2. Materials and Methods

2.1 Minimal Inhibitory Concentration

Minimal inhibitory concentration (MIC) of thyme essential oil for *L. monocytogenes* Scott A and *E. coli* ATCC 25922 was obtained by inoculating these bacteria in Petri dishes (S y M Laboratorios, Puebla, Mexico) with tryptic soy agar, to which previously a specific volume of thyme essential oil solution 49% (see materials and methods 2.3) was added, leaving it to dry for approximately 20 min.

Strains of tested microorganisms *L. monocytogenes* Scott A and *E. coli* ATCC 25922 were obtained from the collection of the Food Microbiology Laboratory of the Chemical and Food Engineering Department at *Universidad de las Américas Puebla*.

Tryptic soy agar (BD Bioxon, State of Mexico, Mexico) was used to prepare a media in which the growth of the microorganisms *E. coli* and *L. monocytogenes* could be observed. The thyme essential oil solution (49%) was prepared in a test tube, adding first 4.9 ml of water, then 200 µl of the emulsifier Tween 80 (Merck, Darmstadt, Germany) and finally 4.9 ml of the thyme essential oil (Hersol, State of Mexico, Mexico).

MICs were obtained for two different temperatures (10°C or 20°C). Thus, the plates inoculated with *E. coli* and *L. monocytogenes* were stored either at 10±1°C or 20±1°C inside an incubator (Thermo Fisher Scientific, Precision, Mexico City, Mexico) for 1 week or 1 day, respectively. After this time, plates were observed with a bacterial colony counter, according to Govaris et al. (2011), to determine if the microorganisms grew and form colonies. An actual count of colonies was not performed, since for this part of the study the interest was on microbial inhibition, and not in which quantity.

More than one test was required to obtain the MIC for each studied microorganism at each tested temperature. The first volumes of thyme essential oil solution used were: 0, 10, 20, 40 or 100 µl, corresponding to concentrations of 0, 0.14, 0.28, 0.56 and 1.4 µl thyme essential oil/g agar (for calculating these concentrations it was taken into account the average weight of the agar, that was 35 g), respectively. Each system (type of microorganism – temperature of incubation – volume of thyme essential oil solution) was made by duplicate. The volumes used for the second test were between the last volume in which the microorganism grew (even smaller volumes, in which microorganisms did grow, were used to try them again and confirm the results) and the first volume in which the microorganism didn't grow, afterwards. So the range of volumes tested was smaller with every test. Tests were performed until it was confirmed, at least by two consecutive tests, that at certain volume of essential oil the microorganisms didn't grow, being this value the reported MIC.

2.2 Cheese preparation and characterization

Fresh cheese was made, according to the method described by Ramírez-López & Vélez-Ruiz (2012), from pasteurized whole cow's milk, of the brand *Alpura*. First, milk was poured into a cooking pot. The pot was placed in a heater until the milk reached 39°C, then heater was turned off, 1 ml of 50% calcium chloride solution (Reactivos Química Meyer, Mexico City,

Mexico) per liter of milk and 0.75 ml of rennin (CHR Hansen, Mexico City, Mexico) per liter of milk, were added to the pot and stirred. Then milk was allowed to stand for 20 min until it curdled. After the curdling was completed, the curd was cut in squares of approximately 2x2 cm and these were allowed to stand for 15 min. Next, the squares were put in a mesh and slightly squeezed to eliminate the whey. To the resulting paste, salt was added in a concentration of 1% of the total cheese mass, taking into account that the cheese yield was 10%. Then the cheese was molded in a circular mold and pressed with a weight of approximately 3.4 kg for 18 to 24 h at 5°C.

The microbiological characterization of the cheese was performed using the Standard Methods agar (BD Bioxon, State of Mexico, Mexico) to determine the microbiological content of the cheese. The agars Violet Red Bile (BD Bioxon, State of Mexico, Mexico), Potato Dextrose (Difco, Maryland, United States), Eosin Methylene Blue (EMB) (BD Bioxon, State of Mexico, Mexico), Oxford (Difco, Maryland, United States), and Chapman 110 (Merck, Darmstadt, Germany) were also used to verify that the cheese was free of coliforms, yeasts and molds, *E. coli*, *L. monocytogenes*, and *Staphylococcus aureus*, respectively.

Soto-Beltran et al. (2015) also carry out a microbiological characterization of fresh cheese, but they analyze for *Listeria* spp., *Salmonella* spp., *E. coli*, Shiga toxin-producing *Escherichia coli* (STEC), and coliforms.

A sample of the cheese was prepared mixing 10 g of the cheese with 90 ml of peptone (BD Bioxon, State of Mexico, Mexico) water. Dilutions 10^{-1} and 10^{-3} of the sample were prepared. In the Standard Methods, Violet Red Bile and Potato Dextrose agars, 1 ml of the dilution was inoculated by pour platting. On the other hand, in the EMB, Oxford and Chapman 110 agars, 100 µl of each of the sample dilutions were inoculated by spreading them on the surface of the corresponding medium. Each system (type of agar – dilution) was made by duplicate. Both spread-plate and pour-plate methods are described by Hunt et al. (2017).

Every plate was incubated at 37±1°C but the time of incubation varied: Violet Red Bile, EMB and Oxford agar plates where incubated for 24 h; the Standard Methods agar plates were incubated for 48 h; and the Potato Dextrose and Chapman 110 agar plates were incubated for 3 days. After incubation time, it was observed if there was growth, and in those were there was, observed colonies were counted using bacterial colony counter. Colonies were counted one by one, having as detection limit 1 log CFU/g (Govaris et al., 2011), when the number of colonies exceeded this limit, only colonies in 5 quadrants of the plate were counted. Then, for both cases, the relevant calculations were made to obtain the number of colony forming units per gram of cheese.

