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Original Research

Antimicrobial Effect of Garlic (*Allium sativum*) and Thyme (Zataria *multiflora Boiss*) Extracts on Some Food Borne Pathogens and Their Effect on Virulence Gene Expression

Mona M El-Azzouny, Azza S El-Demerdash*, Hanan G Seadawy, Sally H Abou-Khadra

Animal Health Research Institute, Egypt

Correspondence to: Dr.azzasalah@yahoo.com

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Abstract: The incrementing scope of pathogenic resistance to antibiotics has encouraged the search for antivirulence natural extracts. Therefore, our study designed to demonstrate the antimicrobial activity of an aqueous-garlic and thyme oil extracts against Gram-positive (*Staphylococcus aureus*) and Gram-negative (*Salmonella* spp.) bacteria by evaluating the influence of sub-inhibitory concentrations on the expression of the most critical virulence genes of the tested isolates. The antibacterial potential of both herbs was checked by the agar well diffusion method and minimum inhibitory concentration (MIC) assay. Interestingly, all isolates were inhibited by both extracts up to 50% concentration. Also, the MIC values of garlic extract (0.125-1µg/ml) against *Salmonella* isolates were lower than the values of thyme extract (0.5- 8µg/ml). But in *S. aureus* isolates, the MIC values of thyme extract (0.25- 2µg/ml) were the lowermost. Conventional PCR investigated that all *S. aureus* isolates carried the *hlg* (hemolysin) and *icaA* (intracellular adhesion) genes, but only six *Salmonella* isolates (three *S.* typhimurium and one each of *S.* kentucky, *S.* anatum, and *S.* lagos) had both the *sopB* (*Salmonella* outer protein B) and *mgtC* (membrane protein) genes. Real-time RT-PCR assays were performed to evaluate the extract's effect on the virulence genes. The thyme-oil extract has significantly repressed *S. aureus* virulence genes expression more than aqueous-garlic extract, which later one has effectively more than thyme-oil extract in downregulating the *Salmonella* virulence genes. In conclusion, garlic and thyme extracts can be used not only as a flavor, but also as potential antimicrobial agents against Gram-positive and negative bacteria.

Key words: Garlic; Thyme; Antimicrobial activity; *Salmonella*; *S. aureus*; Virulence; Gene expression.

Introduction

Food-borne disease (FBD) is characterized by miscellaneous symptoms that arise from the consumption of contaminated foods, mainly meat products, or beverages. Despite the recent advances in food production technology and processing, FBD remains a major cause of morbidity and mortality, constituting both an important public health concern and a significant economic problem at a global level (1, 2). *Staphylococcus aureus* and *Salmonella* species are the most common causes of FBD. Antibiotics and chemical agents may be prescribed for moderate to severe cases food poisoning or foodborne cases (3). However, probably prolonged use of antibiotics led to bacterial adaptation, resulting in the development of multidrug resistance in bacteria and the severity of human diseases related to these pathogens rising in many countries (4, 5, 6). This has significantly limited the delicacy of antibiotics, warranting alternative strategies to combat microbial infections.

Plants have been able to give new compounds of great benefit to humanity. Many approaches have been aimed to find natural biological principles in plants (7). An example of those resources is public medicine as a systematic screening of them may lead to the discovery of new effective antibacterial components (8, 9) to replace or reduce reliance on synthetic food preservatives. In the last 20 years, hundreds of studies demonstrated antimicrobial activity of natural compounds against pathogenic or spoilage organisms. However, few of these have been translated into real food applications (10).

Various societies worldwide have been used garlic to combat infectious disease for many centuries. Also, garlic provided as dietary supplements and belongs to the Liliaceae family. The first one clarified the antibacterial effect of garlic against all bacterial types was Louis Pasteur (11). Many published research articles have mentioned a strong effect of garlic juice against common pathogenic bacteria (12), and against the bacteria that have acquired resistance for antibiotics (13) and also toxin production by some pathogenic bacteria prevented by garlic (14). The antimicrobial activities of garlic are linked to the presence of some bioactive compounds mainly allicin, which is an organosulfur compound (diallyl thiosulfinate) and has multiple inhibitory effects on the microbial cell (15).

