Xylo-oligosaccharide-based prebiotics upregulate the proteins of the Sus-like system in caecal Bacteroidetes of the chicken: evidence of stimbiotic mechanism

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ABSTRACT

The present study was conducted to investigate the stimbiotic mechanism of xylo-oligosaccharide (XOS) in degrading the complex polysaccharides by the caecal bacteria of the chicken, by applying a proteomic approach. A total of 800 as-hatched Ross 308 broiler chicks were equally divided into 4 experimental pens (200 chicks per pen) at a commercial barn, allocating 2 pens per treatment. Birds were fed ad libitum with 2 dietary treatments; CON (without XOS) and XOS (with 0.1g XOS/kg diet) from days 0 to 35. Individual birds were weighed weekly whereas caecal content was obtained on day 35 from 10 of the individually weighed and cervically dislocated birds. The caecal bacteria were lysed and their proteins were quantified using label-free quantitative proteomic mass spectrometry. The results showed that XOS significantly increased ($P<0.05$) bird weight on days 7, 14, 21 and 28, and body weight gain on days 7, 14, 21 and 35 compared to CON. However, no difference ($P>0.05$) in body weight gain was observed from days 0 to 35 between CON and XOS. The proteomic analysis of caecal bacteria revealed that 29 proteins were expressed differently between the CON and the XOS group. Out of 29, 20 proteins were significantly increased in the XOS group compared to CON and 9 of those proteins belonged to the Starch Utilising System (Sus)-like system of the gram-negative Bacteroidetes. Bacteroides thetaiotaomicron (Bt) is a significant constituent of the human gut microbiota, known for its remarkable ability to hydrolyse most glycosidic bonds of polysaccharides. This microorganism possesses a five-protein complex in its outer membrane, named the starch utilization system (Sus), responsible for adhering to, breaking down, and transporting starch into the cell. Sus serves as an exemplar system for numerous polysaccharide utilization loci that target glycans found in Bt and other members of the Bacteroidetes phylum. The proteins of the Sus-like system are involved in the degradation of complex polysaccharides and transportation of the oligosaccharides into the periplasm of the caecal bacteria where they are further broken down

into smaller units. These smaller units are then transported into the cytoplasm of the cell where they are utilised in metabolic pathways leading to the potential generation of short-chain fatty acids, thus improving the nutritive value of residual feed. In conclusion, XOS supplementation upregulates the expression of the proteins of the Sus-like system indicating its role as a stimbiotic.

Key words: Xylo-oligosaccharides, Stimbiotic, Sus-like system, Caecal Bacteroidetes, Proteomics

INTRODUCTION

Monogastric animal diets inevitably contain substantial amounts of arabinoxylans, a major component of the non-starch polysaccharides that form the cell wall of cereal grains such as wheat, maize or barley that make up 50-60% of the monogastric diets (Zannini et al., 2022). It is well-documented that monogastric farm animals; chickens and pigs, do not produce the enzymes needed to break down these arabinoxylans (Petry et al., 2021). Hence, there has been significant interest in monogastric nutrition to enhance the arabinoxylan degrading or fibre fermenting microbiota in the GIT to curtail dysbacteriosis and improve energy extraction from the fibre portion of the feed. The addition of xylanases or xylo-oligosaccharides (XOS) has been shown to improve broilers' production performance and gut health parameters in many studies (Aachary and Prapulla, 2011; Pourabedin and Zhao, 2015). Nonetheless, the presently proposed mechanism by which XOS is quantitatively fermented into short-chain fatty acids (SCFAs) does not explain to account for the elevated concentrations of SCFAs observed, considering the negligible quantities of XOS introduced to poultry diets (Ribeiro et al., 2018). For example, supplementation of broiler diets with 0.1 g/kg and piglet diets with 0.2 g/kg XOS improved performance (Liu et al., 2018; Ribeiro et al., 2018). However, when evaluating the energy contribution of XOS, 0.1 g XOS only provides 0.3 kcal/kg of energy to the diet suggesting that the mechanism cannot consist of quantitative fermentation only (Cho et al., 2020).

The term stimbiotic (STB) was proposed and defined by González-Ortiz et al. (2019), as "an additive that stimulates a fibre-degrading microbiome resulting in an increase in fibre fermentability even though the additive itself contributes little to short-chain fatty acid production." The concept of STBs is that they are not quantitatively fermented by the microbiome like other prebiotics, but instead, they enhance the fermentation of fibre that is already present in the diet (Bedford, 2019).

