Animal Science Papers and Reports vol. 35 (2017) no. 3, 279-299
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Effects of genetically modified maize and soybean meal on the diversity and activity of gut microbiota in broiler chicken*

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(Accepted May 23, 2017)

The effects of genetically modified (GM) soybean meal (SBM) and maize on the diversity and activity of microbiota inhabiting terminal gut segments in broiler chickens were studied. Eight diets were prepared, based on conventional or GM SBM combined with maize cvs Clarica or PR39 F58, or their isogenic MON 810 counterparts cvs Bacilla or PR39 F56. Diets were fed from age 1 to 28 days to 144 Ross broilers, allocated to eight groups of 18 birds each. The microbiota was analysed by terminal restriction fragment length polymorphism (T-RFLP) analysis and its activity was measured. In the ileum and caecum of all groups, members representing the orders *Clostridiales, Lactobacillales* and *Selenomonadales* were present, accompanied by *Bifidobacteriales* in the caecum. The diversity of the order *Lactobacillales* in the ileum and caecum of birds fed GM maize was reduced, while that of *Lactobacillales* in the ileum and *Bifidobacteriales* in the caecum of birds fed GM SBM was higher compared with conventional maize and SBM. The use of GM and conventional maize and SBM did not affect the activity of microbiota measured as bacterial enzyme activity and the concentration of short-chain fatty acids in the ileal and caecal digesta. The GM maize did not change resistance of *E.*

*Financial support was provided by the Polish National Science Centre (grant no. N N311 517540) and by statutory subsidy from the Kielanowski Institute of Animal Physiology and Nutrition, Polish Academy of Sciences.
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coli or ***Clostridium*** **against antibiotics, while GM SBM slightly increased resistance of** ***Clostridium*** **from the ileum against kanamycin and those from caecum against kanamycin and erythromycin compared with conventional feedstuffs. In conclusion the use of GM SBM and maize MON810 in diets did not affect the broiler intestinal ecosystem.**

KEYWORDS: activity / antibiotic resistance / broiler chickens / diversity / genetically modified soybean meal and maize / microbiota

Each year the area cultivated with genetically modified (GM) crops has continuously increased worldwide, reaching 181.5 million hectares in 2014 [ISAAA 2014]. First generation GM crops are the most widely grown: GTS 40-3-2 Roundup Ready^{TM} soybean (a registered trademark of Monsanto Technology LLC) developed for herbicide tolerance and maize developed for insect resistance [ISAAA 2014]. Of the various GM maize lines only MON810 has been authorized for cultivation within the European Union [EFSA 2012], while soybean meal produced from GTS 40-3-2 soybean (GM SBM) is the most common component of feed mixtures for poultry in the EU. However, in many countries public fears are expressed concerning potential direct and indirect effects of GM feeds both on animal health and on the environment. These concerns have given rise to a proposal to ban the use of GM feeds in Poland.

Under EU regulations the potential for gene flow must be considered in novel food safety assessment [OJEC 1997]. The guidelines for testing GM feeds [EFSA 2008] recommend examining their effects on the host and on the host microbiota. The compositional equivalence and lack of adverse effects of first-generation GM SBM and GM maize on animal productivity and health have been demonstrated in many studies [Czerwinski *et al*. 2015 ab, Flachowsky 2013, Świątkiewicz *et al.* 2010, 2011, 2014, Tan *et al.* 2012]. However, a limited number of studies have investigated the influence of GM feeds on the composition and activity of gastrointestinal tract (GIT) microbiota of farm animals. Buzoianu *et al.* [2012 ab] reported that feeding weanling or growing pigs with MON810 maize had no effect on *Enterobacteriaceae, Lactobacillus* or total anaerobe counts in ileal and caecal digesta, but some differences in the counts of total anaerobes and *Proteobacteria* were observed in sows and piglets [Buzoianu *et al.* [2013]. Sieradzki *et al.* [2013] reported that GM soybean meal and MON810 maize did not affect bacterial species or their abundance in the gut of broilers, laying hens, pigs or calves. Einspanier [2013], summarising experiments on the fate of transgenic DNA and newly expressed proteins, concluded that both are degraded in animal gut during the digestion process, similarly to native plant DNA and proteins. No transfer of recDNA or recProteins from commercialised GM plants was found in animal organs or animal products. However, a specific transfer of complete or degraded recDNA or recProteins into the gut microbiota cannot be excluded.

Formerly, in the initial steps leading to the generation of genetically modified plants antibiotics were used as markers for the selection of successful transformants. Gebhard and Smalla [1998] reported data on marker-*nptII* gene rescue by *Acinetobacter* in experiments using DNA from GM sugar beet. De Vries and Wackernagel [1998] reported similar data for transgenic potatoes using *Acinetobacter* and *Pseudomonas*

stutzeri. In the absence of DNA homology to facilitate marker rescue, gene transfer was not detected. Kay *et al.* [2002] extended observations of marker rescue to include GM plants, in which the transgene DNA was located within the chloroplast genome. Nielsen and Townsend [2004] stated that horizontal gene transfer from GM plants to bacteria with subsequent expression of the transgene is a rare event under natural conditions and in the absence of selective pressure, particularly if no homologous sequences are present. However, due to homologous recombination the risk of gene transfer and subsequent integration and expression may be increased by the presence of bacterial sequences within the DNA inserted into the GM plant [Gebhard and Smalla 1998; De Vries *et al.* 2001; Tepfer *et al*. 2003].

Bacteria possess sophisticated mechanisms for the acquisition and rearrangement of genetic material. The transfer of DNA between bacteria may be achieved by conjugation (mediated by direct cell to cell contact between bacteria), transduction (DNA is carried between bacteria by a bacterial plasmid) and transformation (released naked DNA is taken up by bacteria). Fragments of DNA smaller than 200 base pairs are generally considered to be too small to transmit genetic information. On the other hand, even the smallest fragment of DNA can alter existing genetic information. It is well recognized that microbes in the GIT create a unique ecosystem and the appearance or colonization of the GIT by one species may define the appearance or colonization by another. Therefore, even small genetic modifications of bacterial DNA can significantly influence the diversity of the gut ecosystem and its susceptibility to colonization by antibiotic resistant strains. In the production of some transgenic plants, bacterial antibiotic resistance genes are used as markers enabling selection of transformed plant cells. In other cases, resistance genes may be present through incorporation of vector DNA from bacterial constructs. Chambers *et al.* [2002] examined the fate of an antibiotic resistance marker from transgenic maize fed to broiler chicks, and demonstrated that it survives no better than other plant DNA and that gene flow from transgenic maize to the gut microflora is very unlikely.

The aim of the present study was to estimate the effects of feeding Roundup Ready^{TM} SBM and MON810 maize on the diversity and activity of the microbiota inhabiting terminal segments of the gastrointestinal tract in broiler chickens.

