Research Article

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Fermented whey as poultry feed additive to prevent fungal contamination

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Abstract

BACKGROUND: Fungal contamination of poultry feed causes economic losses to industry and represents a potential risk to animal health. The aim of the present study was to analyze the effectiveness of whey fermented with kefir grains as additive to reduce fungal incidence, thus improving feed safety.

RESULTS: Whey fermented for 24 h at 20 °C with kefir grains (100 g L^{-1}) reduced conidial germination of *Aspergillus flavus*, *Aspergillus parasiticus*, *Aspergillus terreus*, *Aspergillus fumigatus*, *Penicillium crustosum*, *Trichoderma longibrachiatum* and *Rhizopus* sp. Poultry feed supplemented with fermented whey (1 L kg^{-1}) was two to four times more resistant to fungal contamination than control feed depending on the fungal species. Additionally, it contained kefir microorganisms at levels of 1×10^8 colony-forming units (CFU) kg^{-1} of lactic acid bacteria and 6×10^7 CFU kg^{-1} of yeasts even after 30 days of storage.

CONCLUSION: Fermented whey added to poultry feed acted as a biopreservative, improving its resistance to fungal contamination and increasing its shelf life.

Keywords: whey; poultry; feed; fungi; kefir; biopreservation

INTRODUCTION

Feed is the main component of total production costs of meat and eggs in the poultry industry. It is also the primary agent through which chickens are exposed to a variety of potentially harmful factors during its passage along the gastrointestinal tract.

Poultry feed can be a substrate for the development of diverse microorganisms, primarily molds, which come from the crop or are introduced during grain harvest, processing, storage or distribution.[1,2] The fungal genera most often encountered in chicken feed globally are *Aspergillus*, *Fusarium* and *Penicillium*.[3–6] The presence of these fungi represents a potential risk to animal and human health, as variations in environmental conditions during production, storage or distribution of feed can trigger fungal growth and/or toxin production. Feed spoilage by fungi may adversely affect its quality through physical damage and alteration of its nutritive value.[7] The ingestion of contaminated feed can cause invasive fungal disease associated with significant morbidity and high mortality in immunocompromised and, to a lesser extent, immunocompetent individuals.[8] In addition, the affected commodity can become contaminated with mycotoxins that cause problems ranging from acute overt disease to chronic insidious disorders that reduce animal productivity.[7] Besides, harmful residues of mycotoxins consumed by animals can appear in derived products destined for human consumption.[9]

Beneficial bacteria, mainly lactic acid bacteria (LAB) and bifidobacteria, can be useful to reduce the incidence of spoilage microorganisms, thus improving food safety, and to act as probiotics with a positive effect on consumer health.[10] LAB are able to produce a wide variety of antifungal metabolites,[11,12] being used for the biopreservation of several varieties of cheese, fermented and non-fermented plant foods, meat, wine, beer, sourdough bread and silage.[10,13] Only a few studies on biopreservation of animal feed have been reported. Murry *et al.*[14] suggested that *Lactobacillus salivarius* and *Lactobacillus plantarum* could be useful to control pathogens on feed, as they inhibit the growth of *Escherichia coli*, *Salmonella typhimurium* and *Clostridium perfringens* on chicken feed media. Heres *et al.*[15] demonstrated that the fermentation of poultry feed with *L. plantarum* prevents its contamination with *Campylobacter* and *Salmonella*. Nevertheless, the application of LAB to prevent fungal contamination of poultry feed is still an unexplored field of study.

Kefir is a beverage obtained by fermentation of milk with kefir grains. The grains are composed of diverse LAB and yeasts incorporated in a polysaccharide and protein matrix.[16] This ancient

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fermented milk has antifungal properties[17,18] and its administration to chickens prevents cecum colonization by *Salmonella kedougou*[19] and *Campylobacter jejuni*.[20] Whey, an inexpensive and highly available by-product of the dairy industry, can be used as an alternative substrate for kefir grain fermentation, yielding a product with inhibitory power against pathogenic bacteria.[21] Considering this background, the present work proposes the addition of fermented whey to poultry feed in order to evaluate its potential application as a biopreservative. For this purpose, the resistance to fungal contamination of supplemented poultry feed and the survival of kefir microorganisms therein were determined.

