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Effect of Primalac® or Enramycin supplementation on performance, intestinal morphology and microbiology of broilers under C*lostridium perfringens* challenge

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Abstract

One performance trial was conducted to determine the effects of supplementing feed or drinking water with a *Lactobacillus*-based probiotic or feed antibiotic on growth parameters, gut health and control pathogen colonization of broilers under C*lostridium perfringens* challenge as compared to unchallenged, unsupplemented diet. One hundred and fifty, 0-d-old male Ross 308 broilers were allocated in 5 experimental treatments for 30 d. The experimental treatments received a corn-soybean basal diet and were as follows: positive control (+CONT) with no additions; negative control (-CONT); antibiotic (ENRA) with addition of Enramycin; Primalac in feed (PF) and Primalac in water (PW). Chicks in treatments 2 to 5 were challenged with *C. perfringens.* Five birds were sampled at 16 and 30 d of age for gross and histologic examination of the duodenum, jejunum and ileum. Overall, feed conversion ratio (FCR) in treatment PF was significantly better ($P < 0.05$) than the +CONT and -CONT treatments, whereas treatments PRIM and PW were intermediate and not different from PF. Feed intake (FI) and body weight gain (BWG) were not influenced by treatment. Birds which had received PF had longer and wider duodenal and jejunal villi than did +CONT, -CONT or ENRA. The changes in *C. perfringens* count appear in parallel to observed changes in villi height and width in the small intestine. The performance of the birds which had consumed PF was better than the birds which consumed the antibiotic treatment (ENRA). The results from this study indicated that Primalac not only helped in the maintenance of beneficial bacteria but also could act as a replacement for antimicrobial growth promoters in broilers.

Key words: *Lactobacillus*-based probiotic, antibiotic, broiler, C*lostridium perfringens* performance, gut health, intestinal mucosa morphometrics.

Introduction

The use of antibiotic in poultry nutrition as antimicrobial growth promoters (AGPs) has been beneficial for the improvement of growth performance and prevention of diseases [1]. However, there are increasing concerns about the risk of developing cross-resistance and multiple antibiotic resistances in pathogenic bacteria in both humans and poultry linked to the therapeutic and sub therapeutic use of antibiotics in livestock [2, 3]. Current trends in poultry production point to reduced use of AGPs and increased use of non-antibiotic feed additives such as direct-fed microbials (DFM)[4].

The intestinal ecosystem contains a very diverse community of microbial cells that influence the host in various ways and some intestinal microflora has beneficial effects on the host. Therefore, it should be possible to shift the microbial community from pathogenic to beneficial bacteria by changing the dietary composition of ingredients [5]. Many researchers are now focused on identifying viable alternatives to antibiotics that offer similar benefits, such as to improve the growth of broilers, improve the utilization of feed and in this way realize better production and financial results. Several groups of these additives are in use such as probiotics, prebiotics, acidifiers, antioxidants and phytogene additives.

Probiotics effect on production can be expressed in reduction of diseases [4, 6], improving the immune system [7, 8] and exhibit influence on morpho-functional characteristics of intestines [9]. These effects lead to better growth of broilers [10, 11], improvement of FCR [7, 8, 11] and improved livability [12]. Probiotics display several ways of action: competitive exclusion which represents competition for locations to adhere to the intestinal mucous membranes, prevention of pathogenic microorganisms from inhabiting the intestinal tract and competition for nutritious substances [6, 13]. Extensive literature reviews have concluded that the benefit of probiotics ranged from no benefits at all to 8% maximum [14]. The inconsistency in the results were explained by the variety of bacterial strains contained in the probiotic preparation, level of inclusion rate in the feed, composition of feed and quality of chickens and environmental conditions [10, 13].

The objective of this study was to determine the efficacy of supplementing Enramycin (ENRA) or DFM, Primalac® on growth performance, histomorphology and bacterial count of small intestinal mucosa in broilers raised in cages under subclinical C. *perfringens* model and to determine the product with the most return and pathogen colonization control.