Additionally, Zataria multiflora Boiss (Common thyme) is a member of the Lamiaceae family, which distributes in areas of Mediterranean, Asia and is cultivated all over the world. Common thyme contains 0.8-2.6 % volatile oil consisting of a highly variable amount of phenols, monoterpene hydrocarbons, and alcohols. Normally thymol considers as a major phenolic component in thyme. Thyme is used as an antispasmodic, carminative, antiseptic, antimicrobial and natural food preservative (16).

The antimicrobial properties of those plants that target cellular viability of bacteria have been adequately discussed previously (17, 18), but very few reviews have highlighted the effects of these compounds in modula-

ting various aspects of bacterial virulence, critical for pathogenesis in the host. Therefore, the objective of this study was to assess the antimicrobial activity of garlic and thyme extracts to further investigate the influence of sub-inhibitory concentrations of those natural plants on the production and expression of the two major virulence genes of *S. aureus* and *Salmonella* spp.

Materials and Methods

Sampling and isolates characterization

A total of 100 retail meat products of bovine origin (minced meat, sausage, burger, and kofta), 25 each, were purchased randomly from different supermarkets in Zagazig, Sharkia Province, Egypt. All samples were subjected to conventional methods for isolation and identification of *S. aureus* and *Salmonella* species (19). *Salmonella* isolates were further identified with API20E identification kits (BioMérieux, Mary l'Etoile, France) and serotyped in the Serology Unit Animal Health Research Institute, Dokki, Giza Egypt using commercial antisera (Difco, Detroit, MIUSA) according to the manufacturer's instructions.

Extracts preparation

Aqueous garlic and thyme oil extracts were prepared according to Onyeagba *et al*. (20) and Betoni *et al*. (21), respectively.

Determination of antibacterial activity of herbal extracts

Agar diffusion assay

Agar well diffusion method was used for initial evaluation of antimicrobial properties of plant extracts against *Salmonella spp.* and *S. aureus* isolates in comparison with ciprofloxacin. The tested isolates were inoculated into 10 mL of sterile nutrient broth and incubated at 37ºC for 8 hours. The cultures were swabbed on the surface of sterile nutrient agar plates using a sterile cotton swab. Agar wells were prepared with the help of sterilized cork borer with 10 mm diameter (22). Using a micropipette, 100ul of different concentrations of each extract and ciprofloxacin (100%, 75%, and 50%) was added to the wells in the plate. In case of thyme, 100µL of 5% dimethylsulphoxide (DMSO) was added to (100µL) of thyme oil extract to solubilize it.

The plates were incubated in an upright position at 37 ºC for 24 hours. The diameter of inhibition zones was measured in mm and the results were recorded. The inhibition zones with a diameter of less than 12 mm were considered as having no antibacterial activity (23).

Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) determination

The broth microdilution method was used with 96-well plates (TPP, Switzerland). Both extracts and ciprofloxacin were diluted twofold in LB broth® (Acumedia, Michigan, USA), and the wells were inoculated with 1×10^6 CFU of bacteria (in a 0.2 ml final volume). The incubation period was 24 h at 37°C. The MIC testing was performed according to the recommendations of the Clinical and Laboratory Standards Institute (24). The range of the concentrations assayed for each antibiotic was 0.062 to 64 µg/ ml.

The MIC value is the lowest antimicrobial concentration that inhibits microorganism growth but subinhibitory concentration (SIC) value is an antimicrobial concentration that below one capable of inhibiting the detectable growth and replication of a microorganism. The minimum bactericidal concentration value (MBC) value was determined according to Khosravi *et al*. (25).

DNA extraction and PCR amplification

DNA extraction of samples was performed using the QIAamp DNA Mini kit (Qiagen, Germany, GmbH). Then, isolates were screened for the presence of the major virulence factors, including hemolysin production (*hlg)* and intercellular adhesion factor (*icaA*) for *S. aureus* and effector protein (*sopB)* and magnesium uptake (*mgtC*) for *Salmonella* spp. The characteristics of all used primers, as well as amplicons length and cycling conditions, are summarized by Ciftci *et al*., (26), Kumar *et al*., (27); Huehn *et al*. (28).

PCR products visualization and analysis

The products of PCR were separated by electrophoresis on 1% agarose gel (Applichem, Germany, GmbH) by running 20 µl of the PCR products. The gel was photographed by a gel documentation system (Alpha Innotech, Biometra) and the data were analyzed by computer software.

