RESEARCH ARTICLE

Nonstarch 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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Abstract

An *in vitro* experiment was carried out to assess how nonstarch polysaccharide (NSP)-degrading enzymes influence the fermentation of dietary fiber in the pig large intestine. Seven wheat and barley products and cultivars with differing carbohydrate fractions were hydrolyzed using pepsin and pancreatin in the presence or not of NSP-degrading enzymes (xylanase and β-glucanase) and the filter retentate was subsequently fermented with sow fecal bacteria. 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. The results showed an increase of nutrient digestibility ($P < 0.001$), whereas fermentability and SCFA production decreased ($P < 0.001$) with addition of the enzyme. SCFA and bacterial community profiles also indicated a shift from propionate to acetate and an increase in cellulolytic *Ruminococcus*- and xylanolytic *Clostridium*-like bacteria. This is explained by the increase in slowly fermentable insoluble carbohydrate and the lower proportion of rapidly fermentable β-glucan and starch in the retentate when grains were incubated with NSP-degrading enzymes. Shifts were also different for the four barley varieties investigated, showing that the efficiency of the enzymes depends on the structure of the carbohydrate fractions in cereal products and cultivars.

Introduction

Indigestible carbohydrates such as nonstarch polysaccharides (NSP) and resistant starch are considered as antinutritional factors for pigs because 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 the 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

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species and type to the other, but also between cultivars of the same grain species and type (Izydorczyk & Dexter, 2008). Indigestible carbohydrate 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 a specific carbohydrate composition in the basal diet. For example, using the variability in the 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 carbohydrate in the intestines might favor intestinal health. For example, butyrate, as a major energy source for the epithelial cells, has been shown to improve the digestive and absorptive capacities of the small intestine in pigs (Bindelle *et al.*, 2008). Indigestible carbohydrate fermentation also increases the 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 a 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 affects the concentration of 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 the addition of a multienzyme 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 the addition of NSP-ases enzymes in the diet disrupts the fiber matrix embedding digestible carbohydrate, 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 carbohydrate for fermentation by intestinal bacteria is increased. Even if depolymerization 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.*, 2011), this increased access can favor small intestinal growth of lactobacilli. Conversely, Rosin *et al.* (2007) observed reduced *Escherichia coli* abundance in cecums 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 carbohydrate 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 carbohydrate fraction of wheat products and barley cultivars and the consequences on intestinal microbial communities, using an *in vitro* model of the porcine gastrointestinal tract.

Materials and methods

Ingredients

The Rovabio^{TM} Excel AP enzyme mixture used in this study was provided by Adisseo (Commentry, France). The enzyme is a complex of NSP-ases (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, two hulless barleys cultivars with a very high content of β-glucan (CDC Fibar and SB94893) and two hulled barley cultivars with a higher content of xylans in their hulls (AC Metcalfe and McLeod). A diet used

Table 1. Chemical composition of the ingredients (g kg^{-1})

Ingredient	Gross energy (MJ kg^{-1})	Crude protein	NDF	ADF	ADL	t-NSP	i-NSP	s-NSP	Starch	β-Glucan
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

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 six 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 hydrolyzed with porcine pepsin (pH 2, 39 °C, 2 h) and porcine pancreatin (pH 6.8, 39 °C, 4 h). Residues were filtered through a 42-μm Nylon cloth, washed twice with 96% ethanol and 99.5% acetone and dried at 60 °C overnight. *In vitro* dry matter digestibility (IVDMD) during hydrolysis was recorded. For each ingredient, two enzymes' addition patterns were implemented: hydrolysis was performed without or with the Rovabio™ enzyme mixture (0.5 mg g^{-1} ingredient) added during the pepsin digestion step. The experimental scheme was as follows: seven ingredients × two enzyme addition patterns × two replicates × four periods.

The residues were incubated in an inoculum prepared from fresh feces of three 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 SCFA are produced by fecal microorganisms. SCFA are buffered by the carbonate ions of the buffer solution to release CO_2. Therefore, in this model, the total gas production reflects the major end products (gases plus SCFA) of microbial fermentation of carbohydrate and is inversely related to substrate disappearance. The released gas volumes (fermentation and buffered gas) were regularly recorded over 72 h of incubation by measuring bottles' inner pressure in order to measure how fast the different ingredients were fermented by the microorganisms (Mauricio *et al.*, 1999). The experimental scheme was as follows: seven ingredients × two enzyme addition patterns × three replicates + three blanks (containing only inoculum).

