Received: 27 September 2023 | Accepted: 13 December 2023

DOI: 10.1111/jpn.13917

ORIGINAL ARTICLE

Poultry

Journal of Animal Physiology and Animal Nutrition WILEY

In vitro evaluation of the application of an optimized xylanase cocktail for improved monogastric feed digestibility

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Funding information

Department of Science and Innovation (DSI) and Technology Innovation Agency (TIA) of South Africa, Grant/Award Number: 2018/FUN/0180 and 2021/FUN236/AA; DSI/National Research Foundation (NRF) of South Africa, Grant/Award Number: SARChI grant 98566; Rhodes University (RC and RRG grants); Research Development Programme from the University of Pretoria, Grant/Award Number: 2896

Abstract

Xylanases from glycoside hydrolase (GH) families 10 and 11 are common feed additives for broiler chicken diets due to their catalytic activity on the nonstarch polysaccharide xylan. This study investigated the potential of an optimized binary GH10 and GH11 xylanase cocktail to mitigate the antinutritional effects of xylan on the digestibility of locally sourced chicken feed. Immunofluorescence visualization of the activity of the xylanase cocktail on xylan in the yellow corn of the feed showed a substantial collapse in the morphology of cell walls. Secondly, the reduction in the viscosity of the digesta of the feed by the cocktail showed an effective degradation of the soluble fraction of xylan. Analysis of the xylan degradation products from broiler feeds by the xylanase cocktail showed that xylotriose and xylopentaose were the major xylooligosaccharides (XOS) produced. In vitro evaluation of the prebiotic potential of these XOS showed that they improved the growth of the beneficial bacteria *Streptococcus thermophilus* and *Lactobacillus bulgaricus*. The antibacterial activity of broths from XOS-supplemented probiotic cultures showed a suppressive effect on the growth of the extraintestinal infectious bacterium *Klebsiella pneumoniae*. Supplementing the xylanase cocktail in cereal animal feeds attenuated xylan's antinutritional effects by reducing digesta viscosity and releasing entrapped nutrients. Furthermore, the production of prebiotic XOS promoted the growth of beneficial bacteria while inhibiting the growth of pathogens. Based on these effects of the xylanase cocktail on the feed, improved growth performance and better feed conversion can potentially be achieved during poultry rearing.

KEYWORDS

anti-nutritional factors, digestibility, feed, prebiotics, xylanases

1 | INTRODUCTION

Chicken feeds are rich in grains, such as corn and wheat, which offer starch, and protein sources, such as soybean meal, as the main ingredients (Choct, 2006; Gous, 2018). However, cereal grains contain antinutritional factors such as nonstarch polysaccharides (NSPs), which cause a 'cage effect' that entraps nutrients in their kernels (Simon, 1998). Nutrient entrapment, in particular, is due to the presence of insoluble NSPs, whereas soluble NSPs impact nutrient availability by increasing digesta

viscosity and reducing nutrient absorption (Khadem et al., 2016; Matthiesen et al., 2021).

Various components of monogastric animal diets, such as NSPs, tannins, phytic acid, lectins and enzyme inhibitors, are antinutritional factors contributing to inefficient feed utilization by livestock (Zentek & Boroojeni, 2020). With the exception of NSPs, all the other antinutritional factors are heat-labile and partially or entirely neutralized by hydrothermal processes used in animal feed production (Zentek & Boroojeni, 2020). The inability of monogastric animals, such as chickens, to produce NSP degrading enzymes, called NSPases, has led to the use of exogenous enzymes as feed additives (Bedford, 2018). The lack of endogenous NSPases in these animals leads to inefficient nutrient availability, often presenting itself as undigested feed commonly found in chicken droppings (Liu & Kim, 2017). Carbohydrate-active enzymes are used for the targeted degradation of NSPs, resulting in better feed conversion and increased substrate availability for the gut microbiota (Bedford, 2018). Meng and Slominski (2005) reported that the supplementation of carbohydrases to cereal-based broiler chicken feeds resulted in the release of entrapped nutrients, increasing their availability to endogenous enzymes.

The most common broiler chicken diet is comprised of corn and soybean meal (Amer et al., 2020). Arabinoxylan is the most abundant NSP in corn, which makes up 50% or more of the total NSP fraction (Knudsen, 1997). Corn xylan is made up of D-xylopyranosyl (Xylp) residues linked by β-1,4-glycosidic bonds (Izydorczyk & Biliaderis, 1995). The Xylp units are substituted with L-arabinofuranose residues through both α-1,2- and α-1,3-linkages, and/or with α-1,2-linked D-glucuronic acid and acetyl groups (Bedford, 1996; Freeman et al., 2017). Corn xylan resides in the layers of the endosperm and bran (testa and pericarp) and varies in complexity in grain tissues (Ward, 2021). The xylan in the peripheral layers contains more galactose and glucuronic acids, and is thus termed glucuronoarabinoxylan (Pedersen et al., 2015). Due to the abundance of xylan in corn, xylanases have become regularly included as part of exogenous enzyme additives for broiler chicken diets (Bedford, 2018, 2019). There are numerous reports on the benefit of supplementing xylanases on their own or as part of multienzyme additives into broiler chicken feeds. Studies have shown that supplementing xylanases to animal feeds reduces digesta viscosity and releases encapsulated nutrients, thus improving the overall feed digestibility and nutrient availability (Matthiesen et al., 2021). Catalytically, the efficiency of xylanases depends on the degree of substitution of the xylan backbone (Ward, 2021). Combinations of xylanases and debranching enzymes (L-α-arabinofuranosidases, α-D-glucuronidases and cinnamoyl esterases) have been used to improve the digestion of different cereal grains such as wheat and corn (Bajaj & Mahajan, 2019; Ravn et al., 2017; Saleh et al., 2019; Ward, 2021). Saleh et al. (2019) reported that a combination of xylanase and arabinofuranosidase improved growth, nutrient digestibility, lipid peroxidation, immune response and expressions of genes related to growth and fatty acid synthesis in broiler chickens when fed low-energy diets based on yellow corn and soybean meal. Xylanases are also part of commercial multienzyme feed additives utilized in the feed industry to improve animal growth performance and reduce feed costs (Bedford, 2018; Saleh et al., 2018).

The most studied and ubiquitous xylanases are classified into two glycoside hydrolase (GH) families: GH10 and GH11, based on amino acid sequence and structural fold similarities (www.cazy.org). GH11 xylanases require three successive unsubstituted xylose residues for their activity, whereas GH10 xylanases require at least two adjacent unsubstituted xylose residues for catalysis to occur (Agger et al., 2010; Ward, 2021).

The complete degradation of the xylan present in plant cell walls often requires the coordinated action of multiple enzymes due to the recalcitrant nature of the biomass and the complex structure of plant cell walls (Zerva et al., 2020). The endosperm cell walls predominantly contain branched xylans and a minimal content of mixed-linked β-glucan and cellulose (Meng & Slominski, 2005; Navarro et al., 2019). On the other hand, the cell walls of the pericarp layer have an abundance of both glucuronoarabinoxylan and cellulose (Meng & Slominski, 2005; Navarro et al., 2019). Malgas and Pletschke (2019) reported on the synergistic degradation of wheat flour arabinoxylan by GH10 and GH11 xylanases. As GH family xylanases target different regions of the xylan backbone, applying xylanolytic enzymes from varying GH families may be required for effective xylan degradation in feeds. An additional benefit to using xylanases as feed additives is the resulting production of prebiotic xylooligosaccharides (XOS), a suitable alternative to antibiotics for positively modulating the gut microbiota (Ding et al., 2018; Kim et al., 2011; Yadav & Jha, 2019).

This study sought to evaluate the application of a binary xylanase cocktail for the efficient degradation of xylan in yellow corn-soybean feed for broiler chickens. Furthermore, an investigation of the prebiotic potential of the XOS produced by the xylanase cocktail during the degradation of the feeds was performed in selected beneficial bacteria and the culture broths of these were tested as antagonists against the extraintestinal infectious bacterium *Klebsiella pneumoniae*.