Material and methods

The tested feedstuffs were four maize cultivars: two cultivars of transgenic (GM) maize MON810 – Bacilla and PR39 F56 and their two non-transgenic counterparts cvs Clarica and PR39 F58, respectively; and two commercial solvent extracted soybean meals (SBM) - GM or conventional, the same as were used in an experiment described in Czerwinski *et al.* [2015a]. Seeds of all maize cultivars were purchased from Hi-Bred Northern Europe Sales Division GmbH, European Commission DG Health and Consumers. They have been licensed for cultivation within the EU as follows: cv Clarica (as Clariti CS) – approvals nos. FR 8197 and IT 345; cv Bacilla – approvals

nos. ES 5052 and FR 10858; cv PR39F58 – approvals nos. CZ 777, DE 8346, HR 63 and PL 48; cv PR39F56 – approvals nos. CZ 409 and DE 8346, respectively. According to the producer's declaration, cv Clarica is a non-GM near-isogenic counterpart line for cv GM Bacilla, while PR39F58 is a non-GM near-isogenic counterpart line for cv GM PR39F56. Two commercial solvent extracted soybean meals (SBM) (HIPRO Brasilia) were used, one from a GM batch of *Glycine max*. L CV A 5403, line GTS 40-3-2, and the other from a non-GM batch (according to the seller's declarations). The chemical composition and quantitative levels of the genetic modification of SBM and maize used in the diets were described in detail in a study by Czerwinski *et al.* [2015a].

The use of the GM feeds in experimental diets was approved by the Polish Ministry of the Environment. The experimental design consisted of a 2×2×2 factorial treatment arrangement. Eight Starter-type (Tab. 1) and eight Finisher-type (Tab. 2) experimental diets were formulated to meet or exceed nutrient requirements for broilers [NRC 1994]. Four diets were based on conventional SBM (S) and: 1. conventional maize cv Clarica (SC); 2. GM maize cv Bacilla (SB); 3. conventional maize cv PR39 F58 (SF58); 4. GM maize cv PR39 F56 (SF56); and four were based on GM SBM (SG) and: 5. conventional maize cv Clarica (SGC); 6. GM maize cv Bacilla (SGB); 7. conventional

Table 1. Composition of Starter-type diets fed from 1 to 21 days of age (g/kg air-dry matter)

Component	Dietary treatment							
	SC	SB	SF58	SF56	SGC	SGB	SGF58	SGF56
Soybean meal								
conventional (S)	384.85	393.45	377.6	371	–	–	–	–
GM (GTS40-3-2) (SG)	–	–	–	–	391	400.4	383.2	377.1
Maize								
conventional cv Clarica (C)	557	–	–	–	552.6	–	–	–
GM (MON 810) cv Bacilla (B)	–	547.8	–	–	–	542.75	–	–
conventional cv PR39 F58 (F58)	–	–	565.55	–	–	–	561.65	–
GM (MON 810) cv PR39 F56 (F56)	–	–	–	572	–	–	–	567.35
Rapeseed oil	20.25	21.0	18.9	18.9	18.5	19.3	17.2	17.3
Limestone	14.3	14.3	14.3	14.2	14.2	14.2	14.2	14.3
Monocalcium phosphate	13.2	13.1	13.3	13.4	13.1	13	13.2	13.3
NaCl	3	3	3	3	3	3	3	3
Vitamin-mineral mixture [1]	5	5	5	5	5	5	5	5
L-Lys (78%)	0.15	0	0.2	0.35	0.25	0	0.25	0.45
DL-Met (98%)	2	2.1	1.9	1.9	2.1	2.1	0.3	1.95
Feed enzyme [2]	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25
Analysed								
dry matter (g/kg)	895	891	890	885	896	888	886	887
crude protein (g/kg DM)	244	255	234	254	228	253	241	261
crude fat (g/kg DM)	57	59	65	61	63	56	63	48
sugars (g/kg DM)	64	54	56	61	65	68	90	85
starch (g/kg DM)	454	432	338	410	433	418	433	460

[1]Provided per kg diet: retinyl acetate 3.75 mg, cholecalciferol 0.069 mg, DL-α-tocopheryl acetate 50 mg, thiamine 2 mg, riboflavin 6 mg, biotin 0.2 mg, pyridoxine 4.5 mg, cyanocobalamin 0.02 mg, menadione 3 mg, niacin 40 mg, folic acid 2 mg, calcium pantothenate 15 mg, choline 528 mg; betaine 75 mg; Mn 80 mg, Zn 60 mg, Se 0.25 mg, Co 0.4 mg, Cu 8 mg, Fe 60 mg, I 1 mg, coccidiostat (Narazin, Nicarbazin) 80 mg and Ca 1.415 g.
[2]Avizyme 1500 (Danisco Cultor) providing per kg of diet: 1000 U subtilisin (protease), 100 U α-amylase, 87 U endo-1,4-β-xylanase, 37 U endo-1,3 (4)-β-glucanase and 6 U pectinase, according to the manufacturer's declaration.

Table 2. Composition of Finisher-type diets fed from 22 to 29 days of age (g/kg air-dry matter)

Component	Dietary treatment							
	SC	SB	SF58	SF56	SGC	SGB	SGF58	SGF56
Soybean meal								
conventional (S)	310	316.8	299.15	292.1	–	–	–	–
GM (GTS40-3-2) (SG)	–	–	–	–	317.6	326	306.5	300
Maize								
conventional cv Clarica (C)	630.15	–	–	–	624.1	–	–	–
GM (MON 810) cv Bacilla (B)	–	622.85	–	–	–	615.4	–	–
conventional cv PR39 F58 (F58)	–	–	643	–	–	–	637	–
GM (MON 810) cv PR39 F56 (F56)	–	–	–	649.6	–	–	–	643.23
Rapeseed oil	18	18.6	15.8	16	16.55	17.1	14.5	14.6
Limestone	14.35	14.35	14.4	14.4	14.35	14.35	14.4	14.38
Monocalcium phosphate	13.85	13.75	13.95	14.05	13.75	13.65	13.85	13.94
NaCl	3	3	3	3	3	3	3	3
Vitamin-mineral mixture[1]	5	5	5	5	5	5	5	5
L-Lys (78%)	2.6	2.5	2.75	2.9	2.6	2.4	2.8	2.9
DL-Met (98%)	2.8	2.9	2.7	2.7	2.8	2.85	2.7	2.7
Feed enzyme[2]	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25
Analysed								
dry matter (g/kg)	895	891	889	889	895	891	892	889
crude protein (g/kg DM)	221	219	226	224	212	215	225	231
crude fat (g/kg DM)	54	54	50	53	53	55	55	52
sugars (g/kg DM)	86	89	89	87	85	79	86	90
starch (g/kg DM)	439	435	426	413	422	420	459	442

[1]Provided per kg diet: retinyl acetate 3.75 mg, cholecalciferol 0.069 mg, DL-α-tocopheryl acetate 50 mg, thiamine 2 mg, riboflavin 6 mg, biotin 0.2 mg, pyridoxine 4.5 mg, cyanocobalamin 0.02 mg, menadione 3 mg, niacin 40 mg, folic acid 2 mg, calcium pantothenate 15 mg, choline 528 mg; betaine 75 mg; Mn 80 mg, Zn 60 mg, Se 0.25 mg, Co 0.4 mg, Cu 8 mg, Fe 60 mg, I 1 mg and Ca 1.415 g.
[2]Avizyme 1500 (Danisco Cultor) providing per kg of diet: 1000 U subtilisin (protease), 100 U α-amylase, 87 U endo-1,4-β-xylanase, 37 U endo-1,3 (4)-β-glucanase and 6 U pectinase, according to the manufacturer's declaration.

maize cv PR39 F58 (SGF58); and 8. GM maize cv PR39 F56 (SGF56). Diets were analysed for their chemical composition [AOAC 1990] in four replicates.

