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Evaluation of antimicrobial activity of oregano oil and assessment of *Listeria monocytogenes* on crab meat during chilled storage

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Abstract
In the current study, Oregano Essential oil, containing potent antimicrobial agents (namely carvacrol and thymol) was used to observe its effect on the cooked crab meat in combination with the age old technology of vacuum packaging when stored at 4 °C for 28 days. In addition, the antimicrobial assay to check the efficacy of the oil against *L. monocytogenes* was done by the disc diffusion method and minimum inhibitory concentration (MIC). The MIC value of was 0.25 µL/mL. The current study found promising results after evaluating the microbiological (TVC and enumeration on PALCAM for *L. monocytogenes*) as well as antimicrobial activity of the essential oil. The mesophilic count of the control sample initially was 2.32 log CFU/mL which reached a final value of 8.9 log CFU/mL whereas for the sample with combination of vacuum packaging and 1% oregano oil (VP+1%O), the value ranged between 2.12 log CFU/mL and 5.02 log CFU/mL which is well below the acceptability range. Also, in the samples inoculated with a known concentration of the bacteria, the sample with vacuum packaging showed a value between 3.69 log CFU/mL and 3.903 log CFU/mL.

Keywords: oregano oil, crabmeat, antimicrobial, chilled storage

Introduction
Food-borne infections are among the most serious and costly public health concerns worldwide. Among food pathogens, *Listeria monocytogenes* is one of the most important pathogens, causing 30% mortality in patients [1-4]. *L. monocytogenes* is a psychrotrophic, Gram-positive, facultative bacterium found in many food products [5] and is considered an environmental contaminant [6]. It is a foodborne pathogen causing listeriosis whose cases occur in immuno-compromised individuals, pregnant women, neonates, and elderly persons [7-9]. *L. monocytogenes* poses a severe microbiological problem in seafood both raw and pasteurized [10]. The ability of *Listeriae* to grow over a wide temperature range (01 and 45 °C), including refrigeration temperatures and even multiply at refrigerated temperatures, is of great concern to the food industry. Prevalence of this pathogen in crab, either raw or pasteurized has been documented by many researchers. The U.S. Department of Agriculture (USDA), Centers for Disease Control (CDC), and the Food and Drug Administration (FDA) have agreed on a "zero tolerance" policy for *L. monocytogenes* in foods not intended for further heat treatment [11]. Western society appears to be experiencing a trend of 'green' consumerism [12-13], desiring fewer synthetic food additives. Due to growing concerns regarding the safety of chemical and synthetic preservatives, alternative mechanisms based on the use of natural compounds have been increasingly tested over the last years [14-17]. Therefore, plant essential oils are gaining interest for their potential as preservative ingredients or decontaminating treatments, as they have Generally Regarded as Safe (GRAS) status and a wide acceptance from consumers(18). Essential oils (EOs) are aromatic and volatile oily liquids obtained from leaves and stems, and commonly concentrated in one particular region such as leaves, bark or fruit of plants(19). The use of natural antimicrobial compounds is important not only for the preservation of food but also for the control of human and plant diseases of microbial origin [20]. Oregano is a leafy perennial herb of the mint family that is indigenous to the Mediterranean region. Usage of oregano EO in meat was found effective in inhibiting spoilage microorganisms [21, 22] Sokovic *et al.*, (2010) found that the greatest activity across the largest range of microorganisms from EOs and components were obtained from *Origanum vulgare* (oregano). The effectiveness of oregano against foodborne pathogens have been demonstrated by numerous *in vitro* studies [23, 24].

Thus, in the present paper evaluation of antimicrobial activity of oregano oil and assessment of *L. monocytogenes* on crab meat during chilled storage was observed.

Materials and Methods

Procurement of the bacterial strain

Listeria monocytogenes was procured from the College of Veterinary and Animal Sciences, Mannuthy, Kerala.

Collection of Essential oil and chemicals

Oregano Essential Oil was obtained from Synthite Industries LTD, Synthite Valley, Kolencherry, Kerala. It is steam distilled oil. The oil (100% pure essential oil) was stored in a dark carton (12x12x30 cm) and kept refrigerated below or at 4 °C as per manufacturer's recommendation until use. All the chemicals of analytical grade and microbiological media used during the course of research were obtained from Merck. Agar powder used in bacteriological media was obtained from HiMedia.