2 | METHODS AND MATERIALS

2.1 | Materials

Soluble (medium viscosity) and insoluble wheat flour arabinoxylan, a rat monoclonal anti-xylan antibody (LM10), XOS standards (xylose (X1), xylobiose (X2), xylotriose (X3), xylotetraose (X4) xylopentaose (X5) and xylohexaose (X6), and a *Geobacillus stearothermophilus* xylanase (XT6, GH10), were purchased from Megazyme™. A xylanase from *Trichoderma viride* (Xyn2A, GH11), pancreatin, α-amylase and Paraplast Plus® were purchased from Sigma Aldrich A. Goat anti-Rat IgG (H + L) antibody conjugated with Alexa Fluor™ 568 was purchased from ThermoFisher Scientific. All other reagents used in this study were obtained from Sigma-Aldrich and MERCK and were reagent grade.

TABLE 1 Composition of the broiler chicken feed diets used in this study (on a % dry weight basis).

Feed component	Starter (%)	Grower (%)
Yellow corn	57.40	70
Soybean	35	20
Yellow corn bran	4	6
Feed lime	1.46	2.34
Premix	0.3	0.3
Salt	0.36	0.3
Kynofos 21 (MDCP)	1.15	0.8
Lysine	0.1	0.16
Methionine	0.23	0.1

Abbreviation: MDCP, mono dicalcium phosphate.

2.2 | Extraction of broiler chicken feed NSPs

NSPs from locally sourced starter and grower broiler chicken feed formulations, with compositions shown in Table 1, were alkaline extracted as described previously by Choct et al. (1996), with modifications.

Briefly, 10 g of the starter and grower feeds were added to 100 mL of deionized water containing 0.1 mg/mL α-amylase and incubated at 50°C for 2 h, followed by addition of 0.5 g of pancreatin and further incubation for 16 h. After incubation, the solution was centrifuged at 1000*g* for 30 min using an Avanti J-E Centrifuge (Life Sciences Division) and the supernatant was decanted. The pellet was resuspended in 100 mL of aqueous 0.2 M sodium hydroxide containing 0.26 M sodium borohydride at 80°C for 2 h and then neutralized with 0.2 M acetic acid. The solution was centrifuged at 1000*g* for 30 min. The supernatant was then decanted and recentrifuged at 13,000*g* for 30 min. The NSP preparation was precipitated from the resulting supernatant by adjusting it to 80% (v/v) ethanol. The precipitate formed was dried to provide an alkali-extractable NSP preparation.

2.3 | Feed compositional analysis

2.3.1 | Moisture and ash content determination

An HG63 Moisture Analyzer (Mettler Toledo) was used to determine the moisture contents of the starter feed, grower feed and the extracted NSPs from the starter and grower feeds. Readings were taken in triplicate and used to determine the dry-matter content of the samples.

To determine the ash content, 300 mg (dry-weight basis) triplicates of the feeds and feed-derived NSPs were weighed in oven-dried pre-weighed porcelain crucibles. Samples were heated to 575°C and charred for 3 h in a muffle furnace using a ramping temperature of 5°C/min. After cooling, the samples were weighed and the difference between the initial and final masses was used to determine the ash content (Sluiter et al., 2012).

2.3.2 | Two-step sulfuric acid hydrolysis

A two-step treatment with sulfuric acid (TSSA) was used to determine the content of carbohydrates and lignin in the feed samples (Sluiter et al., 2012). In the first step (I), 300 mg of milled sample was mixed with 3 mL of 72% (w/w) sulfuric acid in a glass tube and the mixture was then incubated for 1 h at 30°C in a water bath with occasional mixing. The sample was then transferred to a glass flask and 84 mL of ultrapure water was added to achieve a final concentration of 4% (w/w) sulfuric acid. In the second step (II), the glass flask and its content were autoclaved at 121°C for 1 h. After cooling, the sample was filtered through a pre-weighed Pyrex fritted glass crucible (porosity 2, pore size 40–60 μm). The liquid sample was analysed for monosaccharides and acid-soluble lignin (ASL). The solid residue in the glass crucible was washed several times with ultra-pure water and dried overnight in an oven at 105°C. The crucible was weighed after cooling and the difference between the initial and final masses was used to determine the content of Klason lignin. ASL in the filtrate was determined by measuring the absorbance at 320 nm using a UV-1800 spectrophotometer (Shimadzu). An absorptivity constant (ε) of 30 l g^{-1} cm^{-1} was used to determine the ASL content. The monosaccharide content in the liquid fraction was determined by using a high-performance anion-exchange chromatography system, ICS-5000 (Dionex), equipped with an electrochemical detector. A CarboPac PA1 (Dionex) separation column (4 × 250 mm) equipped with a guard column (4 × 50 mm) was used at a flow rate of 1 mL min^{-1} of ultrapure water for 25 min with a postcolumn addition of a 300 mM aqueous solution of NaOH at a flow rate of 0.5 mL min^{-1}. The column was regenerated for 11 min using 60% of the 300 mM NaOH solution and 40% of an aqueous mixture of 200 mM NaOH and 170 mM sodium acetate. This was followed by an equilibration step with ultrapure water for 3 min, in accordance with the procedure described by Tang et al. (2022). The monosaccharides in the samples were quantified by using known concentrations (0.5–30 ppm) of arabinose, galactose, glucose, xylose and mannose as external calibration standards.

2.3.3 | Determination of protein content

The protein content in the feed samples was determined using a previously described procedure by Glaser et al. (2023). Briefly, 200 mg of feed was suspended in 2 mL of a 0.1 M solution of NaOH. The mixture was incubated for 3 h at 80°C in a water bath, allowed to cool and then centrifuged for 10 min at 16,060*g* to separate the liquid fraction from the solid residue. The pH of the liquid fraction was adjusted to 6.0 using 0.5 M HCl. The total protein content in the liquid fraction was determined using the bicinchonic acid assay (BCA) kit following the manufacturer's instructions (Pierce™, Thermo

Scientific). A 25 µL aliquot of the liquid fraction from the sample was added to 200 µL of BCA reagent and incubated without shaking at 37°C for 30 min. The protein content was quantified by measuring the absorbance at 562 nm using a BioTek Epoch Microplate Spectrophotometer (Agilent) with bovine serum albumin (BSA) as the protein standard (0.025–2 mg/mL).

2.4 | Biochemical properties of xylanases used in the study

The temperature optima of Xyn2A, XT6 and the xylanase cocktail (Xyn2A 75%: XT6 25%) were investigated by exposing the xylanases to different temperatures ranging from 30°C to 90°C (on a Labnet AccuBlock™ digital dry bath) in 50 mM sodium citrate buffer (pH 5), with slight agitation. The pH optima of the xylanases at 40°C were determined using buffers at different pH values from 3 to 9. The buffers for pH 3–7 were prepared using a 50 mM citrate-phosphate universal buffer, and for pH values 8 and 9, a 50 mM Tris-base buffer was used. Wheat flour arabinoxylan was used to determine the activity of the xylanases and the reactions were run for 60 min. All enzyme assays were performed in triplicate.

2.5 | Xylanase cocktail formulation and activity assays

The two xylanases, Xyn2A and XT6, were combined on a protein mass basis in a 75:25% ratio to formulate the binary xylanase cocktail as described previously (Malgas & Pletschke, 2019). An aliquot of 100 µL of the enzyme solution was added to 300 µL of 1.33% (w/v) wheat flour arabinoxylan in 50 mM citrate buffer at pH 5.0, giving a final substrate concentration of 1% (w/v). The reaction had a final volume of 400 µL, with a final concentration of 1 mg of protein per gram of biomass. The reaction was incubated at 40°C for 15 min. The reactions were conducted in triplicate and reducing sugars were measured using the 3, 5-dinitrosalicylic acid (DNS) assay (Miller, 1959).

2.6 | Simulated gastrointestinal stability of xylanases

A 100 µL solution composed of a 1:1 (w/w) ratio of the xylanases at 0.04 mg/mL with gut-derived proteases (trypsin, pepsin), mucin or bile salts from porcine at 0.04 mg/mL (Sigma-Aldrich) were mixed (separately) and incubated at 40°C (using the Labnet AccuBlock™ digital dry bath) for 1 h. A 300 µL aliquot of 1.33% (w/v) soluble wheat flour arabinoxylan was added to the samples. Enzyme hydrolysis was performed at 40°C for 30 min and all assays were performed in triplicate. The DNS method was used to quantify the released reducing sugars and the residual specific activity of the xylanases was calculated. Enzyme activity was measured in units (U), where one unit of xylanase activity was defined as the amount of enzyme that liberated 1 µmol of xylose reducing sugar equivalents per minute.

