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Inhibitory Effect of Carob (*Ceratonia siliqua*) Leaves Methanolic Extract on *Listeria monocytogenes*

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ABSTRACT: In recent years, there has been great development in the search for new natural compounds for food preservation aimed at a partial or total replacement of currently popular antimicrobial chemicals. Carob (*Ceratonia siliqua*) offers a natural promising alternative for food safety and bioconservation. In this work, the methanolic extract of carob leaves (MECL) was tested for the ability to inhibit the growth of a range of microorganisms. MECL inhibited the growth of *Listeria monocytogenes* at 28.12 μg/mL by the broth microdilution method. The effect of this bacteriostatic concentration on the growth of this bacterium revealed a pattern of inhibition characterized by (a) a resumed growth phase, which showed a lower rate of growth if compared with controls; and (b) first a lag and then a stationary phase at a lower bacterium concentration. The study of the chemical composition of MECL by high-performance liquid chromatography and liquid chromatography/mass spectrometry showed the presence of gallic acid, (−)-epigallocatechin-3-gallate, myricitrin, isoquercitin, catechin, chlorogenic acid, and malic acid. *L. monocytogenes* growth inhibition was recorded for myricitrin and gallic acid at 450 μg/mL and for (−)-epigallocatechin-3-gallate and isoquercitin, respectively, at 225 and 112.5 μg/mL. Taking into account that proline is a ligand of proline dehydrogenase (PDH), the use of this compound leads us to hypothesize the mode of action of MECL constituents.

KEYWORDS: *antimicrobial, HPLC/MS, gallic acid, food preservation, polyphenols, proline dehydrogenase, Ceratonia siliqua*

INTRODUCTION

Antibiotics are generally beneficial in treating susceptible microbial infections, but such benefits may be offset by the overuse of antibiotics, leading to the emergence of antibiotic-resistant bacteria such as methicillin-resistant *Staphylococcus aureus* (MRSA).[1] Therefore, there is an increased public and scientific need to conduct research on the bactericidal efficiency of phytochemicals and other natural substances as viable alternatives to pharmaceutical and chemical antibiotics. Plant-derived antimicrobial systems have potential use in food preservation with or without additional conditions, such as a controlled packaging atmosphere.

Plant extracts have been traditionally used in folk medicine as well as additives to extend food shelf life.[2] The majority of their properties are due to secondary metabolites such as the volatile constituents and polyphenols, which are a class of higher plant secondary metabolites[3] known for their antioxidant activity of the aromatic ring structure bearing hydroxyl groups. These compounds are also known for their antimicrobial,[4,5] antiparasitic, and anti-insecticidal activities.[6]

Carob (*Ceratonia siliqua*) is a tree widely grown in the Mediterranean region. It belongs to the Caesalpinaceae, a subfamily of Leguminoseae family (Fabaceae).[7] Carob pods are found to contain proteins, fat, carbohydrates, polyphenols, and tannins.[8] The bark and leaves of this tree are used in Turkish folk medicine as an antidiarrheal and diuretic.[9] The leaves are also used for their antioxidant activities.[10] The fruits are traditionally used as an antitussive and against warts.[11] The gum is a galactomannan, a valuable natural food additive for products such as ice cream, sweets, and soups. Carob is also used in the textile and cosmetics industries.[12] The pods of the carob fruit have long been used as a feed for livestock and in human nutrition, including sweets, biscuits, and processed drinks, because of the high sugar content.[13]

Much of the scientific research on carob extracts deals with the antimicrobial activities of different parts of this plant, but to our knowledge and literary survey, there is no report available on the antilisterial activity of carob leaves extracts.

Listeria monocytogenes is a food-borne pathogen that is emerging as a major public health problem. This pathogen causes food-borne infection and can lead to bacteremia, meningitis, and abortion. *L. monocytogenes* is a highly versatile psychrophilic and facultative aerobic microorganism that is able to survive and proliferate in a wide range of substrates and tolerates acidic environments and high salt concentrations.

The aims of the present work were to evaluate the antimicrobial activities of *C. siliqua* leaves methanolic extract as well as its major constituents against a set of bacteria, including *L. monocytogenes*; to find the right storage temperature of the methanolic extract; and finally to understand and hypothesize the mode of antimicrobial action of the carob leaves polyphenols.

