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Non Ruminant Nutrition

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The combination of nutraceuticals and functional feeds as additives modulates gut microbiota and blood markers associated with immune response and health in weanling piglets

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Abstract

This study aimed to evaluate the effects of a combination of feed additives with complementary functional properties on the intestinal microbiota, homocysteine, and vitamins E and B status as well as systemic immune response of weanling piglets. At weaning, 32 litters were assigned to one of the following dietary treatments (**DT**): 1) conventional diet (**CTRL**); 2) CTRL diet supplemented with antibiotics (**ATB**); 3) a cocktail of feed additives containing cranberry extract, encapsulated carvacrol, yeast-derived products, and extra vitamins A, D, E, and B complex (**CKTL**); or 4) CKTL diet with bovine colostrum in replacement of plasma proteins (**CKTL + COL**). Within each litter, the piglets with lowest and highest birth weights (**LBW** and **HBW**, respectively) and two piglets of medium birth weight (**MBW**) were identified. The MBW piglets were euthanized at 42 d of age in order to characterize the ileal and colonic microbiota. Blood samples were also collected at weaning and at 42 d of age from LBW and HBW piglets to measure insulin-like growth factor-1 (**IGF-1**), cysteine, homocysteine, and vitamins E, B_6, and B_{12}, and to characterize the leukocyte populations. At 42 d of age, cytokine production by stimulated peripheral blood mononuclear cells was also measured. In a second experiment, piglets were reared under commercial conditions to evaluate the effects of the DT on the growth performance. At the indicator species analysis, the highest indicator value (**IV**) for *Succinivibrio dextrinosolvens* was found in the CKTL group, whereas the highest IV for *Lactobacillus reuteri* and *Faecalibacterium prausnitzii* was evidenced in the CKTL + COL group ($P < 0.05$). Compared with the other DT, CTRL piglets had higher concentrations of homocysteine, whereas the CKTL and CKTL + COL supplementations increased the concentrations of vitamins E and B_{12} ($P < 0.05$). DT had no effect on IGF-1 concentration and on blood leukocytes populations; however, compared with HBW piglets, LBW animals had lower values of IGF-1, whereas the percentages of γδ T lymphocytes and T helper were decreased and increased, respectively ($P < 0.05$). CKTL + COL also improved the growth performance of piglets reared under commercial conditions ($P < 0.05$). This study highlights the impact of birth weight on piglet systemic immune

defenses and the potential of weaning diet supplemented with feed additives and bovine colostrum to modulate the homocysteine metabolism and the intestinal microbiota.

Key words: alternatives to antibiotics, bovine colostrum, feed additives, gut microbiota, weanling piglets

Abbreviations

ADFI	average daily feed intake
ADG	average daily gain
ATB	antibiotics
BSA	bovine serum albumin
BW	birth weight
CCAC	Canadian Council on Animal Care
CKTL	cocktail of dietary supplements
CKTL + COL	cocktail of dietary supplements + bovine colostrum
ConA	concanavalin A
CTRL	control
DP	double positive
DT	dietary treatments
G:F	gain to feed
HBSS	Hank's balanced salt solution
HBW	high birth weight
HWW	high weaning weight
IGF-1	insulin-like growth factor-1
IL	interleukin
ISA	indicator species analysis
IV	indicator value
LBW	low birth weight
LH-PCR	length heterogeneity polymerase chain reaction
LPS	lipopolysaccharide
LWW	low weaning weight
MBW	medium birth weight
MRPP	multi-response permutation procedure
NK	natural killer
NKT	natural killer T
NMS	nonmetric multidimensional scaling
PBMC	peripheral blood mononuclear cells
PBS	phosphate-buffered saline
PMA	phorbol 12-myristate 13-acetate
Th	T-helper
TNF-α	tumor necrosis factor α

Introduction

Weaning is a stressful period for piglets because they are separated from their mother, put in a new environment with other litters, and fed with a complex solid feed containing new ingredients. These abrupt changes may impair the growth performance of piglets, destabilize intestinal microbiota, and alter intestinal defense mechanisms, which creates favorable conditions for infection (Lallès et al., 2004; Pié et al., 2004). The stress induced by these conditions may be emphasized in low birth weight (**LBW**) piglets, as these animals are at a competitive disadvantage compared with their heavier littermates in terms of preweaning survival, weight gain, intestinal growth, and immune system development (Milligan et al., 2002; Dong et al., 2014). Therefore, for several years, early interventions including the prophylactic use of dietary antibiotics as growth promoters have been used to enhance gut health during the peri-weaning period. Such a practice, however, has been severely criticized because of concerns related to antimicrobial resistance acquired by pathogens and their cross-transfer to humans (Cogliani et al., 2011). Therefore, the search for alternative feeding strategies aiming to improve gut health and resistance to enteric infections is a matter of utmost concern for swine producers. So far, various feed additives such as prebiotics, probiotics, plant extracts, and essential oils have been studied for their potential to improve piglet performance and gut health and to reduce the use of antibiotics as growth promoters (Gresse et al., 2017). Bovine colostrum, a source of growth factors and immunomodulatory components, has also attracted considerable interest as food and feed supplement to improve gut health (Møller et al., 2011; Rathe et al., 2014). However, each of these additives has shown different effects on microbiota and immune response through their influence on bacterial populations, gut defense functions, or both. Therefore, we hypothesized that a cocktail of different feed additives with complementary functional properties may be a promising feeding approach to improve intestinal health and performance of livestock, especially in LBW offspring. In the present study, the feed additives that were chosen as functional food included bovine colostrum, cranberry extract, carvacrol, yeast-derived mannans, and β-glucans. Bovine colostrum contains several bioactive molecules that may have direct antimicrobial and endotoxin-neutralizing effects throughout the gastrointestinal tract as well as other bioactivities that suppress gut inflammation and promote mucosal integrity and tissue repair (Rathe et al., 2014). Cranberry extract is a rich source of phenolic compounds (Vinson et al., 2008), whereas the yeast-derived products and carvacrol have shown complementary prebiotic, antimicrobial, and immunological effects (Wang et al., 2009; Kamiya et al., 2018).

The vitamin and mineral premix was supplemented with selenium yeast and vitamins A, D, E, and B complex above commercial levels because of their known potential in modulating immune functions, especially at the intestinal border (Maggini et al., 2007). Consequently, any restriction in these nutrients during the peri-weaning period may result in a decreased efficiency in responding to environmental challenges.

The main objective of the present study was, therefore, to evaluate the potential of these feed additives to improve piglet gut health by stimulating the establishment of a beneficial commensal microbiota and inhibiting pathobionts. Furthermore, the potential of these dietary treatments (**DT**) to modulate the systemic immune response and to affect the homocysteine metabolism was also evaluated in piglets of different birth weight (**BW**) categories. An additional trial in commercial conditions was also performed to determine the effects of such DT on the growth performance of piglets throughout the nursery period.

Materials and Methods

Animals, housing, and DT

The study was conducted in the Swine Complex of the Sherbrooke Research and Development Centre, Agriculture and Agri-Food Canada (AAFC—Sherbrooke, QC, Canada). The animals were cared for according to a recommended code of practice and procedures reviewed by the Institutional Animal Care Committee in accordance with the Canadian Council on Animal Care (**CCAC**) guidelines on the care and use of farm animals in research (CCAC, 2009). Thirty-two multiparous Yorkshire–Landrace sows and their litters were used in a randomized complete block design. Each block included four sows and their litters, which were randomly distributed into four DT (n = 8 litters per treatment). For the purpose of performance recording, two more blocks were added and a total of 10 litters per treatment were used. Estrus was synchronized before sows were inseminated. When estrus was detected, two inseminations were performed with pooled semen of three Duroc boars provided by a local artificial insemination center (CIPQ Inc., St-Lambert, QC, Canada). Two weeks before expected parturition, sows were housed in two farrowing rooms. Within the first 2 d after birth, the litter size was adjusted to 12 piglets. At 14 d of age, two LBW piglets with poor weight gain during the first week of life and two high birth weight (**HBW**) piglets with superior weight gain during the first week of life were selected within each litter for the measurements of growth performance and plasma metabolites throughout the experimental period, for the characterization of leukocyte populations by flow cytometry and for the measurement of cytokine production by stimulated peripheral blood mononuclear cells (**PBMC**). Average BWs and daily gains during the first week of life were 1.40 ± 0.37 kg and 165 ± 79 g/d for the LBW group and 1.70 ± 0.35 kg and 286 ± 83 g/d for the HBW group, respectively. Two piglets of medium birth weight (**MBW**; 1.51 ± 0.39 kg) were also included in the study to evaluate the effects of DT on intestinal microbiota.

