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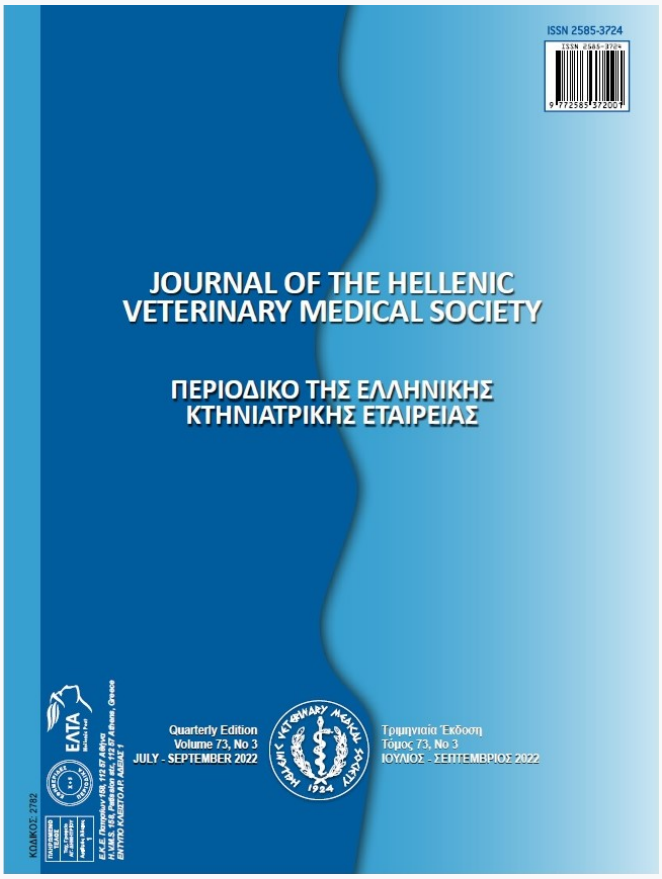

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Research article
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Synergistic effect of feed additives on cell proliferation and morphology in quail (*Coturnix coturnix Japonica*) duodenum

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ABSTRACT: The present study aimed to investigate the effect of a commercial probiotic and a commercial essential oil blend and their mixture, as a natural feed additive, on the expression level of Proliferating Cell Nuclear Antigen (PCNA), proliferation index (PI), and total mucosal thickness of quails. 1-day-old Japanese (*Coturnix coturnix Japonica*) quails, including both males and females, were divided into four groups as follows: (1) control treatment without medication (n: 15); (2) 18 g/ ton-1 probiotic (n: 15); (3) 300 g/ ton-1 essential oil blend (n: 15) and (4) 18 g/ ton-1 probiotic plus 300 g/ ton-1 essential oil blend (n: 15). The study evaluated the effect of natural feed additives added to the quail diet on intestinal health by immunohistochemical and histological analysis. At the end of the experiment, the quail's duodenum was removed, fixed in 10% neutral buffered formalin, and performed a histological examination.

As a result of the study, it was determined that the PCNA expressions, proliferation index, and mucosal thickness were generally significantly increased in the experimental groups compared to the control group ($P<0.001$). The combination of additives caused a synergistic effect for this study. All data were statistically significant in the group where the probiotic and essential oil mixture was used together. It has been determined that these feed additives combination stimulates cell proliferation in the duodenum, where nutrients are absorbed and positively affect intestinal morphology.

Keywords: Duodenum; essential oil blend; PCNA; probiotic; quails.

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INTRODUCTION

The digestive tract of the chicken plays a significant role in its health. Feed additives are widely added to animal diets to increase production performance, improve the welfare and general health of the animal. In general, higher production performance can be achieved in animal production by increasing feed intake, ensuring better digestion and absorption of nutrients, and improving gut health (Van der Aar et al., 2016). Feed additives have recently focused on intestinal health, and the importance of feed additives that have an intestinal health improvement effect has increased. The use of feed additives based on essential oils and probiotics increases poultry production due to several factors, including legislation restricting antibiotic use and consumer preferences for more natural, welfare-friendly poultry production (Emami et al., 2017; Sevim et al., 2020). In recent years, researchers have focused on the effects of probiotics, probiotic combinations, and essential oil use on digestive system morphology and performance parameters in poultry (Yesilbag et al., 2013; Yesilbag et al., 2014; Cao et al., 2018; Grant et al., 2018; Laghouati et al., 2020; Sarmad et al., 2020).

