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Research article

Prebiotic galacto-oligosaccharides improve gut microbiota and secretory immunoglobulins in Sprague Dawley rats

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Abstract: Prebiotic galacto-oligosaccharides (GOS) impart a wide array of health effects on gut microbiota and subsequently improve the host's immunological response; however, their prebiotic potential is affected by several factors, including dose, duration, composition, etc. This study used commercial GOS to evaluate their impact on selected gut microbial populations and secretory immunoglobulin A (IgA). The GOS were supplemented in the basal diet (1% w/w) fed to male Sprague–Dawley rats. Physical parameters such as body weight and organ weights, serum biochemical analysis, and cecal microbial population were determined after 4 weeks of intervention. GOS alone significantly increased bifidobacteria and lactobacilli growth in the intervention group ($p < 0.05$) without changing total bacteria after 4 weeks. Clostridia and coliform counts were significantly reduced only in the GOS group. Bifidobacteria also improved significantly over time, whereas all other microbes remained unaffected by time alone or by diet. The parameters of blood chemistry, serum glucose, AST and ALT, TC, LDL, and TG were not affected significantly ($p > 0.05$) by GOS addition in the diet; however, HDL improved significantly. GOS significantly improved sIgA in cecal and ileal contents ($p < 0.05$) compared to the control group. These findings indicate that GOS supplementation may exert prebiotic effects by increasing selected beneficial bacterial populations and intestinal sIgA levels in animal models.

Keywords: galacto-oligosaccharide; immunoglobulins; sIgA; bifidobacteria; prebiotics

1. Introduction

Functional foods are specific types of foods that, in addition to providing basic nutrition, are involved in the provision of health assistance with a focus on gut health, along with lowering the possibility of diseases and improving the overall health of consumers [1]. The balance of microorganisms in the gastrointestinal tract (GIT) is very important for smooth functioning and the host's overall health. This explains why prebiotics, probiotics, or their combination, synbiotics, have received more attention in the last few years and are being added as food additives in various recipes [2] or produced in food products [3]. Galacto-oligosaccharide (GOS) is a class of prebiotics that positively improve gut microbiota and tightly maintain a balance between beneficial and pathogenic microorganisms in the GIT [4], preventing gut microbiota dysbiosis [5]. The quantity of GOS known to provide bifidogenic effects is higher than 2.5 g per day [6] but can vary depending on age, body weight, and health. Furthermore, the bifidogenic impact of prebiotics is mostly dependent on the type of glycosidic linkage, degree of polymerization, and complexity, as enhanced bifidogenic effects were achieved using a GOS mixture (3.6 g/d) consisting of β-(1→3), β-(1→6), and β-(1→4) linkages as compared to one linkage containing (4.9 g/d), mainly β-(1→4), as well as β-(1→6), after 1 week of consumption [7]. The literature indicates that beta-galactosidase from yeast strains, mainly *Sporobolomyces singularis* and *Kluyveromyces lactis,* produces GOS with β-(1→4) glycosidic linkage, while lactobacilli strains like *Lactobacillus plantarum* [8] and *L. sakei* [9] produce β-(1→3)- and β-(1→6)-containing GOS. Most of the consumed GOS remain unaffected by digestion in the GIT and reach the colon, where they undergo fermentation by selective bacteria [10]. Short-chain fatty acids (SCFA), being end products after fermentation of prebiotics, are involved in several health aspects, including colon environment refinement, selective stimulation of growth of beneficial bacteria, mainly bifidobacteria and lactobacilli in the gut, distribution of energy to colon epithelium, cancer prevention [11,12], lipid profile management, improvement of the immune system, and mineral absorption [13], especially regarding calcium and magnesium.

Healthy microorganisms, improved by prebiotics, are involved in strengthening immunity by fighting against pathogens, including viruses and bacteria, and combating stress in the body and lower GIT problems like inflammatory bowel diseases. These prebiotics not only improve metabolic issues like metabolic syndrome [14] and mineral absorption [13] but also have a significant effect on colorectal cancer prevention [11,12]. Furthermore, GOS also helps to decrease the number of harmful bacteria like Bacteroides and *Clostridium perfringens* and ultimately improves host health and well-being [15].

