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Feasibility of *L. plantarum* and prebiotics on Aflatoxin b_1 detoxification in cow milk

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Abstract

Milk is a key food worldwide prone to mycotoxins contamination. *Lactobacillus plantarum* and prebiotics detoxification ability was evaluated by a Plackett-Burman Design considering the reduction of aflatoxin B_1 (AFB_1) and its bioaccessibility in artificially contaminated ultra-high temperature cow milk. Six variables were evaluated: AFB_1 concentration (from 5.0 to 10.0 μg L^{-1}); incubation time (0 to 6 h); and inulin, oligofructose, β-glucan, and polydextrose concentrations (from 0.00 to 0.75%). The reduction in AFB_1 ranged from 0% to 55.85% and *in vitro* bioaccessibility from 15.62% to 51.09%. The greatest reduction in AFB_1 occurred by adding *L. plantarum* combined with inulin, oligofructose and β-glucan. The greatest reduction in bioaccessibility occurred by adding inulin or oligofructose and *L. plantarum* with a 10.0 μg L^{-1} AFB_1 concentration. A sharp reduction in AFB_1 was accompanied by higher bioaccessibility rates, and in this case, bioaccessibility is considered the main factor to ensure a low AFB_1 absorption by the body. The best experimental condition was 10.0 μg L^{-1} AFB_1, added of *L. plantarum* and inulin or oligofructose (0.75%), ensuring > 16% final bioaccessibility. Such results represent a safe AFB_1 decontamination level for milk.

Keywords: β-glucan, decontamination, inulin, mycotoxin, probiotic.

Practical Application: Probiotic and prebiotic use for a safe AFB_1 decontamination procedure in milk.

1 Introduction

Aflatoxin B_1 (AFB_1) is a mycotoxin produced by *Aspergilus flavus* and *A. parasiticus* identified by the International Agency for Research on Cancer (2012) as a group 1 carcinogenic agent for humans and animals. The indirect milk contamination by mycotoxins occurs by contaminated food consumption by lactating animals allowing for mycotoxins to be carried over to milk (Pimpitak et al., 2020; Zain, 2011). Mycotoxins are resistant to milk`s industrial processing (heat treatments, concentration or drying) justifying their presence at any dairy production chain stage, from raw material attainment to later industrialization stages (Ahmadi, 2020; Campagnollo et al., 2016; Flores-Flores et al., 2015).

AFB_1 is the most prevalent aflatoxin (Bovo et al., 2014), among the 18 identified to date. AFB_1 could suffer hepatic biotransformation and may be converted into aflatoxin M_1 (AFM_1) (Fazeli et al., 2009; Rushing & Selim, 2019) when ingested by animals. However, the literature reports that the conversion from AFB_1 to AFM_1 is only partially performed, and AFB_1 remains detectable in the dairy matrix, reinforcing the detoxification study relevance (Gonçalves et al., 2018; Scaglioni et al., 2014). The AFM_1 incidence in raw and pasteurized milk varied from 61.5% to 100% (Ahmadi, 2020; Cagri-Mehmetoglu, 2018; Hajmohammadi et al., 2020; Öztürk Yilmaz & Altinci, 2019), and incidences of 65%, 40% and 29.6% were reported for kashar (type of cheese), white cheese and butter, respectively (Öztürk Yilmaz & Altinci, 2019). In the same context, the AFB_1 has been also detected in milk and dairy products. An AFB_1 incidence of 41.7% and 13.3% were reported for pasteurized and UHT milk, with an AFB_1 mean content of 1476 ug L^{-1} and 0.690 ug L^{-1}, respectively (Scaglioni et al., 2014). Considering the aflatoxins toxicity, it important to emphasize that AFB_1 is likely to contaminate milk and dairy products become a public health issue, and its evaluation is needed due to its higher resistance to toxicity (Zain, 2011). Thus, the study of procedures that might reduce AFB_1 percentage and its bioaccessibility is made more attractive. The use of probiotics is the most commonly used procedure on mycotoxins decontamination as the lactic acid bacteria species may adsorb mycotoxins from contaminated media. The mechanism is based on a physical binding between the mycotoxins and the bacterial cell wall components, such as polysaccharides and peptidoglycans (Corassin et al., 2013).

