




Antimicrobial resistance of *Escherichia coli* isolates from spray-chilled sheep carcasses during cooling

Resistencia antimicrobiana en aislados de Escherichia coli de canales de ovejas asperjadas durante la refrigeración

Resistência antimicrobiana de isolados de Escherichia coli de carcaças ovinas aspergidas durante o resfriamento

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Abstract

Background: Multidrug-resistant bacteria present in food of animal origin raise human and animal health concerns. **Objective:** To assess antimicrobial resistance of *Escherichia coli* isolates from sheep carcasses subjected to spray-chilling with water (4 and 10 hours) during cooling. **Methods:** Thirty surface swabs were collected from carcasses before and after the last water spray in two slaughter periods. In a first assessment (1st sampling), three spray-chilled carcasses (4 hours), three non-sprayed and one control carcass were sampled. In a second assessment (2nd sampling), the same number of carcasses and treatments were maintained, but spray-chilling was extended to 10 hours. All samples collected were isolated and submitted to susceptibility test using 16 (1st sampling) and 17 (2nd sampling) antimicrobials, respectively. **Results:** Overall, *E. coli* isolates were resistant most antimicrobials. Spray-chilled and control carcasses (10 hours) showed resistance to meropenem. **Conclusion:** *E. coli* isolates from carcasses subjected to spray-chilling with water for 10 hours had higher antimicrobial resistance to one, two, and four antimicrobial classes, characterizing a multidrug resistance profile. These results highlight the need to monitor health status throughout the meat production processes.

Keywords: antimicrobial; antimicrobial resistance; antibiotic; bacterial resistance; carcass; enterobacteria; *Escherichia coli*; microbial resistance; multi-resistant organism; multidrug resistance; public health; sheep; slaughter; spray-chilled; spray-chilling.

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Resumen

Antecedentes: las bacterias multirresistentes presentes en alimentos de origen animal son motivo de alerta para la salud humana y animal. **Objetivo:** verificar la resistencia a antimicrobianos de aislados de *Escherichia coli* en canales ovinas sometidas a aspersión (4 y 10 h) durante la refrigeración. **Métodos:** Luego de dos faenas de sacrificio, treinta hisopos fueron colectados en la superficie de las canales antes y después de la última aspersión. En un primer sacrificio (1era colecta) se recolectaron muestras de tres canales sometidas a aspersión (4 horas), tres sin aspersión y una canal como control. En un segundo sacrificio (2da colecta), el mismo número de canales y tratamientos se mantuvo, y el período de aspersión se extendió a 10 horas. Las muestras recogidas fueron aisladas y sometidas a la prueba de susceptibilidad utilizando 16 (1.ª colecta) y 17 (2.ª colecta) antimicrobianos, respectivamente. **Resultados:** los aislamientos de *E. coli* fueron, en general, resistentes a las principales clases de antimicrobianos. Las canales con aspersión y el control (10 h) presentaron resistencia al meropenem. **Conclusión:** cuando la aspersión duró 10 h, los aislados de *E. coli* presentaron mayor resistencia para una, dos y cuatro clases de antimicrobianos, es decir, fueron multirresistentes a los fármacos utilizados. Esto resalta la necesidad de monitorear el estado de salud durante todos los procesos de producción de carne.

Palabras clave: antibiótico; antimicrobiano; aspersión; canales; enterobacterias; *Escherichia coli*; multiresistencia; organismos multiresistentes; ovejas; resistencia bacteriana; resistencia antibacteriana; resistencia microbiana; resistencia multiple; sacrificio; salud pública.

