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Combined *endo*-β-1,4-xylanase and α-L-arabinofuranosidase increases butyrate concentration during broiler cecal fermentation of maize glucurono-arabinoxylan

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ABSTRACT

Solubilisation of prebiotic arabinoxylooligosaccharides from complex arabinoxylans in *e.g.* maize by xylanases may be increased by addition of auxiliary debranching enzymes. In this study, the hydrolysis and fermentation of maize fibre was investigated *in vitro* using a xylanase and an α-L-arabinofuranosidase. Combining the enzymes induced a higher ($P < .001$) xylose solubilisation and higher ($P < .05$) butyrate production during *in vitro* fermentation of maize fibre with cecal broiler inoculum compared to applying enzymes separately after 48 h. Subsequently, fibre degradation and fermentation was investigated in ROSS 308 broiler chickens supplemented with the enzyme combination to test the effects on gut morphology and microbiota composition along with performance. However, to address the relevance of combining the enzymes *in vivo*, further full factorial studies using individual enzymes at lower dosages are needed. Birds were fed a maize/soy based diet with 100 g/kg maize DDGS and 50 g/kg rapeseed meal. Enzymes supplementation increased ($P < .001$) body weight (+5.4%) and improved ($P < .001$) feed conversion ratio (−5.8%) after 29 days compared to control birds. Non-starch polysaccharide analysis and confocal microscopy of jejunum digesta visualised and confirmed solubilisation of the insoluble maize (glucurono)arabinoxylan. Birds receiving enzyme supplementation had increased ($P < .001$) duodenum villi length (+120 μm) and reduced ($P < .002$) CD3 T-cell infiltration (−22.1%) after 29 days. Cecal butyrate levels were increased ($P < .05$) compared to controls. Although the microbiota composition was not significantly altered, numerical increases in cecal *Ruminococcaceae* and *Lachnospiraceae* genera were observed in birds supplemented with enzymes.

1. Introduction

Degradation and solubilisation of non-starch polysaccharides (NSP) by exogenous enzymes increases available substrates for microbial fermentation in the cecum (Cadogan and Choct, 2015) and thereby the total production of short chain fatty acids (SCFA). SCFA may be used as a direct energy source by broilers (Choct et al., 1996; Steenfeldt et al., 1998) and therefore the NSP fraction

Abbreviations: araF, arabinofuranosidase; AX, arabinoxylan; AXOS, arabinoxylo-oligosaccharide; CD3, cluster of differentiation 3; CLSM, confocal laser scanning microscopy; DDGS, dried distiller's grains with soluble; EP, enzyme protein; GAX, (glucurono)arabinoxylan; GH, glycoside hydrolase; NSP, non-starch polysaccharide; RI, refractive index; SCFA, short chain fatty acids; xyl, xylanase

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represents another potential energy reservoir to increase performance of broilers if rendered fermentable.

The NSP content in maize is around 90 g/kg of the dry matter (DM) on average (Knudsen, 2014). Approximately 64% of the NSP in maize kernels is (glucurono)arabinoxylan (GAX). Only 2% of this GAX is water-soluble (Choct, 1997). Xylanases are well-known for their ability to degrade arabinoxylan (AX) from wheat (Courtin and Delcour, 2001). Maize GAX however, has a higher degree of substitution compared to wheat AX (Knudsen, 1997). The insolubility and complexity of maize GAX structures affects susceptibility to microbial fermentation or hydrolysis by xylanases (Malunga and Beta, 2016). Maize GAX indeed is a heterogenous and highly branched structure heavily decorated with arabinose and several other substituents such as galactose, ferulic acid (Bunzel, 2010) and glucuronic acid. In addition, some of these substituents are often acylated or methylated (Agger et al., 2010). The branched structure sterically impedes xylanases from *e.g.* family 11 and 10 glycoside hydrolases (GH) to bind and cleave the β-1,4-linked xylose backbone (Akin, 2008; Ravn et al., 2016). In general, GH10 xylanases have a higher preference to degrade soluble GAX than GH11 xylanases (Pell et al., 2004; Ravn et al., 2016). Addition of auxiliary de-branching enzymes may increase the solubilisation capacity of xylanases by removing substituents present on the xylan chain, *e.g.* removal of arabinose by arabinofuranosidases (Bachmann and McCarthy, 1991), acetyl groups by acetyl xylan esterases (Selig et al., 2009) or ferulic acid by ferulic acid esterases (Topakas et al., 2005).

Arabinoxylooligosaccharides (AXOS) with a degree of polymerization < 10 are recognized as prebiotic compounds, which pass undigested through the small intestine (Broekaert et al., 2011). They are fermented in the large intestine and promote growth of beneficial bacteria (Gibson and Roberfroid, 1995), such as butyrate-producing bacteria (De Maesschalck et al., 2015; Ravn et al., 2017). AXOS generated from wheat bran AX by *endo*-1,4-β-xylanases improve performance in broilers (Courtin et al., 2008). Soluble AXOS are fermented in the hindgut of broilers into SCFA, *e.g.* acetate, propionate and butyrate (den Besten et al., 2013). Butyrate is the preferred energy source of intestinal colonocytes (Hamer et al., 2008) and the beneficial effects on intestinal gut health and morphology are well described across species (Place et al., 2005; Onrust et al., 2015). To achieve a positive intestinal biological effect of solubilised AXOS, it is necessary that the xylanases are *endo*-acting, cleaving the polymer mid-chain rather than an *exo*-acting enzyme whose action may result in high amounts of monomeric xylose which, at high level, is detrimental to broiler performance (Schutte, 1990). It is therefore necessary to apply enzymes with a suitable substrate activity, efficacy, thermo- and pH tolerance and gastric stability in order to solubilise prebiotic AXOS in the gut. One possible route to increase soluble AXOS from cereal plant cell walls is the addition of xylanases (Courtin et al., 2008; Ravn et al., 2017) along with debranching enzymes. In GAX the most common substituent is arabinose. Removing these arabinoses with arabinofuranosidases may increase accessibility for cleavage by xylanases (Biely, 2012a, 2012b).

