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ORIGINAL RESEARCH ARTICLE

The abundant free-living amoeba, *Acanthamoeba polyphaga*, increases the survival of *Campylobacter jejuni* in milk and orange juice

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Background: *Campylobacter jejuni* is a common cause of human bacterial diarrhea in most parts of the world. Most *C. jejuni* infections are acquired from contaminated poultry, milk, and water. Due to health care costs and human suffering, it is important to identify all possible sources of infection. Unpasteurized milk has been associated with several outbreaks of *C. jejuni* infection. *Campylobacter* has been identified on fresh fruit, and other gastrointestinal pathogens such as *Salmonella, E. coli* O157:H7 and *Cryptosporidium* have been involved in fruit juice outbreaks. *C. jejuni* is sensitive to the acidic environment of fruit juice, but co-cultures with the amoeba, *Acanthamoeba polyphaga*, have previously been shown to protect *C. jejuni* at low pH.

Methods: To study the influence of *A. polyphaga* on the survival of *C. jejuni* in milk and juice, the bacteria were incubated in the two products at room temperature and at 4°C with the following treatments: A) *C. jejuni* preincubated with *A. polyphaga* before the addition of product, B) *C. jejuni* mixed with *A. polyphaga* after the addition of product, and C) *C. jejuni* in product without *A. polyphaga*. Bacterial survival was assessed by colony counts on blood agar plates.

Results: Co-culture with *A. polyphaga* prolonged the *C. jejuni* survival both in milk and juice. The effect of co-culture was most pronounced in juice stored at room temperature. On the other hand, *A. polyphaga* did not have any effect on *C. jejuni* survival during pasteurization of milk or orange juice, indicating that this is a good method for eliminating *C. jejuni* in these products.

Conclusion: Amoebae-associated *C. jejuni* in milk and juice might cause *C. jejuni* infections.

Keywords: *unpasteurized milk*; *fruit juice*; C. jejuni *infection*; *co-culture*; Campylobacter *survival*; *gastrointestinal pathogens*

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Campylobacter jejuni is a leading cause of bacterial, diarrheal disease worldwide (1–3). The majority of *Campylobacter* cases are sporadic, and although poultry is believed to be the main source of infection many other important sources, such as unpasteurized milk, water, and fresh fruit juice, have been identified (4). Although uncommon, outbreaks mainly result from consumption of unpasteurized milk, poultry meat, and contaminated water (5). *C. jejuni* outbreaks from unpasteurized milk are often the result of farmers serving visitors raw milk (6, 7). There are also frequent outbreak reports from certain districts in the United States, where it is legal to sell unpasteurized milk for commercial purposes (8). Due to healthcare costs and human suffering (9–11), it is important to identify all possible sources of infection. Recently, acidic fruit juices such as orange and apple juices have been found to be responsible for multiple outbreaks of *Salmonella*, *E. coli* O157:H7, and *Cryptosporidium* infections (12–14). The consumer's desire for a healthy lifestyle and their demand for fresh produce have with certainty contributed to a number of outbreaks involving unpasteurized juice (15–17). A study from Sobel

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et al. (18) showed an association between children with diarrhea and consumption of freshly squeezed juice. *Campylobacter* has been shown to survive on fresh produce, and eating raw vegetables and fruit has been identified as a risk factor for *Campylobacter* infections (19–22). Little is known about *Campylobacter* contamination of fresh squeezed juice or juice products. In contrast to many other foodborne pathogens, *C. jejuni* is more sensitive to environmental conditions such as aerobic and acidic stress (20, 23). On the other hand, we have previously shown that *C. jejuni* can survive and multiply within free-living amoebae of the genus *Acanthamoeba* and furthermore that co-cultures with *Acanthamoeba polyphaga* can protect *C. jejuni* from acid environments (24–26). *Acanthamoeba* spp. are widespread in various environments, including water, and have been isolated from water distribution systems and potable water around the world (27, 28). The use of potable water both in industry, when producing juice ready for immediate consumption (29), and in households, when adding water to juice concentrate, would make it possible to find *Acanthamoeba* spp. in juice. In harsh conditions, *Acanthamoeba* trophozoites in their vegetative form can transform into a double-walled cyst that are highly resistant to chlorination, antimicrobials, and disinfectants, as well as to changes in pH and osmolarity. Their resistance against disinfection agents (30, 31) and their ability to attach to various surfaces of different origin (32–35) further promote acanthamoebae survival on equipment used for the production/preparation of food. In this study, we have investigated the protective effect of *A. polyphaga* on *C. jejuni* in milk and orange juice. In both products, we found significantly higher bacterial survival in co-cultures, compared to when *C. jejuni* was incubated alone.