The biomedical effects of probiotics are significant due to their inhibition effects against pathogens, optimizing effects on digestive processes, stimulating effects on the immune system, and anti-tumor and anticholesterol activities (Sharif et al., 2017; Emmami et al., 2017). Many previous studies have suggested an increase in the production performance of chickens fed with probiotics (Tekeli et al., 2011; Zafarnejad et al., 2017).

Cylactin® LBC ME20 plus, used as an intestinal flora stabilizer, also reduces the toxin level in the intestines by limiting the growth of harmful bacteria and stimulating the protection of the intestinal wall. It is also a trading name for a feed additive based on dehydrated cells of Enterococcus faecium. Enterococci are gram-positive cocci usually found in the gastrointestinal tract of humans and animals and are resistant to many antibiotics, some disinfectants, high salt concentrations, drying, and heat (Debnam et al., 2005; Wu et al., 2019).

CRINA® Poultry Plus (later abbreviated CPP) is a feed additive consisting of seven active substances: benzoic acid, thymol, eugenol, piperine, isoamyl salicylate, benzyl salicylate, and trans-anethole. In general, CRINA® Poultry Plus is a feed additive intended to be used as a zootechnical additive in a complete feed to positively affect the performance of fattening chickens (Juin and Weber, 2012).

In studies on the digestive system, the most common method used to identify cells proliferating in tissue sections was the immunohistochemistry (IHC) detection of Proliferating cellular nuclear antigen (PCNA) immunoreactivity. Many researchers have obtained definitive results about the treatment method used by determining the increase or decrease in cell proliferation on the digestive system after the treatments are given as supplementary nutrition using this method (Garcia et al., 2007; Tazuke et al., 2011; Zhang et al., 2014; Gesek et al., 2018). This study used the related technique to determine cell proliferation in the intestinal tract after feeding.

The effect of probiotic and essential oil mixture, which is one of the current feed additives, on the morphology and function of the digestive system is known, but the effects of their combined use have not been fully determined. In addition, there is not enough information about the use of combined feed additives on cell renewal and proliferation in the intestines. Therefore, this study was planned to determine first time the effects of adding commercial probiotics (Cylactin LBC ME20 plus), essential oil mixture (Crina Poultry Plus), and especially their combinations to quail diets on the total mucosal thickness and cell proliferation in the duodenum.

MATERIALS AND METHODS

Animals, managements and experimental design

For the feeding study, a total of 200 male and female 1-day-old Japanese (*Coturnix coturnix Japonica*) quails were obtained from the quail breeding unit of Bursa Uludağ University Animal Health and Production, Research and Application Center in Turkey. The quails were randomly allocated to a control and three treatment groups (probiotic group (PRB), essential oil blend group (EOB) and probiotic + essential oil blend group (PRB + EOB)), each containing 50 quails. Each group was randomly divided into five subgroups, comprised of 10 quails each. However, 3 quails were randomly selected from each of 5 subgroups to be used in the histological study.

The study was designed to use a total of 15 quails from each group, including the control group (n: 15), probiotic group (PRB) (n: 15), essential oil blend group (EOB) (n: 15) and probiotic + essential oil blend group (PRB + EOB) (n: 15). Newly hatched

quails in all groups were reared under the same growing conditions in brooding cages (colony type) in an open-sided house with mechanical ventilation. The quails were transferred randomly at the fourth week of age from the growing cages to laying cages (100 cm wide, 45 cm deep, 21 cm high in front, and 17 cm high in the rear) and housed there until the end of the study. All of the quails were brooded and reared at 28 °C for the 1st wk, 27 °C for the 2nd wk, 24 °C for the 3rd wk, and 18-21 °C from the 28th day until the quails reached 42 days of age. The lighting schedule was 23 hr light with 1 hr dark period during the first 3 days, after then, birds were subjected to 16 hr light with 8 hr dark until the end of the experiment.

