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Effect of thymol and environmental factors on growth and biofilm formation by *Listeria monocytogenes*

[Efecto del timol y factores ambientales sobre el crecimiento y formación de biopelícula por *Listeria monocytogenes*]

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Abstract: Thymol (2-isopropyl-5-methylphenol) is an aromatic monoterpene found in essential oils extracted from plants belonging to the Lamiaceae family, such as *Thymus*, *Ocimum*, *Origanum*, *Satureja*, *Thymbra* and *Monarda* genera. Growth and biofilm formation by *Listeria monocytogenes* CLIP 74902 were evaluate using three carbon sources in the presence of thymol. Specific growth rate (h-1) values at 37º with glucose, trehalose and cellobiose with the addition of thymol (µg/mL) 0 (control) and 750, were respectively: 0.22, 0.07; 0.14, 0.04; 0.11, 0.04. Lag periods obtained under the same conditions were (h): 8.19, 13.2; 22.5, 27.5; 23.1, 28.1. A marked antibiofilm activity was observed against the exposure with 750 µg/mL of thymol, showing a high percentage of inhibition: glucose (99 %), trehalose (97 %) and cellobiose (98%), compared to the control. The results suggest that thymol could be used to inhibit the growth and production of biofilms by *L. monocytogenes* in the food industry.

Keywords: Biofilm; Carbon source; Kinetics growth; *Listeria monocytogenes*; Thymol

Resumen: Timol (2-isopropil-5-metilfenol) es un monoterpeno aromático presente en los aceites esenciales extraídos de plantas pertenecientes a la familia Lamiaceae, como los géneros *Thymus*, *Ocimum*, *Origanum*, *Satureja*, *Thymbra* y *Monarda*. El crecimiento y formación de biopelícula por *Listeria monocytogenes* CLIP 74902 fueron evaluados utilizando tres fuentes de carbono en presencia de timol. La velocidad específica de crecimiento (h-1) a 37º con glucosa, trehalosa y celobiosa con la adición de timol (µg/mL) 0 (control) y 750, fueron respectivamente: 0.22, 0.07; 0.14, 0.04, 0.11, 0,04. Los períodos lag obtenidos en las mismas condiciones fueron (h): 8.19, 13.2; 22.5, 27.5; 23.1, 28.1. Una marcada actividad antibiofilm fue obtenida con 750 µg/mL de timol, mostrando un alto porcentaje de inhibición con glucosa (99%), trehalosa (97%) y celobiosa (98%), respecto al control. Los resultados sugieren que timol podría ser usado para inhibir el crecimiento y producción de biopelículas por *L. monocytogenes* en la industria alimentaria.

Palabras clave: Biopelícula; Fuentes de carbono; Cinética de crecimiento; *Listeria monocytogenes*; Timol

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INTRODUCTION

Listeria monocytogenes is a Gram positive rod-shaped, facultative anaerobe, non-spore-forming bacterium, which presents polar flagella that allow it a tumbling motility at room temperature (Orsi & Wiedmann, 2016; Cheng *et al.*, 2018). It is an ubiquitous microorganism isolated from soil, water, wastewater, animals, humans, due to its ability to proliferate over a wide range of temperatures, and a high concentration of salt (NicAogáin & O'Byrne, 2016). *L. monocytogenes* is an important human foodborne pathogen that is widely distributed throughout the environment and that can be isolated from raw or processed food (Farber & Peterkin, 1991). It is the etiological agent of a severe infection called listeriosis, a rare disease which usually occurs in high-risk groups, including elderly, immunocompromised, pregnant women and neonates, and is associated with a high hospitalization and mortality rates (Ferreira *et al.*, 2014). Septicemia, meningitis, miscarriage, and stillbirth are common clinical presentations, although people without these risk factors can also be affected (Lecuit, 2007).

L. monocytogenes is a potentially biofilm-forming bacterium which, once established in processing plants, represents a problem for the food industry. Biofilm is an association of microorganisms where microbial cells adhere to each other on a living or non-living surfaces within a self-produced matrix of extracellular polymeric substance. This mode of growth protects bacteria from environmental stresses, that is, the treatment of biofilms with antibiotics or other biocides is often ineffective for eradication (Van Wolferen *et al.*, 2018). Growth and biofilm formation is dependent on various intrinsic and extrinsic factors such as availability of nutrients, environmental conditions, motility and the presence of inhibitors (Kadam *et al.*, 2013; Oloketuyi & Khan, 2017). Also, biofilm formation by *L. monocytogenes* varies among serotype, lineage and origin of isolation (Nilsson *et al.*, 2011).