Materials and methods

Bacterial and amoebal cultures

The *C. jejuni* strain CCUG 11284 and the *A. polyphaga* strain (Linc Ap-1) were used in all experiments. CCUG 11284 is a wild-type strain that was originally isolated from bovine feces. Before each experiment, bacteria were grown on conventional blood agar plates (Columbia agar II containing 8% vol/vol whole horse blood) at 42°C for 20 h in a microaerobic environment, using a CampyGen gas generating system (CN0025A; Oxoid Ltd., Basingstoke, UK) and a BBL GasPak system (BD, Franklin Lakes, NJ). Bacterial cells were harvested and diluted in a peptone-yeast extract-glucose (PYG) medium and used as stock solution for all treatments. The stock solution was striven to obtain a concentration of approximately 10^7 CFU/ml, as were detected by plate counting. *A. polyphaga* stock cultures were maintained in PYG medium at 27°C in 75 cm^2 culture flasks (Sarstedt, Nürnbrecht, Germany), as described by Axelsson-Olsson et al. (24). For the experiments, *A. polyphaga* were seeded into 12-well culture plates (Fischer Scientific GTF AB, Switzerland) in PYG medium (1 ml/well) and incubated at 27°C for 24 h, until the trophozoites formed confluent layers at the bottom of the wells. Commercially available milk with a pH of 6.4 (protein 3.4 g, sugar 5 g, fat 1.5 g, Ca 120 mg, vitamin A 25 μg, vitamin D 0.38 μg) and orange juice with a pH of 3.9 (protein 0.7 g, sugar 18 g, fat < 0.5 g, Na 0.003 g, vitamin C 30 mg) were used for all experiments. Bolton Broth selective supplement (SR0208; Oxoid Ltd., Basingstoke, UK) was added to the products to inhibit growth of other bacteria than *C. jejuni*.

Survival of C. jejuni *cells co-incubated with* A. polyphaga *in milk and orange juice stored at room temperature and 4°C*

To mimic the conditions of storage in the fridge or at the bench, experiments were incubated at room temperature and 4°C. To test whether the presence of amoeba in two different beverage products, milk and orange juice, influenced the survival of *C. jejuni*, the following three treatments were used: *C. jejuni* preincubated with *A. polyphaga* before the addition of product (treatment A), *C. jejuni* mixed with *A. polyphaga* after the addition of product (treatment B), and *C. jejuni* in product without *A. polyphaga* (treatment C).

For treatment A, 12-well plates with confluent *A. polyphaga* layers in PYG medium were inoculated with 100 μl of the *C. jejuni* stock solution, generating a concentration of 10^6 CFU/ml and a multiplicity of infection (MOI) of one bacteria per amoeba, in each well. Before inoculation with *C. jejuni*, the medium in all wells were gently removed and replaced with 1 ml fresh PYG medium. The plates were incubated for 3 h at 32°C to allow the bacterial cells to attach to and invade amoebae, and thereafter the PYG medium was gently removed and replaced by 2 ml of product. This resulted in an approximately 2-log decrease in bacterial concentration. For treatment B, plates with confluent *A. polyphaga* were prepared by gently removing the PYG medium and replacing it with 2 ml of product. For the control treatment (treatment C), plates without amoebae were prepared with 2 ml product. After the addition of product, the plates for treatment B and C were inoculated with 100 μl of the *C. jejuni* stock solution generating a concentration of 5×10^5 CFU/ml and an MOI of one bacteria per amoeba in treatment B, in each well. Three plates (treatments A–C) were incubated at room temperature and at 4°C, respectively. All plates were incubated in an aerobic environment and each treatment was done in triplicate wells, resulting in three similar wells for each temperature, treatment and product. From each well, a 100-μl sample was taken at time zero (the addition of product) and at 3, 6, 18, 24, and 48 h. All samples were 10-fold serially diluted in PYG medium and spread on blood agar plates for colony counting. Three independent experiments were performed on separate occasions.

