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Original article

Occurrence of Gram-negative bacteria in hens' eggs depending on their source and storage conditions

D. Stępień-Pyśniak

Department of Birds Diseases, Institute of Biological Fundamentals of Animal Diseases, Veterinary Faculty, University of Life Sciences in Lublin, Głęboka 30, 20-612 Lublin, Poland

Abstract

The aim of this study was to analyse the qualitative composition of Gram-negative microbes, mainly of the family *Enterobacteriaceae*, including pathogenic bacteria such as *Salmonella*, in the albumens and yolks and on the shells of hens' eggs, depending on their source and on the temperature and duration of their storage. A total of 375 table eggs were studied, from a large-scale poultry farm, a small-scale poultry farm and a supermarket. Each group was divided into 5 subgroups according to the temperature and duration of their storage during the study. Two serotypes of bacteria of the genus *Salmonella* were identified: *S.* Enteritidis and *S.* Arizonae. Strains of *Salmonella* spp. were also isolated. Apart from *Salmonella* and *Escherichia coli*, among the most frequently isolated bacteria of the family *Enterobacteriaceae* were *Enterobacter* spp., *Klebsiella* spp. and *Citrobacter freundii*. Qualitative analysis of the bacterial microflora of the eggs also showed the presence of other Gram negative bacteria, including *Acinetobacter* spp., *Pseudomonas* spp., *Tatumella ptyseos*, *Providencia stuartii*, *Serratia liquefaciens*, *Flavimonas oryzihabitans*, *Vibrio metschnikovii*, *Leclercia adecarboxylata*, *Kluyvera* spp., *Rahnella aquatilis*, *Proteus mirabilis,* and *Achromobacter* spp. The study demonstrated that the conditions applied, i.e. the temperature and duration of storage, did not significantly influence the prevalence of particular species of Gram-negative bacteria in the eggs. However, based on the analysis of contamination of eggs with *Salmonella* depending on their source, it can be concluded that the system in which the hens are housed affects the risk of contamination of eggs with these pathogens.

Keywords: eggs, bacterial contamination, storage conditions

Introduction

The hen's egg is very rich in nutrients, making it one of the most valuable and most perfect foodstuffs. Eggs can fully meet the requirements of organisms, including humans, for all the nutrients necessary for their development and life functions. At the same time, the many nutrient substances present in eggs create an excellent environment for the development of bacterial microflora, including pathogenic bacteria. The fact that eggs can be contaminated or infected horizontally (through the shell) or vertically (transovarially) makes them a potential source of pathogens participating in the etiology of foodborne diseases in humans.

Correspondence to: D. Stępień-Pyśniak, e-mail: dagmara.stepien@up.lublin.pl

In Poland, according to epidemiological data, food poisoning and foodborne infection following consumption of eggs or dishes containing eggs are usually caused by *Salmonella*, as well as *Staphylococcus* (*St.*) *aureus*, *Escherichia* (*E.*) *coli* and other coli bacilli (Przybylska 2000, 2002, 2003). Microorganisms of the genera *Campylobacter* spp. isolated from eggs or egg products, particularly *Campylobacter* (*C.*) *jejuni* and *Listeria* spp., can also pose a potential threat to the health of consumers (Ibeh and Izuagbe 1986, Shane et al. 1986, Leasor and Foegeding 1989, Farber et al. 1992, Todd 1996, Adesiyun et al. 2005).

Currently, one of the most important factors constituting a risk to consumer health is contamination of eggs with *Salmonella*, which is closely correlated with the spread of these bacteria among poultry (Hasenson et al. 1992, Gast and Beard 1993, Hoszowski and Wasyl 2005). In most EU countries, including Poland, *Salmonella* bacilli have been found in over 1% of table eggs tested (Hoszowski and Wasyl 2005).

Among the zoonotic serovars of *Salmonella*, the most common are *Salmonella* bacteria isolated from birds. The frequency of occurrence of particular *Salmonella* serotypes varies in different parts of the world and changes over time. However, beginning in the 1980s, the dominant serovar causing toxicoinfections in many European countries (including Poland) and in the United States has been *S.* Enteritidis (Rodrigue et al. 1990, Anonymous 2000, 2010, Gonera 2003, Sadkowska-Todys et al. 2006).

