

# A novel microbiome metabolic modulator improves the growth performance of broiler chickens in multiple trials and modulates targeted energy and amino acid metabolic pathways in the cecal metagenome

M. C. Walsh,<sup>\*,1</sup> V. Jacquier,<sup>†</sup> G. Schyns,<sup>‡</sup> J. Claypool,<sup>‡</sup> I. Tamburini,<sup>§</sup> B. Blokker,<sup>†</sup> and J. M. Geremia<sup>§</sup>

*\*DSM Nutritional Products AG, Animal Nutrition & Health, Kaiseraugst, Switzerland; †DSM Nutritional Products, Research Center for Animal Nutrition & Health, Village-Neuf, France; ‡DSM Nutritional Products, Animal Nutrition & Health, Lexington, MA, USA; and §Midori USA Inc., Cambridge, MA, USA*

**ABSTRACT** A meta-analysis of 19 floor-pen trials (579 replicate pen observations) in diverse geographies, basal diets, seasons, and medication programs was carried out to evaluate the effects of 2 precision glycan microbiome metabolic modulators (**MMM1** and **MMM2**) on the performance of broiler chickens. In each trial, negative-control (**NC**) diets were compared with either MMM1 (14 trials) or MMM2 (8 trials), supplemented at an intended dose of 500 g/MT from hatch to 31 to 42 d. A dose response of MMM2 was evaluated in 8 trials at doses of 100, 250, 500, and 1,000 g/MT, not all present in each trial. Linear mixed-effect models were constructed for the final BW, cumulative feed intake, feed conversion ratio (**FCR**) corrected by mortality and BW (**cFCR**), and mortality, with Treatment as the fixed effect, nested random effects of Trial and Block, and adjustments for heterogeneity of variances. A significance level of  $P < 0.05$  was used. In one of the studies, cecal content samples were collected at 42 d for analysis of microbiome gene abundance. Microbiome metabolic

modulator 2 exhibited a reduction of the cFCR of 0.06 g feed/g BW gain compared with the NC and 0.03 g feed/g BW gain compared with MMM1, whereas MMM1 reduced the cFCR by 0.03 g feed/g BW gain compared with NC. Both MMM1 and MMM2 increased the final BW compared with the NC by 43 and 48 g/bird, respectively, with no difference among them. Compared with NC, feed intake was increased by MMM1 (+51 g/bird) and reduced by MMM2 (-74 g/bird). A one-directional dose response of the MMM2 ingredient was observed for the final BW (increasing) and cFCR (decreasing), whereas the feed intake response reached a minimum at 500 g/MT. The metagenomic analysis confirmed an increase in the abundance of genes belonging to the acrylate pathway, which is involved in propionate production, as well as arginine-N-succinyl transferase which is involved in the catabolism of arginine, in response to MMM2. Differential glycan structures of the MMM had an impact on the size and consistency of performance effects in broilers.

**Key words:** microbiome metabolic modulator, broiler chicken, performance, metagenome

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## INTRODUCTION

Advances in molecular biology, analytics, and data science are helping the scientific community gain a deeper understanding of how the metabolic function is linked to microbiome pathways in the gastrointestinal tract of animals (Sergeant et al., 2014; Glendinning et al., 2020) and humans (Qin et al., 2010). In parallel,

the global trend to reduce antibiotic growth promoters in the poultry industry has gathered momentum. These trends are enabling the development of microbiome metabolic modulators (**MMM**), a new category of nutritional feed ingredients that influence targeted metagenomic functions of the microbiome to impact the output of metabolites in the gut, with the final objective of delivering beneficial outcomes for the animal and the environment.

In the past decade, scientists studying the human microbiome found that, microbiota composition varies remarkably between healthy individuals even when corrected for diet and ethnicity; however, their metagenome did not. The functions the different microbiome perform (e.g. their collective metabolic pathways) were found to

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<sup>1</sup>Corresponding author: [Maria.Walsh@dsm.com](mailto:Maria.Walsh@dsm.com)

be stable from person to person (Hutterhower et al., 2012). The same concept is thought to hold true for animals. Unearthing such consistency in the microbiome suggests that the function is more fundamental than the composition. Modern microbiome science has come to view the microbiome as an organ—a group of tissues or cells adapted for specific functions. The detailed makeup can vary without sacrificing its overall role, and like all other organs, the microbiome has its own associated physiology and pathology (Baquero and Nombela, 2012). These insights have led to the hypothesis that nutritional ingredients targeting shifts in microbiome pathways may have more predictable effects than those targeting shifts in microbiota populations.

The biochemistry of glycans from the host and of dietary origin in the gut is exceptionally diverse. Glycans play an important role in shaping both the taxonomy and the functions of the microbiota (Flint et al., 2008; Koropatkin et al., 2012). One of the strategies being developed to modulate core microbiome metabolic pathways is the production and subsequent screening of tailored oligosaccharide populations in *in vitro* systems to modulate microbiome pathways (Geremia et al., 2016, 2020a). The MMM target pathways and metabolic outcomes of the microbiome, rather than modulate the abundance of various taxa (Pourabedin and Zhao, 2015).

Microbial energy and amino acid metabolic pathways are the most important for the host and the environment and therefore are important targets for MMMs. Energy pathways include glycolysis, carbohydrates, and lipid metabolism, as well energy extraction from amino acids. From all metabolites of these pathways, short-chain fatty acids (acetate, propionate, and butyrate) have important roles for animal health and metabolism beyond their contribution as an energy substrate (Byrne et al., 2015; Rios-Covian et al., 2016). Short-chain fatty acids are end-products of a sophisticated interaction between several bacterial groups that involves cross-feeding of intermediate metabolites (Louis and Flint, 2017). Nitrogen pathways cover not only amino acid transport and metabolism and the metabolism of amino acid groups but also *de novo* amino acid synthesis (Jiang et al., 2016). Evidently, strong overlaps exist between energy and amino acid metabolic pathways in the gut microbiome and can therefore be influenced by tailored glycans.

The objective of this study was to evaluate the effects of 2 glycan-based feed ingredients selected as MMM on the performance of broiler chickens. Selection criteria for the glycans were driven by their ability to impact (or influence) core microbiome metabolism. The 2 structurally distinct glycan ingredients were selected for their ability to increase the metabolic output of the C3 and C4 short chain fatty acids (SCFA) biosynthesis pathways (MMM1 and MMM2) and additionally modulate amino acid degradation and amine metabolism (MMM2). Broiler performance was evaluated in 19 independent trials using a control diet and at least one of the MMM

ingredients via feed. A metagenomics analysis of cecal digesta was carried out in a single trial to test the activation of microbiome biochemical pathways involved in the tricarboxylic acid (TCA) cycle and nitrogen metabolism such as amino acid degradations and urea cycle, in response to MMM2. Analysis of targeted pathways of interest is presented in this article, and a detailed description of the functional metagenomic changes will be reported separately.

## MATERIALS AND METHODS

### *Animal Trials*

A total of 19 floor pen trials were conducted using 33,880 broiler chickens. A complete description of the trials is presented in Table 1. All trials used one-day-old male chicks sourced in local hatcheries and raised in floor pens. Birds had *ad libitum* access to feed and water provided by bell feeders and bell or nipple drinkers throughout the experimental period. All breeds were selected from commercially relevant modern genetics (Cobb, Ross, Hubbard).