The experiments were performed in accordance with the guidelines provided by the National Institute of Health for Animal Research and were approved by the ethical committee on animal research at University Animal Experiments Local Ethics Committee (Approval No: 2020-02/05).

Diets and experimental treatments

A basal diet was formulated and considered as a control according to the recommendation of NRC (1994) (2900 Kcal ME/kg and 24% CP). The composition of the diets and the content of nutrients are shown in Table 1. Group feeding was used in all treatment groups. Three experiment diets were formulated by supplemented the basal control diet with CYLACTIN® LBC ME20 plus (18 g/ ton-1), CRINA® Poultry plus (300 g /ton-1), and the mixture of CYLACTIN® LBC ME 20 plus (18 g/ ton-1) + CRINA® Poultry Plus (300 g/ ton-1), respectively. The level of natural feed additives were based on the manufacturer's recommendations (DSM Nutritional Products Ltd., Basel, Switzerland). Cylactin LBC ME20 plus (DSM Nutritional Products Ltd., Basel, Switzerland) is a commercial micro-encapsulated microbial feed additive (probiotic) to stabilize intestinal microbiota. The probiotic contains Enterococcus faecium in the amount of 2.0 × 1010 colony forming units (CFU) per gram. Crina poultry (DSM Nutritional Products Ltd., Basel, Switzerland) is a commercial essential oil blend. According to the information obtained from the manufacturer, the EO components used in this study mainly include thymol, eugenol, piperine and benzoic acid. It is a carefully balanced combination of essential oil compounds of high purity with a content of 872 g/kg in their natural/ nature-identical form on a carrier. The diet was fed to the quails in the form of mash and water ad libitum throughout the entire experimental period (42 d).

Table 1. Ingredients (%) and chemical composition of the basal diet

Ingredients	%
Corn, grain	44.30
Soybean meal	36.0
Wheat	8.0
Corn gluten	5.0
Vegetable oil	3.0
Ca CO_3	1.6
Dicalcium phosphate	0.62
Salt	0.30
L Lysine	0.35
DL Methionine	0.40
L Threonine	0.08
Vitamin mineral premix[a]	0.35
Values analyzed, %	
Metabolisable energy [b], Kcal/kg	2,928
Crude protein	24.69
Crude fiber	3.6
Ether extract	5.07
Ash	7.36
Dry matter	88.03

[a] Supplied the following per kilogram of diet: 3.000.000 IU vitamin A, 1.200.000 IU vitamin D3, 0,36 g vitamin E, 1 mg vitamin K, 3 mg vitamin B1, 4 mg vitamin B2, 3 mg vitamin B6, 0.003 mg vitamin B12, 10 mg pantethonic acid, 20 mg niacin, 40 mg folic acid, 1 g choline, 0.3 mg biotin, 6 mg Cu, 300 mg I, 100 mg Fe, 0.2 mg Se, 60 mg Mn, 50 mg Zn

[b] Metabolisable energy content of diets was estimated using the equation of Carpenter and Clegg (Leeson and Summers, 2001)

Histological procedure and morphometric analysis

At the end of the experimental period, all the quail from each group were slaughtered and duodenum samples were taken out for a histological procedure. The samples were cut to 2 cm in length and washed and fixed in 10% neutral buffered formalin. The tissues were dehydrated with increasing alcohol concentrations, cleared in xylene and embedded in paraffin. Five μm thick sections were cut from paraffin blocks, mounted on slides, and dried overnight. After dewaxing and rehydration, some sections were stained with Crossman's trichrome stain to detect morphological changes (Crossman, 1937). The other sections were processed routinely for immunohistochemical valuation. Total mucosa was measured and micrographs were taken with Nikon 80i microscope. Total mucosa thickness was measured from the top of the villus to the lower limit for randomly 5 mucosa per section.

