.968	0.462	180 fg	17.7 ab	1.45 g	6.2 g	5.5 de
+	Diet	0.830 bc	0.935	0.973	0.537	171 gh	18.6 a	1.51 g	5.6 g	6.4 abcde
-	Wheat	0.839 bc	0.916	0.944	0.095	220 c	11.5 ed	1.80 ef	11.9 c	5.7 de
+	Wheat	0.890 a	0.936	0.989	0.354	192 ef	15.5 c	1.76 ef	7.7 f	7.5 ab
-	Wheat bran	0.567 j	0.784	0.985	0.112	183 f	15.1 c	1.69 f	7.5 f	6.7 abcd
+	Wheat bran	0.607 i	0.816	0.975	0.289	168 h	17.1 b	1.68 f	6.0 g	7.6 a
-	Common barley Mc Leod	0.664 h	0.795	0.820	0.242	207 d	11.7 ed	1.89 de	11.3 d	6.3 bcde
+	Common barley Mc Leod	0.715 f	0.845	0.870	0.475	190 ef	12.5 d	1.88 de	9.6 e	6.7 abcd
-	Common barley AC Metcalfe	0.697 g	0.794	0.860	0.228	201 de	10.8 ef	1.98 d	12.0 c	6.1 cde
+	Common barley AC Metcalfe	0.749 e	0.844	0.897	0.522	183 f	12.1 ed	2.01 cd	9.9 e	7.0 abc
-	Hulless barley CDC Fibar	0.770 d	0.850	0.883	0.836	233 ab	8.5 g	2.15 bc	18.4 a	5.3 e
+	Hulless barley CDC Fibar	0.846 b	0.889	0.939	0.896	220 c	9.9 f	2.26 ab	15.3 b	6.5 abcde
-	Hulless barley SB94893	0.512 k	0.673	0.567	0.064	240 a	10.6 ef	2.13 bc	15.2 b	6.5 abcde
+	Hulless barley SB94893	0.613 i	0.770	0.635	0.287	227 bc	10.4 ef	2.34 a	15.5 b	7.0 abc
	SEM	0.0115	-	-	-	3.64	0.490	0.0412	0.615	0.116
	Sources of variation	**P-values**								
	Ingredient	< 0.001	-	-	-	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001
	Enzyme	< 0.001	-	-	-	< 0.001	< 0.001	0.031	< 0.001	< 0.001
	Ingredient × Enzyme	< 0.001	-	-	-	0.128	< 0.001	0.078	< 0.001	0.219

a-k different letters within a column indicate significant (P<0.01) differences

Table 3. Short-chain fatty acid production (SCFA) (mg/gDM) and molar ratio (%) after 12, 24 and 72 h of fermentation of the ingredients when the NSP-degrading enzymes were added during the pepsin and pancreatin hydrolysis (+) or not (-) (N=3).