After 72 h, the fermentation broth was centrifuged (12 000 ***g*** for 5 min) and the supernatant was removed for analysis of SCFA. The pellet was further used for the 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 g^{-1} DM) denotes the gas accumulation to time, A (mL g^{-1} DM) the maximum gas volume for $t = \infty$, B (h) the time to half asymptote when $G = A/2$ and C is a constant determining the slope of the inflexion point of the profile. From the equation, two additional parameters are calculated: R_{max}, the maximum rate of gas production (mL g^{-1} DM × h), when the microbial population no longer limits the fermentation, and t_{max}, the time at which R_{max} is reached.

Measurement of SCFA production

For each sample, 100 μL of supernatant was mixed in a 1.7-mL Eppendorf tube with 20 μL of a 25% metaphosphoric acid solution to adjust the pH to approximately 2, with 500 μL of an 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 was transferred into a GC vial. Chromatographic analysis was carried out on an Agilent 6890 GC system equipped with a flame ionization detector, a 7683 series liquid injector and an autosampler (Agilent, Germany). Samples were run on a fused-silica capillary column (ZB-FFAP, Phenomenex) with 30 m × 320 μm × 0.25 μm nominal using He as a carrier gas at a flow rate of 1.9 mL min^{-1}. The flow rate of hydrogen and air was 35 and 350 mL min^{-1}, respectively. Electronic pneumatics control was run in the split mode with a split ratio of 100 : 1 at 11.10 psi pressure and a total flow of 191 mL min^{-1}. The temperature program was set as follows: initial temperature, 100 °C for 1 min, ramp with 8 °C min^{-1} and final temperature 200 °C for 13.5 min (Agilent).

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) and centrifuged. The 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 liter: 200 mg RNase, 50 mM Tris-HCl, 50 mM 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^{-1}) and 20 μL proteinase K (20 mg L^{-1}), in order to lyse the bacterial cells. After the addition of 135 μL of lysing buffer B (containing per liter: 3 M 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) 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 3 M 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, redissolved 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 PCR using the universal forward primer S-D-Bact-0008-a-S-20 (AGA GTT TGA TCM TGG CTC AG), labelled with 6-carboxyfluorescein and the reverse primer R3 (TCT ACG CAT TTC AC) (Dorsch & Stackebrandt, 1992). PCR reactions contained 5 μL of 10 × incubation buffer, 1.5 μL of 50 mM $MgCl_2$, 1.5 μL of each primer (10 μM), 1.5 μL of each dNTP (10 mM) and 0.2 μL of Taq-Polymerase (5 U μL^{-1}) and UV-sterilized Millipore water, added until 50 μL. PCR was performed in a Thermolyne Amplitron II temperature cycler (Barnstead/Thermolyne, Dubuque, IA) and the program was 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, and a final extension at 72 °C for 10 min. The size and yield of the PCR products were checked by electrophoresis in a 1.5% agarose gel after staining with ethidium bromide (0.5 μg EtBr mL^{-1} agarose). The PCR product was subsequently extracted from the gel using the Qiagen® PCR Purification Kit (Qiagen, Mississauga, ON, Canada) according to the manufacturer's protocol and the DNA concentration was measured on a NanoDrop® ND-1000 Spectrophotometer (NanoDrop Technologies Inc., Wilmington).

For terminal restriction fragment length polymorphism 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 2 μL reaction buffer and UV-sterilized Millipore water, made up to 20 μL. Two microliters of the digestion solution was 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 the gene scan mode and GENEMAPPER v3.7 software (Applied Biosystems Inc., Foster City, CA). Fragments that were different in $< \pm 3$ bp were considered to be identical.

Profiles were normalized and only terminal restriction fragments (TRFs) with a peak area ratio $> 1\%$ of all TRFs were used for further analyses. TRFs were assigned to bacterial species using the virtual digest tool at the MiCA III website (http://mica.ibest.uidaho.edu/trflp.php). The diversity of the TRFs 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 GC (Varian Star 3400 GC) equipped with a 30 m fused-silica capillary column and a gas flow rate of 36.15 cm s^{-1} after the samples were hydrolyzed with 12 M H_2SO_4 (Englyst & Hudson, 1987). Neutral detergent fibre (NDF) (using heat-stable α-amylase), acid detergent fibre (ADF) and acid detergent lignin (ADL) contents of the raw ingredients were assessed using F57 filter bag in an Ankom Fiber Analyzer (Ankom Technology Corp., Macedon, NY).