Many commercialised prebiotics used in the animal industry such as fructo-oligosaccharides (FOS), galacto-oligosaccharides (GOS) and mannan-oligosaccharides (MOS) are all understood to be quantitatively fermented into SCFAs (Pan et al., 2009). However, dietary supplementation with XOS or in vivo creation of XOS in the gastrointestinal tract (GIT) via the addition of supplemental xylanases likely results in insignificant increments in SCFAs directly but significant increments indirectly by preferentially stimulating the growth and activity of beneficial bacteria such as Bifidobacterium and other lactic acid producing bacteria and lactate consuming butyrate-producing bacteria in the hindgut especially caeca of poultry (Cho et al., 2020).

Several studies have recently suggested that dietary XOS supplementation could improve broiler feed efficiency by improving nutrient digestibility (Ribeiro et al., 2018; Craig et al., 2020) as well as affecting the gut immune system via stimulation of commensal bacteria which stimulate the host's immune system, triggering defensive reactions that thwart the colonization and intrusion of pathogens (Pourabedin et al., 2017; Yuan et al., 2018). The distal ileum, cecum, and colon of broilers are thought to be fermentation sites whose function is influenced by microbiota composition (Sekelja et al., 2012). Firmicutes (up to 75%) and Bacteroidetes (between 10% and 50%) species predominate in the chicken gut microbiota (Dumonceaux et al., 2006). Around 90% of the bacteria in the chicken gastrointestinal tract are still unknown species, indicating that our understanding of chicken intestinal microbiota is limited (Bjerrum et al., 2006). However, it is not known if XOS affects the number of these groups in the distal intestine of chickens. Though Videnska et al. (2014) studying broilers found that Firmicutes dominated the caecal microbiota (76.2%), followed by Proteobacteria (14%), and Bacteroidetes (6.5%), it is yet unclear how dietary XOS supplementation affects bacterial diversity, microbial structure, and gut microbial composition in broilers and what

mechanism exits behind STB effects of XOS to breakdown the fibre or complex polysaccharides.

Bacteroides thetaiotaomicron (Bt), a significant component of the human gut microbiota, is renowned for its impressive capability to cleave glycosidic bonds in polysaccharides. This microorganism possesses a complex in its outer membrane consisting of five proteins, referred to as the starch utilization system (Sus) (Porter et al., 2018). The primary role of Sus is to attach to starch, break it down, and facilitate its transport into the cell. Sus stands as a representative model for various loci involved in utilizing polysaccharides, targeting specific glycans within Bt and other members of the Bacteroidetes phylum (Porter et al., 2018). The proteins within the Sus-like system play a crucial role in decomposing intricate polysaccharides and transporting resulting oligosaccharides into the periplasm of caecal bacteria. These compounds are further degraded into smaller components. Subsequently, these smaller units are conveyed into the cell's cytoplasm, where they become integral to metabolic pathways that produce SCFAs. However, to the best of the authors' knowledge, there is no published evidence on the mechanism of XOS on the gut microbiome of broilers whether there is upregulation or downregulation of proteins identified by the proteomic approach which take part in the breakdown of fibre or complex polysaccharides (e.g., xylans).

The current study was conducted to investigate the stimbiotic mechanism of xylo-oligosaccharide-based prebiotic by a proteomic approach, using a broiler model whether there is upregulation or downregulation of proteins extracted from the caecal microbiome which may take part in the degrading of fibre or complex polysaccharides.