Culture maintenance and inoculum preparation

The strain was maintained at -80 °C in Brain Heart Infusion (BHI) broth supplemented with 20% glycerol until used. Test strain of bacteria was inoculated into BHI broth and incubated at 37 °C for 24 hours. Culture was subjected to three successive 24-hour transfers before use. The culture was transferred to tubes containing 10 mL of BHI and pre-cultured at 37 °C for 24 hours. The bacterial strain was then inoculated in BHI tubes and cultured at the same conditions as mentioned above until early stationary growth phase, that is, approximately 10^9 CFU/mL. Cells were then harvested by centrifugation at 3000 rpm for 20 min at room temperature. The supernatant was decanted, and the pellet was re-suspended in sterile 0.85% saline by vortexing to obtain a strain mixture. Working cultures were adjusted to the required concentration of 10^6 CFU/mL and, which were afterwards used as inoculums in antimicrobial tests.

Antimicrobial activity by disc diffusion method

The antimicrobial activity of Oregano Essential Oil was tested against the bacteria using disc diffusion assay by [25] with slight modifications. BHI was inoculated with 100 µL bacteria 6 log CFU/mL. Oregano EO was diluted with analytical grade ethanol in 5 ratios (1 1/1, 1/10, 1/20 and 1/40; v/v, that is, 20 µL oil; 20 µL oil in 20 µL ethanol; 20 µL oil in 200 µL ethanol; 20 µL oil in 400 µL ethanol and 20 µL oil in 800 µL ethanol respectively), impregnated into 6 mm paper disc and placed on the agar, and the oil was allowed to diffuse into the medium for 30 min at 4 °C. Pure ethanol was used as control. The plates were then incubated at 37 °C for 24 hours and the diameter of the resulting zone of inhibition was measured in mm with a digital caliper. The zone of inhibition was recorded as the mean ± standard deviation (SD) of triplicate experiments. Ampicillin (10 µg/disc), chloramphenicol (30 µg/disc) and streptomycin (10 µg/disc) were used as reference antibiotics for bacteria.

Microtitre Plate Assay (MPA) for Minimum Inhibitory Concentration (MIC) determination and Collection of crab sample

MIC was determined according to the National Committee for Clinical Laboratory Standards [26], a broth microdilution method. Ninety-six well microtitre plates were used for determination. Freshly landed 25 kg blue swimming crab samples were procured from a commercial fish landing center at Thoppumpady in Kochi, Kerala, India. Crabs were brought to the Department of Fish Processing Technology, Kerala University of Fisheries and Ocean Studies (KUFOS) immediately within 1 hour after landing in iced condition (in the ratio of 1:2; w/w) in an insulated high density polyethylene (HDPE) container. Upon arrival, crabs were de-iced and washed with chilled potable water (1 °C – 2 °C) and stored in ice till further processing.

Sample preparation, Enrichment and Isolation

For the detection of *Listeria*, FDA bacteriological and analytical method (BAM) was used. 25 g of the fresh sample of crab was taken and thoroughly blended with 225 mL of sterile Buffered Listeria Enrichment Broth (BLEB) which contains the selective agents. Sample previously homogenised with BLEB (FDA BAM formulation) was incubated at 30 °C for 4 hours. Selective agents were aseptically added to BLEB pre-enrichment to achieve a final concentration of 10 mg/L acriflavin, 40 mg/L cycloheximide and 50 mg/L sodium nalidixic acid after 4 hour incubation which allows injured cells time to recover in a favourable environment. It was followed by thorough mixing and incubation at 30 °C for 24 to 48 hours for enrichment. Enrichment methods, which usually take about 30 to 48 hours, are followed by the identification of the enriched organism. After pre-enrichment period, BLEB enrichment was streaked onto esculin based and chromogenic selective agar (Oxford Agar and PALCAM) and incubated for 24 to 48 hours. Plates were then examined for characteristic colonies. Presence of esculin allows the detection of β-D- glucosidase activity by *Listeria*, causing a blackening of medium. Typical *Listeria* colonies are approximately 2-3 mm in diameter and are black with a black halo and sunken center.