Resumo

Antecedentes: bactérias multirresistentes presentes em alimentos de origem animal são motivo de preocupação e alerta na saúde humana e animal. **Objetivo:** verificar a resistência antimicrobiana em isolados de *Escherichia coli* de carcaças de ovinos pulverizadas ou não (4 e 10 horas) durante a refrigeração. **Métodos:** foram coletados trinta swabs de superfície em carcaças antes e após a última aspersão em dois abates. Em outubro do 2015, três carcaças aspergidas foram amostradas, três sem aspersão e uma carcaça para controle, por um período de 4 horas. Em julho de 2016 (2ª coleta), o mesmo número de carcaças e tratamentos foram mantidos e o período de aspersão foi prolongado em 10 horas. As amostras coletadas foram isoladas e submetidas ao teste de susceptibilidade em 16 (1ª coleta) e 17 (2ª coleta) antimicrobianos, respectivamente. **Resultados:** isolados de *E. coli* foram, em geral, resistentes às principais classes de antimicrobianos. As carcaças e o controle aspergidos (10 h) apresentaram resistência ao meropenem. **Conclusão:** quando a aspersão de água durou 10 horas, os isolados de *E. coli* apresentaram maior resistência antimicrobiana a uma, duas e quatro classes de antimicrobianos, o que é uma multiresistência aos fármacos testados. Isso alerta para a necessidade de monitorar os aspectos de saúde durante todos os processos de produção de carne.

Palavras-chave: abate; antibiótico; antimicrobiano; aspersão; carcaça; enterobacterias; *Escherichia coli*; ovelhas; resistência antimicrobiana; resistência bacteriana; resistência microbiana; organismo multirresistente; saúde pública.

Introduction

Carcass chilling is an essential step to guarantee hygiene, safety, shelf life, and overall appearance of meat. This process reduces the surface temperature of carcasses, preventing growth of unwanted and harmful bacteria (Ockerman and Basu, 2004). Some preventive techniques have been used in slaughterhouses to minimize the negative impact of chilling on carcasses. A common practice consists of spraying carcasses with water during chilling. However, according to Jones and Robertson (1988), and Strydom and Buys (1995), this practice may contribute to increased microbiological contamination under unfavorable conditions.

Antimicrobial resistance can be present in several processes within the food industry and, therefore, it is considered a complex multifactorial event (Santos, 2004). Environmental spread of multiresistant bacteria is pointed out by the World Health Organization as the main responsible for increased human deaths caused by antibiotic-resistant superbugs (O'Brien, 2002). Moreover, indiscriminate use of antimicrobials (Van Boeckel *et al.*, 2015) also contributes to bacterial selection pressure, negatively affecting prevention and treatment of bacterial infections in humans and animals (Arslan and Eyi, 2010).

Growth-promoting antimicrobials are commonly used during several stages of intensive animal production. Nevertheless, the risks to human health outweigh the benefits provided by increased productivity (Oliver *et al.*, 2010), especially involving pathogenic bacteria. Moreover, infections caused by multiresistant bacteria increase the risk of exposure to antimicrobial drugs, particularly considering their toxicological aspects (Safdar and Maki, 2002). This situation applies mainly to the treatment and prophylaxis of animal infectious diseases (Lerma *et al.*, 2014).

Escherichia coli (*E. coli*) is commonly found in human and animal gut, but it can also affect health and lead to serious infections.

E. coli contamination in animal products generally occurs through accidental spillage of fecal material onto the carcasses during slaughter, particularly during the skinning and evisceration operations (Barros *et al.*, 2007). However, cross-contamination occurs by manipulation, inadequate hygiene of facilities and/or equipment (Borch and Arinder, 2002).

This study aimed to assess resistance to antimicrobials in *Escherichia coli* isolates from sheep carcasses subjected to spray-chilling with water (4 and 10 hours) during cooling.

Materials and methods

Ethical considerations

Since this study involved only the sampling and analysis of microbiological materials from sheep carcasses in slaughterhouses, approval of an Ethics Committee on Animal Use was not mandatory. However, we ensure that the sheep were slaughtered within the welfare parameters regulated by the State Inspection Program of Santa Catarina, Brazil.

Experimental Design

Antimicrobial resistance of *E. coli* isolates was assessed by taking samples from randomly selected carcasses in a cold room. Samples were collected by swabbing both sides of each carcass during *postmortem*.

In October 2015, ten carcasses sprayed with chlorinated water (1.5 ppm) for 4 consecutive hours, ten non-sprayed carcasses, and one control carcass were sampled for establishing the current health conditions at that time without the interference of those involved in the study. Therefore, these carcasses were neither included in the experiment nor manipulated by researchers, but only manipulated by the slaughterhouse employees.

In July 2016, the same sampling procedure and sample number of sprayed (n = 10) and non-sprayed carcass (n = 10) were taken. This time the carcasses were sprayed for 10 consecutive

hours, and an additional carcass was added to the control group (n = 2).