The purpose of the present study was to investigate the effect of a combined endoxylanase and arabinofuranosidase enzyme mixture on maize glucurono-arabinoxylan breakdown and cecal microbial fermentation. The hypothesis was that the enzymes could increase butyrate production during cecal fermentation.

The enzyme combination with highest hydrolytic activity and butyrate production was then tested *in vivo* using a fibre-rich diet to support the *in vitro* data.

2. Materials and methods

2.1. Materials

Maize fibre containing 364 g/kg crude fibre, 25.5 g/kg starch, 375 g/kg protein, 98 g/kg fat and 46 g/kg ash was obtained by double de-starching and de-proteinization. A total of 100 kg milled maize grits (Semolina 011, de-germed and milled) were supplied from a local feed mill (Institute of Technology Sdr. Stenderup, Denmark) was incubated in 250 kg tap water heated to 95 °C, pH 6.2 (achieved by addition of 1 M NaOH) with 1 kg Termamyl 120 L (Novozymes, Denmark) for 4 h followed by centrifugation (5300 rpm) and particle separation by decanting. Another de-starching step was performed under same conditions and subsequently, the fibre residue was re-dissolved in 150 kg tap water, heated to 55 °C, pH 8.1 and incubated with 80 g 2.4 L FG Alcalase (Protease, Novozymes, Denmark) for 4 h and then centrifuged (5300 rpm), decanted and freeze dried yielding 2.4 kg of a fibre-rich product. The maize fibre contained 110.5 g/kg xylose. Purified mono component *endo*-β-1,4- xylanase (EC 3.2.1.8): xyl (GH11) was obtained from *B. stearothermophilus* and expressed in *B. subtilis.* Purified mono component α-L-arabinofuranosidase (EC.3.2.1.55): araF (GH62) was obtained from *Penicillium oxalicum* and expressed in *A. Oryzae*. The *endo*-β-1,4- xylanase and the α-L-arabinofuranosidase will henceforth be termed 'xyl' and 'araF'. Both enzymes were supplied from Novozymes, Denmark and are stable under gastrointestinal conditions in the stomach and small intestine (data not shown).

2.2. Activity assay

Xyl activity was tested in a xylan solubilisation assay used to select the best combination of enzymes to test *in vivo*. Maize fibre (400 mg) substrate (n = 4) was incubated with xyl (10 mg EP/kg) with and without araF (5 mg EP/kg). EP = enzyme protein as previously described by Ravn et al. (2016) for 3 h at 40 °C with stirring (500 rpm) in 4 mL sodium acetate (NaOAc) buffer. The xyl dosage can approximately be compared to 10 x commercials dosage and 5 x for the araF. Solubilised AX oligomers and polymers released into the supernatant were hydrolysed to monomers using 1.63 M HCl at 99 °C for 1 h and subsequently cooled and neutralized with 1.3 M NaOH. Total xylose was quantified using a kit from Megazyme (Bray, Ireland), per the manufacturer's instructions.

2.3. In vitro fermentation with cecal inoculum and SCFA determination

Maize fibre (150 mg) in 50 mL falcon tubes were placed in an anaerobic cabinet with non-sealed lids in order to make samples anoxic and for gas exchange to occur during fermentations. Samples were diluted in 15 mL sterile anoxic moura medium prepared as described by Moura et al. (2007) with minor modifications as described by De Maesschalck et al. (2015). The pH was adjusted to 6.5 before placing samples in the anaerobic cabinet. Inoculation with cecal content from 29 day old broilers was mixed and diluted 10 times with Moura medium and 150 µL was added to the maize fibre in a 15 mL final incubation volume to achieve a 1000 x dilution of cecal content. The fermentations were conducted for 48 h at 37 °C, with or without xyl and araF enzymes dosed at 10 and 5 mg EP/kg, respectively. Fermentation supernatants were attained at 6, 24 and 48 h and stored at −20 °C until analysed. All fermentations were done in triplicates and the whole fermentation experiment was repeated on a separate occasion.