2.7 | Visualization of xylan degradation by xylanases using immunofluorescence microscopy

The effects of xylanolytic hydrolysis of yellow corn kernel sections were visualized using immunofluorescence. Yellow corn kernels were fixed using 3.7% (v/v) formaldehyde in water containing 1% (v/v) methanol for 24 h and washed using deionized water. The kernels were dehydrated by incubation in a series of ethanol solutions (50%, 70%, 90% and 100% (v/v) ethanol) for an hour and incubate in 100% (v/v) xylene for an hour. Paraplast plus was melted at 60°C and used to form the sample molds, which were allowed to set for 24 h. Paraffin-embedded samples were sectioned into 5 µm sections using a microtome (using a Leica RM2035, Leica Instruments GmbH). The resulting sections were deparaffinized using absolute xylene for 5 min and a graded series of ethanol (100%, 90%, 70% and 50% [v/v]) for 5 min. The 5 µm yellow corn kernel sections were hydrolysed by immersing the sections in 1 mg/mL of xylanase solutions of the individual enzymes, Xyn2A and XT6, and the combination of Xyn2A to XT6 at 75: 25%. The xylanases were diluted to a 1 mg/mL solution in 50 mM citrate buffer (pH 5) and the control sections were immersed in buffer alone. The kernel sections were treated for 3 h at 40°C, followed by washing with deionized water.

Sections treated with and without xylanase were permeabilized with 1× phosphate buffered saline (PBS) (pH 7.4, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, 137 mM NaCl, 2.7 mM KCl) containing 0.1% (v/v) Triton-X-100 for 15 min. The sections were blocked using sterile PBS containing 1% (w/v) BSA and 0.01% (v/v) Tween for 45 min. A 10-fold dilution of rat monoclonal anti-xylan antibody (LM10) was prepared in PBS containing 0.1% (w/v) BSA and 0.01% (v/v) Tween (this was termed the dilution buffer), and yellow corn sections were incubated with the antibody overnight at 4°C. The sections were washed three times using the dilution buffer for 5 min each time. Sections were incubated with a 300-fold dilution of the secondary antibody (Goat anti-Rat IgG antibody (H + L) conjugated with Alexa Fluor 568) in the dilution buffer for 1 h at 25°C in the dark. The sections were washed with dilution buffer three times for 5 min each time followed by a rinse with deionized water containing 1 µg/mL Hoechst stain (ThermoFisher Scientific). The sections were sealed under a coverslip using clear nail varnish and visualized using an Olympus BX43 fluorescence microscope (Wirsam Scientific & Precision Eq. Ltd).

2.8 | Simulated feed digesta viscosity reduction by the xylanase cocktail

A modified protocol reported by Bedford and Classen (1993) was used to determine the in vitro intestinal viscosity reduction of feed by the application of xylanases. Briefly, 0.6 g of animal feed

(starter and grower) hydrolysed by xylanases was suspended in 900 µL of 0.1 M HCl solution containing 2000 U/mL of pepsin for 45 min at 40°C. Then, 300 µL of 1 M $NaHCO_3$ containing 8 mg of pancreatin was added to the samples and incubated for an additional 2 h. The digesta was centrifuged using the Heraeus Biofuge pico at 16,060*g* for 5 min after incubation and the viscosity was measured. The kinematic viscosity of the digesta samples was determined using a Cannon-Manning Semi-Micro viscometer (size 150, Courtesy of Cannon Instrument Co.). Measurements to estimate the CentiStoke (cSt, mm^2/s) of the samples were performed by multiplying the time in seconds with the constant (0.03146 mm^2/s^2 [cSt/s]) in triplicate.

2.9 | Determination of XOS pattern profiles by thin layer chromatography (TLC) and high-perfomance liquid chromatography (HPLC)

XOS produced from the xylanolytic hydrolysis of chicken feeds were analysed by TLC using a method previously described by Mkabayi et al. (2020). Briefly, XOS were separated on a silica gel 60 F254 plate (Merck) using a mobile phase consisting of 1-butanol, acetic acid and water in a 2:1:1 ratio. A Shimadzu HPLC system (Shimadzu Corp.) equipped with a refractive index detector connected to a CarboSep CHO 411 column (Concise Sci) was used to quantify the XOS as described previously by Mkabayi et al. (2020). The experiment was performed in triplicate.

2.10 | Antioxidant activity of animal feed-derived XOS

The antioxidant activity of XOS produced from the hydrolysis of the substrates was investigated using a method described by Valls et al. (2018). XOS were added to a preformed radical ABTS·+ solution and the remaining ABTS·+ was measured by UV spectrophotometry (BioTeK Epoch 2) after 10 min of incubation with the tested compound. The ABTS·+ was generated by adding 44.5 µL of 140 mM potassium persulfate to 2.5 mL of 7 mM ABTS and incubating the solution for 16 h in the dark. The ABTS·+ absorbance was adjusted to 0.7 ± 0.1 at 730 nm using deionized water. A volume of 225 µL of the ABTS·+ was mixed with 25 µL of 0.5 mg/mL XOS and incubated in the dark for 10 min. The complete discolouration of the samples represented 100% antioxidant activity. The discoloration % was calculated as follows:

$$\text{ABTS}\cdot + \textit{discoloration}\ (\%) = \frac{(Ai - Af)}{Ai} \times 100,$$

where *Ai* represents the ABTS·+ absorbance value of the blank and *Af* is the ABTS·+ absorbance value after incubation with the antioxidant compounds. The % discoloration of ABTS by a compound was considered the % of its antioxidant activity. Gallic acid was used as a positive control and experiments were conducted in triplicate.

2.11 | Prebiotic effect of animal feed-derived XOS

A method described by Magengelele et al. (2023) was used for the prebiotic activity determination of XOS produced from the hydrolysis of the starter and grower chicken feeds by xylanases. Cultures of *Streptococcus thermophilus*, *Bacillus subtilis* and *Lactobacillus bulgaricus* in Luria broth (LB) were harvested by centrifugation at 16,060*g* for 5 min using a Heraeus Biofuge pico and resuspended in 250 µL 50 mM PBS (pH 7.4) before measuring the absorbance readings at 600 nm. The bacterial cells were diluted to an optical density (OD, measured at 600 nm) of 0.1 using 1× M9 minimal media (238.7 mM Na_2HPO_4, 110.2 mM KH_2PO_4, 93.4 mM NH_4Cl and 42.8 mM NaCl). One milliliter of the starter or grower feed-derived hydrolysate with a reducing sugar concentration of 2 mg/mL was mixed with 4 mL of the diluted culture (OD600 of 0.1). The probiotic cultures were incubated at 37°C for 7 h with shaking at 150 rpm (1*g*). Glucose was used as a positive control, while M9 media on its own was used as a negative control. After incubation, the cell viability of the bacterial cultures was determined by mixing 100 µL of the culture with 40 µL of 27 mM resazurin, followed by incubation at 37°C for 10 min and then readings were taken by excitation at 560 nm with monitoring emission at 590 nm. Cell density was determined by centrifuging 1 mL of each cell culture at 16,060*g* for 5 min, discarding the supernatant, resuspending the pellet in 50 mM PBS (pH 7.4) buffer and taking OD600 readings. The enhanced cell viability and cell densities were illustrated by converting the absorbance readings into a percentage relative to the positive control (glucose). All assay reactions were performed in triplicate.

2.12 | Antimicrobial activity of probiotic culture broths

The antibacterial activity of culture broths derived from *S. thermophilus*, *B. subtilis* and *L. bulgaricus* supplemented with XOS from animal feed was assessed against pathogenic bacteria; *K. pneumoniae* and *Staphylococcus aureus*, using a modification of the method reported by Elshikh et al. (2016).

To determine the bacterial log phase, each pathogenic bacterial strain was grown on LB agar (1% (w/w) tryptone, 0.5% (w/w) yeast extract, 1% (w/w) NaCl and 2% (w/w) agar) at 37°C overnight. A single colony of each pathogenic bacteria was inoculated in 5 mL of LB broth and incubated at 37°C overnight with shaking (160 rpm/1*g*). A volume of 100 mL of sterile LB broth was inoculated with 1 mL of the overnight culture and incubated at 37°C for 7 h with shaking at 160 rpm (1*g*). The OD600 was measured every hour and used to plot a graph of OD600 versus time. The log phase of each cell culture was noted in accordance with the calibration curves generated.