Research on plants as potential sources of new and effective antimicrobials and/or antibiofilm with novel modes of action is well established. Essential oils are aromatic and volatile oily liquid extracted from different part of aromatic plants. These oils, have a broad spectrum of pharmacological activity and pleasant odors and taste and can be used in the food industry for their aroma, flavour and preservative properties (Mancini *et al.*, 2015). Although active constituents may occur in plants in low concentrations, plant extracts may, in many cases, represent a superior source of antimicrobial compounds than synthetic drugs (Romero *et al.*, 2016). Thymol (2-isopropyl-5-methylphenol) is a colorless crystalline substance with a characteristic odor. Is the main monoterpene phenol found in the essential oils extracted from plants belonging to the *Lamiaceae* family, such as those of the genera *Thymus*, *Ocimum*, *Origanum*, *Satureja*, *Thymbra*, and *Monarda* (Chouhan *et al.*, 2017). Thymol, and its main natural source, thyme (*Thymus vulgaris*), are employed for their positive antibacterial, antifungal, antioxidant, anti-inflammatory, local anaesthetic, and antiseptic properties as well as for their effects anticancer (Nagoor Meeran *et al.*, 2017). Due to the persistence of *L. monocytogenes* in food and food processing plants, it is important to find natural substances with anti-biofilm activity; in this sense, thymol is promising.

The objective of this study was to evaluate the effect of temperature, different carbon sources and thymol concentrations, on the growth and biofilm formation by *L. monocytogenes*. Currently, natural additive, have acquired great importance in industrial production systems, which guarantees the confidence of consumers. In this paper, we also intend to make a contribution to the possible use of thymol as a natural preservative against *L. monocytogenes* to improve the efficiency and effectiveness of food preservation, according to current market needs and contribute to the reduction of chemical additives.

MATERIALS AND METHODS

Bacterial strains and culture medium

L. monocytogenes CLIP 74902 was stored for long-term in cryovial containing glycerol 20% (v/v) BHI broth at –80°C. From frozen stock it was scraped a small quantity of frozen material using a sterile inoculating loop, inoculated on sterile BHI agar (Difco) plates and incubate overnight (18–24 h) at 37ºC. Then, on single isolated colony of *L. monocytogenes* was transferred into sterile tubes with BHI broth and grown overnight at 37°C (Jones S & D'Orazio, 2013). The growth and production biofilm assays were performed using the following enriched culture medium (EM) (g/L): proteose peptone (Difco), 30; yeast extract (Merck), 5; trypticase (Difco), 5; glucose 2. In this medium glucose was replaced by the following carbon sources (g/L):

trehalose 2, cellobiose 2. The pH was adjusted to 7.0 and sterilized at 121°C for 15 min. Then, thymol, (Sigma-Aldrich Corporation, St Louis, MO, USA), dissolved in 95% ethanol, was added to each medium in a final concentration of (µg/mL): 0 (control), 250 and 750.

Fermenter experiments, inoculum preparation and growth conditions.

Cultures were developed in an 800 mL capacity fermenter containing 700 mL of EM with the different sources of carbon and thymol concentrations. Before the realization of the experiences 1 mL of inoculum previously obtained in BHI broth, was transferred into 250 mL Erlenmeyer flask with sterile BHI broth and incubated overnight (18–24 h) at 37ºC, with orbital shaking (80 rpm) (Thermo Scientific MaxQ 4000). Approximately 5 mL of a 1/25 dilution of this culture was inoculated to the fermenter and incubated at 30ºC or 37ºC for 100 h under aerobic conditions using a sterile air flow of 15 L/h with constant shaking at 80 rpm

Analytical techniques

The cell turbidity was monitored in duplicate samples taken at various times throughout the culture period to measure the optical density in a Spectronic 20 Genesis (Spectronic Instruments) spectrophotometer at 600 nm (OD_{600}). Biomass were estimated at the start and end of the exponential growth phase by dry weight measurement from samples centrifuged at 10,000 x g for 20 min, washed twice with distilled water and dried at 100°C for 16 h. The specific growth rate was calculated using the expression:

$$\mu\ (h^{-1}) = \ln x2/x1 \cdot t^{-1}$$

where x1 and x2 were two determinations of biomass in the exponential growth phase at time t. Lag phase duration (λ) was calculated with the following equation:

$$L\ (h) = t - (\ln x/xo)/\mu$$

where x is the biomass at the end of the logarithmic growth; xo: initial biomass; t: time elapsed until the end of the logarithmic stage and µ: specific growth rate (Pirt, 1975).

Biofilm assay

Biofilm production was measured using crystal violet (CV) staining (Djordjevic *et al*., 2002) with some modifications. In brief, the experiments were performed adding 150 µL of EM with different carbon sources and thymol concentrations in sterile 96 well clear flat bottom polystyrene microplate (Corning® NY USA). Then, 10 µL overnight culture of *L. monocytogenes*, obtained in Erlenmeyer flask, were added into each well and incubated at 37ºC or 30ºC during 100 h under aerobic conditions (shaking 80 rpm). The negative control wells contained broth only. After the incubation period, medium was removed from wells and washed five times with sterile PBS to remove loosely associated bacteria. Microplates were air dried for 45 min and subsequently the remaining attached bacteria were fixed with 200 µL of methanol for 15 min. Then, the cells were stained with 200 µL of 1% (w/v) crystal violet at room temperature for 5 min. After staining, the content of each well was washed with sterile PBS five times. The adhered cells were removed with 33% (v/v) glacial acetic acid, and the optical density at 550 nm (OD_{550}) was measured in a microplate reader (BIO-RAD, Benchmark, California). The tests were carried out in quadruplicate and replicated three times.