At weaning (20 ± 1 d of age), litters were moved to the nursery, housed in a separate pen (1.9 × 1.9 m) for each litter, and assigned to one of the following four DT (one treatment per pen): 1) control weanling diet containing spray-dried plasma proteins at 35 g/kg feed (**CTRL**); 2) control weanling diet + antibiotic chlortetracycline at 1.5 g/kg feed (**ATB**); 3) control weanling diet + a cocktail of dietary supplements (**CKTL**) composed of cranberry extract at 1 g/kg feed (kindly provided by Nutra Canada, Champlain, QC, Canada), encapsulated carvacrol at 0.1 g/kg feed as previously described by Wang et al. (2009), selenized yeast and yeast-derived products such as mannans and glucans at 5 g/kg feed (kindly provided by Lallemand Inc., Montreal, QC, Canada), and extra vitamins A, D, E, and B complex (3 g/kg feed); and 4) control weanling diet + CKTL + defatted bovine colostrum (kindly provided by Sterling Technology, Brookings, SD, USA) at 50 g/kg feed instead of spray-dried plasma proteins (**CKTL + COL**). Because of the higher concentration of proteins in spray-dried plasma, the inclusion level of bovine colostrum was adjusted in order to achieve the same absolute amount of proteins in each diet. Diets were manufactured by Shur-Gain (Regional East Office, Saint-Hyacinthe, QC, Canada) and formulated to meet or exceed nutrient requirements for weaned pigs as recommended by the National Research Council (2012). The composition and calculated chemical analysis of each diet are shown in Table 1. Piglets had ad libitum access to feed and water throughout the trial (from weaning to 42 d of age). Body weight of LBW and HBW piglets was recorded at 1, 7, 14, 20, 35, and 42 d of age; the average daily gain (**ADG**) was subsequently calculated.

Tissue collection and fingerprinting of intestinal bacterial communities

Three weeks after weaning, the MBW piglets of each litter were euthanized for the collection of intestinal digesta and mucosa samples, as previously described (Morissette et al., 2018). Briefly, intestinal segments (10 cm long) were excised from the ileum (30 cm before the cecum) and colon (40 cm after the cecum). After the collection of intestinal digesta, the intestinal sites were individually rigorously washed with ice-cold phosphate-buffered saline (**PBS**) until the mucosa was completely cleaned from digesta. The mucosa was rinsed several times to remove remains of free-floating bacteria, then incubated overnight in 50 mL of PBS + 0.1% Tween 20 (Sigma-Aldrich, St. Louis, MO, USA). Detached bacteria were harvested by centrifugation at 5,000 × g for 25 min at 4 °C, suspended in 1.5 mL PBS, and stored at −80 °C until further analysis. Nucleic acids were extracted from the frozen samples using the bead beating system as previously described (Roy et al., 2009). The amplicon length heterogeneity polymerase chain reaction (**LH-PCR**) of the 16S rRNA genes (Mills et al., 2003) was used to obtain the bacterial community fingerprints as previously described (Morissette et al., 2018).

The following PCR primers were used: fluorescently labeled 27F forward primer (5′-6FAM-AGA GTT TGA TCM TGG CTC AG-3′) and 355R reverse primer (5′-GCT GCC TCC CGT AGG AGT-3′). PCR mixes consisted of: Taq buffer 1× (BioShop Canada Inc., Burlington, ON, Canada), 1.5 mM $MgCl_2$, 0.5 µM each primer, 0.1 mM dNTP, 0.625 U Taq polymerase (BioShop Canada Inc.), and 100 ng DNA in a final 25-µl volume. Thermal cycling was performed in an Eppendorf gradient thermal cycler (Fisher Scientific Company, Ottawa, ON, Canada) as follows: DNA initial denaturation step at 94 °C for 2 min, followed by 25 cycles of denaturation at 94 °C for 60 s, annealing at 60 °C for 60 s, and extension at 72 °C for 60 s, plus a final extension step at 72 °C for 30 min. Each diluted PCR product (1 µL) was mixed with 0.06 µL of GeneScan 500 LIZ Size standard (Applied Biosystems, Foster City, CA, USA) and 12.34 µL of Hi-Di formamide (Applied Biosystems). Capillary electrophoresis was then performed for 40 min on an ABI 3130 Genetic Analyzer (Life Technologies Inc., Burlington, ON, Canada) using POP-4 polymer (Applied Biosystems) and a four-array 36-cm capillary column. Automated amplicon length analysis between 300 and 500 bp and determination of peak height were carried out using the GeneScan Analysis Software (Applied Biosystems).

Cloning and sequencing

Libraries of 16S rRNA gene clones were built to identify the LH-PCR peaks. A total of 12 libraries were constructed from pooled DNA as follows: content and mucosal-detached bacteria isolated from the ileum and colon of CTRL piglets (4 libraries); content from the colon and mucosal-detached bacteria from ileum and colon of ATB piglets (3 libraries); content and mucosal-detached bacteria from the colon of CKTL piglets (2 libraries); and content from ileum and colon and mucosal-detached bacteria from the colon of CKTL + COL piglets (3 libraries). The 27F forward primer (5′-AGA GTT TGA TCM TGG CTC AG-3′) and the 1390R reverse primer (5′-GAC GGG CGG TGT GTA CAA-3′) were used as the PCR primer set. A PCR amplification of the 16S rRNA genes was performed in a 50-µL reaction volume using the same mixture as for LH-PCR. Thermal cycling was performed in an Eppendorf

Table 1. Composition of experimental diets (as-fed basis)

	DT			
Item	CTRL	ATB	CKTL	CKTL + COL
Ingredients, %				
Corn	37.2	36.9	36.5	33.7
Wheat	22.0	22.0	21.4	22.0
Soybean meal, 48% crude protein	17.8	17.8	17.7	19.0
Whey powder	15.0	15.0	15.0	15.0
Vegetal oil and other fat	1.75	1.8	2.05	1.5
Plasma proteins	3.5	3.5	3.5	—
Bovine colostrum	—	—	—	5.0
Cranberry extract	—	—	0.1	0.1
Encapsulated carvacrol[1]	—	—	0.01	0.01
Yeast-derived products (mannans + glucans)	—	—	0.5	0.5
Chlortetracycline	—	0.15	—	—
Mineral and vitamin premix (MVP)[2]	0.2	0.2	—	—
MVP + vitamins A, D, E, B, Se-Met[3]	—	—	0.3	0.3
Calcium carbonate	1.16	1.16	1.16	1.01
Biofos (Dicalcium phosphate 21%)	0.72	0.72	0.72	0.91
Methionine	0.12	0.12	0.12	0.11
Lysine HCl 98%	0.36	0.36	0.36	0.26
L-Threonine	0.11	0.11	0.11	0.09
L-Tryptophan	0.03	0.03	0.03	0.03
Biotin (400 mg/kg)	0.01	0.01	0.01	0.01
Calculated chemical composition				
Metabolizable Energy, kcal/kg	3,172	3,171	3,159	3,158
Crude protein, %	21.6	21.7	21.6	21.8
Fat, %	7.5	7.5	7.5	7.5
Fiber, %	2.5	2.5	2.5	2.5
Ca,%	1.0	1.0	1.0	1.0
P, %	0.8	0.8	0.8	0.8
Na, %	0.2	0.2	0.2	0.2
Total Lys, %	1.32	1.32	1.31	1.32
Standardized ileal digestible, %				
Lys	1.21	1.21	1.20	1.21
Met	0.36	0.36	0.36	0.39
Met + Cys	0.70	0.70	0.69	0.67
Thr	0.78	0.78	0.77	0.78
Trp	0.22	0.22	0.22	0.22
Val	0.81	0.81	0.81	0.81

[1]The capsule contains 90% carvacrol (equivalents to 90 ppm carvacrol in the diet).
[2]MVP provided per kg of diet: Cu as copper sulfate, 30 mg; Fe as ferrous sulfate, 150 mg; Mn as manganous oxide, 44 mg; Zn as zinc sulfate, 80 mg; Se as selenite, 0.3 mg; I as calcium iodate, 2.2 mg; vitamin A as Rovimix A 1,000, 10,000 IU; vitamin D3 as cholecalciferol, 1.1 mg; vitamin E as α-tocopherol, 55 IU; vitamin K as menadione, 2.5; vitamin B_6 (pyridoxine), 2.5 mg; vitamin B_9 (folic acid), 3.0 mg; vitamin B_{12} (cobalamin), 25 µg; niacin, 30 mg; choline, 300 mg; thiamine, 2,7 mg; riboflavin, 5.0 mg.
[3]Provided per kg of diet: same as MVP with the addition of Se as seleno-methionine, 0.3 mg; vitamin A, 30,000 IU; vitamin D_3, 5.0 mg; vitamin E, 250 IU; vitamin K, 4.0; pyridoxine, 4.0 mg; folic acid, 10.0 mg; cobalamin, 100 µg; niacin, 60 mg; choline, 1,000 mg; thiamine, 7.0 mg; riboflavin, 15 mg.

gradient thermal cycler (Fisher Scientific Company) as follows: DNA initial denaturation step at 94 °C for 2 min, followed by 25 cycles of denaturation at 94 °C for 60 s, annealing at 55 °C for 60 s, and extension at 72 °C for 90 s, plus a final extension step at 72 °C for 10 min. Amplicons were purified using the QIAquick PCR purification kit (Qiagen, Valencia, CA, USA), ligated into the pCRII vector TA cloning kit (Invitrogen Canada Inc., Burlington, ON, Canada), transformed into *Escherichia coli* One Shot Top10F′ competent cells (Invitrogen Canada Inc.), and transformants (white *E. coli* colonies) were then isolated as previously described (Barret et al., 2012). Plasmid DNA extractions were performed with the QIAprep Spin Miniprep Kit (Qiagen) according to the manufacturer's instructions and were screened to determine their specific LH-PCR amplicon length. Clones were then sequenced by Eurofins MWG Operon LLC (Huntsville, AL, USA) using the Sanger technology with M13 forward primer (5′-GTA AAA CGA CGG CCA-3′) and M13 reverse primer (5′-CAG GAA ACA GCT ATG AC-3′) (Life Technologies).

