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Effects of dietary calcium, phosphorus and microbial phytase on intestinal morphology in laying hens

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Abstract: Different challenges are being applied in the poultry industry in order to protect animal health and to increase immunity and production. The supplementation of microbial phytase is essential in terms of both reducing the inorganic phytase rate and contributing to the absorption of other minerals. In this study, a newly isolated microbial phytase was added at different concentrations to the diet together with calcium (Ca^{2+}) and available phosphorus (AP), and the effects of this supplementation on intestinal absorption capacity and Ca^{2+} binding capacity were investigated via morphological measurements and immunohistochemical examination of the duodenum and ileum. For this purpose, 90 Lohmann LSL-White laying hens were divided into three main diet groups: 1. Standard Ca^{2+} and AP (Ca+AP), 2. Standard Ca^{2+} and low AP (Ca+low AP), and 3. Low Ca^{2+} and low AP (low Ca+low AP). These three groups were further divided into three phytase subgroups each (without phytase [Phy-], commercial phytase [CP] and microbial phytase [MP]). At the end of the experiment, animals were euthanized, and duodenum and ileum samples were fixed and processed for histological examination. Villus height, crypt depth, total mucosa thickness, and villus width were measured and villus height: crypt depth ratio and villus absorption area were calculated. Caldesmon expression in the duodenum and ileum was also investigated immunohistochemically. The results indicated that villus height, total mucosa thickness, and villus absorption area increased ($p \leq 0.05$) in birds fed with Ca+AP+MP. Stronger caldesmon expression was observed in the MP treated groups. We concluded that MP produced from *Bacillus megaterium* EBD 9-1 bacterium increases the utilization of Ca^{2+} and AP and, thus, can have a beneficial role when these macrominerals are used insufficiently. Ca^{2+}, AP, and MP may have positive effects on the intestinal morphology and absorption area when used at optimum amounts.

Key words: Microbial phytase, calcium, available P, intestinal morphology, caldesmon, laying hens

1. Introduction

The demand for poultry commercial production is increasing every year, as these animals provide a high protein and energy source for humans [1]. In order to ensure efficient production, it is necessary to increase the ability of the animals' feed conversion ratio and the quantity and quality of animal products while protecting animal health. One of the methods used for this purpose is adding feed additives to poultry diets [2].

Phosphorus (P) is the essential macromineral source of the poultry diet and should be used to mediate main metabolic pathways and genomic and physiological processes [3,4]. Although the amount of P gained from plant-based raw materials is sufficient, most of the feeds contain P in the form of phytic acid. The ability of birds to utilize P in this form is very low due to insufficient intestinal phytase enzyme secretion [5]. In addition to problems with digestibility, inorganic P supplements are expensive and cause P pollution in soil and groundwater. Environmental and economic concerns resulted in decreased use of inorganic P and endorsed phytases as a more convenient alternative [6]. Phytate also binds with protein, starch, and other minerals, and inhibits their absorption in the digestive tract [7,8]. The addition of phytase to the diets of laying hens improves the availability of minerals such as Ca^{2+}, Mg, Zn, Fe, K, Cu, and also aminoacids and carbohydrates in addition to increased P utilization [9–12]. However, dietary nutrients, especially Ca^{2+} and P, influence the effectiveness of phytase [13]. Reduction in Ca:P ratio in poultry diets increases

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phytase activity, resulting in improved digestibility and performance [14]. Diet composition and especially the source of phytase in dietary content is particularly important for the efficiency of enzyme activity and affects the resistance of the gastrointestinal tract, the optimum pH value, P release, and the absorption of all other mineral and nutrient content [15].

The most abundant mineral in the body is calcium, and this mineral is absorbed across the intestinal mucosa via two pathways: (i) transcellular with active transport in Ca^{2+} channels, Ca^{2+} pump and intracellular proteins in the duodenum and upper jejunum, (ii) paracellular with passively through tight junction complexes [16,17,18]. Ca^{2+} binds rapidly and reversibly to the calmodulin-actin-myosin I complex. Ca^{2+} can then move to the basolateral region of the cell by microvesicular transport, or ionized Ca^{2+} can diffuse into this area of the cell. As the calmodulin complex becomes saturated with Ca^{2+}, the concentration gradient is not favourable for the movement of Ca^{2+} into the microvillus, which slows down Ca^{2+} absorption. Caldesmon is a calmodulin-binding protein, which is over-expressed when Ca^{2+} levels in absorptive epithelial intestinal cells in microvilli and paracellular area are increased [19,20].

Villus height, crypt depth, villus height:crypt depth (VH:CD) ratio, and villus absorption area are important parameters that provide information about the digestion and absorption potential in the intestines [21,22]. It has been shown that feed additives affect intestinal morphology mainly by the height of the villi and accordingly the VH:CD ratio [23]. Dietary content, particularly enzymes, is known to affect intestinal mucosa and histomorphology. In addition to increasing the nutritive value of feed, phytase addition also prevents villus atrophy and increases the intestinal absorption area and the thickness of the digestive system organs [24,25]. Chickens fed diets containing 500 U/kg microbial phytase had increased villus height and VH:CD ratio in duodenum, ileum, and jejunum [26]. The same amount of phytase has proven to affect intestinal morphology and increase the mucosal thickness in quails [27].