Statistical analysis

Statistical analysis was performed using GraphPad Prism software versión 6.0 for Windows. Analysis of variance (ANOVA) was used, and differences were accepted as significant for $p \leq 0.05$ (Smith, 2014). The data used to analyze the effect of temperature, carbon sources and thymol levels on different growth parameters, were those obtained from tree independent experiments in duplicate. The values used to evaluate the effect of the different variables on biofilm production corresponded to those obtained from three experiences in quadruplicate.

RESULTS

The nutrient composition of the enriched medium used allowed the development of the strain of *L. monocytogenes* studied. Similarly, the cultures systems used was adequate to evaluate the growth parameters and production of biofilm in the presence of various carbon sources, and the effect of the addition of thymol at different temperatures.

***L. monocytogenes* CLIP 74902 kinetics growth using glucose as a carbon source and different thymol concentrations.**

Batch cultures of *L. monocytogenes* CLIP 74902 under different conditions were developed in the fermenter at 37°C or 30ºC for a period of 100 h. Cultures presented a variable lag period according to the temperature tested and the concentration of thymol used. At 37°C lag phase duration showed an increase of 2.41 h when adding 250 µg/mL thymol and 2.60 h with 750 µg/mL thymol, compared to the culture in the absence of thymol. Higher lag periods were observed at 30ºC with similar increases in levels of thymol concentration (3.3 h and 3.7 h respectively, with respect to control) (Table No. 1). Later, the growth curves followed by a logarithmic growth phase and a subsequent period of decline and cell lysis, which lasted until the end of the culture. The magnitude of the growth parameters studied was influenced by the presence of thymol. The specific growth rate (µ) decreased when thymol concentration increased at both 37ºC and 30ºC. A similar effect was observed in the maximum biomass reached with an increase in the times required to reach the maximum values of ODmax (Figure No. 1A, Figure No. 1B). In addition, statistically significant differences were found when comparing the µ and ODmax values between both growth temperatures supplemented with the same concentration of thymol (p=0.005) (Figure No. 1A and Figure No. 1B).

Table No. 1
***L. monocytogenes* CLIP 74902 growth parameters at 30ºC and 37ºC with glucose, trehalose and cellobiose in the presence of different thymol concentrations.**

	TEMPERATURE	THYMOL (µg/mL)	GROWTH PARAMETERS		
			µ (h^{-1})	λ (h)	OD_{max}
Glucose	37º C	0	0.22	8.19	0.68
		250	0.13	10.6	0.55
		750	0.07	13.2	0.40
	30º C	0	0.19	9.80	0.64
		250	0.11	13.1	0.45
		750	0.06	16.8	0.30
Trehalose	37º C	0	0.14	22.5	1.18
		250	0.10	25.6	0.88
		750	0.04	27.5	0.50
	30º C	0	0.11	24.5	1.10
		250	0.04	25.6	0.65
		750	0.02	27.5	0.47
Cellobiose	37º C	0	0.11	23.1	1.10
		250	0.08	26.1	0.74
		750	0.04	28.1	0.46
	30º C	0	0.09	24.5	1.02
		250	0.05	26.5	0.55
		750	0.02	28.5	0.44

µ specific growth rate
λ lag phase duration
OD_{max} maximum optical density at 600 nm
Values are the means of of tree independent experiments in duplicate