Different lactic acid bacteria species, Generally Recognized as Safe (GRAS) used as probiotics, have been studied to reduce AFB_1 contamination and/or its bioaccessibility either in a model system (phosphate buffer saline solution, PBS) or formulated medium (Bovo et al., 2014; Fazeli et al., 2009; Ferrer et al., 2015). However, a single study only evaluated AFB_1 removal and bioaccessibility in milk (Wochner et al., 2019). Moreover, only a small number

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of studies describing the prebiotics effect on the AFB_1 removal and its bioaccessibility (Ferrer et al., 2015; Wochner et al., 2019) are found in the literature. The use of proven potential AFB_1 decontaminants such as probiotics combined with prebiotics is an attractive alternative that ought to be investigated for the mycotoxin decontamination in milk.

This study aimed to evaluate *L. plantarum* action, both isolated and combined with inulin, oligofructose, β-glucan, and polydextrose prebiotics on AFB_1 reduction and its bioaccessibility in whole ultra-high temperature (UHT) milk.

2 Materials and methods

Lyophilized *Lactobacillus plantarum* BG112 (SACCO®, Cadorago, Italy) was activated (0.1%; m v^{-1}) in a Man-Rogosa-Sharpe Broth (MRS, Merck, Darmstadt, Germany) added with 0.05% L-cysteine incubated at 37 ± 1 °C for 12 h (Orion® 502, Fanem, São Paulo, Brazil). The MRS broth containing active microbial cells was centrifuged at 1189 *g* for 5 min (CT-5000R, Cientec, Brazil), the supernatant was then discarded and the microbial pellets were resuspended in milk to obtain a 10^8 CFU mL^{-1} concentration.

2.1 Milk contamination

The AFB_1 standard (Sigma-Aldrich, Saint Louis, Missouri, USA) was resuspended in 100 mL benzene: acetonitrile (98:2; v: v) to obtain a 10 μg mL^{-1} stock solution, which was further diluted with benzene: acetonitrile (98:2; v: v) to obtain the desired spiked concentration (Table 1). The benzene/acetonitrile was evaporated in the oven (45 °C, Cienlab, CTM45, Campinas, Brazil) and the mycotoxin was resuspended in UHT milk obtained from a local market. Beer's Equation (A = εcl) was used to calculate the final solution concentration from the spectrophotometer reading (Lambda XLS, PerkinElmer, Baconsfield, UK) at 360 nm (Scaglioni et al., 2014).

2.2 AFB_1 detoxification in milk

A Plackett-Burman design was used to evaluate AFB_1 reduction and its bioaccessibility in artificially contaminated UHT milk (Table 1). The effects of AFB_1 concentration (5.0 to 10.0 μg L^{-1}), incubation time (0 to 6 h), and inulin (Raftiline GR, Orafti®), oligofructose (Raftilose P95, Orafti®), β-glucan (ProamOatTM, Tate & Lyle®), and polydextrose (Litesse, DuPont-Danisco®) prebiotics were evaluated (each prebiotic from 0 to 0.75%; w v^{-1}). Additionally, three control treatments (CT) - (1) CT1 milk + AFB_1; (2) CT2 milk + *L. plantarum*; and (3) CT3 milk - were performed and incubated for 3 h.

Milk contamination with the desired AFB_1 concentration (Table 1) was carried out using the AFB_1 stock solution. After that, prebiotics were dissolved in the UHT milk as described in Table 1 and sonicated (Elmasonic P60, Elma, Germany) for 5 min (28 ± 2 °C; 80 kHz; 150 W). The *L. plantarum* biomass was added and the runs were incubated (403-3D, Nova Ética, Vargem Grande Paulista, Brazil) at 37 ± 1 °C for the set time (Table 1). A 15 mL aliquot from each run was collected in a falcon tube, frozen at -18 °C for 48 h and lyophilized (25 °C, 24 h, 0.05 mBar, FreeZone 6L, Labconco, Kansas, USA). It was kept frozen and subsequently the AFB_1 extraction and determination were carried out.

Table 1. Plackett-Burman planning matrix with independent variables and response (dependent variable) of AFB_1 reduction (%) and bioaccessibility (%).

