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The effects of xylo-oligosaccharides on performance and microbiota in broiler chickens

1 Running Head: XOS affect broiler performance and colonization

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20 ## Abstract

21 In broiler chickens, feed additives, including prebiotics, are widely used to improve gut health and 22 to stimulate performance. Xylo-oligosaccharides (XOS) are hydrolytic degradation products of 23 arabinoxylans that can be fermented by the gut microbiota. In the current study it was aimed to 24 analyze the prebiotic properties of XOS when added to the broiler diet. Administration of XOS to 25 chickens, on top of a wheat/rye-based diet, significantly improved the feed conversion ratio. XOS 26 significantly increased villus length in the ileum. It also significantly increased numbers of 27 lactobacilli in the colon and *Clostridium* cluster XIVa in the caeca. Moreover, the number of gene 28 copies encoding the key bacterial enzyme for butyrate production, butyryl-CoA:acetate CoA-29 transferase, was significantly increased in the caeca of chickens administered XOS. In this group of 30 chickens, at species level, *Lactobacillus crispatus* and *Anaerostipes butyraticus* were significantly 31 increased in abundance in the colon and caecum, respectively. *In vitro* fermentation of XOS 32 revealed cross-feeding between *L. crispatus* and *A. butyraticus*. Lactate, produced by *L. crispatus* 33 during XOS fermentation, was utilized by the butyrate-producing *Anaerostipes* species. These data 34 show the beneficial effects of XOS on broiler performance when added to the feed, which 35 potentially can be explained by stimulation of butyrate-producing bacteria through cross-feeding of 36 lactate and subsequent effects of butyrate on gastrointestinal function.

37 ## Introduction

38 Cereal fibers are composed of carbohydrate polymers that are resistant to digestion in the small 39 intestine of monogastric animals but are completely or partially fermented in the distal gut, and are 40 believed to stimulate gut health (1). The main components of the cereal fiber fraction are 41 arabinoxylans (AX), pectins, resistant starch, cellulose, β-glucans and lignin (2). Hydrolytic 42 degradation of the heteropolymer AX results in a mixture of arabinose substituted xylo-43 oligosaccharides or arabinoxylan-oligosaccharides (AXOS) and non-substituted xylo-

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44 oligosaccharides (XOS) (3). XOS are oligomers consisting of xylose units, linked through β-(1-4)
45 linkages (4). Selective fermentation of XOS has been shown to induce changes in both the
46 composition and activity of the gastrointestinal microbiota, improving the health and well-being of
47 the host. This suggests that XOS could fulfill the definition of a prebiotic (5). The production of
48 lactate and short chain fatty acids (SCFA), including butyrate, upon fermentation of XOS, has been
49 confirmed in several *in vitro* and *in vivo* studies (3, 6). Lactate can stimulate butyrate production
50 due to cross-feeding between lactate-producing bacteria and lactate-utilizing butyrate-producing
51 bacteria from *Clostridium* cluster XIVa (7). Butyrate has proven beneficial effects on
52 gastrointestinal function, since it has anti-inflammatory properties, fuels epithelial cells and
53 increases the intestinal epithelial integrity. In addition, butyrate has been shown to improve growth
54 performance in production animals and to change the microbiota composition and metabolic
55 activity of the microbial ecosystem in the intestine (8, 9).

56 Beneficial effects of XOS have already been described in rats. In these studies, XOS was shown to
57 significantly increase the bifidobacteria and lactobacilli population in the caecum (10, 11). An *in*
58 *vitro* study using swine faecal microbiota showed the highest SCFA production during fermentation
59 of XOS (12). To our knowledge, there is not much published research on the effect of XOS on the
60 gastrointestinal health of chickens except for the recent publication of Zhenping et al. (13),
61 showing the increased growth performance, enhanced endocrine metabolism and improved immune
62 function in broiler chickens after in feed supplementation of straw-derived XOS. However, the
63 effect of XOS on the microbiota composition in broilers has not yet been described.

64 In the broiler chicken, the distal ileum, the caeca and the colon are regarded as fermentation
65 chambers whose function is determined by the microbiota composition (14, 15). The chicken gut
66 microbiota is dominated by species belonging to the phyla *Firmicutes* (up to 75 %) and
67 *Bacteroidetes* (between 10 % and 50 %) (16-22). Around 90 % of the bacteria in the chicken
68 gastrointestinal tract are unknown species, indicating that the knowledge of the intestinal microbiota

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69 of chickens is incomplete (23-24). The majority of sequences within the *Firmicutes* phylum belong
70 to the families *Ruminococcaceae* and *Lachnospiraceae*, the so called *Clostridium* cluster IV and
71 XIVa, respectively (25). Both families contain numerous members that are known to produce
72 butyrate as a fermentation end product and are therefore linked to beneficial effects on
73 gastrointestinal function (26, 27). Whether the abundance of these groups in the distal gut of
74 chickens is affected by XOS is unclear.

75 In the current study, we analyzed the effect of XOS administration on the performance of broilers.
76 In addition, we aimed to identify the shifts in microbiota composition induced by XOS to explain
77 possible beneficial effects on gastrointestinal health, with emphasis on butyrate production.

78 Materials and Methods

79 Additives/substrates

80 In the *in vivo* study corncob-derived XOS35 (Longlive Bio-technology, Shandong, China) was used
81 as feed additive. XOS35 is a mixture of 35 % XOS with a degree of polymerization (DP) between
82 2-7 and 65% maltodextrin. In the *in vitro* fermentation study XOS35, maltodextrin (Sigma-Aldrich,
83 St. Louis, United States) and XOS95 (Longlive Bio-technology, Shandong, China), a mixture of 95
84 % XOS with DP 2-7 and 5 % xylose, were used. The XOS95 and maltodextrin were used be
85 confirm that the effects of XOS35 in the *in vivo* trial were explained by the XOS.