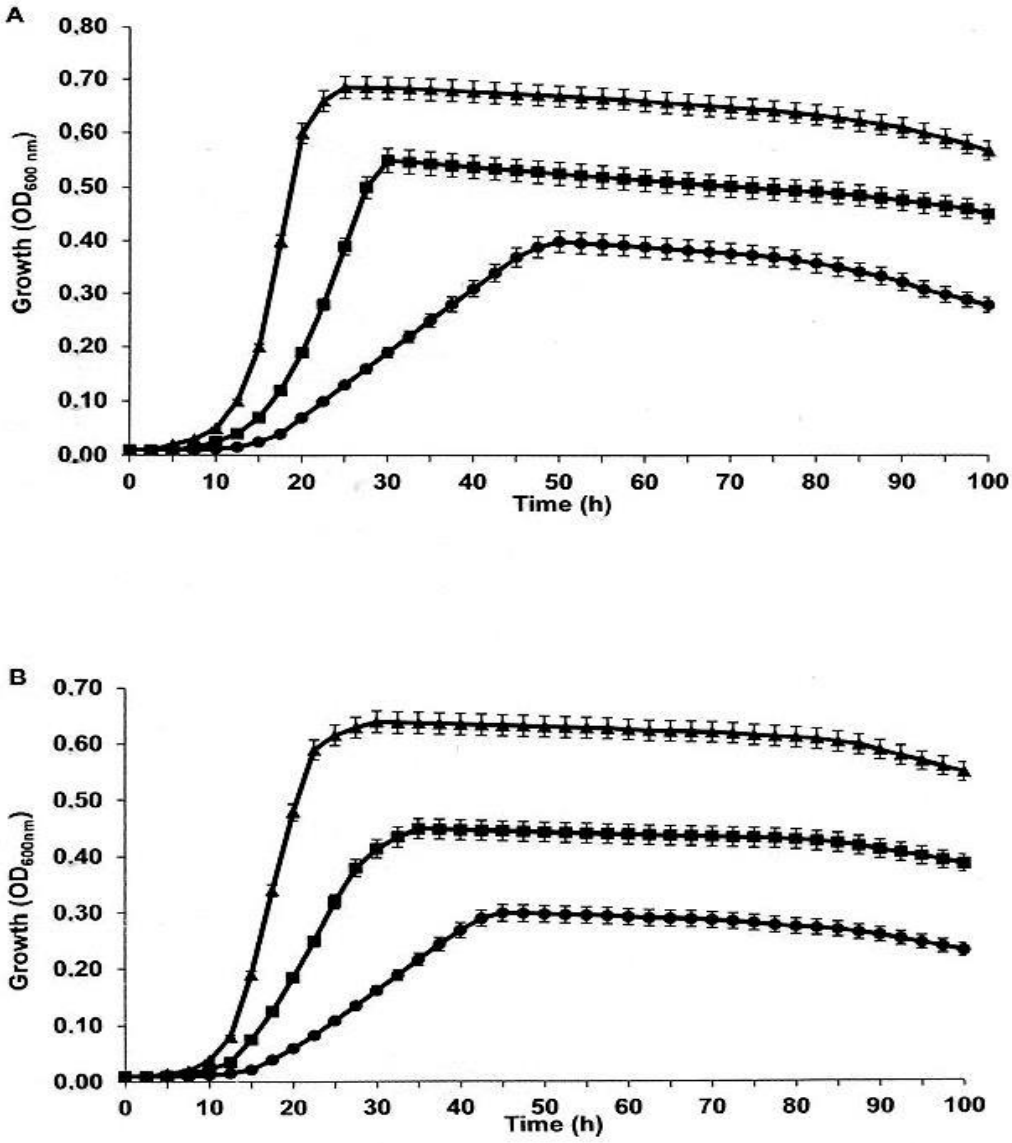

Figure No. 1
***L. monocytogenes* CLIP 74902 kinetics growth with glucose at 37°C (A) or 30ºC (B) in the presence of thymol (µg/mL): 0 (▲), 250 (■) and 750 (●).**
Error bars reflect the deviation of the means.

***L. monocytogenes* CLIP 74902 kinetics growth with trehalose and cellobiose as a carbon source and different thymol concentrations.**

L. monocytogenes CLIP 74902 batch cultures at 30ºC and 37ºC using both disaccharides with the addition of 0, 250 and 750 µL of thymol, presented lag periods higher than 20 h. These values were higher with respect to glucose (Table No. 1). Later, the cultures developed with a prolonged phase of exponential growth at low specific growth rates at both temperatures. The presence of thymol in the culture medium exerted a negative effect on the maximum biomass reached as well as in the time required to obtain it (Figure No. 2A, Figure No. 2B, Figure No. 3A, and Figure No. 3B). When trehalose was used as a carbon source, higher values of

biomass were obtained with respect to cellobiose under the conditions tested. In addition, when the specific rate of growth and ODmax calculated for the disaccharides they were compared with the same parameters obtained with glucose, the results showed significant differences (p=0.0005) (Table No. 1).

Table No. 2
***L. monocytogenes* CLIP 74902 growth parameters at 30ºC and 37ºC with trehalose or cellobiose in the presence of different thymol concentrations**

Parameters	Trehalose						Cellobiose					
	37ºC			30ºC			37ºC			30ºC		
	Thymol (µg/mL)											
	0	250	750	0	250	750	0	250	750	0	250	750
µ (h^{-1})	0.14	0.10	0.04	0.11	0.04	0.02	0.11	0.08	0.04	0.09	0.05	0.02
λ (h)	22.5	25.6	27.5	24.5	25.6	27.5	23.1	26.1	28.1	24.5	26.5	28.5
OD_{max}	1.18	0.88	0.50	1.10	0.65	0.47	1.10	0.74	0.46	1.02	0.55	0.44

µ specific growth rate
λ lag phase duration
OD_{max} maximun optical density at 600nm
Values are the means of independent experiments by duplicate.

Biofilm production

L. monocytogenes was able to produce biofilm in the presence of the carbon sources tested, obtaining higher levels with trehalose and cellobiose compared to glucose in the absence of thymol at 30°C (1.21 and 1.13 times higher, respectively). A similar proportion was observed at 37ºC, and for both temperatures the differences were statistically significant (*p=0.0066*, p=0.0094). When comparing the levels of biofilm produced at 30ºC and 37ºC for each carbohydrate, the increase obtained was 1.43 times higher for the three carbon sources used (*p=0.0045*) (Figure No. 4).

The addition of 250 µg/mL thymol to the culture medium, exerted a moderate inhibitory effect on the production of biofilm by *L. monocytogenes* regardless of the carbon source used at both 30°C and 37°C. The percentage reductions with respect to the control (0 µg/mL thymol) of the sessile biomass at 30°C were the following: glucose 39%, trehalose 32%, cellobiose 35%, (p<0.0001). The same analysis performed at 37°C showed the following percentage reductions: glucose 37%, trehalose 33%, cellobiose 35% (p<0.0001) (Figure No. 4).