2.4. Animals and diets

Broilers were housed and euthanized according to the Belgian animal welfare regulations (2010/63/EC). A total of 480 newly-hatched male Ross-308 broiler chickens were allocated to 16 pens containing 30 chickens per pen with 8 pens pr. treatment group: A control group and a group receiving supplementation with xyl and araF enzymes. Chickens were housed on solid flooring covered with wood shavings and received a light schedule of 18 h/6 h darkness. Room temperature was optimized according to the growing bird's requirements. All birds received the same mash feed starter and grower diet containing either enzyme supplementation or not (control). The starter and grower were fed according to recommended optimized energy and amino acid needs of the birds (Table 3). The starter diet was fed from day 0–7 and the grower diet from day 8–29. The enzymes were sprayed in liquid form (1.5 L enzyme-dilution) onto 30 kg of ground maize premix and kept cool until mixed into the feed rations to achieve the 10 and 5 mg EP/kg concentration.

2.5. Sample collection

Body weight and feed intake were measured at pen level on days 8, 14, 21, and 29. Five chickens per pen were euthanized and weighed individually at days 14 and 29. Jejunum digesta from proximal jejunum to meckel's diverticulum and cecal content from both ceca were collected and snap-frozen in liquid nitrogen. Digesta was pooled per pen and freeze dried before homogenisation. Epithelia tissue from the duodenum loop was obtained from three chickens per pen by sectioning the lower part of the loop followed by washing with sterile water and fixation in 4% formaldehyde solution (BiopSafe ® containers, Ax-lab, Denmark, http://www.axlab.dk/) and stored for further analysis. After 24 h tissue samples were transferred to 70% ethanol before embedding.

2.6. Dysbacteriosis scoring of birds

Immediately after euthanasia and individual weighing, an experienced veterinarian scored birds macroscopically for intestinal inflammation. Each bird was given a score between 0 and 10 for intestinal dysbacteriosis parameters with 0 being normal gastro-intestinal tract and 10 being the most severe dysbacteriosis (Teirlynck et al., 2011). Inflammatory parameters scored were (1) overall gut ballooning; cranial and caudal (2) inflammation/significant redness of the serosal/mucosal side of the gut; (3) fragility of the gut; (4) flaccidity/thickness of the gut; (5) abnormal content; (6) undigested feed particles in the caudal ileum to ileo-cecal junction.

2.7. NSP analysis of digesta

Analysis of insoluble and neutral non-cellulosic polysaccharides (NCP) constituents (NSP constituents without cellulose) was performed on feed rations and pooled, freeze dried and homogenized jejunum digesta in triplicates according to Theander et al. (1995). Solubilisation with 12 M H_2SO_4 was omitted in order to avoid swelling of cellulose.

2.8. Confocal microscopy and immunocytochemistry of digesta

Immunolabeling and confocal microscopy of jejunum digesta samples was performed as described by Ravn et al. (2016). In short, a CLSM SP2 microscope (Leica, Heidelberg, Germany) was used with a 63 x water-immersion objective. Images were processed in the LAS AF Lite (Leica) software. Freeze-dried jejunum digesta was mixed with 2% melted agar, fixated, dehydrated and embedded in paraffin. Immunolabeling with LM27 and LM28 rat primary monoclonal antibody that specifically binds substituted AX regions (Cornuault et al., 2015) and a IgG anti-rat linked to an Alexa-555 fluorophore secondary antibody was purchased from Plant Probes (UK) from http://www.plantprobes.net/index.php. Citiflour AF1 (Agar Scientific, UK) anti-fading agent was added to avoid bleaching of the fluorescent signal.

2.9. Morphological examination

Duodenum epithelium tissue segments sampled at the duodenum loop were fixed in 4% formaldehyde solution (BiopSafe ®, Ax-lab, Denmark). Samples were dehydrated in xylene and a series of graded ethanol, embedded in paraffin and sectioned in 4 µm thick sections. Samples were deparaffinated, stained with hematoxylin and eosin and examined using a Leica DM LB2 digital light

microscope (Leica, Germany) with a Leica DFC 320 camera. Villus length was measured by random measurement of 10 villi per duodenum section from three chickens per pen using the image analysis system LAS v4.0 (Leica Application suite V4; Leica, Germany).

2.10. Immunohistochemical examination of T-cells

Deparaffinated sections of the duodenum from 3 chickens per pen (a total of 24 samples per treatment) were prepared for immune labelling with a pressure cooker antigen retrieval method (Tender Cooker; Nordic Ware, Minneapolis, MN, USA) using 10 mM citrate buffer at pH 6. Immunohistochemical labelling of leucocytes was performed with antibodies specific for CD3 T-cells with Dako CD3 (A0452) (Dako, Glostrup, Denmark). Sections were washed in Dako Autostainer + washing buffer and blocked with peroxidase reagent for 5 mins and rinsed with Dako washing buffer. Sections were incubated with primary antibody for 30 min at room temperature and diluted 100 x in Dako antibody diluent (S3022). Sections were rinsed again in Dako washing buffer and incubated with labelled polymer-HRP (DAB) (K4011) for 30 min at room temperature. Sections were then washed twice with Dako washing buffer and Dako DAB + substrate and DAB + chromogen was added for 5 mins. The staining was stopped and counterstained with haematoxylin for 10 mins and washed for 1 min under running water. Sections were dehydrated using xylene and a graded series of ethanol and mounted. Brown-stained leucocytes were quantified by area% using a color threshold application in the image analysis system LAS v4.0 software (Leica).