86 Animals and diets

87 A total of 192 male and 192 female one-day-old Ross-308 broiler chickens were randomly divided
88 in 12 pens (3 pens of female and 3 pens of male birds per treatment and 32 chickens per pen) and
89 housed on solid floor covered with wood shavings. Light schedule was set to provide an 18h
90 light/6h dark cycle. The infrared bulbs (1 per pen during the first week) together with the central
91 heating system provided optimal temperature. All animals were fed a wheat/rye-based diet with
92 XOS (experimental group) or without XOS (control group) of which the composition is shown in

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93 Table 1. The experimental starter feed (fed from the first day of age until day 13) was supplemented
94 with 0.2 % XOS, the grower feed (fed from day 14 until day 26) and the finisher feed (fed from day
95 27 until day 39) were supplemented with 0.5 % XOS. At 13, 26 and 39 days of age, all broilers and
96 the feed leftovers were weighed per pen to calculate the feed conversion ratio (FCR), weight gain
97 (WG) and feed intake (FI). At 26 days of age, three chickens per pen were euthanized by an
98 intravenous overdose of sodium pentobarbital 20% (Kela, Hoogstraten, Belgium). The complete
99 content of caecum and colon was collected and stored at -70°C, while a part of the ileum at the level
100 of Meckel's diverticulum was fixed in 4% formaldehyde.

101 **Morphological examination**

102 Formalin fixed ileum segments taken at the level of Meckel's diverticulum were dehydrated in
103 xylene, embedded in paraffin and sectioned in 4 µm slides. The sections were deparaffinized (2 x 5
104 min) in xylene, rehydrated in isopropylene (5 min), 95 % alcohol (5 min) and 50 % alcohol (5 min)
105 and stained with haematoxylin and eosin. The sections were examined using light microscopy.
106 Villus length and thickness of *tunica muscularis* were measured by random measurement of 10 villi
107 and 10 measurements of *tunica muscularis* per section using Leica DM LB2 Digital (Leica
108 Microsystems Belgium BVBA, Diegem, Belgium) and a PC-based image analysis system,
109 LAS V3.8 (Leica application Suit V3, Diegem Belgium).

110 **Microbiota composition**

111 DNA extraction

112 DNA was extracted from caecum and colon content using the CTAB method as described
113 previously (29, 30). To 100 mg of intestinal content, 0.5 g unwashed glass beads (Sigma-Aldrich,
114 St. Louis, United States), 0.5 ml CTAB buffer (hexadecyltrimethylammonium bromide 5 % (w/v),
115 0.35 M NaCl, 120 mM K_2HPO_4) and 0.5 ml phenol-chloroform-isoamyl alcohol mixture (25:24:1)
116 (Sigma-Aldrich, St. Louis, United States) were added followed by homogenization in a 2 ml
117 destruction tube. The samples were shaken 6 times for 30 seconds using a beadbeater (MagnaLyser,

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118 Roche, Basel, Switzerland) at 6000 rpm with 30 seconds between shakings. After centrifugation (10
119 min, 8000 rpm), 300 µl of the supernatant was transferred to a new tube. The rest of the tube
120 content was re-extracted with 250 µl CTAB buffer and again homogenized with a beadbeater. The
121 samples were centrifuged for 10 minutes at 8000 rpm and 300 µl supernatant was added to the first
122 300 µl supernatant. The phenol was removed by adding an equal volume of chloroform-isoamyl
123 alcohol (24:1) (Sigma-Aldrich, St. Louis, United States) and a short spin. The aqueous phase was
124 transferred to a new tube. The nucleic acids were precipitated with two volumes of PEG-6000
125 solution (polyethyleenglycol 30 % (w/v), 1.6 M NaCl) for two hours at room temperature. After
126 centrifugation (20 min, 13000 rpm), the pellet was rinsed with one ml of ice-cold 70 % (v/v)
127 ethanol. The pellet was dried and resuspended in 100 µl RNA free water (VWR, Leuven, Belgium).

128 Quantitative PCR for the total bacteria and the butyryl-CoA:acetate-CoA transferase gene

129 The number of total bacteria and butyryl-CoA:acetate-CoA transferase genes was quantified in 3
130 samples per pen (18 samples per treatment). To determine the number of total bacteria, primers Uni
131 331F (5'-TCCTACGGGAGGCAGCAGT-3') and Uni 797R (5'-
132 GGACTAACCAGGGTATCTAATCCTGTT-3') were used (31). Amplification and detection was
133 performed using the CFX384 BioRad detection system (BioRad, Nazareth-Eke, Belgium). Each
134 reaction was done in triplicate in a 12 µl total reaction mixture using 2x SensiMix™ SYBR No-
135 ROX mix (Bioline, Kampenhout, Belgium), 0.5 µM final primer concentration and 2 µl of (50
136 ng/µl) DNA. The amplification program consisted of 1 cycle at 95 °C for 10 min followed by 40
137 cycles of 1 min at 94 °C, 1 min at 53°C and 2 min at 60 °C. The fluorescent products were detected
138 at the last step of each cycle. A melting curve analysis was done after amplification and was
139 obtained by slow heating from 60 °C to 95 °C at a rate of 0.5 °C/5 sec to confirm the specificity of
140 the reaction.

141 To quantify the number of gene copies encoding the butyryl-CoA:acetate-CoA transferase enzyme
142 primers BCoATscrF (5'-GCIGAICATTTCACITGGAAYWS-3') and BCoATscrR (5'-

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143 CCTGCCTTTGCAATRTCIACRA ANGC-3') were used (32). Each reaction was done in triplicate
144 in a 12 µl total reaction mixture using 2x SensiMix™ SYBR No-ROX mix (Bioline, Kampenhout,
145 Belgium), 2.5 µM final primer concentration and 2 µl of (50 ng/µl) DNA. The amplification
146 program consisted of 1 cycle at 95 °C for 10 min followed by 40 cycles of 30 sec at 95 °C, 30 sec at
147 53 °C and 30 sec at 72 °C.