Quantitative analysis of virulence gene expression

The virulence gene expression was analyzed by quantitative real-time PCR (qRT-PCR) and the 16S rRNA housekeeping gene served as an internal control to normalize the expressional levels between samples. Primers were utilized in a 25- µl reaction containing 12.5 µl of the 2x QuantiTect SYBR Green PCR Master Mix (Qiagen, Germany, GmbH), 0.25 µl of RevertAid Reverse Transcriptase (200 U/µL) (Thermo Fisher), 0.5 µl of each primer of 20 pmol concentration, 8.25 µl of water, and 3 µl of RNA template. The reaction was performed in a Stratagene MX3005P real-time PCR with specific conditions mentioned in the Table 1. Amplification curves and Ct values were determined by the Stratagene MX3005P software. To estimate the variation of gene expression on the RNA of the different samples, the Ct of each sample was compared with that of the positive control group according to the "ΔΔCt" method stated by Yuan *et al*.(29).

Statistical data analysis

Data analysis was done using SPSS version 22 for windows. A t-test was used for comparison between fold change of different extracts (i.e. aqueous garlic and thyme oil extracts) and ciprofloxacin against *S. aureus* and *Salmonella* isolates.

Results

The recovery rate of isolation and identification

Of all the meat samples tested, 12 (12%) were found to carry *S. aureus* and only 10 (10%) were infected by *Salmonella* species with a high distribution rate of contaminating pathogens across sausage samples. Serotyping of *Salmonella* isolates revealed that *S.* typhi-

Table 1. Primer sequences, target genes and cycling conditions for SYBR green Rt-PCR.

Target gene	Reverse transcription	Primary denaturation	Amplification (40 cycles)			Dissociation curve (1 cycle)			Reference
			Secondary denaturation	Annealing (Optics on)	Extension	Secondary denaturation	Annealing	Final denaturation	
Staphylococcus 16S rRNA				55˚C 30 sec.			55˚C 1 min.		(30)
Hlg	50˚C 30 min.	94˚C 15 min.	94˚C 15 sec.	55˚C 30 sec.	72˚C 30 sec.	94˚C 1 min.	55˚C 1 min.	94˚C 1 min.	(27)
icaA				49˚C 30 sec.			49˚C 1 min.		(26)
Salmonella 16S rRNA				60˚C 30 sec.			60˚C 1 min.		(31)
sopB	50˚C 30 min.	94˚C 15 min.	94˚C 15 sec.	58˚C 30 sec.	72˚C 30 sec.	94˚C 1 min.	58˚C 1 min.	94˚C 1 min.	
mgtC				58˚C 30 sec.			58˚C 1 min.		(32)

murium predominated (40%) over *S.* anatum (30%), *S.* lagos (20) and *S.* kentucky (10%).

Antimicrobial activity

All tested isolates were inhibited by both extracts up to 50% concentration and the activity was a linear function of concentration. At 100%, the maximum zone of inhibition was observed, but we can see the variation in the size of the inhibition zone among the different group of bacteria. The maximum inhibition zone of *S. aureus* to both extracts was (36 mm), and the minimum was (20 mm). On the other hand, the *Salmonella* inhibition zone range was (18-30)

Additionally, the aqueous garlic and thyme oil extracts possessed MICs and MBCs nearly similar to ciprofloxacin. The aqueous garlic extract was a more effective antibacterial agent (MBCs of 0.25–8 µg/ml) than thyme oil extract (MBCs of 0.5–16 µg/ml). The MIC values of the garlic extract (0.125–1 µg/ml) against *Salmonella* isolates were lower than those of the thyme extract (0.5–8 µg/ml). In contrast, for *S. aureus* isolates, the MIC values of the thyme extract (0.25–2 µg/ml) were lower than those of the garlic extract (0.25–4 µg/ml) as shown in the Table 2.

Detection of some virulence genes in both *S. aureus* and *Salmonella* isolates by conventional PCR

All tested *S. aureus* isolates were carried two virulence genes and gave characteristic bands at 937 bp and 1315 bp for *hlg* and *ica*A, respectively. Additionally, only six *Salmonella* spp. (three *S. t*yphimurium and one each of *S.* kentucky, *S. a*natum, and *S.* lagos) have both virulence genes and gave characteristic bands at 517 bp and 677 bp for *sop*B and *mgt*C, respectively.

