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Effects of Microencapsulated Probiotics on Performance, Organ Development, Diarrhoea Incidences, Blood Parameters, Intestinal Histomorphology and Microflora in Weaning Piglets

Nicoleta Aurelia Lefter [1,†], Mihaela Hăbeanu [1,2], Anca Gheorghe [1,2,*], Mihaela Dumitru [1,*], Claudiu Gal [3] and Petru Alexandru Vlaicu [4,†]

[1] Laboratory of Animal Nutrition and Biotechnology, National Research Development Institute for Biology and Animal Nutrition, 077015 Balotesti, Romania
[2] Research Station for Sericulture, 013685 Bucharest, Romania
[3] Synevovet Laboratory, 077040 Chiajna, Romania
[4] Laboratory of Feed and Food Quality, National Research Development Institute for Biology and Animal Nutrition, 077015 Balotesti, Romania
* Correspondence: anca.gheorghe@ibna.ro (A.G.); mihaela.dumitru@ibna.ro (M.D.)
† These authors contributed equally to this work.

Abstract: The study aimed to assess the effects of the dietary supplementation of microencapsulated *L. acidophilus* and *L. plantarum* and their combination on the growth performance, organ development, diarrhoea incidences, blood profiles, intestinal histomorphology and microflora in weaned piglets. For that, 160 piglets with an average body weight (BW) of 8.52 ± 0.15 kg were divided into four groups (40 piglets/group) and allotted to one of the four dietary treatments as follows: a basal diet (C diet) or a basal diet containing 1×10^8 CFU/g of *L. acidophilus* (LA diet), or a diet containing 3×10^8 CFU/g of *L. plantarum* (LP diet) and a diet with the combination of both bacterial strains (LA + LP diet) for 21 days. On day 14, probiotics significantly increased ADFI, while FCR was higher in the LA and LP groups than the C and LA + LP groups. No effects ($p > 0.05$) on visceral organs weight, intestinal pH and biochemical parameters among treatments were noticed. Treatments significantly lowered diarrhoea incidence compared to control. Villus width was greater ($p < 0.05$) in all small intestinal segments in piglets fed probiotics. In the jejunum and ileum villus length, crypt length, and total villi length were higher ($p < 0.05$), particularly in the LA + LP group. The probiotics, particularly the LA + LP group, modulated the cecal, jejunum and ileum microbial community structure and increased ($p < 0.05$) the amount of *Lactobacillus* spp. while decreasing the populations of *Escherichia coli* and *Staphylococcus*. Our results indicated that dietary supplementation of microencapsulated probiotics, particularly the combination of *L. plantarum* and *L acidophilus* strains, maintained growth performance, lowered diarrhoea incidence and beneficially altered the intestinal architecture and microbial populations of weaned piglets.

Keywords: *L. acidophilus*; *L. plantarum*; microencapsulated probiotics; weaning piglets; performance; microflora; piglets' health

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1. Introduction

In many commercial piggeries, weaning is an important source of production loss and a major stress factor [1]. This effect is generated by actual weaning practices, which involve abrupt separation of the piglets from the sow at a young age, rapid exposure to solid food, littermate, and adaptation to the new environment, among others [1,2]. Further, weaning causes dramatic shifts to the epithelial membranes of the small intestine, such as villus atrophy and crypt hyperplasia, which further leads to a decrease in the capacity for digestion and absorption of available nutrients [2–4]. Under these conditions, the intestine, this fertile environment for beneficial bacteria (such as *Lactobacillus* spaces) and pathogenic

microbes (i.e., *Escherichia coli*, *Salmonella typhimurium*) are affected, and the proliferation of harmful bacteria encouraged with the emergence of infectious diarrhoea [5]. Diarrhoea is a condition in weaned pigs. It can last several days and causes excessive secretion of water and electrolytes into the small intestine, which exceeds the absorptive capacity of the colon. Thus, weaning increases the intestinal oxidative stress in pigs reflected in changes in intestinal architecture and also impairs liver function by translocation of metabolites to the liver, altering the metabolic health status [2]. Frequently, to inhibit pathogenic complications and to prevent the decreasing of feed intake, growth retardation and mortality rate, ZnO or a variety of antibiotics are overused [1,2,6]. However, since it has been unsafe to use antibiotics as growth promoters or due to the main effect of ZnO as co-selects bacteria resistant to antibiotics or environmental implications, scientists have worked to find appropriate replacements [7]. This resulted in the introduction of probiotics as a live microorganism [8] that gives a health benefit to the host when administered in adequate amounts. Their potential modes of action significantly affect the gut's microbial diversity by lowering the luminal pH and bacteriocins production, inhibiting pathogenic strains adhesion and regulating the hosts' immune system [9]. Moreover, positive impacts on the piglets' gut health due to microbial metabolites that directly and indirectly ameliorated piglets' diarrhoea, growth and feed conversion ratio were noticed using *Lactobacilli* compounds (including *L. acidophilus*, *L. plantarum*, *L. reuteri*, *L. acidophilus* and *L. fermentum*) in a meta-analysis of *Lactobacillus*-based probiotics [10]. Furthermore, this new natural re-establishment of beneficial bacteria in the piglets' gut is a more efficient way to reduce the economic loss in this critical phase, as was previously reported [11]. In this context, several in vitro and in vivo studies have tested different lactic acid bacteria (LAB) as probiotics for weaned piglets as the main feed additive source on growth performances, health status, intestinal histomorphology and reducing diarrhoea incidences [12–14]. Among the beneficial bacteria, genus *Lactobacillus* can be considered one of the best candidates as feed additives in piglets' diets due to their high proliferation rate and their ability to colonize or be metabolically active in the intestine, including their potential to survive into stomach acid and throughout the specific digestion process [15].

Lactobacillus spp., the natural producers of nutrients, increase the immunological system and enhance the absorption of micronutrients [16]. They stimulate the generation of organic acids and amino acids and thus affect the physiological functions of animals, such as general health and growth [16]. Several studies have reported that some *Lactobacillus* spp. can change host intestinal microbiota by producing lactic acid and other microbial compounds, and they may prevent the colonization of pathogens via competitive exclusion [14,17,18]. Growing evidence indicates that *L. plantarum* and *L. acidophilus* were found to have probiotic effects on the gastrointestinal site like increased villus high and the villi/crypt ratio of the small intestine due to increasing averaged daily feed intake, average daily gain and the gain to feed ratio of weaning piglets [19]. However, according to some studies [20,21], probiotics, live bacteria, require an encapsulating process to optimize their survival ability and protect them from biological barriers, including stomach acid and bile salts, ensuring their safe delivery. Thus, microencapsulation is an important technological process which helps to delay the quick degradation of drugs in the upper gastrointestinal tract [17,21]. Moreover, spray drying provides a more favorable anaerobic environment for the probiotic bacteria and improves the storage properties. Further, the combination of *L. plantarum* and *L. acidophilus* can synergistically promote the growth of piglets as well as improve their resistance to pathogens by equilibrating the intestinal microflora populations [20]. For these reasons, microencapsulation is an alternative to improve viability during both processing and transit to the distal areas of the intestine.

To the best of our knowledge, there are no studies on the effects of a combined microencapsulated *L. acidophilus* and *L. plantarum* probiotics supplementation in weaning piglets' diets. Thus, we hypothesized that using a mixture of these two microencapsulated probiotics (1:1 ratio) may positively affect growth performance due to complementary effects of antimicrobial properties exerted by probiotics with potential benefits on the

intestinal health of weaning piglets. Therefore, this study aimed to assess the effects of the dietary supplementation of microencapsulated *L. acidophilus* and *L. plantarum* and their combination on the growth performance, organ development, diarrhoea incidences, blood profiles, intestinal histomorphology and microflora in weaned piglets.

2. Materials and Methods

2.1. Ethical Procedure

The study was conducted according to the experimental protocol approved by the Ethics Commission of the National Research-Development Institute for Animal Biology and Nutrition (protocol no. 699/02.2020) and complied with European Directive (2010/63/EU) and Law 43/04.2014 on the protection of animals used for scientific purposes.

2.2. Animals and Housing System

The experiment was conducted on 160 weaned piglets and lasted for 21 days. The hybrid Topigs piglets [♀Large White × Hybrid (Large White × Pietrain) × ♂Talent, mainly Duroc] with average body weight (BW) of 8.52 ± 0.15 kg, age 28 ± 3 days, were randomly divided into 4 treatments, balanced for sex (♀= ♂), weight and litter origin. Each treatment included 40 ear-tagged piglets distributed in four pens with 10 piglets each. Each pen was 175 m × 230 m × 80 m and contained an automatic stainless nipple steel drinker and feeder. The microclimate conditions in the piglets' houses were electronically monitored.

2.3. Probiotics

2.3.1. Strains Isolation, Characterization and Growth Conditions

Lactic acid bacteria (LAB) strains were isolated from healthy pigs' gut digesta (ileum content). After phenotypical and molecular identification [22], the strains were stored at –80 °C with 20% sterile glycerol as a cryoprotectant in Man Rogosa Sharpe broth (MRS, Oxoid CM0361).

2.3.2. Bioreactor Batch Fermentation and Spray Drying Process

Before the experiment, the LAB strain was revitalized in MRS broth to obtain the inoculum starter culture at a concentration of 10^{10} colony-forming units (CFU) per mL. The biomass production was performed in a 5-L bioreactor (BioFlo 320, Eppendorf, one unit, Hamburg, Germany) with a workload of 2 L at 37 °C, 24 h, 150 rpm, pH = 6.5 ± 0.2 as described elsewhere [23].