148 16S sequencing to identify microbiota composition

149 Faecal samples derived from one animal per pen (6 per treatment) were used for 16S sequencing.
150 For each sample, 16S rDNA PCR libraries were generated with the primers E9-29 and E514-430
151 (33) targeting hypervariable regions V1-V3. The oligonucleotide design included 454 Life
152 Sciences's A or B sequencing titanium adapters (Roche Diagnostics, Vilvoorde, Belgium) and
153 multiplex identifiers (MIDs) fused to the 5' end of each primer. The amplification mix contained 5
154 U of FastStart high fidelity polymerase (Roche Diagnostics, Vilvoorde, Belgium), 1x enzyme
155 reaction buffer, 200 µM dNTPs (Eurogentec, Liège, Belgium), 0.2 µM of each primer and 100 ng
156 of genomic DNA in a volume of 100 µl. Thermocycling conditions consisted of a denaturation at 94
157 °C for 15 min followed by 25 cycles at 94 °C for 40 s, 56 °C for 40 s, 72 °C for 1 min and a final
158 elongation step of 7 min at 72 °C. These amplifications were performed on an Ep Master System
159 gradient apparatus (Eppendorf, Hamburg, Germany). Electrophoresis of the PCR products was done
160 on a 1 % agarose gel and the DNA fragments were plugged out and purified using the SV PCR
161 purification kit (Promega Benelux, Leiden, The Netherlands). The quality and quantity of the
162 products were assessed with a Picogreen dsDNA quantitation assay (Isogen, St-Pieters-Leeuw,
163 Belgium). All libraries were run in the same titanium pyrosequencing reaction using Roche MIDs.
164 All amplicons were sequenced using the Roche GS-Junior Genome Sequencer instrument (Roche,
165 Vilvoorde, Belgium), the sequence number of each sample is normalized to 2323 reads.
166 The 16S rDNA sequence reads were processed with the MOTHUR package (34). The quality of all
167 sequence reads were denoised using the Pyronoise algorithm implemented in MOTHUR and
168 filtered with the following criteria: minimal length of 425 bp, an exact match to the barcode and 1

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169 mismatch allowed to the proximal primer. The sequences were evaluated for the presence of
170 chimeric amplifications using Uchime (35). The resulting read sets were compared to a reference
171 dataset of aligned sequences of the corresponding region derived from the SILVA database 1.15 of
172 full-length rDNA sequences (http://www.arb-silva.de/) implemented in MOTHUR (36). The final
173 reads were clustered into operational taxonomic units (OTUs) using the nearest neighbour
174 algorithm using MOTHUR with a 0.03 distance unit cut-off. At the OTU level of analysis (OTU
175 definition level for a 0.02 distance matrix), a total of 3052 OTUs were created. A taxonomic
176 identity was attributed to each OTU by comparison with the SILVA database (80 % homogeneity
177 cut-off). As a secondary analysis all unique sequences for each OTU were compared to the SILVA
178 dataset 1.15 using BLASTN algorithm (37). For each OTU, a consensus detailed taxonomic
179 identification was given based upon the identity (less than 1 % of mismatch with the aligned
180 sequence) and the metadata associated with the best hit (validated bacterial species or not). The raw
181 sequences were deposited in Genbank (accession number: PRJNA277118).

182 ***In vitro* fermentation**

183 Bacterial strains, growth and co-culture studies

184 The butyrate-producing strain *Anaerostipes butyraticus* LMG 24724^T and the lactate producing
185 strain *Lactobacillus crispatus* LMG 9479^T were purchased from the LMG culture collection. *A.*
186 *butyraticus* and *L. crispatus* were grown in M2GSC (38) and Man-Rogosa-Sharpe (MRS) medium,
187 respectively, in an anaerobic chamber (Ruskinn technology, Bridgend, United Kingdom) with 84 %
188 N_2, 8 % H_2 and 8 % CO_2 at 37 °C.

189 The *in vitro* fermentation study was conducted using a nutrient-poor medium described by Moura et
190 al. (39) with minor modifications (0.85 g/l casitone, 0.15 g/l enzymatic digest of soya bean, 0.25 g/l
191 NaCl, 0.125 g/l K_2HPO_4, 5.0g/l bactopeptone, 5.0 g/l yeast nitrogen base and 0.5g/l resazurin. After
192 autoclaving 1 mg/ml cysteine-HCl, 1 % (v/v) of salt solution A (100.0 g/l NH_4Cl, 10.0 g/l
193 $MgCl_2.6H_2O$, 10.0 g/l $CaCl_2.2H_2O$), 1 % (v/v) trace solution (0.025 g/l $MnCl_2.4H_2O$, 0.02 g/l