The number of birds per replicate varied from 9 to 1,800, with a total number of replicate pens per treatment between 6 and 21. The duration of the trials varied between 32 and 42 d, corresponding to commercially relevant market weights. Either commercially approved *in-feed* coccidiostats or a coccidiosis vaccine at hatch at the recommended doses was used in each trial. Vaccination systems varied according to local conditions and included mainly vaccines for coccidiosis, Marek's disease virus, Newcastle disease and avian infectious bronchitis. Light programs varied between 18 and 23 h of light per day along the experiments, according local management systems.

All diets were provided as crumbles in the starter and pellets in grower and finisher diets. Diet compositions and specifications are shown in Table 2. Most diets included a 3-phase program (starter, grower, and finisher), although trials 6, 13, and 19 used only a starter diet and a finisher diet. Five trials (2, 4, 6, 7, and 8) were performed by or in coordination with commercial poultry companies where diet specifications were proprietary and not disclosed (all of which were corn-soybean meal diets).

Animal-based meals were not used in any of the trials, with the exception of trials 1 and 3 which contained 5% animal by-product blend and 5% pork meal, respectively. All trials included either corn or wheat as base grain, soybean meal, and lower inclusions of corn-DDGS (<10%) or rye (<5%) in a proportion of the trials. The number of trials including corn vs. wheat or including fibrous ingredients was similar in trials that included only one of the test ingredients, which avoided bias due to ingredient composition. Nutritional specifications followed the breeder recommendations.

Experimental procedures were conducted in accordance with the applicable animal ethics guidelines for

**Table 1.** Summary of 19 broiler chicken trials included in the performance meta-analysis.<sup>1</sup>

Trial	Research site (country)	Breed	Sex	Facilities	Replicates/ treatment (#)	Birds/ replicate (#)	Feeding periods (day)			Trial duration (day)	Test treatments (dose, g/MT) <sup>2</sup>		
							Starter	Grower	Finisher		Negative Control (NC)	NC + MMM1	NC + MMM2
1	ARS (USA)	Cobb 500	M/F	Pens in house	6	14	15	9	11	35	0	500	—
2	SFAU (USA)	Cobb 500	M/F	Floor pens	12	60	15	9	11	35	0	500	—
3	(Canada) <sup>3</sup>	Ross 708	M	Floor pens	8	60	15	9	11	35	0	500	—
4	SFAU (USA)	Cobb 500	M/F	Floor pens	12	60	15	9	11	35	0	500	—
5	SRUC (UK)	Ross 308	M	Floor pens	8	38	10	14	11	35	0	500	—
6	(USA) <sup>3</sup>	Cobb 500	M/F	Floor pens	12	100	15	—	18	33	0	500	—
7	(USA) <sup>3</sup>	Hubbard M99	M	Pens in house	12	18	15	9	11	35	0	500	—
8	(USA) <sup>3</sup>	Hubbard M99 x Cobb 500	M	Floor pens	8	1,800	14	14	4	32	0	500	—
9	University of Guelph (Canada)	Ross x Ross 708	M	Floor pens	12	17	10	14	18	42	0	500	—
10	Roslin Nutr. (UK)	Ross 308	M	Floor pens	16	35	10	14	18	42	0	500	—
11	Zootests (France)	Ross 308	M	Floor pens	17	30	10	14	18	42	0	500	500
12	AH Pharma (USA)	Hubbard x Cobb	M	Floor pens	21	40	10	14	18	42	0	500	500
13	DSM (France)	Cobb 500	M	Floor pens	12	18	21	—	15	36	0	500	—
14	University of Guelph (Canada)	Ross x Ross 708	M	Floor pens	10	18	10	14	18	42	0	—	100, 250, 500, 1,000
15	AH Pharma (USA)	Hubbard x Cobb	M	Floor pens	14	40	10	14	18	42	0	500	100, 250, 500, 1,000
16	Massey University (New Zealand)	Ross 308	M	Floor pens	12	20	7	14	14	35	0	—	100, 250, 500
17	AgriSearch (Hungary)	Ross 308	M	Floor pens	13	30	14	14	7	35	0	—	250, 500
18	CTPA (France)	Ross 308	M	Pens in house	15	15	10	15	10	35	0	—	500
19	University of Poznan (Poland)	Ross 308	M	Floor pens	17	9	21	—	21	42	0	—	500

<sup>1</sup>All trials used local vaccination programs. Either a coccidia vaccine or a commercial coccidiostat was used in all trials. Industry-standard light programs with day lights from 18 to 23 h were used.<sup>2</sup>Two structurally distinct microbiome metabolic modulators were produced by the catalytic oligomerization of food sugars into tailored glycans (Geremia et al., 2016, 2020a): MMM1 (Glycodex, Midori USA, Inc., Cambridge, MA) and MMM2 (Glycan M2-1, Midori USA, Inc., Cambridge, MA; DSM Nutritional Products, Kaiseraugst, Switzerland).<sup>3</sup>Trial performed at a commercial poultry Company. Company names are not disclosed.

**Table 2.** Main ingredients and nutritional specifications of experimental diets of 19 floor pen trials with broiler chickens<sup>1</sup>.