For the experimental design of drying, a Buchi-Mini Spray Dryer B-290 (BUCHI Labortechnik, Swiss-made, Flawil, Switzerland) was used to microencapsulate LAB strains. As carrier material, maltodextrin (24%, w/v) and glucose (4% w/v) were prepared in distilled water to improve the strains' viability during the process. The survival of powders was done in phosphate-buffered saline pH 7.0 (PBS, Dulbecco A; Oxoid Livingstone Ltd., London, UK) as explained by Dumitru et al. [23], and the final concentration was expressed as colony-forming units (CFU) per gram of spray-dried powder.

2.4. Diets

A basal diet without probiotics was used as control (C) and fed to one group of piglets. The other 3 groups were fed the control diet supplemented with different microencapsulated LAB containing 1% *L. acidophilus* (diet LA) (1×10^8 CFU/kg of feed), 1% *L. plantarum* (diets LP) (3×10^8 CFU/kg of feed) and their mixture (1:1, diet LA + LP). The supplemented probiotics have been mixed with the premix, and after, the premix was added to the basal diet. The ingredients and nutritional composition of the basal diet are shown in Table 1. The content of the feed follows the recommendations specified by the hybrid Topigs guide. Piglets had unrestricted access to food and water (drinkers nipple) throughout the experiment. The mash form feed was used.

Table 1. Ingredients and nutritional value of the basal diet.

Ingredients (g/kg as-Fed Basis)	Basal Diet
Ground corn	667.4
Mustard meal	20.0
Hempseed meal	10.0
Soybean meal	150.0
Corn gluten	30.0
Milk powder	50.0
Hempseed oil	25.0
DL-Methionine	2.2
L-Lysine HCl	4.4
Carbonate calcium	14.6
Monocalcium phosphate	14.3
Salt	1.0
Premix Choline	1.0
Vitamin-mineral premix *&	10.0
Phytase	0.1
Total	1000.0
Nutritional value	
Metabolizable energy (EM, MJ/kg) **	14.0
Crude protein (%)	172.5
Lysine (%)	10.5
Methionine + Cysteine (%)	7.3
Calcium (%)	10.1
Total Phosphorus (%)	7.8

* Vitamin-mineral premix provided per kg of diet: vitamin A, 10,000 IU; vitamin D3, 2000 IU; vitamin E, 30 IU; vitamin K3, 3 mg; vitamin B1, 2 mg; vitamin B2, 6 mg; vitamin B3, 20 mg; vitamin B5, 13.5 mg; vitamin B6, 3 mg; vitamin B7, 0.06 mg; vitamin B9, 0.8 mg; vitamin B12, 0.05 mg; vitamin C, 10 mg; Mn, 30 mg; Fe, 110 mg; Cu, 25 mg; Zn, 100 mg; I, 0.38 mg; Se, 0.36 mg; Co, 0.3 mg; antioxidant, 60 mg. & For experimental diets, probiotics were included in the vitamin-mineral premix. ** Metabolizable energy is a calculated value, while the others are determined values.

2.5. Growth Performance

The growth performances were monitored for 3 weeks (21 days) immediately after the weaning period. The performance parameters were determined as follows: from the first day after weaning up to 14 days, 15 to 21 days and overall period 0 to 21 days, respectively. Body weight (BW, kg) and feed intake (FI, g/day) were recorded and used to calculate average daily weight gain (ADG, g/day), average daily feed intake (ADFI, g/day), and feed conversion ratio (FCR, g feed/g gain) for each group.

2.6. Incidence of Diarrhoea Determination

The animals were monitored daily to identify the piglets with diarrhoea and mortality, and the observations were recorded. The faeces of every animal were examined visually. Diarrhoea incidence (DI%) was calculated using the appropriate formula [24].

$$\text{DI (\%)} = (\text{total number of pigs/total number of pigs with diarrhoea} \times \text{number of experimental days}) \times 100 \quad (1)$$

2.7. Blood Sampling and Analyses

At the end of the trial, 16 piglets/group were selected, and blood samples were collected from jugular venipuncture into 6 mL plain plastic tubes (anticoagulant-free) containing lithium heparin and a gel for plasma separation (Vacutest, Arzergrande, Italy). The samples were centrifuged at 3000 rpm for 15 min at 4 °C for plasma separation. Afterwards, the following plasma parameters: total protein (TP), albumin (ALB), bilirubin (BIL), blood urea nitrogen (BUN), uric acid (UA), creatinine (CRE), alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (AP), creatine kinase (CK), lactate dehydrogenase (LD), gamma-glutamyl transferase (GGT), glucose (GLU), triglyc-

erides (TG), total cholesterol (TC), high-density lipoprotein cholesterol (HDL), low-density lipoprotein (LDL), inorganic phosphorus (IP), magnesium (Mg), and calcium (Ca) were determined with a Spotchem EZ SP4430 (Arkray, Japan) chemistry analyzer and specific test kits were used.

2.8. Intestinal Sampling, Light Microscopy Examination and Intestinal Microflora Analyses

2.8.1. Sampling

After the weaning period of 21 days finished, on the last day of the experimental period 32 piglets (n = 8/group) were euthanised, following exsanguination according to Romanian Law 43/2014 for the handling and protection of animals used for experimental purposes. The contents of the ileum (n = 8/group) and cecum (n = 8/group) were aseptically collected in sterile plastic bags and quickly transferred on ice to the laboratory. One gramme of intestinal content (ileum and cecum) was homogenized with 7 mL Brain Heart Infusion (BHI) broth (Oxoid LTD, UK CM1135) supplemented with 2 mL sterile glycerol and immediately frozen at −20 °C until the analysis, according to the technique described elsewhere [25]. For histological analyses, fragments of the small intestine (middle portion of duodenum, first portion of jejunum and distal part of ileum) were collected, sampled, and transported for further histological analysis to the Synevovet Laboratory (Chiajna, Romania).

2.8.2. Light Microscopy

The duodenum (n = 8/group), jejunum (n = 8/group) and ileum (n = 8/group) sections (5 cm^2) were washed in 0.9% NaCl solution and fixed for 24 h in a 10% buffered (formaldehyde 4% aqueous solution, pH 7.2) formalin solution. Afterwards, parts from the duodenum, jejunum and ileum were embedded in paraffin, and 6 mm transversal sections were stained with the hematoxylin-eosin method. The villi length (VL), villi width (VW), and crypts length (CL) were measured in each section 10 times for each animal from each group using Leica DM3000, Tokyo, Japan microscope with a 40× objective, capturing images, using ×4, ×10, ×20, ×40, and ×100 lenses in succession. The ratios of villi length to the length of the crypt (V/C) and total villi together with crypt length (VCL) were calculated.

2.8.3. Microflora Analyses

After samples defrost, decimal dilutions in phosphate-buffered saline pH 7.0 (PBS, Dulbecco A; Oxoid Livingstone Ltd., London, UK) were performed to enumerate microbial populations. The sample was assessed for Lactic Acid Bacteria (LABs), *Escherichia coli* (*E. coli*; biotype β-haemolytic), *Salmonella* spp., *Clostridium* spp., *Enterococcus* spp., *Coliforms*, and *Staphylococcus* spp. The LABs were cultured on de Man Rogosa and Sharpe agar (MRS; Oxoid CM0361) and incubated in anaerobic conditions at 37 °C for 48 h (Oxoid jar with Anaerogen 2.5 L). *E. coli* biotype β-haemolytic was analysed, as reported by Dumitru et al. [26]. Briefly, it was inoculated 0.01 mL from 10^{-1} dilution on sheep blood agar [Trypticase soy agar (TSA) 5% (w/v)] and incubated at 37 °C for 24 h in aerobic conditions. *Salmonella* spp. was grown on *Salmonella-Shigella* agar (Oxoid CM0099), followed by aerobically incubation at 37 °C for 24 h. *Clostridium* spp. were cultured on Reinforced Clostridial agar (Oxoid CM0151) and incubated anaerobically at 37 °C for 48 h. *Enterococcus* spp. were enumerated on Slanetz-Bartley agar (Oxoid CM0377) incubated at 37 °C for 48 h in anaerobic conditions, according to Sorescu et al. [25] method. The *Coliforms* were cultured on MacConkey agar (Oxoid CM0007) and incubated aerobically at 37 °C for 24 h. Coagulase-positive *Staphylococci* were enumerated on Baird-Parker Agar (BPA; Oxoid LTD, UK) supplemented with egg yolk tellurite emulsion and incubated aerobically at 37 °C for 48 h. Every sample was repeated three times. The microflora enumerations were expressed as $\log_{10}$ colony-forming units (CFU) per gram.

2.9. Statistical Analysis

The results were expressed using the mean and pooled standard error of the mean (SEM). First, we used the Shapiro–Wilk test to check whether the data set was normally distributed. The data were analyzed using one-way ANOVA in IBM SPSS (version 27.0 for windows, SPSS Inc., Chicago, IL, USA) [27]. Means were compared using the Tukey test at 5% and 1% significance levels. The pen was used as an experimental unit for growth performance data and the piglets for other response criteria. The graphs were made in GraphPad Prism software, version 9 (GraphPad Software, La Jolla, CA, USA) and the values were determined to be significant when $p < 0.05$, between groups. The Principal Component Analysis (PCA) was performed using the corresponding function of the Matlab and Simulink (version 2020, MathWorks Inc Bartok B. ut 15/d 1114 Budapest Hungary) software package to determine the relationships between productions performances, diarrhoea incidence and intestinal health parameters corresponding to each experimental group.