Several studies have been performed to investigate the effect of additional microbial and commercial phytase on intestinal morphology, but controversial results have been obtained. In the present study, we used phytase from *Bacillus megaterium* EBD 9-1, which is known to have high phytase production potential. The purpose of the study was to evaluate the duodenal and ileal histomorphology after phytase addition from two sources and to compare these data with caldesmon immunoreactivity in Ca^{2+}-dense regions in laying hen gut.

2. Materials and methods

2.1. Birds, housing and management

All experiments in this work were carried out after the consent of Bursa Uludağ University Animal Research Local Ethics Committee (approval number: 2021-07/05). A total of 90 Lohmann LSL-White laying hens (second term) were used. Birds were housed in three-tier California-type poultry cages, and nipple drinkers were used for providing fresh water continuously. The light was applied between 05:00 am and 09:00 pm (16 h lighting and 8 h darkness). Ventilation was done naturally and by means of fans. Animals were subjected to group feeding. The feeds were placed in trough feeders and animals were fed ad libitum. The nutrient analysis of the feed ingredients and mixed rations used in the preparation of the rations was determined by the method reported in AOAC [28], and the starch content of the rations was determined according to the method reported by Bal et al. [29]. The metabolizable energy levels of laying hen diets were calculated using the formula developed by the Turkish Standardization Institute. Basal ration composition is given in Table 1.

2.2. Treatments

The experiment included three diet groups and each diet group had three phytase subgroups. Groups were as follows: Group 1, standard levels of Ca^{2+} and standard levels of available phosphorus (Ca+AP); Group 2, standard levels of Ca^{2+} and low levels of AP (Ca+low AP); Group 3, low levels of Ca^{2+} and low levels of AP (low Ca+low AP). The subgroups were as without phytase (Phy-), commercial phytase (CP), and microbial phytase (MP).

The commercial phytase (6-phytase derived from *E. coli*) and microbial phytase (3-phytase derived from *Bacillus megaterium* EBD9-1, isolated from soil of Trabzon province in Turkey) were added to the rations at a dose of 300 FTU/kg. Each 300 phytase activity unit is equivalent to 1 gr of P supplied with dicalcium phosphate (DCP). For this reason, 300 units of phytase added to a kilogram of feed provides 0.10% available P in the ration. In addition, it has been reported that the phytase contribution of 300 FTU/kg to the ration equals is 1 g/kg matrix value for Ca^{2+} [30]. The calcium and AP levels in the Ca^{2+}+AP containing group were arranged to be 4.20% and 0.43%, respectively, whereas, in the low Ca+low AP diet group, these values were as 4.10% and 0.33%, respectively.

2.3. Histomorphology and immunohistochemistry

The birds were slaughtered, and duodenum and ileum samples were taken at the end of the experiment. Samples were fixed in 10% neutral buffered formalin, processed routinely, and were embedded in paraffin. Five μm thick sections were cut from paraffin blocks, mounted on slides, and dried overnight. After dewaxing and rehydration, sections were stained with Crossman's triple stain [31]. Villus height, crypt depth, total mucosa thickness, and

Table 1. Ingredients and chemical composition of the basal ration of standard calcium and available phosphorus (Ca+AP) group.

Ingredients	**Rate (%)**
Corn (7.5% CP)	56.57
Soybean meal (44% CP)	5.00
Full fat soybean	17.70
Corn gluten	5.00
Sunflower meal	3.00
Limestone	9.70
Dicalcium phosphate	1.95
Salt	0.25
Vitamin-mineral premix*	0.35
DL-methionine	0.10
L-lysine HCl	0.10
Sodium bicarbonate	0.10
Choline chloride	0.08
Antioxidant	0.10
Analysed concentration	
Metabolisable energy, kcal/kg	2793
Crude protein, %	17.30
Calcium, %	4.20
Available phosphorus, %	0.43
Sodium, %	0.17
Lysine, %	0.83
Methionine, %	0.41
Threonine, %	0.57

*: 3.5 kg premix contains 10,000,000 IU vitamin A; 2,500,000 IU vitamin D_3; 20,000 mg vitamin E; 3,000 mg vitamin K_3; 1,000 mg vitamin B_1; 4,000 mg vitamin B_2; 3,000 mg vitamin B_6; 25 mg vitamin B_{12}; 22,500 mg niacine; 10,000 mg calcium D-pantothenate; 500 mg folic acid; 50 mg biotin; 400,000 mg choline; 100,000 mg Mn; 25,000 mg Fe; 60,000 mg Zn; 5,000 mg Cu; 200 mg Co; 500 mg I; 200 mg Se.

villus width were measured. The villus height was measured from the villus tip to villus-crypt junction level randomly for five villi per section. Crypt depth was measured from the villus-crypt junction to the lower limit of the crypt for five corresponding crypts per section. Total mucosa thickness was measured from the top of the villus to the lower limit of the crypt. Villus width was measured at the midsection of the villi in the longitudinal section (Figure 1). Villus absorption area (2 π [average villus width/2] x villus height) was calculated as described previously [32].