Preparation of crab sample, treatment application, inoculation and packaging

The fresh crab samples were cleaned; shell and claws were removed and were then boiled under steam pressure. The cooked crab was then air cooled to room temperature and then refrigerated at 7.2 ºC (45ºF) or less. The crab meat from crabs was then picked and placed under refrigeration within 2 hours after picking until use. The crab meat was the divided into 6 slots, that is, a control sample; (under aerobic storage in the absence of oregano essential oil); sample under vacuum packaging (VP; in the absence of oregano essential oil); two samples were treated with oregano essential oil (0.5 and 1%; v/w), respectively, under vacuum packaging (VP+0.5%O and VP+1%O); two samples were inoculated with 6 log CFU/mL of *L. monocytogenes* and treated with oregano oil (MIC) under aerobic (A+L+O) and vacuum packaging (VP+L+O) respectively (after pasteurization). Oregano Essential Oil was added to the 4 lots using a micropipette, mixed properly with a sterile glass rod and then all the 6 samples were packed separately in polypropylene pouches of 170x200 mm dimension and 420 mm thickness. Two batches were sealed using an impulse heat-sealing machine (M/s. Sevana Electrical Appliances Pvt. Ltd., Kizhakkambalam, Kerala, India) for aerobic packages. The other four batches were vacuum packaged at -1 bar pressure using a vacuum sealing machine (M/s. Sevana Electrical Appliances Pvt. Ltd.). All the samples after pasteurisation were then stored at 4 °C. Samples were withdrawn from the separately packed slots every 4-5 day interval for microbiological analysis up to 28

days respectively.

Bacteriological analysis
Microbial Enumeration was done by TVC method. Bacteriological analytical method [27] was used.

Statistical Analysis
All experiments were carried out in triplicate and the results were expressed as mean value ± standard deviation (SD). Data excluding sensory were subjected to analysis of variance (ANOVA) using the standard software IBM SPSS-Version 20. P value less than 0.01 (p<0.01) were considered as statistically significant.

Results
Antimicrobial activity by disc diffusion method
The antibacterial activity of Oregano EO against *L. monocytogenes* at different diluted conditions is summarized in Table 1. The results represent the diameter of inhibition zone including diameter of paper disc (6 mm).

Table 1: Antimicrobial activity of Oregano EO against L. monocytogenes by disc diffusion method

L. monocytogenes					
	1[1]	1/1[1]	1/10[1]	1/20[1]	1/40[1]
Essential oil Diameter of inhibition zone (mm)					
Oregano oil	46.1 ±0.01	36 ±0.03	15 ± 0.3	14 ± 0.1	12.8 ± 0.1
Ampicillin	30 ± 0.6				
Chloramphenicol	26.1 ± 0.1				
Streptomycin	13 ± 0.1				
Ethanol	-				

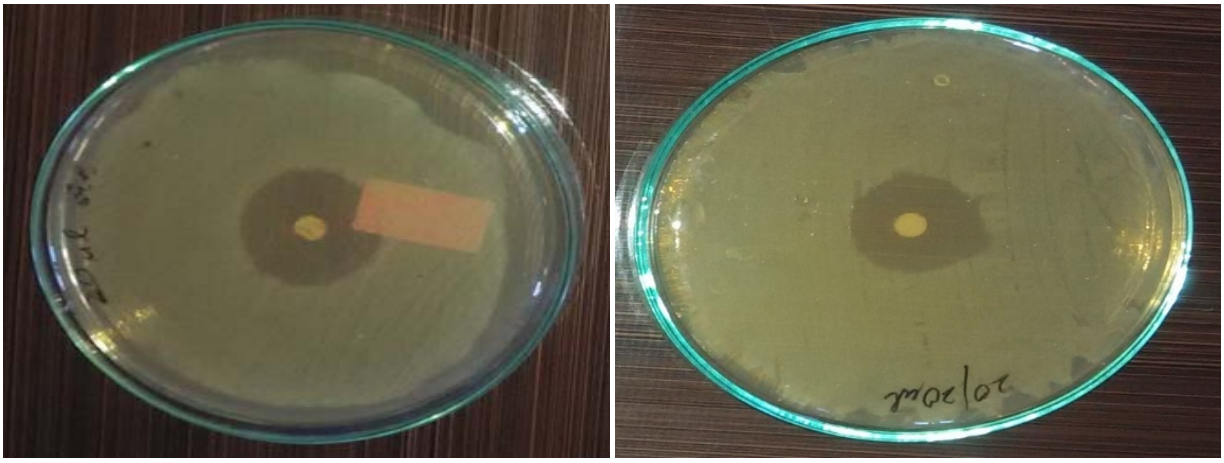

Plate a, b: showing zone of inhibitions for concentrations 1(20µL oil; left) and 1/1 (20 µL oil and 20µL ethanol resp.; right) by disc diffusion method. (-) Diameter of inhibitory zone <7mm considered as no antimicrobial activity
1. Concentrations (1, 1/1, 1/10, 1/20, 1/40) used were v/v.
2. Ampicillin (10µg), chloramphenicol (30µg) and streptomycin (10µg) used as positive control.

Determination of Minimum Inhibitory Concentration (MIC)
The result revealed the potential of oregano EO as natural preservative by inhibiting the growth of pathogenic *L. monocytogenes* at MIC 0.25µL/mL. It is mainly explained by different composition and percentage content of active constituents in essential oils, which found to have an important role in slowing down or stopping the bacterial growth or killing the bacteria.