All experimental procedures were approved by the Local Animal Care and Use Committee, Warsaw, Poland. A total of 192 one-day-old Ross 308 broiler females were obtained from a local commercial hatchery. The birds were randomly assigned to eight experimental treatments, with 24 birds in each group. During the first week of life, chickens were kept in battery cages (three replicates of eight birds per treatment) and fed experimental diets (Tab. 1) *ad libitum.* On day 9, the birds were deprived of feed for 4 h, weighed, and 18 birds per treatment that had a body weight close to the group average were placed in individual cages. From that timepoint each bird was treated as an individual replicate and feed intake was individually measured. From day 22 birds were fed Finisher-type diets without coccidiostats (Tab. 2) until the end of the experiment. Room temperature was maintained at 30°C for the first three days and was gradually reduced thereafter according to standard management practices. The light cycle was 23 h light/1 h darkness during the first week and 18 h light and 6 h darkness from day nine until the end of study. On day 29 the birds were deprived of feed for 4 h, feed remnants and chickens were weighed, then the same diets were

resumed *ad libitum* until slaughter. Feed intake, body weight gain and feed conversion ratio for each bird were calculated for the period from day 9 to day 28.

At 29 or 30 days of age all chickens were weighed and killed by cervical dislocation. The intestinal tract was excised and samples (eight per treatment, each pooled from two chicks) of fresh digesta from the lower ileum (the last 15 cm anterior to the ileocaecal junction) and the caecum were collected into sterile tubes, then mixed and packed into sterile Eppendorf tubes for antibiotic resistance analyses, T-RFLP analyses, microbial enzyme activity measurements and isoflavone analyses. Samples were kept frozen at -20°C. Samples of about 4 g ileal and caecal contents were adjusted to pH 8 with 1 M NaOH and kept frozen at -20°C to assay short chain fatty acids (SCFA).

Frozen samples of digesta from the ileum and caecum (1 g) were thawed, diluted with 9 ml of phosphate-buffered saline (pH 7.2), and homogenized for 2 min with a stomacher lab blender (Interscience, St. Nom, France). The supernatants (0.5 ml) were transferred to sterile Eppendorf tubes. Bacterial cell lysis, DNA extraction and polymerase chain reactions (PCR) were performed using methods described in detail by Czerwiński *et al.* [2010]. The 16S rRNA genes were amplified either using the HPLC-purified 6-carboxyfluorescein-labeled (6-FAM) forward primer Lb008: 5'-6-FAM-AGRGTTTGATYMTGGCTCAG, modified from S-D-Bact-0008-a-S-20 [Leser *et al* 2002] in combination with the reverse primer PH1522: 5'-AAGGAGGTGATCCAGCCGCA [Mikkelsen *et al.* 2003]; or using the HPLC-purified fluorescent-labelled (PET) forward primer Lab158: 5'-PET-TGGAAACAGRTGCTAATACC [Harmsen et al. 1999] in combination with the reverse primer Lac2: 5'-ATTYCACCGCTACACATG [Walter et al. 2001]. The former primer set is considered to be universal (Prokaryotes and Archaea), whereas the latter should specifically target *Lactobacillus* spp. and *Enterococcus* spp. Four replicate PCR reactions performed on each sample were pooled, purified with a QIAquick PCR purification kit (QIAGEN GmbH, Hilden, Germany), and eluted in a final volume of 50 µl. The PCR products were quantified (NanoDrop ND-1000, NanoDrop Technologies, USA) and diluted to similar DNA concentrations. The purified PCR (10 µl) products obtained with the 16S rRNA primers were digested overnight at 37°C with 20 U of restriction endonuclease, either *Hha*I or *Msp*I (Thermo Scientific) in 20-µl reaction mixtures. The purified PCR products from ileal digesta obtained with the *Lactobacillus/Enterococcus* specific primers were digested by *Hha*I or *Mse*I restriction endonucleases. The size of the fluorescently labelled terminal restriction fragments (T-RFs) was determined on an ABI 3130 Genetic Analyzer (Applied Biosystems) using the GENEMAPPER version 3.5 software and an internal size standard LIZ 1200 (Applied Biosystems). The T-RFs obtained were compared to in silico digests of bacterial 16S rRNA gene sequences deposited in the GenBank and/or RDP using the MiCA platform (http://mica.ibest.uidaho.edu/), and some of the most abundant fragments were obtained.

The glycolytic activity in ileal and caecal digesta was measured using the rate of p- or o-nitrophenol release from their nitrophenylglucosides according to the

modified method of Djouzi and Andrieux as modified by Juśkiewicz et al. [2006]. The following substrates (Sigma Chemical Co., St. Louis, MO) were used: for β-glucuronidase, p-nitrophenyl-β-D-glucuronide; for α-galactosidase, p-nitrophenyl-α-D-galactopyranoside; for β-galactosidase, o-nitrophenyl-β-D-galactopyranoside; for α-glucosidase, p-nitrophenyl-α-D-glucopyranoside; and for β-glucosidase, p-nitrophenyl-β-D-glucopyranoside. The reaction mixture contained 0.3 mL of a substrate solution (5 mM) and 0.2 mL of a 1:10 (vol/vol) dilution of the intestinal sample in 100 mM phosphate buffer (pH 7.0) after centrifugation at 10,000 × g for 15 min. Incubations were carried out at 37°C, and p-nitrophenol was quantified at 400 nm and at 420 nm (o-nitrophenol concentration) after the addition of 2.5 mL of 0.25 M cold sodium carbonate. The enzymatic activity (α- and β-glucosidase, α- and β-galactosidase, and β-glucuronidase) was expressed as micromoles of the product formed per minute (IU) per gram of digesta. Analyses were performed in triplicate.

SCFA concentrations in digesta were determined according to the method of Barszcz *et al.* [2011] using isocaproic acid as the internal standard on a HP 5890 AII gas chromatograph equipped with a flame ionisation detector and a Supelco Nukol capillary column (30 m 0.25 mm internal diameter, film 0.25 mm). The initial column temperature was set at 100°C for 2 min, increased to 140°C at 10°C/min and held at the final temperature for 20 min. Analyses were performed in triplicate.