Immunohistochemistry (IHC): streptavidin-biotin-peroxidase complex technique was carried out using the

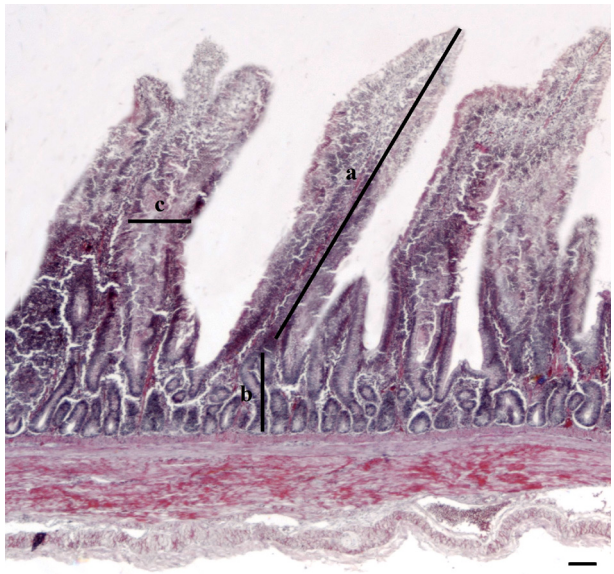

Figure 1. Measurements of the duodenum and ileum; villus height (a), crypt depth (b), total mucosa (a+b), villus width (c). Crossman's triple stain, bar = 100 µm.

ImmPRESS reagent kit (MP 7402; Vector Laboratories, Burlingame, CA, USA). Antigen retrieval was achieved by boiling sections in 1 M sodium citrate buffer (pH 6.1) in a microwave oven three times for 5 min. After cooling, slides were rinsed with phosphate-buffered saline (PBS), and endogenous peroxidase activity was blocked by incubation in 3% H_2O_2 in distilled water for 10 min at room temperature. After blocking with normal horse serum, 2.5%, for 20 minutes to prevent nonspecific antibody binding, sections were incubated with the primary mouse monoclonal antibody to caldesmon (MS-1169-R7, NeoMarkers, Fremont, CA, USA) overnight at 4 °C. Sections then were incubated with a secondary antibody for 30 min at room temperature. Finally, 3,3'-diaminobenzidine (DAB) was used for color development followed by counterstaining with hematoxylin. Sections processed in antibody diluent without primary antibody were included in each case as negative controls. The slides were scored according to the staining intensity as follows: 0: absent, 1: weak, 2: moderate, 3: strong staining. A blinded procedure was applied for scoring by two researchers. All micrographs were taken with Nikon 80i microscope (Nikon Corporation, Tokyo, Japan).

2.4. Statistical analysis

Data were evaluated with a generalized linear mixed model using PROC MIXED of SAS (SAS Institute Inc, 2009). Diet type Ca+AP, Ca+low AP, or low Ca+low AP), phytase source (Phy-, CP or MP), and their two-way interactions were fixed effects of the model. In the model, each sample was assigned as a random effect. Shapiro–Wilk test was

performed to determine the normal distribution of the data using PROC UNIVARIATE of SAS. If data did not show a normal distribution, log-transformation was conducted on the data before the analysis. Also, studentized residuals were determined with all fixed effects and interactions. The results with studentized residue <-4 or >4 were considered outliers and were removed from the model. Degrees of freedom were calculated using between-within approximation. The PDIFF order was used for multiple comparisons with a Tukey–Kramer adjustment due to the total experimental group number. All data are reported as least square means ± pooled SEM in both tables and graphs. The significance level was set at $p \leq 0.05$ for fixed effects, except for Tukey-adjusted p values in multiple comparisons.

3. Results

The effects of different Ca^{2+} and AP amounts and phytase use and/or phytase source on the morphometrical values of the mucosa in duodenum and ileum are presented in Tables 2 and 3, Figures 2 and 3.

In duodenum villus height, total mucosa thickness, villus width, VH:CD ratio, and villus absorption area parameters were significantly higher in animals fed with standard Ca^{2+} and standard AP than in animals fed with low amounts of either Ca^{2+} or AP ($p \leq 0.05$) (Figure 2). Addition of phytase increased villus height, total mucosa thickness, VH:CD ratio, and villus absorption area when compared with the Phy- feeding, and the effect of MP was significantly better regarding the villus height and total mucosa thickness ($p \leq 0.05$) (Figure 2). When the interaction between dietary application and phytase was evaluated highest villi, total mucosa thickness, and villus absorption area were achieved in the Ca+AP+MP fed animals ($p \leq 0.05$) (Table 2). Villus height, total mucosa thickness, VH:CD ratio, and villus absorption area decreased in Phy- groups as Ca^{2+} and AP levels decreased (Table 2). This decrease was partially counterbalanced with the addition of phytase, particularly the MP for villus height and VH:CD ratio.

In ileum villus height, total mucosa thickness, villus width, and villus absorption area were significantly better in Ca+low AP diet administered animals than in the two other diets ($p \leq 0.05$) (Figure 3). The addition of MP resulted in higher villus height, total mucosa thickness, and crypt depth than in the other two groups. VH:CD ratio and villus absorption area values were more optimal after phytase treatment, although this effect could not be substantiated statistically. No difference was observed between the two phytase sources (Table 3).

Caldesmon protein expression was detected in the cytoplasm and apical cell membrane of villus epithelial cells and crypts in duodenum (Figure 4, Table 4) and ileum (Figure 5, Table 5). In the duodenum, the severity of immunoreaction was stronger than in ileum. In the duodenum, the staining intensity was strong in the Ca+AP group, and as the Ca^{2+} and AP levels decreased, the staining intensity also decreased (Figure 4D and Figure 4G). Among all the diet groups, the most severe immunostaining was in MP treatment (Figure 4C, Figure 4F, and Figure 4I). While a slight caldesmon immunoreactivity was observed in the Phy- group where both Ca^{2+} and AP were low (Figure 4G), an increase in the immunostaining was observed after MP application (Figure 4I).