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194 $FeSO_4.7H_2O$, 0.025 g/l $ZnCl_2$, 0.025 g/l $CuCl_2.2H_2O$, 0.05 g/l $CoCl_2.6H_2O$, 0.05 g/l SeO_2, 0.25 g/l
195 $NiCl_2.6H_2O$, 0.25 g/l $Na_2MoO_4.2H_2O$, 0.314 g/l $NaVO_3$, 0.25 g/l H_3BO_3 dissolved in 0.02M HCl)
196 and 1.2 % (v/v) vitamin/phosphate solution (0.0204 g/l biotin, 0.0205 g/l folic acid, 0.164 g/l Ca D-
197 pentothenate, 0.164 g/l nicotinamide, 0.164 g/l riboflavin, 0.164 g/l thiamin HCl, 0.164 g/l
198 pyridoxine HCl, 0.201 g/l *para*-amino benzoid acid, 0.0205 g/l cyanocobalamin dissolved in 54.7
199 g/l KH_2PO_4, filter sterile)) containing a mixture of SCFAs (final concentrations: acetate (31mM);
200 propionate (9mM); isobutyrate, isovalerate and valerate (1mM each)). A 5 % stock solution of
201 XOS35, maltodextrin and XOS95 was prepared in the nutrient-poor medium, filter-sterilized (0.2
202 µm), and diluted in the nutrient-poor medium to a final concentration of 0.5 % (v/v). Un-
203 supplemented nutrient-poor medium was used as control (blank). The final pH of the medium was
204 adjusted to 6.5 ± 0.1. The media were pre-incubated in an anaerobic cabinet until anaerobiosis, as
205 indicated by the colorless state of resazurin in the media. *A. butyraticus* and *L. crispatus,* pre-
206 cultured in M2GSC and MRS broth, respectively, at 37 °C under anaerobic conditions for 24 ± 1 h
207 without shaking, were diluted 100-fold in the supplemented and non-supplemented nutrient-poor
208 medium. The co-culture of *A. butyraticus* and *L. crispatus* was prepared using equal portions of the
209 inoculum (2 times 1/200) from the 2 pure cultures. After 24 h anaerobic incubation at 37 °C,
210 bacterial growth was monitored by measuring the optical density at 650 nm. After measuring the
211 pH, the cultures were centrifuged at 14000 rpm for 10 min at room temperature. The supernatants
212 were stored at -20 °C until lactate and butyrate concentrations were determined using high-
213 performance liquid chromatography (HPLC) analysis. The *in vitro* fermentation assay was done
214 twice in triplicate.

215 <u>Determination of butyrate and lactate concentrations</u>

216 DL-lactate and butyrate were quantified using HPLC with ultraviolet detection, as described by De
217 Baere et al. (40). The supernatant was acidified using concentrated hydrochloric acid and extracted
218 with diethyl ether for 20 min. The upper ether phase was transferred to another extraction tube and
219 extracted again for 20 min with sodium hydroxide. The aqueous phase was transferred to an

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220 autosampler vial and concentrated hydrochloric acid was added. An aliquot was injected on the
221 HPLC-UV instrument. The HPLC instrument consisted of a P1000X type quaternary gradient
222 pump, an AS3000 type autosampler, an UV1000 type ultraviolet detector and a SN4000 type
223 system controller, all from ThermoFisher Scientific (Breda, The Netherlands). Chromatographic
224 separation was achieved using a hypersilGold aQ column (150 x 4.6 mm, particle size: 3 µm,
225 ThermoFisher Scientific). Gradient elution (80/20) was performed using NaH_2PO_4 in HPLC grade
226 water and HPLC grade acetonitrile as mobile phase A and B, respectively. The detector was set at a
227 wavelength of 210 nm. The Chromquest software (ThermoFisher Scientific) was used for data
228 processing.

229 **Statistical analysis**

230 The comparison of the performance data was performed with an independent samples t-test (SPSS
231 22.0). For the qPCR and morphology data were analyzed by means of a linear mixed effect model
232 with pen included as random effect (S-Plus). The differences were considered statistically
233 significant at P value ≤ 0.05 and considered as a tendency at $P \leq 0.1$. Statistical differences in
234 relative abundance in bacterial population between groups were assessed by non-parametric
235 Kruskal-Wallis H test with Benjamin-Hochberg False Discovery Rate screen and Tukey-Kramer
236 post-hoc test. Moreover, differences in specific bacterial population relative abundance based on
237 16S profiling were analyzed with non-parametric Mann-Whitney test using a two-tailed P value
238 calculation. GraphPad Prism software version 5 was used to perform the statistical analysis for the
239 *in vitro* fermentation. All quantitative parameters (pH, OD, SCFA concentrations) were compared
240 using the Kruskal-Wallis test. The Dunns *post hoc* test was applied for multicomparisons of these
241 variables if there was a significant difference with the Kruskal-Wallis test.

242 **<u>Results</u>**

243 **Broiler performance after supplementation of broiler feed with XOS35**

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244 To evaluate the effect of XOS on broiler performance, the body weight and feed intake were
245 measured and FCR and growth were calculated. When considering the starter and grower period
246 together (day 0 to day 26), the FCR was significantly (P = 0.003) more favorable for chickens fed
247 the XOS-supplemented diet compared to the chickens fed the control diet (Table 2). For the whole
248 trial period (day 0 – 39), the FCR was also significantly improved (lower) for the group receiving
249 the XOS-supplemented diet (P = 0.04). The average body weight at the different time points was
250 non-significantly higher for chickens fed the diet supplemented with 0.5 % XOS compared to the
251 chickens given the non-supplemented diet. These results together with the significantly improved
252 FCR show a biologically relevant improved performance for chickens given the XOS-supplemented
253 diet.

254 **Intestinal morphology**

255 Supplementation of 0.5 % XOS to the broiler feed significantly (P = 0.04) increased the villus
256 length in the ileum (Table 3). The *tunica muscularis* was shown (P = 0.38) to be thicker in the
257 group fed the XOS-supplemented diet (Table 3).

258 **Microbiota composition as determined by qPCR and 16S sequencing**

259 There was no difference in the number of total bacteria between the XOS-supplemented and non-
260 supplemented group in both the caecum and the colon (Fig. 1A). The number of gene copies
261 encoding the butyryl-CoA:acetate-CoA transferase was significantly (P = 0.02) higher in the caeca
262 of the chickens that received 0.5 % XOS (Fig. 1B).

263 Significant changes were observed in the abundance of specific 16S sequences in caecum and colon
264 samples at different taxonomic levels (Fig. 2, Table 4). Although 35 bacterial families were
265 detected in the caecal microbiota, only the abundance of *Clostridium* cluster XIVa was shown to be
266 significantly (P = 0.005) increased in animals fed a XOS-supplemented diet as compared to animals
267 fed a control diet (44.29% vs 29.65%, Table 4). Forty one bacterial families were detected in the
268 colon microbiota of which *Lactobacillaceae* was shown to be significantly (P = 0.033) higher in

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269 animals fed a XOS-supplemented diet compared to animals fed a control diet (81.54% vs 41.73%).
270 In the caecal samples a total of 834 species were detected of which 16 were shown to be
271 significantly different between the XOS treated and control animals. A significant higher abundance
272 was observed for *Anaerostipes butyraticus*, a butyrate producing species classified within
273 *Clostridium* cluster XIVa (from 0.4 % to 2.5 %, P = 0.048, Fig. 2). Seven hundred twenty one
274 species were detected in the colon samples of which 11 were shown to be significantly different in
275 animals receiving dietary XOS compared to control diet. XOS supplementation resulted in a
276 significant increase of *Lactobacillus crispatus* (from 4 % to 15 %, P = 0.007, Fig. 2) in the colon.