Main ingredients and specifications (g/kg)	Trial 1	Trial 2 <sup>2</sup>	Trial 3	Trial 4 <sup>2</sup>	Trial 5	Trial 6 <sup>2,4</sup>	Trial 7 <sup>2</sup>	Trial 8 <sup>2</sup>	Trial 9	Trial 10	Trial 11	Trial 12	Trial 13 <sup>4</sup>	Trial 14	Trial 15	Trial 16	Trial 17	Trial 18	Trial 19 <sup>4</sup>
<b>Starter</b>																			
Corn	635.1	ND	634.1	ND	0.0	ND	ND	ND	560.8	0.0	0.0	583.5	439.0	527.0	583.5	0.0	0.0	239.4	320.0
Wheat	0.0	ND	0.0	ND	514.2	ND	ND	ND	0.0	517.8	551.0	0.0	50.0	0.0	0.0	549.2	560.0	368.1	304.8
Soybean meal	273.7	ND	283.0	ND	300.0	ND	ND	ND	341.0	280.0	280.0	300.0	360.0	342.8	300.0	283.1	50.0	332.6	298.5
Corn DDGS	0.0	ND	0.0	ND	0.0	ND	ND	ND	50.0	0.0	0.0	50.0	50.0	50.0	50.0	50.0	0.0	0.0	0.0
Rye	0.0	ND	0.0	ND	0.0	ND	ND	ND	0.0	50.0	50.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
AMEn (kcal/kg)	2,990	ND	2,940	ND	2,844	3,010	ND	ND	2,900	2,900	2,899	2,900	3,083	3,083	2,900	2,900	2,894	2,916	3,010
CP	22.1	ND	20.9	ND	22.7	ND	ND	ND	22.2	21.3	22.5	20.3	22.2	22.5	20.3	22.5	21.7	21.9	22.0
Dig Lys	1.02	ND	0.94	ND	0.91	ND	ND	ND	0.92	0.93	0.92	0.99	0.83	0.92	0.99	0.92	0.95	0.91	0.90
Dig Met	1.35	ND	1.14	ND	1.34	ND	ND	ND	1.24	1.25	1.24	1.33	1.30	1.24	1.33	1.24	1.21	1.21	1.21
Ca	0.90	ND	0.85	ND	0.86	ND	ND	ND	0.96	0.96	0.96	0.92	0.75	0.96	0.92	0.96	0.90	1.00	0.87
Available P	0.45	ND	0.35	ND	0.38	ND	ND	ND	0.48	0.48	0.48	0.35	0.34	0.48	0.35	0.48	0.46	0.47	0.46
<b>Grower<sup>3</sup></b>																			
Corn	685.5	ND	656.0	ND	0.0	—	ND	ND	565.3	0.0	0.0	653.8	—	542.9	653.8	0.0	0.0	189.4	—
Wheat	0.0	ND	0.0	ND	502.8	—	ND	ND	0.0	571.4	557.7	0.0	—	0.0	0.0	573.0	560.0	478.3	—
Soybean meal	219.9	ND	263.0	ND	266.0	—	ND	ND	280.2	170.0	240.0	204.0	—	271.0	204.0	230.5	230.0	266.3	—
Corn DDGS	0.0	ND	0.0	ND	0.0	—	ND	ND	100.0	0.0	0.0	100.0	—	100.0	100.0	60.0	0.0	0.0	—
Rye	0.0	ND	0.0	ND	0.0	—	ND	ND	0.0	50.0	50.0	0.0	—	0.0	0.0	0.0	0.0	0.0	—
AMEn (kcal/kg)	3,060	ND	2,988	ND	2,868	—	—	—	3,000	3,000	2,998	3,040	—	3,107	3,040	3,000	2,952	3,000	—
CP	20.0	ND	19.9	ND	21.4	—	ND	ND	20.8	19.0	20.9	17.5	—	20.5	17.5	20.5	20.6	19.8	—
Dig Lys	0.92	ND	0.86	ND	0.84	—	ND	ND	0.83	0.82	0.84	0.88	—	0.84	0.88	0.84	0.92	0.83	—
Dig Met	1.20	ND	1.06	ND	1.21	—	ND	ND	1.11	1.08	1.11	1.14	—	1.11	1.14	1.11	1.18	1.08	—
Ca	0.90	ND	0.80	ND	0.85	—	ND	ND	0.87	0.84	0.87	0.77	—	0.87	0.77	0.87	0.89	0.79	—
Available P	0.45	ND	0.28	ND	0.38	—	ND	ND	0.43	0.42	0.43	0.30	—	0.44	0.30	0.44	0.42	0.36	—
<b>Finisher</b>																			
Corn	743.2	ND	707.9	ND	0.0	ND	ND	ND	589.5	0.0	0.0	694.1	539.0	577.8	694.1	0.0	0.0	149.4	350.0
Wheat	0.0	ND	0.0	ND	507.5	ND	ND	ND	0.0	599.4	576.7	0.0	100.0	0.0	0.0	620.2	600.0	529.5	318.5
Soybean meal	274.0	ND	28.3	ND	208.0	ND	ND	ND	243.6	100.0	210.0	169.0	262.0	230.2	169.0	176.2	190.0	231.8	242.9
Corn DDGS	0.0	ND	0.0	ND	0.0	ND	ND	ND	100.0	0.0	0.0	100.0	50.0	100.0	100.0	60.0	0.0	0.0	0.0
Rye	0.0	ND	0.0	ND	0.0	ND	ND	ND	0.0	50.0	50.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
AMEn (kcal/kg)	3,150	ND	3,060	ND	2,892	3,200	ND	ND	3,100	3,097	3,099	3,084	3,130	3,130	3,084	3,100	3,000	3,150	3,200
CP	17.9	ND	17.8	ND	19.5	ND	ND	ND	19.4	17.5	19.53	16	18.6	18.8	16	18.8	19.2	18.5	19.9
Dig Lys	0.83	ND	0.86	ND	0.75	ND	ND	ND	0.80	0.78	0.77	0.79	0.74	0.78	0.79	0.79	0.9	0.76	0.82
Dig Met	1.06	ND	0.94	ND	1.08	ND	ND	ND	1.03	1.00	0.99	1.01	1.06	1.00	1.01	0.99	1.0	1.00	1.08
Ca	0.80	ND	0.70	ND	0.84	ND	ND	ND	0.81	0.75	0.79	0.72	0.67	0.79	0.72	0.78	0.9	0.70	0.71
Available P	0.40	ND	0.29	ND	0.39	ND	ND	ND	0.40	0.38	0.38	0.27	0.35	0.39	0.27	0.39	0.4	0.30	0.36

The premixes included vitamins A, D3, E, K3, and B complex vitamins along with manganese, iron, zinc, iodine, copper, selenium, and calcium at commercially relevant levels.

<sup>1</sup>All diets contained 1,000 FYT of phytase per kg (HiPhos, DSM Nutritional Products, Switzerland).

<sup>2</sup>ND = not disclosed.

<sup>3</sup>Five trials were performed by commercial poultry companies. Diet specifications were considered proprietary information and were not disclosed. All of these trials used U.S. corn-soybean meal-based diets with commercially relevant specifications.

<sup>4</sup>Trials 6, 13, and 19 only used a starter diet and finisher diet, not a grower diet.

the relevant country and were approved by the respective university or company animal ethics committee.

## Experimental Design

All trials had a completely randomized block design, testing the main effect of MMM treatments, with blocks intended to capture spatial variation within the barns. In all 19 trials, a negative-control (NC) treatment was compared against one or 2 of the MMM treatments: MMM1 or MMM2. 15 trials included the test ingredients at a single dose of 500 g/MT (Table 1). Four of the trials additionally tested one or more additional doses of MMM2 (100, 250, 750, 1,000 g/MT) during the entire test period.

Independent variables measured in the animal trials included the BW and cumulative feed intake at each time of diet change, as well as at the end of the trials. In each trial, the feed conversion ratio (FCR) was calculated as the pen total feed intake divided by the pen total weight gain. The FCR was mortality adjusted by adding back the weight of dead birds to the total pen weight and then corrected to a common weight (cFCR) using a correction coefficient calculated from published growth data for the corresponding bird genetics.

## Microbiome Metabolic Modulators

Glycan feed ingredients were produced by the catalytic oligomerization of food sugars into tailored glycans as described previously (Geremia et al., 2016, 2020a). The utility of glycans as MMM ingredients is dependent on their chemical composition. As such, 2 structurally distinct glycans were tested to demonstrate resulting differential effects on the host animal (MMM1 and MMM2). Structural characterization of glycans was performed using methods as described previously (Geremia et al., 2020b).

Microbiome metabolic modulator 1 (Glycodex, Midori USA, Inc., Cambridge, MA) was prepared at 1 kg and 1 MT scale from food-grade dextrose syrup (95% DE corn syrup, CAS no. 8029-43-4, Roquette America Inc., Keokuk, IA). The number-average (Mn) and weight-average (Mw) molecular weights of the resulting gluco-oligosaccharide were determined by size-exclusion chromatography or HPLC to be  $762 \pm 12$  g/mol and  $1,154 \pm 14$  g/mol, respectively. The glycosidic linkage distribution of MMM1 was characterized by 2-dimensional heteronuclear single quantum coherence nuclear magnetic resonance spectroscopy. Spectra were analyzed using MestReNova, version 11.0.4-18998 (Mestrelab Research S.L., Santiago de Compostela, Spain) to determine the relative abundance for peaks within the anomeric region of the 2D NMR spectrum. Identifying peaks were characterized as follows: (d1 = 103.39 ppm, d2 = 4.50 ppm) 31.3%  $\pm$  2.7%, (d1 = 102.31 ppm, d2 = 4.64 ppm) 2.4%  $\pm$  1.2%, (d1 = 109.16 ppm, d2 = 5.02 ppm) 2.1%  $\pm$  0.8%, (d1 = 102.45 ppm, d2 = 5.21 ppm) 1.2%  $\pm$  0.5%, (d1 = 99.71 ppm, d2 = 5.34 ppm) 4.2%  $\pm$  3.7%.