The sequences were screened for vector contamination using VecScreen software (http://www.ncbi.nlm.nih.gov/tools/vecscreen/), and M13 primers were eliminated. They were also screened for LH-PCR primers, and if the primer sequences contained over two mismatches, they were rejected. Moreover, sequences had to have a minimum length of 600 bp to be accepted. Chimeras were excluded using Decipher (Wright et al., 2012). The RDP classifier process (Wang et al., 2007) and NCBI BLAST searches (Altschul et al., 1990) were used to match clone sequences with the closest database sequence.

Accepted sequences have a minimum identity of 98% and query coverage of over 97% attributed to the corresponding taxon (Supplementary Table S1). Sequences were deposited in GenBank under accession numbers KX072164 to KX072287.

Measurement of plasma metabolites and characterization of leukocyte populations by flow cytometry

In each litter, blood samples were collected from one LBW and one HBW piglet at weaning (20 ± 1 d of age) and at 42 d of age. On the days of blood collection, after either 1-h or 4-h fasting periods (for suckling and weaned piglets, respectively), 40 mL of blood samples was collected from the jugular by venipuncture using 10-mL Vacutainer tubes spray-coated with K_2EDTA (Becton Dickinson). For each animal, 30 mL of whole blood was then used for PBMC isolation in order to characterize leukocyte populations by flow cytometry at both days of sampling and production of cytokines at day 42 as described below. The remaining 10 mL of whole blood was used to measure plasma concentrations of cysteine, homocysteine, vitamins E, B_6, and B_{12}, and insulin-like growth factor-1 (**IGF-1**). For plasma separation, tubes were centrifuged (1,800 × *g* for 15 min at 4 °C) within 30 min of collection, and plasma was then stored at –20 °C until analysis. Plasma concentrations of cysteine and homocysteine were analyzed by High Performance Liquid Chromatography and as described by Simard et al. (2007). Vitamins E and B_6 were measured in plasma as described by Audet et al. (2004), whereas plasma concentrations of B_{12} were measured with commercial radioassay kits as described by Matte et al. (2012). Plasma concentrations of IGF-1 were measured with a commercial radioimmunoassay kit for humans (ALPCO Diagnostics, Salem, NH) with small modifications as detailed previously (Plante et al., 2011). Validation for a plasma pool from piglets was demonstrated. Parallelism was 101.2% and average mass recovery was 101.3%. Sensitivity of the assay was 0.10 ng/mL. The intra- and inter-assay CVs were 3.87% and 5.29%, respectively.

Isolation of PBMC was performed as previously described (Lessard et al., 2018). Briefly, 30 mL of blood was slowly layered into falcon tubes filled with 15 mL Ficoll-Paque PLUS (density 1,077 g/mL; GE Healthcare Life Science, Baie d'Urfé, QC, Canada). After centrifugation (800 × *g* for 40 min at room temperature), PBMC located over the Ficoll-Paque layer were collected, and the contaminating red blood cells were lysed by hypotonic shock, using water and Hank's balanced salt solution (**HBSS**; Gibco BRL Life Technologies) 10× to restore isotonicity. After two washes in sterile HBSS, cells were adjusted at 5 × 10^6 cells/mL in FACSflow supplemented with 0.5% bovine serum albumin (**BSA**) Fraction V (Bioshop Canada Inc.). Cell suspensions were then immunostained in 96-well round-bottom microplates (VWR international, Mississauga, ON, Canada). Briefly, 10^6 cells/well were centrifuged at 500 × *g* for 5 min at 4 °C, supernatants were discarded, and 100 µL of monoclonal antibodies diluted in washing solution was added in each well according to the immuno-phenotyping panels described in Table 2. Cells were incubated on ice in the dark for 20 min and then washed twice with FACSflow containing 0.5% BSA. The cells were finally suspended in 200 µL and analyzed with a BD FACSCanto II flow cytometer equipped with a three-laser configuration. The BD FACSDiva operating software (BD Biosciences, Mississauga, ON, Canada) was used for the acquisition and data analysis.

PBMC stimulation and cytokine measurement by ELISA

Blood samples collected from the selected LBW and HBW piglets at 42 d of age were also used to measure cytokine production by stimulated PBMC. Briefly, after performing the isolation as previously described, PBMC were resuspended in Roswell Park Memorial Institute 1640 (Wisent Bioproducts, St-Bruno, QC, Canada) supplemented with 10% fetal bovine serum (Wisent Bioproducts) and 1% of an antibiotic solution containing 5,000 units/mL of penicillin and 5,000 µg/mL of streptomycin (Wisent Bioproducts). Cell count and viability were evaluated by Trypan blue exclusion. PBMC were then plated in a 24-well cell culture plate (VWR International, Mississauga, ON, Canada) at a concentration of 2.5 × 10^6 cells/well and stimulated or not with 1 µg/mL concanavalin A (**ConA**), a mixture of 50 ng/mL phorbol 12-myristate 13-acetate (**PMA**) and 500 ng/mL ionomycin, or 250 ng/mL lipopolysaccharide (**LPS**) from *E. coli* 055:B5 + 250 ng/mL LPS from *Salmonella typhimurium* (Sigma-Aldrich, Oakville, ON, Canada). For each condition of each sample, cells were plated in duplicates and incubated for 24 h at 37 °C in a 5% CO_2 humidified incubator. At the end of the incubation period, supernatants were collected for the measurement of interleukin (**IL**) 8, IL10, and tumor necrosis factor α (**TNF-α**) by ELISA using Porcine DuoSet ELISA kit from R&D Systems (Minneapolis, MN). All samples and controls were tested in duplicate according to the manufacturer's instructions.

Experimental trial in commercial conditions

In order to determine the effects of the DT on the growth performance of weanling piglets reared under commercial conditions, a second experiment was carried out in a conventional pig production facility (Centre de formation agricole de St-Anselme, St-Anselme, QC, Canada). All animal procedures were conducted according to the guidelines set by the CCAC (2009), and the experimental protocol was approved by the Laval University Animal Use and Care Committee. A total

Table 2. Monoclonal antibodies used for the flow cytometry immune-phenotyping of the PBMC

Monoclonal antibodies	Clone	Isotype	Fluorochrome	Targeted cells
CD3	BB23-8E6-8C8[1]	IgG_{2a}	Fluorescein isothiocyanate	T lymphocyte subsets
CD4	74-12-4[2]	IgG_{2b}	BD Horizon 450	Th lymphocytes and DP T lymphocytes
CD8α	76-2-11[2]	IgG_{2a}	Allophycocyanin (APC-H7)	Cytotoxic T lymphocytes, DP T lymphocytes, NKT lymphocytes, and NK cells
CD16	FcG7[2]	IgG_1	Streptavidin (Strep-V500)	NKT lymphocytes and NK cells
CD21	BB6-11c9.6[1]	IgG_1	PE (R-phycoerythrin)	Activated B lymphocytes
γδ-T (TCR1-N4)	MAC320[2]	IgG_{2a}	Allophycocyanin	γδ T lymphocytes (subset)

[1]Purchased from Southern Biotech, Birmingham, AL, USA.
[2]Purchased from BD Biosciences, Mississauga, ON, Canada.

of 832 piglets (Génétiporc Fertilis 25 × Génétiporc G Performer 6.0, St-Bernard, QC, Canada) obtained from 80 sows and their litters (adjusted at 11 ± 1 piglets per litter) were used for this study. Eight groups of 104 piglets from 10 different litters were weaned every 3 wk at 21 d of lactation. Body weight at weaning was recorded and piglets were divided into two different weight categories: low and high body weight at weaning (**LWW** and **HWW**, respectively). The mean body weight for the LWW and HWW groups was 5.90 ± 0.40 and 7.20 ± 0.42 kg, respectively. Animals were then immediately moved to two nursery rooms, where for each weight category, 4 pens of 13 piglets were used. In each room, piglets were housed in 1.62 × 1.82 m pens equipped with a self-feeder (four-hole space feeder) and a watering nipple. Starting on the day of weaning, pens of both body weight classes were assigned to the same DT and received the same diets described in Table 1, so that all the four DT were equally distributed in each weight category. This design was repeated for each group of 104 piglets, according to a randomized complete block design. Feed and water were provided ad libitum. Piglets in each pen were weighed at weaning and at 29 and 36 d of age, and ADG was subsequently calculated. Feed consumption was also measured to evaluate the average daily feed intake (**ADFI**) and gain to feed (**G:F**) ratio.

