Effect of a Multi-Strain Probiotic on Growth Performance, Lipid Profile, Antioxidant Status and Immune Response in Piglets at Weaning

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Article

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Abstract: The study aimed to investigate the role of a multi-strain probiotic compound containing *Bacillus mesentericus*, *Bacillus coagulans*, *Enterococcus faecalis* and *Clostridium butyricum* as in-feed zinc oxide (ZnO) alternative on growth performance, diarrhea incidence, antioxidant profile, lipid panel, stress and immunity in piglets at weaning. A total of 72 piglets weaned at 27 ± 1 day were divided randomly into 3 groups with four replicates of six piglets each; (i) negative control group (WC) which fed only basal diet, (ii) probiotic group (WB) which fed basal diet with current probiotic formulation and (iii) positive control (PC) group which fed basal diet with 2500 mg/kg ZnO. The experiment was conducted for 28 days. Probiotic supplementation showed positive effect on growth performance and reduced diarrhea rate. The addition of probiotic in the diet improved lipid panel; the WB group showed significantly higher level of high-density lipoprotein cholesterol and lower levels of total cholesterol and low-density lipoprotein cholesterol as compared to the negative control group. Moreover, probiotic supplementation enhanced antioxidant defense system and gave protection from oxidative damage by increasing the concentration of serum catalase, Glutathione-S-transferase and superoxide dismutase and by decreasing the concentration of serum malonyldialdehyde and total nitric oxide. Heat shock proteins and other stress markers, such as serum cortisol, were reduced in the probiotic-fed group. The probiotic group also displayed higher levels of serum IgG and IgM at all the time points and higher IgA at day 28 as compared to the negative control group. Altogether, these results indicated that feeding of currently used multi-strain probiotic formulation minimizes the weaning stress, thereby improves the growth performance, antioxidant profile, lipid panel and systemic as well as mucosal immunity. Therefore, the multi-strain probiotic compound may be used to replace ZnO in weaned piglets.

Keywords: probiotics; pigs; growth performance; lipid profile; oxidative stress; cytokines; immunity

1. Introduction

In modern intensive farming system, weaning is practiced to enhance the breeding efficiency of sows and economic profit of the farm [1]. In modern farms, it is general practice to wean piglets at three to four weeks of age [2]. Weaning, though a standard management practice, is a stressful and traumatic event for piglets due to sudden dietary, social and environmental changes [3]. Such periods of multiple stressors are linked to severe enteric infection, diarrhea, reduced feed conversion efficiency, loss of weight and death in extreme cases [4,5] leading to enormous economic loss to swine industry. At weaning, as the digestive capacity of a piglet is poor, opportunistic pathogens (mainly *Escherichia coli* and *Salmonella*) residing at the gastrointestinal tract, ferment undigested feed materials and generate toxic metabolites which damage the intestinal mucosa and ultimately results in diarrhea and poor performance of the piglet [3,6]. Moreover, stressors associated with weaning disrupt or weaken antioxidant defense system of the piglets, making them more prone to stress and infection [7]. Sometimes in commercial farming, piglets are weaned at only one or two weeks of age which

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amplifies the detrimental effects of weaning [8]. Therefore, reduction of weaning stress is extremely important and key to profitable pig farming.

In-feed administration of antibiotics and zinc oxide (ZnO) have been widely used to combat post-weaning diarrhea and growth improvement in piglets [9,10]. However, use of some antibiotics as growth promoters has been banned in the European Union, China, Japan and Korea due to increasing occurrence of antibiotic resistance in animals as well as in human, the ultimate consumer of the animal produce [11–13]. In recent years, concern over use of ZnO has been raised as extensive use of ZnO is linked to environmental heavy metal contamination [10]. Moreover, development of antibiotic-resistant microorganisms is a common consequence of application of high doses of dietary ZnO [14,15]. Considering the negative consequences of in-feed ZnO administration on environment and public health, the European Union has recommended to phase out medicinal application of ZnO in pig production by 2022 [16].

Consequently, in the recent past, much emphasis has been paid to find out a safe and practical alternative to ZnO to alleviate weaning stress and to maintain swine health and performance. Among the strategies that have been proposed, probiotics supplementation has proven to be an effective alternative to in-feed ZnO use, because of its potential to stimulate the intestinal immune system, antioxidant status, nutrient digestibility and to increase the production of antimicrobial peptides and cytokines in the intestinal tract [16–18]. Lactic acid bacteria (LAB) which include a variety of bacterial genera like, Lactobacillus, Bacillus, Bifidobacterium, Streptococcus, Enterococcus, and some other microbes, are the most frequently used microorganism as probiotic agents [19]. Among these LAB, spore-forming Bacillus spp. have been considered the most promising as their spores can endure hostile environments and allow for extensive storage at room temperature [20].

The effectiveness of a probiotic is highly strain specific; some strains provide more benefits to the host than others [21]. *B. coagulans* alone [22] or in combination with *E. faecalis* and *C. butyricum* [23] was found beneficial to weaning piglets. However, the combined effect of *B. coagulans*, *E. faecalis*, *C. butyricum* and *B. mesentericus* on weaning piglets has not been investigated extensively. The study was designed with the hypothesis that the multi-strain probiotic compound could be an alternative to ZnO administration in alleviation of weaning stress in piglets. Therefore, the objective of the current study was to investigate the combined effect of multi-strain probiotic supplementation including *B. mesentericus* TO-A, *B. coagulans* SNZ1969, *C. butyricum* TO-A and *E. faecalis* T-110on growth performance, lipid metabolites, antioxidant defense system and serum cytokine profiles of piglets at weaning in an island ecosystem.

2. Materials and Methods

2.1. Experimental area

The present study was conducted at the ICAR-CIARI institute pig farm, Port Blair, South Andaman, a district of Andaman and Nicobar Islands (ANI). The ANI, an archipelago made up of 572 islands and islets, is situated (Lat. 6° to 14° North and Lon. 92° to 94° East) in the meeting point of the Andaman Sea (East Side) and the Bay of Bengal (West Side). It has a total surface area of 8249 sq. km. and a 1,962 km. coastline.

2.2. Experimental Period

The current work was conducted during January to February, 2022. The highest and lowest air temperatures during the study period were 33.5 °C and 21.8 °C, respectively. The average relative humidity ranged from 64.0 % to 79.5 %. Temperature Humidity Index (THI) varied from 75.6 to 81.5 during the study.