3. Results

3.1. Effects of Microencapsulated Probiotics Supplements on Growth Performances of Weaning Piglets

The results regarding the effects of microencapsulated probiotics on the ADFI, ADG, and FCR are presented in Table 2. After the first two weeks of feeding diets supplemented with microencapsulated *L. acidophilus*, *L. plantarum* and their combination, the ADFI was significantly higher ($p = 0.041$) in experimental groups compared to the C group, while the FCR was significantly higher ($p = 0.003$) only in the LA and LP groups compared with both C and LA+LP groups. During the following week, no significant effect ($p > 0.05$) was noted for the production performances, as well as for the overall experimental period. However, the groups supplemented with LA, LP, and the combination of the probiotics (LA + LP) tended to perform better in terms of ADFI, ADG and FCR than the C group.

Table 2. Effects of dietary microencapsulated probiotics supplements on growth performance of weaning piglets.

Items	Treatments				SEM	*p*-Value
	C	LA	LP	LA + LP		
BW (at weaning, kg)	8.53	8.52	8.53	8.52	0.011	0.995
1 to 14 days						
ADFI (g/day)	202 ^{b}	210 ^{a}	215 ^{a}	217 ^{a}	0.013	0.041
ADG (g/day)	92.9	95.7	96.4	98.9	0.083	0.063
FCR (g feed/g gain)	2.15 ^{b}	2.33 ^{a}	2.23 ^{a}	1.99 ^{c}	0.026	0.003
15 to 21 days						
ADFI (g/day)	475	476	476	482	0.021	0.317
ADG (g/day)	341	365	358	359	0.071	0.353
FCR (g feed/g gain)	1.40	1.31	1.33	1.36	0.020	0.255
1 to 21 days						
ADFI (g/day)	263	288	286	289	0.014	0.351
ADG (g/day)	180	186	188	190	0.091	0.694
FCR (g feed/g gain)	1.55	1.64	1.59	1.61	0.018	0.745

$^{a, b, c}$ Means values with different superscripts in the same row differ at $p < 0.05$; SEM—standard error of means; C—control diet; LA—control diet supplemented with microencapsulated *L. acidophilus*; LP—control diet supplemented with microencapsulated *L. plantarum*; LA + LP—control diet supplemented with microencapsulated *L. acidophilus* and *L. plantarum*; BW—body weight; ADFI—average daily feed intake; ADG—average daily gain; FCR—feed conversion ratio.

3.2. Effects of Microencapsulated Probiotics Supplements on Organs Development, Intestinal pH and Diarrhoea Incidence of Weaning Piglets

No significant differences were observed among treatments for any visceral organs weighed (Table 3). The pH measured in the intestinal sections tended to be lower but without significant effect ($p > 0.05$). Out of 21 days, all groups had piglets with diarrhoea during 10 days, but the diarrhoea incidence was significantly lower ($p = 0.001$) in the LP and LA + LP groups compared with the C group.

Table 3. Effect of microencapsulated probiotics supplements on organ development, intestinal pH and diarrhoea incidence of weaning piglets.

Items	Treatments				SEM	*p*-Value
	C	LA	LP	LA + LP		
Carcass yield, %	82.3	81.4	79.6	79.9	0.640	0.098
Liver, g	205.31	226.13	214.63	233.75	4.009	0.593
Spleen, g	21.63	19.58	21.04	19.70	1.249	0.937
Kidneys, g	45.0	45.65	46.33	47.00	1.212	0.960
Heart, g	47.75	50.00	48.00	50.75	1.234	0.818
SI length, cm	1025	1070.75	1086.50	1147.38	17.85	0.094
Intestinal pH						
Duodenum	6.35	6.16	6.10	6.21	0.264	0.337
Jejunum	6.51	6.24	6.30	6.47	0.195	0.061
Ileum	6.82 ^{a}	6.32 ^{b}	6.41 ^{ab}	6.28 ^{b}	0.014	0.023
Days with diarrhoea	10	10	10	10	-	-
Diarrhoea incidence	17.86 ^{a}	10.71 ^{ba}	9.52 ^{bc}	5.95 ^{c}	0.049	0.001

$^{a, b, c}$ Means value with different superscripts in the same row differ ($p < 0.05$); SEM—standard error of means; C—control diet; LA—control diet supplemented with microencapsulated *L. acidophilus*; LP—control diet supplemented with microencapsulated *L. plantarum*; LA + LP—control diet supplemented with microencapsulated *L. acidophilus* and *L. plantarum*; SI—small intestine.

3.3. Effect of Microencapsulated Probiotics Supplements on Biochemical Parameters of Weaning Piglets

Table 4 shows the effect of microencapsulated probiotic bacteria on plasma parameters. No significant difference ($p > 0.05$) was observed in all treatments when lipid, protein, enzymatic and mineral profiles were determined in the experimental animals. From the lipid profile, the GLU parameter tended to be lower in LA and LA + LP groups, compared with the C group, while the HDL tended to be higher. From the protein profile, only the BUN parameter presented a tendency to increase in the LA and LP groups. Lastly, in the enzymatic profile, the AST parameters were slightly higher in the LP group and lowered in the LA + LP group than in the C group. No significant alteration ($p > 0.05$) or tendencies were noted for the mineral profile.

Table 4. Effects of microencapsulated probiotics supplements on plasma parameters of post-weaning piglets.

Items	Parameters	C	LA	LP	LA + LP	SEM	*p*-Value
Lipid profile	GLU, mg dL^{-1}	111.13	104.25	108.88	104.75	3.041	1.035
	TG, mg dL^{-1}	28.15	28.50	29.46	28.89	0.953	0.971
	TCH, mg dL^{-1}	93.00	92.88	92.38	92.01	1.790	0.998
	HDL, mg dL^{-1}	28.63	29.75	29.13	31.63	1.482	0.909
	LDL, mg dL^{-1}	58.87	58.57	58.54	56.63	1.030	1.000

Table 4. *Cont.*

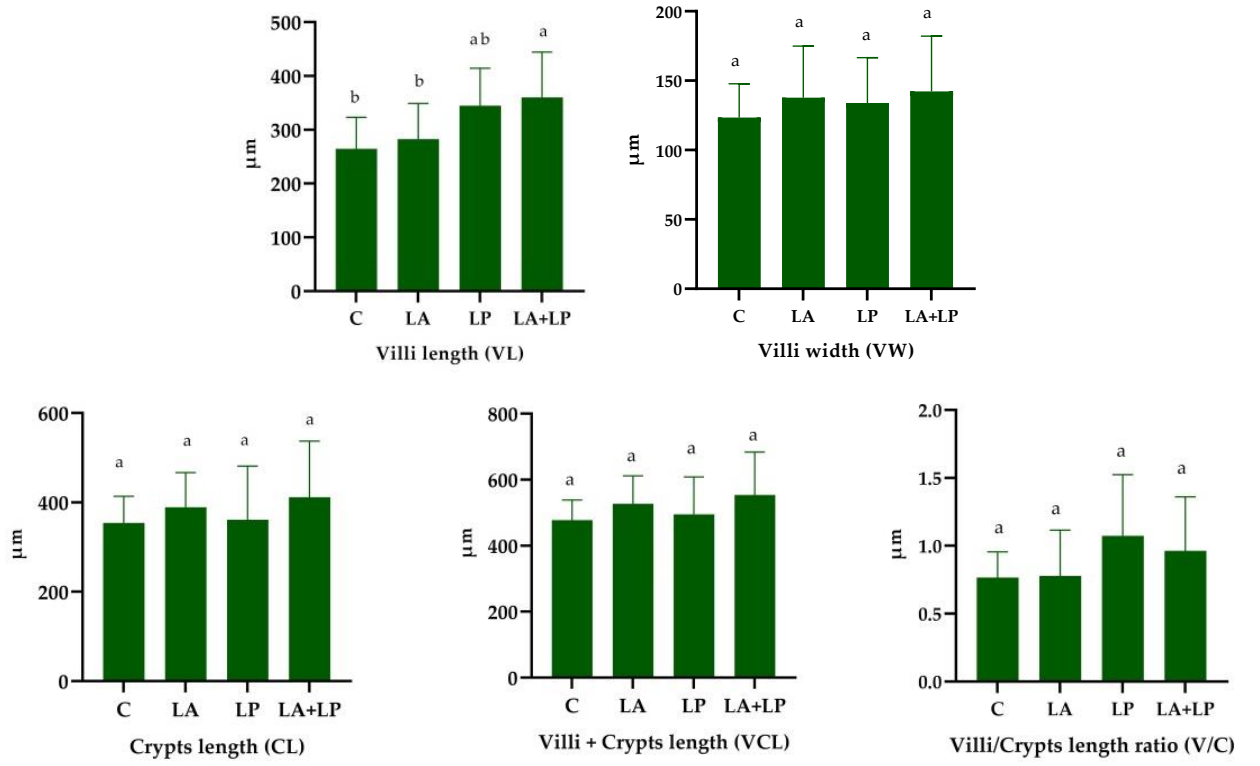
Items	Parameters	C	LA	LP	LA + LP	SEM	*p*-Value
Protein profile	TP, g dL^{-1}	4.76	4.88	4.70	4.80	0.101	0.951
	ALB, g dL^{-1}	2.81	2.88	2.76	2.81	0.063	0.811
	BIL, mg dL^{-1}	0.28	0.24	0.26	0.25	0.010	0.746
	BUN, mg dL^{-1}	16.69	17.75	17.72	16.96	0.622	0.856
	UA, mg dL^{-1}	0.53	0.53	0.54	0.51	0.011	0.941
	CRE, mg dL^{-1}	1.56	1.55	1.53	1.53	0.047	0.981
Enzymatic profile	ALT, U/L	27.75	28.19	28.25	27.50	1.090	0.818
	AST, U/L	25.75	25.25	26.00	24.13	1.168	0.949
	AP, U/L	1200	1120	1122	1222	52.33	0.314
	CK, UI/L	390.00	386.63	392.38	388.50	6.202	0.981
	LD, UI/L	1107.13	1110.63	1115.00	1105.8	34.18	1.000
	GGT, UI/L	45.38	45.63	46.50	46.13	1.921	0.997
Mineral profile	Ca, mg dL^{-1}	11.38	11.36	11.31	11.35	0.261	1.000
	Mg, mg dL^{-1}	2.28	2.29	2.23	2.30	0.053	0.948
	IP, mg dL^{-1}	6.13	6.08	6.04	6.09	0.164	0.998