2.11. DNA extraction and 16S rRNA sequencing of cecal bacteria

Genomic bacterial DNA was extracted from cecal material using a Nucleospin 96 soil kit (Macherey-Nagel, Germany) and a Genie-T vortexer (Scientific Industries Inc., USA) for cell lysis. Quantification and PCR targeting the V3-V4 variable regions of the 16S rRNA gene was performed as previously described by Ravn et al. (2017). Amplicon libraries were purified using the Agencourt® AMpure XP bead protocol (Beckmann Coulter, USA). Samples were sequenced on a MiSeq (Illumina, San Diego, CA). Bioinformatics was performed as previously described by Ravn et al. (2017).

2.12. SCFA analysis of cecal content and in vitro fermentations

Concentrations of SCFA in cecal content and *in vitro* fermentations were quantified by gas chromatography as described by Schäfer (1994). Cecal content from broilers sampled at day 29 were thawed and approximately 100 mg was weighed in before extraction. Supernatants from the *in vitro* fermentations were centrifuged (4000 rpm, 15 min) using 200 µL supernatant mixed with 200 µL MeOH with 10% HCOOH for extraction. Lactate was quantified by HPLC (Dionex, USA) using a Rezex RoA column (Phenomenex, Denmark) equipped with a RI detector.

2.13. Statistical analysis

Analysis of variance was performed using the ANOVA procedure in the statistical package SAS JMP 12.1.0 (SAS Institute Inc., 2015). For performance data, villi length and CD3 T-cell values or SCFA production *in vitro* the effect of Day (or time of incubation), Treatment and their interaction was included in the model. For significant models ($P < .05$) least squared means were separated using the Tukey-Kramer HSD test ($P < .05$) as provided in the ANOVA model. For soluble xylose, NSP data or SCFA concentration in cecal contents the main effect of treatment was tested and comparisons made using the Tukey-Kramer test. It should be noted that for villus length, the average of 10 villi from one bird was used as one data value. Statistical analysis of 16S rRNA metagenomics data was handled in R using ANOVA, as described in detail by Ravn et al. (2017).

Table 1
Total xylan solubilisation (g/kg dry matter) from four replicates of maize fibre incubated in sodium acetate buffer (0.1 M, pH 5) for 3 h at 40 °C without or with xyl and araF dosed at 10 mg EP/kg and 5 mg EP/kg, respectively.

Treatment	Total xylose release (g/kg DM)	SEM[1]
Blank	0.4[c]	0.06
araF	0.4[c]	0.02
xyl	6.2[b]	1.14
xyl + araF	26.1[a]	1.09

[1] SEM = standard error of mean. abc: Mean values within a column not sharing a common letter index differ significantly ($P < .05$; Tukey-Kramer HSD).

3. Results

3.1. Enzyme activity on maize fibre

Solubilisation of total GAX by the enzymes was quantified colorimetrically (n = 4) measuring soluble xylan (Table 1). Combining the xyl with the araF significantly increased (P < .001) xylan solubilisation by a four-fold factor compared to addition of xyl alone. Addition of araF alone did not increase xylose solubilisation compared to blank. The combination of the two enzymes solubilised 23.6% of the total xylose (110.5 g/kg) present in the maize fibre.

3.2. SCFA analysis from in vitro fermentations with cecal inoculum

Fermentation of maize fibre supplemented without and with xyl and araF together with inoculum from pooled cecal content from two 29 d-old broilers was done to investigate SCFA production, in particular butyrate. Addition of xyl in combination with araF dosed at 10 and 5 mg EP/kg, respectively, significantly increased (P < .05) butyrate by 4.16 mM after 24 h of anaerobic incubation compared to control. Furthermore, combination of xyl and araF resulted in significantly higher (P < .05) butyrate levels after 48 h, than using xyl or araF separately, while propionate was significantly lowered (P < .05). No detectable lactate was present in the samples.

3.3. Bird performance

Feed conversion ratio (FCR) was lowered (P < .001) after 14, 21 and 29 days. At day 29 FCR was lowered by −5.8% and body weight (BW) was increased (P < .001) by 5.4% compared to the control group. Feed intake (FI) was not significantly altered by enzyme supplementation (Table 4). Mortality was 5.0% (12 birds) for the control group and 3.3% (8 birds) for the enzyme treatment group during the whole trial period (not significant).

3.4. NSP analysis of jejunum digesta

Analysis of the insoluble NSP fraction of the jejunum digesta showed that ingested xyl and araF enzymes increased GAX solubilisation *in vivo*. Insoluble xylose was lowered (P < .05) in digesta of birds supplemented with enzymes (Table 5).

3.5. Microscopy of cell wall GAX in jejunum digesta

In jejunum digesta of control birds a high fluorescent GAX antibody signal from LM27 and LM28 was observed. The antibody signals were not visible in birds supplemented with xyl and araF enzymes in the diet (Fig. 1), indicating removal of GAX epitopes by the enzymes.

3.6. Dysbacteriosis/inflammation scoring of the intestine

The average macroscopic dysbacteriosis score at day 14 was 1.55 and 1.85 (out of 10) for control and xyl and araF supplemented birds, respectively. Birds scored at day 29 showed an average macroscopic dysbacteriosis score of 4.73 and 4.75 (out of 10) for control and xyl and araF supplemented birds, respectively. There were no statistically significant differences between control and enzyme supplemented birds (data not shown).