Statistical analyses

IVDMD during pepsin and pancreatin hydrolysis, gas fermentation kinetics and TRF profiles and diversity indexes were analyzed using the MIXED procedure of the SAS 9.1 software (SAS Institute, 2004) and comparison of means was performed using the LSMEANS statement with a general linear model using two criteria of classification (ingredient and Rovabio™ addition) and their interaction. SCFA production and molar ratio were analyzed similarly. However, the model included three criteria of classification (ingredient, Rovabio™ addition and sampling time), as well as the two- and three-way interactions. For each grain cultivar, TRF profiles of Rovabio™ 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™ 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, t-NSP, s-NSP and i-NSP contents in the

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, R_{max}, T_{max}) ($N = 3$) 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	IVStarchD	IVβGlucanD	A (mL g^{-1} DM)	B (h)	C	R_{max} (mL g^{-1} DM × h)	t_{max} (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									
	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

Different letters within a column indicate significant ($P < 0.01$) differences.

cereal varieties as well as the presence of Rovabio^{TM} (0 or 1) were imported as explanatory variables. Square root-transformed values for the abundance of TRFs, 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. The significance of the overall ordination model as well as the importance of explanatory variables in the forward selection procedure during the development of the ordination model were tested using the 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 increase 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 Figs 1 and 2 and Table 2. With lower R_{max} and higher B and t_{max} values, ingredients that had been hydrolyzed in the presence of enzymes yielded slower fermentation and less final gas production (A) ($P < 0.05$).

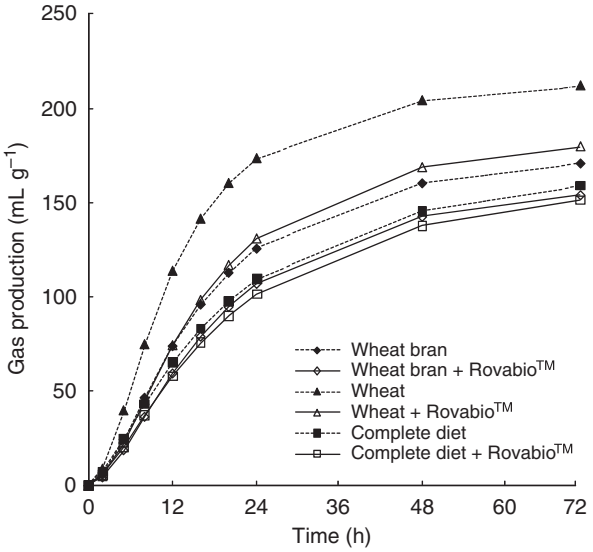

Fig. 1. Gas production curves during *in vitro* fermentation of the pepsin and pancreatin hydrolyzed complete diet and wheat products in the presence of NSP-degrading enzymes (Rovabio^{TM}) or not.

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

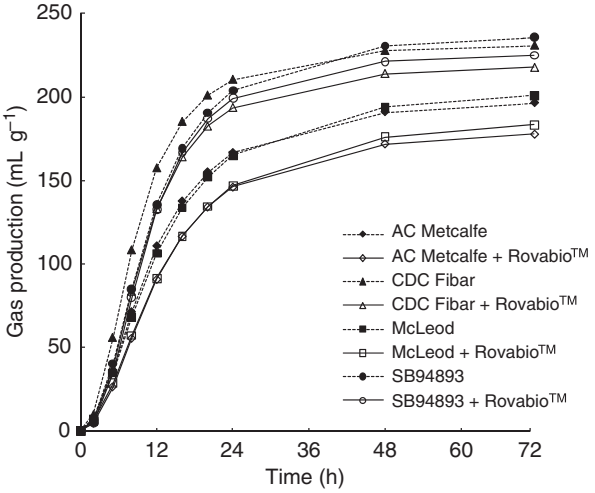

Fig. 2. Gas production curves during *in vitro* fermentation of the pepsin and pancreatin hydrolyzed barley varieties in the presence of NSP-degrading enzymes (Rovabio^{TM}) or not.

and decreased propionate and branched-chain fatty acids (BCFA) of the 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 TRFs 190, 281, 490, 516 and 551 ($P < 0.05$) and the interaction between the ingredient and the enzyme on TRFs 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 could be ascribed to the cereal type or cultivar. Indeed, the differing compositions of these cereal types or cultivars were strongly correlated to the two major axes of the ordination model (Fig. 3a), especially the β-glucan fraction, which is correlated to the first axis, and explains 59.7% of the variability and the insoluble fiber 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^{TM} (Fig. 3b).