A marked antibiofilm activity for *L. monocytogenes* was observed against the exposure with 750 µg/mL of thymol showing high percentages of inhibition compared to the control (0 µg/mL), whose values at 30°C were the following: glucose 98%, trehalose 97% and cellobiose 98% (p<0.05). When the experience was carried out at 37ºC, the percentage reductions obtained for the same concentration of thymol compared to the control were the following: glucose 99%, trehalose 97%, cellobiose 98% (p<0.0001) (Figure No. 4).

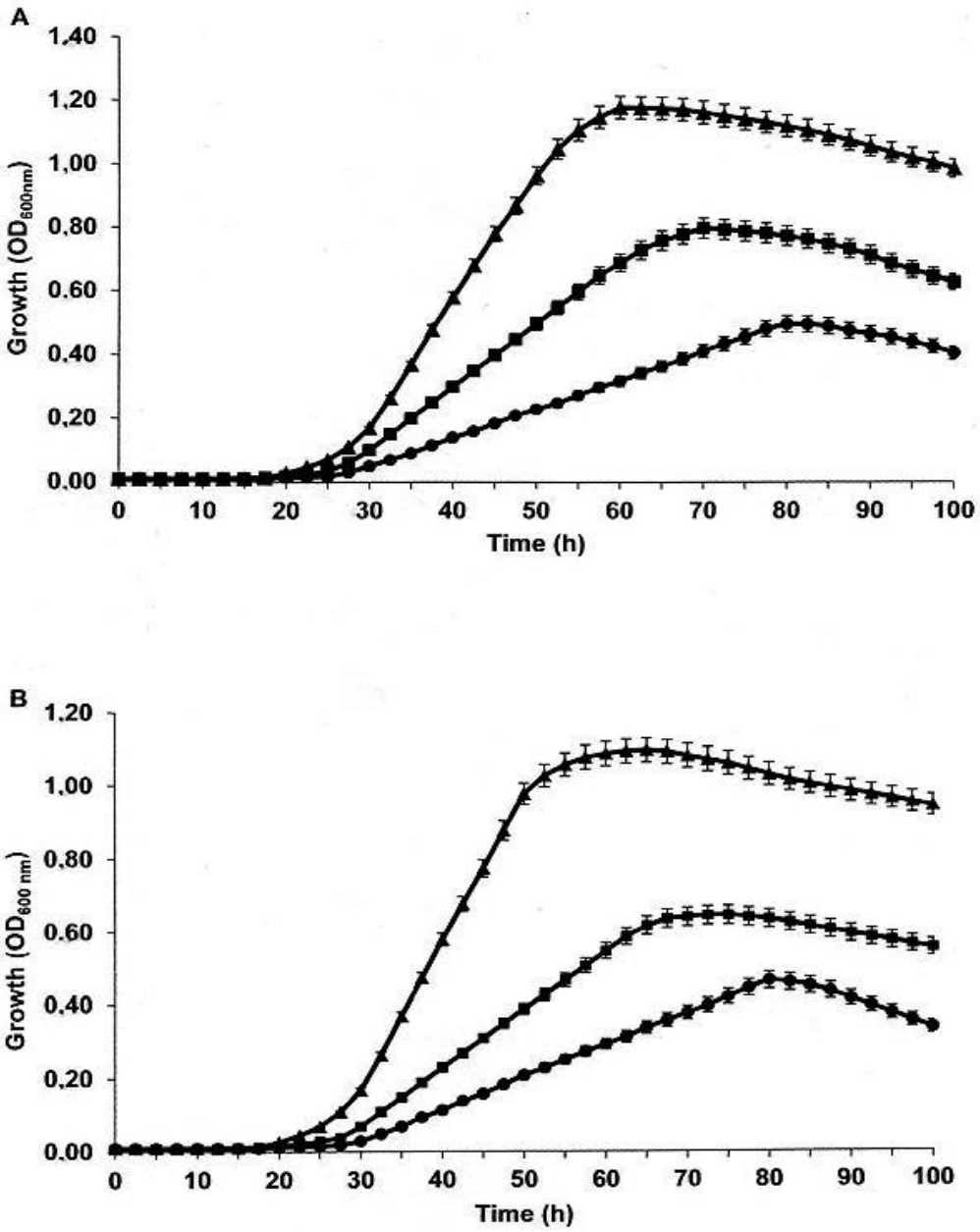

Figure No. 2
***L. monocytogenes* CLIP 74902 kinetics growth with trehalose at 37°C (A) or 30°C (B) in the presence of thymol (µg/mL): 0 (▲), 250 (■) and 750 (●).**
Error bars reflect the deviation of the means.