The defensive role of intestinal mucosa in avoiding attachment of pathogenic bacteria and viruses to gut epithelial cells is provided by secretory immunoglobulin A (sIgA) [16]. The sIgA is considered an antibody that is available in large amounts and present in the intestinal lumen of humans, primarily protecting against pathogens. The sIgA stimulates the removal of pathogens and antigens from the intestinal lumen by hindering their contact with epithelial receptors, entangling them in mucus; this helps to exclude them, and specific activities like peristaltic and mucociliary movements are involved [17]. Prebiotics can play a role in raising sIgA production by improving the intestinal environment by increasing bifidobacteria [18]. Functional foods like prebiotics may increase the production of sIgA and reduce the attachment of pathogens in the GIT, resulting in a reduced burden of diseases caused by pathogenic microbes. Keeping in view the importance of prebiotic GOS as a functional ingredient, the present research was designed with the main objective of evaluating its efficacy in the intestinal microbial ecology of Sprague–Dawley rats and in enhancing immunomodulatory properties.

2. Materials and methods

2.1. Chemicals and substrates

All chemicals were of analytical or gas chromatography grade and were obtained from Merck and Sigma. The β-GOS (i.e., OLIGOMATE® 55N) used in this study was provided as a courtesy by Yakult Pharmaceutical Industry Co., Japan, and contains 4'galactosyllactose (4'-GL) as its main constituent, followed by residual lactose and glucose. The minimum GOS concentration in the final product was 55%.

2.2. Animal housing, treatment, and sample collection

A total of 32 male Sprague–Dawley rats (5 weeks old) were purchased from the National Institute of Health, Islamabad. Rats were kept in an animal room of the institute under controlled conditions, light-dark cycles of 12 h, relative humidity of 55% ± 5%, and temperature of 23 ± 2 ºC. The animals were handled as per guidelines approved by the Institutional Ethical Review Committee for Animal Care (No. ERC-0642 dated 08-02-2023). Rats were allowed to consume water and a standard pellet diet ad libitum. Before the experiment, rats were acclimatized to the environment for 1 week. Then, 32 rats were randomly divided into two dietary treatments: a control group and a GOS group, with 16 rats in each treatment. Rats were housed four per cage, with four cages assigned to each dietary treatment. The control group was fed a basal diet (composition given in Table 1), while the second group was fed the GOS diet (basal diet containing 1% w/w actual GOS, 1.8% commercial product) in replacement of corn starch. The same dietary treatment was maintained for each cage throughout the experimental period. At week 1, two rats from each cage were euthanized with CO_2 for sample collection, while the remaining two rats from each cage were maintained until week 4 for subsequent sample collection. Blood samples were taken through a heart puncture technique, and rats were then decapitated. Blood samples and serum samples were separated into different tubes, followed by the collection of organs and storage at −20℃. The cecal digest was divided into two portions before storage.

Table 1. Formulation and nutritional composition of the basal diet for animal study.

Ingredients	g/kg	Composition (%)	Percent ± SD
Casein	220	Moisture	9.99 ± 0.05
Sucrose	50	Dry matter	90.17 ± 0.14
Corn starch	600	*Crude protein	20.07 ± 0.09
Cellulose	30	*Ether extract	5.4 ± 0.05
Soybean oil	50	*Crude fiber	2.67 ± 0.09
Vitamins mix	10	*Ash	4.74 ± 0.07
Minerals mix	40	*NFE	66.96 ± 0.09

Note: NFE: Nitrogen-free extract; *: on dry weight basis.

2.3. Body and organ weight

Body and organ weights were measured after 1 and 4 weeks. Organ weights, particularly liver, spleen, and kidney, were cleaned and subjected to weight measurements utilizing an electronic balance.

2.4. Serum biochemical analysis

For the determination of serum metabolites that involve glucose (cat. no. 3981), total cholesterol (TC) (cat. no. CH200), high-density lipoproteins (HDL) (cat. no. CH203), triglycerides (TG) (cat. no. TRI210), and low-density lipoproteins (LDL) were calculated using the Friedewald equation LDL = TC − HDL − (0.2 × TG). For liver enzymes like alanine aminotransferase (ALT) (cat. no. AL1205), aspartate aminotransferase (AST) (cat. no. AS1204), and total protein (cat. no. TP4001), based on the Biuret method, commercial diagnostic kits were utilized (Randox Laboratories Ltd., Antrim, UK) according to the manufacturer's instructions.