	Time	SCFA		P-	Acetate		P-	Propionate		P-	Butyrate		P-	BCFA[2]		P-
Ingredient	(h)	-	+	value[1]	-	+	value	-	+	value	-	+	value	-	+	value
Diet	12	164	154	NS	67.8	68.0	NS	22.4	21.7	*	9.1	9.5	NS	0.7	0.8	NS
Diet	24	204	203	NS	69.0	69.5	NS	21.5	20.8	*	9.6	9.8	NS	0	0	NS
Diet	72	317	310	NS	70.8	71.5	NS	19.1	18.6	*	9.0	9.0	NS	1.1	0.9	NS
Wheat	12	267	191	***	68.5	67.1	***	22.1	23.2	**	9.1	9.3	NS	0.3	0.4	NS
Wheat	24	345	264	***	69.2	68.1	***	20.4	21.4	***	10	10.1	NS	0.4	0.4	NS
Wheat	72	451	377	***	69.4	69.9	NS	18.9	18.9	NS	9.8	9.5	NS	1.9	1.7	***
Wheat bran	12	160	151	NS	66.4	65.8	*	24.1	23.6	NS	9.0	9.7	*	0.6	0.8	**
Wheat bran	24	215	205	NS	67.4	67.3	NS	22.2	21.9	NS	9.9	10.3	NS	0.5	0.5	NS
Wheat bran	72	318	320	NS	70.3	70.7	NS	19.5	19.0	*	8.9	9.0	NS	1.3	1.3	NS
Common barley Mc Leod	12	276	248	***	65.7	66.9	***	23.1	21.5	***	10.1	10.8	*	1.1	0.8	***
Common barley Mc Leod	24	348	305	**	65.7	67.3	***	21.2	20.2	***	12.2	11.9	NS	0.9	0.7	***
Common barley Mc Leod	72	452	416	***	67.0	69.2	***	19.6	18.6	***	11.1	10.4	***	2.3	1.9	***
Common barley AC Metcalfe	12	277	235	***	65.8	66.6	***	23.5	22.3	***	9.8	10.4	*	0.9	0.7	*
Common barley AC Metcalfe	24	324	298	NS	65.9	66.6	*	22	20.8	***	11.3	11.9	**	0.8	0.6	***
Common barley AC Metcalfe	72	426	394	***	67.0	68.0	**	20	18.8	***	10.6	11.1	*	2.4	2.2	*

Hulless barley CDC Fibar	12	404	335	***	60.6	62.4	***	31.7	28.8	***	6.6	7.9	***	1.2	1.0	**
Hulless barley CDC Fibar	24	478	425	***	61.1	62.6	***	29.1	26.4	***	8.6	9.9	***	1.2	1.0	***
Hulless barley CDC Fibar	72	554	498	***	60.9	62.6	***	27.2	24.7	***	9.1	10.0	***	2.9	2.8	NS
Hulless barley SB94893	12	333	351	**	64.8	65.8	***	25.6	23.9	***	8.1	9.0	**	1.5	1.3	*
Hulless barley SB94893	24	439	442	NS	62.8	65.1	***	22.7	21.7	***	13.1	11.7	***	1.4	1.5	*
Hulless barley SB94893	72	539	521	*	62.7	64.8	***	21.7	21.1	*	12.5	11.4	***	3.1	2.7	***
SEM		9.83			0.249			0.265			0.116			0.070		
Sources of variation																
Ingredient		<0.001			<0.001			<0.001			<0.001			<0.001		
Enzyme		<0.001			0.003			<0.001			0.019			0.630		
Time		<0.001			<0.001			<0.001			0.018			<0.001		
Ingredient × Enzyme		0.002			<0.001			<0.001			0.906			0.391		
Ingredient × Time		0.456			<0.001			0.059			<0.001			<0.001		
Time × Enzyme		0.942			<0.001			0.775			0.074			0.122		
Ingredient × Enzyme× Time		0.886			0.077			0.601			0.968			0.888		

[1] For one ingredient and one sampling time, significance of the least square means comparison of the influence of the addition of the enzyme on SCFA production and molar ratio (NS, not significant; *, P<0.05;**, P<0.01; ***, P<0.001).

[2] BCFA, branched-chain fatty acids.

Table 4. Terminal restriction fragment length polymorphism (TRFLP) profiles of the microbial communities after 72 h fermentation using a pig fecal inoculum of hydrolyzed ingredients when the NSP-degrading enzymes were added during the pepsin and pancreatin hydrolysis (+) or not (-) (N=3). Values indicate the relative contribution of each TRF to the total community.