Microbiome metabolic modulator 2 (Glycan M2-1, Midori USA, Inc., Cambridge, MA; DSM Nutritional Products, Kaiseraugst, Switzerland) was prepared at multi-kg scale from food-grade glucose monohydrate (CAS no. 14431-43-7, Sigma-Aldrich, St. Louis, MO). The Mn and Mw molecular weights of the resulting gluco-oligosaccharide were determined by size-exclusion chromatography or HPLC to be  $773 \pm 37$  g/mol and  $1,181 \pm 90$  g/mol, respectively. The glycosidic linkage distribution of Glycan M2-1 was characterized by 2-dimensional heteronuclear single quantum coherence nuclear magnetic resonance spectroscopy. Spectra were analyzed using MestReNova, version 11.0.4-18998 (Mestrelab Research S.L., Santiago de Compostela, Spain) to determine the relative abundance for peaks within the anomeric region of the 2-dimensional nuclear magnetic resonance spectrum. Identifying peaks were characterized as follows: (d1 = 103.39 ppm, d2 = 4.50 ppm) 20.8%  $\pm$  0.4%, (d1 = 98.50 ppm, d2 = 4.95 ppm) 30.1%  $\pm$  0.8%, (d1 = 99.71 ppm, d2 = 5.34 ppm) 8.6%  $\pm$  0.2%, (d1 = 100.25 ppm, d2 = 5.39 ppm) 4.9%  $\pm$  0.1%, (d1 = 104.54 ppm, d2 = 4.62 ppm) 2.8%  $\pm$  0.1%.

## Statistical Analyses of Broiler Performance Data

The 2 main objectives of the present study were to 1) perform a meta-analysis of the existing broiler performance information measuring the effects of MMM1 and MMM2 vs. an NC at a single dose and 2) measure the broiler performance effects of different doses of MMM2. All statistical analyses were performed using R, version 4.0.0 (2020-04-24), with packages as described herein.

**Performance Meta-Analysis** A total of 579 replicate pen observations from 19 trials were used (Table 3). All of the 19 studies included the NC treatment (260 replicate pens), 14 trials included MMM1 (14 replicate pens), and 8 trials included MMM2 (127 replicate pens), with 3 trials including all 3 treatments. Linear mixed-effect models were constructed for the final BW, cumulative feed intake, cFCR, and mortality, with MMM treatment and base-grain as the fixed effects and nested random effects across Trial and Block, using the *lme* function from the *nlme* package of R (Pinheiro et al., 2015). The interactions between the base-grain and

**Table 3.** Numbers of pens, birds, and studies included in the statistical analysis.

Treatment <sup>1</sup>	Trials (#)	Replicate pens (#)	Birds (#)
Control	19	260	16,064
MMM1	14	192	14,728
MMM2	8	127	3,361

<sup>1</sup>Two structurally distinct microbiome metabolic modulators (MMM1: Glycodex, Midori USA, Inc., Cambridge, MA; and MMM2: Glycan M2-1, Midori USA, Inc., Cambridge, MA and DSM Nutritional Products, Kaiseraugst, Switzerland) were produced by the catalytic oligomerization of food sugars into tailored glycans (Geremia et al., 2016, 2020a) and tested as a feed ingredient in a subset of a total of 19 trials with broiler chickens.

MMM were tested and removed from the model as nonsignificant. The variance function allowed for different SD by the study site, resulting in the following R notation model:

```
cFCR=lme(cFCR~Treatment,
  random=~1|Trial/Block,
  weights=varIdent(form=~1|Site),
  method="REML",
  control=list(opt="optim"),
  data=data_set)
```

The model was quality checked for normality and against correlated residuals, outlier bias, and excessive leverage. No issues calling into question the model's fidelity were identified. No outliers were excluded from the data set because of statistical criteria. Mortality data were transformed for analysis using the *arcsin* function to allow normality.

Pair-wise comparisons with a Tukey's adjustment were performed using the same model and the multi-comp package of R (Pinheiro et al., 2015). The BW at placement was checked, and it was not significantly different among treatments. Mortality data for individual trials are reported but was not statistically analyzed because of low relevance for individual small-scale trials with low replication and numbers of animals.

The percentage of trials that reached different levels of reduction of the cFCR compared with the NC was plotted to evaluate consistency of response for each of the 2 treatments. The Ls-means for treatments in individual trials are reported for completeness. The ANOVA and Tukey's adjusted contrasts were performed with linear model with spatial blocking included in the model using R (Hothorn et al., 2008), and significant differences among means within trial are reported. A significance level of  $P < 0.05$  was used.

**MMM2 Dose Analysis** A total of 347 replicate pen observations from 8 trials were used. All 8 trials included the NC treatment (119 replicate pens), 3 trials included MMM2 at 100 g/MT (36 replicate pens), 4 trials at 250 g/MT (49 replicate pens), 8 trials at 500 g/MT (119 replicate pens), and 2 trials at 1,000 g/MT (24 replicate pens). The statistical model used the same configuration as the meta-analysis model shown before, with the only difference being the inclusion of the MMM2 dose (0, 100, 250, 500, 1,000 g MMM2/MT of feed) instead of MMM Treatment as the fixed effect.

For each independent variable, dose responses were plotted using a quadratic model with Smooth Conditional Means with the *geom\_smooth* function of the *gglot2* Package of R (Wickham, 2016).

## Metagenomics Analysis of the Cecal Content

The main focus of the overall study was centered on the effect of MMM2 with MMM1 acting as the benchmark. As such, together with a greater magnitude of the cFCR effect for MMM2, it was thought to be

sufficient to focus the whole-genome sequencing and metagenomic analysis of cecal samples to explore more deeply the effects of MMM2 on the microbiome function vs. the control. A partial analysis of microbial metagenome data with focus on pathways of interest is presented in this article, but more detailed analyses of functional metagenome effects will be reported separately.

Cecum digesta samples were collected (1 bird/pen) from the NC and MMM2 treatment (21 reps/treatment) in trial 12 (AHPharma, Hebron, MD) after cervical dislocation on day 42 and frozen at  $-80$  celsius before DNA extraction. Metagenomic DNA was obtained using DNeasy PowerSoil HTP96 according to the recommendations of the manufacturer (QIAGEN, Germany). DNA was sequenced at CoreBiome (New Brighton, MN) under a shallow shotgun technology called Booster-Shot that delivers a sequencing depth around 2M-5 M reads per sample (Illumina HiSeq3000, Illumina Inc., San Diego, CA). After classical treatment of the raw data which included controlling of raw sequence data by FastQC Report software, trimming with CutAdapt to remove adapters and finally mapping to the gene catalog with the Burrows-Wheeler aligner (BWA) were carried out. Gene sequences were then annotated against the chicken gut microbial gene catalog (9.04 million genes, based on 495 intestinal digesta samples; Huang et al., 2018).