The pathogenic bacterial cultures were prepared by inoculating single colonies of bacteria into 5 mL of LB broth and incubating at 37°C overnight with shaking at 160 rpm (1*g*). A 50 µL aliquot of overnight cultures was inoculated into 5 mL LB broth and the bacteria were allowed to grow for 4 h (log phase). The viability assay was

prepared by adding 160 µL of LB broth, 20 µL of the broth from probiotic cell cultures produced in the prebiotic section above, 20 µL of the log phase pathogenic bacterial cultures and 20 µL of 27 mM resazurin to a 96-well plate. The assay was incubated for 15 min and the viability of the bacteria was monitored by fluorescence measurement with excitation at 560 nm followed by emission at 590 nm. The data obtained were presented as percentage viability and the reduction in cell viability indicated antibacterial potential. The following formula was used to calculate the percentage viability:

$$\%\text{Viability} = \frac{(\text{Actual reading} - \text{Background reading})}{(\text{Positive control reading} - \text{Background reading})} \times 100.$$

3 | RESULTS AND DISCUSSION

3.1 | Compositional analysis of broiler chicken feeds

The ability of the formulated xylanase cocktail to alleviate antinutritional effects caused by arabinoxylan was investigated using locally sourced starter and grower feeds mainly composed of yellow corn and soybean. The chemical compositions of the starter and grower feeds, and the extracted NSPs from the respective feeds, were analysed using the TSSA method (Table 2, Sluiter et al., 2012). NSPs in corn make up to 10.3% of dry mass and 21.7% of soybean meal (Knudsen, 1997). The NSPs from cereal grains and soybean meal predominantly contain arabinose, xylose and glucose from the arabinoxylans, β-glucans and cellulose (De Keyser et al., 2018). The high galactose content may be due to the galactans in the soybean meal and alpha-galactosides such as raffinose and stachyose (Kocher et al., 2002). The galactose was higher in the extracted starter NSPs than in the grower NSPs, because the starter contained 35% soybean, significantly more than the 20% soybean meal in the grower. The chemical composition of broiler feeds showed the presence of xylose in the starter, grower, starter NSPs and grower NSPs (1.43%, 1.49%, 6.22% and 6.17% respectively). Xylose is the primary monosaccharide of xylans, making up the backbone of the polysaccharide substituted with arabinose and/or glucuronic acid side chains (Izydorczyk & Biliaderis, 1995). Although yellow corn is the main component of the starter and grower feeds, the major substitution of corn xylan, arabinose, was not detectable when analysing the starter and the grower feeds (Table 2; Ward, 2021). Once the NSPs were extracted from the starter and the grower feeds, arabinose was detected at 8.75% for the starter NSPs and 7.95% for the grower NSPs (Table 2). The resulting arabinose to xylose (A/X) ratios of the extracted NSPs were 1.4 and 1.3 from the starter and grower. These A/X ratios were considerably higher than the reported A/X ratio of 0.72 for corn (Knudsen, 2014). High AX ratios may also be due to the presence of arabinan in the extract, as corn fibre contains 29%–31% arabinan (Doner & Hicks, 1997).

High arabinose content is usually associated with solubility; however, the glucuronoarabinoxylan in the corn has an abundance of

TABLE 2 Chemical composition of broiler chicken feeds and extracted NSPs.[a]

	Arabinose (g/100 g)	Galactose	Glucose	Xylose	Mannose	Klason lignin	Acid-soluble lignin	Ash content	Protein content	Total
Starter feed	ND	2.47 ± 0.11	43.11 ± 0.62	1.43 ± 0.09	ND	1.98 ± 0.21	1.60 ± 0.03	4.88 ± 0.15	22.82 ± 0.35	78.29 ± 0.67
Grower feed	ND	2.76 ± 0.09	43.71 ± 0.75	1.49 ± 0.11	ND	1.98 ± 0.16	1.34 ± 0.04	4.72 ± 0.01	20.54 ± 0.27	76.54 ± 1.06
Starter NSPs	8.75 ± 0.12	9.53 ± 0.17	10.14 ± 0.21	6.22 ± 0.13	ND	1.13 ± 0.18	1.67 ± 0.01	14.32 ± 0.21	14.12 ± 0.74	65.88 ± 0.58
Grower NSPs	7.95 ± 0.08	8.35 ± 0.14	11.82 ± 0.32	6.17 ± 0.06	ND	1.37 ± 0.21	1.32 ± 0.01	15.31 ± 0.05	15.35 ± 0.65	67.64 ± 0.58

Abbreviations: ND, not detectable; NSP, nonstarch polysaccharide.

[a]Mass fractions in %. Arabinose, galactose, glucose, xylose and mannose are reported in anhydrous form. Data are indicated as means ± SD for three replicates.

phenolic crosslinks between protein, cellulose and lignin, reducing its solubility and obstructing enzymatic degradation (Ward, 2021).

3.2 | Biochemical properties of xylanases

The pH and thermal optima of the xylanolytic enzymes (Xyn2A, XT6 and the xylanase cocktail) were established to determine the ideal conditions for their activity. Xyn2A and the xylanase cocktail displayed maximal activity from 40°C to 60°C and XT6 showed the highest activity at 70°C (Table 3). In addition, the assessed pH optima of the xylanases were between 2 and 7—these were consistent with those previously reported by Malgas and Pletschke (2019).

Subsequently, the specific activities of the xylanases were determined using wheat flour arabinoxylan at 40°C and 50 mM sodium citrate buffer (pH 5.0) (Table 3). The xylanase cocktail showed significantly higher hydrolytic activity (396.09 U/mg) on wheat flour arabinoxylan than Xyn2A (356.41 U/mg) and XT6 (327.39 U/mg) (Table 3).

TABLE 3 Physico-chemical properties of xylanases.

	Xyn2A	XT6	Xylanase cocktail
Temperature optima (°C)	40–60	70	40–60
pH optima	2–7	3–7	2–7
Specific activity (U/mg)	356.41	327.39	396.09

3.3 | Simulated gastrointestinal tolerance of the xylanase cocktail

As the xylanases would be supplemented into the chicken feed, they will be ingested along with the feed. The continued benefits of the direct application of the xylanases for chicken ingestion depend on their ability to remain active even when exposed to gastrointestinal

TABLE 4 The effect of gastrointestinal juice components on xylanase activity.[a]

	Control	Trypsin	Mucin	Bile salt	Pepsin
Xyn2A	100 ± 2.8	91.9 ± 2.6	107.7 ± 7.2	98.3 ± 7.6	90.8 ± 4.7
XT6	100 ± 3.2	86.1 ± 4.0	106.8 ± 14.6	68.5 ± 1.9	69.7 ± 10
Xylanase cocktail	100 ± 2.1	83.6 ± 2.7	94.0 ± 7.3	85.3 ± 6.4	81.0 ± 7.7

[a]The residual activity values (in %) are represented as means ± SDs, $n = 3$.