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To make sure that the pH level was not affected, the pH level of the fluid in each well was measured after 48 h, when experiments were completed. Compared to initial pH (milk: 6.41 and juice: 3.89) only a small increase in pH was observed (milk: 0.4 and juice: 0.1).

Pasteurization of C. jejuni *cells co-incubated with A. polyphaga in milk and orange juice*

For pasteurization experiments, the same settings were used, as described above for treatments A, B, and C. Directly after the addition of product, samples of 100 µl were taken from the different treatments, A, B, and C and added to tubes containing 500 µl of milk or juice. The tubes were gently shaken at 1,400 rpm (MS2 Minishaker IKA®, Germany) and then incubated in a water bath (Heto DT Hetotherm, Denmark). Incubation conditions for milk tubes were 72–74°C for 15 sec (equivalent to Swedish low pasteurization guidelines). Incubation conditions for juice tubes were 85°C for 15 sec (equivalent to Swedish pasteurization guidelines). After heating, the sample tubes were put on ice and 100-µl samples were spread on blood agar for colony counting. All experiments were done in triplicates, resulting in three similar wells for each treatment and product.

Statistical analysis

For each well and each time point, a measure of *C. jejuni* cell survival was calculated by dividing the bacterial concentration of the sample (estimated from colony counts) by the bacterial concentration of that well at time 0 h (the addition of product). Statistical analysis was performed using Kruskal–Wallis test with Dunn's multiple comparison test. Data were analyzed using GraphPad Prism version 6 and $p < 0.05$ were considered significant.

Results

Survival of C. jejuni *co-incubated with* A. polyphaga *in milk or orange juice at room temperature*

The experimental setup included three different treatments (A, B, and C); see Materials and Methods section. In milk, the highest *C. jejuni* survival was seen in treatment A, where bacteria were pre-incubated with amoebae before addition of milk (2.8%, 18 h; 3.8%, 24 h; 0.8%, 48 h; Table 1; Fig. 1a). Treatment B showed 0.05% survival at 18 h and reached a fraction of $6.7 \times 10^{-5}$of the inoculum at 48 h (equivalent to 14 CFU/ml; Table 1). After 3 h, the survival of *C. jejuni* without amoebae (treatment C) decreased more rapidly compared to co-cultures (treatments A and B), and the fraction of the inoculum surviving after 18 h was only 3.5×10^{-5} (equivalent to 6 CFU/ml; Table 1). No bacteria could be detected after 24 h. At 18–48 h, treatment A had significantly higher bacterial survival than treatment C (Kruskal–Wallis test with Dunn's multiple comparison test and Bonferroni correction for multiple tests; 18 h, $p = 0.0003$; 24 h, $p < 0.0001$; 48 h, $p = 0.0007$; Fig. 1a).

C. jejuni incubated in juice showed similar trends as in milk albeit with more pronounced differences between co-cultures and bacteria incubated alone. The highest *C. jejuni* survival was seen in treatment A (88.4%, 18 h; 36.9%, 24 h; 0%, 48 h; Table 1; Fig. 1b) and the lowest survival was found in treatment C, *C. jejuni* without amoebae. After 3 h, the survival of *C. jejuni* incubated

Table 1. Mean fraction of the *C. jejuni* inoculum surviving after 18 h, 24 h, and 48 h for treatments A, B, and C when incubated in milk and juice at room temperature (RT) and at 4°C