Using the Kyoto Encyclopedia of Genes and Genomes (KEGG) genomic database (Kaneisha and Goto, 2000; Kaneisha, 2019; [www.genome.jp/kegg](http://www.genome.jp/kegg)), biochemical pathways involved in the TCA cycle and nitrogen metabolism such as amino acid degradations and urea cycle were extracted to build a simplified microbial metagenomic metabolic model. Gene sequences encoding 168 enzymes identified in this model were counted according their annotations. The abundances of the genes were normalized on the abundance of the same DNA sequence in the control sample. Data were analyzed using the nonparametric Mann-Whitney-Wilcoxon test with *P*-value adjust function that applied the *fdr* correction method (Luz Calle, 2019) using the *MultNonParam* statistical package of R.

## RESULTS

### Animal Performance

Table 4 shows the Ls means for the cFCR, cumulative feed intake, and final BW for individual trials. Significant effects ( $P < 0.05$ ) of MMM1 on the cFCR, final BW, and cumulative feed intake compared with the NC were observed in 3, 0, and 1 of 14 trials, respectively. For MMM2, significant effects on the cFCR, final BW, and cumulative feed intake were observed in 3, 0, and 2 of 8 trials, respectively. Mortality was generally less than 10% except for one treatment in one trial with a 12% mortality. Interferences about mortality at individual trial level were avoided because of the low relevance of this analysis in small-scale trials.

**Table 4.** Effects of 2 different microbiome metabolic modulator (MMM1 and MMM2) products<sup>1</sup>, supplemented in the feed in 19 independent trials, on the growth performance of broiler chickens raised in floor pens.

Trial	Trial duration (day)	Treatment	cFCR (g feed/g BW gain)		Cumulative feed intake (g/bird)		Final BW (g/bird)		Mortality <sup>2</sup> (%)
			LS mean	SEM	LS mean	SEM	LS mean	SEM	Mean
1	35	Control	1.612	0.047	3,300	96	2,034	16	4.8
		MMM1	1.574	0.013	3,253	52	2,063	23	1.3
2	35	Control	1.824	0.017	6,095	43	3,263	31	4.8
		MMM1	1.801	0.012	6,078	63	3,302	27	4.8
3	35	Control	1.579	0.014	3,660	46	2,305	30	1.6
		MMM1	1.571	0.020	3,717	43	2,354	22	1.6
4	35	Control	1.859	0.005	5,924	42	3,266	21	4.8
		MMM1	1.851	0.012	5,802	121	3,265	23	3.8
5	35	Control	1.430	0.016	3,532	52	2,490	48	7.7
		MMM1	1.401	0.006	3,616	22	2,669	11	7.1
6	33	Control	1.551	0.014	3,349	59	2,206	22	5.2
		MMM1	1.527	0.009	3,283	39	2,218	17	2.2
7	35	Control	1.726	0.013	5,839	38	3,378	26	9.1
		MMM1	1.702	0.010	5,851	41	3,422	25	3.0
8	32	Control	1.495	0.009	2,560	17	1,711	9	2.7
		MMM1	1.496	0.013	2,619	16	1,742	13	3.0
9	42	Control	1.543	0.015	4,679	61	3,026	44	4.3
		MMM1	1.519	0.016	4,725	72	3,081	52	3.0
10	42	Control	1.65 <sup>a</sup>	0.005	5,090	26	3,095 <sup>b</sup>	15	2.5
		MMM1	1.62 <sup>b</sup>	0.003	5,167	16	3,158 <sup>a</sup>	10	3.1
11	42	Control	1.667	0.009	5,867	47	3,229	20	6.7
		MMM1	1.640	0.013	5,832	76	3,210	30	8.8
		MMM2	1.623	0.006	5,674	43	3,227	21	5.2
12	42	Control	1.85 <sup>a</sup>	0.010	4,511	38	2,507 <sup>b</sup>	16	3.3
		MMM1	1.80 <sup>b</sup>	0.010	4,519	29	2,569 <sup>a,b</sup>	13	6.2
		MMM2	1.79 <sup>b</sup>	0.010	4,508	39	2,581 <sup>a</sup>	16	5.0
13	36	Control	1.562	0.040	4,021	137	2,614	32	7.4
		MMM1	1.524	0.059	3,896	59	2,617	60	7.4
14	42	Control	1.487	0.008	4,486	37	2,981	19	4.3
		MMM2	1.460	0.015	4,616	62	3,016	37	5.6
15	42	Control	2.00 <sup>a</sup>	0.008	4,525	36	2,442	13	0.0
		MMM1	1.95 <sup>b</sup>	0.010	4,531	46	2,502	16	0.0
		MMM2	1.94 <sup>b</sup>	0.014	4,514	38	2,508	17	0.0
16	35	Control	1.423	0.019	3,750	74	2,500	39	6.8
		MMM2	1.385	0.004	3,855	55	2,568	29	11.7
17	35	Control	1.79 <sup>a</sup>	0.009	3,366	16	1,984 <sup>b</sup>	7	3.2
		MMM2	1.68 <sup>b</sup>	0.006	3,222	12	2,026 <sup>a</sup>	5	1.7
18	35	Control	1.594	0.046	2,869	55	1,934	58	1.8
		MMM2	1.547	0.033	3,034	47	2,045	31	3.1
19	42	Control	1.519	0.009	4,951	54	3,297	31	0.0
		MMM2	1.508	0.017	5,024	67	3,350	49	0.0

<sup>a-d</sup>Means with different superscripts differed at  $P < 0.05$ . Superscripts are only shown when main effect of treatment had a  $P < 0.05$  in an ANOVA.

<sup>1</sup>Two structurally distinct microbiome metabolic modulators (MMM1: Glycodex, Midori USA, Inc., Cambridge, MA; and MMM2: Glycan M2-1, Midori USA, Inc., Cambridge, MA and DSM Nutritional Products, Kaiseraugst, Switzerland) were produced by the catalytic oligomerization of food sugars into tailored glycans (Geremia et al., 2016, 2020a) and tested as a feed ingredient in a subset of a total of 19 trials with broiler chickens.

<sup>2</sup>Mortality was not statistically analyzed in individual trials.

**Table 5.** Effects<sup>1</sup> of 2 different microbiome metabolic modulator products<sup>2</sup> supplemented in the feed in 19 independent trials, on the growth performance of broiler chickens raised in floor pens, evaluated with mixed models.

Parameter of interest	Negative control (NC)	MMM1	MMM2	SEM	F Probability
cFCR (g feed/g BW gain)	1.643 <sup>a</sup>	1.616 <sup>b</sup>	1.586 <sup>c</sup>	0.036	<0.001
Feed intake (g/bird)	4,335 <sup>b</sup>	4,386 <sup>a</sup>	4,261 <sup>c</sup>	246	<0.001
Final BW (g/bird)	2,648 <sup>b</sup>	2,691 <sup>a</sup>	2,696 <sup>a</sup>	125	<0.001
Mortality (% pen)	4.17	4.16	3.73	0.61	0.48

<sup>a-d</sup>Means with different superscripts differed at  $P < 0.05$ .

<sup>1</sup>Linear mixed-effect models considered MMM Treatment as the fixed effect and nested random effects across Study and Block, using the lme function from the nlme package of R (Pinheiro et al., 2015). The variance function allowed for different SD by the study site.