Contents of daidzin (D), daidzein (DE), genistin (G) and genistein (GE) in diets and digesta from the ileum and caecum were determined using a modification of the method presented by Wocławek-Potocka *et al.* [2005]. A sample of 150 mg was mixed with 1 ml of 80% methanol, sonicated (30 s), vortexed (30 s) and centrifuged at 10,000 x g for 10 min. Supernatants were decanted into a 10 ml volumetric flask and the residue was re-extracted with 1 ml of 80% methanol and treated as previously described. The procedure was repeated three times. Supernatants were pooled and again centrifuged at 10,000 x g for 15 min. Isoflavones were analysed by reversed-phase HPLC using a Finnigan Surveyor Plus chromatograph (Thermo Scientific, San Jose, USA) with a UV photodiode detector set to scan from 220 to 380 nm. Separations were performed using a C18 Thermo (5μm) stainless steel column (4.6 mm x 250 mm), operating at 25°C with a flow rate of 0.7 mL/min for 32 minutes. A gradient elution was employed with a mixture of two solvents: (A) water/acetonitrile/acetic acid (10/90/0.1 v/v/v) and (B) water/acetonitrile/acetic acid (90/10/0.1 v/v/v). Analyses were performed in triplicate**.**

Antibiotic resistance of the genus *Clostridium* and *Escherichia coli* bacteria isolated from ileal and caecal digesta was assessed by the disc-diffusion method (eight samples per group and gut segment). *Escherichia coli* or *Clostridium* were inoculated in Petri dishes on the solid substrate surface of Chromocult TBX agar (*E. coli*) or DRCM agar (*Clostridium*). Oxoid antibiotic discs (Oxoid Ltd, Basingstoke, UK) of 6 mm in diameter, impregnated with penicillin (10 units), tetracycline (30 μg), erythromycin (30 μg) and kanamycin (30 μg) were placed on the surface. Then the Petri dishes inoculated with *E. coli* were incubated at 37°C for 24 h under aerobic conditions, while those inoculated with *Clostridium* were incubated at 37°C for 72 h under anaerobic conditions. After

incubation the diameter of the inhibition zone was measured. Analyses were performed in triplicate. Antibiotic resistance was expressed as the mean inhibition diameter (mm) and compared with the NCCL Standard [2011].

The distribution analyses were performed using appropriate procedures and SAS software. Outliers were defined as observations, which distance to the location estimate exceeded three-fold the standard deviation. Isoflavones were analysed in a 2 x 2 factorial arrangement (type of soybean meal and intestinal segment) with two-way ANOVA and other data were analysed as a 2x2x2 factorial arrangement with three-way ANOVA using the Statgraphics Plus® ver. 5.1 programme [1994-2001]. When ANOVA indicated significant treatment effects, means were separated using Duncan's multiple range tests. In the Results tables mean values with pooled standard errors are presented. Differences were considered to be significant at $P \leq 0.05$.

Results and discussion

The chemical composition of the conventional and GM soybean meal as well as conventional and GM maize feeds used in the experimental diets was described in detail by Czerwiński *et al.* [2015a]. The quantitative level of RR modification in GTS 40-3-2 SBM used in the experimental diets was 84.91±17.7% (copy GM/copy reference gene). Quantitative analysis of the reference gene and the unique sequence of the GM variety provided quantification of these two sequences expressed in terms of gene copy numbers. The 35S promoter and *nos* terminator were not detected in conventional soybean meal (LOQ <0.05%). The quantitative levels of the genetic modification event MON 810 in the GM maize cvs Bacilla and PR39 F56 used in the experimental diets were confirmed as 56.0% and 50.8%, respectively [limit of quantification (LOQ) <0.1%]. Genetic modification events NK603, Bt11, Bt176, T25, GA21, TC1507, and MON 88017 were confirmed neither in maize cvs Bacilla nor PR39 F56 or in their conventional counterparts, cvs Clarica and PR39 F58 (LOQ <0.1%).

Phytoestrogen concentrations in the diets containing conventional and GM soybean meal are shown in Table 3. The concentration of daidzin (D) in the diets containing conventional SBM was lower than in GM SBM ($P<0.01$), while that of daidzein (DE) did not differ significantly. The DE/D as well as the genistein (GE) to genistin (G) ratio were higher in the diets containing conventional SBM compared with GM SBM ($P<0.01$).

The dietary treatments had no significant effect on the performance of chickens (data not shown). The T-RFLP fingerprints obtained using the universal primers for the *HhaI* and *MspI* digests were compiled separately for ileal digesta in Figure 1, and for caecal digesta in Figure 2. The microbial composition of the caecal digesta showed more diversity than that of the ileal digesta for all dietary treatments, while there were also considerable differences in the microbial communities between individual birds. The results for both intestinal sections revealed that the majority of the fragments/peaks represented members of the orders *Clostridiales*, *Lactobacillales* and

Table 3. Phytoestrogen concentrations in diets containing conventional or GM soybean meal

Diets	Daidzin (D)	Daidzein (DE)		Genistin (G)	Genistein (GE)	
	μmol/g	μmol/g	DE, % D	μmol/g	μmol/g	GE, % G
Soybean meal (S)						
conventional	0.436^{A}	0.134	30.6^{B}	0.537	0.040	7.87^{B}
GM (GTS40-3-2)	0.666^{B}	0.156	23.5^{A}	0.811	0.039	4.79^{A}
pooled SEM	0.163	0.016	8.134	0.194	0.001	2.043

^{AB}Means in the columns with different superscripts differ significantly at P<0.01.

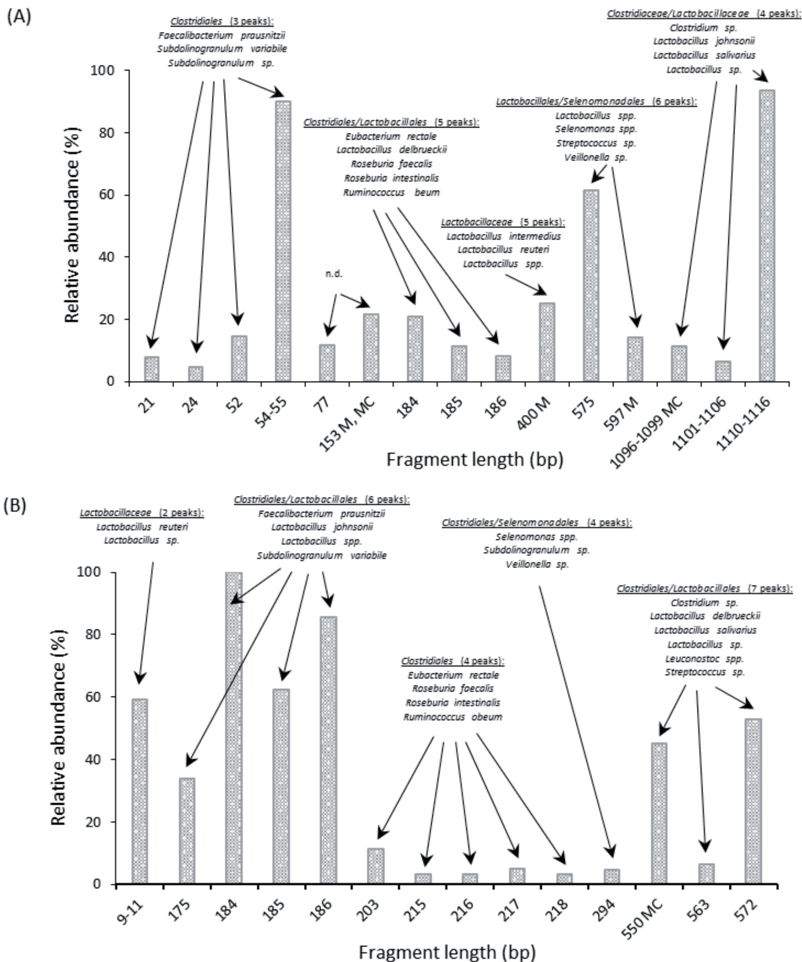

Fig. 1. Compiled data from T-RFLP analysis of ileal digesta samples using a universal primer set. Each bar represents the average value of 62 samples (seven or eight replicates from each treatment group). Some observed fragment lengths could not be identified from available databases – these are indicated by n.d. Top (A) and bottom (B) panels show results of the HhaI and MspI digest, respectively. $^{M,\ S,\ MC}$ indicate a treatment effect (see Materials and Methods) significant at P≤0.05.