2.3. Experimental Animals

The experiment was conducted on indigenous Andaman local pig (ALP). ALP is generally reared by the tribal farmers of these islands on semi-intensive system of management. Under intensive condition, they perform extremely well and attains market weight of 65-70 kg at the age of

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9 months. They provide livelihood and nutritional security to the farmers particularly the tribal farmers of ANI.

2.4. Source of the Probiotic

The probiotic (BIFILAC) used in the present study was purchased from a commercial company (Tablets India Limited, Chennai, India). It is a multi-strain probiotic and contains four microorganisms; the details are presented in Table 1. The probiotic formulation was reported to safe and offers no side effect [24].

Table 1. Composition of the probiotic.

Microbial composition	Strain number	GenBank accession details	Deposition details	Content per gram of product
Enterococcus faecium	T-110	AB687552: 16S DNA CP006030: Complete genome CP006031: Complete plasmid	8936*	3 x 10^7 CFU/g
Clostridium butyricum	TOA	AB687551: 16S DNA CP014704: Chromosome 1 CP014705: Chromosome 2 CP014706: Plasmid	8935*	2 x 10^6 CFU/g
Bacillus mesentericus	TOA	AB687550:16S DNA CP005997: Complete genome	8934*	1 x 10^6 CFU/g
Bacillus coagulans	SNZ 1969	KC146407: 16S DNA	MTCC 5724	5 x 10^7 CFU/g

*All the three strains were deposited with the international deposit agency in Japan.

2.5. Study design

The study included 72 clinically healthy piglets (ALP, weaned at 27 ± 1 day) with initial average body weight of 8.77 ± 0.15 (mean ± SD, kg). The piglets were randomly divided into 3 groups according to sex and body weight with 24 animals in each group (6 piglets per pen; 4 pens/replicates per treatment). Each pen (1.5 x 1.3 m^2) with six animals (3 males and 3 females) was considered as an experimental unit. Throughout the trial, each pen had access to ad libitum feed and drinking water. the pens have concrete floors and height of 0.7 meter which allow adequate natural ventilation. The piglets were grouped as follows; (a) weaned negative control group (WC) received basal diet without probiotics, (b) positive control (PC) group received basal diet with 2500 mg/kg ZnO as reported previously [25] and (c) weaned probiotic group (WB) received basal diet with probiotics (0.1 % with feed). The dose of the probiotics was standardized using a pilot study in which the above-mentioned dose was found most effective in promotion of growth and in alleviation of weaning stress. The composition of the basal diet is provided in Table 2. Probiotics and ZnO were mixed with experimental diets using a feed miller. The experiment was for 28 days.

Table 2. Composition of basal diet and its nutritional levels.

Ingredients	Percentage
Maize	50.00
Wheat bran	15.00

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Soybean meal	29.00
Vitamin & trace min. mix.	2.50
MCP	1.00
Salt	1.00
CaCO3	1.28
DL-Methionine	0.22
Chemical formula of basal diet	
Crude Protein (CP)	20.72
Calcium	0.80
Total phosphorus	0.63
Lysine	0.80
Methionine and Cystine	0.70
Metabolizable energy (ME) (kcal/kg)	3382.31

The basal diet was prepared and given to the experimental piglets according to the nutritional guidelines of National Research Council [26]. Vitamin & trace mineral mix has (per kg feed): vitamin D3 (4000 IU); vitamin K (16 mg); vitamin E (80 IU); vitamin A (20,000 IU); Ca-pantothenate (50 mg); niacin (120 mg); riboflavin (20 mg); pyridoxine (6 mg); thiamine (4 mg); folic acid (2 mg); vitamin B12 (0.08 mg); biotin (0.08 mg); Mn (73 mg); Cu Zn (56 mg); (15 mg); Co (0.5 mg); Se (0.4 mg); I (0.3 mg).

2.6. Estimation of Diarrhea Rate of Piglets

For estimation of diarrhea incidence, feces of piglets were examined visually every morning and afternoon. Scoring of the feces and severity of diarrhea were done as per procedure recommended by Walsh et al. [27] as following; 1 = hard feces; 2 = slightly soft feces; 3 = soft, partially formed feces; 4 = loose, semi-liquid feces; and 5 = watery, mucous-like feces. Fecal consistency score of grade 4 to 5 for 2 consecutive days was considered as diarrhea. Diarrhea rate was calculated as previously described [28].

2.7. Production Parameters

All the piglets in each group were weighed individually on day 0, 7, 14, 21 and 28 with an electronic balance. Daily feed consumption was recorded throughout the study. Average daily feed intake (ADFI), average daily gain (ADG), and feed to gain ratio (F:G) were calculated using standard methodologies.

2.8. Blood Sampling

Each piglet of every group was bled (~ 10 ml) on 0 day, and weekly interval thereafter up to forth week from cranial vena cava into a vacutainer containing clot activator (Hebei Xinle Sci &Tech Co., Ltd. Hebei Province, China), following standard aseptic condition. Serum was separated by keeping the tubes at room temperature for 30 minutes followed by centrifuged at 1200 × g for 10 minutes at 4 °C. Serum was stored at -80 °C till further use.

2.9. Lipid Profile Analysis

Serum lipid profile including concentration of total cholesterol (TC), triglycerides (TG) and high-density lipoprotein cholesterol (HDLc) were determined by enzymatic methods using commercially available kits (Jeev Diagnostics Pvt. Ltd., Chennai, India; Spinreact, S.A., Spain and Pathozyme Diagnostics, Kholapur, India, respectively). The concentration of LDLc was determined as reported previously as: LDLc = TC – HDLc – (TG/5) [29]. Additionally, cardiac risk factor (CRF = TC/HDLc) and atherogenic index (AI= (TC-HDLc)/HDLc were also evaluated as previously described [30,31]. Lipid profile was measured on day 0, 7, 14 and 28; CRF and AI were calculated for day 14 and 28.

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Total antioxidant activity (T-AOC) of serum samples were assessed by commercial kit brought from HiMedia laboratories company (Mumbai, India). Serum T-AOC was detected with reduction of Cu (II)-chromogen complex to Cu (I) complex and absorbance was measured at 460 nm.

Serum activities of glutathione -S-transferase (GSH), superoxide dismutase (SOD), catalase were estimated by commercially available kits brought from Cayman chemicals company (Ann Arbor, Michigan, USA).