3.7. Intestinal morphology

Supplementation with xyl and araF to the diet increased villi length (P < .001) in the duodenum loop with 120 µm in 29 days old birds compared to control birds. No significant difference was observed in 14 day-old birds (Table 6). Furthermore, the area% of CD3 T-cell infiltration was significantly reduced (P < .001) by 22.1% in 29 day-old birds supplemented with xyl and araF (Table 7), indicating a lower inflammation level in enzyme supplemented birds.

3.8. SCFA concentration in cecal content

Cecal butyrate concentration was significantly increased (P < .05) by 3 µmol/g in broilers supplemented with xyl and araF enzymes compared to control (Table 8).

3.9. 16S rRNA analysis of microbiota composition

There was no statistically significant difference in the relative abundance (%) of genera observed between control groups and xyl and araF enzyme supplemented groups. The 10 most abundant genera are showed in Fig. 2. Numerical increases in *Ruminococcaceae* and *Lachnospiraceae* was observed.

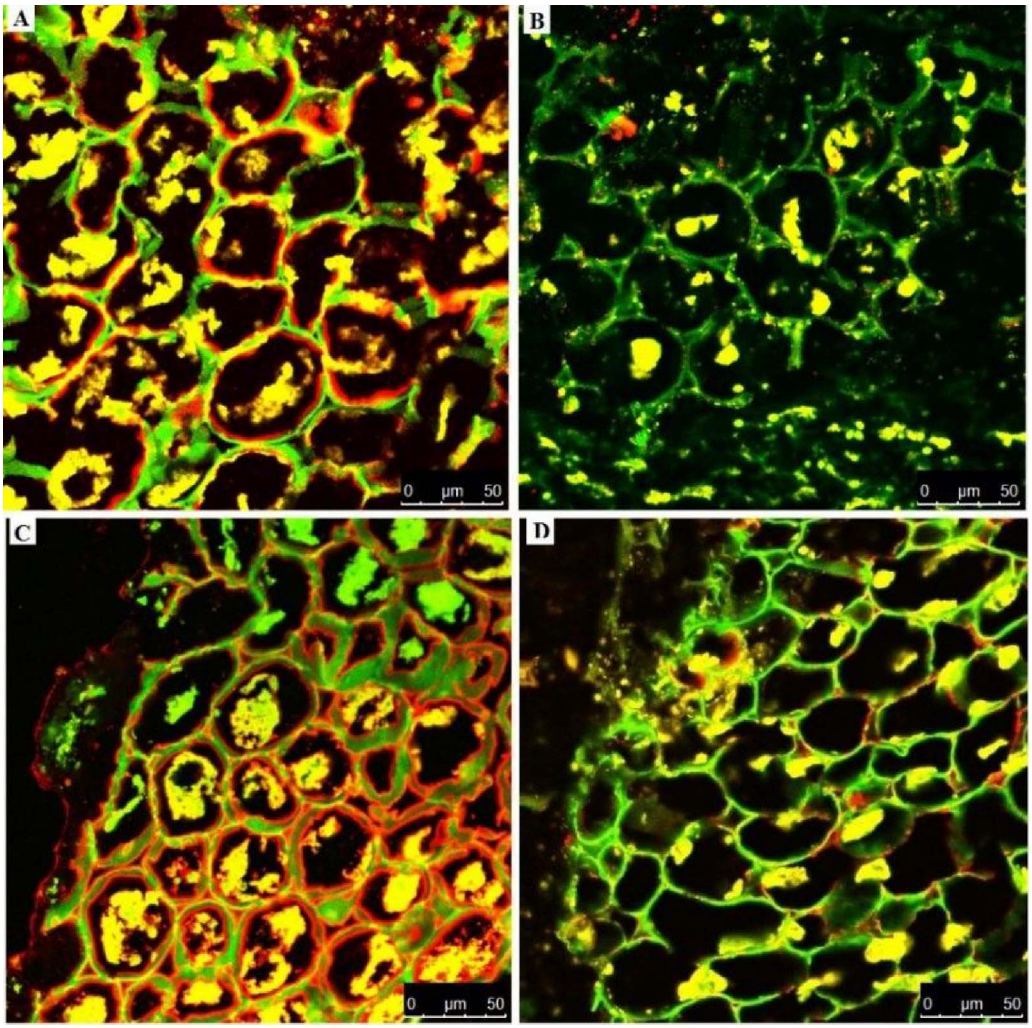

Fig. 1. Confocal microscopy of jejunum digesta from pooled pens receiving control or xyl + araF enzyme supplementation. Control groups with LM27 (A) and LM28 (C). Groups receiving xyl and araF LM27 (B) and LM28 (D). Scale bar = 50 µm.