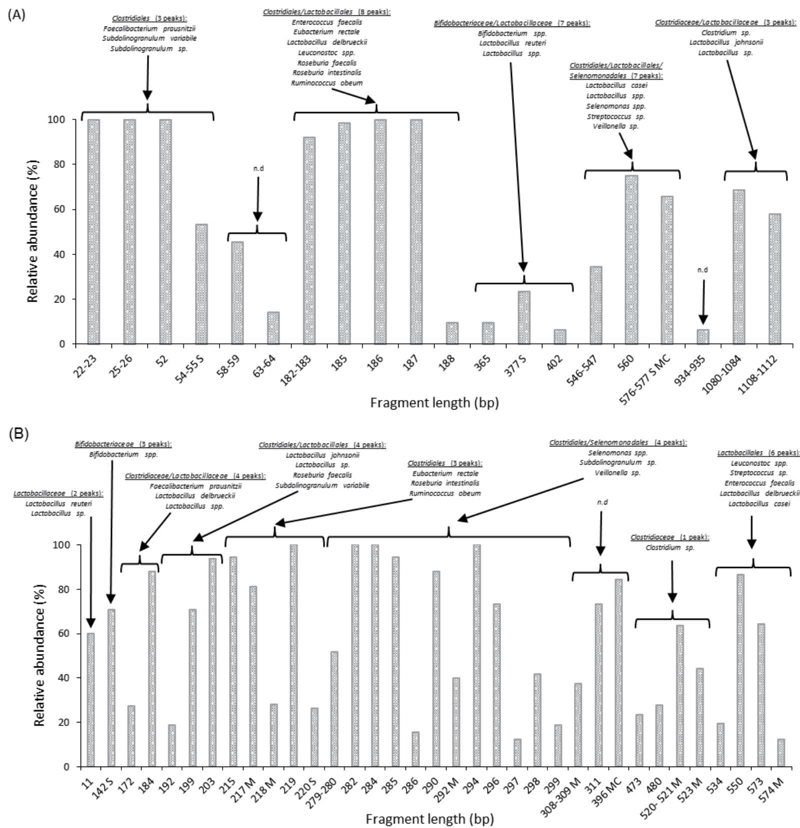

Fig. 2. Compiled data from T-RFLP analysis of caecal digesta samples using a universal primer set. Someobserved fragment lengths could not be identified from available databases – these are indicated by n.d. Top (A) and bottom (B) panels show results of the HhaI and MspI digests, respectively. Each bar represents the average value of 64 samples for HhaI digests (eight per group) and 31 samples for MspI digests (four or five per group). $^{M,\ S,\ MC}$ indicate a treatment effect significant at P≤0.05

Selenomonadales. However, *Bifidobacteriales* in caecal samples were also identified as being among the most abundant peaks in all groups of broilers. For the individual restriction endonuclease digests identification was in most cases possible only at higher taxonomic levels (family or even order). Combining the results of the fingerprints obtained using two different restriction endonuclease digests provided a more precise, yet still tentative identification of the fragments. In particular, in all dietary treatments the most abundant peaks obtained using the universal primers for both digests perfectly matched the in silico digests of several members of the *Clostridiales* order in the ileum (*Eubacterium rectale, Faecalibacterium prausnitzii, Roseburia faecalis, Roseburia intestinalis, Ruminococcus obeum, and Subdolinogranulum variabile*) and in the caecum (*Enterococcus faecalis, Eubacterium rectale, Faecalibacterium*

prausnitzii, Roseburia faecalis, Roseburia intestinalis, Ruminococcus obeum, and Subdolinogranulum variabile). At the same time, in all treatment groups we also found some abundant peaks perfectly matched to the in silico digests of several members of the *Lactobacillales* order in the ileum (*L. delbrueckii, L. johnsonii, L. reuteri, and L. salivarius*) and in the caecum (*L. casei, L. delbrueckii, L. johnsonii, and L. reuteri*). Analysis of variance showed that GM SBM had no significant effect on the ileal bacterial population in comparison with conventional SBM**.** However, in the ileum of birds receiving diets with GM maize compared with birds fed conventional maize (Fig. 1A) peaks of fragment length 153 bp (nd) were more frequent (33.5 vs. 9.4% samples), while peaks of fragment lengths 400 and 597 bp (probably members of the *Lactobacillales* order – *L. intermedius* and *L. reuteri*) were less numerous (13 vs. 37.5% samples and 3.6 vs. 25% samples, respectively, $P<0.05$).

In the caecum (Fig. 2 A and B) of birds fed GM SBM, when compared with birds fed conventional SBM, peaks of fragment length 54/55, 220 and 576/577 (probably members of the *Clostridiales*) were less common (40.6 vs. 65.6, 6.2 vs. 46.2, 53.1 vs. 76.1% of samples, respectively) and peaks of fragment length 142 and 377 (probably members of *Bifidobacteriaceae*) were more common at $P<0.05$ (93.7 vs. 47.5, 34.4 vs. 12.5% of samples, respectively). In birds fed GM maize significant differences ($P<0.05$) were found in peaks of 217 and 218 bp (probably members of the *Clostridiales*), the first being more and the other less frequent (100 vs. 62.5 and 0 vs. 56.2, respectively) compared with birds fed conventional maize.

The T-RFLP fingerprints using a primer set specifically targeting members of the genera *Lactobacillus*, *Enterococcus*, and *Leuconostoc* for *HhaI* and *MseI* digests were obtained only for ileal digesta. They provided some fingerprint patterns that revealed further differences in the *Lactobacillales* composition of the ileal microbiota (Fig. 3). Several fragments potentially representing *L. delbrueckii, L. intermedius, L. johnsonii, L. panis, L. reuteri, L. salivarius* and *Vagococcus fluvialis* were detected in ileal digesta. The T-RFLP fingerprints obtained using this specifically targeting primer set also showed that the use of GM SBM in diets increased the abundance of the order *Lactobacillales* in ileal digesta (including fragments potentially representing *L. delbrueckii, L intermedius, L. johnsonii, L. panis, and L. reuteri*) in comparison with broilers receiving the diet containing conventional SBM (data not shown).