Other Gram-negative bacteria, such as *E. coli*, *Enterobacter* spp., *Citrobacter* spp., *Klebsiella* spp., *Alcaligenes* spp., *Aeromonas* spp, and *Pseudomonas* spp., have also been found in eggs with intact or damaged shells, where they play a role in spoilage and can enter the food chain via eggs, leading to food poisoning or infection in consumers.

With the exception of *S.* Enteritidis, there is currently little information about the distribution of Gram-negative bacteria in particular parts of eggs. Also unknown is the influence of storage conditions and of the source of the eggs on their qualitative bacterial contamination.

The aim of this study was to determine the bacterial contamination of the contents and shells of table eggs depending on their source and on storage duration and temperature. The study comprised a qualitative analysis of Gram-negative microorganisms, mainly from the family *Enterobacteriaceae*, including pathogenic bacteria such as Salmonella, in the albumens, yolks and shells of eggs.

Materials and Methods

A total of 375 hens' eggs were studied. These were table eggs from a large-scale poultry farm (battery cage system), a small-scale poultry farm (litter system) and a supermarket (battery cage system). The eggs from the large-scale and small-scale farms were randomly collected on the day they were laid and placed in sterile cardboard containers using latex gloves disinfected each time with 70% ethanol. In the supermarket, eggs were chosen with an expiry date no less than 3 weeks after the purchase.

Each group was divided into 5 subgroups of 25 eggs each according to the temperature and duration of their storage during the study. The first subgroup consisted of fresh eggs – eggs tested within 3 – 5 hours after collection or purchase (in the case of the eggs from the supermarket). The eggs in the second subgroup were stored at 4°C for 14 days. The third subgroup consisted of eggs stored at 21°C for 14 days, while the eggs in the fourth and fifth groups were stored for 28 days at 4°C and 21°C, respectively.

In the first phase of the study, samples were prepared for microbiological determination. For this purpose each egg was placed whole in a sterile Stomacher bag containing 50 ml of buffered peptone water, where it was twice rinsed for 1 minute each time, with a 5-minute interval. Then the shell was disinfected by immersing the egg in 70% alcohol for 5 minutes and then passing it over a flame. Next the shell was opened using sterile scissors and the albumen and yolk were placed in separate sterile bags. The contents were blended for 60 seconds at normal speed in a Stomacher 400 Circulator (Seward, London, UK).

Detection of *Salmonella*

Pre-enrichment was performed in buffered peptone water at a temperature of 37°C for 18 – 24 hours, followed by selective enrichment in two liquid media, Rappaport-Vassiliadis (OXOID, Hampshire, UK) and Muller-Kauffmann (OXOID, Hampshire, UK), at 42°C for 24 – 48 hours. *Salmonella* bacteria were isolated on two differential-selective media – with phenol red and brilliant green (BGA) (OXOID, Hampshire, UK) and with xylose, lysine and desoxycholate (XLD) (OXOID, Hampshire, UK) – at 37°C for 24 hours (Anonymous 2003).

Biochemical identification of the isolated bacterial colonies was performed using the API 20E test (bioMérieux, France). To determine *Salmonella* serovars, serological identification was carried out using sera specific for somatic antigens and phase I and II ciliary antigens (BIOMED Cracow, Poland).

Table 1. Frequency of occurrence of bacteria in the albumen of eggs depending on their source and storage conditions

Temperature and duration of egg storage	Source of eggs	Type of bacteria isolated									Total **(No./%)** (21 / 4.6%)
		Acinetobacter spp.	Chryzeomonas luteola	Citrobacter freundii	Enterobacter agglomerans	Escherichia coli	Leclercia adecarboxylata	Providencia stuartii	Pseudomonas fluorescens	Tatumella ptyseos	
Fresh eggs	large-scale farm	-	-	-	-	3	1	-	1	-	5/1.1
	supermarket	-	-	1	-	-	-	-	-	1	2/0.4
	small-scale farm	-	-	-	-	-	-	-	-	-	0/0
Eggs stored at 4°C for 14 days	large-scale farm	1	2	-	-	-	-	-	-	-	3/0.7
	supermarket	-	-	-	-	-	-	-	-	-	0/0
	small-scale farm	-	-	-	-	-	-	-	-	-	0/0
Eggs stored at 21°C for 14 days	large-scale farm	-	-	-	1	-	-	-	-	-	1/0.2
	supermarket	-	-	-	-	1	-	-	-	-	1/0.2
	small-scale farm	-	-	-	-	-	-	-	-	-	0/0
Eggs stored at 4°C for 28 days	large-scale farm	-	-	-	-	2	-	-	1	-	3/0.7
	supermarket	-	-	-	-	-	-	-	-	-	0/0
	small-scale farm	-	-	-	-	-	-	-	-	-	0/0
Eggs stored at 21°C for 28 days	large-scale farm	-	-	-	-	-	-	-	1	-	1/0.2
	supermarket	-	-	-	-	1	-	3	-	-	4/0.9
	small-scale farm	-	-	-	-	-	-	-	1	-	1/0.2