Immunohistochemical analysis

The standard streptavidin-biotin peroxidase com-

plex technique was applied. For antigen retrieval, the boiling step was performed in a microwave oven of 750 W with sodium citrate buffer (1 M, pH 6.1) for 3 x 5 minutes. After the sections were washed with PBS, the sections were blocked for 10 minutes for endogenous peroxidase activity. To reduce nonspecific antibody binding, horse serum was applied to sections for 20 min. After that, sections were incubated overnight 4°C with PCNA primary antibody (sc-7907; Santa Cruz Biotechnology, Inc. Texas) diluted 1:100 as recommended by the manufacturer. Samples were washed three times with PBS and incubated with (MP7401; ImmPRESS reagent Vector Laboratories, Inc. CA.) for 30 min at room temperature. Finally, 3.3'-diaminobenzidine (DAB) (Zymed Laboratories San Francisco, CA.) was used for color development, and hematoxylin was used for counterstaining. Sections were cleared with xylol and covered with the entellan.

Proliferative index (PI) rate was calculated as the ratio the number of PCNA positive crypt cells to the total number of crypt cells. The number of proliferating cells per crypt was defined as the mean of proliferating cells in 15 crypts. (Uni et al., 2000; Muskhelishvili et al., 2003; Chen et al., 2013). In addition, two independent observers assessed the intensity and localization of PCNA expression with scoring system: 0, no immunoreaction; 1, weak immunoreaction; 2, moderate immunoreaction; 3, strong immunoreaction (Ergin et al., 2008).

Statistical analysis

The differences between the groups were analyzed using the Kruskal-Wallis and Mann-Whitney U-test of the SPSS 22. The results are presented as means ±SE (Std. Error of Mean).

RESULTS

Morphometric and immunohistochemical results

The intensity of immunostaining, proliferation index, and total mucosal thickness in the duodenum are presented in Table 2 and Figure 1, 2.

The proliferation index was determined by immunodetection of the PCNA antigen. PCNA immunoreactivity was mainly localization in Lieberkühn crypts.

A statistical increase in the experimental groups was determined in PCNA expression intensity, proliferation index, and total mucosal thickness compared to the control group ($P < 0.05$, $P < 0.001$).

Immunreaction of PCNA has a stronger intensity in the PRB+EOB group than the other groups (Table 2, Figure 1, 2). Also, the proliferation index was higher in the PRB+EOB group compared to the other groups (Table 2, Figure 1,2).

The total mucosal thickness in the PRB+EOB group was statistically significant compared to the other groups ($P < 0.001$).

In addition, an increase in total mucosal thickness was determined in the PRB group compared to the EOB group ($P < 0.05$) (Table 2, Figure 1,2)

DISCUSSION

The small intestines have an important place in the poultry's ability to digest and absorb nutrients. The intestinal epithelium is a single cell layer that originates produced by stem cells in the crypt. The integrity of the epithelial layer is crucial for intestinal health and the animal's general health. After the prohibition of antibiotics effective in maintaining intestinal integrity, the focus was on natural performance enhancers in animal nutrition (Awad et al., 2009). Especially recently, the focus has been on the effects of these additives on gut health (Cengiz et al., 2015; Wang et al., 2017). The main additives are probiotics and essential oil blends. In this study, the effects of using these additives separately and together on intestinal health were demonstrated.

Table 2. Evaluation of PCNA expression intensity, proliferation index and total mucosal thickness in the duodenum of the control and experimental groups

Groups	N	PCNA Expression Intensity	Proliferation Index (PI)	Total Mucosa Thickness
Control	15	1,20±0,20[a]	44,69±1,82[a]	986,67±31,85[a]
PRB	15	1,87±0,17[b]	60,27±2,06[b]	1112,20±34,86[b]
EOB	15	1,86±0,19[b]	56,60±1,93[b]	1034,73±12,60[c]
PRB+ EOB	15	2,20±0,20[c]	82,36±1,27[c]	1288,20±25,68[d]
P value		0.008	0.001	0.001

Different letters in the same column show statistical significance ([a,b,c,d]).
Data are means ± SE.