<sup>2</sup>Two structurally distinct microbiome metabolic modulators (MMM1: Glycodex, Midori USA, Inc., Cambridge, MA; and MMM2: Glycan M2-1, Midori USA, Inc., Cambridge, MA and DSM Nutritional Products, Kaiseraugst, Switzerland) were produced by the catalytic oligomerization of food sugars into tailored glycans (Geremia et al., 2016, 2020a) and tested as a feed ingredient in a subset of a total of 19 trials with broiler chickens.

Broiler performance results of the meta-analysis of 19 trials are presented in Table 5. Significant effects of treatment were evident for the final BW, cumulative feed intake, and cFCR. Microbiome metabolic modulator 2 exhibited a reduction of the cFCR of 0.06 g feed/g BW gain compared with the NC and 0.03 g feed/g BW gain compared with MMM1. Microbiome metabolic modulator 1 also presented a reduction of the cFCR of 0.03 g feed/g BW gain compared with the NC.

Compared with the NC, the feed intake was increased by MMM1 (+51 g/bird) and reduced by MMM2 (-74 g/bird). Both MMM1 and MMM2 increased the final BW compared with the NC treatment by +43 and +48 g/bird, respectively, with no significance difference among them. Mortality was not significantly different for MMM treatments.

Figure 1 presents the percentage of trials for MMM1 and MMM2 that reached different levels of reduction of the cFCR compared with the NC. The proportion of trials that reached a reduction of 3 cFCR points (0.03 g feed/g BW gain) was 36% for MMM1 and 75% for MMM2, whereas the proportion of trials reaching a reduction of 4 cFCR points was 21% for MMM1 and 63% for MMM2.

The dose-response analysis for MMM2 demonstrated quadratic responses for the cFCR, cumulative feed intake, and final BW (Figure 2). For cFCR, a negative slope with a positive second derivative was present within this dose range. All tested MMM2 doses significantly reduced the cFCR compared with the NC, but 500 and 1,000 g/MT had a significantly lower cFCR

than 100 g/MT. The regression suggested a plateau between 500 and 1,000 g/MT.

For the final BW, a positive and decreasing slope was evident, with doses of 250, 500, 750, and 1,000 g/MT presenting a significant difference vs. the NC and no difference among their means. The fitted function had not reached a maximum at 1,000 g/MT. The 100 g/MT dose did not influence the final BW ( $P > 0.05$ ).

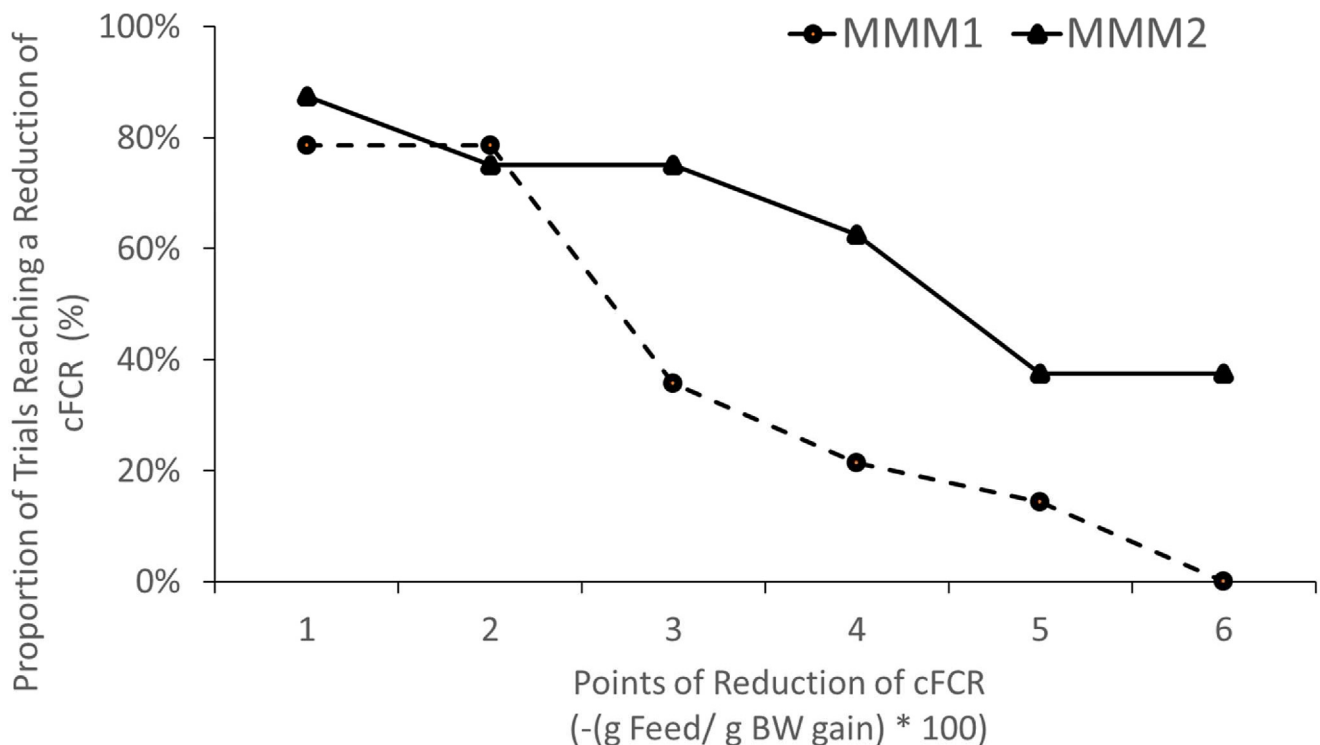
A one-directional dose response of the MMM2 ingredient on the cumulative feed intake was not observed in neither a positive nor a negative direction. However, a minimum was observed at 500 g/MT, which was significantly different from the NC.

### Metagenomics of Cecal Content Samples

Table 6 presents changes in relative abundance of genes involved in the TCA cycle and nitrogen metabolism of the chicken cecum microbiome in trial 12. The normalized abundance of the lactate CoA and propionate CoA transferase (EC 2.8.3.1) was significantly higher in MMM2 treated samples than the NC samples. Equally, the relative abundance of arginine-N-succinyltransferase increased significantly in MMM2 compared with NC samples.