SEM—standard error of means; C—control diet; LA—control diet supplemented with microencapsulated *L. acidophilus*; LP—control diet supplemented with microencapsulated *L. plantarum*; LA + LP—control diet supplemented with microencapsulated *L. acidophilus* and *L. plantarum*; GLU—glucose; TG—triglycerides; TCH—total cholesterol; HDL—high-density lipoprotein cholesterol; LDL—low-density lipoprotein; TP—total protein; ALB—albumin; BIL—bilirubin; BUN—blood urea nitrogen; UA—uric acid; CRE—creatinine; ALT—alanine aminotransferase; AST—aspartate aminotransferase; AP—alkaline phosphatase; CK—creatine kinase; LD—lactate dehydrogenase; GGT—gamma-glutamyl transferase; Ca—calcium; Mg—magnesium; IP—inorganic phosphorus; n = 16/group.

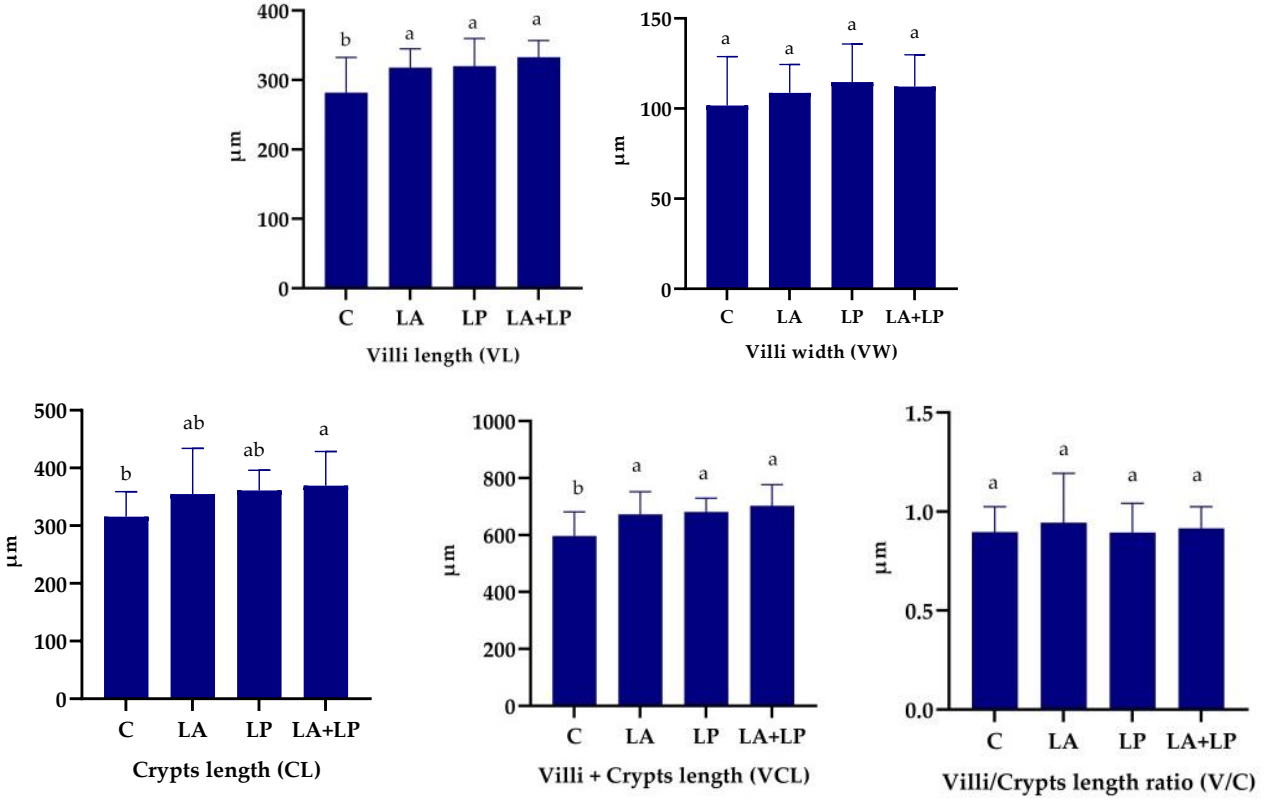
3.4. Effects of Microencapsulated Probiotics Supplements on Intestinal Histomorphology Measurements of Weaning Piglets

The effect of dietary microencapsulated supplements on VL, VW, CL, V/C ratio and VCL in the duodenum, jejunum and ileum segments of the weaned piglets are presented in Figure 1. From the obtained results, we observed that among the measurements made in the duodenum, only the VL was significantly ($p < 0.05$) affected. The LA + LP group had significantly higher ($p < 0.05$) VL compared with LA and C groups as shown in Figure 1A. In the jejunum segment, the VL was significantly higher ($p < 0.05$) in all groups of piglets fed with microencapsulated probiotics, while the CL was significantly higher ($p < 0.05$) only in the LA + LP group compared to the C group. This resulted in significantly higher ($p < 0.05$) VCL (Figure 1B) between the experimental group and the C group. Further, in the ileum segment, VL and CL were significantly higher ($p < 0.05$) in the LA+LP group compared with the C group (Figure 1C). The supplementation with LA resulted in significantly higher ($p < 0.05$) ileum VL compared to the C and LP groups, however, the V/C ratio was significantly higher ($p < 0.05$) only compared to the LA + LP supplemented group. The VCL in the ileum of piglets supplemented with microencapsulated probiotics was significantly higher ($p < 0.05$) only in the LA+LP group, compared to C and LP groups (Figure 1C).

The intestinal morphometry appearances of the duodenum, jejunum and ileum of the weaned piglets are illustrated in Figure 2. The structure of intestinal villi in the three microencapsulated probiotics supplementation groups was more complete and clearer than that of the control group. The measurement results in optical microscopy confirm that supplementation with LA, LP and their combination significantly increased VL of the duodenum but did not have much effect on the ileum and jejunum.