MATERIALS AND METHODS

Animals, Housing and Experimental Treatments

The study trial was approved (Approval No. ARE716) by the Ethical Review Committee of Nottingham Trent University (NTU), with all procedures following the institutional and national guidelines for the care and use of animals (Animal Scientific Procedures Act, 1986). A total of 800 as-hatched Ross 308 broiler chicks were housed at a commercial farm barn, divided into 4 pens, each with the dimensions of 3m × 6m and 200 chicks, allocating 2 pens per treatment and ensuring that the pens within each treatment had similar environmental conditions. In each pen, 60 birds were tagged to record the individual weight of the birds on placement and weekly. The trial was purposefully conducted at a commercial barn rather than a research facility to mimic conditions close to those of a commercial unit. The birds were provided with two dietary treatments; CON (a basal diet without prebiotics) and XOS (a diet supplemented with prebiotic XOS at 0.1 g XOS/kg diet). A prebiotic product, XOS, was supplied by AB Vista, UK, which was corncob-derived with a degree of polymerization between 2 and 7. Feed was provided in feed hoppers and water via bell drinkers. Birds were fed ad libitum from day 0 to 35 and stocked at a commercial stocking density under 38 kg/m^2. Heating was provided via gas brooders with a set point of 32°C at the placement, then decreased by about 0.5°C each day to reach 21°C on day 21, and then maintained according to the Aviagen guidelines for Ross 308 broilers (Aviagen, 2009). The dark period was set to 1 hour on placement day and then increased by 1 hour each day until reached 6 hours (a block of 4 hours and 2 hours) on day 6 which was maintained for the rest of the trial period (days 7-35) according to the commercial guidelines of the poultry industry. Birds were inspected twice daily by NTU poultry research staff primarily to make sure adequate environmental conditions were provided and bird welfare was not compromised. The birds were fed maize-soy diets manufactured to meet the nutritional requirements of birds following the guidelines of Ross 308. The diets were formulated and supplied by AB Vista, UK as starter crumbs, grower pellets and finisher pellets. The study lasted for 35 days with a three-phase feeding

programme; starter diets from d 0 to 14, grower diets from d 14 to 28 and finisher diets from d 28 to 35. The composition of the basal diets is presented in Table 1.

Bird Weight, Body Weight Gain and Sampling

Weekly bird weight and body weight gain of birds were recorded from 60 individually tagged and weighed birds in each pen. For sampling, 5 birds per pen, each weighing nearest to the average weight of 60 tagged birds, were selected on day 35 and transported in the crates from the commercial barn to the NTU Poultry Research Unit where they were humanely euthanised via cervical dislocation by the trained personnel. Both caeca from each bird were excised and collected separately in clean labelled bags. The caeca were snap-frozen on dry ice and then stored at -20°C for further analysis of caecal content.

Bacterial Extraction

Bacterial cells were extracted from the caecal contents following the method described by Tang et al (2014). Briefly, 1.5–2.5g of defrosted caecal content was resuspended in 10 mL sterile phosphate-buffered saline (PBS) containing 0.1% w/v Tween 80 in 50 mL tubes. The samples were spun at 300 rpm at 8°C to separate the bacteria from the caecal digesta and supernatant was collected in fresh 50 mL tubes. Fresh sterile PBS with 0.1% w/v Tween 80 was added to the sample tubes and the tubes were vortexed to resuspend the pellet. This cycle was repeated four times to gather bacterial cells. Approximately 40 mL of supernatant was obtained from each sample. Finally, the pellet was discarded and the cells in the supernatant were pelleted by centrifugation (Centrifuge 5810/ 5810 R - Benchtop Centrifuge, Eppendorf, UK) at 14000 rpm for 20 minutes at 4°C. The recovered cells were washed three times in 50 mL PBS plus 0.1% Tween 80 through resuspension and centrifugation and stored at -20°C.

Protein Extraction and Quantification

The bacterial pellet was lysed by adding 1 ml of lysis buffer containing 8M Urea in 50 mM Tris, and 1µL 1% ProteaseMAXTM (ProteaseMAX™ Surfactant: 50 mM NH_4HCO_3)

(Promega, USA). The pellet was mixed by vortexing. The tubes were then placed in a sonic bath three times for 1 minute at 30-second intervals on ice. The tubes were then centrifuged at 14000 rpm for 10 min at 4°C. The concentration of proteins in the supernatant was determined using the Bicinchoninic Acid (BCA) assay kit (Sigma-Aldrich, UK) using bovine serum albumin as standard according to the manufacturer's instructions. The protein concentration in all samples was normalised to 1.5–2.0µg/µL before Liquid Chromatography-Mass Spectrometry (LCMS) analysis.