277 ***In vitro* fermentation**

278 To investigate cross-feeding between *L. crispatus* and *A. butyraticus* in the presence of XOS, an *in*
279 *vitro* fermentation assay was carried out. XOS95 and maltodextrin were used to confirm the effect
280 of XOS in the *in vivo* trial. Only the monoculture of *L. crispatus* resulted in a small pH drop when
281 XOS35 was added to the medium (6.4 ± 0.04 versus 6.2 ± 0.04, Fig. 3). *A. butyraticus* showed a
282 significantly increased (P = 0.007) proliferation when XOS35 and XOS95 were added to the
283 medium compared to maltodextrin (Fig. 3). The proliferation of *L. crispatus* increased significantly
284 when XOS35 was added to the medium. The proliferation of the strains in the co-culture was higher
285 when XOS35 or XOS95 was added compared to the non-supplemented media (Fig. 3).
286 Supplementation of maltodextrin to the medium did not cause any changes.

287 The concentrations of the fermentation acids butyrate and DL-lactate were determined after
288 incubation, in all monocultures and co-cultures (Fig. 4). It was found that *A. butyraticus* was able to
289 produce butyrate, while *L. crispatus* produced high concentrations of lactate. The concentration of
290 butyrate or lactate produced by *L. crispatus* and *A. butyraticus,* respectively, were below the cut-off
291 values (1mM and 0.5 mM respectively) as determined during optimization of the HPLC method
292 (40). XOS35 and XOS95 significantly stimulated lactate production by *L. crispatus* compared with
293 *A. butyraticus,* which was not able to produce lactate. In the co-culture, lactate concentrations were

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294 very low, even when XOS35 or XOS95 were added to the medium, while the butyrate
295 concentration was higher as compared to the concentrations in the monoculture of *A. butyraticus*
296 with XOS35 (3.3 ± 0.8 versus 2.3 ± 0.6, Fig. 4), but non-significant. A similar observation was
297 made for XOS95 (2.4 ± 0.8 versus 1.6 ± 0.6).

298 **Discussion**

299 It is generally accepted that shifts in the intestinal microbiota composition may be the result of
300 dietary changes, such as the addition of cereal fibres (22, 41,42). In the current study, we
301 demonstrated that administration of XOS to broiler feed altered the microbiota composition in the
302 gut, with butyrate-producing bacteria and lactobacilli being more abundant in caeca and colon,
303 respectively.

304 In the chicken gut, lactobacilli are one of the predominant genera (43). These bacteria have the
305 ability to adhere to the mucosal layers and epithelium, promoting colonisation (44, 45). Through
306 interaction with the intestinal epithelial cells, lactobacilli can cause immunomodulation and offer
307 protection to the intestinal barrier by antagonistic activities against pathogens (45-47). In addition,
308 the probiotic use of lactobacilli has been shown to beneficially affect performance in broilers.
309 Broilers fed diets containing a mixture of 12 *Lactobacillus* strains or a single *Lactobacillus*
310 *acidophilus* strain had a better weight gain and a better FCR (48, 49). Lactobacilli are known to
311 ferment carbohydrates into lactic acid as major end-product which may lower the pH of the
312 intestinal environment resulting in the inhibition of growth of acid-sensitive pathogenic bacteria.
313 However, this pH effect may be rather limited as lactic acid is absorbed from the intestine or used
314 as a substrate for lactate-utilizing bacteria, such as representatives of the genera *Eubacterium*,
315 *Anaerostipes*, *Veillonella* and *Megasphaera* (50, 51).

316 In the present study, in addition to the significant higher abundance of lactobacilli in the colon, we
317 found an increased number of butyryl-CoA:acetate CoA-transferase gene copies in the caeca of

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318 chickens that received a XOS supplemented diet. Butyryl-CoA:acetate CoA-transferase is a key
319 enzyme in the major pathway for bacterial butyrate production in the gut (7). Hippe et al. showed
320 that this enzyme is a suitable marker for the butyrate producing capacity of the intestinal microbiota
321 which mainly belong to *Clostridium* cluster IV and XIVa (52),(53). We observed a significant
322 increase of members from both clusters in the caeca of chickens that were administered XOS.

323 The increased abundance of both lactobacilli and butyrate-producing bacteria can partly be
324 explained by cross-feeding mechanisms. Bacteria related to *Eubacterium hallii* and *Anaerostipes*
325 *caccae*, both members of *Clostridium* cluster XIVa, are able to convert acetate and lactate into
326 butyrate (7, 54). This metabolic cross-feeding between lactate producing and lactate utilizing
327 bacteria may help to stabilize the luminal pH and may be a factor in the butyrogenic effect of
328 certain dietary substrates (55). Our *in vivo* study showed a significant increase of the lactate
329 producing species *Lactobacillus crispatus* in the colon and the lactate-utilizing butyrate-producing
330 species *Anaerostipes butyraticus* in the caeca. The lactic acid produced by *L. crispatus* in the colon
331 may reach the caecum and become available for *A. butyraticus* due to antiperistalsis (56, 57). The *in*
332 *vitro* fermentation assay showed that reference strains of both species metabolized XOS resulting in
333 production of high concentrations of lactic acid by *L. crispatus*, which were supposed to be
334 consumed by the butyrate-producing bacterium *A. butyraticus*. Most likely also many other strains
335 can carry out a similar cross-feeding reaction in order to generate high butyrate levels in the chicken
336 hindgut.