Statistical analysis

Diversity indices (richness, evenness, and Shannon index) were calculated from LH-PCR data as described by Roy et al. (2009) and analyzed using ANOVA and the Friedman test in SAS (SAS Statistical Analysis System, Release 9.4, 2002 to 2012, SAS Institute Inc., Cary, NC, USA). Multi-response permutation procedure (**MRPP**), indicator species analysis (**ISA**), and nonmetric multidimensional scaling (**NMS**) were performed using Bray–Curtis distance measures in the PC-ORD v. 6.0 software (MjM software, OR; McCune and Mefford, 1999). MRPP was used to test for the significance of group differences between DT. The ISA (Dufrêne and Legendre, 1997) was used to identify amplicons responsible for the differences observed between the dietary groups. For each amplicon, the proportional abundance in a particular group relative to the abundance in all groups and the relative frequency within a group were calculated. Indicator values (**IVs**; range from 0 to 100, absent to exclusively present, respectively) were obtained by multiplying the relative abundance by the relative frequency of each amplicon in a given group, as determined from fingerprint data. Amplicons present at a frequency of at least 50% in one of the four dietary groups were retained for these analyses.

Growth performance, data from flow cytometry, and concentrations of plasma metabolites and PBMC cytokines were analyzed using the MIXED procedure of SAS. The model included the factors: DT (CTRL, ATB, CKTL, and CKTL + COL), BW (HBW and LBW), and AGE (weaning and day 42) as fixed effects, with the piglet litter as the experimental unit, while the block was considered as a random effect. DT were then compared using Tukey's test. When the interactions between DT or BW and AGE reached significance and required further investigations, a separate ANOVA was performed for each day of sampling. When necessary, percentage data of leukocyte populations as determined by flow cytometry were standardized by angular transformation prior to analysis (Steel and Torrie, 1980). Growth performance data from the second experiment carried out in commercial conditions were analyzed using the MIXED procedure of SAS with the pen as an experimental unit. The fixed effects of the model included factors such as DT, weaning weight, and AGE, and the block was considered as a random effect. DT were then compared using Tukey's test. The results were considered significant at $P < 0.05$ or as a trend at $0.05 \leq P < 0.10$.

Results

Growth performance

Body weight and ADG of suckling and weanling piglets from the first trial are shown in Table 3. As expected, HBW piglets were heavier than LBW piglets during the whole experimental period ($P < 0.001$). The ADG of HBW piglets was superior to that of LBW piglets not only from birth to weaning ($P < 0.001$) but also from 28 to 35 d ($P < 0.001$) and from 35 to 42 d ($P = 0.06$) of age. However, there were no differences in the first week after weaning (day 20 to 28). Conversely, no dietary effect was observed on the growth performance of piglets ($P > 0.10$).

Indices of intestinal microbial diversity

As estimated by the richness, evenness, and Shannon indices, no differences were detected in the ileal microbial diversity of

Table 3. Effects of the DT and BW on the body weight and ADG of piglets during the lactation and nursery periods

	DT						BW			
Days of age	CTRL	ATB	CKTL	CKTL + COL	SEM	P-value	HBW	LBW	SEM	P-value
Body weight, kg										
1	1.52	1.52	1.65	1.51	0.08	0.43	1.70	1.40	0.05	<0.001
7	2.95	2.75	3.02	2.90	0.19	0.56	3.42	2.39	0.15	<0.001
14	4.92	4.75	5.11	5.00	0.26	0.67	5.78	4.10	0.20	<0.001
20 (weaning)	6.44	6.18	6.57	6.57	0.34	0.70	7.49	5.39	0.27	<0.001
28	8.42	8.05	8.38	8.41	0.38	0.84	9.41	7.21	0.27	<0.001
35	11.62	11.12	11.37	11.96	0.54	0.65	12.85	10.18	0.38	<0.001
42	14.21	12.99	13.27	14.53	0.63	0.24	15.19	12.36	0.39	<0.001
ADG, g/d										
1 to 7	238	205	228	232	22	0.42	286	165	19	<0.001
1 to 20	259	245	259	266	15	0.66	305	210	12	<0.001
20 to 28	247	233	226	231	25	0.83	240	228	22	0.44
20 to 35	345	329	320	359	25	0.48	357	320	20	0.002
28 to 35	458	439	427	506	31	0.17	491	424	22	<0.001
35 to 42	400	352	346	404	40	0.06	389	362	38	0.06

piglets fed different experimental diets ($P > 0.10$). Conversely, colonic microbial diversity was differently modulated by DT (Table 4). Indeed, the number of amplicons in LH-PCR profiles, which refers to the richness index, was significantly greater in the colonic lumen of piglets fed the CKTL diet than in piglets fed the ATB diet ($P < 0.05$). Conversely, the number of bacterial amplicons in colonic mucosa was reduced in the CKTL + COL group compared with the CTRL and CKTL groups ($P < 0.05$) and similar to the ATB group. Furthermore, as shown by the Shannon index, there was an increase of the microbial intestinal diversity in the colonic mucosa of piglets fed the CKTL diet compared with the ATB group ($P < 0.05$), while the presence of bovine colostrum in weaning diet attenuated the influence of the cocktail supplementation on the Shannon index.

MRPP analysis and NMS representation of bacterial LH-PCR fingerprint profiles

In order to further compare ileal and colonic composition of microbiota between piglets fed different experimental diets, NMS analysis and MRPP tests were performed. The results are summarized in Figure 1. First, there was a significant distance between bacterial populations of pigs fed ATB and CKTL + COL diets in the luminal ileum (Figure 1A; $P < 0.01$), and a less pronounced separation between microbiota of ATB and CKTL piglets ($P = 0.07$). Piglets receiving the CKTL + COL diet also showed differences in the mucosa-associated ileal microbiota when compared with CTRL ($P < 0.05$) and ATB piglets ($P < 0.05$) (Figure 1B). On the other hand, the analysis of the luminal microbial populations in the colon (Figure 1C) revealed some trends in terms of difference in bacterial populations between CTRL and ATB ($P = 0.06$), CKTL and CTRL ($P = 0.07$), as well as CKTL + COL and ATB DT ($P = 0.07$). In addition, the mucosa-associated colonic microbiota from piglets fed CKTL and CKTL + COL diets were different when compared with piglets fed CTRL and ATB diets ($P < 0.05$; Figure 1D). Furthermore, differences in the mucosa-associated colonic bacteria between CKTL and CKTL + COL groups were also present ($P < 0.01$).

IV and taxonomic identification of increased LH-PCR amplicons

The presence of specific LH-PCR amplicons in different intestinal sites was demonstrated (Table 5). In CTRL piglets, amplicons with increased IV were mainly found in the colon. For instance, the IV of a 367-bp amplicon, classified as *Lactobacillus amylovorus* during the taxonomic identification, was increased in both the colonic lumen and mucosa of the CTRL group compared with piglets fed the other experimental diets ($P < 0.05$ and $P = 0.05$, respectively). A 332-bp amplicon identified as *Helicobacter equorum* was also associated with the CTRL diet in ileal mucosa ($P < 0.01$). In the ATB group, significant differences were mainly found in the lumen of the small intestine and colon. Results showed increased IV for the 353-bp amplicon in the luminal ileum ($P < 0.05$) that was associated with bacteria belonging to the genus *Streptococcus*, mainly *Streptococcus alactolyticus* and *Streptococcus hyointestinalis*. A second 333-bp amplicon represented by bacteria from *Clostridium* cluster XI, such as *Clostridium mayombei*, was increased in both ileal and colonic lumen of ATB piglets compared with the other experimental diets ($P < 0.05$ and $P = 0.05$, respectively). In piglets fed the CKTL diet, differences were mainly found in the colonic mucosa. The 335-bp amplicon isolated from this site, subsequently identified as *Succinivibrio dextrinosolvens*, was increased in this group compared with piglets receiving the other DT ($P < 0.05$). Most differences were then observed for piglets receiving the CKTL + COL diet. First, the IV of a 377-bp amplicon, represented by *Lactobacillus* species such as *Lactobacillus reuteri* and *Lactobacillus mucosae*, was increased in both the ileal lumen and the colonic mucosa of this group when compared with piglets fed the other experimental diets ($P < 0.05$). Other differences were mainly found in the colon. More specifically, a 347-bp amplicon with increased IV, mainly represented by uncultured Erysipelotrichaceae and *Faecalibacterium prausnitzii* (a member of the Ruminococcaceae family), was found in both the colonic lumen and mucosa ($P < 0.05$ and $P < 0.001$, respectively). Members of the Prevotellaceae family, with *Prevotella stercorea* as the main representative, were also associated with this amplicon in the colonic lumen. Finally, the colonic 342-bp and 380-bp

Table 4. Microbial diversity analysis[1] of intestinal populations in 42-d-old piglets fed different experimental diets

	DT					
Index	CTRL	ATB	CKTL	CKTL + COL	SEM	*P*-value
Ileum, lumen						
Richness	15.3	16.9	13.8	12.9	1.3	0.13
Evenness	0.66	0.60	0.62	0.58	0.03	0.23
Shannon	1.76	1.67	1.59	1.49	0.10	0.30
Ileum, mucosa						
Richness	19.6	23.4	18.7	17.8	1.6	0.10
Evenness	0.55	0.65	0.60	0.67	0.05	0.30
Shannon	1.65	2.03	1.74	1.83	0.16	0.39
Colon, lumen						
Richness	23.9^{ab}	20.0^{b}	25.6^{a}	23.1^{ab}	1.3	0.03
Evenness	0.64	0.60	0.67	0.66	0.03	0.29
Shannon	2.04	1.79	2.17	2.08	0.11	0.10
Colon, mucosa						
Richness	22.0^{a}	18.6^{ab}	22.4^{a}	16.9^{b}	1.3	0.01
Evenness	0.62	0.56	0.67	0.66	0.03	0.05
Shannon	1.90^{ab}	1.63^{b}	2.07^{a}	1.84^{ab}	0.10	0.03

[1]ANOVA: $n = 16$ for each group (except for ATB, $n = 15$).
^{a,b}Results of the Tukey's test: within a row, means without a common superscript differ ($P < 0.05$).