Serum malonyldialdehyde (MDA) levels were measured to determine the degree of lipid peroxidation as previously described [30]. The MDA concentration of serum samples were estimated with 2-thiobarbituric acid and the variations in absorbance were read at 534 nm.

T-AOC and MDA were measured at day 0 and weekly interval thereafter throughout the study whereas SOD, catalase and GSH levels were evaluated on day 0, 7, 14 and 28.

2.10. Measurement of Stress Biomarkers

Total nitric oxide (TNO) and heat shock proteins (HSPs) were observed at day 0 and at weekly interval till the end of experiment, whereas serum cortisol concentration was measured on day 0, 7, 14 and 28.

2.11. Antioxidant and Oxidative Profile

2.11.1. Nitric Oxide Assay

TNO level was analyzed by commercially available NO Estimation kit (HiMedia laboratories, Mumbai, India).

2.11.2. Serum Cortisol Assay

Level of cortisol in serum was measured by commercial cortisol detection kit by Arsh Biotech company (Life Technologies, Delhi, India) using biotin double antibody sandwich technology.

2.11.3. Determination of serum HSPs

Serum heat shock proteins (HSP90, HSP70, HSP40, and HSP20) were determined by commercial double antibody sandwich ELISA kits by Arsh Biotech company (Life Technologies, Delhi, India).

2.12. Immune Parameters

Serum immunoglobulin concentrations including IgM, IgG and IgA were determined by commercial kits brought from Arsh Biotech company (Delhi, India).

Serum interleukin concentrations including IL-1β, IL-2, IL-4, IL-6, IFN-γ and IL-12 were determined by porcine ELISA based kits brought from Arsh Biotech company (Life Technologies, Delhi, India). All the parameters were evaluated on day 0, 7, 14 and 28.

2.13. Statistical Analysis

Before analysis, Shapiro-Wilk statistics were performed to check the data for normality. Data had a homoscedastic distribution and a normal shape. One-way repeated measures analysis of variance (ANOVA) or a within-subjects ANOVA was applied to determine the significant differences be-tween groups at a particular time point in GraphPad Prism software (http://www.graphpad.com). Pen was served as an experimental unit in analysis of diarrhea rate, F:G and ADFI while, each pig was treated as the experimental unit for other experimental parameters of this study. The analyzed data of each parameter was presented as mean (M) ± standard deviation (SD). The statistical significance was defined as the mean values with a significance level of $p < 0.05$.

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3. Results

3.1. Production Parameters and Diarrhea Incidence

Effect of currently used probiotic formulation on production parameters and diarrhea incidences are presented in Table 3. No differences in body weights among three groups at day 0 and day 7 were observed. Thereafter, significantly higher body weights in WB and PC groups as compared to WC group were recorded on day 14, day 21 and day 28. But, it did not show any changes between WB and PC group. WB and PC groups showed greater average daily weight gain on 0-14 day and 14-28 days as compared to those of WC group. Moreover, the overall ADG (0-28 D) of WC group was significantly lower than WB and PC group. Significantly ADFI in PC as compared to WC was recorded for 0-14 D whereas it did not vary between WB and PC group. Overall ADFI (0-28 D) did not differ significantly among the groups. WB and PC groups showed significantly lower feed to gain ration (F:G) as compared to the control group (WC) for 0-14 D, 14-28 D and overall (0-28 D). The diarrhea rates in piglets supplemented with the probiotic compound (WB) or in-feed ZnO (PC) were lower than that of the negative control group (WC).

Table 3. Production Parameters and diarrhea incidence of piglets.

Parameters	WC	WB	PC
		Body Weight/kg	
0 D	8.84 ± 0.12	8.79 ± 0.14	8.75 ± 0.12
7 D	9.87 ± 0.33	10.11 ± 0.24	10.38 ± 0.18
14 D	11.25 ± 0.13A	11.79 ± 0.11B	11.78 ± 0.14B
21 D	12.58 ± 0.17A	13.63 ± 0.34B	13.71 ± 0.38B
28 D	13.80 ± 0.22A	14.88 ± 0.43B	14.97 ± 0.45B
		ADG/g	
0-14 D	171.90 ± 10.70A	214.17 ± 14.14B	216.2 ± 15.98B
14-28 D	182.26 ± 17.69A	220.48 ± 28.47B	228.1± 31.70B
0-28 D	177.08 ± 7.21A	217.32 ± 15.54B	222.1 ± 16.76B
		ADFI/g	
0-14 D	417.73 ± 26.01A	454.03 ± 29.98AB	459.2 ± 33.94B
14-28 D	466.59 ± 45.30	485.05 ± 62.63	499.2 ± 69.32
0-28 D	442.16 ± 18.45	469.54 ± 33.97	479.2 ± 36.78
		F:G	
0-14 D	2.426 ± 0.03A	2.11 ± 0.01B	2.12 ± 0.01B
14-28 D	2.57 ± 0.02A	2.20 ± 0.01B	2.18 ± 0.01B
0-28 D	2.49 ± 0.01A	2.16 ± 0.01B	2.16 ± 0.01B
		Diarrhea rate (%)	
0-28 D	11.67 ± 1.31A	4.67 ± 1.07B	4.53± 0.78B

Analyzed data were shown as M ± SD. ^{A,B}Values with different superscripts in a same row differ significantly. WC designates weaned negative control group, WB designates weaned probiotic group and PC designates positive control (ZnO) group.

3.2. Lipid Panel Analysis

TC did not vary significantly among the groups up to day 14 (Figure 1a). At day 28, TC was significantly greater in WC group than those of other two groups (WB and PC), whereas WB and PC did not record any difference.

Down-regulated HDLc concentration (Figure 1b) was observed in WC group as compared to WB and PC groups on day 14 and 28 whereas the concentration was higher in WB group than PC group on day 28.

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At day 7 and day 14, TG concentration in WC group was significantly greater as compared to other two group (WB and PC), whereas, it did not vary between WB and PC group (Figure 1c). Day 28 recorded no significant difference in TG concentration among the groups.

Regarding LDLc concentration (Figure 1d), significantly higher value on Day 28 was observed in WC as compared to the other two groups, whereas, WB and PC was unchanged. On the other time points, there was no significant change among the groups.

