

# Effects of *Bacillus subtilis*, butyrate, mannan-oligosaccharide, and naked oat ( $\beta$ -glucans) on growth performance, serum parameters, and gut health of broiler chickens

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**ABSTRACT** Four nonantibiotic alternative growth promoters for broiler chickens were evaluated. Ross 308 chicks were fed a control diet (mainly corn and soybean meal) or a diet supplemented with a probiotic (*Bacillus subtilis* Gallipro DSM 17299), encapsulated butyric acid (Novyrate C), mannan-oligosaccharide (Actigen MOS) or formulated with 20% naked oat (starter diet) and 30% naked oat (grower and finisher). The study was carried out as a complete random blocked design with 10 pens for each diet, 45 birds per pen. Compared to the control, the naked oat diet improved the average daily gain by 16% during the starter phase (up to d 10). The probiotic did so during the grower phase as did butyric acid in the finisher phase (up to d 34). For the experiment overall, the probiotic decreased average daily gain

slightly. The best improvement in feed conversion ratio was obtained in the butyrate group (5%). No significant treatment effect on crop pH or on mortality was observed. The naked oat diet gave a slightly lower cecum pH on d 34. The MOS supplement decreased jejunal mass on d 34 and increased villus length (34%) and villus height/crypt depth ratio (32%) measured on d 10. Naked oat, butyric acid and MOS diets all reduced serum endotoxin levels. The probiotic increased serum C-reactive protein. All noncontrol diets reduced serum malondialdehyde. The naked oat diet reduced d 34 litter pH by about 0.3. Some effects of the proposed non-antibiotic growth promoters have been observed and could contribute to livestock performance. Their exact modes of action remained to be defined.

**Key words:** broilers, antibiotic, alternative to antibiotic growth promoters, growth performance, gut health

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## INTRODUCTION

For least 50 yr, antibiotic growth promoters (AGP) have been commonly used to enhance weight gain in poultry production (Engberg et al., 2000). This practice has participated to the development of antibiotic resistance which has driven many countries to reduce their use, and led to the withdrawal of antibiotic growth promoters in the European Union in January 1, 2006 (Castanon, 2007). In Canada, as of May 15, 2014, Canadian Chicken farmers are no longer permitted to use preventatively Category I antibiotics, while the preventive use of Category II antibiotics has been stopped in January 2019. The goal is to eliminate the preventive use of Category III antibiotics by the end of 2021 (Chicken Farmers of Canada, 2021). Antibiotic

resistance is defined as the ability of microorganisms to proliferate in the presence of an antibiotic that generally inhibits or kills microorganisms of the same species (Ruma, 2016). The resulting growth rate reduction with AGP removal impacts production efficiency and may influence food safety and broiler health (Zhao et al., 2001). Consequently, research efforts have been done to find alternatives to maintain feed efficiency and broiler health in the absence of AGP (Diarra and Malouin, 2014). The efficacy of an alternative to AGP may be characterized by FCR maintenance, low mortality rate, and a good gut health status (Yegani and Korver, 2008). Many alternatives to AGP have been proposed to improve animal health, growth performance, and immune response. With the objective of determining the most studied and most promising alternatives by quantifying their effects, a meta-analysis of 79 scientific articles published from 2000 to 2017 has been performed (Rouissi et al., unpublished data). It showed that the most frequently studied alternatives to AGP in broilers are the following classes (with the most studied in percentage) probiotics (76% *Bacillus subtilis*), prebiotics (82% Mannan-oligosaccharides), organic

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acids (67% butyrate), and essential oils (52% oregano-based). Significant positive effects on average daily gain (7–9%) and FCR (2.4–8%) have been observed for these AGP. It was however not possible to look at the underlying mode of action with this approach. Organic acid supplementation of broiler diets, particularly butyric acid, has drawn attention because of its antimicrobial properties, effects on gastrointestinal mucosa growth improving cell proliferation epithelial and villi height (Mehdi et al., 2018). Furthermore, the organic acids in poultry might have been showed to have direct effect on the gastrointestinal tract (GIT) bacteria population, reducing the level of some pathogenic bacteria (Khan and Iqbal, 2016). Probiotics which is viable microorganisms, such as *Bacillus*, *Bifidobacterium*, *Enterococcus*, *Escherichia*, *Lactobacillus*, *Lactococcus*, and *Streptococcus*, used as feed additives are another category that receives a lot of attention. The modes of action of probiotics were extensively reviewed (Simon et al., 2001; Ghadban, 2002; Edens, 2003) and consist in; 1) competitive exclusion including competition for substrates, production of antimicrobial metabolites that inhibit pathogens, and competition for attachment sites and, 2) immune modulation. Another category of alternative to AGP is prebiotics, which are nondigestible food ingredients that beneficially affect the host by selectively stimulating the growth and/or activity of intestinal bacteria and fermentation (Choct, 2009; Huyghebaert et al., 2011). There is a plethora of commercially available prebiotics such as fructo-oligosaccharides (FOS), oligofructose, inulin, mannan-oligosaccharides (MOS), arabinoxylo oligosaccharides (AXOS), xylo-oligosaccharides (XOS), and neoagaro-oligosaccharides (NAOS), (Femia et al., 2010; Patel and Goyal, 2012). However, there are also natural prebiotics such as naked oats, which are too often overlooked. For example, naked oats contain beta-glucans ( $\beta$ -glucans), nonstarchy polysaccharides with known prebiotic effects (Svihus and Gulford, 2002; Osek et al., 2003).

Based on the results of previous studies (Rouissi et al., unpublished data), the hypothesis that is possible to maintain growth performance in a context of antibiotics growth promoter withdrawal with natural alternatives was tested. The objective of this study was to evaluate and compare in similar conditions the in vivo effects of butyric acid, mannan-oligosaccharide, *Bacillus subtilis* and naked oat on growth performance, selected serum parameters of health status and gut morphology of broilers, as well as litter quality characteristics that provide proxy evidence of bird wellbeing.