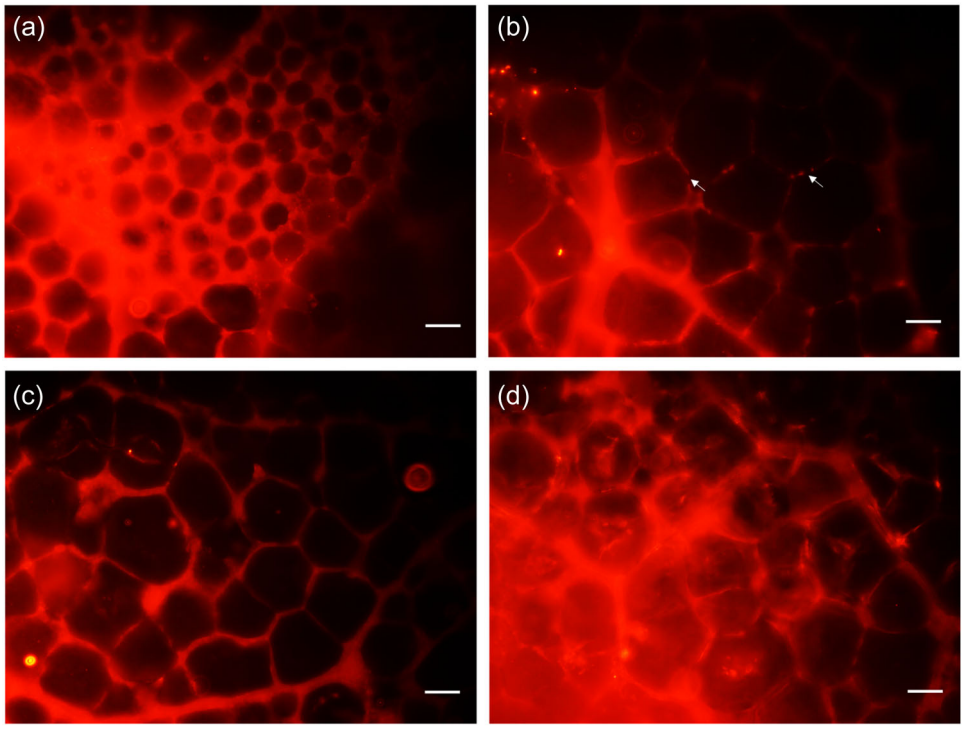

FIGURE 1 Indirect immunofluorescence micrographs of transverse sections of yellow corn kernels to illustrate xylan (red) deconstruction by (a) buffer and enzyme treatments; (b) Xyn2A, (c) XT6 and (d) xylanase cocktail (Xyn2A 75%: XT6 25%). Arrows indicate gaps resulting from the xylanolytic degradation of the xylan in the cell wall. Scale bar = 100 µm. [Color figure can be viewed at wileyonlinelibrary.com]

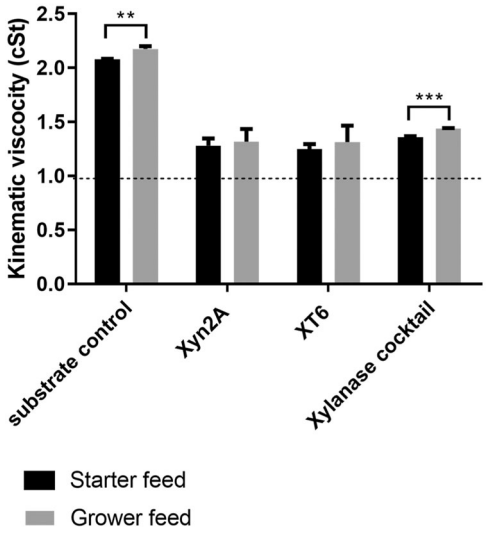

FIGURE 2 In vitro viscosity after digestion simulation. Before the gut simulation, the starter and grower feeds were treated with xylanases and the resulting viscosities were recorded. Experiments were conducted in triplicate. The kinematic viscosity of water was determined to be 1 cSt and is represented by the dotted line in the figure above. Analysis of variance was conducted (**$p < 0.005$ and ***$p < 0.0005$). Data points represent mean values ± SD ($n = 3$).

conditions. Therefore, the susceptibility of xylanases to gastrointestinal conditions was investigated by exposing the enzymes to trypsin, pepsin, mucin and bile salts (Table 4). Only Xyn2A retained more than 90% of its activity after exposure to trypsin, mucin, bile salts and pepsin for an hour (Table 4). However, XT6 showed a 30% reduction in activity upon exposure to pepsin and bile salts.

The activity of the xylanase cocktail was maintained above 80% when exposed to trypsin, mucin, bile salts and pepsin (84%, 94%, 85% and 81% activity respectively). These results are consistent with those reported for a GH11 xylanase that retained up to 80% activity after an hour of exposure to proteases (trypsin and pepsin) (Luo et al., 2009). The retained activity of the xylanase cocktail was slightly lower than that of the Xyn2A enzyme alone. This phenomenon could be due to the composition of the cocktail in which most of the xylanase combination consists of 75% Xyn2A. XT6 was particularly susceptible to the bile salts and pepsin, which was reflected in the reduced activity of the cocktail, composed of 25% XT6 (Table 4). The retained activities of the xylanases when exposed to gastric conditions suggest continued (beneficial) hydrolysis of the feed in the animal's gut, leading to improved overall nutrient release and utilization.

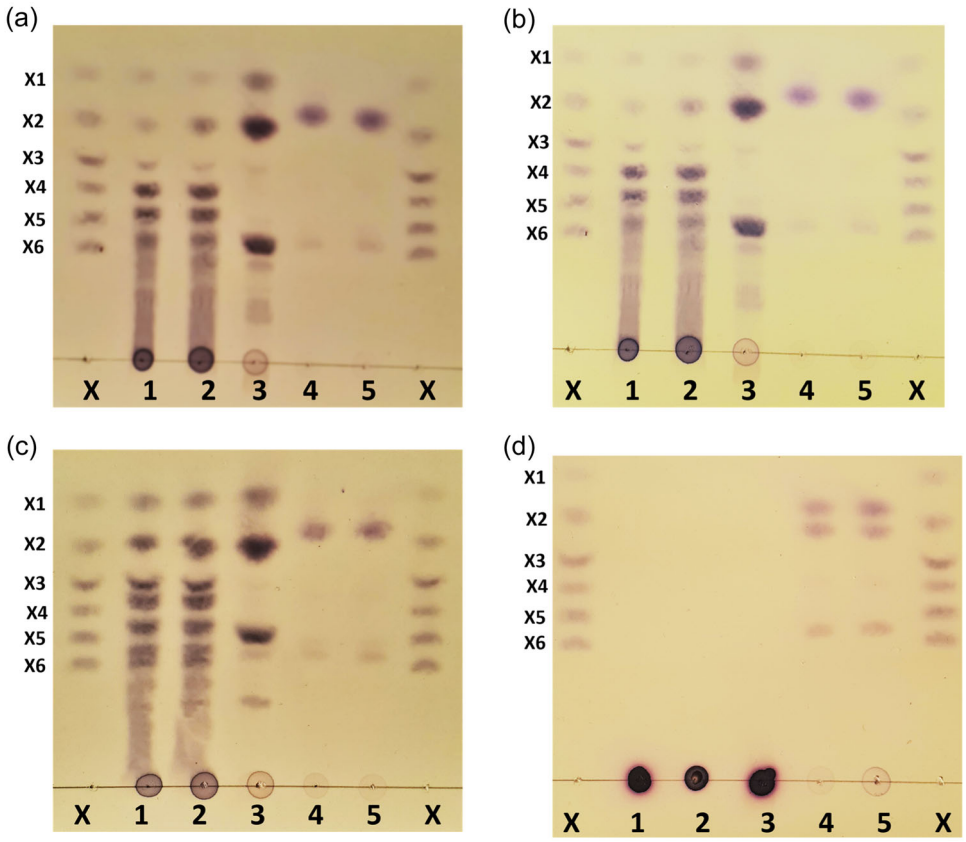

FIGURE 3 Thin layer chromatography profile of xylooligosaccharides produced from wheat arabinoxylan, beechwood xylan (glucuronoxylan and/or glucuronoarabinoxylan) and chicken feeds hydrolysed by xylanases. The xylanases (a) Xyn2A, (b) XT6 and (c) the xylanase cocktail (Xyn2A 75%: XT6 25%) were used to hydrolyse 1% (w/v) of: (1) soluble wheat arabinoxylan, (2) insoluble wheat arabinoxylan, (3) beechwood xylan, (4) starter feed and (5) grower feed. (d) represents substrate controls (no enzyme treatment). X, Xylo-oligosaccharides standards; X1, xylose; X2, xylobiose; X3, xylotriose; X4, xylotetraose; X5, xylopentaose; X6, xylohexaose (Megazyme™). [Color figure can be viewed at wileyonlinelibrary.com]