Discussion

The *in vitro* model showed a positive influence of the Rovabio^{TM} 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 were incubated with pig feces. However, the positive action of Rovabio^{TM} 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 fiber 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^{TM}, 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 with 46% amylose found for SB94893 (Pieper *et al.*, 2009). This 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 organized tertiary structure that reduces its solubility and digestibility, compared with 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} (approximately 98%), which is consistent with previous observations (Bindelle *et al.*, 2007b).

Interestingly, there was a high variability in β-glucan digestibility between the four 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, solubilization 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 with waxy, high-amylopectin starches (CDC Fibar), whereas grains with a normal starch structure (approximately 75% amylopectin and 25% amylose) showed an intermediate β-glucan digestibility, which supports this hypothesis. The slower pancreatin hydrolysis of amylose could thus reduce the accessibility and/or the efficiency of β-glucan hydrolysis by the exogenous NSP-degrading enzymes. Furthermore, the decreased fermentability of hydrolyzed grains with the addition of Rovabio^{TM} (Figs 1 and 2) can be ascribed to lower contents in highly fermentable substrates such as starch (average of

Table 3. SCFA production (mg g^{-1} DM) 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 (−) ($N = 3$)

		SCFA			Acetate value†			Propionate			Butyrate			BCFA		
Ingredient	Time (h)	−	+	*P*-value	−	+	*P*-value	−	+	*P*-value	−	+	*P*-value	−	+	*P*-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		

†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; *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$).

160 g kg^{-1} DM with enzymes vs. 191 g kg^{-1} DM without) and possibly β-glucan (average of 6.1 g kg^{-1} DM with enzymes vs. 6.9 g kg^{-1} DM without) in the hydrolyzed grains, as well as to the increase in insoluble NSP.

The major influence of carbohydrate composition on the 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 R_{max}) and less extended (A) fermentation. Consistent with Diebold *et al.* (2004), using another xylanase source with wheat-base diets, more acetate and less propionate were produced in Rovabio™ hydrolyzed grains and fibrolytic species were favored by the enzymes as indicated by the increase in the profile of TRF 281 (Fig. 3a). This fragment length was assigned to *Ruminococcus flavefaciens*-like or *Clostridium xylanolyticum*-like phylotypes, described previously to possess capabilities to degrade cellulose and xylans (Rogers & Baecker, 1991; Flint *et al.*, 2008). The grain types also played a significant role in the changes in the bacterial populations observed after the addition of Rovabio™. The influence of the NSP-ases was particularly high in CDC Fibar because the diversity in the composition of the bacteria population was significantly reduced by the enzymes, as measured through the Shannon index. The entire TRF profile for CDC Fibar tended ($P < 0.09$) to change subsequently after Rovabio™ addition, as indicated by the χ^2-test. Wheat-fermenting microbial populations showed an increase in *Eubacterium hallii*- and *Eubacterium 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 the 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 (Fig. 3a), TRF 516 was associated with soluble-NSP and β-glucan, carbohydrate fractions that were decreased after Rovabio™ 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 (R_{max}), SB94893

Table 4. Terminal restriction fragment length polymorphism 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 (−) ($N = 3$)

		TRF (bp)										
Enzyme	Ingredient	32*	139	190	218	281	292	465	490	516	551	Shannon index
−	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	3.62E − 3	4.95E − 3	3.67E − 3	4.41E − 3	1.79E − 2	6.46E − 3	4.56E − 3	5.00E − 3	1.24E − 2	7.63E − 3	1.35E − 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

Values indicate the relative contribution of each TRF to the total community.

Different letters within a column indicate significant ($P < 0.05$) differences.

*Most likely species identity TRF 32, *Butyrivibrio fibrisolvens*; TRF 139, *Clostridium innocuum*; TRF 190, *Clostridium aminovorans*, *Clostridium bogorii*; TRF 218, *Eubacterium hallii*, *Eubacterium limosum*; TRF 281, *Ruminococcus flavefaciens*, *Clostridium xylanolyticum*; TRF 292, *Clostridium ramosum*; TRF 465, uncultured; TRF 490, uncultured; TRF 516, *Clostridium butyricum*, *Clostridium botulinum*, *Clostridium cellulovorans*, *Clostridium tyrobutyricum*, *Clostridium acetobutylicum*; TRF 551, *Streptococcus*, *Leuconostoc*.