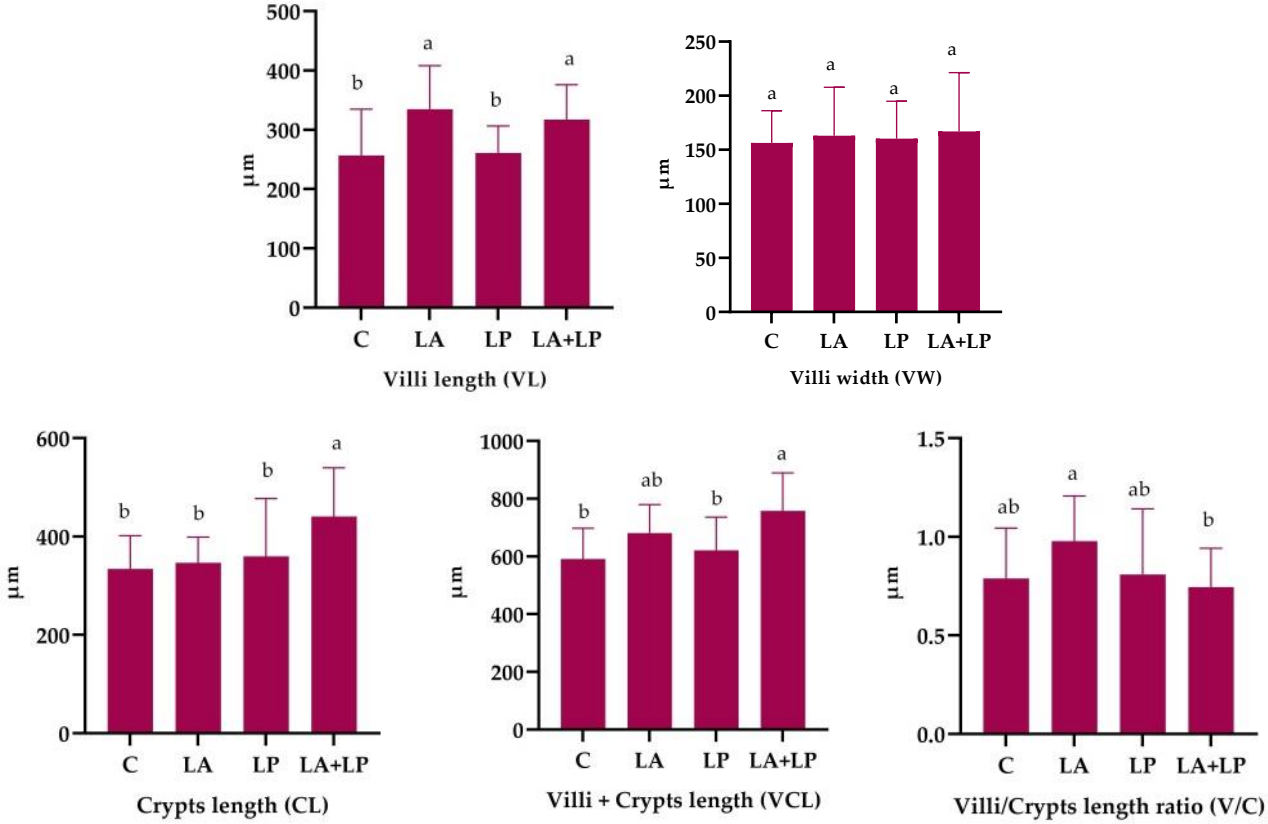


(**A**) **Duodenum**



(**B**) **Jejunum**

Figure 1. *Cont.*



(**C**) **Ileum**

Figure 1. Effects of microencapsulated probiotics supplements on intestinal histomorphology measurements in the duodenum (**A**), jejunum (**B**) and ileum (**C**) of the weaning piglets (n = 8/group). C—control diet; LA—control diet supplemented with microencapsulated *L. acidophilus*; LP—control diet supplemented with microencapsulated *L. plantarum*; LA + LP—control diet supplemented with microencapsulated *L. acidophilus* and *L. plantarum*. $^{a, b}$ superscripts withing each figure bars represents significant difference among the groups at $p < 0.05$.

3.5. Effects of Microencapsulated Probiotics Supplements on Intestinal Microbiota of Jejunum, Ileum and Caecum of Weaning Piglets

Figure 3 presents the results regarding the impact of microencapsulated probiotics on selected parts intestinal microflora of weaning piglets. In the jejunum part (Figure 3A), the *Lactobacilli* count was significantly higher ($p < 0.05$) only in LA and LA + LP groups compared to the C group. The *E. coli* was not detected in the LP and LA + LP ($p < 0.05$) groups, and the *Enterococcus* spp. was not influenced by the dietary supplements ($p > 0.05$). The *Coliforms* were significantly lower ($p < 0.05$) in the LA+LP group compared with all three (C, LA, LP) experimental groups. At the same time, the *Staphylococcus* was significantly lower ($p < 0.05$) only compared with C and LA groups. The *Clostridium* spp. was significantly higher in the LA group compared with the LP and LA + LP groups. Further, in the ileum intestinal segment (Figure 3B), the count of *Lactobacilli* spp. was significantly higher ($p < 0.05$) in the groups supplemented microencapsulated probiotics (LA, LP and LA + LP) compared to the C group. *E. coli* was significantly decreased ($p < 0.05$) only in the LA + LP group compared to the C group. The *Coliforms* were significantly decreased in the LA group compared with the other groups, while the *Clostridium* spp. was significantly decreased ($p < 0.05$) only in the LA + LP group. *Enterococcus* spp. was lowered in the LA+LP group compared with the C group, while *Staphylococcus* spp. parameter, both LP and LA+LP groups were significantly ($p < 0.05$) affected compared with the C and LA groups (Figure 3B). In the caecum segment, the count of *Lactobacilli* was significantly ($p < 0.05$) lower in the C group compared with all experimental groups. No significant

effect was observed for the *Coliforms* and *Enterococcus* ($p > 0.05$). The *E. coli* was significantly lower only in the LA + LP group compared with the C group, while the *Staphylococcus* was significantly lower ($p < 0.05$) compared with all other experimental groups (Figure 3C). *Salmonella* was absent in all cases.

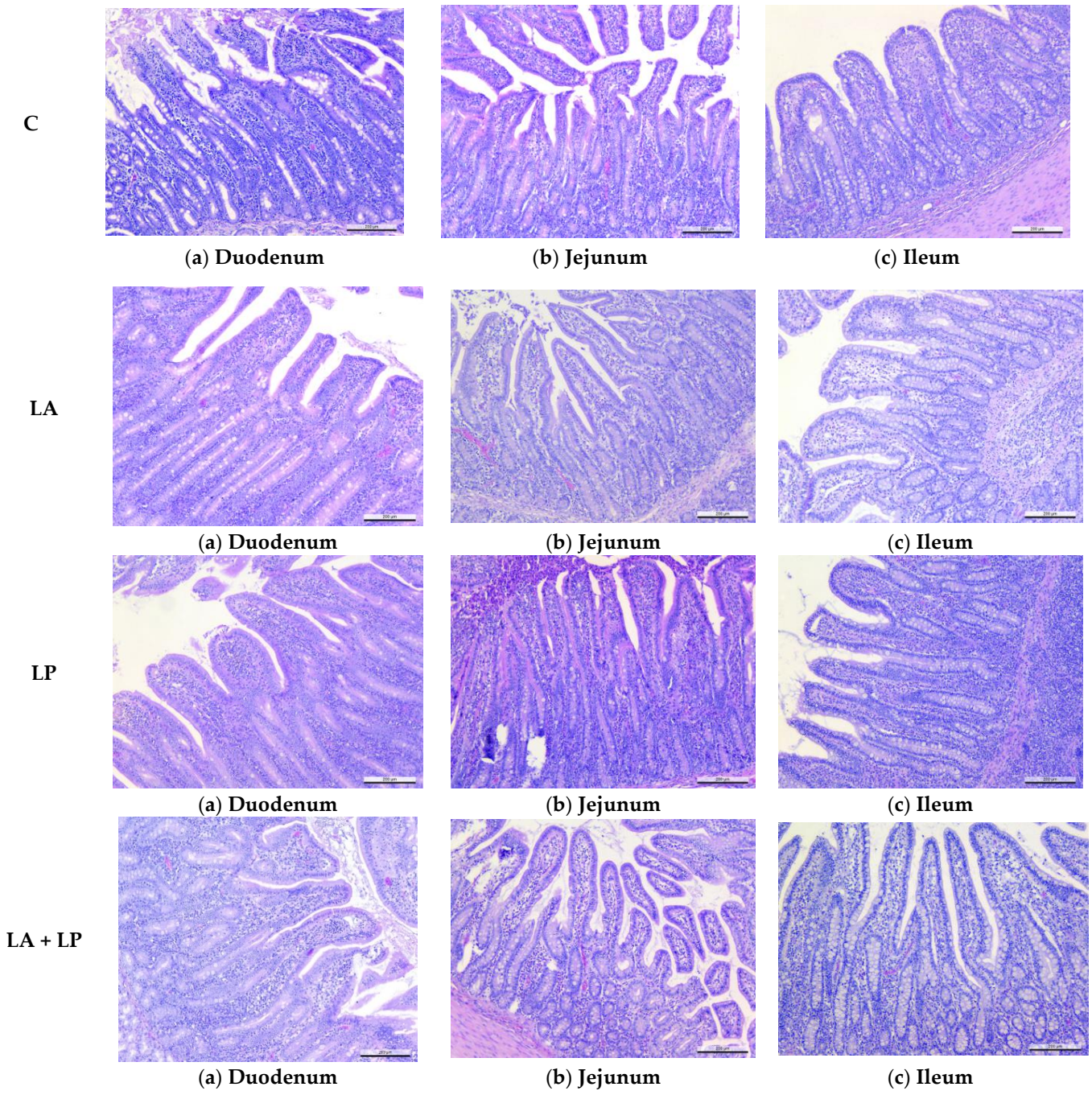
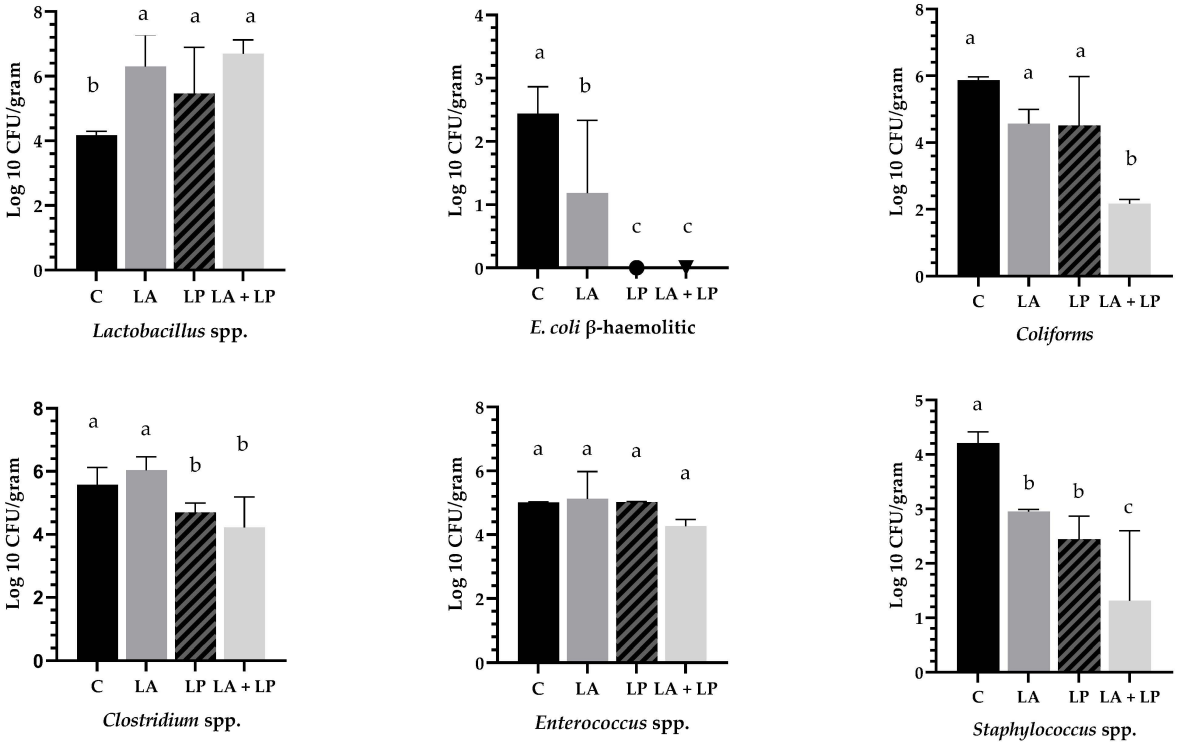
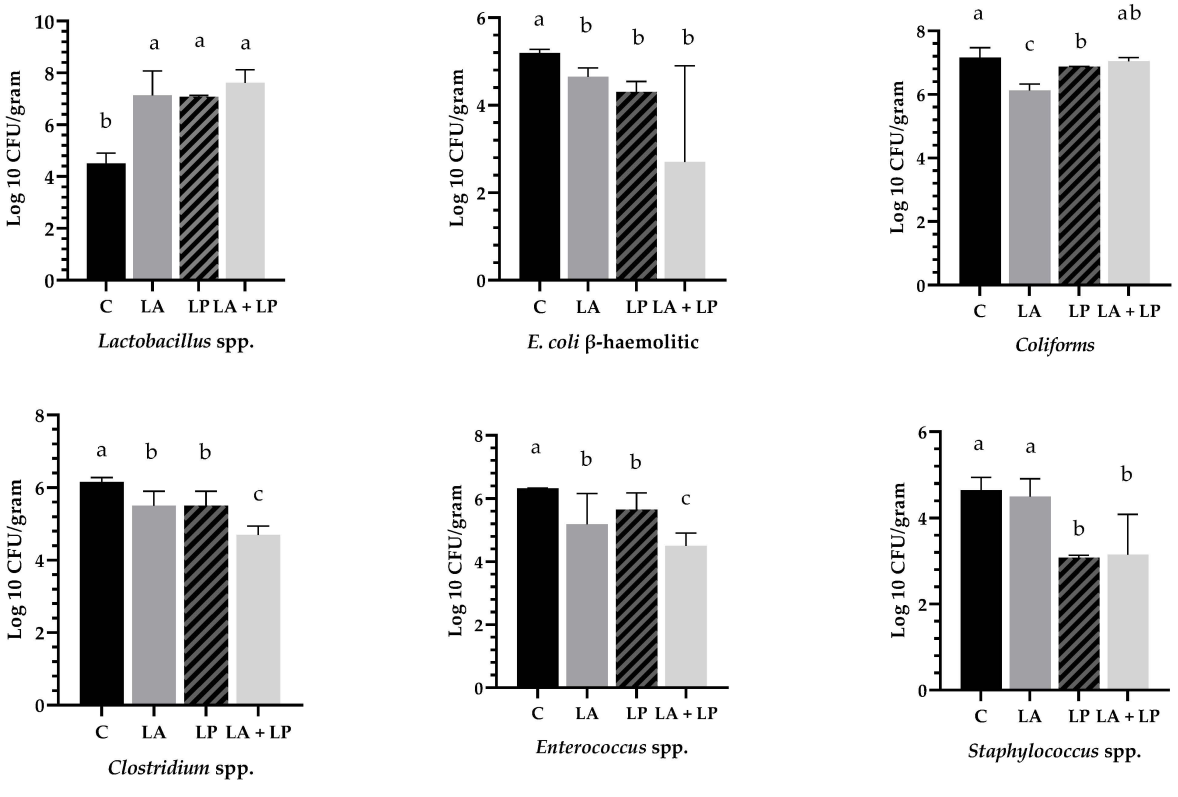