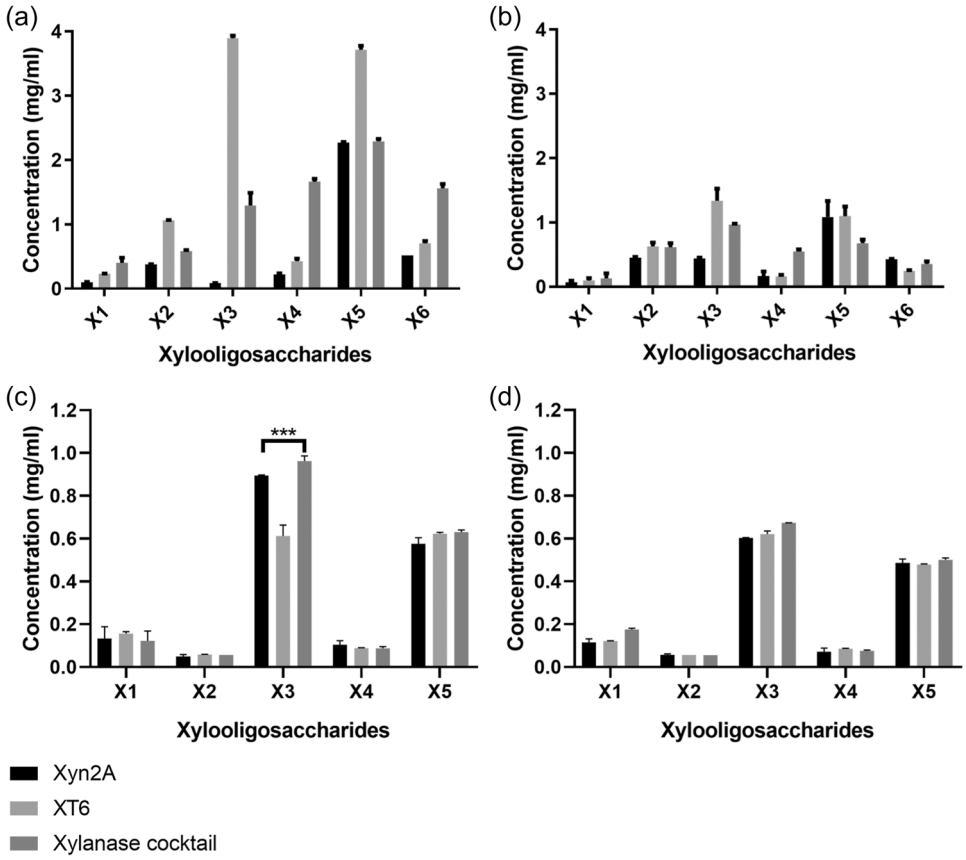

FIGURE 4 High-perfomance liquid chromatography analysis of the xylooligosaccharides produced from wheat arabinoxylan and chicken feeds (via hydrolysis by the xylanases). The xylanases Xyn2A, XT6 and the xylanase cocktail were used to hydrolyse 1% (w/v) of (a) soluble wheat arabinoxylan, (b) insoluble wheat arabinoxylan, (c) starter feed and (d) grower feed. Analysis of variance was conducted (***$p < 0.0005$). Data points represent mean values ± SD ($n = 3$).

3.4 | Immunofluorescence microscopic visualization of feed digestion by the xylanase cocktail

Previous studies have visualized the specific degradation of xylan in wheat, barley and rye grains by a GH11 xylanase (Ravn et al., 2016, 2017). Considering that most chicken diets consist of corn supplemented with a protein source such as soybean meal, the degradation of xylan was visualized using yellow corn as substrate. The effect of hydrolysis of xylan present in the cell walls of yellow corn endosperm by Xyn2A, XT6 and the xylanase cocktail was observed by immunofluorescence staining. Specific degradation of the cell wall by the xylanases was visualized using a xylan-specific primary rat monoclonal antibody, LM10 (Figure 1; red staining). The untreated sections were immersed in 50 mM citrate buffer (pH 5) and showed intact thicker cell walls compared to the xylanase-treated samples (Figure 1a–d). The samples treated with Xyn2A showed degradation of the xylan surrounding the cells with visible disruptions (gaps) in the cell wall (Figure 1b, arrows). Hydrolysis of the samples by the formulated xylanase cocktail resulted in morphological disruption of the endosperm cells and reduced emission of the fluorescent label (Figure 1d). Treatment with the xylanase cocktail resulted in the breakdown of cell structure and release of cell contents, which reduced the cage effect produced by the NSPs.

3.5 | Simulated feed digesta viscosity reduction by the xylanase cocktail

One of the main anti-nutritional factors of the NSPs is their contribution to digesta viscosity that results in reduced digestibility of nutrients and animal growth performance (Bedford, 2018). The supplementation of feeds with enzymes has become a common practice to reduce the adverse effects of increased viscosity (Bedford, 2018). The effects of hydrolysis of the starter and grower feeds by the xylanases on the intestinal viscosity were investigated using an in vitro digestion model of the broiler chicken gut (Bedford & Classen, 1993; Figure 2).

The hydrolysis of the starter and grower feeds by the xylanases resulted in significant in vitro intestinal viscosity reduction (Figure 2). There was no significant difference in the extent of viscosity reduction when the activity of the xylanase cocktail was compared with the activity of Xyn2A and XT6 enzymes on their own. The reduction in intestinal viscosity by xylanases has been reported to improve the nutrient digestibility of cereal grains (Matthiesen et al., 2021). The viscosity reduction in the starter and grower digesta indicates successful degradation of the xylan, resulting in the production of prebiotic XOS.

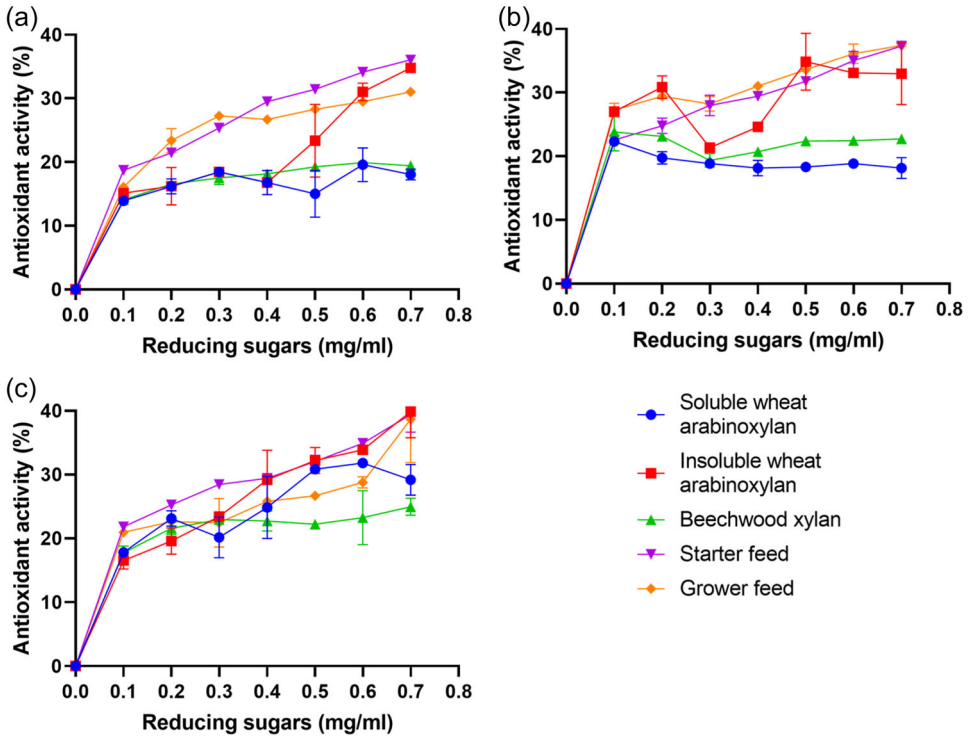

FIGURE 5 Antioxidant activity of the oligosaccharides from wheat-based model substrates, beechwood model substrate and animal feeds hydrolysed by (a) Xyn2A, (b) XT6 and (c) the xylanase cocktail. The resulting hydrolysates (oligosaccharides) were incubated with ABTS·+ and the reduction was calculated with the measured ABTS·+ absorbances after incubation. Data points represent mean values ± SD ($n = 3$). [Color figure can be viewed at wileyonlinelibrary.com]

3.6 | Determination of XOS hydrolysis profiles by TLC and HPLC

The main xylans in yellow corn are arabinoxylan and glucuronoarabinoxylan (Hamaker et al., 2019). The effect of the hydrolysis of soluble arabinoxylan, insoluble arabinoxylan and glucuronoarabinoxylan by the xylanases was determined by analysing products from the xylanolytic hydrolysis of soluble wheat arabinoxylan, insoluble wheat arabinoxylan and beechwood xylan (Figure 3). Hydrolyses of soluble and insoluble wheat arabinoxylan by xylanases produced X1, X2, X3, X4, X5 and X6 (Figure 3a–c). Beechwood xylan was selected as another model substrate as it is mainly composed of glucuronoxylan and glucuronoarabinoxylan (Malgas et al., 2019, 2021). The hydrolyses of the beechwood xylan by Xyn2A, XT6 and the xylanase cocktail produced X1 and X2 oligosaccharides (Figure 3a–c). However, the TLC showed the migration of products between X5 and X6 from the hydrolysis of beechwood by Xyn2A and XT6. The xylanase cocktail also produced X5 and the product between X5 and X6. The starter and the grower feeds showed the presence of three sugars that appeared between X1 and X2, X2 and X3, and X5 and X6, prior to hydrolysis. The hydrolyses of the feeds by the xylanases resulted in two products shown between X1 and X2, and X5 and X6; the xylanase cocktail additionally produced X5 oligosaccharides from the starter and grower feeds. A previous report by Malgas and Pletschke (2019) showed a similar sugar profile, with oligosaccharides migrating between XOS standards. The migration pattern may be due to aldouronic acids derived from the hydrolysis of the glucuronoarabinoxylan present in the yellow corn fraction of the starter and grower feeds (Izydorczyk, 2009; Karlsson et al., 2021). The hydrolysis of glucuronoarabinoxylan by GH10 and GH11 xylanases has been reported to produce aldotetraouronic acids MeGlcA3Xyl3 and MeGlcA3Xyl4 respectively (Puchart et al., 2021).