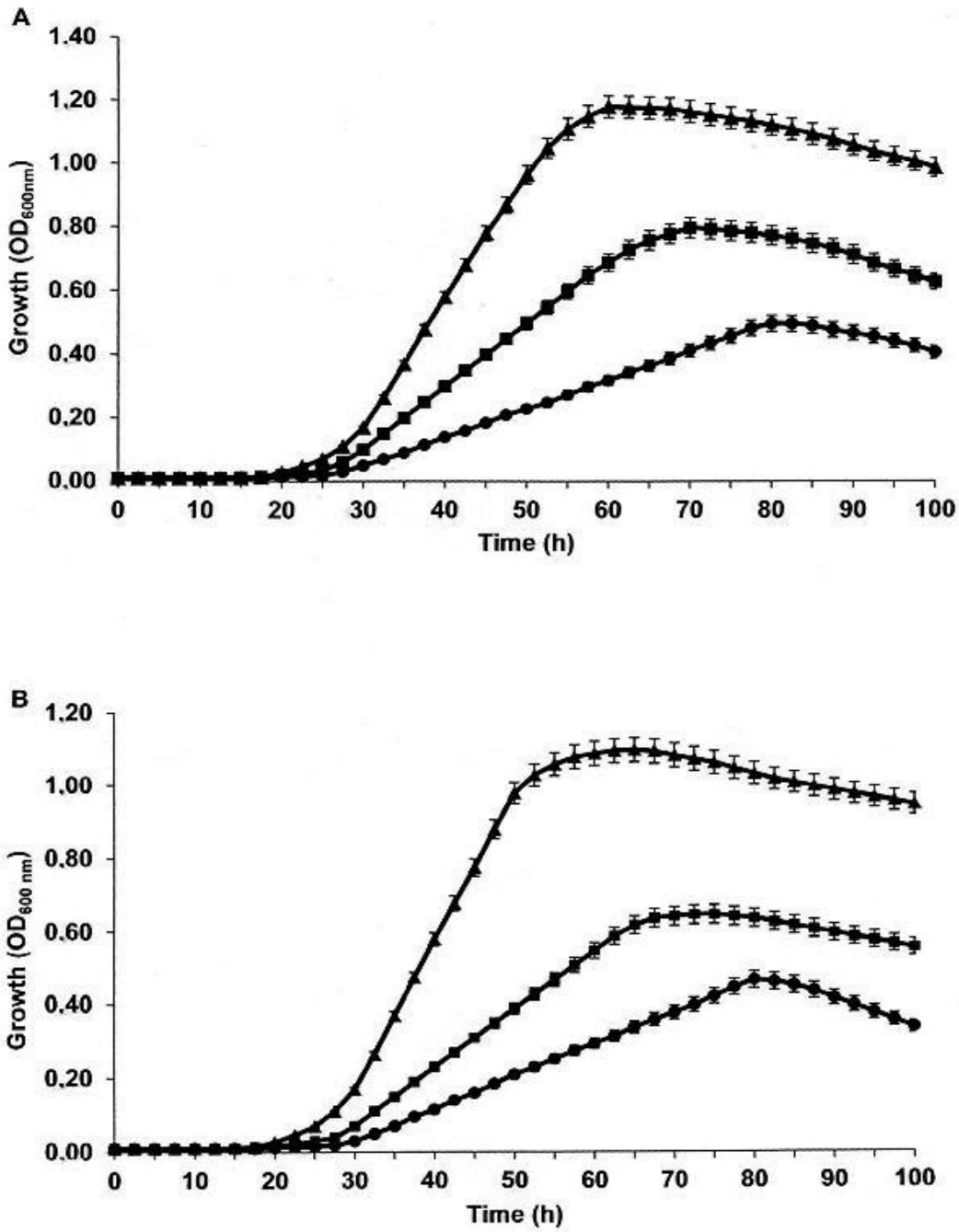

Figure No. 3
***L. monocytogenes* CLIP 74902 kinetics growth with cellobiose at 37°C (A) or 30ºC (B) in the presence of thymol (µg/mL): 0 (▲), 250 (■) and 750 (●).**
Error bars reflect the deviation of the means