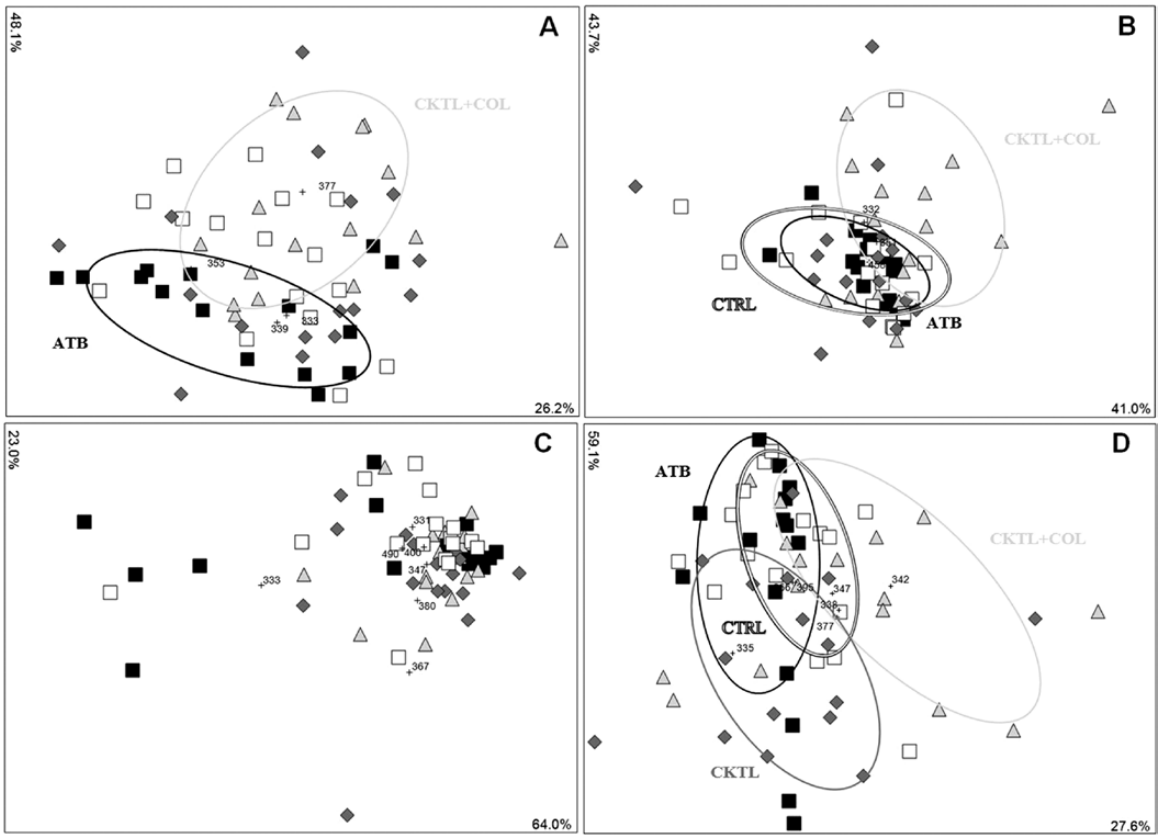

Figure 1. NMS-2D graphic representation of the intestinal bacterial LH-PCR fingerprint profiles in the ileal lumen (A), ileal mucosa (B), colonic lumen (C), and colonic mucosa (D) of piglets receiving the following DT: CTRL (□); ATB (■); CKTL (♦); CKTL + COL (Δ).

amplicons showing greater IV in piglets of the CKTL + COL group ($P < 0.01$) were represented by uncultured bacteria.

Measurements of plasma metabolites

There were significant DT × AGE interactions ($P < 0.05$) for all the plasma concentrations of vitamins and amino acids, and data are reported by day of sampling in Table 6.

Results revealed that vitamin E concentrations in plasma decreased from weaning to 42 d of age, but this decrease was less pronounced in piglets fed CKTL and CKTL + COL diets than in piglets receiving CTRL and ATB diets (DT × AGE interaction, $P < 0.01$). Over the same period, vitamin B_6 concentrations in plasma increased in all groups, except for piglets fed CTRL diet, in which it remained stable (DT × AGE interaction, $P < 0.001$). Indeed, increases of 35% and 47% of vitamin B_6 over time were observed in the CKTL and CKTL + COL groups, respectively, whereas the response was much more pronounced in pigs fed ATB diet, with an increase of 58%. For plasma concentrations of vitamin B_{12}, decreases of 28% and 24% were observed in CTRL and ATB piglets, respectively, whereas increases of 59% and 18% occurred in piglets fed CKTL and CKTL + COL diets, respectively (DT × AGE interaction, $P < 0.001$). Plasma concentrations of homocysteine decreased between weaning and 42 d of age, except in piglets fed CTRL, in which it remained high and stable (DT × AGE interaction, $P < 0.001$). Indeed, in piglets fed CKTL and CKTL + COL diets, homocysteine concentrations declined by 47% and 55%, respectively, whereas a decrease of 40% was observed in piglets fed ATB. For cysteine, DT × AGE and BW × AGE interactions were detected ($P < 0.05$) but as the differences among means were less than 5%, these responses were not considered biologically significant and were not further described and discussed.

Regarding the influence of BW on the concentrations of the selected vitamins and amino acids, no effect was observed. At weaning, plasma vitamin B_6 concentrations were lower by approximately 7% in HBW than in LBW piglets (1.06 vs. 1.14 μM, respectively; $P < 0.05$), but this effect disappeared by 42 d of age. Finally, there was also an effect of piglet BW on IGF-1 concentrations throughout the postweaning period regardless of the DT, with concentrations being lower in LBW than HBW piglets ($P < 0.001$; Table 7).

Characterization of leukocyte populations by flow cytometry

As shown in Table 7, most effects on leukocyte populations were related to BW. The percentage of γδ T lymphocytes ($CD3^+$ $\gamma\delta^+$) was greater in HBW piglets when compared with their lighter counterparts ($P < 0.01$). In addition, T-helper (**Th**) lymphocyte and B lymphocyte populations were affected by BW, but this effect was dependent upon piglet age (BW × AGE interactions: $P < 0.05$ and $P < 0.001$, respectively). When ANOVA was performed separately for each day of sampling, LBW piglets showed a higher percentage of Th lymphocytes ($CD3^+$ $CD4^+$ $CD8\alpha^-$) at weaning, but this value decreased from days 20 to 42, whereas it remained stable in the HBW group (BW effect at weaning, $P < 0.01$). Moreover, the percentage of B lymphocytes ($CD3^-$ $CD21^+$), whose values at weaning were higher in HBW than in LBW piglets (BW effect at weaning, $P < 0.05$), increased over time in both classes of body weight. However, the increase was more pronounced in the LBW group with a greater

Table 5. Taxonomic identification of LH-PCR amplicons with marked IV increase in piglets fed different experimental diets

Intestinal site	LH-PCR amplicon	IV, %[1,2]				P-value[5]	Identity	No. of clones[3]
		CTRL	ATB	CKTL	CKTL + COL			
Ileum, lumen	333 bp	23	**35**	19	16	0.04	*Clostridium mayombei*	13/14
	353 bp	28	**44**	10	14	0.01	*Streptococcus alactolyticus*	15/19
							Streptococcus hyointestinalis	3/19
	377 bp	24	7	27	**40**	0.01	*Lactobacillus reuteri*	12/18
							Lactobacillus mucosae	6/18
Ileum, mucosa	332 bp	**44**	11	13	7	0.007	*Helicobacter equorum*	3/3
Colon, lumen	367 bp	**37**	0	9	9	0.01	*Lactobacillus amylovorus*	10/10
	333 bp	21	**41**	18	15	0.05	*Clostridium mayombei*	15/16
	347 bp	14	9	15	**40**	0.01	*Faecalibacterium prausnitzii*	10/23
							Prevotella stercorea	4/23
							Uncultured Prevotellaceae[4]	3/23
							Uncultured Erysipelotrichaceae[4]	2/23
							Lactobacillus reuteri	2/23
	380 bp	1	6	0	**34**	0.004	Uncultured Bacteria	2/2
Colon, mucosa	367 bp	**30**	1	7	8	0.05	*Lactobacillus amylovorus*	13/13
	335 bp	12	0	**35**	8	0.01	*Succinivibrio dextrinosolvens*	7/7
	342 bp	10	1	3	**45**	0.002	Uncultured Bacteria	4/8
							Uncultured Erysipelotrichaceae[4]	2/8
	347 bp	18	21	16	**45**	<0.001	Uncultured Erysipelotrichaceae[4]	16/26
							Faecalibacterium prausnitzii	5/26
							Uncultured Bacteroidetes[4]	3/26
	377 bp	25	12	17	**37**	0.03	*Lactobacillus reuteri*	11/12

[1]IV = relative abundance in a given group compared with the other groups × relative frequency within a given group. The maximum IV across the four clusters is indicated in bold.
[2]Monte Carlo test, $n = 16$ for each group (except for ATB, $n = 15$).
[3]Number of clones that correspond to the LH-PCR amplicon identity.
[4]Identity found by the RDP classifier process (Wang et al., 2007).
[5]Only peaks with P-value ≤ 0.1 and corresponding to at least one sequenced clone are presented.