## MATERIALS AND METHODS

The trial was conducted at the Deschambault Animal Science Research Center (120 Roy street, Deschambault, QC G0A 1S0, Canada) from January 2019 to February 2019 and approved by Animal Care Committee of Laval University (CPAUL) that is accredited by the Canadian Council of Animal Care (CCAC) (Guidelines of the Canadian Council of Animal Care, 2009).

### Birds and Housing

Day-old (male Ross 308) chicks (n = 2,250) obtained from a local commercial hatchery were assigned randomly to one of 50 floor pens (7.2 m<sup>2</sup>; 45 birds each). Each pens come with an automatic Hanging Waterer and chick's galvanized Poultry Feeder 5L.

Ambient temperature was maintained at 33°C during the first week and reduced gradually to 22°C by the end of the third week and maintained at 22°C until the end of the trial. The lighting program met Ross 308 guidelines, as did the starter phase diet (Ross 308 Broiler Nutrition Specifications, 2014). During the grower and finisher phases, metabolizable energy and standardized ileal digestible (SID) lysine were set at 96% of the

**Table 1.** Broiler chicken basal diet composition (ingredient %).

Ingredient	Diet					
	Starter		Grower		Finisher	
Corn meal	55.018	39.4	56.66	31.82	61.4	36.56
Naked oat <sup>1</sup>	-	20	-	30	-	30
Soybean meal 48%	26.1	22.1	17.6	15	12.2	9.5
Wheat	0	0	10	10	10	10
Soybean meal 44%	10	10	9.3	7	10.5	8.3
Meat/bone meal	5	5	4.6	4.1	4.1	3.6
Calcium carbonate	0.66	0.69	0.49	0.61	0.55	0.67
Sodium chloride	0.22	0.21	0.21	0.19	0.22	0.19
Sodium bicarbonate	0.21	0.22	0.21	0.25	0.21	0.26
L-lysine	0.25	0.33	0.25	0.34	0.22	0.31
DL-methionine	0.37	0.34	0.29	0.25	0.24	0.2
Threonine	0.09	0.12	0.07	0.1	0.06	0.08
Micronutrient premix <sup>2</sup>	0.2	0.2	0.2	0.2	0.2	0.2
Others <sup>3</sup>	0.15	0.15	0.12	0.14	0.1	0.13
Calculated content						
Metabolizable kcal/kg	3026	3027	3029	3025	3075	3075
Crude protein (N × 6.25)	23.3	23.2	20.2	20.3	18.2	18.3
Lysine digestible (%)			1.0580	1.0601	0.9303	0.9294

<sup>1</sup>Values used for formulation are: crude protein, 14.9%, Fat, 5.5%, NDF, 8.3%, AMEn, 3 200 Kcal/kg.

<sup>2</sup>vitamins and trace elements.

<sup>3</sup>phytase, choline, vitamin E, HyD.

**Table 2.** Description of the experimental design and dietary treatments.

Items	Control	Butyrate1	MOS2	Bs3	N-Oat4
Number of pens per treatment	10	10	10	10	10
Incorporation rate, kg/t					
Starter (1–10 d)	-	1,00	0,800	0,125	200
Grower (11–21 d)	-	0,500	0,400	0,125	300
Finisher (22–35 d)	-	0,250	0,200	0,125	300

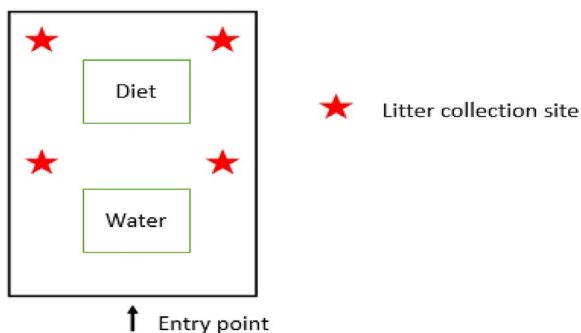
requirements (Table 1) to ensure that any growth promotion effect would be observable.

## Experimental Design

All birds were fed as pellet 3 basal diets (Table 1): starter (0–10 d), grower (11–21 d), and finisher (22–34 d) diets that differed only within each phase by the addition of one of the supplements at manufacturer recommended dose (Table 2) as follows: 1) a Control diet (basal diet), 2) Control + an encapsulated butyric acid butyric acid (SSFA, NUTRI-AD, Dendermonde, Belgium) with 30% of C4:0, 3) Control + Actigen prebiotic, (Alltech Inc, St-Hyacinthe, QC, Canada) a second generation MOS, 4) Control + probiotic, Gallipro, (Chr. Hansen, Denmark, distributed by DCL, St-Hyacinthe, QC, Canada) a *Bacillus subtilis*-based probiotic (strain DSM17299), and 5) naked oat as a natural  $\beta$ -glucans source (SEMICAN Inc. Plessisville, QC, Canada). The products were added manually at the feed mill without specific premix or carrier.

## Data Collection

**Litter Moisture and pH** A 100 g litter sample was collected from all 50 pens on d 9, 20 and 30 for moisture content and pH measurement. The pen was virtually divided into 4 similar zones and one sample per zone was taken while avoiding being too close to the drinkers and feeders and they were homogenized before taking the 100 g (Figure 1). Litter samples were oven-dried at 100°C for 2 d and moisture content percent was calculated with the following formula:  $[100 - (\text{dry weight} - \text{content weight}) / (\text{wet weight} - \text{content weight})] * 100$ . For pH measurement (Rajkovich et al., 2011), 1 g of ground litter sample (<1 mm) was added to 20 mL of deionized water. The suspension was put on a shaker (Boekel

**Figure 1.** Diagram of a parquet floor and litter collection points.

Scientific, Orbitron II Laboratory Mixer, Model 260250, Pennsylvania Blvd, PA) for an hour and then left to stand for one hour for the liquid and solid phases to separate. The pH was measured in the liquid phase using a portable pH meter (Ross, Orion Star A221, Thermo Scientific, Beverly, CA).