	Fraction of the *C. jejuni* inoculum surviving at different time points		
Treatment	18 h	24 h	48 h
Milk RT			
A	0.028 (±0.029)	0.038 (±0.030)	8.1×10^{-3} ($\pm 8.5 \times 10^{-3}$)
B	5.2×10^{-4} ($\pm 5.1 \times 10^{-4}$)	5.1×10^{-4} ($\pm 2.7 \times 10^{-4}$)	6.7×10^{-5} ($\pm 7.5 \times 10^{-5}$)
C	3.5×10^{-5} ($\pm 5.5 \times 10^{-5}$)	0	0
Juice RT			
A	0.884 (±0.666)	0.369 (±0.298)	0
B	0.041 (±0.037)	0.020 (±0.011)	2.8×10^{-4} ($\pm 3.1 \times 10^{-4}$)
C	0	0	0
Milk 4°C			
A	0.903 (±0.515)	0.343 (±0.332)	4.1×10^{-3} ($\pm 3.9 \times 10^{-3}$)
B	0.192 (±0.093)	0.182 (±0.090)	6.8×10^{-3} (±0.017)
C	0.241 (±0.177)	0.201 (±0.053)	0.033 (±0.025)
Juice 4°C			
A	0.906 (±0.608)	0.567 (±0.042)	3.5×10^{-3} ($\pm 4.8 \times 10^{-3}$)
B	0.074 (±0.058)	0.034 (±0.023)	1.0×10^{-4} ($\pm 1.7 \times 10^{-4}$)
C	0.033 (±0.027)	0.020 (±0.027)	7.4×10^{-6} ($\pm 2.2 \times 10^{-5}$)

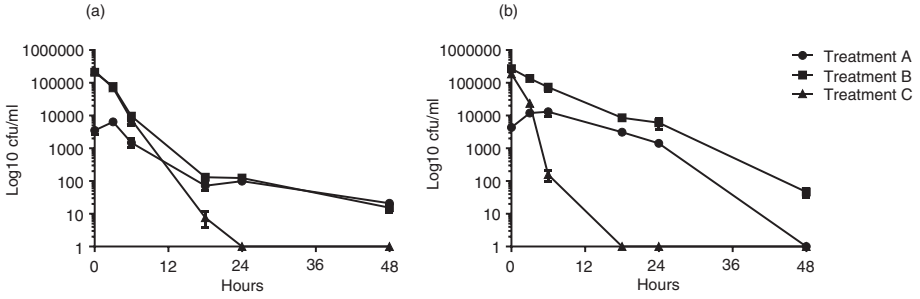

Fig. 1. Survival of *C. jejuni* co-incubated with *A. polyphaga* in milk (a) or orange juice (b) at room temperature after 0 h, 3 h, 6 h, 24 h, and 48 h. Data are based on three independent experiments with *C. jejuni* treated in three different ways treatment A (dots), *C. jejuni* preincubated with *A. polyphaga* before the addition of product; treatment B (squares), *C. jejuni* inoculated to *A. polyphaga* after the addition of product; and treatment C (triangles), *C. jejuni* in product without *A. polyphaga*. To use the log10 scale, a constant 1 had to be added to all cfu values to manage zeros. Means ± SEM (missing data points: one out of nine replicates for: milk treatment B at 0 h, milk treatment A at 3 h and juice treatment B at 3 h).

without amoebae decreased rapidly compared to co-cultures (treatments A and B) and no viable bacteria could be detected after 18 h (Table 1, Fig. 1b). The relative survival of *C. jejuni* in treatment B (4.1%, 18 h; 2.0%, 24 h; 0.02%, 48 h; Table 1) was lower than in treatment A but higher than in C. However, due to the higher start concentration in treatment B, compared to treatment A, the bacterial concentration in the juice was still 45 CFU/ml at 48 h. Statistically significant differences in survival were seen between: A and B (Kruskal–Wallis test with Dunn's multiple comparison test and Bonferroni correction for multiple tests: 18 h, $p = 0.0428$; 24 h, $p = 0.0428$; 48 h, $p = 0.0029$; A and C: 18 h, $p < 0.0001$; 24 h, $p < 0.0001$; B and C: 18 h, $p = 0.0428$; 24 h, $p = 0.0428$; 48 h, $p = 0.0029$; Fig. 1b; Table 1).