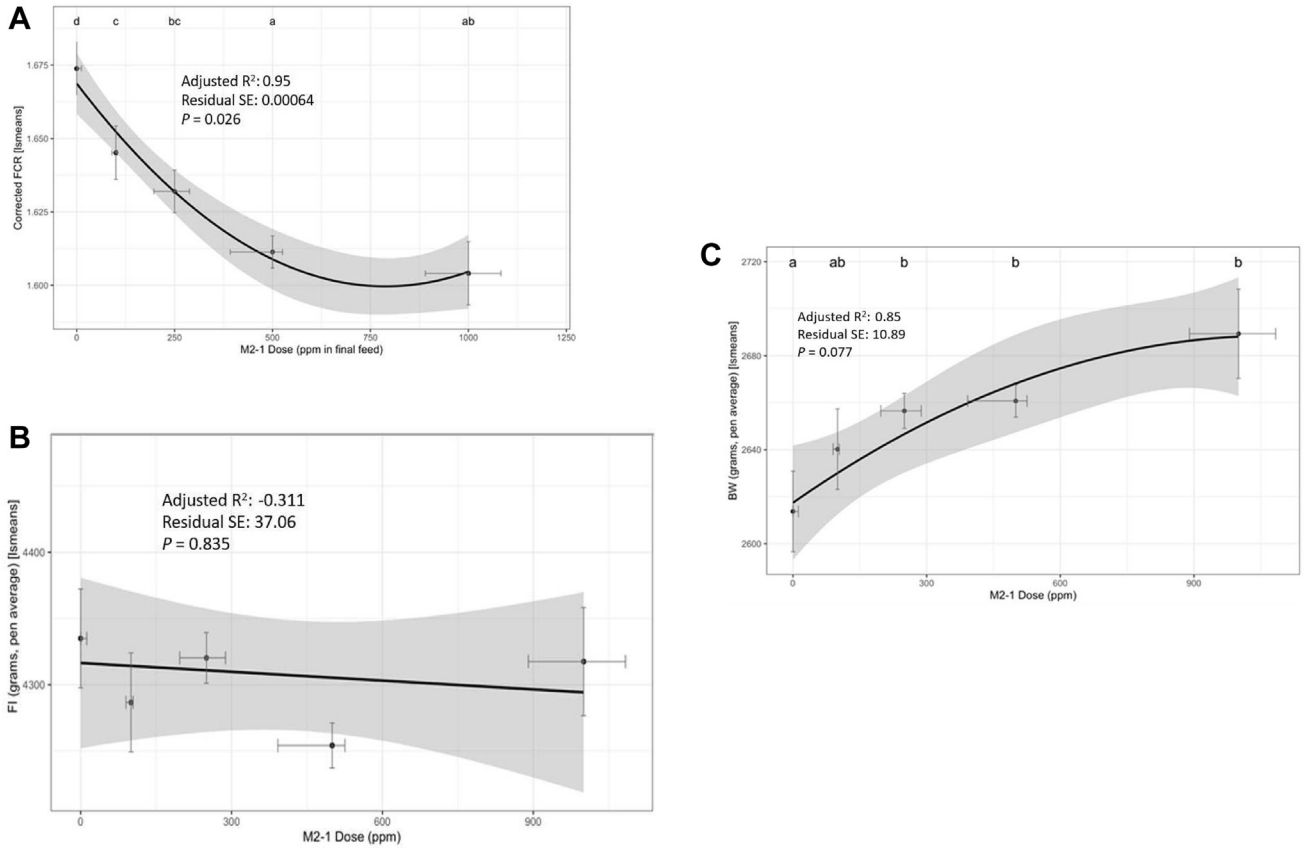
## DISCUSSION

The primary objective of this study was to evaluate the effects of 2 glycan feed ingredients selected as MMM1 and MMM2 on the performance of broiler chickens. The 2 structurally distinct glycan ingredients



**Figure 1.** Percentage of broiler chicken trials reaching different levels of the cFCR (mortality and BW-corrected FCR) for 2 distinct microbiome metabolic modulator products (MMM1 and MMM2) applied in feed at 500 g/MT.





**Figure 2.** Tukey’s corrected Ls mean comparisons and smooth regressions of the effect of dose in feed of microbiome metabolic modulator MMM2 (M2-1) on (A) the BW and mortality-corrected FCR (cFCR); (B) cumulative feed intake, and (C) the final BW in 8 trials with broiler chickens from 0 to 32-42 days. Vertical error bars reflect the SE in the mean. Horizontal error bars reflect the estimated range of doses formulated into feed for each intended dose level. Inclusion of the glycan microbiome metabolic modulator (MMM2) in the final feed was determined by liquid chromatography with tandem mass spectrometry (LC-MS/MS) using methods described previously (Geremia et al., 2020b). For each dose level, the error bars were determined as the (asymmetric) 1-sigma CI obtained from in-feed assaying of pelleted samples drawn from n = 35 independent diet formulations (i.e., study, treatment group, and phase). <sup>a-d</sup>Means with different superscripts differed at P < 0.05.

were selected for their ability to increase metabolic output of the C3 and C4 SCFA biosynthesis pathways (MMM1 and MMM2) and additionally for their ability to limit (or reduce) amine biosynthesis linked to amino acid degradation (MMM2).

Animal data from 19 trials allowed an evaluation of animal trial results through a mixed model that included random effects and accounted for differences in intratrial variation. This approach provided a robust estimation of animal performance results in small-scale trials to be validated at commercial scale. It contrasts with the common approach of publishing single performance trials,

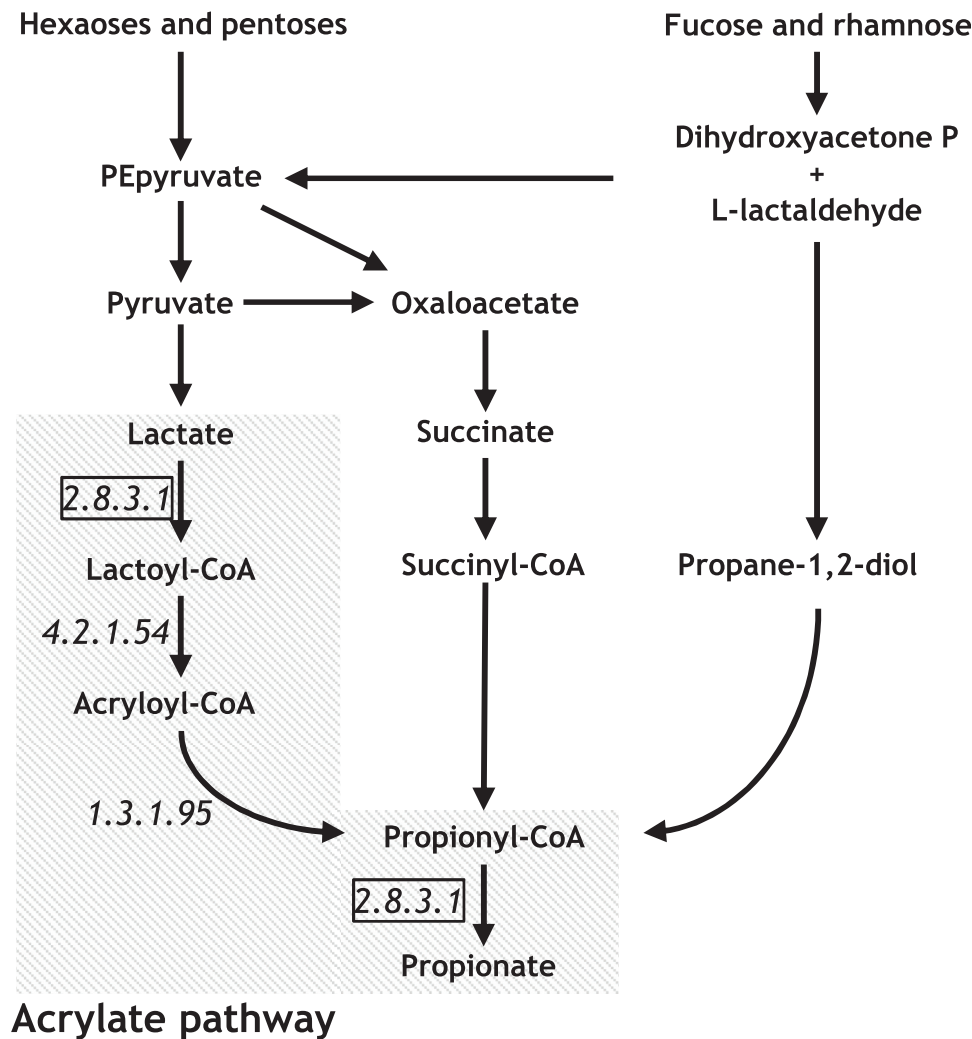
which suffers from publication bias when positive results are prioritized for publication. For instance, Blajman et al. (2014) carried out a meta-analysis of 46 articles evaluating broiler performance in response to probiotics supplementation and reported evidence of publication bias.