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Table 1. Analyte HPLC-MS/MS (−) Transitions and Instrument Conditions

compd	MW	precursor mass (m/z)	first transition		second transition		R_t (min)	R^2	concn in MECL (μg/g) ± SD
			mass (m/z)	CE (V)	mass (m/z)	CE (V)			
gallic acid	170.1	$[M - H]^-$	169.1 → 124.8	12.5			10.517	0.9995	544 ± 310
(−)-epigallocatechin-3-gallate	458.37	$[M - H]^-$	457.4 → 304.9	17.5	457.9 → 468.7	15.5	13.012	0.9991	541 ± 135
isoquercitin	464.00	$[M - H]^-$	463.0 → 300.9	19.0	463.0 → 270.7	41.5	16.794	0.9998	131 ± 84
myricitrin	464.00	$[M - H]^-$	463.0 → 317.0	21.0	463.0 → 286.8	40.5	16.643	0.9996	1660 ± 999
chlorogenic acid	354.30	$[M - H]^-$	653.3 → 190.8	16.0			13.712	0.9979	9.70 ± 5.55
malic acid	134.00	$[M - H]^-$	133.0 → 071.0	13.5	133.0 → 114.8	09.0	07.848	0.9935	163 ± 53
catechin	290.00	$[M - H]^-$	289.0 → 202.8	18.0	289.0 → 244.7	14.0	14.642	0.9980	1205 ± 176

MATERIALS AND METHODS

Chemicals. Standards of gallic acid, (−)-epigallocatichin-3-gallate, myricitrin, isoquercitin, malic acid, catechin, chlorogenic acid, and dimethyl sulfoxide (DMSO) were obtained from Sigma-Aldrich (Milano, Italy), and L-(−)-proline (Carlo Erba Milano, Italy), methanol, phosphoric acid, acetonitrile, formic acid, and water were of high-performance liquid chromatography (HPLC)-grade.

Extraction and Chemical Characterization. *Plant Materials.* Leaves of *C. siliqua* were collected from Cagliari (Sardinia, Italy) in March, 2012, and were dried in the absence of light at room temperature. They were then sealed in paper bags, stored at room temperature, and kept in the dark until use. Voucher specimens were deposited at the Department of Life and Environment Sciences, University of Cagliari, Cagliari, Italy, for species identification.

Carob Leaves Methanol Extracts. Dried leaves (100 g) were ground and extracted with methanol (1:10, w/v) in a sonicator apparatus for 15 min, filtered through Whatman no. 40 filter paper, and centrifuged for 15 min at 13000 rpm. Then, the extract was sterilized by filtration using 0.45 μm filter.

HPLC Analysis. Phenolic compounds were determined using a modification of a previously described HPLC technique.[14] An Agilent 1100 series HPLC system, provided with a diode array detector (DAD) model G 1315 A, an G 1313 A autosampler, and a G 1311 A pump, was used. The separation was achieved on a Waters Spherisorb 5 μm (ODS2 250 mm × 4.6 mm) at 40 °C. The mobile phase consisted of (A) aqueous phosphoric acid (0.22 M) and (B) acetonitrile. The solvent gradient started at 10% A reaching 35% A in 20 min, and 100% A in 60 min followed by post-time isocratic conditions for 10 min at 10% A before the next injection. The flow rate was 1 mL/min, and the injection volume was 10 μL. The monitoring wavelengths were 280 and 350 nm with a scan from 200 to 600 nm. The identification of each compound was based on a combination of retention time and spectral matching with reference phenolics. Quantification of the identified phenolic compounds was performed by correlating the measured peak area with the calibration curves obtained with reference compounds.