Materials and Methods

***Animals, husbandry and treatments*:** Ross 308 chicks were obtained from a commercial hatchery [15]. Chicks were sexed, grouped by weight in such a way as to reduce variation in mean body weight, a total of 150, 0-d old male broiler chicks allotted to 25 cages (50 cm length, 60 cm width and 36 cm depth) in a four-deck cage system and received the experimental diets in electrically

heated battery brooders with raised wire floors. The chicks had been vaccinated for Marek's disease, Newcastle and infectious bronchitis. Birds were maintained at 24 h light schedule.

The DFM used in this experiment, Primalac, is commercially available and contains primarily *Lactobacillus acidophilus, Lactobacillus casei, Bifidobacterium thermophilum* and *Enterococcus faecium* [16]. Birds received one of the following five treatments: T1 = Control diet (+CONT); T2 = T1 + *C. perfringens* challenge (-CONT); T3 = T2 + Enramycin 0.1 g/kg of feed (ENRA); T4 = T2 + Primalac administered at 0.9 g/kg feed (starter) and 0.6 g/kg feed (finisher) (PF) and T5 = T2 + Primalac administered in water at 0.12 g/kg water (starter) and 0.06 g/kg water (finisher) (PW). On d 16, birds in treatments 2 to 5 were challenged by *C. perfringens* using overdose of anticoccidial vaccine namely Paracox-8 (10-fold dose) on day 16, on days 18 and 20 chicks were gavaged with 1 ml of a cocktail containing *C. perfringens* inoculations (4 x 10^8 CFU) [17]. Culture of *C. perfringens* was obtained commercially [18] and propagated under anaerobic conditions for 16 hours at 37°C in screw cap tubes, and cells were harvested by centrifugation (4500 g at 4°C) and diluted in physiological saline. A typical isocaloric and isonitrogenous starter (0-16 d) and finisher (17-30 d) diets based on corn-SBM diets were formulated in mashed form according to Table 1 which met or exceeded the recommendations in commercial practice in Saudi Arabia. Ambient temperature and relative humidity were concurrently and continuously recorded at 3 hours interval using two data loggers [19] which were placed inside the chamber. The average temperature and relative humidity for the whole period were 24.95±0.26°C (SD) and 26.63±3.30% (SD), respectively. The study was conducted in Nov and Dec, 2011 under a protocol approved by King Saud University and complies with the current laws of Saudi Arabia.

Table 1. Dietary composition of broiler chick starter and finisher diets.

Ingredients, %	Starter			Finisher		
	Treatment					
	1,2 &5	3*	4*	1,2 & 5	3*	4*
Yellow corn	56.00	55.99	55.91	57.75	57.74	57.96
Soybean meal	36.10	36.10	36.10	34.0	34.0	34.0
Palm oil	3.80	3.80	3.80	4.80	4.80	4.80
Dicalcium phosphate	2.30	2.30	2.3	2.0	2.0	2.0
Ground limestone	0.72	0.72	0.72	0.64	0.64	0.64
Choline chloride	0.10	0.10	0.10	0.05	0.05	0.05
DL-methionine	0.23	0.23	0.23	0.16	0.16	0.16
L-lysine	0.15	0.15	0.15	-	-	-
Salt	0.30	0.30	0.30	0.30	0.30	0.30
Vitamin premix[1]	0.25	0.25	0.25	0.25	0.25	0.25
Trace mineral mix[2]	0.05	0.05	0.05	0.05	0.05	0.05
Enramycin	-	0.01	-	0.00	0.01	0.00
Primalac FG	-	-	0.09	-	-	0.06
Total	*100*	*100*	*100*	*100*	*100*	*100*
Calculated analysis						
ME, kcal/kg	3000	3000	3000	3100	3100	3100
Crude protein, %	22.0	22.0	22.0	21.0	21.0	21.0
Non phytate P, %	0.45	0.45	0.45	0.40	0.40	0.40
Calcium, %	1.0	1.0	1.0	0.9	0.9	0.9
Lysine, %	1.25	1.25	1.25	1.1	1.1	1.1
Methionine, %	0.55	0.55	0.55	0.47	0.47	0.47

*Diet 3 had 0.01% Enramycin, diet 4 had 0.09% (starter period) and (0.06% finisher period) Primalac on the expense of corn. [1]Vitamin mix supplied per kg of diet: retinyl acetate, 3.41 mg; cholecalciferol, 0.07 mg; DL-α-tocopheryl acetate, 27.5 mg; menadione sodium bisulphate, 6 mg; riboflavin, 7.7 mg; niacin, 44 mg; panthotenic acid, cyanocobalamin, 0.02; choline 496 mg; folic acid, 1.32 mg; pyridoxine HCl, 4.82 mg; thiamine mononitrate, 2.16 mg; D-biotin, 0.11 mg. [2]Mineral-mix supplied per kg of diet: manganese, 67 mg; zinc, 54 mg; copper, 2 mg; iodine, 0.5 mg; iron, 75 mg; and selenium, 0.2 mg.