4. Discussion

In recent years, phytases have been widely used in broiler and laying hen diets with the aim of increasing the utilization of phosphorus [33]. However, phytase metabolism is related to the presence of Ca^{2+} and P in ideal dietary ratios. In this study, the effect of diets with different Ca^{2+} and P levels and phytase from two different sources on intestinal morphology and absorption capacity of laying hens was investigated via histomorphometric examination of the duodenum and ileum.

Intestinal morphological parameters were significantly influenced by varying ratios of Ca^{2+}, AP, and MP. Regarding the Ca^{2+} and AP levels, villus height and total mucosa thickness were at the highest values when standard levels of Ca^{2+} and AP were applied. Morphometric values were negatively affected when Ca^{2+} or AP was provided at suboptimal levels. Villus height and VH:CD ratio are associated with the digestive and absorptive capacity because of the increased intestinal surface area and the number of lactobacilli in the intestinal lumen [26]. In previous studies, phytase was added to the poultry diets at different ratios, resulting in different growth parameters, Ca-P digestibility, and intestinal absorptive capacity [26,34,35]. It has been shown that phytase should be limited to 250–300 FTU/kg doses, which is equivalent to 0.8 g of P in laying hens [36]. In the study of Nourmuhammadi et al. in which 1,000 U/kg of dietary microbial phytase was added to broiler diets, although there was an increase in crypt depth in the duodenum, VH:CD ratio and total mucosa thickness decreased in duodenum, jejunum, and ileum [26]. In another study, male broilers were fed with a P deficient diet which resulted in shorter VH, higher CD, and lower VH:CD ratio; the intestinal parameters were restored after the birds were fed with 500 FTU/kg phytase [37]. In contrast to our study, Catalan et al. observed no effect on villus height, crypt depth, and VH:CD ratio in duodenum, jejunum, and ileum after addition of phytate phosphorus and also phytase at a concentration of 500 FTU/kg [38]. In our study, we observed that 300 FTU/kg MP application was more effective than CP in duodenum and ileum as evidenced by increased villus height, total

Table 2. Morphometric measurements of duodenum after Ca^{2+} and AP variable diet and phytase application. Values are expressed as mean.

Variables	Duodenum												
	Ca+AP			Ca+low AP			low Ca+low AP				P-values		
	Phy-	CP	MP	Phy-	CP	MP	Phy-	CP	MP	SEM	Diet	Phytase	DxP
Villus height (µm)	1690.60^{bc}	1821.70^{b}	2628.0^{a}	1213.0^{ef}	1488.50^{cd}	1313.20^{de}	943.80^{g}	1092.40^{fg}	1242.30^{ef}	43.08	<0.0001	<0.0001	<0.0001
Crypt depth (µm)	312.90^{b}	383.70^{ab}	405.50^{a}	341.50^{ab}	313.10^{b}	354.90^{ab}	351.50^{b}	336.70^{ab}	332.10^{ab}	18.11	0.14	0.14	0.01
Total mucosa (µm)	2015.10^{c}	2661.80^{b}	3147.90^{a}	1690.90^{d}	1905.40^{cd}	1958.10^{c}	1370.10^{e}	1490.0^{e}	1839.60^{cd}	47.05	<0.0001	<0.0001	<0.0001
Villus width (µm)	239.50^{abc}	307.66^{a}	285.85^{ab}	278.27^{ab}	227.05^{bc}	221.40^{bc}	191.15^{c}	194.41^{c}	184.26^{c}	16.93	<0.0001	0.85	0.01
Villus height:crypt depth	5.51^{ab}	4.97^{bc}	6.66^{a}	3.65^{d}	4.80^{bc}	3.73^{d}	2.75^{e}	3.26^{de}	3.81^{cd}	0.26	<0.0001	0.001	<0.0001
Villus absorption area (mm^2)	1.275^{cd}	1.768^{b}	2.360^{a}	1.065^{de}	1.074^{cde}	0.911^{def}	0.566^{f}	0.670^{ef}	0.724^{ef}	0.10	<0.0001	0.0001	<0.0001

Ca: Calcium, AP: Available phosphorus, Phy: Phytase, CP: Commercial phytase, MP: Microbial phytase, SEM: standard error of mean; DxP: diet-phytase interaction.
$^{a–g}$: Different letters on the same row indicate statistical difference among the groups.

Table 3. Morphometric measurements of ileum after Ca^{2+} and AP variable diet and phytase application. Values are expressed as mean.