HPLC-ESI-MS/MS Analysis. A Varian 1200 L triple-quadrupole tandem mass spectrometer (Palo Alto, CA) coupled with a ProStar 410 autosampler and two ProStar 210 pumps and a 1200 L triple-quadrupole mass spectrometer was used with an electrospray ionization (ESI) source. The Varian MS workstation version 6.7 software was used for data acquisition and processing. Chromatographic separation was performed on a Zorbax Column Synergi 4 μ MAX-RP 80A (150 mm × 4.6 mm) (Phenomenex). The mobile phase consisted of (A) double-distilled water and (B) methanol containing 0.1% formic acid. The solvent gradient started at 10% B reaching 100% in 20 min and 100% in 25 min followed by a post-time isocratic conditions for 5 min at 10% B before the next injection. The mobile phase, previously degassed with high-purity helium, was pumped at a flow rate of 0.3 mL/min, and the injection volume was 10 μL. ESI was operated in negative ion mode. The electrospray capillary potential was set to −40 V, the needle was set at −4500 V, and the shield was set at −600 V. Nitrogen at 48 mTorr and 41 °C was used as a drying gas for solvent evaporation. The atmospheric pressure ionization (API) housing was kept at 50 °C. Parent compounds were subjected to collision-induced dissociation using argon at 2.40 mTorr in the multiple reaction monitoring (MRM) in the negative mode. Table 1 reports the observed mass transitions and collision energy used for quantitation of different phenolics. The scan time was 1 s, and the detector multiplier voltage was set to 2000 V, with an isolation width of m/z 1 for quadrupole 1 and m/z 1.9 for quadrupole 3.

Microbiological Assay. The antimicrobial activity of carob leaves methanolic extract was studied. The experiments were carried out using a set of microorganisms: *Escherichia coli* (ATCC 25922), *Salmonella typhimurium* (ATCC 14024), and *Pseudomonas aeruginosa* (ATCC 9027) strains for Gram(−), *L. monocytogenes* (ATCC 35152) and *S. aureus* (ATCC 25932) strains for Gram(+), and *Aspergillus niger* (ATCC 16404), *Fusarium oxysporum*, *Fusarium graminearum*, and *Penecillium* spp. as fungal strains. We also used *L. monocytogenes* strains isolated in our laboratory for this study. For each of the above-mentioned strains, the minimum inhibitory concentration (MIC) was determined using the broth microdilution method. Stock standard phenolics solutions at 1% (v/v) in DMSO were prepared. Working solutions were prepared by dilution in microplates at concentrations between 3.5 and 900 μg/mL. The bacterial suspensions were added in the microwells at a concentration of 10^5 colony-forming units/mL (CFU/mL). The plates were incubated aerobically at 37 °C for 24 h. Bacterial growth was revealed by the presence of turbidity and a "pellet" on the well bottom. MICs were determined as the first well that did not produce a pellet.

Inhibited microorganisms were then tested using standard disk[15] to determine the MBC (minimal bactericide concentration). The MBC is the lowest concentration of a substance with a bactericidal effect of 99.99%. The experimental determination of MBC consists of measuring the vitality percent (%) without visible growth. The same experiment is repeated with carob leaves compounds deduced by HPLC and liquid chromatography/mass spectrometry (LC/MS), first checked individually and then in polyphenolic association to look for synergic activities, and in the presence of proline (1 mM).

For fungal strains, the tests were carried out by insemination, with mycelia fragments of 6 mm in diameter (10 days hold), in Petri dishes containing potato dextrose agar (PDA). After the addition of the extract (450 and 900 μg/mL), the plates were sealed with parafilm and incubated in the dark at 22 °C. Control samples with the mycelia in PDA and distilled water were incubated under the same conditions. The effectiveness of the treatments was evaluated by measuring the average diametric growth of the colonies after 4, 8, and 12 days of the inoculum. The percentage of inhibition (I) was calculated according to the formula of Zygadlo and Guzman[16,17]

$$I\ (\%) = [(C - T)/C] \times 100$$

where C = average diameter of fungi grown in PDA + water, and T = average diameter of fungi cultivated in PDA with the extract.

When no mycelium growth was observed, the latter was transferred to a plate containing only PDA and incubated for 48 h, to determine if the inhibition was fungistatic or fungicide. All experiments were repeated three times to confirm the MIC.

For the assessment of listerial growth with and without inhibitor (MECL), a suspension of 10^3 CFU/mL of the bacterium was made in

which we added the desired concentration of MECL, and 100 μL was plated in agar ALOA (Microbiol-Cagliari). This action was repeated in different plates after dilutions every 3 h with reference to a control without extract. The bacterium was incubated overnight at 37 °C, and then, the number of survived bacteria was counted.