Note: fresh eggs – eggs tested within 3 – 5 hours after collection or purchase (in the case of supermarket eggs).

Isolation of enterobacteriaceae bacteria other than *Salmonella*

Qualitative determination of Gram-negative microorganisms, with particular consideration given to the family *Enterobacteriaceae*, was carried out on Salmonella-Shigella agar (OXOID, Hampshire, UK) and MacConkey agar (OXOID, Hampshire, UK) at a temperature of 37°C for 24 – 48 hours (Papadopoulou et al. 1997). The isolated bacteria were then tested for oxidase production. An appropriate commercial test, i.e. API 20E (bioMérieux, France) or API 20NE (bioMérieux, France), was used for further biochemical identification.

Results

Detailed data on the species and number of Gram-negative bacteria isolated from the albumens, yolks and shells of eggs depending on their source and storage conditions are shown in Tables 1 – 3. Of the 1,125 tested samples of albumens, yolks and shells of eggs from all sources and storage conditions, Gram-negative bacteria were found in 453 cases (40.3% of samples tested). There were 21 strains isolated from albumens (4.60% of all isolates), of which 13 were isolated from eggs from the large-scale farm, 7 from eggs purchased in the supermarket, and 1 from eggs from the small-scale farm (Table 1). Gram-negative bacteria were found more often in yolks than in albumens – 32 strains (7.10% of all isolates), of which 13 strains were isolated from the yolks of eggs from the large-scale farm, 7 from eggs from the small-scale farm and 12 from eggs purchased in the supermarket (Table 2). The highest percentage of bacterial contamination was noted on egg shell surfaces – 400 strains (88.30% of all isolates), with 134 strains noted on the shells of eggs from the large-scale farm, 123 on the shells of eggs from the small-scale farm, and 143 on the shells of eggs purchased in the supermarket (Table 3).

Table 2. Frequency of occurrence of bacteria in yolks of eggs depending on their source and storage conditions

Temperature and duration of egg storage	Source of eggs	Type of bacteria isolated											Total (No./%) (32 / 7.1%)
		Acinetobacter spp.	Enterobacter agglomerans	Enterobacter cloacae	Escherichia coli	Leclercia adecarboxylata	Proteus mirabilis	Providencia stuartii	Pseudomonas fluorescens	Salmonella Enteritidis	Tatumella ptyseos	Vibrio metschnikovii	
Fresh eggs	large-scale farm	-	-	-	2	1	-	-	1	-	-	-	4/0.9
	supermarket	-	-	-	-	-	-	-	-	-	1	-	1/0.2
	small-scale farm	1	-	1	1	-	-	-	-	-	-	-	3/0.7
Eggs stored at 4°C for 14 days	large-scale farm	-	1	-	1	-	-	-	-	-	-	-	2/0.4
	supermarket	3	-	-	-	-	-	-	-	-	-	-	3/0.7
	small-scale farm	-	-	-	1	-	-	-	-	-	-	-	1/0.2
Eggs stored at 21°C for 14 days	large-scale farm	2	-	-	-	-	-	-	-	**1**	1	-	4/0.9
	supermarket	-	-	-	-	-	-	-	-	-	-	1	1/0.2
	small-scale farm	-	-	-	1	-	-	-	-	-	-	-	1/0.2
Eggs stored at 4°C for 28 days	large-scale farm	1	-	-	-	-	-	-	-	-	-	-	1/0.2
	supermarket	-	-	-	-	-	-	-	-	-	2	-	2/0.4
	small-scale farm	-	-	-	1	-	-	-	-	-	-	-	1/0.2
Eggs stored at 21°C for 28 days	large-scale farm	-	-	-	1	-	-	-	1	-	-	-	2/0.4
	supermarket	-	-	-	1	-	1	3	-	-	-	-	5/1.1
	small-scale farm	-	-	-	1	-	-	-	-	-	-	-	1/0.2

Note: fresh eggs – eggs tested within 3 – 5 hours after collection or purchase (in the case of supermarket eggs).