Table 6. Plasma metabolites changes from weaning (day 20 ± 1) to 42 d of age in piglets fed different experimental diets

Metabolite	Day	DT				SEM	P-value
		CTRL	ATB	CKTL	CKTL + COL		
Cysteine, μM	20	210[b]	201[a]	208[ab]	203[ab]	4	0.03
	42	208	207	199	208	4	0.06
Homocysteine, μM	20	25.7	27.9	27.2	23.6	1.5	0.21
	42	26.2[b]	16.7[a]	12.3[a]	12.5[a]	1.3	<0.001
Vitamin E, μM	20	1.33[b]	1.04[a]	1.20[ab]	1.35[b]	0.07	0.004
	42	0.58[a]	0.49[a]	0.78[b]	0.91[b]	0.07	<0.001
Vitamin B_6, μM	20	1.13	1.10	1.11	1.05	0.06	0.72
	42	1.05[a]	1.74[c]	1.50[b]	1.54[b]	0.05	<0.001
Vitamin B_{12}, pM	20	202	212	186	210	17	0.70
	42	145[a]	160[a]	295[c]	248[b]	16	<0.001

[a,b,c]Results of the Tukey's test: within a row, means without a common superscript differ ($P < 0.05$).

percentage of B lymphocytes than the HBW group at the end of the trial ($P < 0.05$). Furthermore, there was a significant DT × BW interaction for the cytotoxic T lymphocyte subset ($CD3^+$ $CD4^-$ $CD8\alpha^{high}$), indicating that piglet BW had a differential effect depending on dietary treatment ($P < 0.01$). The percentage of this leukocyte population was higher in HBW piglets receiving CKTL + COL diet when compared with LBW piglets of the same group, whereas no difference was observed between LBW and HBW piglets fed the other DT (Supplementary Figure S1). Finally, as low percentage values (<2%) were found for NKT lymphocytes ($CD3^+$ $CD8\alpha^+$ $CD16^+$) and double-positive (**DP**) T lymphocytes ($CD3^+$ $CD4^+$ $CD8\alpha^+$), data from these leukocyte subsets are not shown.

Measurement of cytokine production in stimulated PBMC

The production of IL8, IL10, and TNF-α by activated PBMC in response to ConA, PMA, or LPS stimulation is shown in Table 8. No effect of DT or BW on the production of IL8 or IL10 was detected. Conversely, TNF-α production tended to be modulated by DT

($P = 0.06$), and its production after ConA or PMA stimulation was decreased in piglets fed CKTL + COL compared with piglets fed the ATB diet ($P < 0.05$ and $P = 0.06$, respectively).

Growth performance of piglets reared under commercial conditions

The effects of the DT on the growth performance of piglets reared under commercial conditions are shown in Table 9. No significant difference was detected between CKTL-supplemented and non-supplemented piglets. However, when the overall period was considered (days 21 to 36), piglets fed the CKTL + COL diet showed greater ADG compared with all the other DT ($P < 0.05$) and a higher G:F ratio compared with piglets of the CTRL and CKTL groups ($P < 0.05$). During the first postweaning week (21 to 29 d), piglets fed the CKTL + COL diet had growth rates similar to piglets fed the ATB diet and showed higher ADG and G:F ratio than CKTL, but not CTRL, piglets ($P < 0.05$). Such effects of the CKTL + COL supplementation were amplified during the second postweaning week (29 to 36 d); piglets of this group had better ADG and G:F ratio compared with both CKTL and CTRL animals ($P < 0.05$), whereas no significant difference between the ATB group and the other DT was detected. Furthermore, the CKTL + COL supplementation also increased feed consumption on the overall period when compared with both CTRL and CKTL groups, as well as on the second postweaning week when compared with the CTRL group ($P < 0.05$).

As expected, there were significant differences between LWW and HWW piglets for ADG, G:F ratio, and feed consumption ($P < 0.05$; data not shown). Furthermore, results showed no significant interaction between DT and weaning weight for these parameters.

Discussion

Weaning is one of the most stressful periods in the pig's life and it results in important physiological, immunological, and microbial gut changes that impair defense mechanisms against enteric infections and reduce growth performance (Lallès et al., 2004). Such effects induced by weaning stress may be exacerbated in LBW piglets. There is evidence that BW influences the growth and development of the small intestine as well as the neonatal immune responsiveness (D'Inca et al., 2010; Hu et al., 2015; Lessard et al., 2018) and also the growth performance at all the stages of production (Fix et al., 2010).

Table 7. Effect of age and BW on the percentage of different blood mononuclear immune cell subsets and on the plasma concentrations of IGF-1 in HBW and LBW piglets

		BW			P-value		
Variable	Day	HBW	LBW	SEM	AGE	BW	AGE × BW
T lymphocytes ($CD3^+$)							
Helper ($CD4^+CD8\alpha^-$)	20	20.5[b]	24.2[a]	1.1	0.09	0.05	0.03
	42	20.6	21.1	1.0			
Cytotoxic ($CD4^-CD8\alpha^{high}$)	20	6.1	6.0	0.4	0.80	0.89	0.44
	42	6.0	5.9	0.4			
γδ ($\gamma\delta^+$)	20	19.7	16.7	0.9	0.59	0.006	0.57
	42	19.0	16.6	0.9			
B lymphocytes ($CD3^-CD21^+$)	20	19.8[a]	18.2[b]	1.2	0.003	0.73	<0.001
	42	20.6[b]	22.7[a]	0.7			
NK cells ($CD3^-CD8\alpha^{low}CD16^+$)	20	15.7	16.6	1.4	0.65	0.95	0.39
	42	16.1	15.3	1.6			
IGF-1, ng/mL	20	77.4	61.3	2.8	<0.001	<0.001	0.99
	42	102.5	86.3	4.8			

[a,b]Analysis by day: within a row, means without a common superscript differ.

Table 8. Production of IL8, IL10, and TNF-α by PBMC in response to ConA, PMA, or LPS stimulation in 42-d-old piglets fed different experimental diets[1]

		DT					
Variable	Stimulation	CTRL	ATB	CKTL	CKTL + COL	SEM	P-value
IL8	ConA	0.89	0.75	0.60	0.57	0.28	0.71
	LPS	2.57	3.21	2.56	2.30	0.65	0.54
	PMA	2.75	2.92	2.80	2.08	0.54	0.47
IL10	ConA	3.52	4.17	3.18	2.43	0.77	0.12
	LPS	4.17	4.93	4.09	3.38	0.85	0.25
	PMA	3.58	4.36	3.65	2.76	0.78	0.25
TNF-α	ConA	2.39[ab]	2.85[a]	2.08[ab]	1.59[b]	0.43	0.06
	LPS	2.10	2.87	2.35	1.96	0.41	0.19
	PMA	3.36	3.97	3.71	2.60	0.56	0.06

[1]Data are reported as delta values relative to the basal condition and expressed as $\log_2$ pg/mL.
[a,b]Results of the Tukey's test: within a row, means without a common superscript differ ($P < 0.05$).

Table 9. ADG, ADFI, and G:F ratio of piglets reared under commercial conditions and fed different experimental diets

	DT					
Days	CTRL	ATB	CKTL	CKTL + COL	SEM	P-value
Body weight, kg						
Initial	6.59	6.55	6.58	6.59	0.15	0.95
Final	9.26^{b}	9.53^{b}	9.17^{b}	10.03^{a}	0.29	0.001
ADG, g/d						
21 to 29	127^{abc}	140^{ab}	111^{c}	147^{a}	12	<0.001
29 to 36	248^{b}	287^{ab}	258^{b}	328^{a}	27	<0.001
21 to 36	181^{c}	209^{b}	179^{c}	233^{a}	14	<0.001
ADFI, g/d						
21 to 29	186	186	173	191	10	0.11
29 to 36	349^{b}	370^{ab}	357^{ab}	390^{a}	27	0.002
21 to 36	261^{b}	272^{ab}	259^{b}	284^{a}	15	0.002
G:F ratio						
21 to 29	0.704^{ab}	0.758^{a}	0.632^{b}	0.775^{a}	0.044	0.002
29 to 36	0.706^{b}	0.768^{ab}	0.729^{b}	0.830^{a}	0.040	<0.001
21 to 36	0.696^{b}	0.763^{a}	0.691^{b}	0.818^{a}	0.030	<0.001

^{a,b}Results of the Tukey's test: within a row, means without a common superscript differ.