Protein Digestion and Clean-up

A 50µg of protein (approximately 25µL) from each sample was transferred to an individual 1.5mL lo-bind microtube (Eppendorf, UK). The protein lysate solution was then dried in a vacuum concentrator (Concentrator Plus, Eppendorf, UK) at 60°C. Each tube was then reconstituted in 5% SDS in 50 mM triethylammonium bicarbonate (TEAB) at pH 7.5. The sample tubes were then placed in a sonicating water bath for 10 min and centrifuged at room temperature (~25°C) for 4 min at 13,000 rpm to remove insoluble matter. The proteins were then reduced and alkylated by adding 1uL of 0.5M dithiothreitol (DTT) to each tube and then incubated at 56°C for 20 min on a shaking thermomixer (Bioer Mixing Block MB-102, Profcontrol GmbH, Germany). After cooling to room temperature, 2µL of 0.5M iodoacetamide (IAA) was added and the tubes were incubated in the dark for 15 min at room temperature (~25°C).

The proteins were then cleaned and digested using the Protifi S-Trap protocol (Protifi, USA). In brief, acidified samples were precipitated with 6 volumes of S-Trap binding buffer (MeOH:TEAB 9:1) and pipetted onto the S-Trap micro spin column and centrifuged for 4 min at 4000 rpm until the suspension passed through the column. The protein suspension now trapped on the S-Trap was then washed 4 times by adding 150µL of S-Trap binding buffer and

spinning at the same parameters as previously to flow through to remove contaminants, salts and any detergents remaining from the extraction steps.

Trypsin solution (5µg trypsin at 1:10 trypsin:protein ratio, in 20µL) was added to the S-Trap as per the manufacturer's instructions and loosely capped and placed in a thermomixer at 47°C for 90 min (not shaking). Peptides were then eluted from the S-Trap by sequential addition and centrifugation to the microtube of 40µL 50 mM TEAB followed by 40µL 0.2% formic acid and finally 35µL of 50% acetonitrile containing 0.2% formic acid. Samples were dried at 60°C in a vacuum concentrator, reconstituted in 30µL 5% acetonitrile/0.1% formic acid, and transferred to a high recovery plastic HPLC vial for mass spectrometry.

Mass Spectrometry

Samples were analysed on a Sciex TripleTOF 6600 mass spectrometer coupled in line with an Eksigent ekspert nano LC 425 system running in microflow (5µL/min) mobile phase B (100% acetonitrile + 0.1% formic acid) over mobile phase A (0.1% formic acid). Samples were analysed as described previously (Gruet et al., 2020) in two different modes; 1) 3 injections of a pool of all samples in Information Dependent Acquisition or IDA (also known as Data Dependent Acquisition/DDA) to generate a list of protein/peptide identifications to use a spectral/ion library for subsequent SWATH analysis. 2) Individual samples in SWATH-MS mode (also known as Data Independent Acquisition or DIA). In brief, 4µL (~6.67µg based on pre-digest protein level) of the reconstituted sample was injected and trapped onto a YMC Triart-C18 pre-column (5 mm, 3µm, 300µm ID) at a flow rate of 10µL min mobile phase A 100% for 2 min. The sample was then eluted off the trap column by valve switching and running a gradient and onto a YMC Triart-C18 analytical column (15cm, 2µm, 300µm ID) that was in line with the Sciex TripleTOF 6600 Duospray Source using a 50µm electrode in positive mode, +5500 V for both IDA (87 min run) and SWATH (57 min run).

Protein Quantitation

Following analysis with MarkerView 1.2 Software (SCIEX), and generation of a volcano plot of Log2 fold change vs P-value, after threshold cut-offs for significance, "interest lists" of proteins that had increased in the microbiota of the XOS-fed birds compared to the corresponding proteins in the microbiota of the control group was generated.

Data Processing

Spectral library generation, alignment and fold change analysis were performed using PeakView 2.1 software (SCIEX, Framingham, USA) and the SWATH microapp. In brief, IDA data were searched using ProteinPilot 5.02 (iodoacetamide alkylation, biological modifications emphasised in a thorough search) against the UniProt TrEMBL unreviewed all bacteria database (October 2019, uniprot.org). The group file output from ProteinPilot was imported as an "ion library" into the SWATH microapp in PeakView 2.1 and aligned against the SWATH using 9 endogenous peptides present in all samples.