**Feed Analysis** Feed was sampled during the batch bagging process (500 g) and at the beginning of each dietary phase. Dry matter, crude protein, calcium, phosphorus, sodium, fat, and energy (Table 3-1) were analyzed according to AOAC (2000) methods.

**Performance Data** Birds and feed were weighed (on d 0, 10, 21, 28, and 34) to evaluate average daily feed intake (ADFI), average daily gain (ADG), and feed conversion ratio (FCR) for each growing diets. Mortality was recorded daily. All dead birds and those euthanized for tissue sampling (10, 21, and 34 d) were individually weighted and growth performance data were corrected for mortality.

## Intestinal Morphology and Histomorphometry

**Parameters** At 10, 21, 28, and 35 d of age, one bird per pen (10 per treatment) were euthanized by cervical dislocation. The pH of the crop, distal ileum, and cecum was measure using a portable pH meter (Orion Star A221, Thermo Scientific, Saint-Laurent, QC, Canada). The duodenum, jejunum, ileum, and caeca were weighed. Once emptied of its contents with distilled water using Nalgene LDPE wash bottle, a section of the distal ileum was collected for intestinal villi and crypts measurements. A first cross-section was made 1 cm from Meckel's diverticulum and the second 1.5 cm distally. Each tissue was individually fixed in a formalin solution and stored at room temperature (Marković et al., 2009). Multiple 5  $\mu$ m sections of paraffin embedded tissues were obtained from each sample with a microtome and stained with hematoxylin and eosin (Scheuer and Chalk, 1986). Villus and crypt measurements were based on 18 villi and crypt per bird using a microscope (Microscope Central, Nikon Eclipse E600 Fluorescence, Feasterville, PA) (objective lens: X4) and a PC-based image analysis system and software analysis (Q-Capture, Fiji).

## Cecal Short-Chain Fatty Acid Profile in the Cecum

One gram of cecum content was extracted upon euthanasia with an alcohol-cleaned spatula, placed in a 15 mL conical tube, acidified with 2 mL of H<sub>2</sub>SO<sub>4</sub> (1.5%), vortexed then frozen at -20°C. The frozen sample was homogenized and centrifuged at 10,000 RPM for 15 min at 4°C. The supernatant was analyzed on a Hewlett Packard 6890N gas chromatograph (Agilent Technologies, Wilmington, DE) equipped with a flame ionization detector and an autosampler (Hewlett Packard, Avondale, PA) for volatile fatty acid.

**Fecal Analysis** On d 34, 150 g of fresh droppings was collected, placed in a tube and stored at -20°C. DNA was extracted from thawed samples using the Bioline Fecal DNA Extraction kit according to the manufacturer's protocol (*Clostridium perfringens* was quantified in triplicate) by PCR (Wu et al., 2011). Results for each sample were reported as number of copies per g of feces.

**Biochemical Analysis** Blood sample was collected (in red cap tube, 6.0 mL with coagulation activator) from the

**Table 3.** Growth performance of broiler chickens fed with different alternatives to antibiotics growth promoters at different time points.

Parameter	Dietary treatments					<i>P</i> value vs. control <sup>1</sup>				
	Control	Butyrate	MOS	Bs	N-Oat	SEM	Butyrate	MOS	Bs	N-Oat
Starter (0–10 d)										
ADG (g)	17.29	17.54	17.62	17.70	20.08	0.168	0.242	0.203	0.141	<0.001
ADFI (g/kg)	25.76	26.31	26.06	26.21	27.35	0.191	0.097	0.433	0.256	<0.001
FCR	1.495	1.496	1.501	1.466	1.369	0.0120	0.758	0.639	0.114	<0.001
Mortality <sup>2</sup>	0.650	0.650	1.300	0.650	0.650	0.391	0.553	0.253	1.000	1.000
Grower (10–21 d)										
ADG (g)	64.96	63.80	65.62	63.85	69.30	0.522	0.242	0.556	0.319	<0.001
ADFI (g/kg)	81.08	81.27	82.43	81.48	82.67	0.748	0.887	0.371	0.789	0.297
FCR	1.239	1.268	1.248	1.274	1.194	0.006	0.009	0.429	0.002	<0.001
Mortality <sup>2</sup>	0	0.990	0.470	0.310	1.090	0.307	0.126	0.343	0.478	0.0631
Finisher (21–34 d)										
ADG (g)	105.98	110.66	108.01	98.26	105.60	0.931	0.002	0.182	<0.001	0.7835
ADFI (g/kg)	165.13	164.60	164.65	163.8	168.25	1.181	0.784	0.784	0.4655	0.0811
FCR	1.5570	1.4890	1.5250	1.658	1.5930	0.015	0.004	0.165	<0.001	0.1147
Mortality <sup>2</sup>	0.5100	0.5400	0.6400	0.320	0.7600	0.271	0.990	0.796	0.6766	0.5960
Overall (0–34 d)										
ADG (g)	65.84	67.26	66.77	62.88	67.73	0.550	0.080	0.306	0.001	0.041
ADFI (g/kg)	95.59	95.09	96.00	94.72	97.87	0.700	0.542	0.619	0.299	0.007
FCR	1.451	1.422	1.437	1.52	1.441	0.008	0.035	0.311	0.0001	0.475
FBW (kg)	2.3223	2.3173	2.3508	2.385	2.3329	0.026	0.878	0.385	0.0594	0.7459
Mortality	1.73	2.39	2.82	2.17	3.261	1.20	0.537	0.305	0.680	0.153

Abbreviations: ADG, average daily gain (g); ADFI, average daily feed intake (g/kg); FCR, feed conversion ratio; FBW, final body weight (kg).