Carcass measurements: At d 30, five birds per treatment were selected, after euthanasia, feather, heads, necks, and shanks were removed, and the remaining carcasses were dissected to breast and leg quarter and weighed. The percentage of yield of each part was calculated on the basis of dressed weight.

Histopathology and morphometric measurements: At 16 and 30 d of age, the entire GI tract from five birds per treatment was removed aseptically, small intestine weighed and the total length was measured and separated into duodenum, jejunum (proximal to Meckel's diverticulum) and ileum (proximal to ileo-caecal junction) and for each part measurements of length and weight were taken. From each portion of the small intestine 2-cm-long samples were collected for histology measurements. Samples were fixed in phosphate-buffered formalin for at least 48 h, after which they were embedded in paraffin. Sections of 5 mm were cut and stained with haematoxylin and eosin. Measurements of height and width were based on at least 5 well-oriented villi per section per broiler using an IX71 Inverted Olympus Microscope (Eyepiece WH10X, Objective lens 4X) and a PC-based image analysis system [20] with software analysis [21].

Enumeration and identification of bacterial cells: Ileal digesta contents were aseptically emptied in a new sterile bag and kept in ice until time of analyses. Samples were diluted in 0.9% saline, and 0.1 ml of each sample was plated on duplicates by using selective agar media for enumeration of target bacterial groups. *C. perfringens* was counted on tryptose sulfite-cycloserine (TSC) agar for *C. perfringens* (Oxoid CM587 with the addition of SR88 and SR47). Colonies on TSC agar that were suspected to be *C. perfringens* were plated secondarily on blood agar [22]. Enterobacteriaceae were isolated on MacConkey agar (Oxoid CM7) after incubation time of 24 h in an aerobic atmosphere at 37° [22]. Isolates of Enterobacteriaceae and *Salmonella* were identified by API 20E. The API 20E strips (BioMerieux Vitek) were inoculated, incubated at 37°C for 18 to 48 h, and interpreted as recommended by the manufacturer. Results were expressed as $\log_{10}$ colony-forming units per ileal digesta ($\log_{10}$ CFU/ g).

Statistical analysis: All statistical analysis was performed using the Statistical Analysis System [23]. A cage constituted the experimental unit. Three treatments were replicated 5 times in a randomized complete block design. Means for measurements showing significant differences in the analysis of variance were tested using the PDIFF option. Mean ± standard error of the mean (SEM) was presented in the tables and differences were considered statistically significant at $P < 0.05$.

Results

Table 2 shows the effect of treatment on broiler performance. During the starter period, BWG, FI and FCR were not influenced ($P > 0.05$) by treatment. During the finisher period, no significant differences in BWG or FI were observed, however, FCR was affected by dietary treatment ($P < 0.05$); birds which had received ENRA, PF or PW had better FCR as compared to +CONT or -CONT. Cumulative FCR was affected ($P < 0.05$) by treatment. Birds which had received PF had the best FCR among all groups with no significant differences from ENRA or PW. Primalac in feed resulted in 11 and 14 points improvement in FCR as compared +CONT and -CONT,

Table 2. Body weight gain (BWG), feed intake (FI) and feed conversion ratio (FCR) of broiler chickens given experimental diets at different ages.