2.5. Cecal microbial populations

For calculating microbial populations in cecal samples of rats, the plate culture method was employed [19]. A sample (1 g) was taken, followed by 10-fold serial dilutions in saline solution. After dilution, a 100 µL sample was inoculated onto Petri plates containing selective media. Specific types of media for different bacterial growth, and specific conditions for temperature and time, are mentioned in Table 2. The calculations for colony-forming units (cfu) were performed, and $\log^{10}$ values were utilized for statistical purposes. All samples' readings were taken in triplicate.

Table 2. Culture conditions for the selected bacterial groups.

Bacterial group	Medium	Atmosphere	Temperature	Time	Enumeration
Bifidobacteria	Bifidobacterium agar	Anaerobic*	37°C	48 h	CFU/g
Lactobacilli	MRS	Anaerobic	37°C	48 h	CFU/g
Clostridia	Reinforced clostridial agar	Anaerobic	37°C	40–48 h	CFU/g
Coliforms	MacConkey	Aerobic	37°C	24 h	CFU/g
Total bacteria	Plate count agar	Aerobic	30°C	48–72 h	CFU/g

Note: *: Strict anaerobic conditions in a jar containing anaerobic sachets (Oxoid, UK) were used.

2.6. Secretory immunoglobulin A (sIgA)

Serum immunoglobulin levels and IgA in cecal content were measured using commercial ELISA kits (cat. no. E0235Ra, Bioassay Technology, Shanghai, China) for rats according to the manufacturer's protocols. Cecal (140 mg) and ileal (10 mg) contents were centrifuged (4000 × g, 30 min, 20°C), then mixed and incubated with bovine serum albumin (BSA) in PBS to collect supernatants, which were kept at −20°C. For coating 96-well microtiter plates, 100 µL of rabbit anti-rat secretory component antibody was added, previously diluted to 1:1000 and incubated at 4°C overnight. Before application of samples, blocking was done with BSA (40 g/L) in Tween solution for approximately 1 h, followed by washing. Further addition of 100 µL of horseradish peroxidase-conjugated goat monoclonal anti-rat IgA solution was done after 2 h of incubation at 37°C. After another 2-h incubation period, peroxidase substrate was added, and incubation was done for 30 min in the dark. The stop solution for the enzymatic reaction was 1 mol/L H_3PO_4. Absorbance at wavelength 450 nm was utilized for the estimation of sIgA.

2.7. Statistical analysis

The data were represented as mean ± SE. For the animal study, resulting organ weights, body weights, and cecal and ileal sIgA means were compared using a Student's t-test. For bacterial populations, the cage was considered the experimental unit for dietary treatment because the diet was assigned at the cage level, with four cages allocated to each dietary treatment. Two rats from each cage were sampled at week 1, while the remaining two rats from the same cages were sampled in week 4. The two rats sampled from each cage at each time point were therefore considered subsamples of the corresponding cage. Cage-level mean values were used for statistical analysis. A two-factor analysis of variance was performed, with dietary treatment (control and GOS) as the between-cage factor and sampling time (1 and 4 weeks) as the within-cage factor, including the diet × time interaction. Statistical significance was considered at $p < 0.05$.

3. Results and discussion

3.1. Effect of GOS supplementation on body weights and organ weights

There were no significant changes ($p > 0.05$) in body weight and internal organ weight depending on treatment group after 4 weeks (Table 3). These findings indicate that GOS did not affect organ weight during the study period. The prebiotic GOS is undigestible due to its β-configuration in glycosidic linkage, providing less than 50% of energy per gram as compared to carbohydrates, as its main purpose is to improve beneficial bacteria instead of providing calories. Impure prebiotic GOS at up to 5% in the diet of Sprague–Dawley rats had no significant impact on body weight, even after 4 weeks [13]. In the current study, only 55% pure GOS was used; even such high-purity GOS does not show any significant improvement in the weight of the liver, spleen, and kidney in relation to the body mass of rats [20].

Table 3. Effect of prebiotic GOS on body and organ weights in grams/100 g of rat after 4 weeks.