Survival of C. jejuni *co-incubated with* A. polyphaga *in milk and orange juice at 4°C*

At 4°C, *C. jejuni* generally survived better in both milk and juice and the differences were less pronounced between co-cultures and bacteria incubated alone. In milk, the highest *C. jejuni* survival was seen in treatment A for time point up to 24 h (90.3%, 18 h; 34.3%, 24 h; Table 1; Fig. 2a). At these time points, treatment C (24.1%, 18 h; 20.1%, 24 h; Table 1) gave a higher survival than treatment B (19.2%, 18 h; 18.2%, 24 h; Table 1). At 48 h, the highest *C. jejuni* survival was seen in treatment C (3.3% Table 1), and the survival in treatments A and B were 0.4 and 0.6%, respectively (Table 1). Statistically significant differences in survival were seen at 18 h between: A and B as well as A and C (Kruskal–Wallis test with Dunn's multiple comparison test and Bonferroni correction for multiple tests: A and B:18 h, $p = 0.001$; A and C: 18 h, $p = 0.0045$; Fig. 2a).

When *C. jejuni* were incubated in juice at 4°C, the differences seen between co-cultures (treatments A and B) and *C. jejuni* incubated without amoebae (treatment C), were similar to those seen in juice at room temperature, although the bacteria in treatment C survived longer compared to incubation at room temperature. The highest *C. jejuni* survival was seen in treatment A: 90.6%, 18 h; 56.7%, 24 h; 0.35%, 48 h; Table 1) and the lowest survival was found in treatment C: 3.3%, 18 h; 2.0%, 24 h; 0.00074%; 1 CFU/ml; 48 h; Table 1). The survival of *C. jejuni* in treatment B: 7.4%, 18 h; 3.4%, 24 h; 0.01%, equivalent to 18 CFU/ml; 48 h, Table 1) was lower than A but higher than C. Statistically significant differences in survival were seen at 18–24 h between A and B, and 18–48 h between A and C (Kruskal–Wallis test with Dunn's multiple comparison test and Bonferroni correction for multiple tests;

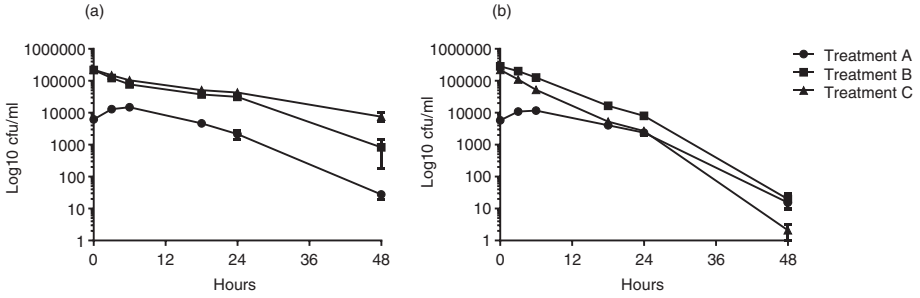

Fig. 2. Survival of *C. jejuni* co-incubated with *A. polyphaga* in milk (a) or orange juice (b) at 4°C after 0 h, 3 h, 6 h, 24 h, and 48 h. Data are based on three independent experiments with *C. jejuni* treated in three different ways treatment A (dots), *C. jejuni* preincubated with *A. polyphaga* before the addition of product; treatment B (squares), *C. jejuni* inoculated to *A. polyphaga* after the addition of product; and treatment C (triangles), *C. jejuni* in product without *A. polyphaga*. To use the log10 scale, a constant 1 had to be added to all cfu values to manage zeros. Means ± SEM (missing data points: one out of nine replicates for juice treatment C at 18 h).

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A and B: 18 h, $p = 0.0125$; 24 h, $p = 0.005$; A and C: 18 h, $p = 0.0001$; 24 h, $p = 0.0002$; 48 h, $p = 0.0489$; Fig. 2b).

Survival of C. jejuni *co-incubated with* A. polyphaga *at pasteurization temperatures*

A. polyphaga did not show any protective effect on *C. jejuni* when heated to recommended pasteurization temperatures, neither in milk nor in orange juice (data not shown). Growth was totally inhibited in all samples from treatments A, B, and C. Results were based on three independent experiments. Each experiment included three similar wells for each treatment and product.