As shown in Table 4, neither the presence of GM SBM meal or GM maize in the diets, nor the maize cultivar affected resistance of *E. coli* isolated from the ileum and caecum against erythromycin, kanamycin and tetracycline. Similarly, feeding birds with GM maize had no effect on resistance of *Clostridium* isolated from the ileum and caecum to erythromycin, kanamycin, penicillin and tetracycline. Only in birds fed with GM SBM resistance of *Clostridium* isolated from the ileum to kanamycin was approx. 9.3% higher ($P<0.01$) and resistance of *Clostridium* isolated from the caecum to kanamycin and erythromycin was about 9.1 and 7.1% higher ($P<0.05$), respectively, in comparison with the groups fed conventional SBM.

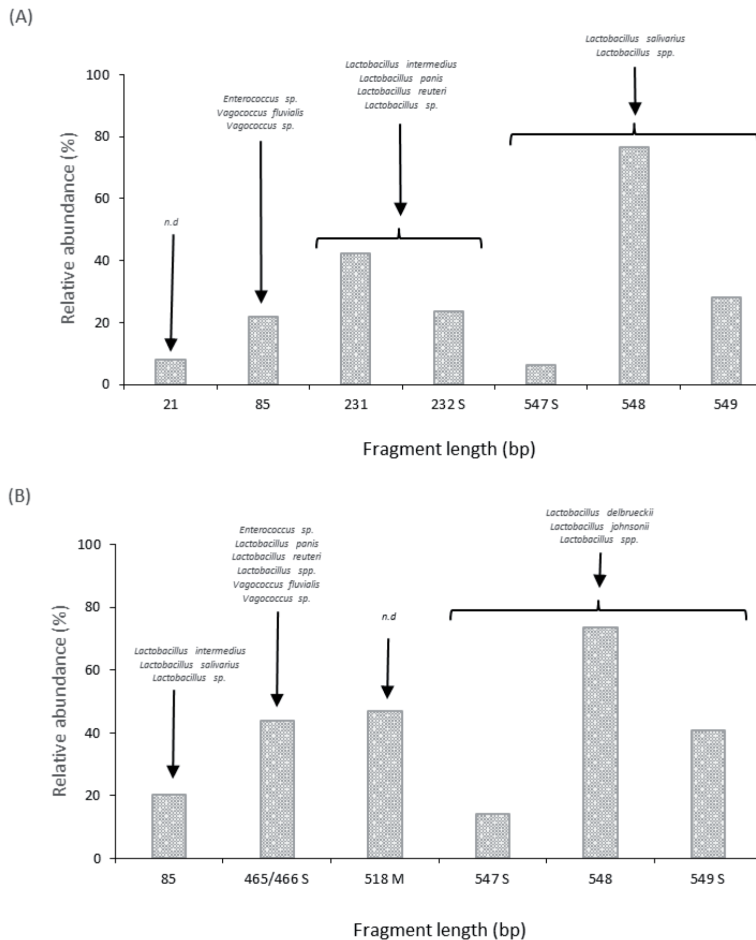

Fig. 3. Compiled data from T-RFLP analysis of ileal digesta samples using a *Lactobacillus/Enterococcus* specific primer set. Each bar represents the average value of 62 samples (seven or eight replicates from each treatment group). Some of the observed fragment lengths could not be identified from available databases – these are indicated by n.d. Top (A) and bottom (B) panels show results of the HhaI and MspI digests, respectively. $^{M,\ S,\ MC}$ indicate a treatment effect (see Materials and Methods) significant at P≤0.05.

The main effects of feeding GM or conventional feeds on bacterial enzyme activity in the ileum and caecum are presented in Table 5. Neither GM maize nor GM SBM had a substantial effect on bacterial enzyme activity in the ileum or caecum in comparison with conventional maize and SBM, respectively. Among the five bacterial enzymes evaluated, only the activity of β-glucosidase in ileal and caecal digesta was significantly changed, being lower by 12 and 13% (P<0.05), respectively, in birds fed with maize cv PR39 compared with maize cv Clarica/Bacilla.

Table 4. Main effects of dietary treatments on in vitro susceptibility tests of *E. coli* sp. and *Clostridium* sp. isolated from ileal and caecal digesta, diameter of the inhibition zone in mm[1]

Main effect	*E. coli sp.*			*Clostridium sp.*			
	erythromycin	kanamycin	tetracycline	erythromycin	kanamycin	tetracycline	penicillin
				Ileal digesta			
Soybean meal (S)							
conventional	15.66	19.56	11.16	9.72	9.75^{B}	9.28	8.81
GM (GTS40-3-2)	15.59	19.56	10.34	9.25	8.84^{A}	8.88	8.56
pooled SEM	0.38	0.54	0.63	0.25	0.22	0.17	0.18
Maize (M)							
conventional	15.88	19.31	10.25	9.59	9.44	9.19	8.88
GM (MON 810)	15.38	19.81	11.25	9.38	9.16	8.97	8.50
pooled SEM	0.38	0.54	0.63	0.25	0.22	0.17	0.18
Maize cv (MC)							
Clarica/Bacilla	15.44	19.84	10.19	9.34	9.28	9.09	8.50
PR39	15.81	19.28	11.31	9.63	9.31	9.06	8.88
pooled SEM	0.38	0.54	0.63	0.25	0.22	0.17	0.18
				Caecal digesta			
Soybean meal (S)							
conventional	13.41	19.50	10.81	9.59^{b}	9.59^{b}	8.97	9.13
GM (GTS40-3-2)	14.03	19.81	9.81	8.91^{a}	8.72^{a}	9.06	8.94
pooled SEM	0.36	0.62	0.40	0.19	0.28	0.18	0.27
Maize (M)							
conventional	13.84	19.00	10.53	9.50	9.41	9.06	8.97
GM (MON 810)	13.59	20.31	10.09	9.00	8.91	8.97	9.09
pooled SEM	0.36	0.62	0.40	0.19	0.28	0.18	0.27
Maize cv (MC)							
Clarica/Bacilla	13.78	19.56	10.44	9.72^{B}	9.47	9.09	9.41
PR39	13.66	19.75	10.19	8.78^{A}	8.84	8.94	8.66
pooled SEM	0.36	0.62	0.40	0.19	0.28	0.18	0.27

[1]According to NCCL Standards [2011] an inhibition zone in mm: ≤15, ≤13, ≤11, ≤14 - resistance, 16-20, 14-17, 12-14 - mild susceptibility, ≥21, ≥18, ≥15, ≥15 - susceptibility to erythromycin, kanamycin, tetracycline and penicillin, respectively.
[aA...]Within main effects means in columns bearing different superscripts differ significantly at:small letters – P<0.05; capitals – P<0.01.
All interactions were non-significant.

As shown in Table 6, neither the GM maize nor the maize cultivar affected SCFA concentrations in the ileum or caecum of broilers. GM SBM did not influence the SCFA concentration in the ileum, while the concentration of propionate and isobutyrate (P<0.05) and valerate (P<0.01) in the caecum was lower by about 32, 33 and 36%, respectively, when compared with birds fed conventional SBM.