Figure 2. The intestinal morphometric appearances of the duodenum (**a**), jejunum (**b**) and ileum (**c**) of the weaned piglets (n = 8/group). The scale bars in each image indicate 200 µm. Villus height was measured from the tip of the villus to the crypt-villus junction and the crypt depth was measured from the crypt-villus junction to the base of the crypt. C—control diet; LA—control diet supplemented with microencapsulated *L. acidophilus;* LP—control diet supplemented with microencapsulated *L. plantarum*; LA + LP—control diet supplemented with microencapsulated *L. acidophilus* and *L. plantarum*.



(A) Jejunum



(B) Ileum

Figure 3. *Cont.*

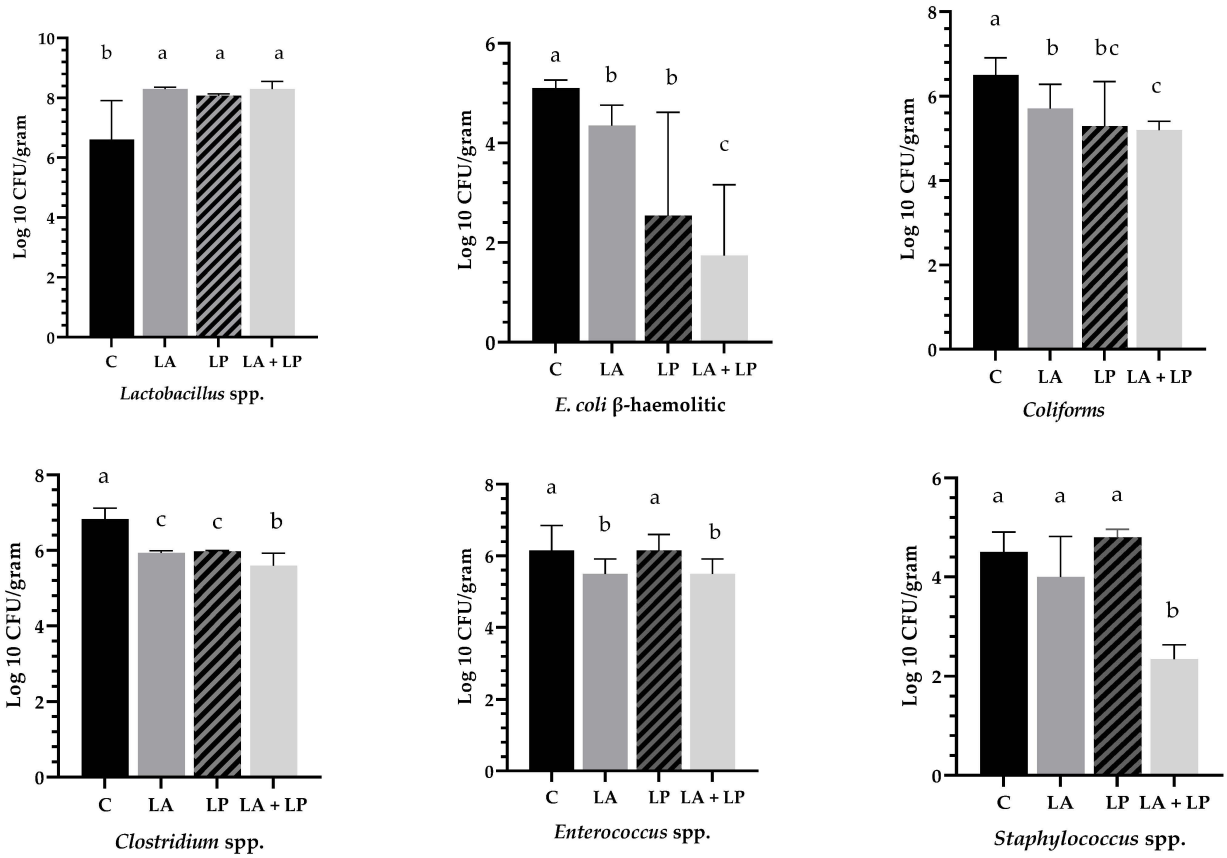
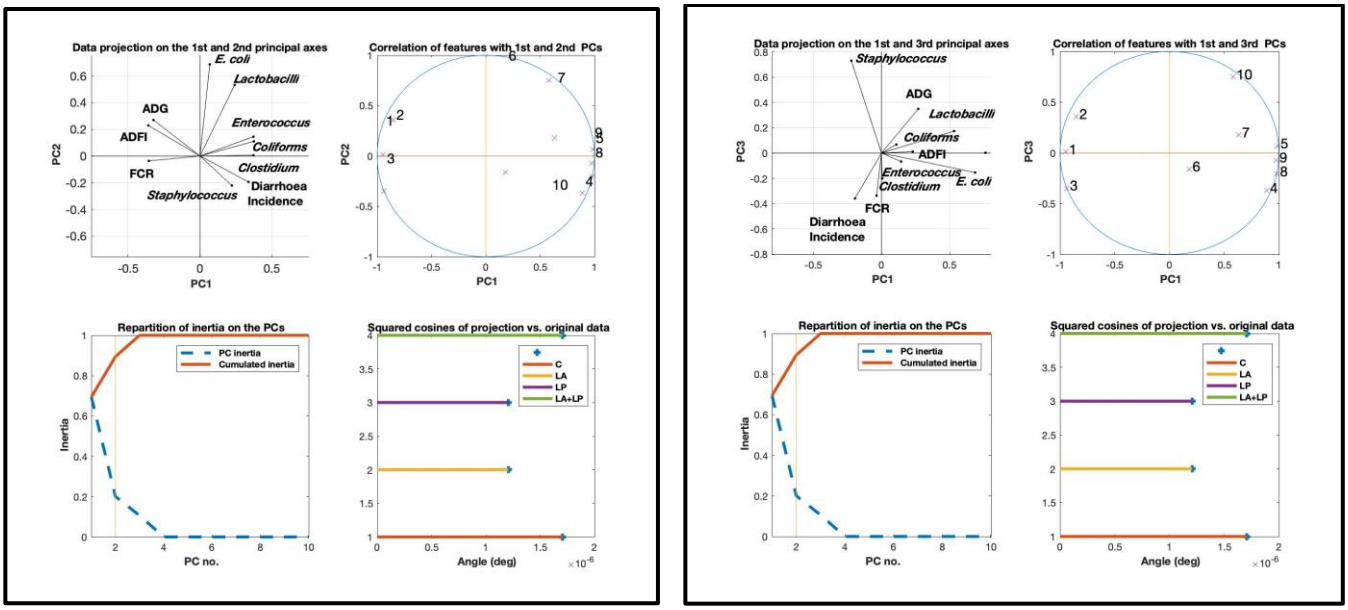