The WC group showed significantly higher values of CRF (Figure 1e) and AI (Figure 1f) on day 14 and 28 than the WB and PC group.

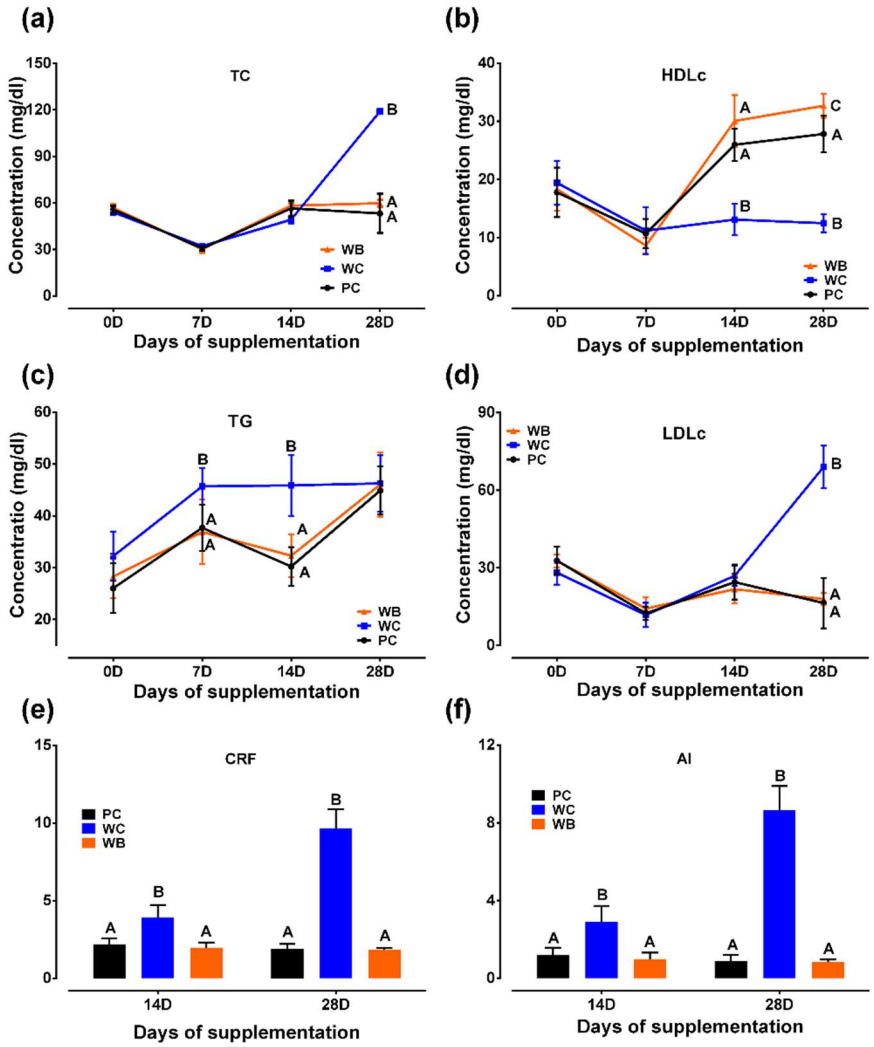

Figure 1. Effect on serum lipid panel of weaned piglets. (a) Total cholesterol (TC) concentration; (b) High-density lipoprotein cholesterol (HDLc) concentration; (c) Triglycerides (TG) concentration; (d) Low-density lipoprotein cholesterol (LDLc) concentration; (e) Cardiac risk factor (CRF); (f) Atherogenic index (AI). Analyzed data were shown as M ± SD. ^{A,B,C}Values in a particular time point having different superscripts. WC: weaned negative control group, WB: weaned probiotic group and PC: positive control (ZnO) group.

3.3. Antioxidant Profiles and Oxidative Stress Indicators

Significantly lower T-AOC (Figure 2a) in WC group than that of other two groups (WB, PC) was observed throughout the study period, whereas no significant difference between WB and PC was detected.

Activities of SOD (Figure 2b), catalase (Figure 2c) and concentrations of GSH (Figure 2d) in PC and WB were significantly greater than the WC group on both day 14 and 28, whereas it did not vary

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among the two groups (WB and PC) except for SOD and catalase at day 7, in which both were found higher in PC group than those of WB group.

At every week under consideration, the WC group showed significantly greater MDA concentration than WB and PC group. No significant difference in MDA concentration between WB and PC was observed throughout the study period except at day 7 in which WB group showed significantly higher MDA concentration than PC group (Figure 2e).