Treatments	Initial body weight	Final body weight	Liver	Kidney	Spleen
Control	108.78 ± 3.86	234.68 ± 2.22	8.82 ± 0.32	2.26 ± 0.09	0.68 ± 0.04
GOS	106.36 ± 4.21	235.70 ± 1.77	8.36 ± 0.42	2.27 ± 0.05	0.62 ± 0.09

Note: Values are expressed as mean ± standard error. N = 8 animals.

3.2. Effect of GOS on serum lipid profile

The effect of prebiotics on the serum lipid profile is variable, as some animal models show that the utilization of functional foods with prebiotics [21] and probiotics lowers TC, TG, and LDL, while increasing HDL; in humans, opposite and variable results were found [22]. This effect seems to be modulated by several factors, including dose, type of pre- and probiotics, overall composition of the functional food, duration of use, health condition of the consumer, and lifestyle factors. Table 4 shows that a diet with only 1% GOS for 4 weeks significantly ($p < 0.05$) improved rats' HDL in serum as compared to the control group. Various studies confirm that prebiotics, including GOS, reduce hypercholesterolemia and increase good cholesterol in a dose-dependent manner through several

mechanisms, including lower reabsorption of bile salts in enterohepatic circulation, increased production of SCFA after fermentation by beneficial bacteria, low glycemic response due to the complex nature, and increased growth of probiotics [23]. Further, probiotics have proven hypocholesterolemic effects, with several modes of action [24]. Here, serum levels of glucose, AST and ALT, TC, LDL, and TG showed nonsignificant results ($p > 0.05$) compared to the control after 4 weeks of intervention.

Table 4. Effect of GOS on serum glucose, AST, ALT, total protein, and lipid profile of rats after four weeks.

Groups	Glucose (mg/dL)	Total protein (g/L)	AST (U/L)	ALT (U/L)	TC (mg/dL)	HDL (mg/dL)	LDL (mg/dL)	TG (mg/dL)
Control	109.5 ± 2.7	17.9 ± 1.0	73.9 ± 1.3	18.3 ± 1.1	78.5 ± 2.8	51.8 ± 3.0[b]	11.0 ± 0.7	76.3 ± 1.0
GOS	110.8 ± 4.0	18.8 ± 0.6	74.4 ± 3.1	18.8 ± 0.6	76.3 ± 4.2	53.1 ± 2.5[a]	11.0 ± 1.0	75.9 ± 1.5

Note: Values are shown as mean ± standard error, N = 8 animals; means showing different letters inside the column are significantly different.

The prebiotic's impact on cholesterol levels might be more pronounced when given to hypercholesterolemic subjects at a high dose of 20 g inulin/day for 3 weeks [25,26]. Various studies have found an improvement in lipid metabolism in humans, although some show opposite results both in humans and in animal trials. Alliet et al. [27] revealed no significant response in lipid profiles after utilizing GOS and FOS in infant formula. Another work [28] also revealed a nonsignificant effect, even after prolonged exposure for 6 months, of inulin-type fructans on the lipid profile in human subjects. Similarly, Giacco, Clemente, and Luongo et al. [26] reported no significant response of FOS at 10 g/day after two months of use in human subjects.

3.3. Effect of GOS incorporation on selected bacterial populations

Gut health can be improved by the consumption of prebiotics in the diet, as there is an improvement in selected culturable bacterial populations. The prebiotics reach the colon without digestion in the upper GIT and are selectively fermented by healthy microbes, resulting in the growth of beneficial bacterial populations. These beneficial gut microbes play important roles in the gut fermentation and metabolism process and produce vitamins, antioxidants, SCFA [29], and immunoglobulins [18]. They have a protective effect against harmful opponents and exchange signals at the molecular level among varied genera and intestinal epithelial cells [30], ultimately enhancing the consumers' health. In the current study, results revealed that total bifidobacterial and lactobacilli counts were significantly ($p < 0.05$) improved by GOS as compared to the control after 4 weeks (Table 5). This further confirms the prebiotic role of GOS, which increases the population of beneficial bacteria in the gut. There are multiple evidence that pre- and probiotics result in health benefits ranging from increased absorption of minerals [13,31] to cancer protective and preventive roles [11,12,32].