Enzyme	Ingredient	TRF (bp)										Shannon index
		32[1]	139	190	218	281	292	465	490	516	551	
-	Diet	0.000 b	0.034 c	0.054 ab	0.047 cde	0.506 a	0.094 bc	0.043 b	0.047 bc	0.150 ef	0.025 ef	0.704 b
+	Diet	0.000 b	0.037 bc	0.067 a	0.062 bc	0.504 a	0.101 bc	0.058 b	0.020 c	0.132 fg	0.019 ef	0.702 b
-	Wheat	0.000 b	0.046 bc	0.034 bcd	0.034 def	0.415 bcd	0.150 ab	0.063 b	0.037 c	0.203 de	0.017 f	0.730 b
+	Wheat	0.000 b	0.047 bc	0.053 ab	0.067 b	0.495 ab	0.124 abc	0.068 b	0.021 c	0.109 fg	0.016 f	0.712 b
-	Wheat bran	0.000 b	0.057 bc	0.042 bc	0.094 a	0.466 abc	0.080 c	0.119 a	0.009 c	0.124 fg	0.010 f	0.680 b
+	Wheat bran	0.041a	0.046 bc	0.070 a	0.093 a	0.443 abc	0.100 bc	0.066 b	0.025 c	0.086 g	0.030 ef	0.786 ab
-	Common barley Mc Leod	0.000 b	0.041bc	0.009 e	0.006 g	0.389 cd	0.098 bc	0.053 b	0.032 c	0.315 a	0.058 cde	0.679 b
+	Common barley Mc Leod	0.009 b	0.037 bc	0.019 cde	0.018 fg	0.421 bcd	0.097 bc	0.051 b	0.039 c	0.273 abc	0.036 de	0.713 b
-	Common barley AC Metcalfe	0.000 b	0.043 bc	0.024 cde	0.021 fg	0.341 d	0.101 bc	0.052 b	0.050 b	0.296 ab	0.072 cd	0.761 ab
+	Common barley AC Metcalfe	0.030 ab	0.044 bc	0.030 bcde	0.025 fg	0.354 d	0.105 abc	0.056 b	0.034 c	0.283 ab	0.040 d	0.767 ab
-	Hulless barley CDC Fibar	0.000 b	0.057 bc	0.015 de	0.030 ef	0.220 e	0.139 abc	0.062 b	0.103 a	0.203 de	0.169 a	0.855 a
+	Hulless barley CDC Fibar	0.008 b	0.114 a	0.008 e	0.015 fg	0.342 d	0.138 abc	0.044 b	0.025 c	0.214 cd	0.092 bc	0.688 b
-	Hulless barley SB94893	0.000 b	0.074 abc	0.011 e	0.049 bcd	0.175 e	0.168 a	0.079 ab	0.079 b	0.248 bcd	0.116 b	0.862 a
+	Hulless barley SB94893	0.000 b	0.082 ab	0.025 cde	0.045 cde	0.207 e	0.144 abc	0.063 b	0.079 b	0.268 abc	0.087 bc	0.853 a
	SEM	3.62^{E-3}	4.95^{E-3}	3.67^{E-3}	4.41^{E-3}	1.79^{E-2}	6.46^{E-3}	4.56^{E-3}	5.00^{E-3}	1.24^{E-2}	7.63^{E-3}	1.35^{E-2}
	Sources of variation											
	Ingredient	0.652	0.028	< 0.001	< 0.001	< 0.001	0.041	0.143	0.001	< 0.001	< 0.001	0.008
	Enzyme	0.105	0.372	0.012	0.090	0.027	0.789	0.299	0.032	0.040	0.009	0.745
	Ingredient × Enzyme	0.652	0.508	0.508	0.035	0.218	0.950	0.480	0.033	0.199	0.054	0.010

[1] most likely species identity TRF 32 - *Butyrivibrio fibrisolvens*, TRF 139 - *Clostridium innocuum*, TRF 190 - *Clostridium aminovorans, C. bogorii*, TRF 218 - *Eubacterium hallii, E. limosum*, TRF 281 - *Ruminococcus flavefaciens*, *Clostridium xylanolyticum* TRF 292 - *Clostridium ramosum*, TRF 465 - uncultured, TRF 490 - uncultured, TRF 516 - *Clostridium butyricum, C.botulinum, C.cellulovorans, C.tyrobutyricum, C.acetobutylicum*, TRF 551 – *Streptococcus*, *Leuconostoc*