Samples

The sampling technique was an adaptation of the non-destructive method by NZFSA (2008), using sterile swabs. The swabs were taken by swabbing vertically, horizontally and diagonally over a 100 cm² surface area delimited by a sterile template between the 12 and 13th ribs on the left and right carcass halves (Figure 1). In the first sampling, seven carcasses were selected and distributed, as

follows: three carcasses subjected to spray-chilling using water (CWS 4 h, n = 36), three non-sprayed carcasses (NSC 4 h, n = 36) and one control carcass (COC 4 h, n= 12), totaling 84 plates of *E. coli* isolates. In the second sampling, eight sprayed carcasses (CWS 10 h, n = 42), three non-sprayed carcasses (NSC 10 h, n = 42) and two control carcasses (COC 10 h, n = 28) were selected, totaling 112 plates of *E. coli* isolates. All samples were packed in sterile Brain Heart Infusion (BHI) test tubes and immediately taken to the laboratory for processing and analysis.

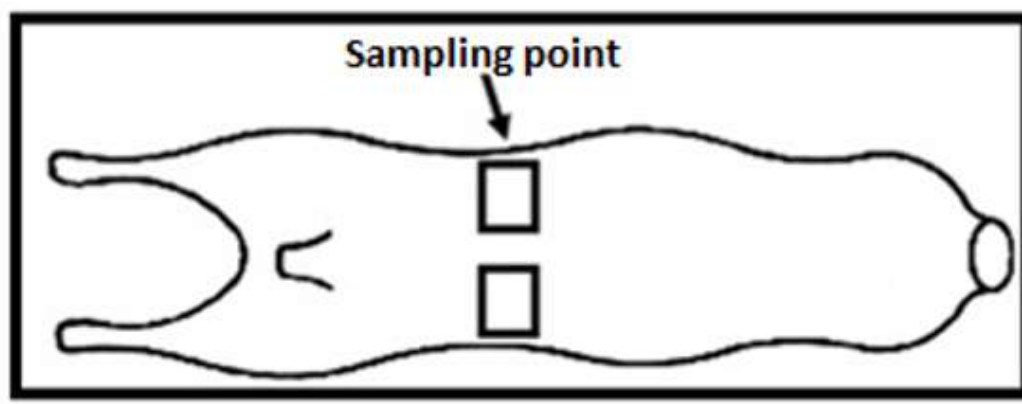


Figure 1. Carcass sampling areas.

Isolation and identification of *Escherichia coli*

The microbiological samples were homogenized in the laboratory. Then 1 mL was pipetted and incubated on six Petri dishes containing MacConkey agar medium (using the following serial dilutions: 10¹, 10², 10³, 10⁴, 10⁵ and 10⁶ CFU mL⁻¹) (KASVI, São José dos Pinhais, PR, Brazil). In the second step, the process was repeated using seven Petri dishes (10¹, 10², 10³, 10⁴, 10⁵, and 10⁶ CFU mL⁻¹). A total of 84 and 112 plates of *E. coli* isolates were obtained in the first and second samplings, respectively. Six characteristic *E. coli* colonies (CFU/cm²) were isolated from each plate using the dilutions indicated above. Samples were then incubated at 37°C for 18-24 hours according to the method described by Lenahan *et al.* (2009).

Antimicrobial susceptibility test

Samples were diluted in a 2 mL saline solution (0.85%), and turbidity was estimated with the McFarland's scale. Subsequently, samples were incubated on Müller-Hinton agar medium (Oxoid Brasil Ltda, Pinheiros, SP, Brazil) to perform the susceptibility test using the disk diffusion method (Bauer *et al.*, 1966).