<sup>1</sup>Control versus butyric acid (T2), mannan-oligosaccharide (T3), *Bacillus subtilis* (T4), or naked oat (T5).

<sup>2</sup>Percentage of birds at this stage, not cumulative.

brachial vein at d 10, 21, and 34 on 10 birds/treatment prior to euthanasia by cervical dislocation for tissue collection described before. Blood samples were centrifuged at 1,500 RPM at 4°C for 10 min and the serum was stored at –20°C until analysis. Total protein was analyzed with commercially available diagnostic kits (Thermo Scientific Pierce BCA Protein Assay Kit, 23227, Waltham, MA). The serum endotoxin levels were analyzed by a quantitative Chromogenic End-point Tachypleus Amebocyte Lysate Endotoxin Detection Kit as per the manufacturer's instructions (PyroGene Recombinant Factor C Endotoxin Detection Assay, LONZA, Morristown, NJ). C-reactive protein (CRP) was analyzed using an assay kit, Chicken C-reactive protein Elisa kit (Elabscience Biotechnology Inc., Houston, TX). Uric Acid was measured with a commercially available diagnostic kit (QuantiChrom™ Uric Acid Assay Kit, BioAssay Systems, Hayward, CA). Malondialdehyde (MDA) a lipid peroxidation marker that is used as an indicator of oxidative stress, was determined by a biochemical determination according to [Ermiš et al. \(2005\)](#). Due to a problem with the freezer, only CRP was analyzed at d 34.

## Statistical Analyses

The study was carried out as a randomized complete block design. For measures that has been repeated many times (intestinal section pH and weight and SCFA), time and treatment effects, as well as their interactions, were tested with the MIXED procedure (SAS 9.4, SAS, Cary, NC). For the other measurements, feed additives were compared to the control using the MIXED procedure and the Dunnett test (SAS 9.4, SAS, NC). Significance was declared at  $P < 0.05$ , and a tendency was declared at  $0.05 \leq P \leq 0.10$ .

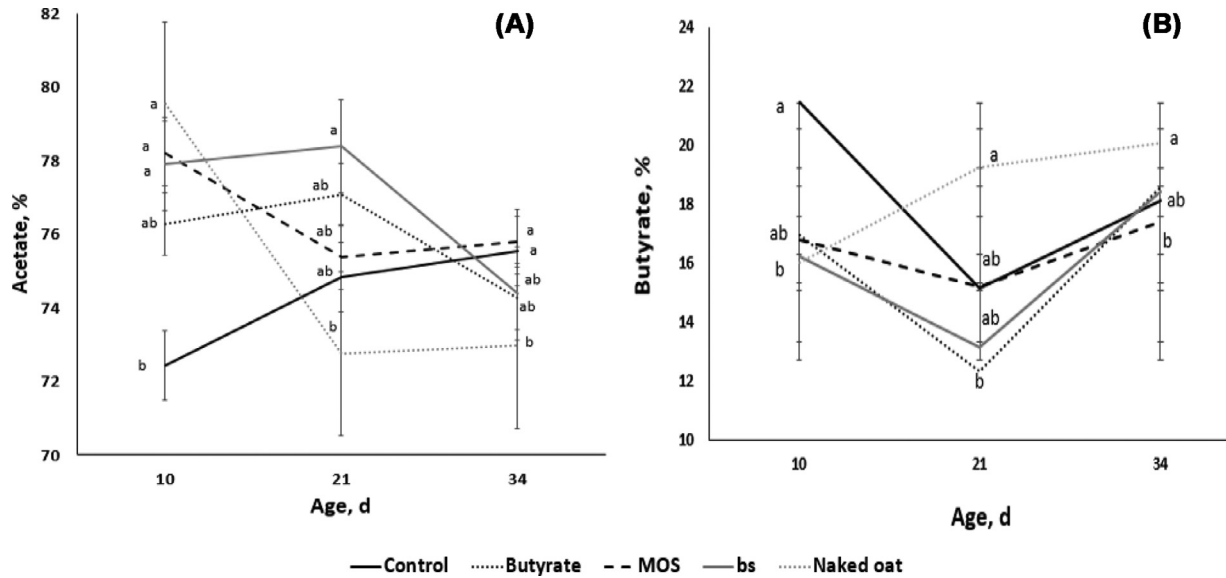
## RESULTS

### Growth Performance

At the end of the starter phase, the ADFI and ADG were respectively 6% and 16% greater in the naked oat diet group compared to the control diet and the FCR was 9% lower ( $P < 0.001$ ). No significant difference in growth performance was associated with any other diet at this phase ([Table 3](#)). No diet had any significant effect on mortality. In the grower phase, the ADG remained greater and the FCR lower in the naked oat group ( $P < 0.001$ ) while the *Bacillus* diet appeared to increase the FCR by 2.8% compared to the control diet ( $P = 0.002$ ). No significant effect on ADFI was observed between the treatments. Mortality tended to be higher in the naked oat group than in the control group ( $P = 0.06$ ). In the finisher phase, the butyric acid supplement brought a 4% increase in ADG ( $P = 0.002$ ) and a 5% decrease in FCR ( $P = 0.005$ ). The *Bacillus* supplement had the opposite effect, with an 8% decrease in ADG ( $P < 0.001$ ) and a 6% increase in FCR ( $P < 0.001$ ). Dietary treatments did not modify ADFI and mortality. For the 34-d trial overall, ADG was lower and FCR was higher in the *Bacillus* group ( $P < 0.001$ ), the ADFI was increased by 2% in the naked oat group and no significant effects were noted for the other diets ([Table 3](#)). The best (i.e., lowest) FCR was observed in the butyrate group ( $P < 0.005$ ) and no significant treatment effect on mortality was observed.