MATERIALS AND METHODS

Kefir grains and whey fermentation

Kefir grains CIDCA AGK10 belonging to the Centro de Investigación y Desarrollo en Criotecnología de Alimentos (CIDCA, La Plata, Argentina) collection were added to whey at a concentration of 100 g L^{-1}. Sweet whey powder (Lactogal SA, Porto, Portugal) reconstituted in water at 100 g L^{-1} was used. The reconstituted whey contained 91.3 g water, 0.8 g ash, 1 g proteins, <0.3 g lipids and 6.5 g lactose per 100 g and had a pH of 6.3. Fermentation was conducted at 20 °C for 24 h. Then kefir grains were separated from the fermentation products by filtration through a plastic sieve. The grains were cultured in whey for 20 days before the assays. The fermented whey contained $(1.0 \pm 0.7) \times 10^7$ colony-forming units (CFU) mL^{-1} of LAB and $(4.2 \pm 1.7) \times 10^6$ CFU mL^{-1} of yeasts,[22] and its chemical composition has been reported by Londero *et al.*[21]

Fungal strains

Aspergillus flavus AFUNL5 was isolated from cereals at Laboratorio de Micología, Universidad Nacional del Litoral (Argentina). *Penicillium crustosum* CMUNLP4 was isolated from cheese at Cátedra de Microbiología, Facultad de Ciencias Exactas, Universidad Nacional de La Plata (Argentina). *Aspergillus terreus* CMUNLP1, *Aspergillus fumigatus* CMUNLP2, *Trichoderma longibrachiatum* CMUNLP5 and *Rhizopus* sp. CMUNLP6 were isolated from the commercial chicken food Nutrisur® BB (Nutrisur, La Plata, Argentina). *Aspergillus parasiticus* NRRL 2999 was obtained from the Agricultural Research Service Culture Collection (Washington, DC, USA). Fungi were maintained at 4 °C in agar (2 g agar L^{-1} water) until used.

Supernatant preparation

Supernatant of fermented whey was obtained by centrifugation at 13 000 × *g* for 15 min and filtered through a 0.45 μm membrane (Orange Scientific, Braine-l'Alleud, Belgium). The resulting sterile filtrate was stored at −20 °C until use. By the same procedure, supernatants were obtained from fresh whey acidified with 2 mol L^{-1} HCl (Merck, Darmstadt, Germany) to pH 3.6 and from fresh whey supplemented with 8.6 g L^{-1} DL-lactic acid and 0.8 g L^{-1} acetic acid (Sigma Chemical Co., St Louis, MO, USA) to give the same concentration as measured in the fermented product.

Conidial suspension preparation

Fungal cultures were performed on potato dextrose agar (Britania, Buenos Aires, Argentina) slants at 30 °C for 7 days. Aliquots (10 mL) of conidial solution (0.1 g L^{-1} sodium lauryl sulfate and 10 g L^{-1} glucose) were added to the tubes. Conidia were loosened by gently scraping the surface of the agar with a sterile loop and counted in a Neubauer chamber. Appropriate dilutions were done to adjust the suspensions to 10^4 or 10^5 conidia mL^{-1} or L^{-1} depending on the assay.

Antifungal activity of fermented whey supernatants

Fungi conidial germination reduction was analyzed by a microdilution test[23] on 96-well sterile plates (Corning Incorporated, Corning, NY, USA). Each well was loaded with 190 μL of supernatant and inoculated with 10 μL of conidial suspension. Conidial germination was measured after 48 h at 30 °C by determining the optical density at 580 nm with a spectrophotometer (ELISA Plate Reader SLT Lab instruments Rainbow Reader, Vienna, Austria). Conidial suspension inoculated into fresh whey supernatant was used as germination control. Antifungal activity was expressed as the percentage of germination reduction compared with the control. Inhibition of conidial germination greater than 70% was considered high, between 40 and 70% moderate and between 20 and 40% low.[24] Three independent determinations with four replicates per sample were made. Differences were statistically tested using one-way analysis of variance (ANOVA) and the Tukey test to determine significant differences ($\alpha = 0.05$).