The low consistency of performance responses in empirical trials is frequently mentioned as an issue for probiotics and prebiotics feed additives aiming to modulate intestinal microbiota (Ducatelle et al., 2015). It is hypothesized that one reason for the inconsistent response of feed additives with mechanisms of action

**Table 6.** Changes in the abundance of selected cecal microbial genes involved in tricarboxylic acid cycle and nitrogen metabolism in response to a microbiome metabolic modulatorsupplemented in feed compared with the control.

End product	Related metabolites	Enzyme	EC code enzyme	Relative abundance gene	P Value	Q value
Propanoate	(R)-lactate	Lactate CoA transferase	2.8.3.1	1.17	0.001	0.039
	(R)-lactoyl-CoA	Lactoyl-CoA dehydratase	4.2.1.54	ND <sup>1</sup>		
	Acryloyl-CoA	Acryloyl-CoA reductase	1.3.1.95	ND		
	Propanoyl-CoA	Propionate coA transferase	2.8.3.1	1.17		
N2-succinyl-L-arginine	Succinyl-CoA	Arginine-N-succinyl transferase	2.3.1.109	3.05	1.4 E-6	1.6 E-4

<sup>1</sup>ND = not determined.



**Figure 3.** Schematic representation of the known pathways for propionate formation in gut bacteria (based on the study by Reichardt et al., 2014), with emphasis on the acrylate pathway, highlighted in gray.

that aim to modulate the abundance of specific taxa of bacteria like species from the *Enterobacteriaceae* or *Lactobacillaceae* families is that those responses are subject to a high level of variation in the taxonomic profile of intestinal microbiota in different trial conditions and geographies. It has been reported that performance effects for probiotics are generally greater when challenge conditions are present, whether those are direct pathogen or dietary challenges (Ducatelle et al., 2015). Other feed ingredients that are traditionally considered prebiotics such as yeast cell walls also elicit a significant stimulation of the innate immune system in the intestinal mucosa (Broadway et al., 2015). Those effects provide benefits particularly in periods of greater stress or when factors promoting dysbacteriosis are present.

The approach of activating metabolic pathways of the microbiome with precision ingredients has the advantage of being independent on the composition of the microbial communities in the gastrointestinal tract, which are highly variable, but relying on always present microbiome pathways. The pivotal observation made by Turnbaugh et al. (2007) that metagenomic carriage is

stable among individuals despite variation in community structure endorses that assumption. Diverse groups are currently replicating that observation in chickens, and it has already been documented in broadly diverse organisms (Jiang et al., 2016).

In the present study, the meta-analysis (Table 5) indicated clear differences in the performance of chickens when supplemented with MMM2 vs. MMM1 at an equal dose, with MMM2 exhibiting greater responses on the cFCR and final BW. In addition, a greater consistency of response was observed for MMM2 than MMM1, with proportionally more trials reaching reductions of the cFCR that are considered biologically and economically relevant (>0.03 g feed/g BW gain). These observations confirmed the hypothesis that structural differences in glycan structure among MMM ingredients result in differences in the extent and the consistency of the performance response.

A clear dose response for MMM2 on the final BW and cFCR was present, suggesting a plateau for the cFCR at a dose between 500 and 1,000 g/MT. Those types of dose effects on broiler performance are rarely seen with probiotics and prebiotics in the absence of a challenge model.

Although both MMM ingredients increased the final BW and feed efficiency, a divergent feed intake response between MMM ingredients was observed, with the MMM1 ingredient increasing and MMM2 decreasing the feed intake compared with the NC. It is possible that structural glycan differences may have affected differentially microbiome metabolic pathways with effects on appetite, although there is not enough information in the present study to accept or reject that hypothesis. Differences in the ability of SCFA, in particular butyrate vs. propionate and acetate, to elicit anorexigenic signaling have been reported in mammals (Byrne et al., 2015). Similarly, changes in the production of biogenic amines by the microbiome can have a significant impact on appetite and energy balance (Nelson and Gehlert, 2006). Given the fact that these MMM ingredients were selected for their ability to influence microbial pathways of both energy and nitrogen utilization, it is possible that a combination of these effects on systemic metabolism was present. This approach to feed additive screening and selection opens new opportunities for targeted modulation of animal metabolism.

The analysis of changes in the microbiome pathways of energy and nitrogen metabolism in the cecum of chickens in one of the trials (Table 6) offered a glimpse of the changes that occurred at the microbiome environment level. Only a subset of the metagenomics data on pathways of interest is presented in the present article, and a more detailed view of metagenomic effects of MMM2 will be reported separately. Inferences presented here are still preliminary and aim to provide initial evidence of the mode of action. Lactate CoA and propionate CoA transferase are the main signature enzymes of the acrylate pathway, one of the 3 pathways that produces propionate in the gut (Figure 3; Reichardt et al., 2014). These enzymes were upregulated in the microbiome of birds receiving MMM2 supplementation. The propionate produced by the microbiome in the gut can be uptaken by the animal and transferred to the liver to support gluconeogenesis (Ringer, 1912) but also contribute to changes in energy balance affecting neuroendocrine feedback mechanisms systemically (Richards and Proszkowiec-Weglarz, 2007; Byrne et al., 2015). This mode of action may be one of the drivers of the performance results in response to MMM2 supplementation in this set of broiler trials.

Equally, it is interesting to highlight changes in the relative abundance of the arginine-N-succinyl transferase gene. This enzyme catalyzes the first step of one of the catabolic pathways of arginine, ultimately allowing the carbon flux to re-enter the TCA cycle at the  $\alpha$ -ketoglutarate step from glutamate (Cynober, 1994). TCA is the major energy-yielding metabolic pathway in the cell and ultimately delivers most of the ATP production of the cell. Apart from the possibility of redirecting amino acid metabolism toward energy yielding pathways of the microbiome, this finding suggests that it is possible to modulate amino acid metabolism pathways in the chicken intestine with designed glycans, either in the direction of deamination or decarboxylation (Fan et al., 2015).

The positive impact of MMM2 on the productivity of chickens compared with the NC and MMM1 was supported by in vivo metagenomic changes in SCFA and amino acid metabolism pathways, corresponding to the in vitro selection criteria of this ingredient. This study demonstrated that differential glycan structures in glycan-based MMM ingredients have an impact on the magnitude and consistency of performance effects in broilers chickens and offer the opportunity for consistent targeting of metabolic pathways in the microbiome that positively impact animal productivity, welfare, and environmental sustainability.

## DISCLOSURES

The authors disclose that there are no conflicts of interest that the reviewers should be made aware of.

## SUPPLEMENTARY DATA

Supplementary data associated with this article can be found in the online version at <https://doi.org/10.1016/j.psj.2020.10.054>.

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