### Short Chain Fatty Acid Content of the Cecum

The total short chain fatty acid (SCFA) content of the cecum decreased in all groups from starter to grower (72.8–55.1 mmol/L,  $P < 0.001$ ; [Figure 2A](#)) and then



**Figure 2.** Concentrations of acetate (A) and butyrate (B) in the caeca of growing broiler chickens fed alternative to antibiotic growth promoters. Means  $\pm$  SE ( $n = 10$ ); a,b within time, means without a common letter differ ( $P < 0.05$ ); A, B within time, means without a common letter have a tendency to differ ( $P < 0.10$ ). Abbreviation: Bs, *Bacillus subtilis*.

rose sharply during the finisher phase (55.1–102 mmol/L,  $P < 0.001$ ). Acetate proportion was influenced by age and dietary treatment ( $P = 0.02$ ) showing that birds receiving naked oat had the highest acetate proportion at d 10 and then the lowest at d 21 and 34 (Figure 2A). A similar tendency (day  $\times$  treatment,  $P = 0.07$ ) was observed for butyrate, but with opposite results, showing an increased butyrate in the naked oat group (Figure 2B) while the other treatments resulted in a decreased butyrate proportion from d 10 to d 21.

### Weight and pH of the Intestinal Segments

Crop pH was not influenced by dietary treatments but decreased with age ( $P < 0.001$ ). However, ileum pH increased with age and tended to decrease in birds fed with butyrate treatment ( $P = 0.06$ ; Figure 2B) when compared to controls (6.9 at d 10 vs. 7.2 at d 34). A time per treatment interaction was observed for cecal pH ( $P = 0.02$ ) with the highest pH measured at d ten and the lowest at d 34 in the naked oat diet group. The opposite was observed for the *Bacillus*-supplemented diet group (Figure 2C). As expected, weight of digestive tract section increase with age ( $P < 0.001$ ) without any treatment effect except for a decrease in jejunal weight at d 34 in the MOS group (Figure 2A) (Interaction day  $\times$  treatment,  $P = 0.027$ ).

### Litter Properties

As expected, litter moisture increased with age ( $P < 0.001$ , Table 4) and no treatment effect was observed. Litter pH also increased with age ( $P < 0.001$ ) but at a different rate with the naked oat treatment group that reduced pH at d 30 in comparison to Control (6.52 vs. 6.82; Interaction Day  $\times$  Treatments,  $P = 0.011$ ; Figure 2C).

### Ileum Histology

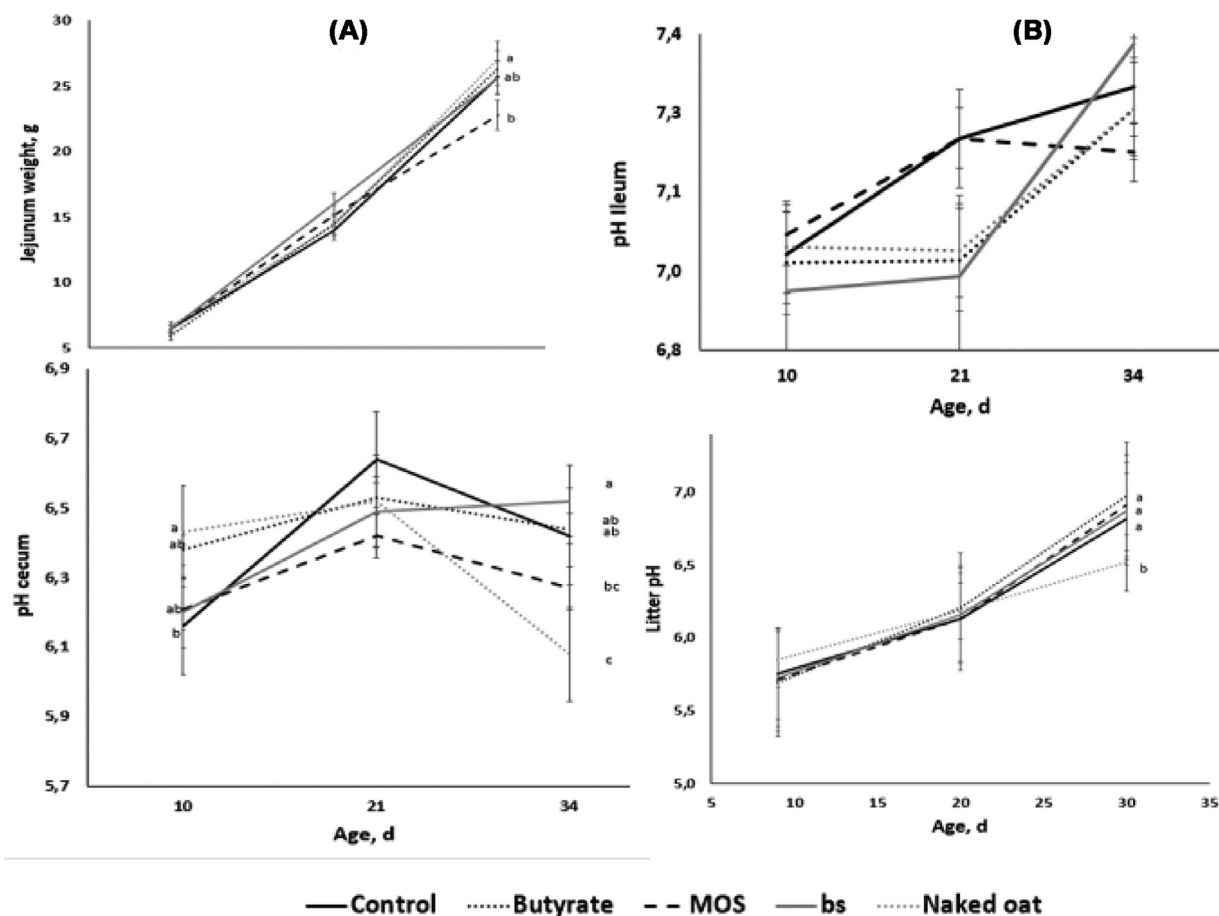
No treatment effect on ileum crypt depth was apparent on d 10 (Table 5). However, villus length and villus height/crypt depth ratio were higher in birds receiving mannan-oligosaccharides compared to the control birds (+34%;  $P < 0.001$  and +32%;  $P = 0.05$ ). At d 21, villus length was also higher in birds receiving mannan-oligosaccharides (15%;  $P = 0.03$ ) but there were no significant differences between the Control and the other treatment groups for crypt depth and villus height/crypt depth ratio, for a tendency to a higher villus height/crypt depth ratio (14%,  $P = 0.08$ ) in naked oat in comparison to Control birds.