Stability and Heat Inactivation of Carob Leaves Extract. To assess the effects of storage on the activity of carob leaves extract, aliquots were stored at different temperatures (−18, +4, and 37 °C and at room temperature) for 24 days. Afterward, the MIC was redetermined.

Effect of Carob Extract on Some Enzyme Activity. The changes of different cellular enzymatic activities of treated or untreated bacteria, collected from the inhibition and the exponential growth phase culture, were evaluated using the API-ZYM system (BioMérieux). The API-ZYM system is a set of ready for use small tubes, which serve for identification of microorganisms by a rapid miniaturized biochemical test.

One hundred microliters was plated in agar TSYEA and incubated for 24 h at 37 °C, and then, a suspension of 10^5 CFU/mL was made. Each API-ZYM gallery was inoculated with 100 μL of this last and incubated overnight at 37 °C.

Statistical Analysis. All tests of the assessment of listerial growth were run in triplicate and averaged. Means, standard errors, standard deviations, and degrees of significance (using Student's *t* test) were calculated from replicates within the experiments, and analyses were done using Microsoft Excel XP 2010. Differences of $P < 0.05$ were considered to be significant.

RESULTS AND DISCUSSION

Because of the complexity of various natural mixtures of phenolic compounds, many extraction methods have been reported in the literature using different solvents and a range of selective chromatographic separation steps. In the present work, we used a simple methanolic extraction method. The phenolic compounds of carob leaves methanolic extract were fractionated and analyzed by HPLC-DAD and HPLC-MS. The use of DAD allows not only the peak identification but also peak purity determination. The purity of all identified peaks in our experiments generally reached 1.00, and the corresponding chromatogram obtained at 210 nm is presented in Figure 1.

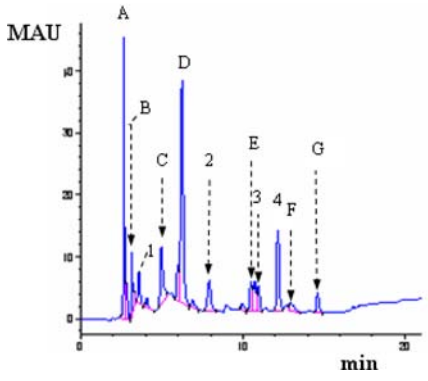

Figure 1. HPLC profile of carob leaves methanolic extract with detection at 210 nm. Identified peaks as compared to standard compounds: 1, gallic acid; 2, (−)-epigallocatechin-3-gallate; 3, myricitrin; and 4, isoquercitin. Unidentified peaks: A−G.

These compounds were identified by comparison of the retention times and UV/visible spectra with the available standards corresponding peaks (Table 1). Four of the 12 observed peaks matched with the standard compounds used in this work were attributed to gallic acid, (−)-epigallocatechin-3-gallate, myricitrin, and isoquercitin, which correspond to compounds found in carob leaves.[18−20] Performing mass spectrometric analysis in the negative ion mode resulted in mass chromatograms for deprotonated ions $[M - H]^-$ at m/z ratios similar to those obtained with authentic standards. The ESI-MS technique allowed confirmation of and completion of the identification of the phenolic compounds based on their specific and characteristic molecular ions described in the literature. With this technique, we observed three different phenolic compounds (Table 1).

Using the well microdilution method, MECL showed a high antimicrobial activity against both reference strain *L. Monocytogenes* (ATCC 35152) and isolated strains with MIC at 28.12 μg/mL (Table 2). This is the first report on the antilisterial activity of carob leaves methanolic extract. According to the Table 1, the (−)-epigallocatechin-3-gallate represents 12.71% of all polyphenols present in MECL or 114.39 μg/mL, while it is active against *S. aureus* at 200 μg/mL.[21] We can explain this resistance by the phenomenon of antagonism or synergism between all polyphenols present in MECL. No inhibition zone was detected when the disk diffusion method was used. This can indicate that carob leaves methanolic extract has a bacteriostatic activity against this bacterium. Bacteria that grow around the disk are those that may metabolize polyphenols present in MECL.