The quantification of the XOS produced from the xylanase hydrolysis of the starter and grower feed was performed using HPLC. Hydrolysis of the starter and grower feeds showed the production of predominantly X3 and X5 XOS by the xylanases (Figure 4a–d). The xylanase cocktail and Xyn2A released significantly higher X3 XOS from the starter feed compared to XT6 (Figure 4c). However, there were no significant differences in the XOS produced from the grower feed by the xylanases.

3.7 | Antioxidant activity of animal feed-derived oligosaccharides

The antioxidant activity of the oligosaccharides produced during hydrolysis of the feeds and model xylan substrates by the xylanases was determined by quantitative analysis of ABTS·+ radical discoloration. The oligosaccharides (XOS and aldouronic acids) from the starter, grower feeds and insoluble wheat arabinoxylan showed significantly higher antioxidant activity compared with XOS produced from the soluble wheat arabinoxylan model substrates (Figure 5a,b).

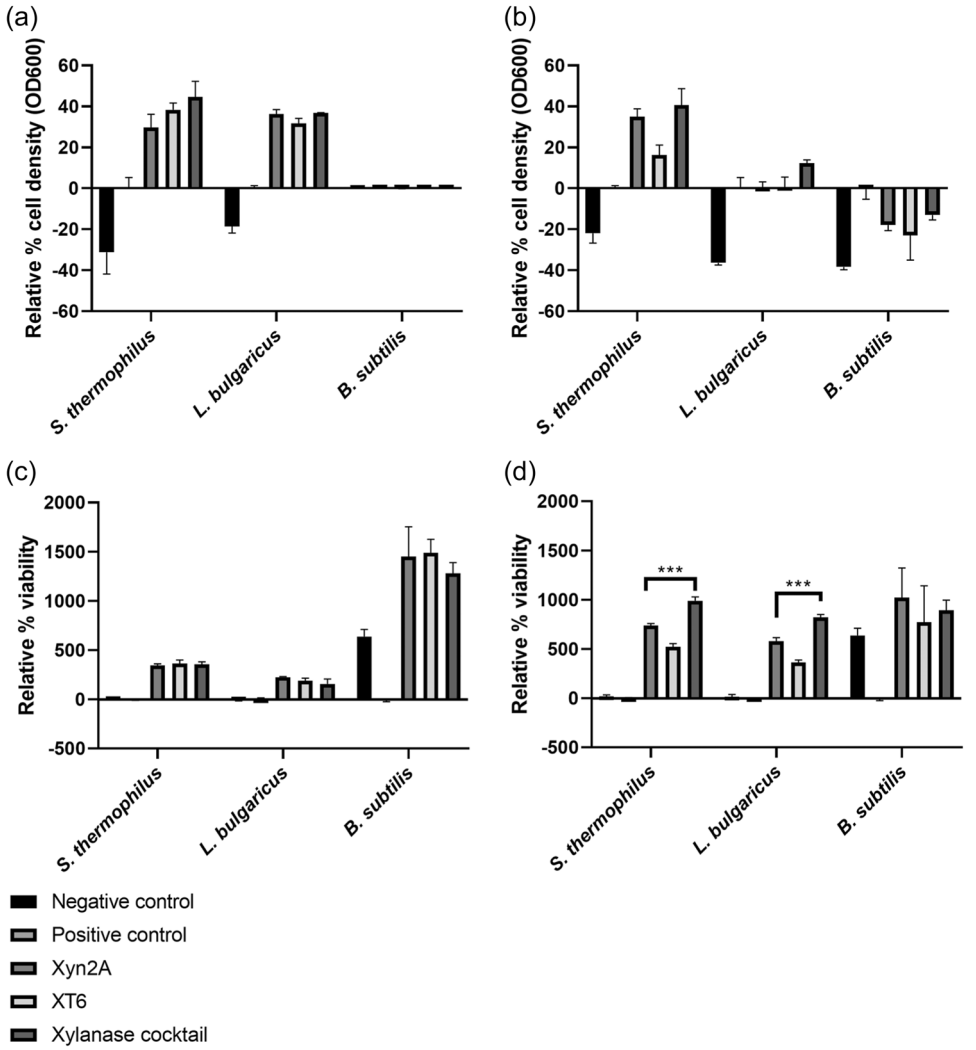

FIGURE 6 The effect of xylooligosaccharides (XOS) produced from the hydrolysis of local chicken feeds by xylanases on probiotic cultures. XOS produced from the hydrolysis of chicken feeds were supplemented into various bacteria cell cultures and incubated for 7 h. (a) Starter cell density (OD600), (b) grower cell density (OD600), (c) starter cell viability and (d) grower cell viability. Analysis of variance was conducted (***$p < 0.0005$). Data points represent mean values ± SD ($n = 3$). Negative control = M9 media, positive control = glucose.

The activity of the xylanase cocktail enhanced the antioxidant activity of soluble wheat arabinoxylan (Figure 5c). The hydrolysis of the starter feeds, grower feeds and beechwood xylan resulted in XOS substituted with methyl glucuronic acid (MeGlcA). Acidic XOS are reported to have higher antioxidant activity than unsubstituted XOS (Valls et al., 2018). However, the products from the beechwood xylan (containing aldouronic acids) generally showed low antioxidant activity. In addition, the degree of polymerization of the XOS is linked to increased antioxidant activity (Valls et al., 2018). The starter and grower animal feeds produced more X5 XOS with a high degree of polymerization, reflected in the observed increased antioxidant activity (Figure 5).

3.8 | Prebiotic effect of chicken feed hydrolysates

Several studies have reported the prebiotic effects of XOS in promoting the growth of various probiotics (Chapla et al., 2012; Ding et al., 2018; Lin et al., 2016; Moura et al., 2007). The prebiotic potential of the oligosaccharide mixture derived from the hydrolysis of feed by the xylanases was investigated by adding sugars to monocultures of different gut-inhabiting bacteria (Figure 6). Three gut-inhabiting probiotics (*S. thermophilus*, *L. bulgaricus* and *B. subtilis*) were used to investigate the prebiotic effect of XOS as a carbon source. Supplementation of the bacterial cultures with XOS produced by the starter increased probiotic cell densities of *S. thermophilus* and *L. bulgaricus* (Figure 6a). The XOS produced from the grower showed enhanced growth of *S. thermophilus* (Figure 6b). Only the oligosaccharides produced by the xylanase cocktail from the grower feed increased the cell density of *L. bulgaricus* (Figure 6b). Chapla et al. (2012) reported a similar enhancement of cell densities of *Lactobacillus* species by XOS produced from corncobs by a xylanase. In addition, Li et al. (2015) reported the ability of XOS to selectively stimulate the growth of multiple *Lactobacillus* species.