Preparation and characterization of supplemented poultry feed

A 200 mL aliquot of fermented whey was added to 200 g of poultry feed Nutrisur® BB. Then the supplemented feed (termed FW) was dried in a convection oven at 50 °C until a water activity (a_w) of 0.5 ± 0.05 was achieved. By the same methodology, fresh whey acidified with HCl to pH 3.6 and fresh whey supplemented with organic acids to give the same concentration as the fermented product were added separately to poultry feed (termed W + HCl and W + OA respectively). Feed supplemented with water and dried by the same procedure was used as control.

To analyze the survival of kefir microorganisms in supplemented poultry feed, samples of 10 g were taken at 0, 15 and 30 days of storage and homogenized with 90 mL of tryptone (1 g L^{-1})/glycerol (200 mL L^{-1}) in a Stomacher 400 Circulator (Seward Medical, London, UK). Appropriate dilutions in 1 g L^{-1} tryptone were plated on De Man/Rogosa/Sharpe (MRS) agar (Difco, Detroit, MI, USA) for LAB and on yeast extract/glucose/chloramphenicol (YGC) agar (Merck, Darmstadt, Germany) for yeasts. Plates were incubated at 30 °C for 48 h and colonies were counted. Results were expressed as CFU g^{-1} poultry feed.

The water activity (a_w) of supplemented poultry feed was measured at 20 °C with an AquaLab Series 3 TE meter (AquaLab, Pullman, WA, USA). The pH was measured using a pH211 pH meter equipped with an HI 1330B microelectrode (Hanna Instruments, Ann Arbor, MI, USA). Organic acids were determined both qualitatively and quantitatively by high-performance liquid chromatography (HPLC). Acid separation was performed with an Aminex HPX-87H ion exchange column (Bio-Rad Labs, Richmond, CA, USA), and organic acids were detected in the UV at 214 nm. Acid identification was based on matching the retention times with standard acids. Samples (10 g) of supplemented poultry feed were added to 40 mL of distilled water, shaken for 5 min and centrifuged at 10 000 × *g* for 10 min. The resulting supernatants were filtered through a 0.45 μm membrane filter (Orange Scientific). The resulting filtrates were injected (20 μL) into the chromatograph (Waters 717, Millipore, Milford, MA, USA). Analyses were conducted at a flow rate of 0.7 mL min^{-1} at 60 °C using 0.005 mol L^{-1} H_2SO_4 as mobile phase. Quantification was performed using a calibration curve of HPLC-grade standard acids (Sigma Chemical Co.). Results were expressed as g acid L^{-1} filtrate.

Resistance of poultry feed to fungal contamination

To analyze the resistance of poultry feed to fungal contamination, an adaptation of the method described by Gerez *et al.*[24] was used. All poultry feeds were fractionated on Petri sterile plates, with 10 g of feed per plate. Each plate was sprayed with 0.1 mL of conidial suspension containing 10^5 conidia mL^{-1}. Plates were incubated at 20 °C and checked daily to determine the shelf life, defined as the time (in days) at which, as a consequence of mold growth, the appearance of the food was altered.

All treatments were analyzed in three triplicate independent trials. For statistical comparisons, ANOVA and the Tukey test at the 0.05 level of significance were applied.

RESULTS AND DISCUSSION

Antifungal activity of fermented whey

The antifungal activity of supernatants of fermented whey with a pH of 3.6, fresh whey with the same pH and concentration of lactic and acetic acids as fermented whey, and fresh whey acidified with HCl to pH 3.6 was evaluated by determination of conidial germination inhibition (Fig. 1). Whey fermented with kefir grains showed a high percentage of conidial germination inhibition (≥70%) on all fungal species evaluated. The pH was not the causal agent of the inhibition, since unfermented whey acidified with HCl was not inhibitory (<20%) on *Rhizopus* sp. and had a low (20–40%) or moderate (40–70%) antifungal effect on most of the species studied. Only *P. crustosum* was sensitive to low pH, and a high (≥70%) reduction of conidial germination was observed. In contrast, the percentage of conidial germination inhibition produced by fermented whey and by artificially acidified whey containing the same concentration of lactic and acetic acids was not significantly different ($P > 0.05$) (Fig. 1). These results indicate that organic acids are largely involved in the antifungal effect of whey fermented with kefir grains. Acetic acid is more effective than lactic acid in preventing the growth of filamentous fungi,[24–26] but these two acids exhibit a synergistic effect.[27,28] Therefore the presence of both lactic and acetic acids is probably implicated in the strong antifungal effect of the fermented whey.