Detection of *L. monocytogenes*
The presence of *L. monocytogenes* in raw crab sample was monitored and it was found that none of the samples tested were positive for *L. monocytogenes.* The results were revealed as such because of proper sanitation procedures and catching the crabs from cleaner waters.

Bacteriological analysis
Determination of Total Viable Count (TVC)
The initial mesophilic count of the control sample was 2.32 log CFU/mL which reached a final value of 8.9 log CFU/mL which is above the acceptability range. For the samples treated with oregano oil (0.5% and 1%), increase in the bacterial count remained below the acceptability limit even after 28 days storage as is seen by the Figure 1. The count varied significantly (p<0.01) in the oregano oil treated samples when compared to control.

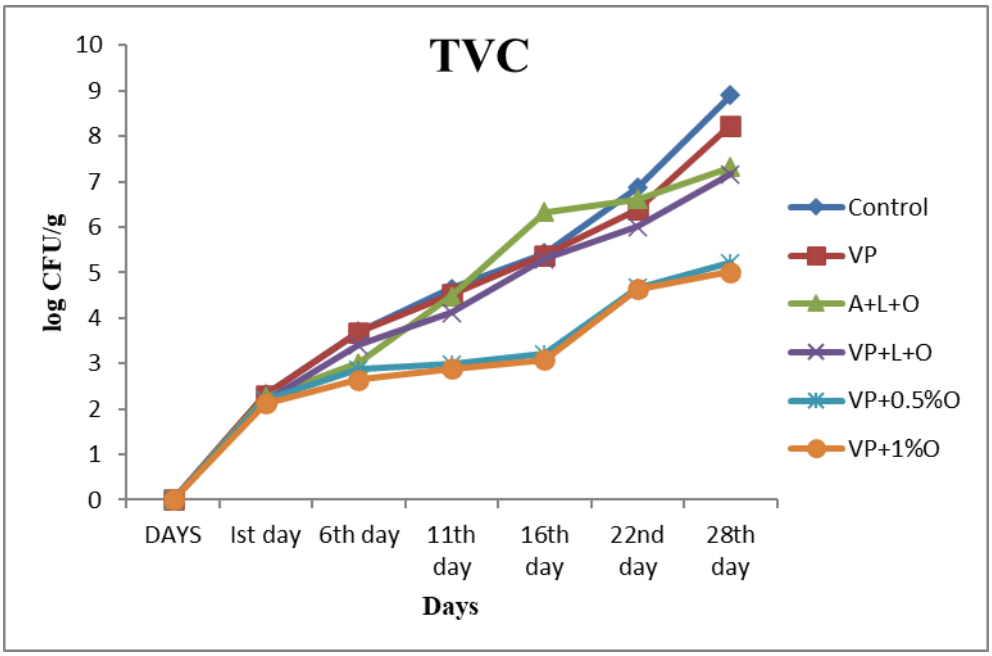

Fig 1: Mesophilic bacteria count of crab meat treated with oregano oil and vacuum packaging and their combination and control during 28 days of storage at 4 °C. Control showing increasing trend as compared to treated sample ($p<0.01$).

Table 2: Behavior of *L. monocytogenes* during storage. The values are expressed in log CFU/g.

	Days of storage					
Treatment	1	6	11	16	22	28
A+L+O	3.83	3.81	3.849	3.84	3.83	3.829
V+L+O	3.69	3.687	3.67	3.65	3.61	3.603

The total viable count of the samples A+L+O and V+L+O reached just above the acceptability limit by the end of storage period and was somewhat significantly ($p<0.01$) lower than the control. The count of *L. monocytogenes* in two of the samples which contained *L. monocytogenes* had values ranging between 3.83 and 3.829 CFU/mL in aerobic oregano oil treated sample and between 3.69 and 3.603 CFU/mL for vacuum packed oregano oil treated sample on the 28th day of storage**.** The count was enumerated by plating in a similar way as for the total viable count only that the plating was done on PALCAM agar which is a *Listeria* selective agar. The results were promising as the count of *L. monocytogenes* was low (Table 02) at the end of storage and showed a somewhat decreasing trend.

Conclusion

The present results indicate that there is considerable decrease in the amount of *L. monocytogenes* in the samples treated with Oregano essential oil in different concentrations. Addition of Oregano oil at concentrations of 0.5% and 1% in combination with vacuum packaging resulted in a strong bacteriostatic effect against *L. monocytogenes.* Treatment with oregano oil efficiently retarded microbial development and the values of the treated samples were below the acceptable microbial limit.

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