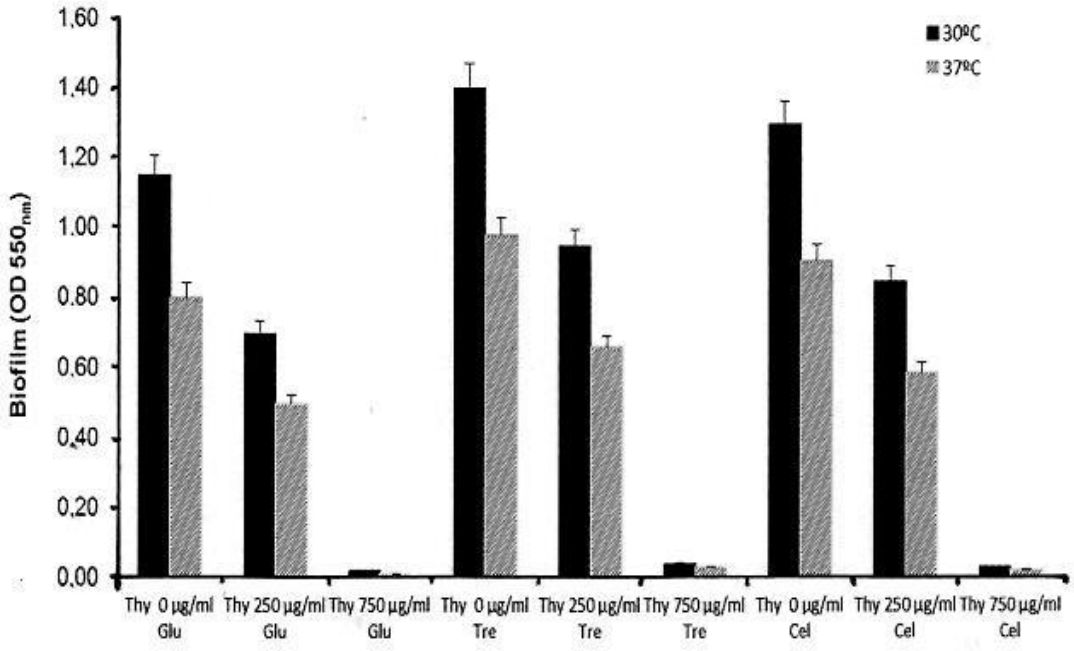

Figure No. 4
***L. monocytogenes* CLIP 74902 biofilm production with glucose, trehalose, cellobiose at 30°C or 37ºC in the presence of thymol (µg/mL) 0, 250 and 750.**
Error bars reflect the deviation of the means.

DISCUSSION

The liquid culture media used in the present work has not been previously tried for the growth of *L. monocytogenes*. This culture medium was initially optimized for growth and toxin type G production of *Clostridium argentinense*. Six factorial experiments were performed to assess the effect of nutrient composition, incubation conditions and associated microorganisms (*Bacillus subtilis, Lactobacillus plantarum*) on the toxinogenesis of *C. argentinense* (Calleri de Milan *et al*., 1992). Some variables were modified, such as pH, carbon sources and incubation temperatures, to adapt it to the growth of *L. monocytogenes* CLIP 74902. Batch cultures at 30°C or 37ºC, using different sugars, showed a typical bacterial growth curve shows distinct phases of growth: lag phase, the delay before the start of exponential growth; exponential phase, where cell division proceeds at a constant rate; stationary phase, when conditions become unfavorable for growth and bacteria stop replicating; death phase, when cells lose viability; and, finally, long term stationary phase. The differences observed in the lag phase duration with the different carbon sources can be attributed to the time during which the cells adjust to a new substrate, what includes the synthesis of cellular components necessary, before the onset of exponential growth (Madigan *et al*., 2000; Muñoz-Cuevas *et al*., 2010). Characterizing the lag phase in microbial growth curves has importance in food sciences, environmental sciences, bioremediation and in understanding basic cellular processes (Yates & Smotzer, 2007).

Temperature growth affected significantly lag phase duration and the specific growth rate (Figures 1, 2, 3). It is known that *L. monocytogenes* can grow in a large temperature interval, between 0°C to 45°C, but much slower at low temperatures than near the temperature optimum of 37°C (Tienungoon *et al*., 2000). This environmental factor is able to influence in the expression of virulence genes in *L. monocytogenes*. It has been well documented that the expression of virulence factors, such as transcriptional activator protein (PrfA), internalin, listeriolysin, phospholipases, metalloprotease, and actin polymerization proteins, is influenced by

bacterial growth phase and temperature (Leimeister-Wächter *et al.*, 1992; Scortti et al., 2007; Mc Gann *et al*., 2007; Ivy *et al*., 2010).

The growth phases of *L. monocytogenes* were also affected by the addition of different concentrations of thymol. By increasing the concentration of 2-isopropyl-5-methylphenol, the growth curves showed a prolongation of the lag phase and decreases in the specific growth rate and maximum biomass obtained. The antimicrobial activity of thymol-rich essential oils have been evaluated for their possible benefits in medical applications. In particular, several *in vitro* studies have shown that thymol possesses antibacterial and antifungal properties both Gram-negative (*Escherichia coli* O157:H7, *Proteus mirabilis*, *P. vulgaris*, *Salmonella typhimurium*, *Serratia marcescens*, *Yersinia enterocolitica*, *Pseudomonas fluorescens*, *P. putida*) and Gram-positive (*Micrococcus* spp., *Sarcina* flava, *Staphylococcus aureus*, *Bacillus licheniformis*, *B. thuringiensis*) (Marino *et al*., 1999; Miladinovic *et al*., 2015). The antifungal effects of thymol and other compounds, such as carvacrol, eugenol and menthol, against different fungi have been examined to investigate the application of these compounds as possible natural alternatives for controlling the growth of food-relevant fungi and to reduce the use of synthetic and toxic fungicides. The antifungal effect has been evaluated for *Aspergillus niger*, *A. fumigatus*, *A. flavus* (Abbaszadeh *et al*., 2014). Other authors have evaluated the effect of thymol against *C. albicans*, *C. krusei* and *C. tropicalis* (De Vasconcelos *et al*., 2014; De Castro *et al*., 2015).

In this work it was observed that the growth kinetics of *L. monocytogenes*, at the temperatures evaluated, was significantly affected by the carbon sources used. With glucose, the specific rate of growth was higher than that obtained with trehalose and cellobiose. However, the maximum biomass was higher with the disaccharides compared with glucose. This effect would be linked to the rate of absorption and transport of carbohydrate by *L. monocytogenes*. It is known that *L. monocytogenes* possesses a phosphoenol pyruvate (PEP)-dependent phosphotransferase systems (PTSs), involved in the transport, phosphorylation of carbohydrates and in the regulation of different metabolic pathways of various sugars that may or may not be transported by it (Wang *et al*., 2014). When the bacterium is outside the host cell, extracellular carbon sources such as glucose and cellobiose, are transported by PTSs. *L. monocytogenes* can effectively adapt to environmental conditions outside the eukaryotic host cells and is able to multiply using various carbon sources. In contrast, this microorganism can utilize non-PTS-dependent carbon sources available in the cytoplasm of eukaryotic cells, such as phosphorylated glucose and glycerol, for intracellular growth (Stoll & Goebel, 2010; Balay *et al*., 2018).