Nowadays, awareness of the inhibition of filamentous fungi by kefir is scarce. Cevikbas *et al.*[17] reported that kefir has antifungal activity against *Microsporum* sp. and *Trichophyton* spp. A recent publication describes the ability of kefir to inhibit the growth of *Fusarium graminearum* and the germination and aflatoxin production of *A. flavus*.[18] In the present work the capacity of whey fermented with kefir grains to inhibit the conidial germination of seven filamentous fungi frequently implicated in poultry feed spoilage is demonstrated.

Characterization of supplemented poultry feed

In poultry feed supplemented with fermented whey, 64% of the LAB and 13% of the yeasts supplied survived the drying process. Moreover, kefir microorganisms were strongly resistant to storage at 20 °C, since the viability decreased 1 log during the first 15 days and thereafter the concentration remained constant up to 30 days (Table 1).

In whey fermented with kefir grains, the presence of *Lactobacillus kefiranofaciens*, *Lactobacillus kefir*, *Lactobacillus parakefir*, *Lactococcus lactis*, *Kluyveromyces marxianus*, *Saccharomyces unisporus* and *Saccharomyces cerevisiae* has been reported.[22] Certain strains of these species have probiotic potential, being capable of down-regulating the intestinal epithelial innate response[29] and protecting epithelial cells against pathogens.[30] The effective dose of probiotics included in chicken feed is generally between 10^8 and 10^9 CFU kg^{-1},[31–33] but it varies between products. In poultry feed

Table 1. Concentration of lactic acid bacteria (LAB) and yeasts in poultry feed supplemented with whey fermented with kefir grains during its storage at 20 °C

Days of storage	LAB (CFU g^{-1})	Yeasts (CFU g^{-1})
0	$(5.9 \pm 2.3) \times 10^6$	$(5.0 \pm 1.9) \times 10^5$
15	$(1.1 \pm 0.9) \times 10^5$	$(6.8 \pm 1.4) \times 10^4$
30	$(1.0 \pm 0.9) \times 10^5$	$(6.0 \pm 2.5) \times 10^4$

Values are mean ± standard deviation of three independent determinations.

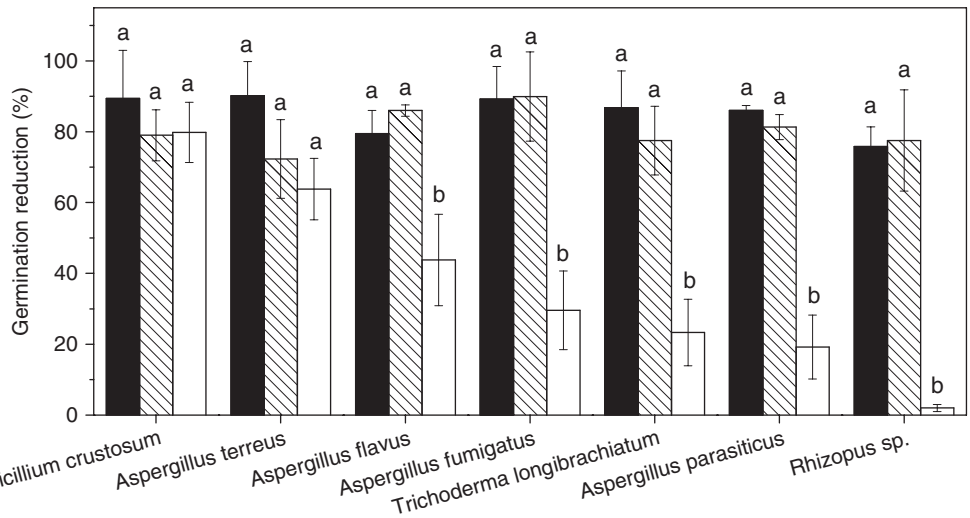

Figure 1. Percentage of fungi conidial germination reduction by supernatants of: whey fermented for 24 h with 100 g L^{-1} kefir grains at pH 3.6 (FW, ■); whey supplemented with lactic and acetic acids to give the same concentration and pH as fermented whey (W + OA, ▨); and whey acidified with HCl to pH 3.6 (W + HCl, □). Different letters above bars corresponding to the same fungal species indicate statistically significant differences ($P < 0.05$).