Table 2. Activity of Carob Leaves Methanol Extract against Bacterial and Fungal Strains[a]

	technique	
	microdilution method	disk diffusion
microorganisms	MIC (μg/mL)	diameter of the inhibition zone (mm)
	Gram (−)	
E.coli (ATCC 25922)	>900	NA
S. typhi (ATCC 14024)	>900	NA
P. aeruginosa (ATCC 9027)	>900	NA
	Gram (+)	
L. monocytogenes (ATCC 35152)	28.12	NA
S. aureus (ATCC 25932)	>900	NA
	fungal strains	
A. niger (ATCC 16404)	−	NA
F. oxysporum	−	NA
F.graminearum	−	NA
Penecillium spp.	−	NA

[a]NA, not active; −, not tested.

To more specifically determine which components have inhibitory effects, pure phenolic compounds were examined. Results of MIC are presented in Table 3. All compounds with an aromatic ring similar to the gallic acid structure have an inhibitory effect against *L.monocytogenes*.

Gallic acid is a phenolic acid that was found distributed in many plants[22] and exhibits inhibitory activity against many species of bacteria, fungus, and yeast.[23,24] To study the effect of the MECL on bacterial growth, different concentrations (28.12 and 100 μg/mL) of MECL were tested. As compared with the control cell suspensions without carob extract, we observed a modification of the typical cell growth curves of *L. monocytogenes* (Figure 2). The addition of these concentrations of our extract at cell density of 10^3 CFU/mL evidenced a longer

Table 3. MICs of Polyphenols Found in MECL on *L. monocytogenes*[a]

	technique		
	microdilution method		disk diffusion
polyphenols	MIC (μg/mL)	+1 mM proline	diameter of inhibition zone (mm)
isoquercitin	112.5	–	NA
gallic acid	450	++	NA
myricitrin	450	–	NA
(−)-epigallocatechin-3-gallate	225	++	NA
synergic activity			
isoquercitin + gallic acid	450	–	NA
isoquercitin + myricitrin	–	–	NA
isoquercitin + (−)-epigallocatechin-3-gallate	–	–	NA
gallic acid + myricitrin	900	–	NA
gallic acid + (−)-epigallocatechin-3-gallate	–	–	NA
myricitrin + (−)-epigallocatechin-3-gallate	–	–	NA

[a]NA, not active; ++, active; and –, not tested.

inhibition phase (phase Lag), cell growth phase resumed by a rate lower than the control cell suspensions. In addition, cultures exposed to MECL entered in a stationary phase at a lower bacteria concentration. When the concentration of the extract was increased, from 28.12 to 100 μg/mL, the duration of the inhibition phase increased, whereas the rate of the growth after inhibition as well as the cell concentration at which stationary phase was entered decreased. The observation that the resumed growth rate is substantially lower than the uninhibited culture growth rate confirms that the inhibited cells are not totally able to metabolize compounds in the carob extract or recover from their inhibition. This phenomenon was found also on treatment of *Salmonella hadar* with aqueous garlic extract.[25] The lower secondary growth rate could indicate either the presence of some unrepaired lesions in the cells or the depletion of limiting nutrients from the medium during the inhibition phase.

The antimicrobial activity evolution of MECL during storage shows that carob extract could be stored at +4 or −18 °C because no detectable big losses of antibacterial activity at these temperatures were noticed over 24 days (Figure 3). These results suggest that the observed antimicrobial activity is probably due to the constituent polyphenols of MECL that are stable at low temperatures.

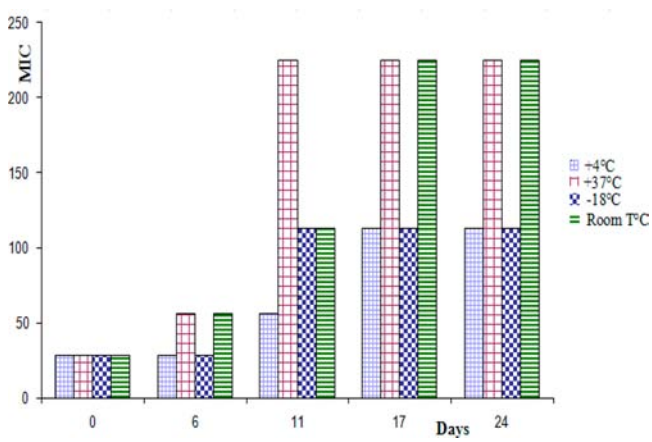

Figure 3. MIC evolution of carob leaves methanolic extract against *L. monocytogenes* during storage at different temperatures.