4. Discussion

Understanding the interactions between exogenous fibre degrading enzymes added to animal feedstuffs and the resulting intestinal fermentation patterns in the animal may help optimize gut health and ultimately performance. In the present study, highly substituted maize GAX solubilisation was shown to be increased by addition of araF together with xyl. Combination of araF with xyl clearly increased ($P < .001$) total GAX solubilisation from maize fibre *in vitro* by more than four times compared to the xyl alone (Table 1). Other α-L-arabinofuranosidases have previously shown high affinity towards AX (Wang et al., 2014). In the current study, arabinose substitution removal apparently increased the catalytic activity of the xyl. The synergistic effects on GAX solubilisation by combining xylanases with arabinofuranosidases is well-documented (de Vries et al., 2000; Jia et al., 2016). However, those studies were based on wheat-derived GAX, which are far less branched and complex compared to maize GAX (Yang et al., 2014). Higher enzymatic mediated solubilisation has also been observed in wheat GAX compared to maize GAX (Rose and Inglett, 2011). Studies on enzymatic hydrolysis of maize GAX often include pre-treatments (acid, alkali hydrolysis, mechanical or heat) to remove side groups and render the structure more accessible for enzymatic hydrolysis (Agger et al., 2010; Appeldoorn et al., 2010; Rose and Inglett, 2010). However, the araF used in this study targeted and cleaved arabinose from maize GAX without the need of such pre-treatments.

Although *in vitro* fermentation of maize fibre with cecal inoculum showed no difference in total SCFA production with xyl and araF addition, butyrate levels were increased ($P < .05$) by 4.16 mM at 24 h after addition of the enzyme combination (Table 2) compared to control. After 48 h, butyrate levels were still higher ($P < .05$) with the addition of xyl and araF combined while propionate was lowered ($P < .05$). The advantage of combining xyl and araF compared applying single xyl was marginal and butyrate levels were only slightly, but significantly higher ($P < .05$) after 48 h (Table 2). Considering the difference in solubilisation values of xylan obtained *in vitro*, the combination of xyl and araF was chosen for investigation in broiler chickens to test he effect of

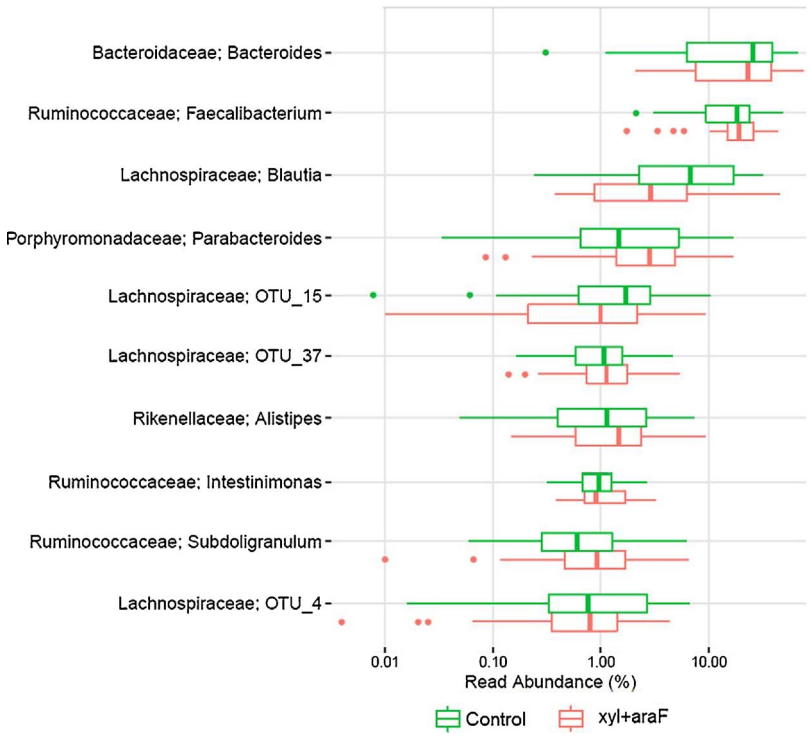

Fig. 2. Relative abundance of 10 most abundant bacterial genera as present in control (green) and xyl + araF enzyme supplemented (red) samples. Lines besides boxes indicate standard deviations and dots indicate outliers. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 2
SCFA production (mM) during *in vitro* fermentation for 6 h, 24 h and 48 h of maize fibre at 37 °C using cecal broiler content inoculum in triplicates, incubated without or with xyl and araF enzymes dosed at 10 and 5 mg EP/kg, respectively.

SCFA	Control	xyl	araF	xyl + araF	Pooled SEM[1]
Acetate					
6h	16.9^{a}	3.9^{a}	18.0^{a}	11.0^{a}	15.24
24h	19.9^{b}	47.3^{ab}	49.2^{a}	37.7^{ab}	22.16
48h	88.7^{a}	70.9^{a}	81.5^{a}	93.1^{a}	30.89
Propionate					
6h	5.4^{a}	2.3^{a}	2.3^{a}	2.9^{a}	2.99
24h	8.0^{b}	15.9^{a}	8.7^{ab}	12.1^{ab}	6.68
48h	24.7^{a}	20.3^{ab}	13.8^{b}	13.0^{b}	9.18
Isobutyrate					
6h	0.4^{a}	0.1^{a}	0.2^{a}	0.3^{a}	0.31
24h	1.4^{ab}	2.4^{a}	0.9^{b}	1.8^{ab}	0.85
48h	3.7^{a}	3.0^{ab}	2.3^{b}	2.9^{b}	1.01
Butyrate					
6h	1.0^{a}	0.7^{ab}	0.6^{b}	0.8^{ab}	0.27
24h	3.1^{c}	7.0^{ab}	4.8^{bc}	7.3^{a}	1.59
48h	5.3^{c}	7.1^{b}	5.5^{c}	7.7^{a}	1.20
Isovalerate					
6h	0.4^{a}	0.2^{a}	0.2^{a}	0.3^{a}	0.19
24h	2.8^{b}	4.5^{a}	2.9^{b}	3.6^{ab}	1.01
48h	6.6^{a}	5.3^{ab}	3.9^{b}	5.2^{ab}	1.60
Valerate					
6h	0.4^{a}	0.1^{a}	0.2^{a}	0.3^{a}	0.25
24h	0.6^{b}	1.1^{a}	0.6^{b}	0.8^{ab}	0.33
48h	11.0^{a}	8.8^{ab}	5.7^{b}	8.5^{ab}	3.14
Total SCFA (6 h)	24.6^{a}	7.3^{a}	21.5^{a}	15.5^{a}	18.81
Total SCFA (24 h)	35.8^{a}	75.9^{a}	67.0^{a}	62.0^{a}	25.37
Total SCFA (48 h)	140.0^{a}	115.5^{a}	112.6^{a}	131.5^{a}	38.47