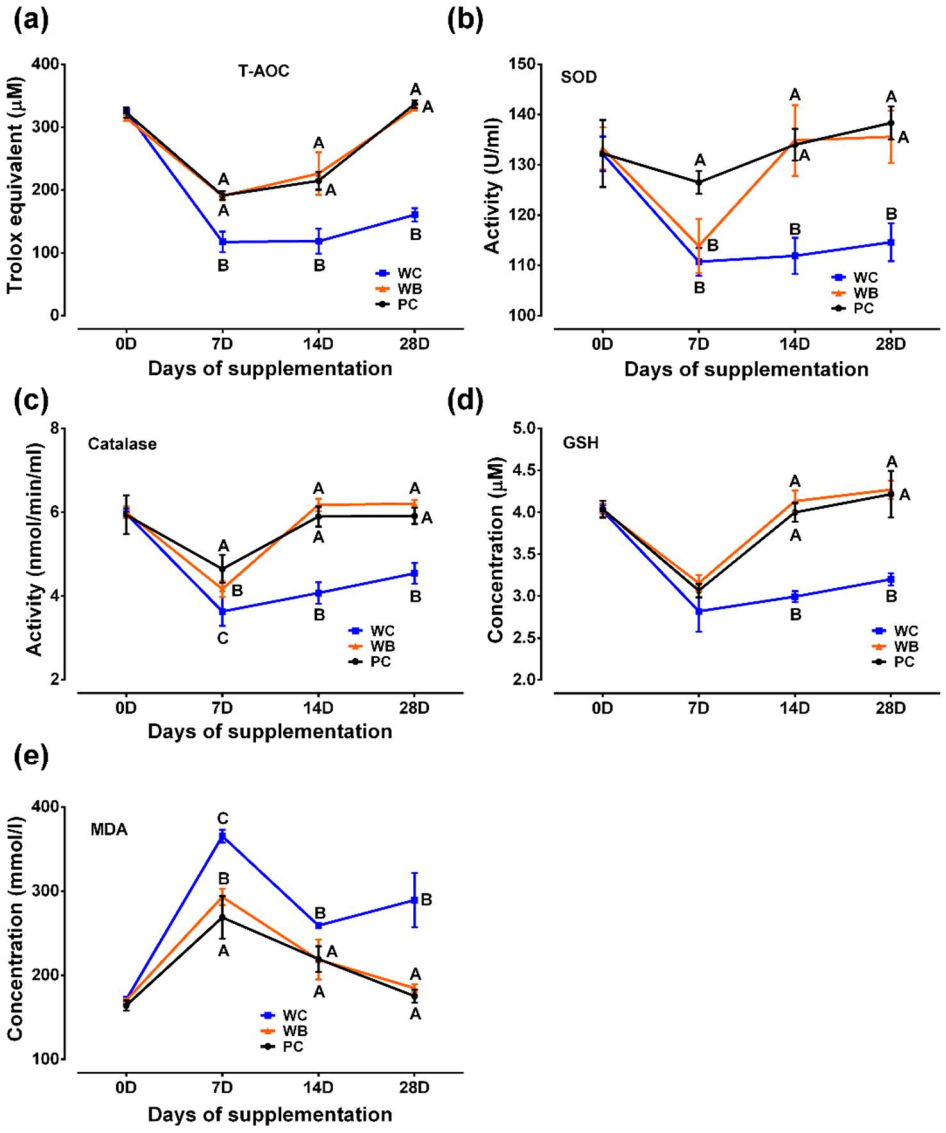

Figure 2. Results of probiotic administration on antioxidant activity and oxidative stress markers of weaned piglets. (a) Total antioxidant capacity (T-AOC); (b) Superoxide dismutase (SOD) activity; (c) Catalase activity; (d) Glutathione-S-transferase (GSH) concentration; (e) Malonyldialdehyde (MDA) concentration. Analyzed data were shown as M ± SD. ^{A,B,C}Values in a particular time point having different superscripts. WC: weaned negative control group, WB: weaned probiotic group and PC: positive control (ZnO) group.

3.4. Evaluation of Stress Parameters

3.4.1. Total Serum Nitric Oxide Concentration

WC group showed significantly higher serum TNO concentration (Figure 3a) on day 7 and 14 in comparison to WC and PC group whereas, there was no significant difference among the three groups on day 21 and 28.

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3.4.2. Serum Cortisol

Significantly higher cortisol concentrations (Figure 3b) in WC and WB as compared that of PC were recorded on day 7. At day 14 and 28, significantly reduced concentrations of cortisol in WB and PC groups as compared to WC group was observed while, there was no significant difference between WB and PC on those days.

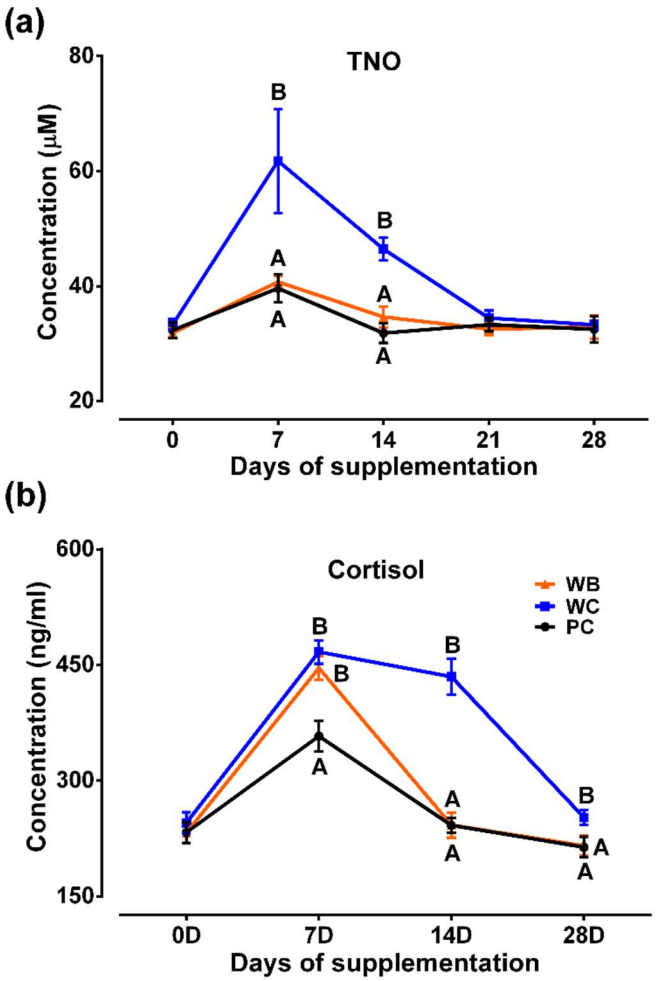

Figure 3. Results of probiotic supplementation on total nitric oxide (TNO) and cortisol concentration in weaned piglets. (a) Serum TNO level, (b) Serum cortisol level. Analyzed data were shown as M ± SD. ^{A,B,C}Values in a particular time point having different superscripts. WC: weaned negative control group, WB: weaned probiotic group and PC: positive control (ZnO) group.

3.4.3. Serum Heat Shock Proteins (HSPs)

Serum concentrations of four HSPs (HSP20, HSP70, HSP40 and HSP90) were evaluated (Figure 4) in this study. Concentrations of all four HSP isoforms in WC group were found up-regulated in all the time points as compared to those of WB and PC groups except for HSP20 and HSP70 on day 7 and HSP90 on day 28. HSP20 and HSP70 levels didn't vary significantly between WC and WB at day 7. Concentrations of all four HSPs between WB and PC groups were found insignificant in all the time points.