Variables	Ileum												
	Ca+AP			Ca+low AP			low Ca+low AP				P-values		
	Phy-	CP	MP	Phy-	CP	MP	Phy-	CP	MP	SEM	Diet	Phytase	DxP
Villus height (µm)	746.45	880.78	1044.62	916.99	1000.05	1165.88	808.78	823.68	940.58	41.46	<0.0001	<0.0001	0.30
Crypt depth (µm)	280.10	307.20	334.20	332.10	291.10	348.50	290.70	296.30	320.00	16.10	0.27	0.01	0.33
Total mucosa (µm)	965.20	1079.40	1206.90	1120.90	1230.70	1388.00	1035.00	1050.90	1197.20	41.32	<0.0001	<0.0001	0.65
Villus width (µm)	144.12^{c}	210.85^{ab}	144.12^{c}	169.09^{abc}	226.66^{a}	177.81^{ab}	179.28^{abc}	141.04^{c}	151.40^{bc}	11.68	0.0003	0.03	<0.0001
Villus height:crypt depth	2.72	2.93	3.23	2.84	3.47	3.46	2.85	2.58	2.97	0.20	0.07	0.05	0.53
Villus area (mm^2)	0.335^{c}	0.592^{ab}	0.481^{bc}	0.490^{b}	0.714^{a}	0.654^{a}	0.453^{bc}	0.366^{c}	0.443^{bc}	0.043	<0.0001	0.002	0.0003

Ca: Calcium, AP: Available phosphorus, Phy: Phytase, CP: Commercial phytase, MP: Microbial phytase, SEM: standard error of mean; DxP: diet-phytase interaction.
$^{a–c}$: Different letters on the same row indicate statistical differences among the groups.

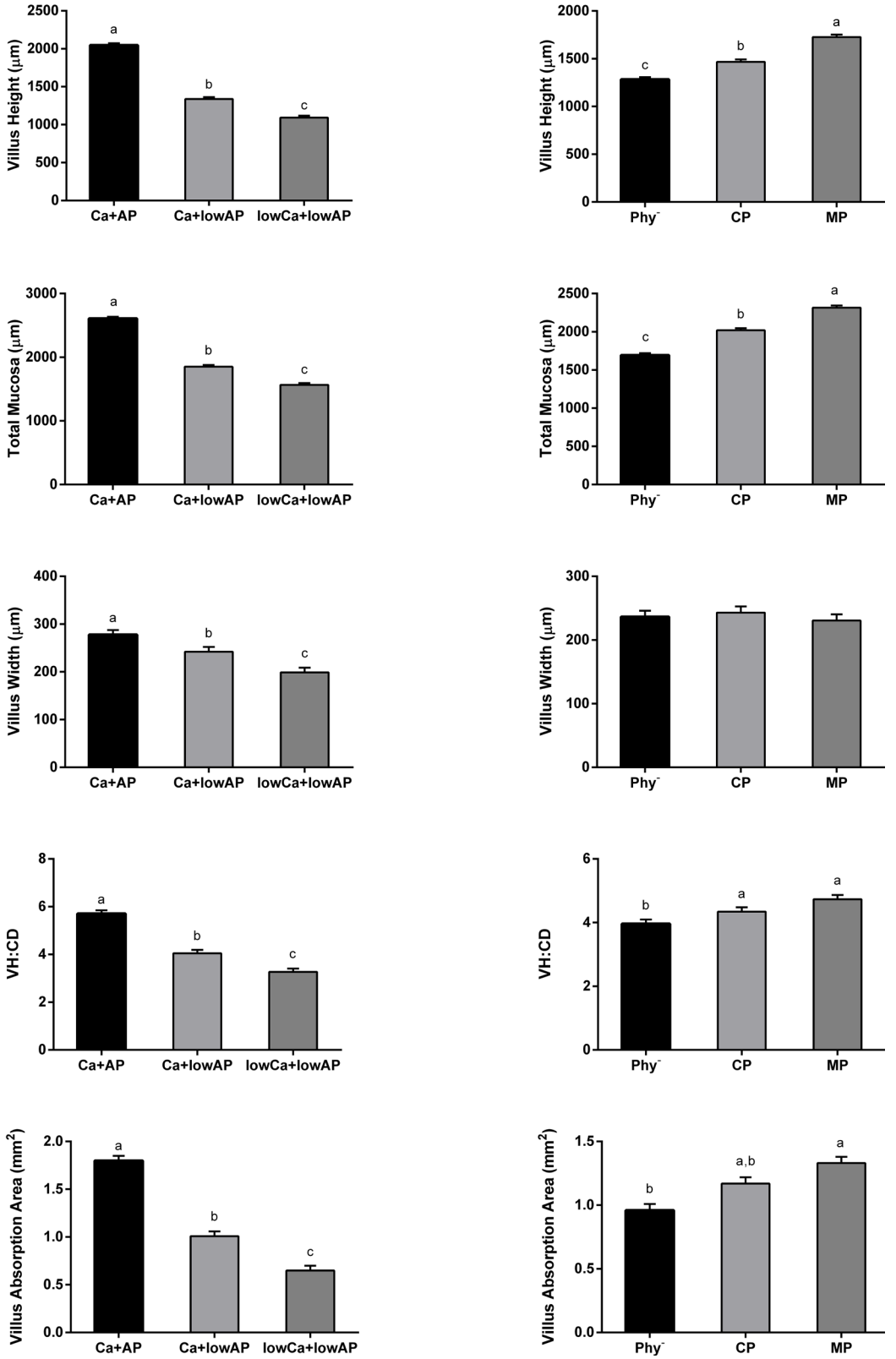

Figure 2. Morphometric results after diet and phytase treatment in duodenum ($p \leq 0.05$). VH:CD: Villus height: crypt depth, Ca: Calcium, AP: Available phosphorus, Phy: Phytase, CP: Commercial phytase, MP: Microbial phytase. [a–c]: Different letters on the same graph indicate significant difference among the groups.