Quantitative assessment effect of aqueous garlic extract and thyme oil extract on some virulence genes in both *S. aureus* and *Salmonella isolates* using the qRT-PCR

By RT-PCR, comparing the amount of examining virulence gene products (CDNA) before and after treatment with a sub-inhibitory concentration of aqueous

Table 3. Results of RT-PCR showing expression of *hlg, icaA* genes in *S. aureus* isolates before and after treatment with SIC of aqueous garlic extract, thyme oil extract, and ciprofloxacin.

Genes	Isolate No.	Fold change		
		Aqueous garlic extract	Thyme oil extract	Ciprofloxacin
Hlg	1	0.7120	0.0552	0.1142
	2	0.7474	0.0708	0.0638
	3	0.6830	0.0780	0.1111
	4	0.5396	0.1869	0.0304
	5	0.7738	0.0743	0.2717
icaA	1	0.2793	0.0361	0.1349
	2	0.5471	0.0733	0.0454
	3	0.1446	0.0684	0.5743
	4	0.1708	0.0703	0.1150
	5	0.2517	0.0280	0.7792

Table 2. SIC, MIC, and MBC of aqueous garlic extract and thyme oil extract against *S. aureus* and *Salmonella* spp. isolates.

Isolates	Concentration of SIC, MIC,MBC(µg/ml)								
	Aqueous garlic extract			Thyme oil extract			Ciprofloxacin		
	SIC	MIC	MBC	SIC	MIC	MBC	SIC	MIC	MBC
S. aureus									
1	2	4	8	1	2	4	0.5	1	2
2	1	2	4	0.5	1	2	0.25	0.5	1
3	1	2	4	0.5	1	2	0.125	0.25	0.5
4	0.25	0.5	1	0.25	0.5	1	0.125	0.25	0.5
5	0.25	0.5	1	1	2	4	0.5	1	2
6	0.5	1	2	0.5	1	2	0.25	0.5	1
7	0.25	0.5	1	0.125	0.25	0.5	0.125	0.25	0.5
8	0.125	0.25	0.5	0.5	1	2	0.25	0.5	1
9	2	4	8	0.5	1	2	0.5	1	2
10	0.5	1	2	0.5	1	2	0.125	0.25	0.5
11	0.25	0.5	1	0.25	0.5	1	0.25	0.5	1
12	0.25	0.5	1	0.5	1	2	0.5	1	2
***Salmonella* spp.**									
S. typhimurium	0.5	1	2	4	8	16	0.5	1	2
S. typhimurium	0.125	0.25	0.5	0.25	0.5	1	0.25	0.5	1
S. typhimurium	0.062	0.125	0.25	0.5	0.5	1	0.125	0.25	0.5
S. typhimurium	0.25	0.5	1	0.5	1	2	0.125	0.25	0.5
S. lagos	0.25	0.5	1	1	2	4	0.5	1	2
S. lagos	0.25	0.5	1	1	2	4	0.125	0.25	0.5
S. anatum	0.5	1	2	1	2	4	0.25	0.5	1
S. anatum	0.25	0.5	1	0.5	1	2	0.25	0.5	1
S. anatum	0.125	0.25	0.5	0.25	0.5	1	0.125	0.25	0.5
S. kentucky	0.25	1	2	2	4	8	0.5	1	2

Table 4. Results of RT-PCR showing expression of *sopB, mgtC* in *Salmonella* spp. isolates before and after treatment with SIC of aqueous garlic extract, thyme oil extract, and ciprofloxacin.