There were 33 species of Gram-negative bacteria isolated from the eggs tested. *E. coli* and *Ps. fluorescens* were dominant in the albumens, while *E. coli* and *Acinetobacter* spp. dominated in the yolks, irrespective of their source and storage conditions. Among the bacteria isolated from egg shells, *E. coli, Citrobacter* (*Citro.*) *freundii*, *Acinetobacter* spp. and the genera *Enterobacter* and *Klebsiella* were dominant. In addition, *Salmonella* bacteria were found on the shells of 3.2% (4/125) of eggs tested from the large-scale farm and on the surface of 3.2% (4/125) of eggs purchased in the supermarket, including *S.* Enteritidis and *S.* Arizonae, as well as other *Salmonella* strains whose species could not be determined (Table 3). *S.* Enteritidis was also isolated from 0.8% (1/125) of yolks from the large-scale farm (Table 2).

Discussion

An important factor influencing quantitative bacterial contamination of eggs is the temperature at which they are stored, because the safety of eggs depends on the number of bacterial cells on the shells and in the content of the egg and on the rate at which they multiply within it.

The risk of illness resulting from consumption of contaminated eggs depends not only on the number of bacterial cells in the contents or on the shells of the eggs, but also on the type of bacteria (Bradshaw et al. 1990). Among the most widespread foodborne infections directly connected with egg consumption are *Salmonella* infections.

In this study, *Salmonella* bacteria were found on the shells of 3.2% of eggs tested from the large-scale farm and on the surface of 3.2% of eggs purchased in the supermarket. Moreover, *S.* Enteritidis was also isolated from 0.8% of yolks from the large-scale farm. The results of the present study were similar to data published by Indar et al. (1998). They found the frequency of *Salmonella* occurrence on the shell (4.7%) to be significantly higher than in the contents (1.2%).

A study determining the type of microflora in eggs depending on their source was conducted by Adesiyun

Table 3. Frequency of occurrence of bacteria on the shells of eggs depending on their source and storage conditions.

Temperature and duration of egg storage	Source of eggs	Achromobacter spp.	Acinetobacter spp.	Aeromonas spp.[a]	Alcaligenes spp.	Chryzeomonas luteola	Citrobacter freundii	Enterobacter spp.[b]	Erwinia nigrifluens	Escherichia coli	Escherichia spp.[c]	Flavimonas oryzihabitans	Klebsiella spp.[d]	Kluyvera spp.	Leclercia adecarboxylata	Proteus mirabilis	Providencia stuartii	Pseudomonas spp.[e]	Rahnella aquatilis	Salmonella Arizonae	Salmonella Enteritidis	Salmonella spp.	Serratia liquefaciens	Tatumella ptyseos	Vibrio metschnikovii	Total **(No./%)** (400 / 88.3%)
Fresh eggs	large-scale farm	-	4	-	-	-	3	7	-	12	-	1	3	-	1	-	-	1	-	**-**	**1**	**-**	-	-	-	33 7.3
	supermarket	-	2	-	-	-	4	8	-	16	-	-	-	-	-	-	-	-	2	**-**	**-**	**-**	-	-	-	32 7.1
	small-scale farm	-	1	2	-	-	2	8	1	6	-	-	3	-	-	-	-	-	-	**-**	**-**	**-**	1	-	-	24 5.3
Eggs stored at 4°C for 14 days	large-scale farm	-	1	-	-	2	3	7	-	8	-	1	8	-	1	1	-	-	-	**-**	**-**	**1**	-	-	-	33 7.3
	supermarket	-	2	-	-	-	1	5	-	15	-	-	-	-	-	-	-	-	-	**3**	**-**	**-**	-	-	2	28 6.2
	small-scale farm	-	-	-	-	-	1	18	-	2	-	-	2	1	-	-	-	-	-	**-**	**-**	**-**	-	-	-	24 5.3
Eggs stored at 21°C for 14 days	large-scale farm	-	1	1	-	-	6	8	1	7	-	-	-	-	-	-	-	-	-	**-**	**-**	**-**	-	2	-	26 5.7
	supermarket	-	3	1	-	-	-	11	-	7	-	-	2	-	-	-	-	4	-	**1**	**-**	**-**	-	1	-	30 6.6
	small-scale farm	5	3	-	-	-	-	10	-	4	-	-	1	1	-	-	-	1	-	**-**	**-**	**-**	-	-	-	25 5.5
Eggs stored at 4°C for 28 days	large-scale farm	-	5	-	-	-	-	4	-	2	-	-	-	-	-	-	-	3	-	**-**	**1**	**-**	-	-	1	16 3.5
	supermarket	-	2	1	-	-	1	7	1	10	2	1	-	-	-	-	-	-	-	**-**	**-**	**-**	-	2	-	27 6
	small-scale farm	-	1	3	2	-	1	6	-	4	1	-	3	1	-	1	-	-	-	**-**	**-**	**-**	-	-	-	23 5.1
Eggs stored at 21°C for 28 days	large-scale farm	-	6	1	-	-	1	2	-	8	-	-	-	-	-	-	-	7	-	**-**	**1**	**-**	-	-	-	26 5.7
	supermarket	-	2	-	-	-	-	7	1	6	2	-	1	-	-	2	3	1	-	**-**	**-**	**-**	1	-	-	26 5.7
	small-scale farm	-	-	2	-	-	5	3	-	4	3	-	6	1	-	2	-	1	-	**-**	**-**	**-**	-	-	-	27 6