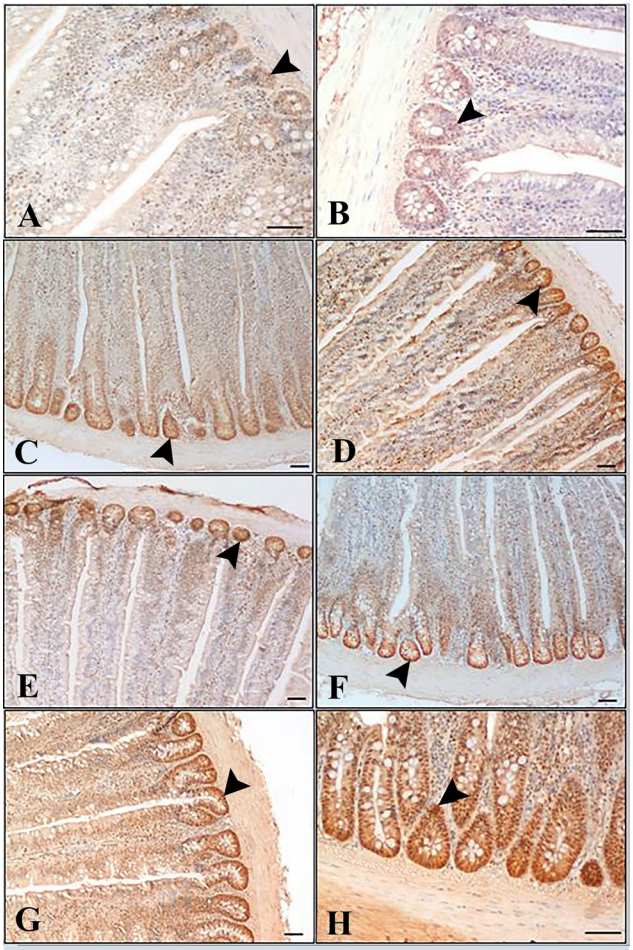

Figure 1. PCNA expression in duodenum of control and treatment groups Control group (A-B); Probiotic treatment group (C-D); Essential oil blend treatment group (E-F); Probiotic+ Essential oil blend treatment group (G-H). PCNA expression (arrow head). Bar 50 µm.

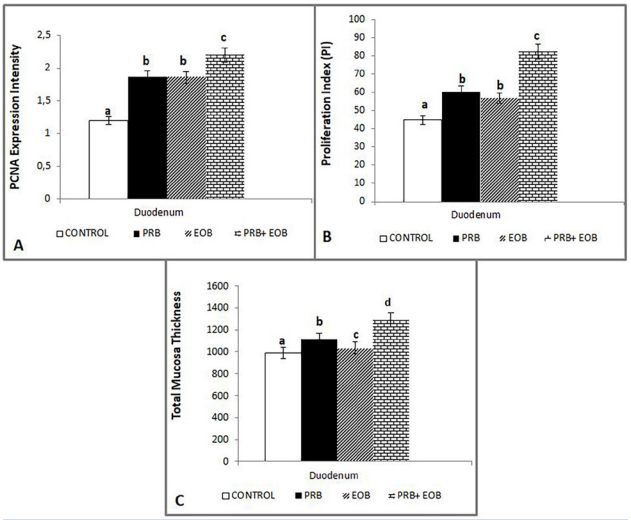

Figure 2. Comparison of PCNA expression intensity (A), proliferation index (B) and total mucosal thickness (C) in duodenum of control and experimental groups. Probiotic group (PRB), Essential oil blend group (EOB), Probiotic + Essential oil blend group (PRB+EOB)

Studies on different types of livestock have shown that adding the essential oil blend and probiotic supplement combination to feed diets has a positive effect on growth performance. Tan et al. (2020) reported that pigs receiving dietary supplements (essential oils or a combination of essential oils and probiotics) had better growth performance. Also, Diao et al. (2015), in a study on weaned piglets, showed that adding an essential oil mixture containing benzoic acid and thymol to their nutritional diet increased nutrient digestion and absorption, reduced diarrhea, and maintained a favorable gut microenvironment. Probiotics improve the health and performance of the animal, mainly by regulating the intestinal microbiota, inhibiting the growth of pathogenic microorganisms, stimulating feed intake and digestion, and supporting the immune system (Ekim et al., 2020). Previous studies showed that, after supplementation of the probiotic supplement, E. faecium, which was used in our study, improved production performance was observed in broiler chick diets, and a decrease in pathogenic bacteria such as Escherichia coli and Enterobacteriaceae (Kralik et al., 2004).