In the main experiment of the present study, LBW piglets had reduced ADG during the whole experimental period, and this effect was associated with a reduction in IGF-1 concentrations. These findings are in agreement with previous results indicating that IGF-1 concentrations are reduced in LBW compared with HBW pigs (Michiels et al., 2013). The hormones of the IGF family are known to exert positive effects on intestinal mucosa development, enterocyte proliferation, and cell apoptosis (Hoeflich and Meyer, 2017), and IGF-1 has a prominent role in the regulation of immunity and inflammation (Ni et al., 2013; Spaziani et al., 2014). Therefore, these results suggest that the influence of BW on the development of the piglet immune system after birth could be related to IGF-1, as further discussed below.

In the second trial conducted in commercial conditions and with larger-sized experimental groups, CKTL supplementation with the addition of bovine colostrum increased ADG, ADFI, and G:F ratio of piglets during the nursery period. These results are in agreement with previous findings showing that bovine colostrum positively affects the growth performance of suckling and weanling piglets (Huguet et al., 2012; Rasmussen et al., 2016). Furthermore, several reports have already shown increases in the digestive and absorptive capacities of the small intestine after feeding bovine colostrum, which may explain the improved ADG and G:F ratio observed here in commercial conditions (Huguet et al., 2006; Rasmussen et al., 2016). This growth-promoting capacity of bovine colostrum may be ascribed to a synergistic effect of its different components that are known to enhance intestinal development, preserve intestinal integrity after weaning, and improve gut health and sanitary status (Boudry et al., 2008), as our findings further suggest.

Dietary effects on intestinal microbiota

The main objective of the present study was to investigate the potential of different combinations of feed additives to modulate piglet gut health and growth performance. Results indicate that microbial communities in the colon were more affected by dietary supplementation than those in the ileum. Variations in the bacterial populations between compartments of the pig gastrointestinal tract may at least partially explain this difference. Crespo-Piazuelo et al. (2018) observed a low α-diversity and a high β-diversity in the midgut (duodenum, jejunum, and ileum), probably due to a lower number of microorganisms in these regions when compared with the large bowel (proximal and distal colon). In the present study, a higher richness index in the colonic lumen and a higher microbial diversity (as measured by the Shannon index) in the colonic lumen and mucosa were observed in piglets fed CKTL diet compared with the ATB diet. This is in line with previous studies showing that broad-spectrum antibiotics, such as chlortetracycline, may reduce bacterial diversity while expanding and collapsing membership of specific indigenous taxa (Modi et al., 2014). Conversely, the CKTL + COL diet did not have the same effects as the CKTL diet on the microbial indices. Bovine colostrum can reduce the number of intestinal Enterobacteriaceae and increase *Lactobacillus* spp. and *Bifidobacterium* spp. (Boudry et al., 2008; Sugiharto et al., 2015). In the present study, the decrease of richness index in the CKTL + COL group was possibly due to a reduction of potentially pathogenic microorganisms and an enhancement of lactic acid bacteria (**LAB**). This effect may be ascribed to the antibacterial and prebiotic properties of the several bioactive molecules present in bovine colostrum, such as β-defensin, lactoferrin, lysozyme, and oligosaccharides (Stelwagen et al., 2009). Similarly to present results, Poulsen et al. (2017) showed a decrease of the fecal diversity index in piglets fed bovine colostrum, even though these animals had a higher abundance of potentially beneficial bacteria such as LAB.

Current results indicate that the piglet intestinal microbiota after weaning was differently modulated by the DT. The microbial prevalence in the CTRL and ATB groups suggested an important intestinal disturbance due to the stress of weaning. In these groups, bacterial populations that are normally present at a high extent in the intestine of weaned piglets, such as *Clostridium* spp., were markedly enhanced and, therefore, may have contributed to dislodging other intestinal bacteria and to reducing the microbial diversity, as mentioned above for the ATB group. As a matter of fact, most studies conducted during the weaning period usually report, together with a loss of microbial diversity, an increase of facultative anaerobes such as Proteobacteriaceae, (including *E. coli*) as well as members of the genera *Clostridium*, *Streptococcus*, *Lactobacillus*, and *Helicobacter* (Dowd et al., 2008; Gresse et al., 2017). In line with these reports, CTRL animals in the present study had a marked IV increase of *Helicobacter*

species (*H. equorum*) in the ileal mucosa and *L. amylovorus* in colonic lumen and mucosa, whereas in the ATB group, microbial indicators belonged to *C. mayombei* and *Streptococcus* species such as *S. alactolyticus* and *S. hyointestinalis*. *Helicobacter equorum* may be considered as an opportunistic colonizer, closely related to other species widely distributed in nature, such as *H. pullorum* and *H. canadensis* (Moyaert et al., 2007; Mladenova-Hristova et al., 2017), whereas *L. amylovorus* is one of the most dominant members of piglet intestinal *Lactobacilli* (Janczyk et al., 2007). Furthermore, *Clostridium* is ubiquitous in the small intestine, and *C. mayombei* is an abundant member of this group in the terminal ileum, particularly in the presence of high amylose concentrations (Tajima et al., 2013; Luo et al., 2015). Similarly, *Streptococcus* is one of the major genera of the porcine intestinal microbiota and its members *S. alactolyticus* and *S. hyointestinalis* predominate in intestinal and fecal samples of pigs of different ages (Devriese et al., 1994; Isaacson and Kim, 2012).

Feeding the CKTL and CKTL + COL diets was associated with the prevalence of different microbial populations that may exert beneficial effects on the intestinal health. The CKTL group showed a prevalence of the microbial indicator *S. dextrinosolvens*. This bacterium plays an important role as a starch digester, with the production of acetic and succinic acids as the main fermentation end-products (Santos and Thompson, 2014). Reductions in its relative abundance have been related to alterations in carbohydrate metabolism, induced, for example, by a shift in the colonic microbiota due to infections (Li et al., 2012). Concerning the CKTL + COL group, *Lactobacillus* species such as *L. reuteri* in both ileum and colon, as well as *F. prausnitzii* and *P. stercorea* in the large intestine, were increased. *L. reuteri* is well known for its probiotic properties and its ability to prevent enterotoxigenic E. coli infection, as well as for possessing the complete genes necessary for de novo synthesis of vitamin B_{12} (Santos et al., 2008; Wang et al., 2016). The health benefits ascribed to this microorganism are in part mediated by the synthesis and secretion of reuterin, an antimicrobial compound active against Gram-positive and Gram-negative bacteria, yeasts, molds, and protozoa (Schaefer et al., 2010). Furthermore, *F. prausnitzii* is a commensal bacterium and a major member of adult human and porcine microbiota, whose prevalence is often decreased in conditions of intestinal dysbiosis (Miquel et al., 2014). It is a butyrate producer with anti-inflammatory and immunomodulatory capacities and, for all these reasons, it is considered an important contributor to the functions of the microbiota and intestinal health (Qiu et al., 2013; Foditsch et al., 2015). Moreover, *Prevotella* was already shown to be one of the most abundant genera in postweaning pigs, and *P. stercorea* is one of the most abundant species during the weaning transition and the postweaning period (Pajarillo et al., 2014). Finally, certain microbial indicators were related to unclassified bacteria. Pajarillo et al. (2014) showed that the abundance of unclassified bacteria increased as piglets undergo weaning. However, whether the shift in these populations due to DT can be associated with intestinal metabolic functions and defense remains unknown.

The different shaping of the intestinal microflora due to the DT was also confirmed at the NMS analysis and MRPP tests. Piglets fed the CKTL diet differed from the CTRL and ATB groups in terms of colonic bacterial populations, and the addition of bovine colostrum strongly affected the luminal microbiota in both the ileum and colon when compared with the CTRL and ATB groups, as well as the mucosal colonic microbiota when compared with the CKTL group.

Overall, the current results strongly suggest that the dietary incorporation of bovine colostrum increased potentially beneficial bacteria in both ileum and colon of piglets. Similarly, other studies have shown that the administration of bovine colostrum, because of its antimicrobial and prebiotic properties, may increase the presence of beneficial microbial populations such as LAB genera and reduce bacteria, such as *E. coli*, *Clostridium*, and other potential pathogenic species (Menchetti et al., 2016; Poulsen et al., 2017). On the other hand, the effects of the CKTL supplementation on the microbial populations of the large intestine are likely due to the prebiotic and antimicrobial properties of its components. In fact, yeast-derived products such as mannans and β-glucans were shown to help protect pigs against ETEC infections (Stuyven et al., 2009; Halas and Nochta, 2012). Furthermore, cranberry extracts can display inhibitory activity against pathogens such as *E. coli* because of their high content in phenolic acids (Lacombe et al., 2010), whereas carvacrol, as one of the main component of oregano oil, has gained interest for its antimicrobial potential against a wide range of microorganisms, including Gram-positive and Gram-negative bacteria, molds, and yeasts (Nostro and Papalia, 2012). Therefore, these feed additives included in the CKTL supplementation have the potential to act synergistically at the expense of harmful bacteria and to shift the composition of the microbial environment of the gut. The action of one or more of them may be responsible for the effects on the intestinal microflora evidenced for the CKTL group.