Figure 3. Effect of microencapsulated probiotic supplements on intestinal microflora in the jejunum (**A**), ileum (**B**) and caecum (**C**) of weaning piglets (n = 8/group). C—control diet; LA—control diet supplemented with microencapsulated *L. acidophilus*; LP—control diet supplemented with microencapsulated *L. plantarum*; LA+LP—control diet supplemented with microencapsulated *L. acidophilus* and *L. plantarum*. The results are expressed as ($\log_{10}$ CFU/g). $^{a, b, c}$ different superscripts within each figure represents significant difference among the groups at $p < 0.05$.

3.6. Principal Component Analysis (PCA)

The application of PCA enabled easier analysis and comparison of similarities between groups by lowering the number of variables. We considered a centred and normalized data version to obtain the PCA representation (Figure 4). The cumulated inertia of the PC1 + PC2 and the inertia of PC1 + PC3 dimensions showed that there are strong relationships between variables and suggests the number of dimensions that should be studied. The first and second components (PC1 and PC2) showed eigenvalues higher than 1 (6.91 and 2), covering 69% in PC1 and 20% in PC2 of the global variance of the data (Figure 4A). However, because the eigenvalue of PC3 was higher than 1 (1.02%), Cattell's scree plot confirmed that the usage of PC3 is also appropriate, showing a variance of the data of 10% (Figure 4B), and together explaining 99% of the data variation.



(**A**) (**B**)

Figure 4. Results of the principal component analysis (PCA), where: (**A**) is the graphical representation of the first and second principal components (PC1 and PC2), covering 89% of the global variance, correlation of features with first 2 PCs, inertia and angle degree. (**B**) is the graphical representation of the first and third principal components (PC1 and PC3), covering 79% of the global variance, correlation of features with first and 3rd PCs, inertia and angle degree. ADG—average daily gain; ADFI—average daily feed intake; FCR—feed conversion ratio.

4. Discussion

4.1. Effects of Microencapsulated Probiotics Supplements on Growth Performances of Weaning Piglets

The microencapsulated probiotics supplements used in this study showed positive effects on the ADFI, ADG and FCR during the first two-week period, while no significant modifications were observed for the overall period. Different studies have shown that probiotics are suitable substitutes for antibiotics in feed [28,29] because they can synergistically promote the growth of animals as well as improve their immune systems [30,31]. In previous research studies it was reported that utilization of *L. acidophilus* or *L. plantarum* did not affect the growth performances of weaned piglets [32,33], while others suggested that *L. plantarum* increased ADG in weaned pigs [29]. Moreover, it was reported that *Lactobacillus* (5×10^{10} CFU/kg) had positive effects on the growth performance of piglets by effectively improving barrier function [34] due to their probiotic effect. This can be explained by the exposure of the piglets to stress in connection with weaning, which produces major changes in the gut, and the time needed for adaptation to the new situation. In this regard, the efficiency of probiotics should be expected to be higher when the animals are confronted with stress during the first 2 weeks after weaning. When the most critical phase after weaning has passed and a normal gut function has been re-established, the impact of probiotic supplements should be expected to be less important for piglet performance [19]. In the present study, supplementation with microencapsulated *L. acidophilus* (1×10^8 CFU/kg of feed) or *L. plantarum* (3×10^8 CFU/kg of feed) had no significant influence on the overall production performances, which concludes other results where different concentrations of *L. acidophilus* (7.5×10^8 CFU/kg) or *L. plantarum* (2×10^8 CFU/g) were used [33,35]. However, the supplementation with the combination of microencapsulated *L. acidophilus* and *L. plantarum* significantly affected final production performances. This can be attributed to the variability in the viable counts of probiotics in the feed and the high survival rate of *Lactobacillus* colonizing in the intestine, which may produce organic acids and vitamins to improve growth performance. Probiotics also may exert their beneficial effects only when

their viable counts reach a certain quantity in the gastrointestinal tract. Literature data showed that combined probiotics supplementation with *L. reuteri* and *L. plantarum* did not affect ADG and FCR in weaned piglets [36]. However, the comparison of our results with those of others is complicated by the fact that different studies have used different bacterial strains and pigs of different ages, housing, and health status, but the utilization of combined microencapsulated probiotics needs further investigation. Moreover, in the current study, the piglets were fed microencapsulated supplements, while previous studies have used non-encapsulated administration methods. Although this aspect needs further investigation, we believe it leads to different results.

4.2. Effects of Microencapsulated Probiotics Supplements on Organs Development, Intestinal pH and Diarrhoea Incidence of Weaning Piglets

Feeding microencapsulated probiotics to the weaning piglets resulted in no modifications in the weight of the measured organs. This result was expected considering the young age of the animals and the fact that BW and ADG were similar among the groups. To our knowledge, literature data are lacking on reporting these results in weaning piglets, maybe because visceral organs do not present commercial interest for this category of piglet. Similarly, it has been reported that feeding piglets with tannic acids as antibiotics replacements for the development of the liver, spleen, kidney, heart and small intestine were not influenced [37]. For the intestinal pH determined in different intestinal segments it was observed that all experimental groups tended to have lower values, but only in the ileum, significant differences were observed in the LA and LA + LP groups compared to the C group. These results are very important because intestinal pH is one of the controlling factors for maintaining microbial balance and gut health in pigs. Weaning piglets have a limited capacity for secretion of hydrochloric acid and pepsin activity which further negatively influenced digestion and nutrient absorption of some nutrients, especially vegetal proteins. Therefore, a low pH is beneficial to prevent the growth of harmful bacteria, enhancing nutrient digestion and improving growth performance. Previously it was reported that lower gastric pH maintains a healthy gut because it hinders the passage of pathogenic bacteria into the small intestine [38–40], while a higher pH favours the introduction of food-borne pathogens to colonize the gut, resulting in the initiation of diarrhoea in piglets after weaning and decreased production performances [41]. In the study of [42], it was found that supplementing dietary probiotics bacteria to the weaning piglets increased the production of short-chain fatty acids, which helps to reduce the pH values and subsequently depress the growth of pathogenic bacteria. Literature data show that feeding microencapsulated *Enterococcus faecalis* for 21 days to weaning piglets' pH values were not different among treatments [43] but showed less incidence of diarrhoea. Similarly, tannin acids were ineffective in reducing pH in the intestinal segments [37]. Further, in this study dietary microencapsulated probiotics were shown to alleviate the detrimental effects of the weaning crisis by reducing diarrhoea, especially in the LP and LA+LP groups compared to the C group. Overall, the beneficial effects of the LAB strains are consistent with some recent findings using other probiotic strains, including *Enterococcus faecium* and multispecies probiotics such as *L. acidophilus*, *L. casei*, *Bacillus thermophilum*, and *E. faecium*, or microencapsulated *L. plantarum* or *Pediococcus acidilactici* which have been shown to reduce the severity of diarrhoea in weaning piglets [44,45]. However, to our knowledge, no other studies reported the effects of *L. acidophilus* and *L. plantarum* combined on organ development or reducing diarrhoea incidence in weaning piglets.

4.3. Effect of Microencapsulated Probiotics Supplements on Biochemical Parameters of Weaning Piglets

In the current study, supplementing weaning piglets with microencapsulated probiotic bacteria resulted in no significant effect on biochemical parameters. Only tendencies to decrease or increase some parameters were observed (Table 4). The fact that lipid, protein, mineral and enzyme levels were not significantly affected suggests that neither the oxidative challenge induced by weaning stressors nor the experimental diets induced circulating

abnormal metabolite levels. Similarly, Aiyegoro et al. [46] found no significant difference in biochemical parameters such as total serum protein, cholesterol and glucose among all treatment groups in piglets supplemented with different probiotics such as *L. reuteri, S. salivarius* or a combination of the same probiotics. These results suggest that the piglets without probiotics addition are more susceptible to infection and dehydration, as Busanello et al. [47] observed. Other authors [29–31] reported that probiotics could synergistically promote the growth of animals as well as improve their resistance to infections by stimulating the immune system.