	Treatment					SEM	p*
	T1	T2	T3	T4	T5		
Performance at 16 d							
BWG (g)	479.2	481.2	515.2	507.2	483.8	±12.4	NS
FI (g)	638.0	645.6	652.6	647.2	658.4	±19.0	NS
FCR(g: g)	1.333	1.342	1.267	1.276	1.361	±0.03	NS
Performance at 30 d							
BWG (g)	886.3	832.1	869.5	861.1	818.3	±26.9	NS
Feed (g)	1489.6	1468.9	1432.5	1362.4	1354.0	±39.0	0.07
FCR (g: g)	1.718^{a}	1.770^{a}	1.656^{ab}	1.581^{b}	1.658^{ab}	±0.04	0.04
Cumulative							
BWG (g)	1345.3	1313.3	1384.6	1368.2	1302.1	±32.7	NS
Feed (g)	2127.4	2114.5	2084.8	2009.1	2012.4	±37.9	NS
FCR(g: g)	1.583^{ab}	1.611^{a}	1.511^{bc}	1.468^{c}	1.547^{abc}	±0.03	0.02
Liv[1] (%)	100.0	92.0	92.0	100.0	84.0	±6.69	NS

[1]Liv = Livability ^{abc}means in the row with different superscripts differ significantly (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, N.S: Not significant).

respectively ($P < 0.05$). On the other hand, birds which had received the -CONT treatment produced the poorest FCR. No differences in cumulative BWG or FI due to treatment were observed.

The mean percentage of carcass parts in different treatments is documented in Table 3. Treatment had a significant effect on dressing percentage and relative liver weight ($P < 0.05$). Dressing percentage and relative liver weight were lower for birds which had received -CONT treatment. No difference in breast muscle yield, leg quarter yield or abdominal fat was noticed between treatments ($P < 0.05$).

The morphometric measurements of the intestinal epithelium samples at 16 d of age are given in Table 4. Duodenal, jejunal and ileal length showed significant differences due to treatments ($P < 0.01, 0.001, 0.001$, respectively). Duodenum and jejunum from birds which had received +CONT were longest among all treatments while the ileum from the same group was shortest. Intestine weight and relative weight were not affected by any treatment ($P > 0.05$). Ileal villus height was significantly longer for birds which had received +CONT, PF or PW as compared to -CONT or ENTRA. On the other hand, ileal villi were wider in the PF group ($P < 0.05$). The morphometric measurements at 30 d of age are given in Table 5.Shorter small intestine was obtained from birds which had received +CONT as compared to all other treatments ($P < 0.05$). On the other hand, birds which had received PF had longer and wider villi in the duodenum and jejunum than did the other groups ($P < 0.05$). Ileal villus height and width were similar among all treatments ($P > 0.05$).

Data related to ileal bacterial count in broilers at 16 and 30 d of age are presented in Table 6. Similar bacterial count of *C. perfringens* and Gram negative bacilli were found in the starter period (before the challenge). At the end of the experiment, the ileal *C. perfringens* count in the PF birds tended to be the lowest among all groups ($P < 0.001$). No differences in Gram negative bacilli were found because of the treatment.

Table 3. Effect of different treatments on parts yield as percentages of broiler dressed weight at d 30.

	Treatment					SEM	p*
	T1	T2	T3	T4	T5		
Dressed yield (%)	70.9^{a}	69.9^{b}	70.8^{a}	70.9^{a}	70.2^{ab}	±0.25	*
Breast (%)[1]	35.6	35.2	34.3	35.5	34.1	±0.52	NS
Leg quarter (%)[1]	40.4	40.3	40.2	40.9	42.3	±0.72	NS
Abdominal fat (%)	1.05	0.80	1.26	1.51	1.86	±0.31	NS
Liver (g/100 g)	0.31^{a}	0.24^{b}	0.28^{ab}	0.31^{a}	0.34^{a}	±0.02	*

[1]Breast and leg quarter were expressed as percentage of the carcass weight. ^{abc}means in the row with different superscripts differ significantly (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, N.S: Not significant).

Table 4. Intestinal morphology and histology of broilers at d 16.