Table 5. Main effects of dietary treatments on bacterial enzyme activity in ileal and caecal digesta (U[1]/g)

Main effect	α-glucosidase	β-glucosidase	α-galactosidase	β-galactosidase	β-glucuronidase
			Ileal digesta		
Soybean meal (S)					
conventional	0.177	0.139	0.147	0.0735	0.145
GM (GTS40-3-2)	0.187	0.139	0.151	0.0735	0.146
pooled SEM	0.004	0.003	0.004	0.0016	0.004
Maize (M)					
conventional	0.184	0.140	0.148	0.0746	0.147
GM (MON 810)	0.180	0.138	0.151	0.0723	0.144
pooled SEM	0.004	0.003	0.004	0.0016	0.004
Maize cv (MC)					
Clarica/Bacilla	0.182	0.143[b]	0.152	0.0756	0.150
PR39	0.182	0.134[a]	0.146	0.0713	0.141
Pooled SEM	0.004	0.003	0.004	0.0016	0.004
			Caecal digesta		
Soybean meal (S)					
conventional	0.582	0.343	0.790	0.211	1.62
GM (GTS40-3-2)	0.607	0.338	0.727	0.222	1.54
pooled SEM	0.023	0.017	0.050	0.009	0.09
Maize (M)					
conventional	0.599	0.337	0.768	0.211	1.59
GM (MON 810)	0.589	0.345	0.749	0.222	1.57
pooled SEM	0.023	0.017	0.050	0.009	0.09
Maize cv (MC)					
Clarica/Bacilla	0.617	0.367[b]	0.763	0.225	1.61
PR39	0.571	0.314[a]	0.754	0.209	1.56
pooled SEM	0.023	0.017	0.050	0.009	0.09

U – µmol of *p-(o-)*nitrophenol formed per min per g of digesta.
[aA...]Within main effects means in columns bearing different superscripts differ significantly at P<0.05.
All interactions were non-significant.

The major effects of conventional vs. GM SBM used in diets on phytoestrogen concentrations in digesta are shown in Table 7. Similar to the concentrations in the diets (Tab. 3) the concentrations of daidzin (D) and genistin (G) in the digesta of birds fed diets with GM SBM were higher than in birds fed conventional SBM (P<0.01). However, the concentrations of daidzein (DE) and genistein (GE) in the diets did not differ significantly, while in digesta they were higher in birds fed GM SBM when compared with conventional SBM (P<0.01 and P<0.05, respectively). The DE/D and GE/G ratios were significantly lower in caecal digesta compared with ileal digesta (P<0.05). The DE/D and GE/G ratios were significantly lower in diets containing GM SBM, while in digesta they did not differ in comparison to conventional SBM.

The gastrointestinal tract of chickens consists of the oesophagus, crop, proventriculus, gizzard, small intestines (duodenum, jejunum and ileum), caecum, colon and the cloaca. All parts of the GIT are populated by bacteria, but due to the fast passage rate and unfavourable pH of digesta the bacterial density in the proximal gut is low. The ideal habitats for a diverse microbiome are the distal parts of the GIT, i.e. the ileum and caecum. The microbiome of the distal GIT parts and the products of its metabolism, i.e. SCFA, have considerable effects on the whole gut

Table 6. Main effects of dietary treatments on SCFA concentration in ileal and caecal digesta (µmol/g)

Main effect	Total SCFA	Acetate	Propionate	Butyrate	Isobutyrate	Valerate	Isovalerate
				Ileal digesta			
Soybean meal (S)							
conventional	46.3	40.7	2.87	1.45	0.871	-	-
GM (GTS40-3-2)	40.1	36.5	1.87	0.93	0.695	-	-
pooled SEM	3.9	2.8	0.73	0.28	0.107	-	-
Maize (M)							
conventional	44.4	39.3	2.72	1.37	0.838	-	-
GM (MON 810)	41.9	38.0	2.02	1.02	0.728	-	-
pooled SEM	3.9	2.8	0.73	0.28	0.107	-	-
Maize cv (MC)							
Clarica/Bacilla	48.1	42.4	2.86	1.41	0.885	-	-
PR39	38.3	34.8	1.88	0.98	0.681	-	-
pooled SEM	3.9	2.8	0.73	0.28	0.107	-	-
				Caecal digesta			
Soybean meal (S)							
conventional	128.0	96.2	5.09^{b}	23.64	0.942^{b}	1.449^{B}	0.827
GM (GTS40-3-2)	120.9	92.1	3.46^{a}	23.66	0.635^{a}	0.927^{A}	0.566
pooled SEM	6.4	5.1	0.44	1.11	0.0898	0.104	0.100
Maize (M)							
conventional	126.9	96.3	4.45	23.65	0.810	1.265	0.693
GM (MON 810)	122.0	92.0	4.10	23.65	0.767	1.111	0.699
pooled SEM	6.4	5.1	0.44	1.11	0.0898	0.104	0.100
Maize cv (MC)							
Clarica/Bacilla	126.6	96.2	4.74	23.23	0.882	1.192	0.738
PR39	122.3	92.1	3.82	24.07	0.695	1.185	0.655
pooled SEM	6.4	5.1	0.44	1.11	0.0898	0.104	0.100

[aA...]Within main effects means in columns with different superscripts differ significantly at: small letters –P<0.05; capitals –P<0.01.
All interactions were non-significant.

and litter microbiome as well as the bird's health. The diversity and activity of the gut microbiome depend on many factors, including the composition and structure of the feed, nutrient availability, feed particle size, physicochemical properties of feed components, feed additives, as well as the pH of the digesta and the activity of gut-associated lymphatic cells. The gut microbiota in chickens reaches a relatively stable, yet dynamic state at the age of about 14 days post-hatching and plays an important role in inhibiting the establishment of intestinal pathogens [Deusch *et al.* 2015; Pan and Yu 2014, Rehman *et al.* 2007].

Table 7. Main effects of conventional or GM SBM and gut segment on phytoestrogen concentrations in digesta

Main effect	Daidzin (D) µmol/g	Daidzein (DE) µmol/g	DE, % D	Genistin (G) µmol/g	Genistein (GE) µmol/g	GE, % G
Soybean meal (S)						
conventional	0.436^{A}	0.069^{A}	15.8	0.272^{A}	0.053^{a}	19.3
GM (GTS40-3-2)	0.553^{B}	0.103^{B}	18.6	0.456^{B}	0.069^{b}	15.2
pooled SEM	0.015	0.004	1.224	0.017	0.013	1.364
Intestinal segment (I)						
ileum	0.411^{A}	0.095^{b}	23.2^{b}	0.621^{B}	0.107^{B}	17.2^{b}
caecum	0.578^{B}	0.077^{a}	13.2^{a}	0.107^{A}	0.015^{A}	14.1^{a}
pooled SEM	0.016	0.005	2.209	0.020	0.005	1.672
Interaction						
S×I	>0.001	ns	0.038	>0.001	ns	ns

[aA...]Within main effects means in columns bearing different superscripts differ significantly at: small letters –P<0.05; capitals –P<0.01. ns – non-significant.