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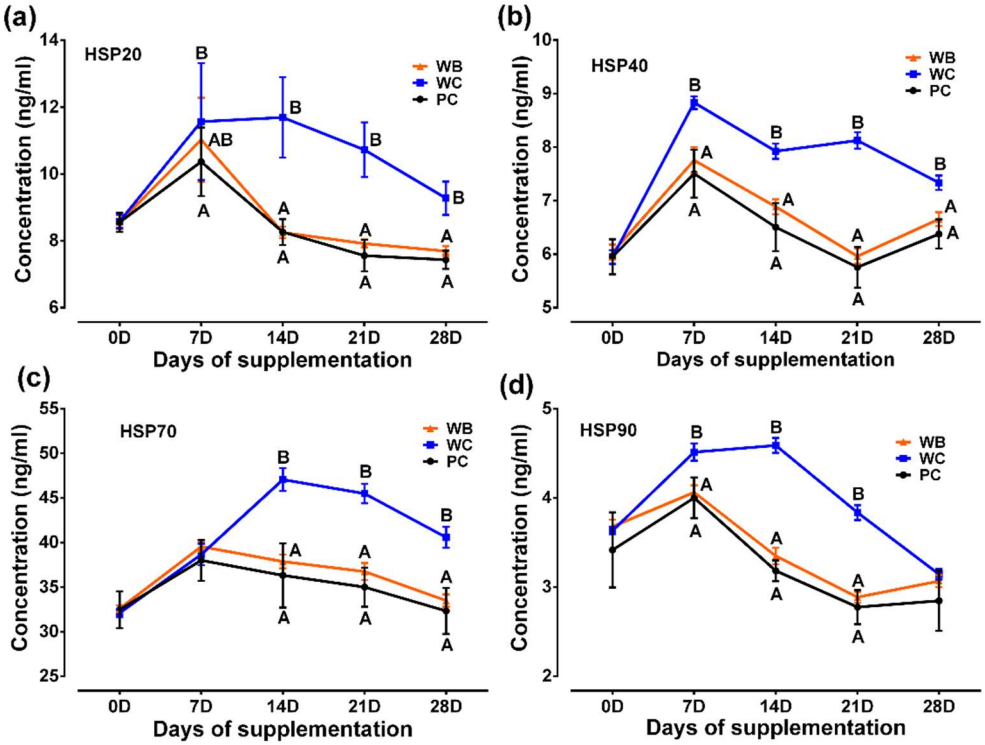

Figure 4. Results of probiotic supplementation on heat shock proteins (HSPs) in weaned piglets. (a) HSP20; (b) HSP40; (c) HSP70; (d) HSP90. Analyzed data were shown as M ± SD. ^{A,B,C}Values in a particular time point having different superscripts. WC: weaned negative control group, WB: weaned probiotic group and PC: positive control (ZnO) group.

3.5. Immune Parameters of Serum

WB group recorded significantly greater levels of IgM and IgG on day 14 and 28 and IgA on day 28 than that of the other two groups, whereas the values in the WC group were found significantly lower in all the time points for IgM and IgG and day 7 for IgA than those of other two groups (Figure 5a-c).

On day 14 and day 28, IL-1β, IL-2, IL-6, IFN-γ and IL-12 concentrations were lower in WB and PC group than the WC group, while it did not vary among WB and PC group. WC showed reduced serum IL-4 concentrations at day 7 and day 14 as compared to the other two groups. At day 28, in WB group showed significantly greater concentration of IL-4 than the other two groups (WC and PC) (Figure 5d-i).

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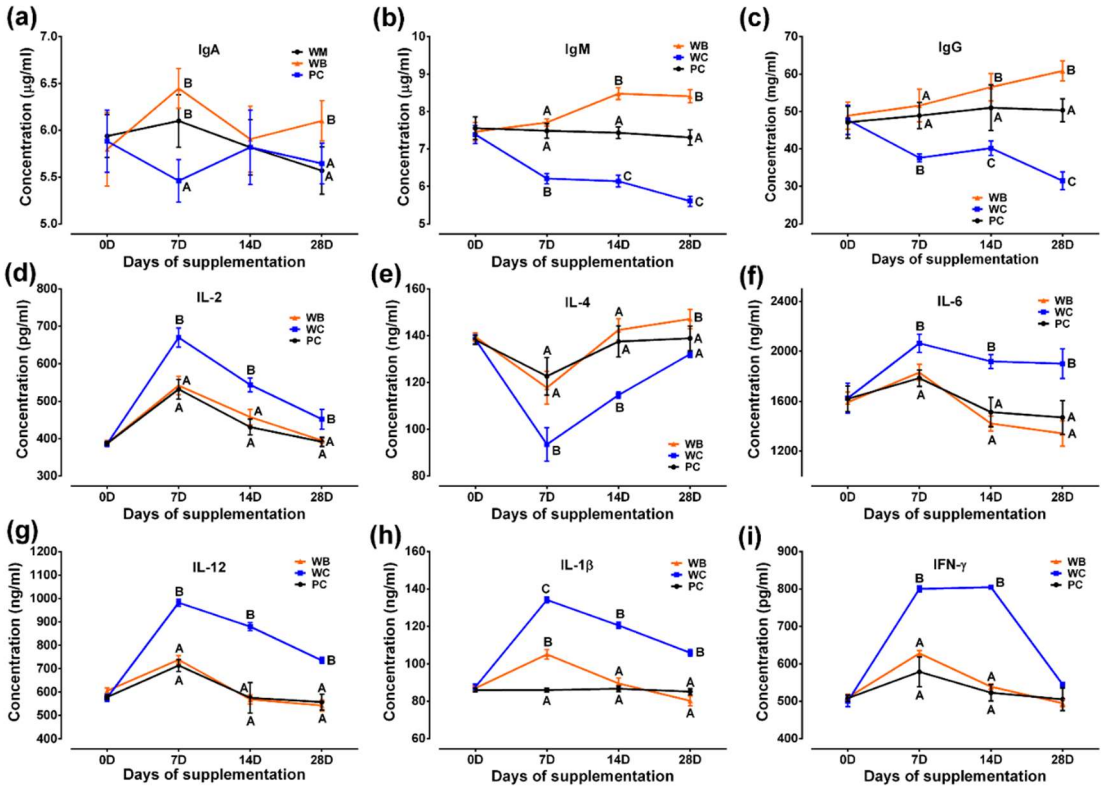

Figure 5. Results of probiotic supplementation on serum immunoglobulins and cytokines in weaned piglets. (a) IgA; (b) IgG; (c) IgM; (d) IL-2; (e) IL-4; (f) IL-6; (g) IL-12; (h) IL-1β; (i) IFN-γ. Analyzed data were shown as M ± SD. ^{A,B,C}Values in a particular time point having different superscripts. WC: weaned negative control group, WB: weaned probiotic group and PC: positive control (ZnO) group.