	Treatment					SEM	p*
	T1	T2	T3	T4	T5		
Intestine length (cm)	129.7	132.0	127.0	129.8	133.0	±7.74	NS
Duodenum length (%)	19.5^{a}	17.4^{b}	16.8^{bc}	15.8^{bc}	15.1^{c}	±0.67	**
Jejunum length (%)	47.8^{a}	41.6^{b}	41.5^{b}	40.8^{b}	41.9^{b}	±0.67	***
Ileum length (%)	32.7^{c}	41.1^{b}	41.7^{ab}	43.4^{a}	42.9^{ab}	±0.71	***
Intestine weight (g/ cm)	0.28	0.21	0.27	0.24	0.24	±0.02	NS
IRW[1] (g/100g BW)	7.4	7.6	7.2	6.9	7.5	±0.74	NS
Ileal Villus height[2] (µm)	4509^{a}	3765^{b}	3804^{b}	4147^{ab}	4253^{ab}	±172.0	*
Ileal Villus width[2] (µm)	692.6^{b}	766^{ab}	653^{b}	902^{a}	691^{b}	±58.2	*

[1]IRW: intestine relative weight. [2]Measurements of height and width were based on at least 5 well-oriented villi per ileum per broiler for a total of 5 birds per treatment. ^{abc}means in the row with different superscripts differ significantly (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, N.S: Not significant).

Discussion

The results revealed a significant improvement in FCR 30 d of age for birds which had received the PF treatment. This could be explained by the numeric reduction in FI which was associated with this group, while BWG was similar among all groups. There was no significant difference in FCR in birds which had received PF as compared to the ENRA. Evidence has been presented that *Lactobacillus*-based DFM improves performance and FCR in broilers. The results are in general agreement with earlier reports [4] that the overall FCR for probiotic treatment in feed and water and probiotic in feed was not different from antibiotic treatment. The probiotic which was used in the experiment contained *Lactobacillus*, *Bifidobacterium*, *Enterococcus* and *Pediococcus* strains. Broiler chickens were challenged with *C. perfringens*, normal gut flora products such as Aviguard were found to reduce gross intestinal lesions and improve FCR [24]. Similarly, Eckert *et al.*[25] used the same commercial DFM product and reported that broilers administered *Lactobacillus*-based Primalac by intermittent drinking water application increased BWG and improved FCR through d 40 compared with control birds. Angel *et al.* [26] reported that the addition of Primalac to the moderate nutrient density diet overcame the negative effect of nutrient concentration on performance but not when the low density diet was fed, chicks fed pelleted feed containing DFM had greater BWG, improved FCR and improved nutrient retention at 18, 32 and 42 d. ENRA, on the other hand, BWG was improved by 3.0% and FCR by 2.4% of broilers on the floor [27]. In other studies *Lactobacillus*-based DFM did not have any effects[28,29]. The contradictory results can partly be explained by presence of subclinical health problems prevalent in the testing facility.

In this experiment, we challenged the birds with *C. perfringens* to mimic a challenge that a bird could face in rearing facilities. *C. perfringens* among the most gut-specific pathogens which is assumed to be the main health problem associated with removing the antibiotics from feed [30]. *C. perfringens* infection of

Table 5. Intestinal morphology and histology of broilers at 30 d of age.

	Treatment					SEM	p*
	T1	T2	T3	T4	T5		
Intestine length (cm)	152.0[b]	178.0[a]	176.2[a]	182.0[a]	185.5[a]	±6.62	*
Duodenum length (%)	15.7[ab]	16.6[a]	14.8[bc]	16.3[a]	14.5[c]	±0.29	*
Jejunum length (%)	52.1	41.8	43.2	42.3	42.6	±3.85	NS
Ileum length (%)	32.2	41.7	42.1	41.4	42.9	±3.85	NS
Intestine weight (g/ cm)	0.36	0.44	0.49	0.45	0.52	±0.03	NS
IRW[1] (g/100g BW)	4.7	5.7	5.5	5.7	6.2	±0.35	NS
Villus height[2] *(μm)*							
Duodenum	8964[ab]	8315[c]	8480[bc]	9313[a]	8523[bc]	±189	*
Jejunum	7208[d]	8449[c]	9467[b]	10504[a]	996[ab]	±320	*
Ileum	5869	6643	4692	6454	5580	±454	NS
Villus width[2] *(μm)*							
Duodenum	1245[b]	1298[b]	1647[ab]	1910[a]	1780[a]	±139	*
Jejunum	1109[b]	938[b]	1215[b]	1796[a]	1254[b]	±144	*
Ileum	843	882	851	1019	794	±156	NS

[1]IRW: intestine relative weight.[2]Measurements of height and width were based on at least 5 well-oriented villi per section per broiler for a total of 5 birds per treatment. [abc]means in the row with different superscripts differ significantly (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, N.S: Not significant).