337 Production of butyrate most probably plays a role in the beneficial effects on gut morphology and
338 growth performance observed in the current study. In poultry, butyrate enhances non-specific
339 intestinal defence mechanisms against pathogens that can affect performance, such as *Clostridium*
340 *perfringens*, by stimulating the mucin glycoprotein expression in intestinal epithelial cells (58-60).
341 Butyrate is a major energy source for the colonocytes and exerts anti-inflammatory activities by
342 several mechanisms (61). One of these mechanisms is the suppression of nuclear factor kappa B

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343 (NF-κB) that regulates the expression of pro-inflammatory cytokines (62). Butyrate has also been
344 shown to interfere with signalling by interferon-γ (IFN- γ) through its inhibitory effect on the
345 activation of signal transducer and activator of transcription 1 (STAT1) (63). Butyrate also
346 upregulates the expression of peroxisome proliferator-activated receptor-γ (PPAR-γ), a transcription
347 factor that belongs to the nuclear hormone receptor family. PPAR-γ inhibits the expression of
348 inflammatory cytokines and directs the differentiation of immune cells towards anti-inflammatory
349 phenotypes (64-66).

350 We observed longer villi in the ileum of chickens that were fed a XOS-supplemented diet as
351 compared to chickens fed a control diet. This effect on the small intestinal morphology may at least
352 partly be due to butyrate production by *Clostridium* cluster IV and XIVa species in the hindgut
353 through its effect on the expression of glucagon-like peptide-2 (GLP-2). Butyrate indeed appears to
354 be a strong stimulator of GLP-2 production. This hormone is secreted by entero-endocrine L-cells
355 and acts indirectly through multiple downstream mediators (67). Its receptor (GLP-2R) is localized
356 on distinct subpopulations of gut endocrine cells in the stomach, small intestine, and colon but also
357 on subepithelial myofibroblasts (68, 69). Hu *et al*. showed a beneficial effect of intravenous GLP-2
358 injection in broilers on growth performance, intestinal morphology, villi height and crypt cell
359 proliferation (70).

360 In conclusion, XOS, supplemented to the broiler diet, improved broiler performance by improving
361 the feed conversion ratio. Administration of XOS resulted in an increased abundance of butyrate-
362 producing bacteria in the caeca and lactobacilli in the colon at day 26 of age. It is hypothesized that
363 microbial cross-feeding, in which lactic acid produced by the lactobacilli is consumed by butyrate-
364 producing bacteria in the caeca stimulates gut heath and consequently performance, through the
365 beneficial effects of butyrate. Whether this cross-feeding also occurs in the complex gut ecosystem,
366 needs to be clarified in further *in vivo* work.

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581 **Tables**

582 **TABLE 1:** The composition and nutrient content of the wheat/rye diet administered to chickens. Starter diet was given from day 1
583 until 13, grower was given from day 14 until 26 and finisher was given from day 27 until 39.

584 **TABLE 2:** The effect of XOS supplementation on the growth performance of the chickens. Feed conversion ratio (FCR), body
585 weight (BW), feed intake (FI) and weight gain (WG) were measured at three time intervals for animals fed a wheat/rye-based diet
586 without or with supplemented with 0.2% XOS (day 1-13) and 0.5% XOS (day 14-26; 27-39). Values are the mean of 6 pens with 32
587 chickens ± standard error of the mean and n is the number of the chickens in each group. Statistical analysis was done with SPSS.
588 Independent samples t-test was used to determine statistical differences between groups receiving non-supplemented and XOS
589 supplemented diet. P-values less than 0.05 and 0.001 were considered significant (*, **).

590 **TABLE 3:** The effects of XOS supplementation on the intestinal morphology of chickens on day 26. The data shown are the mean
591 length of the villi (µm) and mean thickness of the tunica muscularis (µm) in ileal sections taken at day 26 of animals fed a wheat/rye-
592 based diet, without or with supplemented with 0.5 % XOS (n=18). The length and the thickness were measured of 10 randomly
593 selected villi and 10 different places for the *tunica muscularis* using a PC-based analysis system. Statistical analysis was done with
594 S-plus, using a linear mixed effects model with pen as random factor. P-value less than 0.05 were considered significant.

595 **TABLE 4:** List of Clostridium cluster XIVa and cluster IV members identified in the caeca of chickens at day 26 of which the
596 relative proportion was significantly different between the XOS supplemented and unsupplemented group. The results are based on
597 the sequencing data of 6 chickens per treatment (ns, not significant).

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598 **Figures**

599 **FIGURE 1:** Number of total bacteria (A) and butyryl-CoA:acetate-CoA transferase gene copies (B) expressed as log10 copy number
600 of the gene per g of wet content in the caecal and colonic content of 26-day old chickens fed a wheat/rye-based diet either or not
601 supplemented with 0.5% XOS (18 chickens for each treatment). Statistical analysis is done with S-plus using a linear mixed effects
602 model with pen as random factor to determine statistical difference between groups of animals fed a wheat/rye- based diet without
603 and with XOS. * $P \leq 0.05$

604 **FIGURE 2:** Box plots showing mean relative sequence abundance of the *Clostridium* cluster XIVa and *Anaerostipes butyraticus* in
605 the caecum (A) and of the *Lactobacillaceae* and *Lactobacillus crispatus* in the colon (B) of 26-day old chickens fed without or with
606 XOS-supplemented feed (6 chickens for each treatment). The plus represents the mean value and the whiskers are the median, the
607 min/max value and 1[ste]/3rd quartiles.

608 **FIGURE 3:** pH values and optical densities (650 nm) after 24 h of *in vitro* fermentation of different substrates by *A. butyraticus, L.*
609 *crispatus* and both in co-culture. All the *in vitro* fermentation experiments were done twice in triplicate. Statistical analysis was done
610 with GraphPad Prism 5, using a Kruskal-Wallis test with a Dunns *post hoc* test. P-values ≤ 0.05 (*) and ≤ 0.01 (**) were considered
611 significant.