Note: fresh eggs – eggs tested within 3 – 5 hours after collection or purchase (in the case of supermarket eggs).

Identified bacteria species among particular bacteria genus:

[a] *Aeromonas* spp. (*A. hydrophila, A. salm. salmonicida)*

[b] *Enterobacter* spp. (*Ent. agglomerans, Ent. amnigenus, Ent. cloacae, Ent. sakazakii)*

[c] *Escherichia* spp. (*E. herm annii, E. vulneris*)

[d] *Klebsiella* spp. (*K. ozaenae, K. rhinoscleromatis)*

[e] *Pseudomonas* spp. (*Ps. alcaligenes, Ps. fluorescens, Ps. mendocida, Ps. stutzeri)*

et al. (2005). They isolated *Salmonella* bacteria from 6.5% of shells and 6.5% of contents of eggs taken directly from farms, and from 2.8% of shells and 7.5% of contents of eggs purchased in supermarkets. Moreover, in some of the eggs from the supermarket these bacteria were found both on the surface and in the content (0.9%), which was not observed in the case of the farm eggs. Other authors have found that the frequency of *Salmonella* isolation from eggs in naturally infected flocks of laying hens ranges from 0.03% to 0.05% (Kinde et al. 1996, Ebel and Schlosser 2001).

In 2008, the European Union Member States (MSs) (including Poland) implemented new *Salmonella* control programmes in laying hen flocks of *Gallus gallus* providing eggs intended for human consumption in accordance with Regulation (EC) No 2160/2003. The control programmes consist of proper and effective measures for prevention, detection, and control of *Salmonella* at all relevant stages of the egg production line, particularly at the level of primary production, in order to reduce *Salmonella* prevalence and the risk to public health.

In Poland, from 2004 to 2005 a baseline survey was conducted concerning prevalence of *Salmonella* in flocks of laying hens. The prevalence of salmonella ranges from 57% to 94%. In 2008 *Salmonella* spp. was found in 169 laying hens flocks among 1533 tested. *S.* Enteritidis was found in 140 flocks, *S.* Typhimurium in 8 flocks. *S.* Hadar, *S.* Infantis and *S.* Virchow were found in 9 flocks and unspecified *Salmonella* spp. in 22 flocks. The percentage of infection in adult laying hen flocks was 9.36%, while in 2008 *Salmonella* was found in 0.33% of eggs and egg products tested. The most frequent serovars in eggs and egg products were *S.* Enteritidis and S*almonella* spp., unspecified. In addition, fifteen MSs reported data from investigations of table eggs. In total, 0.5% of the tested units were positive for *Salmonella*, which is a reduction compared to 2007 (0.8%) (Anonymous 2010).