Discussion

Campylobacter causes approximately 200,000 human infections annually in the European Union, with sometimes very severe chronic complications such as Guillain–Barre syndrome (GBS) (36, 37). In this study, we investigated the protective effect of the free-living amoeba *A. polyphaga* on *C. jejuni* survival in milk and orange juice. It is well known that drinking unpasteurized milk is a risk for acquiring *C. jejuni* infections (38); however, the consumption of unpasteurized milk and milk products (39, 40) as well as unpasteurized juices is common in many countries (12). The acidic pH in orange juice has been considered lethal to *C. jejuni* (23, 41), and therefore orange juices have not been considered a risk factor for acquiring campylobacteriosis. On the other hand, *C. jejuni* seem well adapted to survive the acidic milieu of the human stomach as well as disinfection with acid in poultry stables (42, 43). We have previously shown an increased acid tolerance of *C. jejuni* CCUG 11284 in co-cultures with *A. polyphaga* (26). Free-living amoebae are common inhabitants of potable water plumbing systems (44, 45) and fruit squeezing machines have surfaces that are difficult to clean, with possible formation of biofilms inhabited by amoebae as a result (32, 34–46). Hence, such systems could provide entry ports where the presence of amoeba could increase the *C. jejuni* survival and cause infections.

As differences in bacterial survival were observed in a previous study depending on weather the bacteria were added to *A. polyphaga* before (treatment A) or after addition of acidified medium (treatment B), we evaluated the effect of these two treatments in juice and milk (26). The effect of temperature was studied by incubation at room temperature and 4°C. We found significantly higher bacterial survival in co-cultures compared to when *C. jejuni* were incubated alone (treatment C). In both products and at both temperatures, the highest survival was found in co-cultures where *C. jejuni* were added to *A. polyphaga* before the addition of the product (treatment A). The effect of co-culture was most pronounced in juice stored at room temperature, as no *C. jejuni* survival was detected after 18 h in cultures with *C. jejuni* alone. Also milk stored at room temperature and juice stored at 4°C showed significantly higher bacterial survival in co-cultures, compared to when *C. jejuni* were incubated alone. However, in milk stored at 4°C the bacterial survival at 24 h was not significantly affected by co-culture with amoebae and at 48 h, *C. jejuni* incubated alone (treatment C) actually survived better than in co-culture. This is consistent with previous studies reporting good survival of *Campylobacter* in refrigerated milk (41, 47). In our previous study assessing *C. jejuni* survival in an acidified medium, we found the highest bacterial survival in co-cultures where *C. jejuni* were added after the addition of acidic media. In that study we found that the acid milieu triggered *C. jejuni* motility and uptake into the amoebae. However, the products tested here are more complex than a defined bacterial growth medium and other constituent of milk and juice may likely have affected the results. In the majority of cases, the concentration of amoebae in juice or milk is most likely very low. This was not studied by us, and hence possible *C. jejuni* protection from amoebae present in low concentrations needs to be evaluated in the future. High concentrations of amoebae could be present in contaminated water or by growth of amoebae in beverages stored at room temperature for a longer period of time. In all experiments the survival curves for *C. jejuni* in treatment A were characterized by an increase in bacterial concentration at the beginning of the experiments (3 and 6 h). This increase might be explained by extracellular *C. jejuni* residing in the adherent trophozoite layer. When the medium was changed from PYG to milk or juice these bacteria did probably gradually disperse into the liquid creating a transient increase in bacterial concentration. Together, our results suggest that *A. polyphaga* can prolong *C. jejuni* survival both in milk and juice.

It has been shown that legionellae increase their thermal resistance when co-cultured with acanthamoebae, and that intracellular *legionellae pneumophila* can survive temperatures up to 80°C (48). Acanthamoebae cysts alone have been shown to survive at temperatures up to 80°C and even up to 95°C for at least 10 min (48–50). We studied if *C. jejuni* in co-culture with *A. polyphaga* could survive heating to recommended pasteurization temperatures for milk (72–74°C) and juice (85°C). However, *A. polyphaga* did not have any effect on *C. jejuni* survival during pasteurization of milk or orange juice, confirming that this is a good method for eliminating *C. jejuni* in these products.

In conclusion, amoebae associated *C. jejuni* in milk and juice survived better than free bacteria both at room temperature and at 4°C, but *A. polyphaga* could not protect the bacteria from pasteurization.

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Conflict of interest and funding

The authors declare that they have no conflicts of interest.

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