Table 2. Water activity (a_w), pH and concentration of lactic and acetic acids in supplemented poultry feed during its storage at 20 °C

Parameter	Days of storage	Supplemented poultry feed[a]		
		FW	W + OA	Control
a_w	0	0.527 ± 0.009a	0.531 ± 0.006a	0.500 ± 0.001a
	10	0.567 ± 0.003a	0.572 ± 0.003a	0.546 ± 0.003a
	20	0.572 ± 0.003a	0.579 ± 0.001a	0.560 ± 0.003a
	30	0.574 ± 0.001a	0.582 ± 0.002a	0.557 ± 0.004a
pH	0	5.37 ± 0.03a	5.33 ± 0.03a	6.25 ± 0.03b
	10	5.31 ± 0.04a	5.27 ± 0.03a	6.26 ± 0.03b
	20	5.25 ± 0.03a	5.20 ± 0.04a	6.21 ± 0.04b
	30	5.27 ± 0.03a	5.23 ± 0.04a	6.17 ± 0.04b
Lactic acid (g L^{-1})	0	3.62 ± 0.14a	3.11 ± 0.14a	ND
	10	3.85 ± 0.09a	3.44 ± 0.08a	ND
	20	3.24 ± 0.15a	2.95 ± 0.15a	ND
	30	3.41 ± 0.14a	2.95 ± 0.10a	ND
Acetic acid (g L^{-1})	0	0.82 ± 0.08a	0.85 ± 0.05a	ND
	10	0.97 ± 0.09a	0.87 ± 0.07a	ND
	20	0.93 ± 0.07a	0.79 ± 0.12a	ND
	30	0.92 ± 0.18a	0.87 ± 0.14a	ND

Values are mean ± standard deviation of two independent determinations. Within each parameter, different letters indicate statistically significant differences ($P < 0.05$) according to the Tukey test. ND, not detected.
[a] Poultry feed supplemented with: whey fermented for 24 h with 100 g L^{-1} kefir grains (FW); whey containing the same pH and concentration of lactic and acetic acids as fermented whey (W + OA); or sterile water (control).

Table 3. Days elapsed until fungal growth became visible in artificially contaminated poultry feed

Contaminant	Supplemented poultry feed[a]			
	Control	FW	W + OA	W + HCl
Rhizopus sp. CMUNLP6	10.0 ± 5.2a	23.0 ± 6.0b	12.0 ± 2.8a	9.0 ± 4.2a
Trichoderma longibrachiatum CMUNLP5	9.7 ± 4.9a	23.0 ± 2.8b	20.0 ± 6.0b	8.0 ± 2.8a
Aspergillus fumigatus CMUNLP2	10.0 ± 4.4a	24.0 ± 1.4b	20.0 ± 6.0b	7.5 ± 3.5a
Aspergillus terreus CMUNLP1	10.0 ± 7.0a	25.5 ± 6.4b	19.5 ± 4.9b	10.5 ± 0.7a
Penicillium crustosum CMUNLP4	6.7 ± 4.7a	27.0 ± 4.4b	15.5 ± 3.0a	14.5 ± 6.3a
Aspergillus parasiticus NRRL 2999	11.0 ± 7.0a	27.3 ± 6.4b	14.5 ± 3.5a	13.0 ± 1.4a
Aspergillus flavus AFUNL5	9.4 ± 5.5a	28.7 ± 4.1b	15.5 ± 6.4a	12.5 ± 0.7a

Values are mean ± standard deviation of three independent determinations. Within each row, different letters indicate statistically significant differences ($P < 0.05$) according to the Tukey test.
[a] Poultry feed supplemented with: sterile water (control); whey fermented for 24 h with 100 g L^{-1} kefir grains (FW); whey containing the same pH and concentration of lactic and acetic acids as fermented whey (W + OA); or whey acidified with HCl to pH 3.6 (W + HCl).

supplemented with fermented whey, the product contained 1×10^8 CFU kg^{-1} of LAB and 6×10^7 CFU kg^{-1} of yeasts after 1 month of storage. Therefore it would be of interest to determine in future studies if the species of kefir microorganisms present in the supplemented poultry feed provide some health benefit to chickens.