To obtain bifidogenic and other benefits, the dose, purity of prebiotics, and duration of intervention are critical. GOS at high purity (1%) not only improves the growth of bifidobacteria in mice but also enhances the gene expression of glucagon-like peptide-1 and peptide YY, responsible for decreased blood pressure and serum cholesterol [29]. Similarly, Santos et al. [33] evaluated the

bifidogenic effect of eight different types of prebiotics and noticed that only 1% oligosaccharides can quickly improve healthy bacteria; however, this impact can be reversed by a basal diet.

Human studies show similar trends toward bifidobacteria and lactobacilli growth, but the former is more linked and dependent on substrates like prebiotics. That can be significant from a health point of view, to define dietary approaches for specific microbiota in the gut; particular organisms grow with specific carbon sources and can then compete with other groups of bacteria. Also, the degree of conversion of the substrate into bacterial cell mass, as prebiotic and probiotic activity, is affected by the size of the cell population. The biological activity of oligosaccharides is influenced by their structural characteristics, including monosaccharide composition, degree of polymerization, glycosidic linkage, and branching pattern. Structural differences can affect the accessibility and utilization of oligosaccharides by specific intestinal microorganisms, consequently influencing microbial and host responses. Recent studies have demonstrated structure-dependent differences in the biological effects of oligosaccharides, including effects on gut microbial composition and intestinal inflammatory responses [34]. The oligomate product utilized in the current study mainly offers β-(1→4) linkages. The results in Table 5 show that lactobacilli are significantly improved by GOS compared to the control, but no effect of time was observed. This clearly shows that GOS affects lactobacilli growth, but as the population has not increased or decreased throughout the duration of the study, time has not contributed much to its growth. The combination of bifidobacteria along with some *Lactobacillus* species is considered to play a significant part in the eco-physiology of the colonic microbiota, as they are beneficial and impart health effects by improving immunity and resistance to infection and diarrheal diseases [10].

In the present study, total bacteria showed no significant differences between diets, not even after the intervention period. Clostridia and coliforms tended to get significantly lower ($p < 0.05$) compared to the control group. The increased population of bifidobacteria can be helpful, as it lowers the performance and number of clostridia, considered putrefactive bacteria, so the resultant toxic fermented products are reduced [35]. Due to its selectively fermentable characteristics, prebiotic GOS suppresses the growth of pathogenic microbes like *Clostridium difficile* and reduces toxin production in in-vitro fermentation [36] and rat studies [11].

Table 5. Effect of galacto-oligosaccharide on cecal microbiota population.

Bacterial groups	Bacterial numbers		Statistical effect		
	Control	GOS	Diet	Time	Diet × Time
Bifidobacteria					
1 week	7.41 ± 0.24[b]	7.45 ± 0.20[a]	*	*	*
4 weeks	8.63 ± 0.20[a]	9.85 ± 0.29[b]			
Lactobacilli					
1 week	8.93 ± 0.80[b]	9.46 ± 0.20[a]	*	*	*
4 weeks	9.03 ± 0.39[b]	9.66 ± 0.96[a]			
Total bacteria					
1 week	10.67±1.29[a]	10.82 ± 0.32[a]	ns	ns	ns
4 weeks	10.73 ±0.23[a]	10.73 ±0.23[a]			
Clostridia					
1 week	7.82 ± 0.25[a]	7.34 ± 0.59[b]	*	ns	ns
4 weeks	8.03 ± 0.28[a]	6.81 ± 0.27[b]			
Coliforms					
1 week	6.08 ± 0.60[a]	5.3± 0.43[b]	*	ns	ns
4 weeks	5.76 ± 0.37[a]	5.15 ± 0.31[b]			

Note: Values are expressed as mean ± standard error of four cages per dietary treatment. The two rats sampled from each cage at each time point were averaged to obtain the cage-level value. * shows statistical significance ($p < 0.05$).

3.4. Effect of GOS incorporation on cecal and ileal sIgA

Different prebiotics can show varied responses related to microbiota, metabolites, and other biological functions due to varied structure, purity, dose, and duration of use. GOS can be beneficial in terms of the immune response it offers by increasing gram-positive bacteria and lowering gram-negative bacteria in the GIT, along with elevated fecal sIgA [37].