Three groups of antimicrobials (Laborclin) were selected as standard antimicrobials. Group 1 from the first sampling (October 2015) consisted of amikacin (30 µg), amoxicillin + clavulanate (20/10 µg), ceftriaxone (30 µg), ceftazidime (30 µg), and gentamicin (10 µg). This antimicrobial combination was aimed to confirm the presence of ESBL. Group 2 included nitrofurantoin (300 µg), cefepime (30

µg), nalidixic acid (30 µg), norfloxacin (10 µg), and ciprofloxacin (5 µg). Group 3 consisted of cephalothin (30 µg), ampicillin (20 µg), meropenem (10 µg), sulfonamide (300 µg), and tetracycline (30 µg). The same antimicrobials used in the susceptibility test for the first sampling were used in the second sampling (July 2016), but chloramphenicol (30 µg) and trimethoprim (25 µg) were added to the second group. *E. coli* strain ATCC 25922 was used as a control. Reading and interpretation of the results were performed according to the Clinical and Laboratory Standards Institute (CLSI, 2015). Multiresistant isolates demonstrated resistance to three or more classes of antimicrobials (Magiorako *et al.*, 2012). Determination of multiple antibiotic resistance (MAR) index was conducted according to the methodology described by Krumperman (1983).

Statistical analysis

A bivariate analysis using Pearson's correlation with $p < 0.05$ as significant was performed to test for heterogeneity or linear trend between treatments, considering the prevalence of bacterial resistance in each class of antimicrobials and their respective multi-drug resistance. A 95% confidence interval was considered for all tests. Additional calculations were performed using the Epi Info 7 software.

Results

The slaughtering process involves several critical points that could influence the results obtained in this study in terms of microbial contamination. Carcasses sprayed for 4 hours (CWS 4 h) showed resistance to cephalothin (94%, 34/36), nalidixic acid (19%, 7/36), and sulfonamide (25%, 9/36), as shown in Table 1.

Isolates from non-sprayed carcasses (NSC 4 h) had the highest percentage of resistance to cephalothin (97%, 35/36) and the lowest percentage of resistance to ampicillin + sulbactam (17%, 6/36), amoxicillin + clavulanate (11%, 4/36), nitrofurantoin (19%, 7/36), and nalidixic acid (14%, 5/36). The following percentages of resistance were obtained for isolates from the

control group (COC 4 h): cephalothin (83%, 10/12), ampicillin + sulbactam (42%, 5/12), amoxicillin + clavulanic acid (33%, 4/12). Resistance to nalidixic acid (14%, 5/36) of isolates from non-sprayed carcasses was higher than 10%, which warns public health risk, especially regarding the presence of residues in meat.

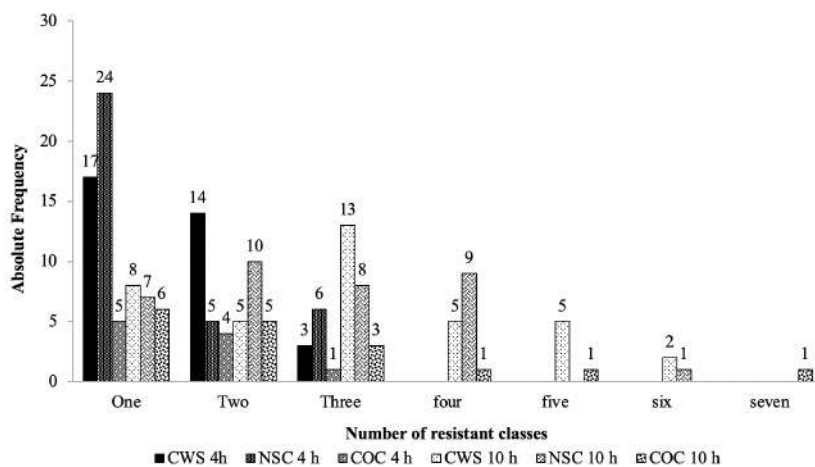
All 17 antimicrobials tested in *E. coli* isolates from carcasses sprayed for 10 h (10 h CWS) during the second sampling presented antimicrobial resistance higher than 10% (Table 1). Antimicrobials amikacin (7%, 3/42), gentamicin (4%, 2/42) ciprofloxacin (0%), norfloxacin (0%), and sulfonamide (7%, 3/42) showed low resistance in carcasses of the treatment NSC 10 h. The highest percentage of resistance was observed to cephalothin (83%, 35/42), followed by ceftriaxone (31%, 13/42), ampicillin + sulbactam (60%, 25/42), amoxicillin + clavulanate (83%, 35/42), nitrofurantoin (60%, 25/42), nalidixic acid (31%, 13/42). Four out of 17 antimicrobials studied belong to the class of β -lactams. The control group (COC 10 h) showed higher percentage of resistance to cephalothin (18%, 5/28), ceftazidime (18%, 5/28), ceftriaxone (14%, 4/28), meropenem (14%, 4/28), nitrofurantoin (18%, 5/28), nalidixic acid (11%, 3/28), tetracycline (11%, 3/28) and trimethoprim (14%, 4/28). Resistance to meropenem was observed in spray-chilled carcasses (19%, 8/42) and the control group (14%, 4/28).