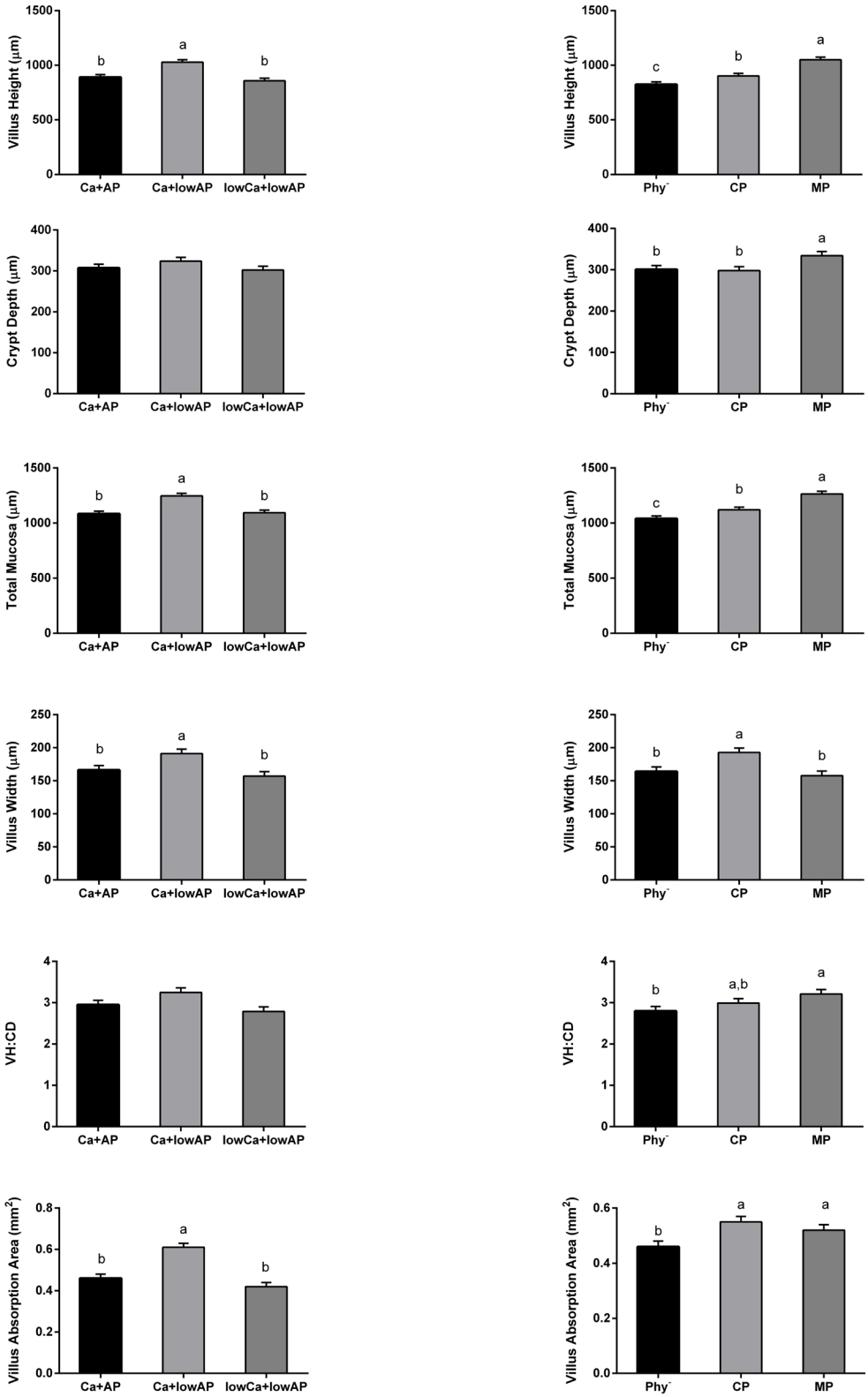

Figure 3. Morphometric results after diet and phytase treatment in ileum ($p \leq 0.05$). VH:CD: Villus height:crypt depth, Ca: Calcium, AP: Available phosphorus, Phy: Phytase, CP: Commercial phytase, MP: Microbial phytase. [a–c]: Different letters on the same graph indicate significant differences among the groups.