The use of the API-ZYM system does not show differences in the enzymatic profile of the bacterium. This result leads us to search and propose a mode of action of the phenolic compounds present in MECL on *L. monocytogenes*. With the use of proline, we tried to evaluate if phenolic metabolites behave as proline analogues or proline mimics. If this is the

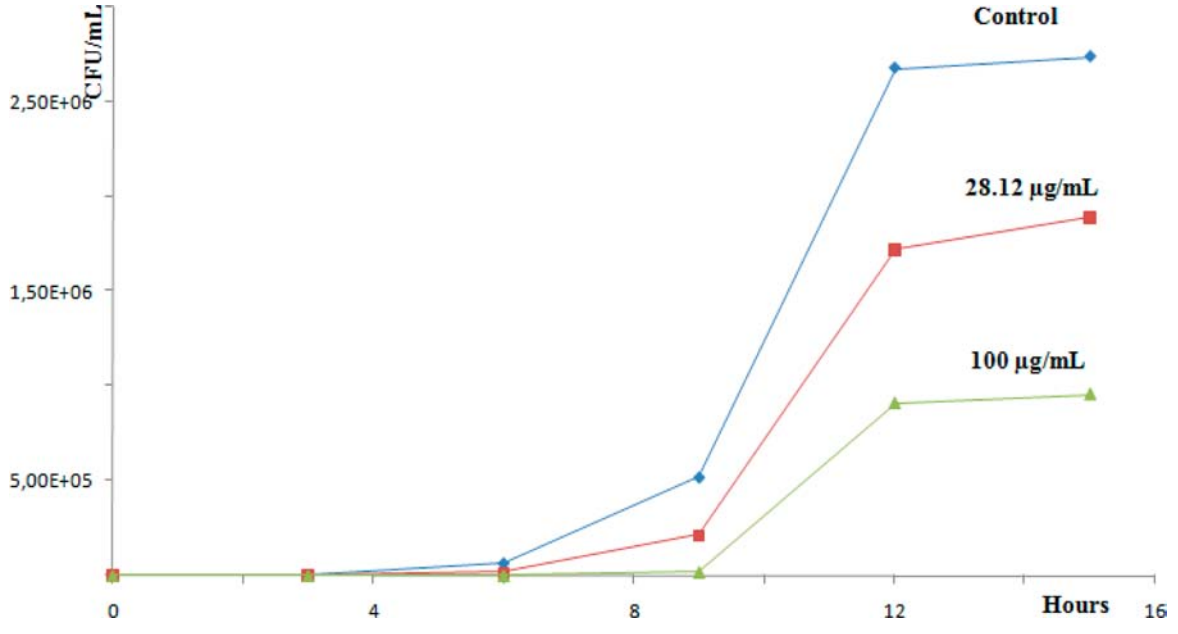

Figure 2. Growth curves of *L. monocytogenes* at 37 °C with and without treatment at different concentrations (MIC = 28.12 and 100 μg/mL) of carob leaves methanol extract.

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case, polyphenols can inhibit proline oxidation by inhibition of proline dehydrogenase (PDH) at the cytoplasm membrane in the prokaryotic cell and therefore inhibit the bacterium growth.[26] In this case, the addition of proline could overcome the inhibition of proline analogues with aromatic ring structures.

The antimicrobial effect of gallic acid and (−)-epigallocatechin-3-gallate was removed when 1 mM proline was added into the well. These results can give insight into the mode of action of polyphenols that might act as inhibitors of PDH and disturb the pentose phosphate pathway, confirming previous studies for other pathogenic bacteria such us *H. pylori*.[26]

This paper deals with the study of the antimicrobial activity of carob leaves methanol extract against *L. monocytogenes*. Leaves of the carob tree contain high levels of polyphenolic compounds with antilisterial activity that may act by inhibiting the PDH. In the future, we will study the antilisterial activity of carob leaves methanol extract in vivo using real substrates such us meat and fish samples.

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Notes

The authors declare no competing financial interest.

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