Contrary to our results, multi-probiotic *Lactobacillus* supplementation has been reported to alter lipid fractions in pigs [48,49]. Although we did not find any studies following similar dietary supplementation on weaned piglets, it is difficult to confirm that probiotics derived from microencapsulated LAB contribute significantly to the immune system of the weaned piglets. The main reason behind this is that probiotics differ from antibiotics; they are not intended to eradicate invasive pathogens, only to protect them. Therefore, observed improvements or positive effects are often reported in sow or fattening-growing pigs [46,50] and are linked to the various applied situations.

4.4. Effects of Microencapsulated Probiotics Supplements on Intestinal Histomorphology of Weaning Piglets

Three critical components compose the gut ecosystem: the immune system, intestinal epithelial cells, and the microbial population. The nutrition, gender, background genotype, housing conditions, litter size, and age of the animals might all have a favourable or unfavourable impact on these three factors. Probiotics like *L. acidophilus* and *L. plantarum* or their combination may improve not only the integrity of intestinal epithelial cells but also the intestinal microbiome, which will further boost nutrient absorption and enhance animal development performance. In the present study, the supplementation of a microencapsulated *L. acidophilus* and *L. plantarum*, as well as their combination, improved the morphological parameters after 21 days of feeding in the weaning period of piglets, especially VL in the duodenum, and VCL in all segments with various changes for the other parameters. This result is benefic to the animals because, as it was previously reported, longer VL indicate a better absorptive capability of the small intestine and hence a healthy gut [51], while on the opposite, a reduction in VW has been associated with higher incidences of diarrhoea [41]. Additionally, the overall intestinal morphology, including VW, VL, CL, C/D, and VCL, represents the intestine's capacity to digest and absorb nutrients in relation to a healthy intestinal gut [52]. Wider villi are connected with an increased surface area capable of better absorption of nutrients accessible because intestinal villi are the main area for nutrient absorption [53]. The villus factory, or new epithelial cell production, is referred to as the crypts. Faster tissue turnover is typically associated with deeper and longer crypts to ensure villus renewal in response to natural sloughing or inflammation induced by infections or related toxins [54]. Moreover, the modifications we found in the current study regarding the effect of microencapsulated probiotics on the weaning piglets are correlated with improved growth performance, as presented in Table 2. Although there are fewer studies on the effect of microencapsulated LAB on the intestinal morphology of piglets, it has been reported that *L. plantarum* increased the VL and V/C ratio in the duodenum with no significant effect in the jejunum of the piglets [55]. Others [56] reported that combined chitosan and microencapsulated *Enterococcus faecalis* resulted in a tendency to increase VL but significantly affected the V/C ratio.

All in all, as observed in the present study and in agreement with the studies referenced above, microencapsulation of the LABs had a significant positive effect on intestinal morphology development, while the C group piglets had the shortest VL and higher incidences of diarrhoea. Nevertheless, comparing data on intestinal morphology from different experiments is difficult because of differences in the diets, breed, age, experimental conditions and as well as no known standards for the measurements of VL, VW, CL or V/C ratio [13]. Therefore, the positive effect on the intestinal morphology observed in the study

may be because the probiotics used were microencapsulated and presented better stability compared with other probiotics reported in the literature. This aspect leaves a door open for further studies to compare the parallel effect of these probiotics as microencapsulated products versus non-encapsulated ones in the weaning piglets' diets.

4.5. Effects of Microencapsulated Probiotics Supplements on Intestinal Microbiota of Jejunum, Ileum and Caecum of Weaning Piglets

The stressor factors, especially in the early weaning stage of piglets, induce changes in gut microbiota and epithelial barrier [57,58]; hence various studies have indicated that supplementation of probiotics could help to balance the bacterial community in weaned piglets [29,59]. In the current study, the microencapsulated probiotics used in weaning piglets showed some promising effects. The increased number of *Lactobacilli* in the intestinal segments represents a beneficial effect for weaning piglets, to defeat the overgrowth of pathogens. Moreover, the alteration of *E. coli* and *Streptococcus* spp. was favourable to creating an anaerobic environment for establishing other colonizers such as *Lactobacillus* and *Clostridium*. Previously, it was reported that in severely stressed animals the gastrointestinal microflora was significantly affected by decreasing total *Lactobacillus* populations during the neonatal and weaning period of untreated piglets [60]. Similarly, other studies conducted during the weaning transition of untreated piglets have reported a decrease in bacteria of the *Lactobacillus* group and a loss of microbial diversity, whereas *Clostridium* spp., *Prevotella* spp. or facultative anaerobes such as *Proteobacteriaceae*, including *E. coli*, were favoured [61–64]. Opposite to these studies, it was found that *Lactobacillus* strains with specific probiotic traits would decrease diarrhoea severity at various life stages and alleviate weaning stress syndrome [65], which is in line with our results. Moreover, the increased number of *Lactobacilli* in the intestinal segments is also reflected in the decreased number of *E. coli* and infections with *Salmonella* spp. It was reported that the administration of *L. plantarum* to newly weaned pigs appeared to inhibit the growth of opportunistic pathogens, as reported by other authors [66]. In a trial where *L. casei* combined with maltodextrin was given to the pigs, the count of *E. coli* in the jejunal mucosa was significantly inhibited [67]. As in our case, this inhibitory effect was probably caused by *Lactobacillus* addition which presents antibacterial substances and stimulates the host immunity system. However, besides the supplements used to alleviate the weaning crisis, the host's genetic background also plays a key role in driving the settlement of the gut microbiota, presenting a predisposing factor to piglet infections. Regarding the use of probiotic LABs in weaning piglets, recent studies have reported similar results in terms of improving intestinal health. Zhang et al. [68] reported that *L. reuteri* increased the beneficial species richness of the microbiota in the jejunum, colon, and cecum of weaning piglets. Orally administrated *L. casei* and *Enterococcus faecalis* or a combination of *L. casei* and *E. faecalis* at a ratio of 3:1 probiotic improved gut microbiota and increased microbial [24]. A greater villus height and abundance of *Lactobacillus* was detected in the intestinal segments of piglets supplemented with *L. salivarius* compared with the piglets from the control group, as reported in a recent study [1]. Different *L. plantarum* has been reported to beneficially modulate the intestinal microbiota in weaning piglets [69,70]; however, limited results are reported for *L. acidophilus* [34,71]. To our knowledge, no other studies reported the combined effect of *L. plantarum* and *L. acidophilus*. Although we obtained significant results from this probiotic supplement, further investigations are required.

4.6. Principal Component Analyses

The principal component analysis (PCA) was conducted in order to explore the relationship between dietary supplements and production performances, diarrhoea incidence and the intestinal microflora population of the weaning piglets (Figure 4). After mean-centering and scaling to unit variance, the PCA on these attributes explained about 99% of the variability in the data. The loading of PC1 and PC2 which occupied 89% of the data variance (Figure 4A), revealed that production performances and intestinal health as well

as diarrhoea incidence are oppositely correlated. These variables are positioned in opposite directions alongside the axis of PC1. From the same analyses, it was clear that the intestinal microflora population was strongly correlated with the diarrhoea incidence (Figure 4A), all these parameters being grouped on the same side of the PC1 axis, while the production performances were placed on the left side of the PC2 axis. However, by analyzing the data variance from PC 1 and PC3, which occupied about 79%, it was noted that the variables were differently distributed. The *Staphylococcus* was placed in the opposite direction (PC3) with ADG and *Lactobacilli*, with the total count of *E. coli* in the second dimension and diarrhoea incidence in the third dimension of PC1 (Figure 4B). These results revealed that the total count of *Lactobacilli* and *Coliform* might have an influence on ADG and ADFI. The values of *E. coli*, *Lactobacilli*, *Staphylococcus* diarrhoea incidence, ADG and FCR were higher in both plotting areas of PC1, PC2 and PC3. Overall, according to the PCA results, the three supplemented diets had a significant impact on the intestinal microflora population and diarrhoea incidence of the animals, with a low impact on production performances.

5. Conclusions

This study's findings revealed that microencapsulated probiotic bacteria such as *L. acidophilus*, *L. plantarum*, or their combination had no significant effect on production performances. However, the diarrhoea incidence was significantly decreased, especially in the *L. plantarum* and combination of *L. acidophilus* and *L. plantarum* groups. Further, the usage of the microencapsulated *L. acidophilus*, *L. plantarum* and their combinations were very effective in decreasing intestinal pH in the ileum segment and improving intestinal health by significantly increasing the beneficial bacteria such as lactobacilli and decreasing the counts of *E. coli* and *Staphylococcus* in the jejunum, ileum and caecum. In conclusion, the utilization of combined microencapsulated probiotics such as *L. acidophilus* and *L. plantarum* has been proven to be the most effective in weaned piglets' diet, in terms of reducing the diarrhoea incidence, improving the intestinal morphology and microflora.

Author Contributions: Conceptualization, N.A.L. and M.H.; methodology, N.A.L., M.D., M.H. and C.G.; software, A.G. and P.A.V.; validation, N.A.L., M.H. and M.D.; formal analysis, N.A.L., M.D., A.G. and C.G.; investigation, N.A.L. and M.D.; resources, M.H.; data curation, N.A.L. and M.D.; writing—original draft preparation, N.A.L. and P.A.V.; writing—review and editing, N.A.L., M.H. and P.A.V.; project administration, M.H.; funding acquisition, M.H. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: The study was conducted according to the experimental protocol approved by the Ethics Commission of the National Research-Development Institute for Animal Biology and Nutrition (protocol no. 699/02/2020) and complied with European Directive (2010/63/EU) and Law 43/04.2014 on the protection of animals used for scientific purposes.

Data Availability Statement: Not applicable.

Conflicts of Interest: The authors declare no conflict of interest.

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