The chicken GIT contains more than 900 species of bacteria living in complex communities. This diverse microbiota helps in the breakdown and digestion of food and plays an important role in the health of the host, but only a fraction of the bacteria (10-60%) can be grown in the laboratory using culture-based methods [Deusch *et al.* 2015, Apajalahti *et al.* 2004]. Therefore, in our study a survey of ileal and caecal bacterial populations was performed using T-RFLP analysis of DNA isolated from the digesta. We found that in all dietary treatment groups members representing the orders *Clostridiales*, *Lactobacillales* and *Selenomonadales* were found in the ileum and caecum, while in the caecum *Bifidobacteriales* were also present. The microbiota of the caecum showed more diversity than that of the ileum for all dietary treatments, which is typical of these ecosystems. However, there were very large bird-to-bird variations within treatment groups, thus confirming the reports of Deusch *et al.* [2015] and Tan *et al.* [2012].

We found that GM soybean meal had no significant effect on the composition of the ileal microbiota, but despite great intragroup variation in broilers fed GM maize some members of the *Lactobacillales* order, most probably *L. intermedius* and *L. reuteri*, were less common compared with birds fed conventional maize. In the caecum of birds fed GM SBM some members of the *Clostridiales* were less plentiful and some members of *Bifidobacteriaceae* were more plentiful compared with birds fed conventional SBM. In birds fed GM maize differences were found in the abundance of some *Clostridiales* members compared with conventional maize.

The number of studies investigating the effect of feeding GM feedstuffs on the composition of gut microbiota in animals is limited. Tan *et al.* [2012] reported that feeding transgenic MON 40-3-2 SBM had little effect on the species number in the intestinal microbiota of broilers compared with conventional SBM. Buzoianu *et al.* [2012a] studied gut microbiota in weanling pigs after 31 days of Bt maize exposure. In a long-term study, Buzoianu *et al.* [2012b] demonstrated that feeding MON810

maize to pigs for 110 days had no effect on the abundance of *Enterobacteriaceae*, *Lactobacillus* or total anaerobes in ileal or caecal digesta or in faeces except for the abundance of the genus *Holdemania* in the caecum. In another experiment Buzoianu *et al.* [2013] demonstrated no adverse effects of dietary GM Bt maize on the intestinal microbiota of sows and their offspring, although some differences were observed in total anaerobe and *Enterobacteriaceae* counts and differences in the abundance of *Proteobacteria* between treatment groups. Schroder *et al.* [2007] reported that in rats fed Bt rice expressing the Cry1Ab protein coliform counts were higher in the ileum and counts of *Bifidobacterium* were lower in the duodenum, whereas no effect on faecal coliforms, *Lactobacillus* or total anaerobes was observed in comparison to rats fed non-transgenic parental wild type rice.

Intestinal bacteria are capable of producing and excreting many metabolites, e.g. inhibitory substances. These included SCFA, lactic acid, ammonia, hydrogen peroxide, bacteriolytic enzymes and bacteriocins, as well as several well designated or undesignated inhibitory substances exhibiting antimicrobial activity against a wide spectrum of microorganisms [Rehman *et al.* 2007; Van der Wielen *et al.* 2000]. Among others, the order *Lactobacillales*, particularly the *Lactobacillus* genus, are important beneficial microorganisms relevant to the healthy intestinal microbiota of chickens. Some *Lactobacilli* show specific activities, e.g. *L. salivarius*, *L. acidophilus* and *L. delbrueckii* produce significant amounts of both lactic acid and hydrogen peroxide [Yuksekdag *et al.* 2014] as well as bacteriocins active against some pathogenic bacteria [Messaoudi *et al.* 2012]. For these reasons, not only the counts and diversity of intestinal microbiota are important, but also their metabolic activity.

In our study we found that the use of GM maize and GM soybean meal had no influence on bacterial enzyme activity in the ileum and caecum, the only significant difference being found in β-glucosidase activity, which was higher when cv Clarica/Bacilla was used, and lower when cv PR39 was used. This last finding may indicate a difference in the content or structure of some non-starch polysaccharides between the maize cultivars used. The lack of differences in bacterial enzyme activity between birds fed GM and conventional feeds is consistent with the stable SCFA concentrations found in ileal and caecal contents also observed in this study. In ileal and caecal digesta concentrations of the total SCFA and its major component acetic acid did not differ between dietary treatments. In caecal digesta only the concentrations of propionic, isobutyric and valeric acids were lower in birds fed GM SBM when compared with conventional SBM.

Soybean meal contains isoflavones (ISF), diphenolic compounds that exist in conjugated (daidzin and genistin) or unconjugated (aglycone) forms. ISF exhibit several biological activities, as they e.g. may act as antioxidants and enhance immune system activity [Payne *et al.* 2001]. Only the aglycone forms of ISF, i.e. daidzein and genistein, are absorbed in the small intestine and exhibit biological activity in broilers [Jiang *et al.* 2007]. Intestinal flora plays a significant role in the biotransformation of isoflavones from glucosides into aglycone forms, while differences in the concentrations

of isoflavones and their metabolites in the intestinal tract may be due to the enzymatic activity of microbiota. Otieno *et al.* [2006] reported that *Bifidobacterium animalis*, *Lactobacillus acidophilus* and *Lactobacillus casei* cause a significant increase in the concentration of isoflavone aglycones via the β-glucosidase-catalysed hydrolysis of isoflavone glucoside conjugates**.** GM SBM meal used in the present study contains higher levels of daidzin and genistin, but a lower relative ratio of their metabolites than conventional SBM. However, the ratio of daidzein and genistein to daidzin and genistin, respectively, in the digesta of birds fed GM SBM did not differ from that in birds fed conventional SBM, confirming previous findings that GM feeds had no significant effect on the metabolic activity of microbiota.

The horizontal transfer of transgenes from GM feed to the microbiota is a very important issue considering its potential impact on consumer health. In first generation GM plants transgenic constructs were often of bacterial origin, suggesting that homologous DNA sequences could facilitate the incorporation of plant transgenes into the bacterial genome after transformation [Gebhard and Smalla 1998]. Thus transgenic crops had been considered as possible donors of transgenes that could be taken up by microorganisms under appropriate conditions. Additionally, they could potentially transfer their newly acquired resistance genes to other microorganisms [Guiemonde *et al.* 2014]. At present antibiotic resistance marker genes are not introduced into transgenic crop lines, including those used in this study: GM soybean GTS 40-3-2 and GM maize MON810 [CERA 2015].

Our results indicated that *E. coli* from the ileum and caecum of chickens were resistant to erythromycin and tetracycline, but susceptible to kanamycin, while *Clostridium* from the ileum and caecum were resistant to all the antibiotics used. The effect of GM feeds on the results of susceptibility tests was negligible. In birds fed with GM SBM resistance of *Clostridium* isolated from the ileum to kanamycin was about 9.3% higher, while resistance of *Clostridium* isolated from the caecum to kanamycin and erythromycin was about 9.1 and 7.1% higher, respectively, in comparison with the groups fed conventional SBM. It supports the conclusions of Chambers *et al.* [2002] that in broiler chicks gene flow from transgenic maize to the gut microbiota is very unlikely, as the antibiotic resistance markers from transgenic maize survived no better in their digestive tract than other plant DNA.

It may be concluded that the use of genetically modified soybean meal GTS 40-3-2 and genetically modified maize MON810 has little effect on bacterial community structure or its metabolic activity in broilers.

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