Table 6. Ileal bacterial count in broilers at 16 and 30 d of age.

	Treatment					SEM	p*
	T1	T2	T3	T4	T5		
Starter	Mean (log_{10} CFU/g)						
C. perfringens	4.2	3.9	4.3	4.8	4.7	±0.18	NS
Gram negative bacilli	3.8	4.3	4.3	3.8	3.6	±0.28	NS
Finisher							
C. perfringens	4.7[a]	5.0[a]	4.8[a]	0.0[b]	4.8[a]	±0.22	***
Gram negative bacilli	4.8	4.8	5.0	4.9	4.6	±0.15	NS

Measurements of were based on 5 broilers per treatment. [abc]means in the row with different superscripts differ significantly (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, N.S: Not significant).

broilers may cause impairment of production performance [31] and subclinical disease associated with necrotic enteritis which is characterized by damage to the intestinal mucosa that decreases digestion, absorption and reduces weight gains [32]. It was suggested that the growth suppressing effect of intestinal bacteria was due to the production of toxic metabolites that irritate the gut mucosa, thereby inhibiting nutrient absorption. We noted a total elimination of *C. perfringens* in the ileum of PF group, while, the Gram negative bacilli was not responsive to any treatment. This elimination could be explained by competitive exclusion and stimulation of the immune system. According to Bedford [33], most AGPs target Gram positive bacteria suggests that this group of bacteria is an important cause of impaired performance. On the other hand, Pedroso *et al.* [27] reported that ENRA induced changes in the intestinal microbiota composition in broilers, however, the number of bacterial genotypes found in the intestinal tract of chickens was not reduced. This agrees with our results, since ENRA failed to lower the *C. perfringens* or bacilli counts. The effect of bacteria such as *Lactobacillus* spp. to reduce or prevent colonization of undesirable bacteria such as *Salmonella* in the intestinal tract of poultry has been mostly positive [34, 35], however, no effect for Primalac was reported in this experiment on *Salmonella*. None of the challenged birds produced overt clinical signs of necrotic enterities and there were no mortalities associated with oral exposure to high doses of *C. perfringens*. However, many of the challenged birds showed distinctly pronounced pathological changes in the intestinal tissue. The gross examination of the responses in birds challenged orally with *C. perfringens* showed sub-clinical inflammatory responses throughout various sections of gizzard, duodenum, jejunum, ileum and ceca associated with intestinal lesions and hemorrhages.

Long villi are usually equated with excellent gut health, high absorptive efficiency and healthier intestinal tract of chickens [36]. According to Cera *et al.* [37], maximum absorption and digestion capacity is provided by a large luminal area with villus height and mature enterocytes and is essential to animal development. Primalac supplementation to the feed (PF) improved duodenal and jejunal villi height by 9.8 and 10.9%, respectively, and jejunal width by 41.1% as compared to the ENRA. Evidence has been presented that *Lactobacillus*-based DFM as Primalac improves intestinal morphology in poultry [38-40]. These data suggest that DFM like PF increase metabolic efficiency via changes in intestinal physiology and metabolism. Cant *et al.* [41] estimated that 25% of total energy needs of the animal are consumed by the gastrointestinal tract. Oliveira *et al.* [42] explained the low villi height and perimeter was due to antibiotic feeding, antibiotic suppress the beneficial bacteria in the gut, as *Lactobacillus* and bifidobacteria. No differences in total and relative small intestine weight were found in this experiment.

Conclusions

Changes in intestinal morphology and ileal *C. perfringens* as observed in this study support the idea that broiler gut health and function can be improved by dietary supplementation with DFM products such as Primalac which can serve as a safe alternative to antibiotic in broiler diet. Primalac in feed was found to be effective in improving the performance by eliminating *C. perfringens* and changing the villi height and width in the small intestine under necrotic enteritis infection model used in this experiment. Under conditions in the present study, Primalac administration in drinking water was not effective method in lowering *C. perfringens* count and improving the performance compared to Primalac in feed.

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