### Serum Biochemistry

On d 10, no treatment effects on plasma C-reactive protein or malondialdehyde were apparent, whereas the butyrate diet appeared to lower the endotoxin concentration ( $P = 0.02$ ) as did mannan-oligosaccharide ( $P = 0.05$ ) and naked oat ( $P = 0.03$ ) in comparison with the control diet (Table 6). On d 21, a significant increase (52%,  $P = 0.036$ ) in C-reactive protein appeared in the *Bacillus* treatment group in comparison to the control group, and malondialdehyde was lowered in all noncontrol groups ( $P < 0.05$ ).

## DISCUSSION

There is a worldwide interest in finding alternatives to antibiotics growth promoters which can both optimize growth performance and poultry health. The main objective of this study was to validate the results of our meta-analysis we conducted (Rouissi et al., unpublished data) of 79 previous studies published from 2000 to 2017, on the impact of alternatives to AGP on growth performance. The second objective was more specifically



**Figure 3.** The weight of jejunum (A), pH of ileum (B), caeca, (C) and litter (D) of growing broiler chickens fed with alternative to antibiotic growth promoters. Means  $\pm$  SE (n = 10); a,b,c within time, means without a common letter differ ( $P < 0.05$ ); A, B within time, means without a common letter have a tendency to differ ( $P < 0.10$ ). Abbreviation: Bs, *Bacillus subtilis*.

to directly measure the effects of alternatives to AGP on growth and intestinal morphology and to evaluate easily performed analyses of serum and pen litter as proxies of bird health. Results obtained from the meta-analysis (Rouissi et al., unpublished data) showed no effect on

feed intake, whereas average daily gain increased linearly and quadratically ( $P < 0.05$ ) with incremental doses of butyric acid and mannan-oligosaccharide, and linearly with *Bacillus subtilis* by up to respectively 7%, 8% and 9% at recommended doses varying from  $10^6$  to  $10^8$

**Table 4.** Probability value for gastrointestinal segment weight and pH and SCFA production in the caeca in broiler chicken fed alternative to antibiotic growth promoters.<sup>1,2</sup>

Item	Means at the end of the feeding phase <sup>3</sup>			P-value			
	Starter	Grower	Finisher	SEM	Treatment	Time	Treatment $\times$ Time
GIT section weight, g							
Duodenum	4.87	11.8	19.4	0.37	0.268	<0.001	0.084
Jejunum	6.69	14.7	25.5	0.47	0.118	<0.001	0.026
Ileum	4.91	1.30	20.00	0.40	0.066	<0.001	0.169
Caeca	1.49	2.51	6.60	0.17	0.122	<0.001	0.823
pH							
Crop	4.16	2.51	2.86	0.17	0.885	<0.001	0.513
Ileum	6.94	7.06	7.28	0.04	0.731	<0.001	0.061
Caeca	6.25	6.44	6.31	0.07	0.373	0.009	0.021
SCFA							
Total SCFA, mmol	72.8	55.1	102.0	7.04	0.331	<0.001	0.556
Acetate, %	77.2	75.4	74.4	0.82	0.190	0.002	0.024
Propionate, %	3.30	5.60	4.75	0.38	0.377	<0.001	0.136
Butyrate, %	17.3	15.3	18.4	0.89	0.071	<0.001	0.072
Litter							
pH	5.77	6.18	6.84	0.04	0.307	<0.001	0.011
Moisture	12.2	19.2	25.7	0.77	0.365	<0.001	0.976

<sup>1</sup>Means are presented in Figures 2 and 3 when treatment or treatment  $\times$  time was significant or tended to be.

<sup>2</sup>Abbreviation: SCFA, short chain fatty acid.

<sup>3</sup>GIT section weight: the gastrointestinal tract, pH and SCFA were measured at d 10, 21 and 34; Litter pH and moisture were measured at d 9, 20 and 30.

cfu/g. Improvement in feed conversion ratio reached 2.4%, 8%, and 3% respectively for butyric acid, mannan-oligosaccharide, and *Bacillus subtilis*. In the current study, energy and lysine supply were reduced in the grower and finisher phases based on a strategy used in many publications of the meta-analysis. Field experiments have shown that reducing energy and lysine contents to 96% of their requirements results in a measurable decrease in growth performance.