In *E. coli* isolates of the second sampling, the highest resistance to antimicrobial classes were detected for nitrofurans (60, 60 and 17%), sulfonamides (7, 7 and 25%) and β -lactams (24, 18 and 16%) in sprayed, non-sprayed carcasses, and control group, respectively. When comparing the number of resistant antimicrobial classes between *E. coli* isolates of the first and second samplings (Figure 2), the most significant values were observed for carcasses sprayed and non-sprayed for 10 hours.

Table 1. Percentage (%) and absolute frequency (AF) of resistant *E. coli* isolates from sheep carcasses subjected to spray-chilling with water (CWS) and non-sprayed carcasses (NSC) at different sampling moments (2015 and 2016).

Antimicrobial	Treatments											
	CWS 4 h		NSC 4 h		COC 4 h		CWS 10 h		NSC 10 h		COC 10 h	
	AF	%	AF	%	AF	%	AF	%	AF	%	AF	%
AMI (30 µg)	0	0	0	0	0	0	3	7	1	2	1	3
GEN (10 µg)	0	0	0	0	0	0	2	4	0	0	1	3
CFL (30 µg)	34	94	35	97	10	83	36	86	35	83	5	18
CAZ (30 µg)	0	0	0	0	0	0	6	14	0	0	5	18
CRO (30 µg)	3	8	1	3	1	8	13	31	20	48	4	14
CPM (30 µg)	0	0	0	0	0	0	6	14	2	5	0	0
AMC (20/10 µg)	2	6	4	11	4	33	30	71	35	83	3	10
ASB (20 µg)	2	6	6	17	5	42	23	55	25	60	3	7
MER (10 µg)	0	0	0	0	0	0	8	19	1	2	4	14
NIT (300 µg)	2	6	7	9	4	33	25	60	25	0	5	8
CLO (30 µg)	(-)	(-)	(-)	(-)	(-)	(-)	2	5	4	9	1	4
NOR (10 µg)	0	0	0	0	0	0	0	0	0	0	0	0
CIP (5 µg)	0	0	0	0	0	0	0	0	0	0	0	0
NAL (30 µg)	7	19	5	14	0	0	14	33	13	31	3	11
SUL (300 µg)	9	25	5	14	1	8	3	7	3	7	7	25
TET (30 µg)	2	6	0	0	0	0	6	14	1	2	3	11
TRI (25 µg)	(-)	(-)	(-)	(-)	(-)	(-)	24	57	16	38	4	14

CWS 4 and 10 h = carcasses subjected to spray-chilling using water for 4 and 10 h; NSC 4 and 10 h = non-sprayed carcasses; COC 4 and 10 h = control carcass; AF = absolute frequency; (-) = antimicrobial not tested; AMI = amikacin; GEN = gentamicin; CFL = cephalotin; CAZ = ceftazidime; CRO = ceftriaxone; CPM = cefepime; AMC = amoxicillin + clavulanate; ASB = amoxicillin + subactam; MER = meropenem; NIT = nitrofurantoin; CLO = chloramphenicol; NOR = norfloxacin; CIP = ciprofloxacin; NAL = nalidixic acid; SUL = sulfonamide; TET = tetracycline; TRI = trimethoprim.

**Figure 2.** Absolute frequency of antimicrobial-resistant *E. coli* isolates per class tested in the first (October 2015) and second sampling (July 2016).

The multiple antibiotic resistance (MAR) indexes of spray-chilled, non-sprayed, and control carcasses were 0.3, 0.1, and 0.1, respectively. These values reinforce concerns regarding cross-contamination during the manipulation of animal products. Relative to samplings periods, the values were statistically significant (Table 2) ($p < 0.05$) only when

comparing NSC 4 h x NSC 10 h ($p = 0.006$) for one class, CWS 4 h x CWS 10 h ($p = 0.0577$) for two classes and NSC 4 h x NSC 10 h ($p = 0.0211$) for four classes, in which spray-chilled carcasses were more multiresistant. No significant results were found for comparisons within treatments.