Table 3
Composition and nutrient contents of the starter and grower diets.

Parameter	Starter diet +4% VM[1]	Grower diet +4% VM[1]
Feedstuffs (g/kg)		
Maize	493.4	454.7
Soya Hipro	293	218.5
DDGS maize	50	100
Rapeseed meal	50	100
Soy oil	49.5	73.5
Lysine 50% (liquid)	6	6
DL-Methionine	3.7	3.1
L-Threonine (*via* VM[2])	1.6	1.3
Monocalcium	16.4	14.5
Limestone	14	8.7
Salt	3	3
Premix vit, min mix	5	5
Wheat[2] (as support of Threonine)	14.4	11.7
Total	1000	1000
Calculated energy content		
MEbr (kcal/kg)	2810	2897
ME (MJ/kg)	11.8	12.1
Nutrient composition, g/kg		
Crude protein	215	205
Crude fat	79	104
aNDF	101	94
LYSav	11.9	10.9
M + Cav	9.2	8.4
THREO av	8	7.3
TRYav	2.1	1.9
Ca	10.9	8.4
Pav	4.6	4.3

VM[1] = 100% maize as support of the enzymes. VM[2] = 10% premix of Threonine based on wheat.

Table 4
Effect of enzyme supplementation on growth performance of birds over a period of 29 days.

Measurements (day)	8		14		21		29		
Treatment	Control	xyl + araF	Control	xyl + araF	Control	xyl + araF	Control	xyl + araF	Pooled SEM[1]
FCR	1.13	1.11	1.42	1.34	1.47	1.40	1.55	1.46	0.01
P value[2]		.9		< .01		< .01		< .01	
FI (g/day)	21.4	21.2	54.6	52.4	91.1	91.8	133.49	136.7	8.04
P value[2]		.7		.09		.9		.4	
BW (g)	191.0	190.1	421.3	424.6	854.1	883.0	1545.8	1633.5	55.86
P value[2]		.9		.8		.03		< .01	
LWG (g/day)	19.0	18.9	38.4	39.1	62.13	65.5	86.45	93.8	5.54
P value[2]		.9		.5		< .01		< .01	

[1] SEM = standard error of mean. Feed conversion ratio (FCR), feed intake (FI), body weight (BW) and live weight gain (LWG) were calculated at four time intervals. Values are the means for 8 pens with 30 chickens (25 and 20 chickens after day 14 and 29, respectively).
[2] P-value: Pairwise comparison of means (Tukey-Kramer HSD test) for control and enzyme supplementation up to days 8, 14, 21 and 29, respectively.

increased xylan solubilisation and butyrate production.

In vivo, the NSP analysis of jejunum digesta showed that the xyl and araF enzyme combination solubilised insoluble maize GAX ($P < .05$). Almost equal amounts of xylose (4.7 g/kg DM) and arabinose (3.1 g/kg DM) were solubilised from the insoluble digesta fraction. The ratio of ara/xyl was 0.97 (close to 1:1) in the remaining insoluble xylan (Table 5) after enzyme treatment. This indicates that the enzyme treatment had indeed been able to mediate solubilisation of the xylan chain leading to an increased ara/xyl ratio compared to that of the control (0.91) in the insoluble fraction. A high degree of arabinose moieties in the xylan polymer represents a more difficult substrate for degradation by xylanases (Sørensen et al., 2007). Phenolic compounds substitution as well as acylation of the xylan polymer can also impede xylanases (Boukari et al., 2011; Biely, 2012a,b). Confocal microscopy on jejunum digesta using commercial antibodies (LM27 and LM28) targeting branched grass xylan structures (Cornuault et al., 2015) visualised solubilisation of GAX in maize cell walls by the enzymes indicating target epitopes of the enzymes. Fluorescent antibody epitopes were almost completely removed by the enzymes (fig. 1), corresponding well with the NSP wet chemistry results.

Overall performance (FCR, body weight) was improved in broilers supplemented with the xyl and araF combination. Other studies have also reported improved performance using a combination of *endo*-xylanasess and de-branching enzyme such as

Table 5
Average individual non-starch polysaccharide content (g/kg DM) in the insoluble fraction, as well as their total insoluble content in pooled broiler jejunum digesta from 3 control pens and 3 xyl and araF supplemented pens sampled at day 29.