Genes	Isolate No.	Fold change		
		Aqueous garlic extract	Thyme oil extract	Ciprofloxacin
*sop*B	*S.* anatum	0.0884	0.4204	0.0374
	S. kentucky	0.0587	0.1349	0.0451
	S. lagos	0.0559	0.5176	0.0791
	S. typhimurium	0.0575	0.3322	0.0121
*mgt*C	*S.* anatum	0.0718	0.4263	0.0981
	S. kentucky	0.0954	0.2535	0.0201
	S. lagos	0.0612	0.3737	0.0396
	S. typhimurium	0.0670	0.2117	0.0433

* P-value<0.05 was considered statistically significant.

garlic and thyme oil extracts (**Tables 3, 4**). Results revealed that the amount of examining gene products was relatively increased in untreated samples with both extracts than those treated, that leads to high threshold cycle (Ct) value in treated than untreated. Interestingly, we found that thyme oil extract was more effective in significantly reducing the expression of *S. aureus* virulence genes than aqueous garlic extract counter to *Salmonella* spp., in which aqueous garlic extract repressed virulence genes expression higher than thyme oil extract

The fold changes in *mgtC* and *sop*B genes expression after treatment with SIC of aqueous garlic extract were (0.061:0.095 fold) and (0.055:0.088 fold), respectively, which were nearly to a fold change in same gene expression after treatment with SIC of ciprofloxacin that were (0.020:0.098 fold) and (0.012:0.079 fold), respectively. But in case *of S. aureus*, downgrading of the virulence genes expression *hlg* and *ica*A after treatment with SIC of thyme oil extract were expressed by fold changes (0.061:0.095 fold) and (0.02:0.07 fold), respectively, and were close to fold changes of ciprofloxacin SIC that were (0.03:0.2 fold) and (0.045:0.77 fold), respectively.

Discussion

Contaminated food is well established as the main source of transmission for pathogenic bacteria. It is the major cause of several diseases in developing countries resulting in many cases of mortality and morbidity (33). Beef, one of the main sources of foodborne infections, has a great impact on food safety. In this study, the presence of *Salmonella* and *S. aureus* was established to be approximately 10% and 12%, respectively, in retail meat products. Our findings were compatible with those previously reported by other researchers (34, 35)**.**

In the present work, the most commonly recovered serovar from different retail meats was *S. typhimurium* followed by *S.* anatum, *S.* lagos*,* and *S.* kentucky involved primarily in human salmonellosis as previously mentioned by Jakee *et al.* (36)**.** Detection of four *Salmonella* serovars in this study reflects the possibility of cross-contamination from various sources in slaughterhouses and poor hygiene during the butchering and processing of meat.

In recent years, the use of natural compounds has gained attention due to increasing concerns over the safety of synthetic chemicals and emerging antibiotic resistance in bacteria (37). In the food industry, a negative consumer reaction to chemical preservatives has prompted an increased interest in natural alternatives (38). For these reasons, plant extracts, because of their established antimicrobial activities as well as their relatively lower toxicity and reduced number of side effects, may be potentially useful replacements for chemical preservatives (39)**.** Also, plant extracts have a multi-component nature, so it is more difficult for bacteria to develop resistance than many commonly used antibiotics, which have a single target site (40)**.**

Of interest, all obtained *S. aureus* and *Salmonella* isolates were inhibited by aqueous garlic and thyme extracts up to 50% concentration. It has been proposed that the mechanism of herbal antimicrobial effects involves the inhibition of various cellular processes, followed by an increase in plasma membrane permeability and finally ion leakage from the cells (41)**.**

Cavalitto and Bailey (42) were the first to demonstrate that the antibacterial action of garlic is mainly due to allicin which primarily inhibits the acetyl CoA forming system lead to inhibit DNA and protein synthesis, then inhibits RNA synthesis as a primary target (43). Therefore, the development of the organism will not occur because proteins are essential for all parts of cell structure.

However, allicin is rapidly oxidized, unstable and volatile, meaning it rapidly breaks down after raw garlic is cracked. It has been reported that aqueous garlic extract has more potent antibacterial activity than an equal amount of allicin. This may be because a water-based extract of garlic stabilizes allicin, at least partially, due to the hydrogen bonding between water and the reactive oxygen atom in illicit that lessens its instability and/or there may be water-soluble ingredients in cracked garlic that destabilize the molecule (44)**.**

The results of this study indicate that the aqueous garlic extract (AGE) had a high antibacterial activity for gram negative (*Salmonella* spp.) than gram positives (*S. aureus*). This results justified by Belguith *et al*.(45) who said that aqueous garlic extract acts mainly on Gram-negative bacteria, and their outer membrane (OM), which is absent in Gram-positive bacteria, seems to be one of its main targets. It seems the AGE alters the ultrastructure of the OM, whereas changes are not seen in the cytoplasmic membrane.