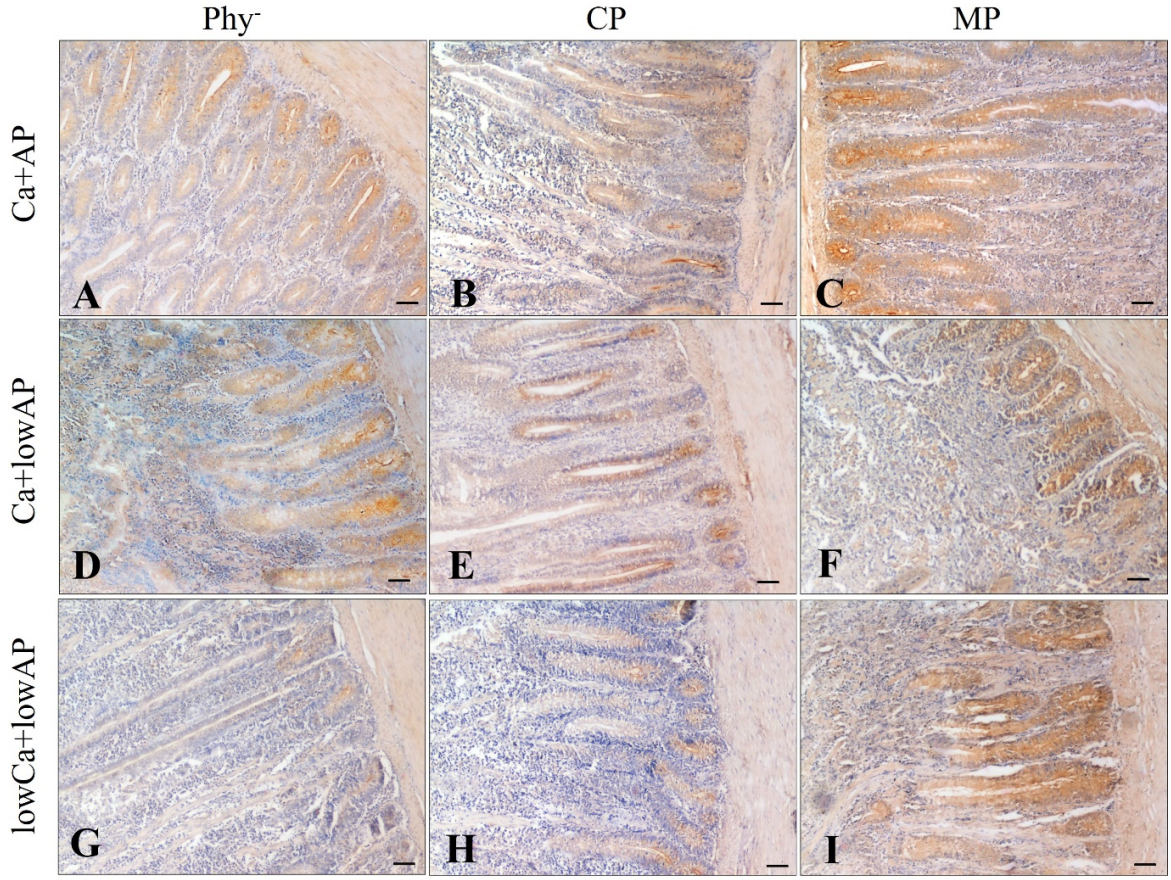

Figure 4. Caldesmon positive enterocytes and crypt cells in duodenum, streptavidin-biotin-peroxidase staining, DAB chromogen, bar = 100 µm. Ca: Calcium, AP: Available phosphorus, Phy: Phytase, CP: Commercial phytase, MP: Microbial phytase.

Table 4. Score of Caldesmon expression in duodenum after Ca^{2+} and AP variable diet and phytase application.

	Phy-	**CP**	**MP**
Ca+AP	$1.00 \pm 0.32^{c,B}$	1.60 ± 0.24^{b}	2.80 ± 0.20^{a}
Ca+lowAP	1.40 ± 0.24^{A}	2.00 ± 0.32	2.40 ± 0.40
lowCa+lowAP	$0.20 \pm 0.20^{c,BC}$	0.60 ± 0.24^{b}	2.60 ± 0.24^{a}

Data are means ± SEM. Ca: Calcium, AP: Available phosphorus, Phy: Phytase, CP: Commercial phytase, MP: Microbial phytase.
$^{a-c}$: Different letters on the same row indicate statistical difference among the groups, $p \leq 0.05$.
$^{A-C}$: Different capital letters on the same column indicate statistical difference among the groups, $p \leq 0.05$.

mucosa thickness, VH:CD ratio, and villus absorption area.

There is also an interaction between phytase efficiency and Ca^{2+} concentration. It has been shown that in broilers treated with 1,500 FTU/kg phytase, P digestibility was increased when Ca^{2+}, P, and phytase were added to diets [39]. However, very high Ca^{2+} in the diet raises the pH value of the digestive content and negatively affects phytase utilization and absorption of other minerals [40]. Amerah et al. reported that P digestibility was better at low Ca:AP ratio in the diet, and when this ratio rises above 2.14, phytase can hydrolyse the phytate in proximal intestine [33]. In addition, it has been reported that the amount of P can be reduced in diets consisting appropriate Ca^{2+} content, but there may be problems especially in bone mineralization in diets where both Ca^{2+} and P are reduced [41]. These results are in agreement with our study. In the current study, the difference between all groups in the interaction of diet-phytase showed that there was a synergistic effect between Ca^{2+}, AP, and MP in the duodenum. When the phytase-free groups were examined, the highest villi heights were measured in the Ca+AP group, whereas lower heights were observed in the Ca+lowAP and low Ca+low AP groups. In the interaction of MP with Ca^{2+} and AP, the highest villus height was measured in the Ca+AP+MP group, while the height decreased significantly when the amount of AP was decreased. Presence of MP in the low Ca+low AP group