The small intestinal epithelium contains nutrient transporters and digestive enzymes, secreting hormones and functioning as a physiological barrier against pathogens by producing glycoproteins and defensins (Zhang et al., 2009). It is crucial in terms of animal performance to demonstrate the effectiveness of probiotics in protecting the integrity of the intestinal epithelial structure. Previous studies showed that a diverse variety of probiotics might be used to maintain intestinal integrity (Awad et al., 2009; Zhang et al., 2016). Intestinal epithelial cells need to be constantly renewed due to their short lifespan. In our study, the total mucosal thickness, including intestinal epithelium, increased, especially in the PRB-EOB group. These results suggest an increase in the specific activities of brush-edged enzymes such as lactase and sucrase and mucosal enzymes such as peptidase and aminopeptidase Induction of these mechanisms promotes an increase in the surface area of absorption in the intestine and maximizes nutrient utilization by inducing absorption.

Intestinal stem cells located at or near the crypt base of its mucosa play an essential role in the growth and regeneration of the intestinal mucosa and maintenance of intestinal epithelial homeostasis (Yilmaz et al., 2012; Mah et al., 2014). The precursor or proliferative cells in the crypt region continuously differ-

entiate into secretory cells (goblet, enteroendocrine, and Paneth) and absorptive enterocytes cells (Van der Flier and Clevers, 2009). These proliferating cells can be observed by immunohistochemical staining of an endogenous substance called ''PCNA'', also known as cyclin or DNA-polymerase delta auxiliary protein (Tzora et al., 2017).

In addition, the recovery of the intestinal mucosa after various insults is highly dependent on the proliferation and the differentiation of Intestinal stem cells (Barker et al., 2008; Chen et al., 2009; Barker, 2014). In the literature, studies on intestinal stem cells have often investigated the changes in the crypts of the intestines, including crypt cell proliferation and the proliferation index such as proliferating cell nuclear antigen (PCNA) in the crypt region (Guo et al., 2013; Peng et al., 2015). Lieberkühn Crypts containing stem cells are the proliferation center of the intestinal epithelium. The newly created enterocytes then migrate upward and are expelled from the tip of the villi. In our study, PCNA expression and proliferation index were increased in the Probiotic + Essential oil blend group compared to other groups. In addition, an increase in total mucosal thickness was observed in the PRB+EOB group. The increase in mitotic activity induces cell regeneration in the intestine and has a supportive effect on intestinal health. Also the increase in cell proliferation and proliferation index in the combined nutritional supplement group is directly related to the increase in total mucosal length and directly supports nutrient absorption and growth performance. Consistent with our study, Wu et al. (2019) found that adding E. faecium probiotics to the broiler diet significantly increased PCNA positive cells and the intensity of PCNA expression. Also, it indicated that probiotics could promote the proliferation of intestinal cells and accelerate the development of the intestine tract (Wu et al., 2019). Similar to our study, Silva et al. (2009) observed an increase in PCNA expression in the intestinal cells of quails fed essential oil. They suggest that essential oil treatment was improved bird performance, nutrient utilization and absorption. Also, Timmerman et al. (2006) studied the effects of chicken-specific probiotics, consisting of 7-Lactobacillus species, on broiler chickens and observed reduced mortality and increased productivity in birds with probiotic supplementation compared to the control group. Similar observations were reported by Khambualai et al. (2010) in broiler chickens after commercial probiotic product supplementation with higher cell mitosis in crypts in the duodenum and jejunum in probiotic groups compared with the control group. They found that probiotic supplementation also changed the mitotic index in the crypts, and proliferation activity of the crypt cells was higher in treated groups.

CONCLUSIONS

In the present study, the combined nutritional supplement (PRB+EOB) group had higher proliferative activity in crypts and total mucosa of the duodenum from the other groups, which is clear indicates better digestibility and absorption. This study emphasizes the importance of the further evaluation of the combined feed additives in order to maximize their positive effects on the gut tissue of qualis and, consequently, the overall health of qualis. This is the first histological study on these combine feed additives, suggesting that further studies should be carried out by using different animal species or relative combined feed additives.

ACKNOWLEDGEMENT

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APPROVAL OF THE ETHICS COMMITTEE

This study was approved by the Bursa Uludag University Animal Experimentation Local Ethics Committee (2020-02/05).

CONFLICT OF INTEREST

The authors declared that there is no conflict of interest.

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