Run	x_1	x_2	x_3	x_4	x_5	x_6	Reduction (%)	Bioaccessibility (%)
1	+1 (10)	-1 (0)	+ 1 (0.75)	- 1 (0)	- 1 (0)	- 1 (0)	7.57^{d} ± 1.56	15.92^{h} ± 0.79
2	+1 (10)	+1 (6)	-1 (0)	+ 1 (0.75)	- 1 (0)	- 1 (0)	22.98^{c} ± 0.60	15.62^{h} ± 0.55
3	-1 (5)	+1 (6)	+ 1 (0.75)	- 1 (0)	+ 1 (0.75)	- 1 (0)	0.00	26.53^{f} ± 2.13
4	+1 (10)	-1 (0)	+ 1 (0.75)	+ 1 (0.75)	- 1 (0)	+ 1 (0.75)	0.00	21.25^{g} ± 1.60
5	+1 (10)	+1 (6)	-1 (0)	+ 1 (0.75)	+ 1 (0.75)	- 1 (0)	0.00	20.00^{g} ± 2.00
6	+1 (10)	+1 (6)	+ 1 (0.75)	- 1 (0)	+ 1 (0.75)	+ 1 (0.75)	30.18^{b}± 1.97	30.76^{e} ± 1.74
7	-1 (5)	+1 (6)	+ 1 (0.75)	+ 1 (0.75)	- 1 (0)	+ 1 (0.75)	0.00	35.63^{d} ± 0.28
8	-1 (5)	-1 (0)	+ 1 (0.75)	+ 1 (0.75)	+ 1 (0.75)	- 1 (0)	55.85^{a} ± 0.66	51.09^{b} ± 0.99
9	-1(5)	-1 (0)	-1 (0)	+ 1 (0.75)	+ 1 (0.75)	+ 1 (0.75)	5.48^{d} ± 0.41	39.34^{c} ± 0.77
10	+1 (10)	-1 (0)	-1 (0)	+ 1 (0)	+ 1 (0.75)	+ 1 (0.75)	25.43^{c} ± 3.42	26.78^{f} ± 0.81
11	-1 (5)	+1 (6)	-1 (0)	- 1 (0)	- 1 (0)	+ 1 (0.75)	0.00	30.55^{e} ± 0.74
12	-1 (5)	-1 (0)	-1 (0)	- 1 (0)	- 1 (0)	- 1 (0)	31.45^{b} ± 2.94	27.12^{f} ± 0.93
13	0 (7.5)	0 (3)	0 (0.38)	0 (0.38)	0 (0.38)	0 (0.38)	25.64^{c} ± 1.54	28.01^{ef} ± 0.27
14	0 (7.5)	0 (3)	0 (0.38)	0 (0.38)	0 (0.38)	0 (0.38)	21.71^{c} ± 2.57	21.00^{g} ± 0.39
CT_1	(7.5)	(3)	-	-	-	-	< LOQ	101.0^{a} ± 6.3
CT_2	(0)	(3)	-	-	-	-	< LOQ	< LOQ
CT_3	(0)	(3)	-	-	-	-	< LOQ	< LOQ

Independent variables: x_1 = AFB_1 concentration (μg L^{-1}); x_2 = time (h); x_3 = inulin (%); x_4 = oligofructose (%); x_5 = β-glucan (%); x_6 = polydextrose (%); CT1: positive control (milk + AFB_1); CT2: negative control (milk + *L. plantarum*); and CT3: negative control (milk only); < LOQ = below the limit of quantification; different superscript letters in columns indicate differences by Tukey test ($P < 0.05$).

2.3 AFB_1 extraction and determination

AFB_1 was extracted and purified following the QuEChERS method (Quick, Easy, Cheap, Effective, Rugged and Safe) as previously described by Sartori et al. (2015) with some modifications. The lyophilized runs were resuspended in 15 mL water, added with 10 mL hexane and 15 mL acetonitrile acidified with acetic acid 1% (v: v) and vortex-stirred for 30 s (LS Logen, Diadema, Brazil). After that, 6 g magnesium sulfate (Êxodo Científica, Hortolândia, Brazil) and 1.5 g sodium chloride (Synth Ltda, Diadema, Brazil) were added to the mixture. The tubes were vortex-stirred for 1 min and centrifuged at 1189 g for 7 min at 25 °C. The hexane phase was discarded and a 5 mL aliquot of acetonitrile phase was collected and oven-dried at 45 °C. For AFB_1 determination, the dry sample was resuspended in a 500 µL acetonitrile:methanol:water solution acidified with 1% acetic acid (35:10:55) and centrifuged at 3473 g.