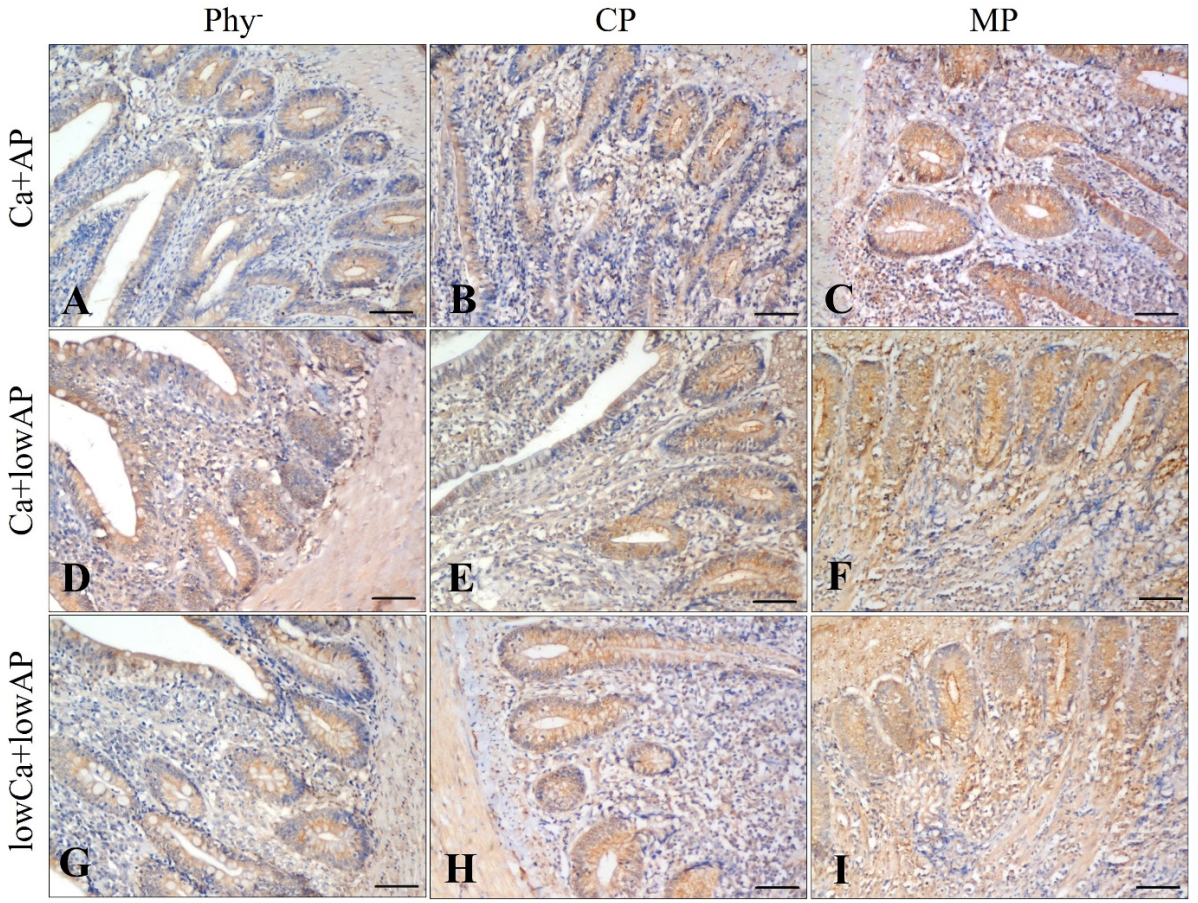

Figure 5. Caldesmon positive enterocytes and crypt cells in ileum, Streptavidin-biotin-peroxidase staining, DAB chromogen, bar = 100 µm. Ca: Calcium, AP: Available phosphorus, Phy: Phytase, CP: Commercial phytase, MP: Microbial phytase.

Table 5. Score of caldesmon expression in ileum after Ca^{2+} and AP variable diet and phytase application.

	Phy-	**CP**	**MP**
Ca+AP	0.60 ± 0.24^{bc}	1.40 ± 0.24^{b}	$2.60 \pm 0,24^{a}$
Ca+lowAP	0.80 ± 0.20^{c}	1.60 ± 0.24^{b}	2.00 ± 0.32^{ab}
lowCa+lowAP	0.40 ± 0.24^{b}	1.40 ± 0.24^{ab}	2.00 ± 0.32^{a}

Data are means ± SEM. Ca: Calcium, AP: Available phosphorus, Phy: Phytase, CP: Commercial phytase, MP: Microbial phytase.
$^{a-c}$: Different letters on the same row indicate statistical difference among the groups, $p \leq 0.05$.

slowed this downward trend. Therefore, while sufficient Ca^{2+} and AP levels are an important factor in the intestinal environment, it is evident that MP increases the utilization of Ca^{2+} and AP, and acts as a compensator in cases where Ca^{2+} and AP are provided insufficiently.

In the ileum, the diet and phytase interaction had an effect on villus width and villus absorption area. The fact that other parameters are not affected confirms that phytase, $Ca^{2+,}$ and P absorption occur mostly in the proximal intestines, as stated in previous studies.

Caldesmon expression also paralleled the morphological results. Severe caldesmon expression was observed in all three diet groups receiving MP, indicating that phytase contributed significantly to the absorption of Ca^{2+}. In addition, especially in the low Ca+low AP group, the expression severity was quite weak in the Phy- and CP groups. The increase in caldesmon expression suggests that absorption of Ca^{2+} increases after MP application, even when calcium is available in the intestinal milieu at low levels.

As a conclusion, we observed that phytase application was effective on the histomorphology of the duodenum and ileum and that the application of newly isolated MP favourably affected the villus height, crypt depth, total mucosa, VH:CD ratio, villi width, and villus surface area. Providing standard levels of Ca^{2+} and AP increased the efficiency of the added MP and, thus, improved the intestinal mucosa morphology and absorptive capacity.

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