The viability of the probiotics was increased by incubation with XOS from the hydrolysates of the starter and grower feeds

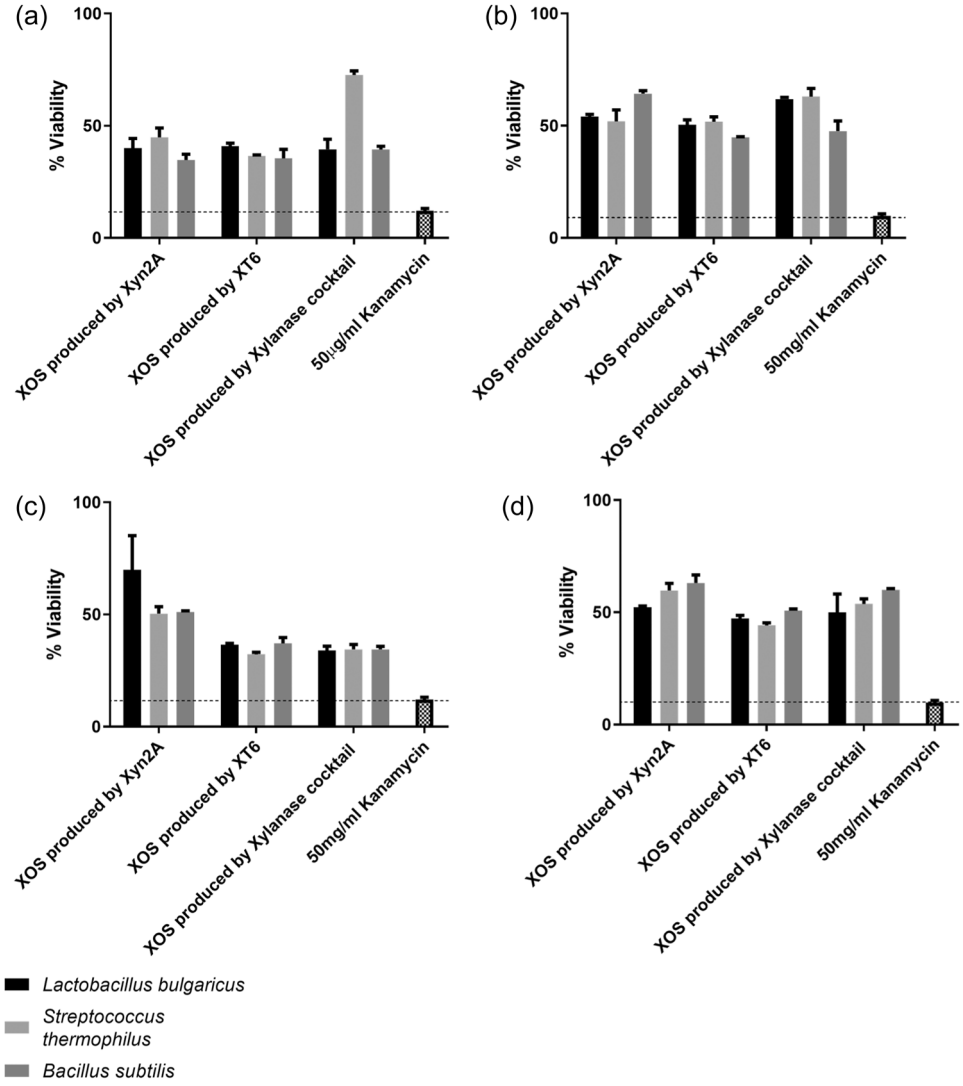

FIGURE 7 Antibacterial activity of the broths of probiotics supplemented with xylooligosaccharides (XOS) from starter feeds against pathogenic bacteria. Demonstration of the effect when the supernatants of probiotics supplemented with XOS from the hydrolysis of starter (a, b) and grower feed (c, d) are exposed to the pathogenic bacteria *Klebsiella pneumoniae* (a, c) and *Staphylococcus aureus* (b, d). Values are represented as mean values ± SD ($n = 4$).

(Figure 6c,d). Interestingly, the addition of XOS produced by the xylanase cocktail from the grower feed showed the highest cell viability for *S. thermophilus* and *L. bulgaricus* cultures.

3.9 | Antibacterial activity of probiotics against pathogenic bacteria

The antibacterial effect of metabolites produced by probiotic cells against pathogenic bacteria has already been reported (Akpinar et al., 2011). This property is important for the in vivo interaction of intestinal bacteria, as it suggests that probiotics may be able to displace pathogenic bacteria in their natural environment. The antibacterial effect of probiotics that had consumed XOS from chicken feed as a carbon source was investigated against *K. pneumoniae* and *Staphylococcus aureus* (Figure 7). The presence of *S. aureus* has been found in broiler chickens and is a common contaminant of broiler chicken products (Sood et al., 2020; Syed et al., 2020). Additionally, *K. pneumoniae* is known to cause respiratory infections in both chickens and humans (Tantawy et al., 2018). Both *S. aureus* and *K. pneumoniae* have been reported to show resistance to antimicrobial agents (Sood et al., 2020; Tantawy et al., 2018). The supernatants from the probiotic cultures successfully inhibited the growth of *K. pneumoniae* and *S. aureus* (Figure 7). These results are consistent with a report by Abdelhalim et al. (2022), who demonstrated the antimicrobial activity of cell-free supernatants of various *Lactobacillus* species on *K. pneumoniae*. A study by Basit et al. (2020) reported the antimicrobial effect of phytobiotics or phytogenic feed additives on various pathogenic bacteria, including *S. aureus*, isolated from the caecal microbiota of broiler chickens.

Probiotics have been reported to produce antimicrobial proteins called bacteriocins that suppress the growth of other bacteria (Benítez-Chao et al., 2021; Negi et al., 2018).

The presence of bacteria potentially pathogenic to humans in the intestines of chickens poses a risk of product contamination if the intestinal contents are spread (Pan & Yu, 2014). Suppressing the growth of potential human pathogens in broiler chickens protects the consumer from exposure (Pan & Yu, 2014). In addition, the antimicrobial activity of probiotics may be a solution for the reduction of antibiotic resistance by providing a non-antibiotic option for suppressing intestinal pathogenic bacteria in livestock (Tantawy et al., 2018).

4 | CONCLUSION

In summary, the formulated xylanase cocktail alleviated the antinutritional effects of xylan by hydrolysing its insoluble fraction in the endosperm cell walls of yellow corn and reducing the viscosity of the feed digesta as a result of the soluble fraction. XOS produced by the xylanase cocktail showed significant prebiotic activity by improving the viability of the probiotic bacteria *S. thermophilus* and *L. bulgaricus*. In addition, the culture broths of the probiotics supplemented with XOS showed antibacterial activity against the potential pathogens *S. aureus* and *K. pneumoniae*. This is likely to lead to a healthy intestinal tract, affecting various systems in the bodies and the general health of monogastric animals. The XOS also exhibited antioxidant activity that could reduce the risk of many diseases in these animals. In general, the optimized xylanase cocktail developed in this study showed great potential as a feed additive for yellow corn-soybean chicken feeds.

ANIMAL WELFARE STATEMENT

The authors confirm that the ethical policies of the journal, as noted on the journal's author guidelines page, have been adhered to. No ethical approval was required as this study only involved an in vitro evaluation and no animals were therefore used throughout this study.

AUTHOR CONTRIBUTIONS

Lebogang Ramatsui: Methodology, visualization, formal analysis, investigation, writing—original draft, writing—review and editing. **Tariro Sithole**: Methodology, visualization, formal analysis, investigation, writing—review and editing. **Madhavi L. Gandla**: Methodology, validation, formal analysis. **Leif J. Jönsson**: Investigation, writing—review and editing. **Adrienne Edkins**: Methodology, formal analysis, investigation, writing—review and editing. **Samkelo Malgas**: Conceptualization, supervision, project administration, formal analysis, validation, writing—review and editing. **Brett I. Pletschke**: Conceptualization, formal analysis, methodology, funding acquisition, supervision, project administration, formal analysis, visualization, writing—review and editing.

ACKNOWLEDGEMENTS

The authors appreciate the financial support received from the Department of Science and Innovation (DSI) and Technology Innovation Agency (TIA) of South Africa (SIIP: Enzyme and Microbial Technology (EMT) grants 2018/FUN/0180 and 2021/FUN236/AA), the DSI/National Research Foundation (NRF) (SARChI grant 98566 to Adrienne Edkins), as well as Rhodes University. Samkelo Malgas was supported by the Research Development Programme from the University of Pretoria (grant 2896).

CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

Data are available upon request.

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How to cite this article: Ramatsui, L., Sithole, T., Gandla, M. L., Jönsson, L. J., Edkins, A. L., Malgas, S., & Pletschke, B. I. (2024). *In vitro* evaluation of the application of an optimized xylanase cocktail for improved monogastric feed digestibility. *Journal of Animal Physiology and Animal Nutrition*, *108*, 596–610. https://doi.org/10.1111/jpn.13917