AFB_1 determination was carried out by Ultra-high-pressure liquid chromatography (UHPLC) (Wochner et al., 2019). The UHPLC (Ultimate 3000, Thermofisher, Germering, Germany) was equipped with an automatic sample injector, quaternary pump, oven, and a fluorescence detector (FLD) and controlled by Chromeleon 7.2 software. The samples were injected (20 µL) in a reverse-phase column (C18 Acclaim PA2, 5 µm Analítica, 4.6 x 250 mm) at a 35 °C oven temperature. The mobile phase comprised of acetonitrile: methanol: water acidified with 1% acetic acid (35:10:55) at a 1.0 mL min^{-1} flow rate. The excitation and emission wavelengths were 360 and 450 nm, respectively and the chromatographic run time was 10 min. AFB_1 identification was based on retention time and a co-chromatography was carried out using a spike that increased the signal for confirmation. AFB_1 quantification was performed by external standardization using a 6-point calibration curve with measurements in triplicate ($R^2 \geq 0.998$ and $P < 0.001$). The recovery test was performed in triplicate based on samples and 10 µg mL^{-1}spike level. The methodology was validated and the linearity, limit of detection (LOD), limit of quantification (LOQ), recovery, and relative standard deviation (RSD) parameters are seen in Table 2.

2.4 AFB_1 reduction and its bioaccessibility determination

The evaluation of AFB_1 runs removal percentage was estimated taking into account the difference between AFB_1 expected and real concentrations, divided by real concentration and multiplied by 100% (El Khoury et al., 2011). For bioaccessibility evaluation, the runs were submitted to an *in vitro* digestibility analysis (Kabak & Ozbey, 2012; Wochner et al., 2019). Milk from each run (4.5 mL) was collected, heated, and kept at 37 °C. The following steps were used: (1) 6 mL saliva was added to milk and incubated for 5 min; (2) 12 mL gastric juice was added and the mixture was stirred in a shaker for 2 h (55 rpm); (3) simultaneous addition of 12 mL duodenal juice, 6 mL bile, and 2 mL 1 mol L^{-1} $NaHCO_3$ and stirred in a shaker for 2 h (55 rpm); (4) centrifuged at 2750 g for 5 min at 25 °C. The supernatant obtained was lyophilized for further AFB_1 extraction, purification, and determination as previously described. Bioaccessibility was estimated by AFB_1 concentration after chyme divided by the initial AFB_1 concentration, multiplied by 100%.

2.5 Statistical analysis

The results were shown by average ± standard deviation and subjected to Analysis of Variance (ANOVA) and Tukey Test ($p < 0.05$), using the Statistica 8.0 software. The studied variables effects on the Plackett-Burman Design were also estimated by the same software ($p < 0.10$).

3 Results and discussion

The results for probiotic and prebiotics detoxification ability on AFB_1 reduction in UHT milk are seen in Table 1. The highest AFB_1 reduction was obtained for run 8 reaching 55.85%, while the reduction for run 12 containing only *L. plantarum* was 31.45%. In contrast, Wochner et al. (2019) observed that *L. acidophilus* combined with prebiotics was as efficient or less (13.53% to 35.53%) than only probiotic action (34.96%) on AFB_1 reduction for whole milk. The difference might be due to the AFB_1 reduction ability by *Lactobacillus* strain in the presence of prebiotics.

Moreover, Peltonen et al. (2001) evaluated the potential of twelve *Lactobacillus* strains to remove AFB_1 in phosphate buffer saline, with results ranging from 17.3% to 59.7%, highlighting 28.4% AFB_1 reduction for *L. plantarum*. Additionally, isolated *L. plantarum* reduced AFB_1 by 56% using phosphate buffer saline (Fazeli et al., 2009). The AFB_1 reduction results from this article are within the range described in the literature for isolated *L. plantarum,* even when testing contaminated milk, which plays a more real condition compared to the phosphate buffer saline medium. Moreover, the use of prebiotics improves the probiotic development in milk and increases AFB_1 reduction under specific conditions.