It is also pertinent to point out that a_w, pH and organic acid concentrations were not modified during the storage of supplemented poultry feed (Table 2). Chemical modifications caused by the addition of fermented whey to poultry feed do not adversely affect its nutritional value. Whey proteins are a rich source of leucine, isoleucine, methionine, cysteine and valine,[34] thus whey might provide proteins with high biological value to poultry feed. Moreover, lactose is added to feed with the fermented whey. It has been reported that the inclusion of lactose in chickens' diet reduces *Salmonella enteritidis* invasion[35,36] and the symptoms of necrotic enteritis.[37] Therefore, with the fermented whey, in addition to potentially probiotic microorganisms of kefir, nutrients and bioactive compounds present in whey would be added to the feed.

Resistance to mold contamination

The resistance to mold contamination was evaluated in poultry feed supplemented with water (control), fermented whey (FW), whey with organic acids (W + OA) and whey with HCl (W + HCl) (Table 3). On feed used as control, fungal growth became visible after 6–11 days of storage. There was no significant ($P > 0.05$) delay in fungal growth on feed supplemented with whey acidified with HCl. Feed supplemented with whey fermented with kefir grains presented strong resistance to fungal contamination, since, for all seven species evaluated, the storage time without fungal growth was 23–29 days, thus extending by two to four times the shelf life of the feed (Table 3). The time without fungal growth of feed containing lactic and acetic acids was not significantly extended, except for *T. longibrachiatum*, *A. terreus* and *A. fumigatus*, so the

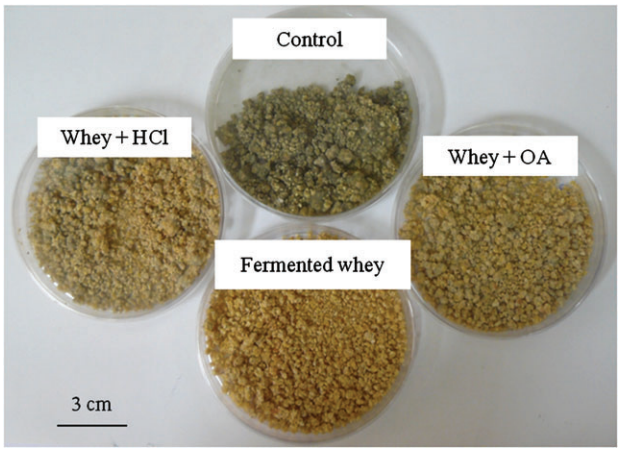

Figure 2. Visual appearance of poultry feed artificially contaminated with *Penicillium crustosum* CMUNLP4 after 20 days of storage. Feed was supplemented with: sterile water (control); whey fermented for 24 h with 100 g L^{-1} kefir grains at pH 3.6 (FW); whey containing the same pH and concentration of lactic and acetic acids as fermented whey (W + OA); or whey acidified with HCl to pH 3.6 (W + HCl).

organic acids were not solely responsible for the antifungal effect. These results differ from those of conidial germination inhibition, in which organic acids had the same effect as fermented whey. Feed supplemented with fermented whey contains kefir LAB and yeasts that were not present in the supernatant assayed in the first experimental design. These microorganisms constitute a source of enzymes and metabolites that could interfere with conidial germination and fungal growth, thus increasing the antifungal capacity of feed supplemented with fermented whey with respect to feed supplemented with organic acids.

Figure 2 shows the appearance of feed contaminated with *P. crustosum* after 20 days of storage. One can see pronounced fungal growth in feed used as control, the early development of fungi in feed supplemented with HCl or organic acids and the visual absence of fungi in feed supplemented with fermented whey. Similar differences between treatments were observed for the other fungal species analyzed.

CONCLUSIONS

There are very few precedents of the application of LAB or their metabolites to preserve animal feed. In the present study the efficacy of the addition of whey fermented with kefir grains to chicken feed in fungal spoilage control is demonstrated. The antifungal capacity reported here is relevant because it opens up perspectives for this innovative application of whey fermented with kefir grains. Moreover, feed formulated with fermented whey contains viable kefir LAB and yeasts, the probiotic effect of these microorganisms on chicken being a point of interest for future studies.

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