The anti-biofilm activity of essential oils against *Listeria monocytogenes and Salmonella* Enteritidis

La actividad anti-biofilm de los aceites esenciales sobre *Listeria monocytogenes*y *Salmonella* Enteritidis

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Abstract

The presence of biofilms is common in food industry. Biofilms can exist on all types of surfaces in food plants ranging from plastic, glass, metal, wood, to food products. The attachment of the bacteria to the food product or the product contact surfaces leads to serious hygienic problems and economic losses due to food spoilage. In addition to that, biofilms persist on food contact surfaces. For these reasons, it is considered that the presence of biofilms in the food systems is a serious public health risk. Chemical products are not recommended because the bacteria can acquire resistance, which lead to a significant increase in the number of microbial strains becoming resistant. Therefore, it is important to investigate the use of natural products (essential oils), known for their antimicrobial activity, in order to control biofilm development by foodborne pathogens.

Keywords: food; attachment; foodborne pathogens.

Resumen

La presencia de biofilms es habitual en la industria alimentaria. Los biofilms pueden presentarse en todo tipo de superficies de las plantas de procesado de alimentos, desde plásticos, vidrio, metal o madera, hasta los propios alimentos. La fijación de las bacterias a los alimentos o a las superficies conduce a serios problemas higiénicos y pérdidas económicas debidas a la alteración de alimentos. Además, los biofilms persisten en las superficies en contacto con los alimentos. Por estas razones, se considera que la presencia de biofilms en los sistemas alimentarios constituye un riesgo grave para la salud pública. El uso de productos químicos no está recomendado ya que las bacterias pueden desarrollar resistencia a los mismos, que puede llevar a un aumento significativo del número de cepas microbianas con resistencia adquirida. Así pues, resulta conveniente investigar el uso de productos naturales(aceites esenciales), conocidos por su actividad antimicrobiana, para el control de los biofilms causados por microorganismos patógenos.

Palabras clave: Alimentos; adhesión; patógenos alimentarios.

1. INTRODUCTION

Listeria monocytogenes is a Gram positive bacterium that is commonly present in the environment, and also in food processing facilities [1, 2].It is a foodborne pathogen of a particular concern because of its high mortality rate. It can be found in many raw foods such as fruits, vegetables and meats as well as processed food [2]. Also, it is a serious problem in the processing facilities because it forms biofilms, which are difficult to dislodge during cleaning [3].Among the most virulent foodborne pathogens are also *Salmonella* spp that can attach and form biofilms on surfaces found in food-processing plants including plastic, cement, and stainless steel [4,5].

Biofilms formed on processing surfaces in food industry have always been a major cause for food spoilage and contamination [6], although their importance has only been recognized quite recently. They are an association of microorganisms in which microbial cells adhere to each other within a self-produced matrix of extracellular polymeric substance mainly composed of polysaccharides [7].In order to control biofilms, several strategies have been developed to prevent food contamination and spoilage, such as cleaning-in-place (CIP), disinfection by physical processes, and chemical-based decontamination [8]. Unfortunately, chemical products have been ineffective to eradicate biofilms because the excessive use of disinfectant agents to remove them allowed microorganisms to acquire resistance, which leaded in a sharp and dangerous increase in the number of microbial strains [9]. So, it is necessary to look for new alternatives that are natural, effective and unable to generate resistance. Essential oils have always been among the best options to choose as they have been generally recognized as safe (GRAS) [10]. Bacteria included in biofilm structure are generally more resistant to antimicrobial agents than planktonic cells which make the issue even more serious [11]. Therefore, the aim of this study is to investigate, the anti-biofilm effect of essential oils on *Listeria monocytogenes* and *Salmonella* Enteritidis.

2. MATERIAL AND METHODS

2.1 Bacterial culture

Listeria monocytogenes CECT 4032 and *Salmonella enteric* serovar Enteritidis CECT 4300 will be used in this study and will be provided by the Spanish Type Culture Collection (CECT, Valencia, Spain).

2.2 Essential oils

Essential oils of onion, garlic, cinnamon, geranium and clove will beused and will be dissolved in 95% v/v ethanol. Onion, garlic and cinnamon essential oils will be provided by Iberchem (Murcia, Spain). Geranium and clove essential oils will be obtained from Tunisia.