It is noteworthy that in our study some differences were also evidenced between luminal and mucosal microbiota, regardless of the DT. As a matter of fact, the physicochemical conditions and substrate availability of mucosa and lumen create diversified microenvironments that support diverse microbial populations (Zhang et al., 2018). Proximity to the mucosal layer exposes the mucosa-associated microbiome to a host-derived oxygen source, promoting the growth of oxygen-tolerant populations (Albenberg et al., 2014; Kelly et al., 2017). In accordance with our study, some authors observed that the mucosal layer of the intestine is enriched with bacterial families such as Helicobacteraceae, in comparison to the lumen where they are almost completely absent (Kelly et al., 2017; Zhang et al., 2018). These microbes are adept at survival within the outer mucus layer, owing to their microaerophilic metabolism, effective flagella-propelled motility in a viscous environment, and mucin-colonizing abilities (Kelly et al., 2017; Zhang et al., 2018). Conversely, because of their intolerance to oxygen, obligate anaerobic bacteria from the Clostridiaceae and Prevotellaceae families were found mostly in the intestinal lumen, as previously reported (Kelly et al., 2017; Zhang et al., 2018).

Effects of DT and BW on plasma metabolites

Analysis of the plasma metabolites showed some important effects due to the DT. Age and diet effects on vitamin E in blood reflect a negative balance between dietary supplies of vitamin E and the metabolic utilization of this vitamin, which is directed primarily toward antioxidant protection and immune function development in the weanling pig (Wilburn et al., 2008). For homocysteine, feeding CKTL and CKTL + COL diets led to decreases of 47% to 55%, respectively, of this metabolite from weaning to 42 d of age. Such responses were expected because the CKTL supplement increased the provision of vitamins, such as folic acid, vitamin B_{12}, and vitamin B_6, which are involved in the metabolic disposal of homocysteine (Giguére et al., 2008; Zhang et al., 2009). The disposal of this intermediate amino acid may proceed through remethylation, via a reaction that depends on folate and vitamin B_{12}, or through the transsulfuration

pathway, which requires vitamin B_6 as a cofactor (Hoffman, 2011). In suckling piglets, there is a rapid postnatal development of hyperhomocysteinemia (3 to 30 μM from birth to 21 d of age), a condition persisting during the postweaning period (Simard et al., 2007) and attenuated by adequate provisions of folic acid and/or vitamin B_{12} (Giguére et al., 2008; Audet et al., 2015) and vitamin B_6 (Zhang et al., 2009). Results, therefore, suggest that the extra supplies of vitamins related to the fate of homocysteine in the CKTL and CKTL + COL diets contributed to the disposal of this metabolite, as indicated by its decreased peripheral concentrations. However, an unexpected dietary effect on plasma homocysteine was the 40% decrease between 21 and 42 d of age in piglets fed the ATB diet, while corresponding values remained high and stable for piglets fed the CTRL diet. This ATB effect on homocysteine was in line with a 58% increase of plasma vitamin B_6, whereas the corresponding values were stable in CTRL piglets. Such responses strongly suggest that the presence of dietary ATB favored either the growth of gut microbiota involved in the enteric synthesis of vitamin B_6 and/or the elimination of gut microbiota requiring a large amount of enteric vitamin B_6. It is noteworthy that the majority of Actinobacteria, Bacteroidetes, and Proteobacteria in human gut microbiota have the ability to synthesize pyridoxal 5′-phosphate, the coenzyme form of vitamin B_6 (Magnúsdóttir et al., 2015). It is also well recognized that the predominant uptake of such microbially produced vitamins occurs in the colon via specific carrier-mediated processes, in contrast to dietary vitamins, which are adsorbed in the proximal tract of the small intestine (Said, 2011). This source may contribute not only to the cellular nutrition and health of the local colonocytes but also to the total body vitamin nutrition (Said, 2011). The ATB impact on homocysteine likely came from the B_6-dependent transsulfuration pathway because peripheral B_{12} (involved in the remethylation pathway) was similar for ATB and CTRL piglets. To the best of our knowledge, such findings on homocysteine blood concentrations have never been reported before and may improve our understanding of homeostasis and control of this detrimental metabolite across species.

Effects of DT and BW on the circulating leukocyte populations

In the present study, the analysis of leukocyte populations in blood revealed that the percentages of Th lymphocytes, γδ T lymphocytes, and B lymphocytes were more affected by BW and AGE than by DT. Such findings are in accordance with a previous study showing that BW and growth performance during lactation markedly affect the development of piglet's immune system (Lessard et al., 2018). The association between growth performance and certain immune traits in growing pigs has also been previously demonstrated (Galina-Pantoja et al., 2006; Clapperton et al., 2009). Higher percentages of monocytes, B lymphocytes, and NK cells were associated with lower daily weight gains, feed efficiency, and health status in growing-finishing pigs, whereas the proportion of γδ T lymphocyte was positively correlated with daily gains (Clapperton et al., 2008, 2009). Similarly, the present results indicated that proportions of B lymphocytes, γδ T lymphocytes, and Th lymphocytes were differently modulated in HBW and LBW piglets. These cells not only play an important role in the immune response against pathogens through the generation of antibodies, antigen presentation, and recruitment and activation of other immune cells, but they are also involved in a wide range of other functions, such as epithelial wound repair and tolerance induction (Zhu and Paul, 2010; Ferreira, 2013; Sinkora and Butler, 2016). Therefore, the immune response development in piglets is clearly affected by BW and growth performance during the first weeks after birth, and these effects may be related to differences in the gut exposure to microbial antigens (Galina-Pantoja et al., 2006; Clapperton et al., 2009; Morissette et al., 2018). In addition, the lower concentrations of IGF-1 in LBW piglets may have contributed to this modulation of the immune defenses. Indeed, many authors have demonstrated the immunostimulatory activity IGF-1, especially in times of growth or stressful challenge to the organism (Dorshkind and Horseman, 2000). Relatively high numbers of IGF-1 receptors were identified on monocytes, NK cells, and Th lymphocytes, and also lower numbers on cytotoxic T lymphocytes and B lymphocytes. This highlights the potential of IGF-1 to stimulate the activation and proliferation of many immune cell subsets (Weigent, 2013).

Current observations showed that replacing plasma proteins with bovine colostrum may affect the responsiveness of the immune system. Piglets fed the CKTL + COL diet seemed to react differently to inflammatory stimuli when compared with the ATB group, as showed by the dietary treatment effect on TNF-α production from stimulated PBMC. TNF-α is a major pro-inflammatory cytokine and plays an important role in the regulation of processes such as cellular communication and cell differentiation (Brenner et al., 2015). Therefore, the generation of an inflammatory response after pathogenic stimulation might be differently modulated in animals receiving the CKTL + COL diet. Similarly, coculture with bovine colostrum was shown to inhibit TNF secretion from LPS-stimulated human PBMC (Shing et al., 2009), as well as the expression of many early and late inflammatory genes induced by enteric pathogens (such as *E. coli* and *Salmonella enterica*) in porcine intestinal epithelial cells (Blais et al., 2015). This is in line with the abovementioned effects of this particular treatment on the growth performance of piglets reared in commercial conditions.

Conclusions

Supplementing weaning diets with a combination of feed additives possessing prebiotic, antimicrobial, and immunological properties and with bovine colostrum affected the establishment of bacterial populations in the piglet gastrointestinal tract, as evidenced by the high levels of potentially beneficial bacteria, such as *L. reuteri* and *F. prausnitzii*. In the main trial, the combination of feed additives, whether supplemented with bovine colostrum or not, decreased the concentrations of homocysteine, a powerful generator of reactive oxygen species, and also increased the levels of metabolites playing an important role in the disposal of this amino acid, such as vitamins B_6 and B_{12}. These DT also showed the potential to affect the responsiveness of stimulated PBMC, as showed by the TNF-α production after ConA stimulation. Furthermore, the combination with bovine colostrum also increased piglet growth performance after weaning, as evidenced in the second trial performed in commercial conditions. However, regardless of the administered DT, BW and weaning were the factors that had the strongest influence on the leukocyte populations identified by flow cytometry. Indeed, an LBW was associated with lower percentages of γδ T lymphocytes during the whole experimental period, a higher proportion of Th lymphocytes at 20 d of age, and a different modulation of B lymphocytes before and after weaning.

Further studies are required to better understand the potential of modulating the gut microbiota and the immune response of piglets by supplementing weaning diets with

various nutrients and feed additives. The most appropriate and effective doses of bovine colostrum to use in piglet diets also need to be determined. Furthermore, research should also focus on the detrimental impact of BW on the immune defenses and its long-lasting effects on animal health and performance.

Supplementary Data

Supplementary data are available at *Journal of Animal Science* online.

Supplementary Figure S1. Effect of DT and body weight interaction (DT × BW) on the blood percentage of cytotoxic T lymphocytes ($CD3^+$ $CD4^-$ $CD8\alpha^{high}$) in LBW and HBW piglets fed different experimental diets. Means and SEM are shown. $^{**}P < 0.01$.

Supplementary Table S1. Analysis and identity of the clones associated with the intestinal bacterial LH-PCR amplicons in piglets fed different diets. Only peaks with P-value ≤ 0.05 in ISA are presented

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Conflict of interest statement

The authors declare that they have no conflict of interest.

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