4. Discussion

Weaning is the most stressful event a piglet [3]. Weaned piglets must adapt to the stressful condition rapidly to increase their growth and performance [4]. Probiotics can help the weaning piglets adapt to this stressful condition by modulating the intestinal microbial population and stimulating the immune system of the host, which can then reduce diarrhea incidence, enhance gut health and growth performance [21,22,32]. A number of bacterial species have been used as probiotics. Among them lactic acid bacteria and butyric acid bacteria are the most commonly used in swine production [33]. In the present study the multi-strain probiotic compound containing *B. mesentericus* TO-A, *B. coagulans* SNZ1969, *C. butyricum* TO-A and *E. faecalis* T-110showed beneficial effect on growth parameters, lipid profile, antioxidant defense system and immune parameters in weaning piglets; thus, may be a good alternative to in-feed ZnO supplementation.

Body weight, ADFI, F:G and ADG are vital parameters of animal performance in pig industry. Here, the weaned piglets supplemented with probiotics (WB) demonstrated higher body weight, fortnightly ADG and overall ADG than those of the weaned negative control group suggesting beneficial effects of currently used multi-strain probiotic formulation. Moreover, as compared to the negative control group, the probiotic group had significantly lower diarrhea rate as well as F:G . Probiotics supplemented group and positive control group did not show any significant difference in growth and incidence of diarrhea. Similar results were reported by Cai et al. [34] who observed improved growth performance in weaned piglets supplemented with Bacillus based probiotics. Our findings were also supported by a former study in which weaned pigs received a complex probiotic formulation (*L. paracasei*, *E. faecium* and *B. subtilis)* had greater body weight, improved ADG and lower F:G than negative control [35]. Probiotics produce various enzymes, including arabinose, α-amylase, maltase, cellulase, levansucrase, dextranase, alkaline protease, β-glucanas, and neutral protease which enhance the nutrient digestibility in the gut [36,37]. However, the results of probiotic

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supplementation are inconsistent. Previous studies of Giang et al. [38] and Méndez-Palacios et al. [39] reported that the inclusion of Bacillus-based or Lactobacillus-based feed additives were failed to enhance the growth and production parameters of newly weaned piglets. The outcome of these studies may have been altered due to several other factors including diet composition, feed form and their interaction with probiotics, stains, probiotics doses, age of the pigs, surrounding environment and strategies of probiotics supplementation [40].

At weaning, piglets experience fasting due to abrupt change in feed; this stimulates fat mobilization from its reservoirs to support energy deficit [41]. High cholesterol concentration especially high concentration of LDL, and high level of TG in the bloodstream are closely related with atherosclerotic cardiovascular disease [42,43]. In the present study, current probiotics formulation improved the lipid profile of WB group which had significantly greater HDLc and lower TC and LDLc concentration as compared to the WC group. Our observations were in accordance with previous studies. Yu et al. [44] found that selenium in combination with complex probiotics (*L. acidophilus*, *L. pentose*, and *B. subtilis*) tended to reduce TC, VLDL, and TG and increase HDLc concentrations. Kim al. [45] found that cholesterol concentration was significantly decreased in the pigs fed with Lactobacillus-based probiotic. From this, it may be inferred that probiotic supplementation in weaning piglets improves the lipid profile by decreasing the TC and LDL concentration in serum and by increasing the HDLc concentration.

Weaning stress often induces oxidative stress [10,46]. In normal cellular metabolism, NO synthase (NOS) and NAD(P)H oxidase isoforms generate reactive oxygen species (ROS) and reactive nitrogen species (RNS) respectively [47]. At low/moderate concentrations, ROS and RNS are involved in a variety of physiological roles including cell signaling pathways and mitogenic response [48]. But when ROS and RNS concentration exceeds the normal cellular level, they cause potential damage to essential biomolecules (DNA, proteins, and lipids), starting a free radical chain reaction [49]. Therefore, oxidative stress and subsequent overproduction of ROS and RNS reduce immune response, increase susceptibility to pathogenic microorganisms, induce enterocyte apoptosis with cell cycle arrest in the gastrointestinal tract and eventually decrease production performance [10,50]. The current study observed that the serum TNO concentration between WB and PC did not vary throughout the experimental period, whereas the concentration was significantly increased on day 7 and 14 in WC than the other two groups. Furthermore, we determined MDA concentration to investigate whether weaning stress led to oxidative damage. Cell membranes or plasma membranes are rich in polyunsaturated fatty acids that make susceptible to free radical assault because of their multiple double bonds [51]. As a result of this oxidation of lipid molecules, MDA is produced, which interacts with biomolecules and exerts cytotoxic and genotoxic effects [52]. So, MDA is used as a biomarker of oxidative damage [53]. In this study, WB group recorded lower serum MDA concentration than WC group. These results suggest that the probiotics supplementation can reduce lipid peroxidation and oxidative damage. Similar findings were reported in previous reports [54,55].

Decrease in total NO and MDA concentration in probiotics supplemented piglets over un-supplemented piglets indicated improvement in the antioxidant system. The improved serum T-AOC offered additional support for an improvement in the antioxidant defense system for probiotic treated piglets. The antioxidant defense system in the body comprises of several antioxidant enzymes such as superoxide dismutase (SOD), glutathione peroxidase (GSH-Px), and catalase [56]. SOD converts superoxide radicals to less toxic H_2O_2 and then it is further processed to non-toxic water either by GSH-Px or catalase [57]. Probiotics can enhance the T-AOC by increasing the production of these antioxidant enzymes [58]. In our study, WB group showed elevated T-AOC, SOD, catalase and GSH levels on day 14 and 28 than WC group. Increased T-AOC and antioxidant enzyme levels in the probiotic supplemented group further supported the idea that currently used probiotic formulation may contribute to improve the antioxidant profiles of weaned piglets. Similar observations were reported earlier [54,55]. LAB can degrade free radicals by producing intracellular enzymatic (SOD and catalase) and non-enzymatic antioxidants (glutathione and thioredoxin) [59,60]. This explains how the current probiotics improved the antioxidant status of weaned piglets.