The aligned data were then processed in PeakView to generate quantitative data based on peak areas for each protein using the following summed parameters: 12 peptides per protein, and 6 transitions per peptide at a peptide confidence threshold of 97%. A false discovery level of 5% (peptide) was used and modified peptides were excluded. An XIC width of 30ppm was used and the retention time window was finally set to 5 min. The processed data were exported (Peak Areas) into MarkerView 1.2 (SCIEX) which was used to carry out a comparative analysis of the peak areas per protein and a T-Test and fold change analysis was carried out. Results were then plotted as log2 fold change vs P-value and an interest list of significantly changed proteins was generated with a cut-off of Log2 fold change ±0.3, $P<0.05$. Significantly changed protein intensity data were exported to a software program 'heatmapper' (Babicki et al., 2016), to generate a heatmap and carry out hierarchical clustering analysis (distance measurement method 'Pearson', clustering method 'complete linkage', with no grouping).

RESULTS

Bird Weight and Body Weight Gain

The results (Table 2) showed that bird weight was significantly increased on days 7 ($P<0.010$), 14 ($P<0.010$), 21 ($P<0.010$) and 28 ($P=0.004$) in XOS fed group compared to the CON group. However, further with age, the difference in bird weight on day 35 ($P=0.812$) was evened out between the CON and XOS groups. Body weight gain of birds was significantly higher ($P<0.01$) in XOS fed compared to the CON group for the first, second, third and fifth weeks. However, no difference in body weight gain was observed during the fourth week ($P=0.257$) and overall experimental period; d 0-35 ($P=0.818$).

Proteomics

A ProteinPilot search of the 3 injections of the pooled sample identified 421 protein groups at a 1 % False Discovery Rate (FDR). Generating a spectral library in PeakView (SWATH microapp) and removing shared and modified peptides resulted in a library of 418 unique proteins for SWATH quantitation, with 382 reaching the quality threshold for quantitation. The protein with the highest coverage and most peptides identified was Collagen triple helix repeat (20 peptides), species BACUN (Bacteroides uniformis) from which its peptides were used for library retention time alignment.

Proteomic analysis showed that a total of 20 proteins out of 29 (Table 3) were significantly upregulated in the XOS group compared to the CON. Out of these 20 upregulated proteins, 9 proteins are part of the Sus system (including SusC, SusE and the biopolymer transport complex ExbB) in Bacteroides spp. The number of uncharacterised proteins and protein domains of unknown function (DUF) also increased in the XOS group. Interestingly, proteins quantified with increased expression and involved in the sus-like system were SusC proteins, predominantly cell outer membrane proteins in the current study. These were SusC/RagA

family TonB-linked outer membrane protein in Bacteroides vulgatus dnLKV7, SusC/RagA family TonB-linked outer membrane protein in Faecalibacterium spp. An121, Biopolymer transporter ExbB in Bacteroides vulgatus CL09T03C04, SusC/RagA family TonB-linked outer membrane protein in Bacteroides massiliensis B84634 (UniProt Accession: I9IW03 and R9HTP3), SusE domain-containing protein in Bacteroides dorei CL03T12C01, SusC/RagA family TonB-linked outer membrane protein in Anaeromassilibacillus spp. An200, TonB-dependent receptor (generic) in Bacteroides stercoris CC31F, and TonB-dependent receptor in Bacteroides vulgatus dnLKV7.

The hierarchical clustering heatmap (Figure 1) shows that the samples were clustered clearly concerning most of the significantly changed proteins. The upregulated 20 proteins in the XOS group are represented from numbers 26 to 22 in the bottom right corner of the given heatmap. The UniProt Accession and Protein IDs of these proteins with significantly increased expression are given in Table 3.

DISCUSSION

The sole aim of the study was to investigate the stimbiotic mechanism of XOS where a proteomic approach was applied to evaluate the range of proteins produced by the entire caecal microbiota in response to XOS supplementation in the diets and explore the mechanism lying behind the effects of XOS on birds. The increase in bird weight may partly be explained by the upregulation of the Sus-like and the transporter proteins in the XOS-fed group. The upregulation of these Sus-like and the transporter proteins in the XOS group which were significantly increased in fold change relative to the CON group may have played a vital role in degrading the complex polysaccharides and absorbing nutrients more efficiently compared to the CON group with no XOS, and this could have been reflected in increased bird weight or body weight gain at certain age points. Suo et al. (2015) supported the idea that