Table 2. Statistical significance of antimicrobial-resistant classes tested in *E. coli* isolates from sheep carcasses subjected to spray-chilling with water in the *postmortem* period.

Treatments			Antimicrobial Classes						
			One	Two	Three	Four	Five	Six	Seven
CWS 4 h	x	NSC 4 h	NS	NS	NS	NS	NS	NS	NS
CWS 4 h	x	COC 4 h	NS	NS	NS	NS	NS	NS	NS
NSC 4 h	x	COC 4 h	NS	NS	NS	NS	NS	NS	NS
CWS 4 h	x	CWS 10 h	NS	0.0577*	NS	NS	NS	NS	NS
NSC 4 h	x	NSC 10 h	0.006*	NS	NS	0.0211*	NS	NS	NS
CCO 4 h	x	COC 10 h	NS	NS	NS	NS	NS	NS	NS
CWS 10 h	x	CWS 10 h	NS	NS	NS	NS	NS	NS	NS
CWS 10 h	x	COC 10 h	NS	NS	NS	NS	NS	NS	NS
NSC 10 h	x	COC 10 h	NS	NS	NS	NS	NS	NS	NS

CWS = Carcasses subjected to spray-chilling using water for 4 and 10 h; NSC = Non-sprayed carcasses; COC 4 and 10 h = Control carcass, NS = not significant, *Significance declared at $p < 0.05$.

Discussion

We found high levels of antimicrobial resistance in carcasses subjected to long spray-chilling with water. Regardless of treatment, it is noteworthy that antimicrobial resistance was significant for most antimicrobials, mainly for β -lactams. A previous study showed that antimicrobial resistance might involve three mechanisms: decreased accumulation of the drugs by the cell, hydrolysis of antimicrobials, and/or reductions in drug affinity due to alterations in binding proteins (Miró *et al.*, 1994). In addition to the ability of bacteria to produce β -lactamases, no extended-spectrum β -lactamases (ESBL) were found among *E. coli* isolates in this study.

In the first sampling, carcasses sprayed for 4 hours were resistant to β -lactams, quinolones, and sulfonamides, while non-sprayed carcasses showed resistance to β -lactams, quinolones, and

nitrofurans. According to Phillips *et al.* (2004), antimicrobials can select resistant mutant bacterial strains through resistance transferred from other bacteria. The resistance to quinolone in gram-negative bacilli is mainly attributed to gene mutations that encode quinolone targets and alterations in external membrane proteins or efflux pumps (Strahilevitz *et al.*, 2009; Lindgren *et al.*, 2003). Overall, these results corroborate previous studies conducted by Sáenz *et al.* (2001) and Dontorou *et al.* (2003), which indicate resistance to the main classes of antimicrobials in *E. coli* isolates from animal products.

Studies by Rahamathulla *et al.* (2016) showed that resistance to meropenem is an increasing public health problem because this antibiotic is one of the last resources for the treatment of infections in hospitals. Rasmussen *et al.* (1996) reported that this is associated with genes encoding carbapenemases on plasmids or

transposons in carbapenem-resistant bacteria. In *E. coli*, these genes are responsible for resistance to carbapenems due to the presence of an outer membrane protein deficiency and expression of plasmid-mediated class C beta-lactamase gene (Stapleton *et al.*, 1999). The *E. coli* isolates from the control carcasses showed resistance to meropenem, possibly due to cross-contamination of bacteria with the resistance gene for this carbapenem during slaughter.

The present study indicated that *E. coli* isolates from carcasses subjected to spray-chilling with water had greater antimicrobial resistance, especially in carcasses sprayed for 10 h, even with the recommended chlorine level. Longer spray-chilling time facilitated the dispersion of bacteria on carcasses, which may increase contamination of carcass surfaces. Moreover, spray-chilling increased both colonization of bacteria and their survival under cooling conditions.

Declarations

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Conflicts of interest

The authors declare they have no conflicts of interest with regard to the work presented in this report.

Author contributions

All authors contributed extensively to all aspects of this work, including the conception and design of the study, implementation, data analysis, and manuscript writing.

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