In the current study, butyrate treatment tended to increase ADFI during the starter phase, followed by a negative effect on FCR during the grower phase, and then a significant improvement of ADG and FCR during the finisher phase. Some studies showed that FCR was increased in butyrate supplemented diets during the grower phase, (Moquet et al., 2018; Gonzalez-Ortiz et al., 2019) and growth performance was improved in others (Abd El-Hakim et al., 2009; Raza et al., 2017). In the present study, the higher growth performances in the finisher phase are not associated with modification of the intestinal health parameters measured except for ileal pH at d 21. Because fat-coated butyrate is slowly released (Chamba et al., 2014; Sikandar et al., 2017), this could explain why the butyrate used in this study was effective in reducing ileal pH. Nevertheless, parameters related to microbiota modifications (Mehdi et al., 2018) that have not been measured, may have been involved in the growth promoter effect observed. Probiotics influence health through modulation of immunological factors, intestinal permeability and bacterial translocation, and secretion of various metabolites (De Vrese and Schrezenmeir, 2008). Surprisingly, *Bacillus subtilis* diet decreased ADG and increased FCR during the grower (11–21 d) and the finisher (22–34 d) phases. Previous publications have shown a positive effect of *Bacillus subtilis* supplementation on growth performance (Gadde et al., 2017; Gong et al., 2018; Park et al., 2018). Abramowicz et al. (2019) have shown that *Bacillus subtilis* PB6 added in drinking water increased growth performance, as well as many other indicators such as the total number of aerobic bacteria, the length of the intestinal villi and the depth of the crypts, and immunoglobulin A. In the present study, concomitantly with the reduced growth performance, the serum concentration of C-reactive protein (CRP) was increased at d 21 and at d 34 (Table 5) in birds receiving *Bacillus subtilis* in comparison to the

Control group. Because CRP is an acute-phase protein in inflammatory responses, and it is stimulated by the presence of both interleucine-1 (IL-1) and interleucine-6 (IL-6), such increase might indicate the development of a local inflammatory process (Eckersall and Bell, 2010). An increase in the concentration of CRP is associated with cell damage caused by pathogens, that within 36 to 48 h postexposure to such stimulus (Jarosz et al., 2019). The reduced growth performance in *Bacillus subtilis* treatment coincides with the reduction in AMEn and digestible Lys and other amino acids supplied in ratio with Lys in grower and finisher diet. From a nutritional viewpoint, substrates such as amino acids and energy are needed to support immune system, with amino acids and energy deficiency being one of the major causes of immunodeficiency globally (Field et al., 2002; Ruth and Field, 2013). Moreover, both innate and adaptive immune systems are highly dependent upon an adequate availability of amino acids (Kim et al., 2007). One of the hypotheses for the lower performance in birds receiving the probiotic could thus be that the energy and/or protein deficiency would have induced a global inflammatory state or targeted in the intestine or both. This could lead to a probiotic-induced dysbiosis decreasing absorption due to alteration of the gut microflora and intestinal mucosal integrity (Suez et al., 2018) or to a use of energy for the increased immune response (Lei et al., 2015) both leading to a decrease in growth performance.

The positive effect of the naked oat diet could be due to the reduced soybean meal and hence antinutritional factor content. The higher pH and higher proportion of acetate in the cecum on d 10 but no longer apparent on d 21 suggest that less substrate and probably different substrates passed from the ileum to the cecum during these early growth phases in birds fed the naked oat diet (Svihus and Gullord, 2002) and that digesta arrived in the cecum with relatively little unabsorbed nutrient. In fact, although the naked oat starter diet was formulated to meet all requirements, values of energy or other nutrients may have been underestimated, leading to higher gain and lower FCR compared to the corn-based diet. The apparent metabolizable energy or AMEn value (zero-nitrogen corrected) of naked oat is higher than that of wheat, due presumably due to its high oil content and absence of husks (MacLeod et al., 2008) and it contains about half as much neutral detergent fiber as corn

**Table 5.** Ileal histomorphological parameters of broiler chickens fed with different alternatives to antibiotics growth promoters after the starter and the grower phases.

Parameter	Dietary treatment					P value vs. control diet				
	Control	Butyrate	MOS	Bs	N-Oat	SEM	Butyrate	MOS	Bs	N-Oat
D 10										
Villus length ( $\mu\text{m}$ )	165	188	221	180	193	13.98	0.150	< 0.001	0.320	0.210
Crypt depth ( $\mu\text{m}$ )	39.4	41.1	39.2	38.0	40.4	2.753	0.630	0.961	0.680	0.770
Villus length/Crypt depth	4.82	4.46	6.35	4.97	5.51	0.615	0.560	0.050	0.100	0.230
D 28										
Villus length ( $\mu\text{m}$ )	287	298	330	318	310	15.09	0.560	0.030	0.100	0.230
Crypt depth ( $\mu\text{m}$ )	27.5	27.8	27.6	27.7	24.3	1.840	0.890	0.940	0.910	0.20
Villus length/Crypt depth	11.2	11.4	12.6	12.5	12.7	0.877	0.830	0.220	0.260	0.080

**Table 6.** Serum parameters of broiler chickens fed different alternatives to antibiotics growth promoters.

Parameter	Dietary treatments					P value vs. control diet				
	Control	Butyrate	MOS	BS	N-Oat	SEM	Butyrate	MOS	BS	N-Oat
Starter (0–10 d)										
CRP (ng/mL)	0.93	1.01	0.66	0.87	0.67	0.195	0.718	0.255	0.788	0.257
MDA ( $\mu$ M/L)	7.06	8.56	9.38	9.13	9.59	1.24	0.302	0.115	0.157	0.085
Endotoxin (EU/mL)	4.52	3.06	3.47	4.03	3.23	0.41	0.016	0.050	0.411	0.031
Grower (10–21 d)										
CRP (ng/mL)	1.38	1.86	1.60	2.09	1.58	0.27	0.147	0.487	0.035	0.523
MDA ( $\mu$ M/L)	9.16	5.77	6.46	6.43	6.93	0.87	0.005	0.026	0.024	0.085
Endotoxin (EU/mL)	5.61	7.30	6.56	5.65	6.35	0.63	0.050	0.274	0.955	0.392
Finisher (22–34 d)*										
CRP (ng/mL)	0.200	0.205	0.203	0.284	0.157	0.046	0.159	0.6630	< 0.001	0.467
MDA ( $\mu$ M/L)						NA				
Endotoxin (EU/mL)						NA				

Abbreviations: CRP, C-reactive protein; MDA, malondialdehyde; endotoxin.