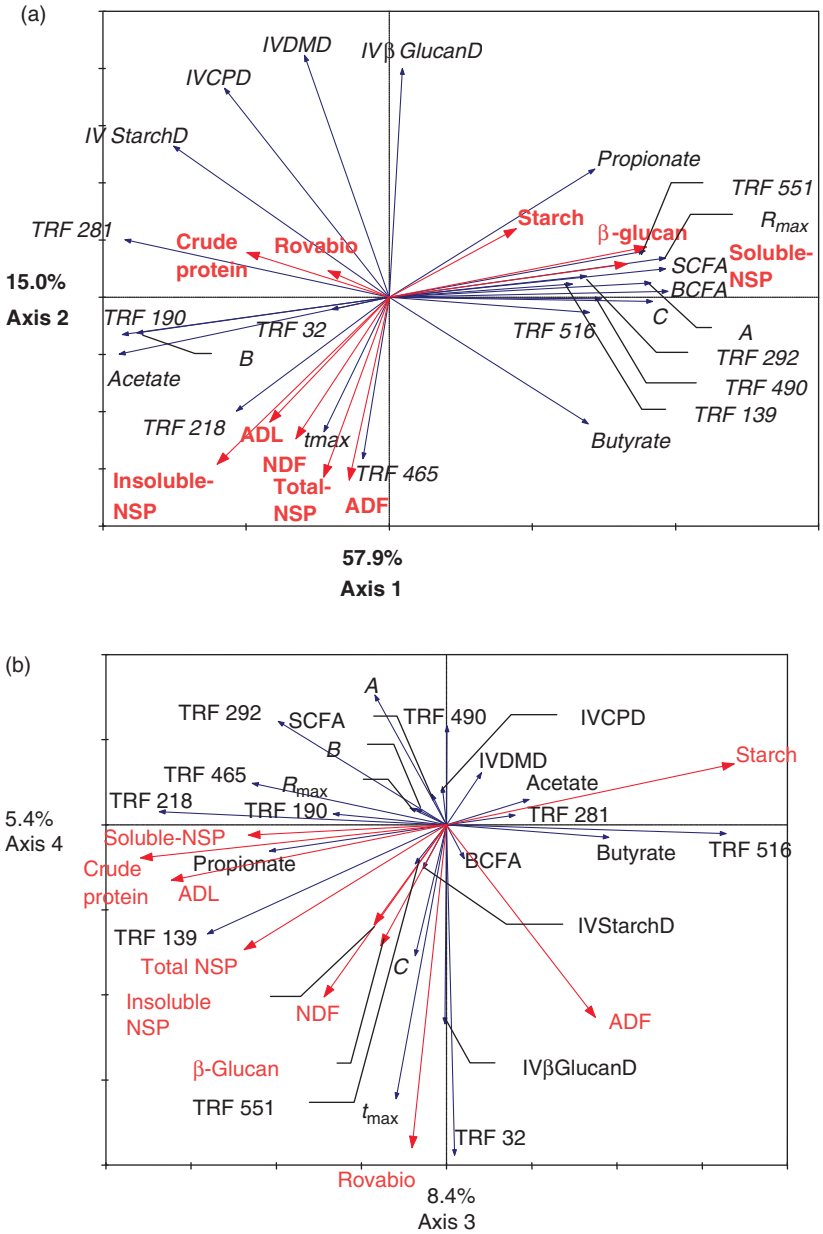

Fig. 3. RDA [(a) axis 1 and 2; (b) axis 3 and 4] of the influence of the composition of the wheat and barley products and cultivars and the presence of NSP-degrading enzymes (Rovabio^{TM}) during *in vitro* pepsin and pancreatin hydrolysis on *in vitro* digestion and fermentation parameters, SCFA production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

with Rovabio^{TM} showed an unusual increase in SCFA production after 12 h of fermentation (333 mg g^{-1} DM without enzymes vs. 351 mg g^{-1} DM with enzymes, $P < 0.01$), as compared with 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 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 before fermentation through solubilization 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 in preventing *Salmonella* colonization in the intestines of pigs support this assumption.

In addition to changes in carbohydrate structure, the indigestible protein content decreased in all grain types and cultivars with the addition of Rovabio™. This increase in CP digestibility consecutive to enzyme addition was higher in barley compared with wheat and wheat bran. As a consequence, reduced protein fermentation metabolites (BCFA) were observed in the Rovabio™ 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 drastic impact on digestibility, SCFA and bacterial population compared with 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™ (Fig. 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 (t_{max} 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 the nutrient composition entering the large intestine and subsequently alter the bacterial composition. However, the extent to which this relationship between the prebiotic potential of NSP in the grains and the addition of Rovabio™ 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 the one hand, and starch, CP and NSP digestibility, as well as bacterial community, on the other.

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Authors' contribution

J.B. and R.P. contributed equally to this work.

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