In this study 2 serotypes of *Salmonella* bacteria were identified – *S.* Enteritidis and *S.* Arizonae. Other *Salmonella* spp. strains were also identified. Musgrove et al. (2004) also isolated *S.* Arizonae from the shells of table eggs kept for 5 weeks in cold store. Adesiyun et al. (2005) distinguished 4 different serotypes among the *Salmonella* bacteria isolated from the farm eggs, represented by *S.* Enteritidis, *S.* Mbandaka, *S.* Javiana, and *S.* Caracas, and 4 serovars from the supermarket eggs – *S.* Enteritidis, *S.* Braenderup, *S.* Georgia, *S.* Ohio – as well as a strain of undetermined species belonging to group C_1.

In the present study, other bacteria of the family *Enterobacteriaceae* occurred besides *Salmonella,* including *E. coli*. The data obtained show that *E. coli* was the bacterium most often isolated, mainly from the shells of eggs purchased in the supermarket. Its frequency of occurrence gradually fell under cold store conditions, and underwent a considerable decrease during storage at room temperature. This relationship was not observed in the case of eggs from the large-scale and small-scale poultry farms, where the number of eggs on which *E. coli* was found first decreased and then increased or remained constant. This bacillus was isolated much less often from the egg contents. Musgrove et al. (2004) observed similar relationship when the type of bacteria on eggshells during 6 weeks of storage was analysed weekly. Adesiyun et al. (2005) obtained similar results. They found this bacterium on 58.7% of shells, in 4.3% of egg contents and in all parts at the same time in 8.7% of farm eggs. In supermarket eggs, with the exception of the contents (2.8%), *E. coli* was isolated much less frequently, i.e. from 15.9% of the shells and 1.19% of entire eggs.

In the present study, apart from *Salmonella* and *E. coli*, the most frequently isolated *Enterobacteriaceae* bacteria included *Enterobacter* spp., *Klebsiella* spp. and *Citro. freundii*. Adesiyun et al. (2006) obtained similar results when they analysed the frequency with which bacteria occurred in eggs depending on their source. They noted the presence of bacteria of the genera *Enterobacter* and *Klebsiella* in 23 and 17 eggs respectively out of 184 tested. Results obtained by Papadoupoulu et al. (1997) and by Musgrove et al. (2004) also confirm that the bacteria most frequently isolated from eggs are Gram-negative bacteria such as *Enterobacter* spp., *E. coli* and *Klebsiella* spp.

Qualitative analysis of the bacterial microflora of the eggs also showed the presence of other Gram-negative bacteria, including *Acinetobacter* spp., *Pseudomonas* spp., *Tatumella ptyseos*, *Providencia stuartii*, *Serratia liquefaciens*, *Flavimonas oryzihabitans*, *Vibrio metschnikovii*, *Leclercia adecarboxylata*, *Kluyvera* spp., *Rahnella aquatilis*, *Proteus mirabilis,* and *Achromobacter* spp. These microbes were isolated infrequently, mainly from eggshell surfaces, irrespective of storage conditions or the source of the eggs. Musgrove et al. (2004) made similar observations when bacterial microflora of the family *Enterobacteriaceae* on the shells of three successive batches of farm eggs were analysed weekly during 6 weeks of storage.

The present study found that Gram-negative bacteria were isolated less often from yolks, with only some of the species noted, and sporadically from albumens. Among the most frequently isolated *Enterobacteriaceae* bacteria on eggshell surfaces were *E. coli*, *Enterobacter* spp., *Klebsiella* spp. and *Citro. freundii*. The conditions applied, i.e. the temperature and duration of storage, were not found to significantly influence the prevalence of particular species of Gram-negative bacteria in the eggs. However, based on the analysis of *Salmonella* contamination of eggs depending on where they were purchased, it can be

concluded that the system of housing of the hens affects the risk of infection with these pathogens.

Although the enteric bacteria isolated from eggs in this study are not universally considered to be pathogens, they may present a health risk for consumers using raw or undercooked eggs in their diet. WHO predicts that serious problems with opportunistic infections may appear in the 21st century. For this reason it is necessary to educate the public about good sanitary practices in handling eggs and preparing them for consumption.

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