2.3 Agar disk diffusion assay

The test will be performed, following the procedure described by[12]. 100 µL bacterial inoculum of approximately 1–2×10^8CFU/mL will be applied to the surface of a Brain Heart Infusion agar plate (BHIA; Scharlau Chemie, Barcelona, Spain). Then, 3 sterile disks of 6 mm (BD sensi-disc, Becton Dickinson GMBH Heidelberg) will be placed on the inoculated agar surface. One will be impregnated with 20 µL of essential oil, the other with ethanol (negative control) and the last one with erythromycin (7.5 µg / disc; positive control; Duchefa Biochemie. The Netherlands).The plates will then be incubated for 24 h at 37°C prior to determination of results. Finally, the zones of growth inhibition around each of the disks will be measured in mm.

2.4 Determination of minimal inhibitory concentration (MIC)

This procedure described by [13], involves preparing two-fold dilutions of essential oils ranging from 0.2 mg/mL until 0.00625 mg/mL in 5 mL of Tryptic Soy Broth (TSB; Scharlau Chemie) liquid growth medium dispensed in test tubes. The essential oil-containing tubes will

then be inoculated with a 0.1 mL standardized bacterial suspension of 10^6 CFU/mL. Following overnight incubation at 37°C, the tubes will be examined for visible bacterial growth as evidenced by turbidity. The lowest concentration of essential oil that prevents growth represents the minimal inhibitory concentration (MIC).

2.5 Inhibition of initial cell attachment

The method was adopted by [14]. Therefore, solutions of antimicrobials (equivalent to 0.5 MIC, 1 MIC and 2 MIC) will be prepared. 100 µL of each solution will be added to the flat-bottom 96-well polystyrene microtiter plates, and equal volumes of ethanol and erythromycin will be added as negative and positive controls, respectively. A standardized culture of 10^6 CFU/mL will be added into the wells in order to achieve 200 µL as a final volume in each well. The cultures will be added into the wells in triplicate, and sterile TSB will be added as an additional control to guarantee the sterility of the medium during the experiment. The plates will be sterile sealed and incubated at 37°C for 24 h to allow cell attachment and biofilm formation. After one night incubation, the modified crystal violet assay [16] will be performed to assess biofilm biomass, and the results will be expressed as percentage inhibition (Eqn 1).

Percentage inhibition = [(OD Negative control – OD Experimental)/OD Negative control]*100

2.6 Inhibition of preformed biofilm

Biofilms will be allowed to form for 6 h before adding the essential oils on the microtiter plates, by pipetting 100 µL of standardized culture of 10^6CFU/mL into selected wells of the microtiter plate in triplicate and incubating them for 6 hours at 37°C, allowing the biofilm to be formed. Then, 100 µL of each stock solution of the essential oils will be added to each well to achieve a final volume of 200 µL. Equal volumes of ethanol and erythromycin will be added as negative and positive controls respectively. After the treatment of preformed biofilms with antimicrobials, the plates will be incubated over a series of time intervals (1, 5 and 20 h). At each time interval, biofilm biomass will be measured using the crystal violet assay [15].

2.7 Biofilm biomass (Crystal violet assay)

For the cell attachment assessment (Section 2.5) and the 1 h, 5 h and 20 h incubation (Section 2.6), culture medium from each well will be gently removed and the plates will be washed five times with sterile distilled water to remove planktonic bacteria. They will then be air-dried for 45 min. Afterwards, 100 µL of 1% crystal violet will be added to each well to stain the adherent bacteria. Microtiter plates will then be incubated at room temperature for 45 min to allow staining. Each microtiter plate will then be washed five times with distilled water, shaking out as much liquid as possible in order to remove the crystal violet solution. This step will remove any crystal violet that is not specifically staining the adherent bacteria. 125 µL of 95% ethanol will then be added to the wells to destain the wells, allowing the dye to solubilize by covering plates and incubating them for 10 to 15 min at room temperature. 100 µL of the destaining solution from each well will then be transferred to a new plate, and the absorbance will be measured at 595 nm using a microplate reader (Bioscreen C; Lab Systems, Helsinki, Finland). The mean absorbance (OD595 nm) will be used for determining the percentage inhibition of biomass formation for each concentration of antimicrobial according to the equation mentioned above in section 2.2

2.8 Statistical treatment of the data

All experiments will be performed at least in triplicate. ANOVA test will be performed to ascertain the statistical significance of the data.

3. EXPECTED RESULTS

Essential oils should have more effect on the initial cell attachment than on preformed biofilm because once the biofilm is formed, bacteria present in it have more chance to survive stress conditions.

If the results found are promising, the identification of the bioactive components in the essential oils would allow the inclusion of these compounds in novel disinfectant and sanitizer formulations

4. ACKNOWLEDGEMENT

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