\*we did not do the analysis for MDA and endotoxin at the finisher phase because of a lack of samples due to problem with freezer.

and mostly (i.e., 62%) the soluble NSP type (Sauvant et al., 2004; Bach Knudsen, 2014). However, since none of these possible advantages were apparent at the end of the finisher phase, perhaps only young broilers benefit from them. Although corn and wheat AMEn values for chickens appear to increase linearly with bird age (Yang et al., 2020), this might not be the case for naked oat. Whereas the mass of the small intestine nearly quadrupling overall during grower and finisher phase, bird weight increased by a factor of 6 over these 2 periods. The absorption capacity relative to body weight thus may have decreased with age. Meanwhile, the cecum grew faster during the finisher phase, by more than 4-fold over the 2 phases. Although the cecum was 60% smaller on d 10 than on d 21, total SCFA content was 1.3 times higher, suggesting that it received more unabsorbed nutrients from the ileum on d 10. These results are consistent with previous observations (Van den Borne et al., 2015). The intestinal mucosa of broiler chickens undergoes major structural development during the first 7 d post hatch, especially in terms of villus height or relative length in the jejunum (Iji et al., 2001), which is known to be the principal site of nutrient absorption (Collins et al., 2019). The disappearance of the positive effects of the naked oat diet by the end of the finisher phase is reflected in the lower pH measured in the cecum and the litter. Naked oat fiber is highly fermentable and yields larger amounts of butyric acid that do other types of dietary fiber (Casterline et al., 1997). The proportion of butyrate increased from 10 to 21 d of age and stays higher in comparison to other diets. Proportion of SCFA also changed with age, with reduction in acetate and propionate and increase of butyrate showing a switch over time to more butyrogenic microflora in the caeca as previously observed (Gonzalez-Ortiz et al., 2017). It is worthy to note that SCFA production is influenced by many factors (e.g., diet, feeding phase duration, pen or cages) other than just age. Oat  $\beta$ -glucan might thus behave as a prebiotic: a no digestible feed ingredient that affects the host by selectively stimulating the growth and/or activity of one strain or a limited number of bacterial strains in the colon and thus improves host health

(Gibson and Roberfroid, 1995). The modification cecal fermentation can lead to change of litter characteristics. Thus, the reduction of litter pH with naked oat diet at d 30 (6.19 vs. 6.23) is probably the result of higher fermentability of oat  $\beta$ -glucan (Daou and Zhang, 2012). Low litter pH is a good indicator of bird health condition. Hardin and Roney (1989) showed that the litter pH reduction resulted in a decline in microbial populations, including *E. coli*, *Salmonella*, and *Clostridium*, to below detectable limits. Additionally, Payne et al. (2007) showed that achieving a low pH led to the fastest reduction in *Salmonella* populations when compared with higher pH. The current results showed another positive effect of naked oat on broiler health. In addition to modifying the cecal SCFA content and the pH, naked oat tended to increase villus height/crypt depth ratio (+14% vs. control) during the grower phase that may be attributed again to its high level of  $\beta$ -glucan that has been showed to increase this ratio (Teng and Kim, 2018). This higher ratio could improve the nutrient absorption capacity of birds in this group and then growth performances. These results indicate that naked oat can be incorporated in broiler diets at 20 to 30% without negative effects. Previous studies have shown that naked oat can substitute other cereals in broiler diets (Kamińska, 2003; Osek et al., 2003, Szymczyk et al., 2005).

The MOS treatment had no effect on growth performances during different phases of experiment. These results are opposite to those obtained in several studies, including those reported in the meta-analysis of Roussi et al. (2020). Although MOS had no effect on performance, an increase of the villus height was observed during the starter and grower phase (0–21 d) and villus height/crypt depth ratio during the starter phase (0–10 d). These results are consistent with previous studies showing that mannan-oligosaccharides increase villus height and surface area, decrease crypt depth, induce numbers of sulphated-acidic goblet cells, and upregulate gene expression of MUC, which is related to mucin secretion (Baurhoo et al., 2009; Chee et al., 2010). Longer villi correspond to a larger intestinal surface area and higher digestive enzymes activities, therefore, increased nutrient



absorption and improved digestibility (Gao et al., 2008). Additionally, it was proposed that a decreased crypt depth indicates a lower epithelial cell turnover, therefore better efficient energy use by the host for growth and enhanced productivity (Murugesan et al., 2015). However, this change in the morphology of the mucosa did not improve the growth of the birds, suggesting that the height of the villi was not a factor limiting absorption in this study. In fact, the absorption of nutrients depends on the villi but also on the length of the intestine and the presence of specific transporters.

## CONCLUSIONS

As regulations and the rise of consumer demand for poultry “Raised without antibiotics” increase pressure to abandon the use of antibiotics as growth promoters (AGP), the search for alternative approaches to stimulating the growth of broiler chickens intensifies. The objective of the current study was to compare the most studied alternative to antibiotics to assess their effects as growth promoter and to measure parameters informing about health of broilers. Results showed that butyric acid and naked oat, an alternative feedstuff rich in prebiotic, have a growth promoting effect in finisher for the first one and mainly in starter and grower phase for naked oat. Besides, MOS had no impact on growth performance while Bs have reduced it. On the biomarkers studied, caeca pH and SCFA production are promising to explain growth promoter effects but should probably be combined with other markers of microbiota population and intestinal membrane integrity. The exact modes of action still need to be defined more precisely for successful replacement of antibiotics growth promoter. It should be noted, that optimal combinations of various alternatives will probably be the key to maximizing performance and maintaining productivity.

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## DISCLOSURES

There is no conflict of interest.

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