Table 2. Validation parameters of AFB_1 determination (n = 3).

Matrix	AFB_1 spike (µg L^{-1})	Recovery (%)	RSD (%)	LOD (µg L^{-1})	LOQ (µg L^{-1})	Linearity	R^2
Milk	0.5	64.80	8.1	0.1	0.3	y = 96.9 x + 0.39	0.998
	5.0	66.72	4.1				
	10.0	68.25	0.2				
Biologic fluid [(a)]	0.5	87.54	3.9	0.9	2.8	y = 96.9 x + 0.39	0.998
	5.0	62.83	8.3				
	10.0	60.4	1.6				

RSD: relative standard deviation; LOD: limit of detection; LOQ: limit of quantification.

Some *Lactobacillus* strains have demonstrated potential for aflatoxin reduction due to their ability to reduce the carcinogenic or toxic effect of food carcinogens. The mechanism is correlated to physical binding with either carcinogenic or metabolic transformation into less toxic and carcinogenic degradation products (El-Nezami et al., 1998). The most accepted theory states that a physical bind occurs between the mycotoxin and bacterial cell wall components such as polysaccharides, peptidoglycans, lipoteichoic acid, and teichoic acid (Bovo et al., 2014; Serrano-Niño et al., 2015; Wochner et al., 2018).

In contrast, the interaction between LAB and some mycotoxins buildup could occur by specific phenomena such as binding. Thus, for runs without AFB_1 reduction or with lower reduction than run 12 (Table 3), the binding between the mycotoxin and probiotic may have been undone, since it is reversible and the prebiotics might have affected the binding (Oatley et al., 2016). The reversibility of the binding suggests an implication of noncovalent type of bounds such as Van der Waals and hydrogen bonds (Assaf et al., 2019).

Taking into account Plackett-Burman Design, the use of variables within the studied range resulted in statistically similar AFB_1 reductions (Table 3), reinforcing the efficacy of *L. plantarum* and presenting a promising approach combined with prebiotics for AFB_1 reduction. Moreover, the addition of prebiotics to the runs could foster their functionality on the human body improving the AFB_1 reduction.

The mycotoxin concentration had no significant effect on AFB_1 reduction, thus the 5:1 to 10:1 μg g^{-1} mycotoxin: probiotic rate did not affect the decontamination process. Similarly, Wochner et al. (2019) reported that 3.25 to 6.0 μg L^{-1} AFB_1 concentrations did not influence its adsorption rate by *L. acidophilus* in whole milk. It is noteworthy that the combination of probiotics and prebiotics presented reduction effects on AFB_1 even at higher mycotoxin concentrations.

Incubation time had no reduction effect on AFB_1 within the studied range; which corroborate with the theory described in the literature. The aflatoxin-microorganism binding is considered a rapid process that occurs within the first minutes of contact. A physical adsorption process occurs between the probiotic cell wall components and AFB_1 instead of covalent binding or degradation by bacteria metabolism (Bovo et al., 2013; El-Nezami et al., 1998; Shetty & Jespersen, 2006).

All runs presented an AFB_1 bioaccessibility reduction when compared to the positive control with the lowest values observed for run 1 (15.92%) and run 2 (15.62%). The use of isolated *L. plantarum* (run 12) obtained a higher bioaccessibility (27.12%), proving the higher inulin or oligofructose efficiency when combined with probiotic. On the other hand, the highest AFB_1 bioaccessibility level was obtained for run 8 (51.09%) (Table 1). The obtained bioaccessibility values were lower than those reported by Wochner et al. (2019) that described values from 23.68% to 72.67% for whole milk treated with *L. acidophilus* and prebiotics, and 34.96% for isolated *L. acidophilus*. In this way, *L. plantarum* presented a higher bioaccessibility reduction efficiency, and a lower bioaccessibility leads to a further reduction of toxins available for absorption in the intestine.