612 **FIGURE 4:** Butyrate and DL-lactate concentration after 24 h of *in vitro* fermentation of different substrates by *A. butyraticus*, *L.*
613 *crispatus* and both in co-culture. The *in vitro* fermentation experiments were done twice in triplicate. Statistical analysis was done
614 with GraphPad Prism 5, using a Kruskal-Wallis test followed by a Dunns *post hoc* test. P-values ≤ 0.05 (*), ≤ 0.01 (**) and < 0.001
615 (***) were considered significant.

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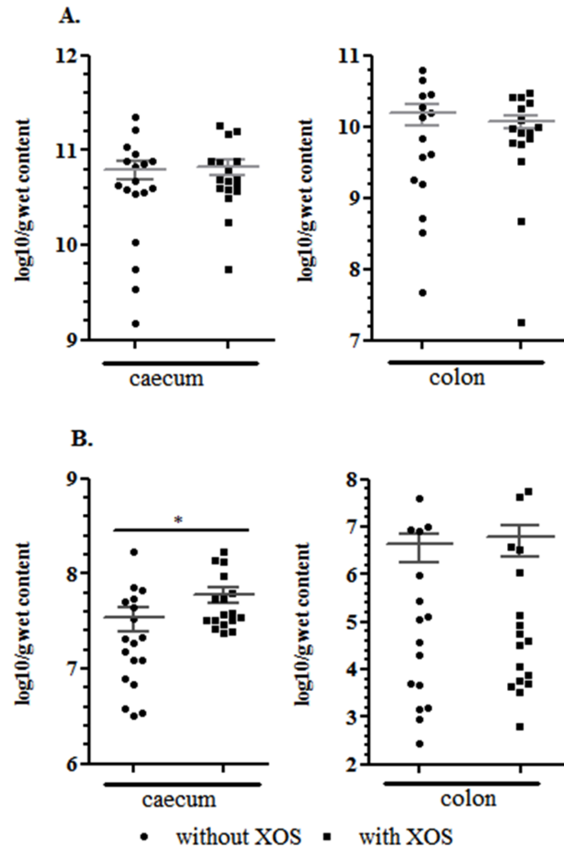

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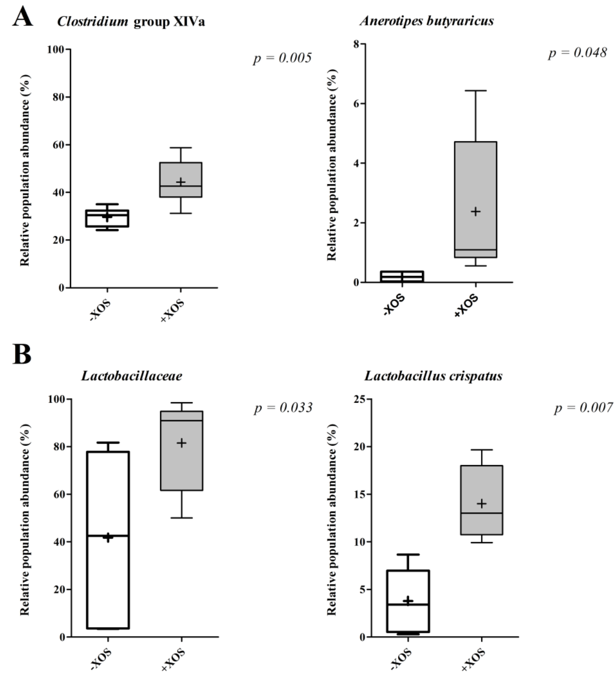

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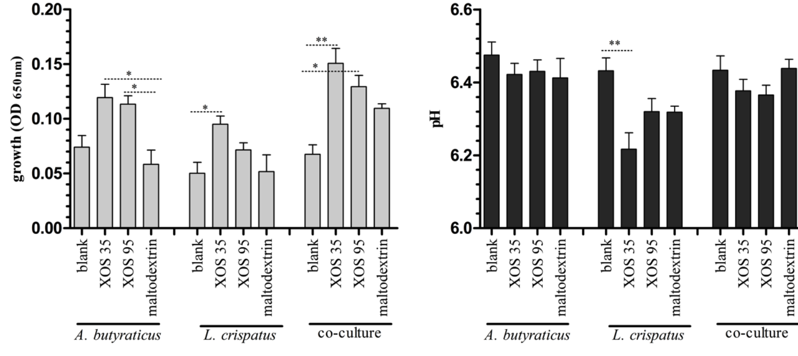

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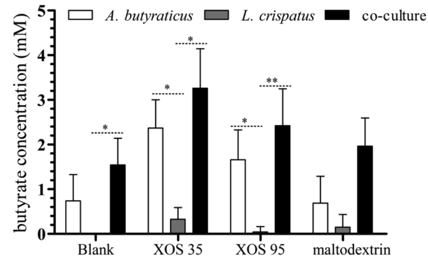

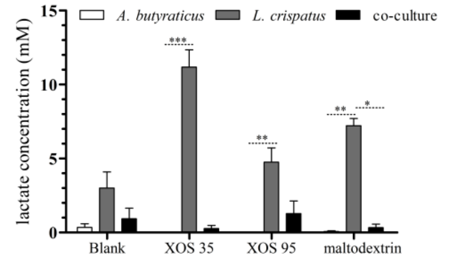

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1 **Tables**

2 **TABLE 1:** The composition and nutrient content of the wheat/rye diet administered to chickens**.** Starter diet was given from day 1
3 until 13, grower was given from day 14 until 26 and finisher was given from day 27 until 39.