The persistence of *L. monocytogenes* in the environment can be explained by the ability to use available nutrients from diverse ecosystems. The disaccharide trehalose (α-D-glucopyranosyl-1,1-α-D-glucopyranoside) is widely distributed in nature and found in shellfish, insects, plants, mammals, bacteria, and fungi. Trehalose also aids in the survival of many organisms during exposure to adverse conditions caused by temperature extremes, desiccation, high osmolarity, and oxidative stress (Ells & Truelstrup Hansen, 2011). Cellobiose is a disaccharide consisting of two glucose units in a beta (1-4) glycosidic linkage and is a microbial breakdown product from plant material. This carbon source comes from the enzymatic degradation of cellulose and hemicellulose, which are the main types of complex carbon compounds present in nature, and its degradation results a key step in the terrestrial carbon cycle. In this way, the disaccharide cellobiose is present in large amounts in the soil environment, where *L. monocytogenes* can grow saprophytically (Gilbreth *et al*., 2004; López-Mondéjar *et al*., 2016).
The persistence of *L. monocytogenes* in the premises and equipment used in the food industry for many years, even when adequate hygienic measures are implemented, is related to the capacity of this microorganism to form biofilms. This implies a risk of contamination of ready-to-eat products (Reis-Teixeira *et al*., 2017). The results obtained in this work showed that *L. monocytogenes* biofilm production on polystyrene surface was notably influenced according to the available carbon sources, incubation temperature, and the presence of the aromatic monoterpene thymol. Higher sessile biomass was obtained with cellobiose and trehalose, compared with glucose, at both temperatures. We could suggest that the biofilm-forming processes mainly affected metabolic pathways for energy production and transport as well as metabolism of carbohydrate. This effect could be due to processes of

regulation by catabolic repression (CR), where the predominant mechanism in *L. monocytogenes* involves repression mediated by catabolite control protein A (CcpA). CcpA has been shown to coordinate central metabolism and biofilm formation in some grampositive bacteria, such as *B. subtilis*, *S. aureus* and *S. epidermidis* (Zhou *et al*., 2012).

Biofilm formation by *L. monocytogenes* resulted significantly different according to growth temperatures tested. It's known that bacterial attachment to surfaces is influenced by the physicochemical properties of the environment (temperature and pH), surface (hydrophobicity) among other factors. In addition, it has been suggested that the flagellar motility is required or initial cell attachment during biofilm formation by overcoming any repulsive interfacial forces (Kocot & Olszewska, 2017). Previous studies have shown that flagellar motility gene expression in *L. monocytogenes* is regulated by temperature. *L. monocytogenes* strains are highly flagellated and motile at low temperatures, 30°C and below, and are typically not motile at temperatures of 37°C or above (Lemon *et al*., 2007).

Thymol markedly reduced the formation of *L. monocytogenes* biofilm and its effect resulted concentration-dependent. The highest antibiofilm activity was observed with the addition of 750 µg/mL of monoterpene. It has been amply demonstrated the efficacy of several plant-derived compounds, including thymol and its isomer carvacrol, in inhibiting biofilm formation both gram-positive and gram-negative microorganisms as well as fungi (Marchese *et al*., 2016). Particularly for *L. monocytogenes*, the antibiofilm effect of 2-isopropyl-5-methylphenol on the formation of sessile biomass and the inactivation of mature biofilms at different incubation temperatures in polystyrene and stainless steel, has been proven. Monoterpene sub-inhibitory concentrations were effective in preventing the biofilm synthesis, reduction of exopolysaccharide production and the down-regulation of biofilm-associated genes. Using minimum bactericidal concentrations the inactivation capacity of preformed biofilms of *L. monocytogenes* was demonstrated (Upadhyay *et al*., 2013).

To explain the antimicrobial properties of monoterpenes, it has been proposed that these compounds they are able to change the cellular permeability by inducing various morphological and physiological changes in stability, hydrophobicity, fluidity and fatty acid composition of the cytoplasmic membrane. In addition, monoterpenes are capable of causing an uncontrolled flow of hydrogen ion that alter the movement of the solutes, dependent on the proton pump, through the cell membrane and the intracellular pH modification. Both factors exert an inhibitory effect on cell attachment to surfaces, in the early stages of biofilm formation (Nieto, 2017).

CONCLUSIONS

L. monocytogenes represents a risk to food safety in relation to ready-to-eat (RTE) products, since this microorganism is capable of growing in a wide thermal range, including cooling temperatures. The manufacture of minimally processed fresh products presents important challenges for their quality and safety, which is why antimicrobial agents have been widely used for the conservation of RTE foods. The results suggest that thymol could potentially be used to control the formation of biofilms by *L. monocytogenes* in the food industry.

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