297 XOS directly reached the mucosa of the small intestine to boost nutritional absorption and
298 that XOS might improve animal health when they found significantly improved FCR in the
299 XOS-fed group compared to the control. However, the current findings regarding bird weight
300 or body weight gain were not in agreement with the previous findings as those authors did not
301 observe improved body weight ($P>0.05$) of the birds which may be due to the dosage rate or
302 age of the birds of XOS as they fed the birds with 0, 25, 50, 75, 100 XOS mg per kg of diet
303 from days 0 to 42. Similarly, Maesschalck et al. (2015) found no difference ($P>0.05$) in the
304 bird weight or body weight gain at days 0-26 and 0-39, where the birds were fed a wheat-rye
305 diet with or without 0.2% XOS (days 1–13) and 0.5% XOS (days 14–26 and 27–39). Singh et
306 al. (2021) also found no difference in bird weight gain from day 0 to 42 between the XOS-fed
307 and CON groups on corn-soy diets. Both studies used different inclusion rates of XOS
308 compared to the current one. The difference in findings may be due to the inclusion rate or
309 composition of XOS, the age of the birds or diet formulation. Interestingly, the final bird
310 weight at the end of the trial on day 35 was evened out between XOS and CON groups.
311 Firstly, this is probably because mature birds possess more diverse and intricate microbial
312 populations (Awad et al., 2016; Ocejo et al., 2019), which could aid them in more efficiently
313 using nutrients in later growth stage. Secondly, compared to the control group, introducing
314 XOS supplements during early stages might initially facilitate the growth of young birds
315 which can lead to the adaptation of gut microbial communities to utilize arabinoxylan or
316 glycans effectively, thereby promoting growth (Bautil et al., 2020).
317 The current study's main objective was to explore the mechanism of the caecal bacteria
318 involved in the degradation of polysaccharides, by applying a proteomic approach behind the
319 effect of XOS rather than the efficacy of XOS. It was found that Sus-like proteins e.g.,
320 SusC/RagA family TonB-linked outer membrane protein, and SusE domain-containing
321 protein, were significantly higher in the XOS group compared to the CON group. Out of the

20 significantly increased proteins, 12 were belonging to Bacteroids spp. In addition, both the TonB-dependent receptor and the biopolymer transport protein ExbB were also found to be higher in the same spp. SusC-like proteins are members of the TonB receptor family involved in the transport of oligosaccharides across the outer membrane into the periplasmic space with the help of energy derived from proton motive force and the TonB-ExbBD complex (Martens et al., 2009).

The Sus-like system is an operon of 8 genes located within the polysaccharide utilisation loci (PULs) on the chromosome of Gram-negative Bacteroides. The Starch Utilisation System (Sus) was discovered in Bacteroides thetaiotaomicron, a prominent human gut Bacteroidete by Slayers et al. (1977a; 1977b), through their work on starch degradation by the bacterium (Figure 2). Subsequent research in microbial genome sequencing uncovered the derivatives of the prototypic (Sus) system which was called the “Sus-like system”. They are particularly well represented in the genomes of B. thetaiotaomicron and many other Bacteroidetes. The proteins expressed by the Sus-like system are either outer membrane or periplasmic proteins. An important characteristic of these proteins is their synchronised action involved in polysaccharide binding and degradation (Martens et al., 2009). Essentially they confer the ability to Bacteroides (a genus of Bacteroidetes) to metabolise a single glycan or a group of related glycans (Shipman et al., 2000; Martens et al., 2008; Sonnenburg et al., 2010; Dodd et al., 2010).

The current study showed significantly increased expression of SusC and SusE proteins in the XOS compared with the CON group which were mainly outer membrane proteins. Amongst 9 proteins involved in the sus-like system, SusC was observed with the highest expression in the current study. Bacteroidetes species bind oligosaccharides and transport them through the outer membrane for further digestion using cell surface proteins associated with the starch utilisation system (Sus) proteins SusC and SusD. To promote the utilisation of different

polysaccharides, these bacteria often develop hundreds of SusC-like porins and SusD-like oligosaccharide-binding proteins.

Each Sus-like system contains at least one pair of outer membrane proteins homologous to SusC and SusD, which are essential for the import and degradation of starch in the prototypic system, Sus (Shipman et al., 2000). SusC-like proteins are predicted in TonB-dependent receptors that span the outer membrane and transport oligosaccharides in an energy-dependent manner. SusD-like proteins are outer membrane lipoproteins that are oriented towards the external environment; they bind directly to specific glycans and contribute to the capture and delivery of oligosaccharides to the SusC transporter (Koropatkin et al., 2008; Sonnenburg et al., 2010). SusC- and SusD-like proteins work in a coordinated manner with other outer membrane glycan-binding proteins and polysaccharide degrading enzymes (glycoside hydrolases, polysaccharide lyases, and carbohydrate esterases), which are grouped into sequence-based families in the Carbohydrate Active Enzymes (CAZy) database (Cantarel et al., 2009).