	Starter diet	Grower diet	Finisher diet
Feedstuff (%)			
Wheat	44.00	46.51	49.78
Rye	5.00	5.00	5.00
Soybean meal (48)	23.30	19.78	16.61
Soybeans	7.50	5.00	5.00
Sunflower meal 27	2.50	2.50	2.50
Rapeseed meal	7.50	10.00	10.00
Animal fat	3.90	5.00	5.12
Soy oil	2.80	2.82	2.61
Vitamin + trace (vitamix)	1.00	1.00	1.00
$CaCO_3$	0.55	0.56	0.75
Di-Ca-phosphate	0.90	0.62	0.37
NaCl	0.21	0.21	0.19
Na-bicarbonate	0.10	0.10	0.10
L-Lys-HCl	0.14	0.15	0.20
DL-Methionine	0.50	0.70	0.70
L-Threonine	0.04	0.03	0.03
Phytase	0.02	0.02	0.02
Nutrient composition			
Crude protein (%)	23.00	21.50	20.50
Crude fat (%)	10.23	10.66	10.91
Non-soluble polysaccharides (%)	13.87	13.98	13.83
Metabolisable energy (MJ/kg)	11.72	12.15	12.25
D-Lysine (%)	1.12	1.03	1.00
D-Sulfur amino acids (%)	1.10	0.77	0.75
D-Threonine (%)	0.73	0.67	0.65
D-Valine (%)	0.84	0.76	0.72
Ca (%)	0.85	0.80	0.75
Available P (%)	0.40	0.35	0.30
NaCl + KCl (mEq/kg)	247	225	208
Linoleic acid (18:2) (%)	3.34	3.15	3.17

4

5 **TABLE 2:** The effect of XOS supplementation on the growth performance of the chickens. Feed conversion ratio (FCR), body
6 weight (BW), feed intake (FI) and weight gain (WG) were measured at three time intervals for animals fed a wheat/rye-based diet
7 without or with supplemented with 0.2% XOS (day 1-13) and 0.5% XOS (day 14-26; 27-39). Values are the mean of 6 pens with 32
8 chickens ± standard error of the mean and n is the number of the chickens in each group. Statistical analysis was done with SPSS.

9 Independent samples t-test was used to determine statistical differences between groups receiving non-supplemented and XOS
10 supplemented diet. P-values less than 0.05 and 0.001 were considered significant (*, **).

Intervals in days		FCR	BW (g)	FI (g/d)	WG (g/d)
0-26	-XOS	1.50 ± 0.01	1364 ± 15.39	67.3 ± 2.3	44,8 ± 1.50
	+ XOS	1.45 ± 0.01	1421 ± 16.97	69.4 ± 2.04	47,8 ± 1.72
	P-value	P = 0.003 (**)	P = 0.30	P = 0.50	P = 0.19
0-39	-XOS	1.66 ± 0.01	2401 ± 60.01	100.0 ± 2.75	60.4 ± 1.53
	+ XOS	1.63 ± 0.01	2446 ± 57.26	100.4 ± 2.43	61.6 ± 1.46
	P-value	P = 0.04 (*)	P = 0.60	P = 0.93	P = 0.60

11

12 **TABLE 3:** The effects of XOS supplementation on the intestinal morphology of chickens on day 26. The data shown are the mean
13 length of the villi (μm) and mean thickness of the tunica muscularis (μm) in ileal sections taken at day 26 of animals fed a wheat/rye-
14 based diet, without or with supplemented with 0.5 % XOS (n=18). The length and the thickness were measured of 10 randomly
15 selected villi and 10 different places for the *tunica muscularis* using a PC-based analysis system. Statistical analysis was done with S-
16 plus, using a linear mixed effects model with pen as random factor. P-value less than 0.05 were considered significant.

	– 0.5% XOS	+ 0.5% XOS	
length of villi (μm)	1059 ± 40.00	1228 ± 59.79	P = 0.04
thickness of *tunica muscularis* (μm)	167.0 ± 11.01	178.9 ± 6.32	P = 0.38

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17 **TABLE 4:** List of *Clostridium* cluster XIVa and cluster IV members identified in the caeca of chickens at day 26 of which the relative proportion was significantly different between the XOS
18 supplemented and unsupplemented group. The results are based on the sequencing data of 6 chickens per treatment (ns, not significant).

				Highest 16S rRNA gene sequence similarity	
	- 0.5 % XOS	+ 0.5 % XOS	P-value	Type strain of validly named species (% 16S rRNA gene sequence similarity)	Accession number
Clostridium cluster XIVa	29.64	44.29	0.004		
Blautia_RL199	0.04	0.15	0.04	*Blautia faecis* (95.96)	DQ793371
Lachnospiraceae_cc142	1.27	5.47	0.04	*Blautia schinkii* (93.93)	DQ057372
Lachnospiraceae_ic1296	0.34	0.79	0.03	*Blautia producta* (92.33)	DQ057459
Lachnospiraceae_GRC80	0.68	0.27	0.02	*Eubacterium contortum* (94.27)	DQ673545
Lachnospiraceae_B5-F3	1.19	7.28	0.01	*Blautia producta* (93.77)	EF025241
Lachnospiraceae_TS29	0.22	0.92	0.04	*Eubacterium hallii* (95.71)	FJ367509
Clostridium cluster IV	29.12	29.31	ns		
Ruminococcaceae_BY13	0.69	0.05	0.04	*Pseudoflavonifactor capillosus* (96.23)	DQ342336
Ruminococcaceae_CFT19C1	0.00	0.08	0.04	*Clostridium alkalicellulosi* (84.4)	DQ455843
Ruminococcaceae_CFT212F12	0.05	0.16	0.01	*Oscillibacter valericigenes* (95.73)	DQ456381
Ruminococcaceae_RL246	0.19	0.78	0.02	*Clostridium alkalicellulosi* (85.44)	DQ793581
Ruminococcaceae_TS1	1.78	0.29	0.03	*Clostridium aldrichii* (85.66)	FJ365262
Ruminococcaceae_ELU0008	0.04	0.30	0.01	*Subdoligranulum variabile* (92.69)	HQ740050

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