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Stresses associated with weaning especially oxidative stress enhance the production of heat shock proteins (HSPs) [61]. HSPs are highly conserved intracellular proteins that are conserved across the species, but cell death or tissue injury caused by physiological stress may increase their release into serum [62]. HSPs are involved in a variety of physiological functions including, protein synthesis and homeostasis, improve antioxidant defense system, protect gut epithelium from oxidative stress and inflammation and inhibit apoptotic pathways [63]. In our study, the piglets with probiotic supplementation (WB) showed significantly decreased serum levels of HSPs (HSP70, HSP40, HSP90 and HSP20) as compared to the un-supplemented group (WC). This indicates downregulating effect of probiotics supplementation on serum HSPs levels in weaning piglets. Weaning increases formation of free radicals which induce the production of HSPs [61,64]. So, the down regulating effect of probiotic on serum HSPs might be due to decreased formation of free radicals and increase in total antioxidant capacity. Similar results were also reported Gan et al. [65] in which selenium-enriched probiotic supplementation reduced mRNA expression of HSPs in heat stressed piglets.

Cortisol is an important biomarker of stress. Weaning is a stressful event for a piglet and most frequently cause a marked rise in cortisol level [66]. Cortisol is assumed to be produced by the hypothalamic-pituitary-adrenal (HPA) axis [67]. Under stress like weaning stress, HPA axis activates and increases the production of cortisol hormone which is a healthy adaptive response of body to cope up with the situation [68]. Cortisol, up to a certain level, is beneficial to the host, but at high level and chronic persistence may have deleterious effects on productivity [69]. In our study, serum cortisol concentration significantly decreased in probiotic group on day 14 and 28 as compared to the negative control group, whereas there was no difference with positive control group. These findings clearly indicate that weaning stress enhances the production of cortisol hormone, which can be down regulated by probiotic supplementation. These findings provide another evidence that dietary probiotic supplementation can alleviate the weaning stress. Our findings were in accordance with previous studies; Burdick Sanchez et al. [70] found that serum cortisol concentration was reduced in pigs fed with *Lactobacillus acidophilus* fermentation product. Similarly, Wang et al. [71] observed that dietary supplementation of *L. fermentum* I5007 in weaned piglets could decrease the diquat induced plasma cortisol level.

Immune system modulation is one of the crucial roles of probiotics. It has been reported that probiotics interact with gut microbiota, epithelial cells and immune cells which in turn stimulate the immune function and antibody production [72,73]. IgAs are the predominant isotype expressed in all the mucosal tissues and aids in mucosal immunity. The most prevalent antibody, IgG, is present in blood and extracellular fluid and plays important roles in systemic immune response. IgM is the main antibody produced at the initial stage of antibody mediated immune response and the major component of natural antibody [74]. In the present study, the probiotic group displayed higher levels of serum IgG and IgM at all the time points and higher IgA at day 28 as compared to the negative control group. When compared with the positive control group, higher serum levels of all the Igs were detected in the probiotic group at the end of the experiment. These findings were in accordance with previous studies. Dong et al. [75] demonstrated that the serum IgM and IgA levels were improved in weaned piglets treated with complex probiotic containing *L. plantarum* and *B. subtilis*. Dlamini et al. [76] also observed increased IgG levels in weaned piglets upon supplementation of a combined probiotics. Our results suggested that direct-fed complex probiotics enhanced mucosal as well as humoral immunity of the weaned piglets. These significant increases may be due to the persistence of current probiotic bacteria in the intestinal tract and acting as an immune adjuvant to the humoral immune system and therefore stimulating antibody production. We next determined the concentrations of serum pro-inflammatory cytokines including IL-2, IL-1β, IFN-γ, IL-6, and IL-12 and anti-inflammatory cytokine IL-4 to assess the effects of weaning stress on intestinal or systemic inflammatory response. Cytokines are small and cell signaling molecules which plays major roles in the immune and inflammatory responses and overall homeostasis of the body [77]. Excess production of cytokines especially pro-inflammatory cytokines has negative influence on immune response and gut integrity which ultimately reduce the growth performance [78,79]. There is an intricate balance between pro and anti-inflammatory cytokines; anti-inflammatory cytokines suppress the production

of pro-inflammatory cytokines and thus protect against intestinal inflammation and maintain the gut integrity [77]. Their harmony is therefore essential for the host immunological and inflammatory response. Abrupt changes in the dietary and environmental factors during weaning may lead to changes in the cytokine network and cause transient inflammation of the gut which may disrupt the barrier function [80]. In current study, as compared to un-supplemented group (WC), the concentrations of pro-inflammatory cytokines were lower in probiotics supplemented group. This was in agreement with a previous finding where Bacillus based compound probiotics (*C. butyricum*, *B. subtilis*, and *B. licheniformis*) modulated the inflammatory process in weaned piglets by decreasing serum pro-inflammatory cytokines (IL-1β, IL-6, and TNF-α) [81]. In case of IL-4, the anti-inflammatory cytokine, significantly increased levels on day 7, 14 and 28 in the probiotic group than the levels in negative control group were recorded. Similar results were published by Laskowska et al. [82] that showed the multi-microbial probiotic formulation "Bokashi" could raise the serum IL-4 level in pregnant sow. The experimental data of this study indicated that currently used multi-strain probiotic combination can reduce the transient inflammation and improve intestinal barrier function which eventually contribute to improved growth performance of weaned piglets.

5. Conclusions

It may be concluded that supplementation of the multi-strain *Bacillus*-based probiotic formulation containing *Bacillus mesentericus*, *Bacillus coagulans*, *Enterococcus faecalis* and *Clostridium butyricum* minimized the weaning stress, thereby improved feed intake, body weight, antioxidant activity, lipid profile, and systemic as well as mucosal immunity and overall growth performance of weaned piglets. Therefore, the multi-strain probiotic compound may be used to replace ZnO in weaned piglets.

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Institutional Review Board Statement: The animal study protocol was approved by the Institutional Ethics Committee of ICAR-Central Island Agricultural Research Institute (ICAR-CIARI), Port Blair, Andaman and Nicobar Islands, India (protocol code ICAR-CIARI/AS/23468 and date of approval 23.12.2021). Humane animal care was practiced throughout the study and every effort was made to minimize the suffering of the animals. All procedures were carried out in conformity with the relevant national regulations and guidelines.

Data Availability Statement: All data are available within the manuscript.

Conflicts of Interest: There is no competing interest amongst the authors. The study's design, data collection, analysis, or interpretation; the preparation of the manuscript; or the choice to publish the findings were all made independently of the funders.

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