	Jejunum digesta		
Non-Starch Polysaccharides	Control	xyl + araF	Pooled SEM[1]
Insoluble			
Rhamnose	3.9[a]	3.8[a]	0.27
Fucose	1.8[a]	1.8[a]	0.21
Arabinose	41.3[a]	38.2[a]	2.26
Xylose	46.6[a]	41.9[b]	2.45
Mannose	7.5[a]	7.5[a]	0.20
Galactose	21.5[a]	22.4[a]	0.90
Glucose	20.6[a]	21.2[a]	1.35
Sum of insoluble NSP	143.3[a]	139.1[a]	5.27
Ratio: Arabinose/Xylose	0.91[a]	0.97[b]	0.01

[1] SEM = Standard error of mean. ab: Means within a row not sharing a common letter index differ significantly (P < 0.05; Tukey-Kramer HSD).

Table 6
Effects of xyl and araF supplementation on broiler villi length[1] in the duodenum.

	Day14		Day29		
Treatment	Villi length (μm)	*P* value[2]	Villi length (μm)	*P* value[2]	Pooled SEM[3]
Control (n = 24)	1644	–	1918	–	56.6
xyl + araF (n = 24)	1667	.47	2039	< .01	40.4

[1] Random measurements of 10 villi per duodenum section (24 sections) was performed by a computer-based LAS v.4 software (Leica) analysis system.
[2] *P* value[1]. Pairwise comparison of means (Tukey-Kramer HSD test) for control and enzyme supplementation at days 14 and 29, respectively.
[3] SEM = Standard error of mean.

Table 7
Effect of xyl and araF enzyme supplementation on CD3 T-cell infiltration in the duodenum as quantified by area%[1.]

	Day14		Day29		
Treatment	CD3 T-cell (area%)[1]	*P* value[2]	CD3 T-cell (area%)[1]	*P* value[2]	Pooled SEM[3]
Control (n = 24)	8.4	–	11.8	–	1.21
xyl + araF (n = 24)	7.5	.30	9.2	< .01	1.08

[1] The area% of CD3 T-cells in villi tissue (brown stained) was quantified using a color threshold tool in the LAS v.4 software (Leica).
[2] *P* value. Comparison of means for control and enzyme supplementation at days 14 and 29 (Tukey-Kramer HSD test).
[3] SEM = Standard error of mean.

Table 8
SCFA concentrations (μmol/g) in cecal content of broilers supplemented without (n = 24) and with xyl and araF enzymes (n = 24).

Group	Control	xyl + araF	Pooled SEM[1]
Acetate	51.3[a]	58.0[a]	4.87
Propionate	9.2[a]	9.4[a]	0.97
Iso-butyrate	0.7[a]	0.6[a]	0.09
Butyrate	10.9[b]	14.0[a]	1.59
Iso-valerate	1.3[a]	1.3[a]	0.12
Valerate	1.2[a]	1.3[a]	0.14
Total SCFA	76.0[a]	84.4[a]	7.11

[1] SEM = Standard Error Mean. ab: Mean values within a column not sharing a common letter differ significantly (P < .05; Tukey-Kramer HSD).

arabinofuranosidases (Lei et al., 2016) however, using wheat-based diets only. The rationale for the increased performance observed in the present study remain inconclusive without data on total tract digestibility of nutrients, but may in part be due to increased hindgut fermentation caused by enzyme solubilised AXOS (Choct, 2006).

In line with the *in vitro* fermentations, butyrate concentrations were increased (P < .05) by 3.04 μmol/g in the cecal content of broilers supplemented with xyl and araF enzymes (Table 8). The increased (P < .002) villus length (+120 μm) and lowered (P < .001) T-cell infiltration (−22.1%) observed in the duodenum in 29 days old broilers (Tables 6 and 7) supplemented with the

enzyme combination can be hypothesized to be linked to the increased butyrate fermentation in the cecum. Higher levels of butyrate act anti-inflammatory (Place et al., 2005) and stimulate multiplication and differentiation of the intestinal epithelial cells (Timbermont et al., 2010; Guilloteau et al., 2010). One mechanism underlying a systemic effect of increased butyrate concentration may be that butyrate affects enteroendocrine L-cells to secret glucagon-Like peptide-2 which stimulates intestinal cell growth (Onrust et al., 2015; Tappenden et al., 2003). *Ruminococcaceae* and *Lachnospiraceae,* which are known butyrate-producing bacteria from Clostridium cluster IV and XIVa (Hippe et al., 2011) were numerically increased with addition of the enzyme combination. These observations indicate that the xyl in combination with a de-branching enzyme increases butyrogenic AXOS in a maize-based diet, which may contribute to an increased performance and a healthy gut.

5. Conclusion

Combining a xylanase with an arabinofuranosidase increased maize GAX solubilisation and butyrate production during maize fibre fermentation *in vitro*. The difference in butyrate production when combining the enzymes compared to applying xylanase alone was marginal. The enzyme combination increased performance in an *in vivo* trial with broilers fed a maize/soy fibre-rich diet. Addition of the enzyme combination also resulted in improved gut morphology most likely due to increased fermentation of AXOS into butyrate in the hindgut. The relevance of the combination of the enzymes to enhance xylose solubilisation, and explain the *in vivo* response observed, needs to be investigated.

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