The mycotoxin and β-glucan concentration variables presented both a negative and positive effect, respectively ($p < 0.10$) (Table 3). Thus, a higher AFB_1 concentration and a lower β-glucan concentration within the studied range reduced the bioaccessibility. Conversely, Meca et al. (2012), when evaluating the effect of β-glucan, chitosan, fructooligosaccharides, galattomannan, inulin, and pectin added at 1% and 5% concentrations on beauvericin bioaccessibility in wheat crispy breads, found that mycotoxin binding ability with the prebiotics was higher at a mycotoxin concentration of 25 mg L^{-1} than at 5 mg L^{-1}. However, the type and concentration of mycotoxin, and the food matrix studied followed a different approach, which justifies the differences observed in the current study. Such variation could be linked to the occurrence of specific binding sites to prebiotics that prevents binding with aflatoxins at the highest concentration.

Time had no effect on bioaccessibility ($p > 0.10$) which is interesting when considering a minimum time for the milk decontamination process. On the other hand, the decontamination process by *L. acidophilus* on AFB_1 contaminated whole milk was significantly influenced by time (Wochner et al., 2019). The differences could be linked to the AFB_1 concentration studied and the potential for *Lactobacillus* strain binding to mycotoxins.

Although run 1 (inulin) and 2 (oligofructose) showed the lowest bioaccessibility values, inulin, oligofructose, and polydextrose

Table 3. Effect of the variables studied in the *Plackett-Burman* Design on the AFB_1 reduction and bioaccessibility.

Variables	Effect	Standard deviation	t(7)	*p*-value	Effect	Standard deviation	t(7)	*p*-value
	AFB_1 reduction				*AFB_1 bioaccessibility*			
Mean	16.18	5.40	2.99	0.020*	27.49	1.67	16.46	0.000*
AFB_1 concentration (μg L^{-1})	-1.06	11.70	-0.09	0.930	-14.11	3.62	-3.90	0.006*
Time (h)	-12.14	11.70	-1.04	0.334	-2.95	3.62	-0.82	0.441
Inulin (%)	1.34	11.70	0.11	0.912	4.41	3.62	1.22	0.262
Oligofructose (%)	-0.22	12.58	-0.02	0.987	4.71	3.89	1.21	0.265
β-glucan (%)	9.20	11.70	0.79	0.458	7.28	3.62	2.01	0.084*
Polydextrose (%)	-9.43	11.70	-0.81	0.447	3.89	3.62	1.08	0.318

* $P < 0.10$.

prebiotics had no significant effect within the studied range, which reinforces a greater effect of aflatoxins concentration than prebiotics. Runs 1 and 2 carried out with higher AFB_1 concentrations (10.0 μg L^{-1}) showed bioaccessibility rates from 15.62% to 30.76%, lower values compared to those obtained with 5.0 g L^{-1} AFB_1 concentration (26.53% to 51.09%). Likewise, Meca et al. (2012) when studying soluble dietary fibers added to the model solutions reported a large beauvericin bioaccessibility reduction to a higher mycotoxin concentration (25 mg L^{-1}).

Run 8 was ideal for aflatoxin concentration reduction. However, the higher bioaccessibility (51.09%) obtained in this run may allow the body to absorb the available AFB_1. It was noted that a higher AFB_1 reduction is not always followed by a lower bioaccessibility, which justifies the choice of runs where low bioaccessibility prevails over a higher mycotoxin concentration reduction, considering the aflatoxins cumulative effect in the body. The use of prebiotics inulin or oligofructose (0.75%) plays an important role in bioaccessibility reduction, confirmed by the percentages obtained in run 1 and 2. Despite the effects of probiotics in AFB_1 reduction are extensively mentioned, (Bovo et al., 2014; El-Nezami et al., 1998; Fazeli et al., 2009; Kabak et al., 2009; Wochner et al., 2019) their combination with prebiotics is an alternative, especially on reducing bioaccessibility.

4 Conclusion

The sharpest AFB_1 reduction (56%) occurred by adding *L. plantarum* individually or combined with inulin, oligofructose and β-glucan. The lowest bioaccessibility occurred by adding inulin or oligofructose individually with the probiotic. It was noted that a sharper AFB_1 reduction resulted in a higher bioaccessibility rate, which was in this case, the prevalent factor. In this respect, the optimal experimental condition was achieved using a 10.0 μg L^{-1} AFB_1 concentration added with *L. plantarum* and inulin (0.75%) or oligofructose (0.75%) ensuring a < 16% final bioaccessibility. The results portrayed a safe decontamination procedure with milk production chain application potential.

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