Concerning xylan degrading, Sus-like machinery was reported by Dodd et al. (2010) in Prevotella bryanatii B14, a Bacteroidete that has been frequently isolated from the rumen microbiome. The authors reported the discovery of an invariant 6 gene cluster flanked by either biochemically categorised or predicted glycoside hydrolases and carbohydrate esterases in P. bryantii B14. This gene cluster was found to be critical to xylan utilisation in this bacterium and more importantly was highly conserved in other xylanolytic Prevotella and Bacteroides spp. derived from the bovine rumen and the human colonic microbiomes which suggests “a conserved mechanism for xylan utilisation by xylanolytic Bacteroidetes” (Dodd et al., 2010). However, no protein expression belonging to Prevotella bryanatii B14 was observed in the current study in contrast to Dodd et al. (2010) which may be due to the differences in animal species, diets or even the microbial ecologies between the two species.

In addition, the SusC gene is a part of the core xylan utilisation system which is conserved among certain species within the phylum Bacteroidetes (Dodd et al., 2010). The presence of two xylan PULs, the large xylan PUL (PUL-xylL) and the small xylan PUL (PUL-xylS) in Bacteroides ovatus were described by Rogowski et al. (2015), both of which encode SusC-like proteins. Mendis et al. (2018) used three different SusC transcripts as a proxy for the expression of PUL-xylL and PUL-xylS in B. ovatus growing on xylan substrates differing in their degree of polymerisation and degree of substitution. The increased expression of SusC-like proteins in the present study may, therefore, indicate the upregulation of the entire Sus-like system which includes glycoside hydrolases (GH) responsible for the breakdown of oligosaccharides encoded by either one or both xylan-PULs which supports the proposed stimbiotic mechanism of XOS (Foley et al., 2016).

Following the hypothesis, the current study explored the mechanism behind the stimbiotic effect of XOS in meat poultry that XOS upregulates the proteins, specifically involved in the sus-like system for the degradation of complex polysaccharides. To the authors' best knowledge, this is the first study providing evidence that a sus-like system is involved in the chicken caecal microbiota for the breakdown of complex polysaccharides. However, it was not possible to measure the feed intake for individual pens due to the set-up of a commercial barn which remains the limitation of the current study and might have confounded the results but needs further investigation. Furthermore, future work to support this study would involve the isolation of several different Bacteroides from the caeca of chickens reared under fully controlled conditions, and growing them on media containing XOS used in this study as the sole carbon source. The levels of the SusC transcript from these bacteria can then be determined using RT-PCR which would indicate the expression of the cognate PULs. Furthermore, the activity of xylosidases released in the growth media could be quantified

spectrophotometrically by continuous monitoring of xylose released using a D-xylose detection kit.

In conclusion, this study for the first time using label-free proteomic mass spectrometry indicated that supplementation of XOS in broiler diets stimulated the Gram-negative Bacteroidetes to upregulate their xylan degrading (Sus-like) system via upregulation of SusC-like and other membrane proteins involved in the transport of complex polysaccharides across the outer membrane. Understanding the mechanism of XOS prebiotics, highlighting their potential to upregulate the proteins related to the sus-like system, can promote our understanding regarding the breakdown of polysaccharides by the microbiome, leading to advantageous outcomes for broiler birds. Supplementing XOS can help the birds utilize diets with complex polysaccharides more efficiently by transporting and breaking them down into smaller units in the periplasm and then cytoplasm of caecal microbiota where they may be utilised in metabolic pathways leading to the potential generation of SCFAs, which may impede the growth of intestinal pathogens, thus promoting gut health, growth performance, and nutritive value of residual feed. Understanding the mechanism of XOS can also promote the scope to use non-traditional feed resources rich in fibre or complex polysaccharides economically to manufacture poultry diets. However, further evidence from in vitro studies using pure cultures of Bacteroides from the chicken caecum is required for further confirmation.

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