With respect to thyme extract, the major active compound is thymol, which exerted its antimicrobial action

through binding to membrane proteins by hydrophobic bonding and hydrogen bonding and then changing the permeability of the membranes. Thymol also decreased intracellular adenosine triphosphate (ATP) content of bacteria and increased extracellular ATP, which could disrupt the function of plasma membranes (46).

our data revealed that thyme oil extract had highly antimicrobial effect against *S. aureus* (Gram-positive) with MICs (4-16µg/ml) than *Salmonella* spp. (Gram-negative) with MICs (8-64µg/ml) as mentioned previously by Ghasemi *et al.* (16) that found the antimicrobial activity of the thyme essential oil is more effective on Gram-positive than Gram-negative bacteria and this justified by Goodarzi (47) as these compounds act by interfering with the cell wall. Gram-positive bacteria have a thick cell wall, high in the peptidoglycan and teichoic acid. But, Gram-negative bacteria contain an outer membrane and a periplasmic space, which contains peptidoglycan, protein ingredients. Therefore, the essential oil of the Zataria multiflora may be able to spread easily through the loose outer wall of Gram-positive bacteria, but must go throughout the narrow channels of the Gram-negative outer membrane.

The pathogenicity of both pathogens is dependent, to a great extent, upon the secretion of a variety of extracellular and intracellular virulence factors (48, 30). PCR profile revealed that all obtained *S. aureus* isolates (100%) were carrying genes involving in producing biofilm formation (*ica*A) and gamma-hemolysin (*hlg*). It has been reported that the dose of antibiotics needed to kill biofilm bacteria was up to 1000-fold greater than the dose needed to kill planktonic bacteria (49).Therefore, we propose that our extracts are a cost-effective antimicrobial agent for the treatment of biofilm infection.

Regarding *Salmonella*, *sop*B and *mgt*C genes were shown to be present in 60% of isolates. The *mgt*C magnesium transport protein is a putative P-type ATPases which encodes a membrane protein that is indispensable for *Salmonella* survival in macrophages (50). *Sop*B gene implicated in the translocated effector protein of T3SS for *SPI*-5 translocated into the host cytosol, where it mediates inflammation and fluid secretion in the intestinal mucosa (51).

Both extracts used for the treatment of *S. aureus* and *Salmonella* infections not only depends on the respective bacteriostatic or bactericidal effects but also on the ability to prevent the release of virulence factors by dying or stressed bacteria (52, 53). To address this, a real-time RT-PCR assay was used to assess the impact of garlic and thyme extracts on the expression level of the examined virulence genes.

Based on our findings, these extracts at sub-inhibitory concentrations are capable of decreasing biofilm formation and hemolysin production by *S. aureus* via significantly down-regulating *ica*A and *hlg* genes. These suppressing effects were more remarkable for thyme oil extract in comparison with aqueous garlic extract. The same impact was detected concerning to *Salmonella* virulence expression after exposure to sub-inhibitory concentrations of both extracts with high efficiency of garlic.

Several studies have shown that some herbal extracts have inhibitory effects on virulence expression of Gram-positive and Gram-negative bacteria. For example, Kollanoor-Johny *et al.*(54) who found that trans-cinnamaldehyde (TC) and eugenol (EG) reduced the motility and invasive abilities of *S. Enteritidis* and down-regulated expression of the motility genes (*flhC* and *mot*A) and invasion genes *(hilA*, *hilD*, and *invF)*. And confirmed also in another bacterial type by Wojnicz *et al*. (55) who detected that the *Urtica dioica* extracts significantly reduced the motility of the *E. coli* rods and biofilm activity and other extracts of *Vaccinium Vitis-idea* decreased the bacterial survival and virulence factors involved in tissue colonization and biofilm formation of the uropathogenic *Escherichia coli*. Also, Sakuragi and Kolter (56) stated that A. sativum extract resulted in over 4-fold down-regulation of the pelF gene expression in *P. aeruginosa*.

In conclusion, our study supports the prospects for the use of garlic and thyme extracts as an antipathogenic remedy in combined therapy with antibiotics against resistant bacteria.

Conflict of interest

The authors manifested that they have no conflicts of interest.

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