The role of sIgA is very important and related to immunity enhancement in the body. Prebiotics affect the mucosa-associated immune system, and sIgA is considered to be present in large numbers in the mucosa of the intestine. The ability of sIgA to provide defense against toxins and pathogenic organisms by blocking their approach to epithelial receptors [38], and removing and deactivating viruses, is related to its amount in feces. Table 6 shows that rats fed GOS had significantly increased sIgA in cecal and ileal content ($p < 0.05$) in comparison to the control group after 4 weeks. This is similar to the study by Vulevic et al. [37], in which B-GOS was administered to overweight human subjects at 5.5 g/day for 12 weeks and raised fecal sIgA along with an improved lipid profile. Similarly, Faseleh et al. [39] revealed that prebiotics (OligoPKC) increased IgA and other immunoglobulins in serum and cecal contents of male rats after 4 weeks. The immunological effect of GOS is due to its fermentability and production of beneficial bacteria, bifidobacteria and lactobacilli [40], in the colon. Prebiotics can indirectly play a role in sIgA concentration by digesting it in the caecum, affecting bacterial strains like clostridia that are considered to be involved in deteriorating IgA [41] due to the possession of protease action, which has an ideal pH within the neutral range. Hence, raised cecal secretion of IgA might be due to enhanced cecal bifidobacteria and lactobacilli (Table 5). The increase in sIgA observed following GOS supplementation occurred along changes in selected bacterial

populations. Previous studies have suggested that prebiotic-associated changes in the intestinal microbial environment may influence mucosal IgA responses [42]. However, SCFAs, intestinal pH, and other potential immunological mediators were not measured in the present study; therefore, the observed increase in sIgA should be interpreted as an association rather than evidence of a direct mechanistic relationship.

Table 6. Effect of GOS on sIgA levels in cecal and ileal samples.

Immunoglobulin	Control	GOS
sIgA (μg/g cecal content)	230.16 ± 17.26^{b}	321.66 ± 21.14^{a}
sIgA (μg/g ileal content)	227.66 ± 15.38^{b}	319 ± 18.72^{a}

Note: Values are mean ± standard error of eight biological replicates. Means with different letters between columns differ significantly ($p < 0.05$).

4. Conclusions

The present study evaluated the effects of adding commercial galacto-oligosaccharides to the diet of healthy young male Sprague–Dawley rats and revealed that GOS significantly improved selected beneficial culturable bacterial populations, particularly bifidobacteria and lactobacilli, while reducing clostridia and coliform populations. Serum HDL levels increased significantly relative to control and other lipid markers. The research findings for immune parameters showed that GOS supplementation significantly increased sIgA levels in cecal and ileal content after 4 weeks. The present study evaluated specific culturable bacterial populations, while the specific mechanisms underlying the observed increase in sIgA were not investigated. Therefore, these findings should be interpreted within the context of animal model and 4-week intervention. Further studies, including mechanistic investigations and appropriately designed human clinical trials, are required to establish the relevance of these findings to human health.

5. Study limitations

First, the microbiological assessment was based on culture-dependent enumeration of specific bacterial groups and therefore does not represent the complete intestinal microbial community or its diversity. Second, detailed structural characterization of the GOS, including degree of polymerization, molecular weight distribution, and individual composition, was not performed in the present study; these characteristics were therefore described based on available manufacturer and published information. Third, although changes in selected bacterial populations occurred with changes in sIgA, potential mechanistic mediators such as short-chain fatty acids, intestinal pH, and cytokines were not measured, and therefore causal relationships cannot be established.

Author contributions

Amna Ashraf Bajwa conducted the trial, collected the data, and drafted the initial manuscript; Sanaullah Iqbal conceptualized, designed the study, supervised, and reviewed the manuscript; Muhammad Nasir performed the statistical analysis and critically reviewed the manuscript; Aftab

Ahmad Anjum performed the microbiology trial, critically reviewed the manuscript, and gave significant inputs. All authors contributed to manuscript revisions and approved the final version.

Use of AI tools declaration

The authors declare they have not used Artificial Intelligence (AI) tools in the creation of this article.

Ethics approval and consent to participate

Ethical approval was obtained from the Institutional Ethical Review Committee for Animal Care (No. ERC-0642 dated 08-02-2023). Consent to participate is not applicable.

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Conflict of interest

The authors declare no conflict of interest.

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