Two cheeses were made (following the process described above), and each of them was subjected to a physicochemical characterization, which consisted in an ash, moisture, protein, and fat quantification.

Ash was determined by ignition of the cheese according to the method defined by the Association of Official Analytical Chemists (AOAC, 1995) 935.42 (33.7.07). The method is detailed below.

Approximately 3 g of cheese were placed in a porcelain crucible, previously carried to constant weight. The crucible with the sample of cheese was accurately weighed on an analytical balance (AY220, Shimadzu, Japan). Then, the crucible was placed above a burner in order to carbonize the sample, always taking care that the sample didn't ignite. Afterwards, the crucible was placed inside a muffle furnace at a temperature of 550°C for 24 h to complete ignition. Then, the crucible with the ashes was allowed to cool in a desiccator and then was accurately weighed on the analytical balance, and the ash content was expressed on wet basis:

$$\%\ Ash\ (wet\ basis) = \frac{Mass\ of\ the\ ashed\ sample}{Initial\ mass\ of\ the\ wet\ sample} x100 \qquad (Eq.\ 1)$$

The ash determination was made by triplicate for each of the two cheeses.

Moisture was determined by oven drying of the cheese according to the AOAC (1995) official method 926.08 (33.7.03). The method is detailed below.

Approximately 2 g of cheese were placed in an aluminum dish, previously carried to constant weight that contained sand and a crystal paddle. The dish with the sand, the paddle and the wet sample was accurately weighed (Weight A) on an analytical balance (AY220, Shimadzu, Japan). Then, the paddle was used to spread the sample across the dish in order to attain a greater sample surface area and so a better moisture removal. The sample was dried by placing the dish inside an oven (Riossa, Distrito Federal, México) at 100°C for 24 h. After this time, the dish was removed from the oven and then it was allowed to cool inside a desiccator for 15 min. Then, the dish with the sand, the paddle and the dry sample was accurately weighed (Weight B) on an analytical balance. The moisture content was calculated as follows:

$$Moisture\ (\%) = \frac{Weight\ A - Weight\ B}{Initial\ mass\ of\ the\ wet\ sample} x100 \qquad (Eq.\ 2)$$

The moisture content determination was performed six times for each of the two cheeses.

Protein was determined by the Kjeldahl method according to the AOAC (1995) official method 920.123-1920 (33.7.12). The method is detailed below.

First, 0.2 g of cheese and 0.8 g of digester mix (88.89% potassium sulfate, 8.89% copper sulfate pentahydrate and 2.22% selenium dioxide) were placed inside a Kjeldahl flask. Then, 5 ml of sulfuric acid (Reactivos Química Meyer, Mexico City, Mexico) were added to the flask. The flask was placed on a heater and the sample was allowed to digest until the contents of the flask turned emerald green. Then, the mixture was neutralized with a 45% sodium hydroxide solution (Merck, Darmstadt, Germany). Distillation was carried out to this mixture until approximately 50 ml of distilled were obtained. This distilled was received in another flask with 5 ml of 5% boric acid solution (Sigma-Aldrich, Toluca, Mexico) and 0.5

ml of 0.5% methyl red solution (Sigma-Aldrich, Toluca, Mexico). Finally, the distilled was titrated with a 0.1N hydrochloric acid solution (JT Baker, Mexico City, Mexico). The protein content was calculated as follows:

$$Protein\ (\%) = \frac{ml\ HCl * N * 0.014 * 6.38}{Initial\ mass\ of\ the\ sample} x100 \qquad (Eq. 3)$$

Where N refers to the normality of the hydrochloric acid solution. The protein determination was made by duplicate for each of the two cheeses.

Fat was determined by extraction of the fat of the cheese according to the method defined by the AOAC (1995) 933.05 (33.7.17). The method is detailed below.

Approximately 1 g of sample was placed inside a Mojonnier flask. The sample was accurately weighed on an analytical balance. Then 9 ml of water and 1 ml of ammonium hydroxide (J.T Baker, Mexico City, Mexico) were added to the flask. The content of the flask was mixed and then the mixture was warmed at low heat until the casein was well softened. The mixture was then neutralized with 10 ml of hydrochloric acid (J.T Baker, Mexico City, Mexico), adding it slowly to the flask to prevent a violent reaction. The mixture was gently boiled for 5 min and then it was cooled. 10 ml alcohol (Reactivos Química Meyer, Mexico City, Mexico), 25 ml ether (J.T Baker, Mexico City, Mexico), and 25 ml petroleum ether (Reactivos Química Meyer, Mexico City, Mexico) were added to the flask, mixing thoroughly for 1 minute after adding each reagent. Then the solution was left to stand until the phases separated, the solution was decanted. The same procedure was performed again but using 5 ml alcohol, 15 ml ether, and 15 ml petroleum ether. The ethereal phases of both extractions were transferred into a beaker (pre-weighed). The beaker was then placed on a heater in order to evaporate the solvents. The beaker with the fat (that remained in the beaker) was accurately weighed on an analytical balance. The fat content was calculated as follows:

$$Fat\ (\%) = \frac{Mass\ of\ the\ flask\ with\ fat - Mass\ of\ the\ flask}{Initial\ mass\ of\ the\ sample} x100 \qquad (Eq. 4)$$

The fat determination was performed by duplicate for each of the two cheeses.

2.3 Determination of MIC in fresh cheese

For the realization of this part of the experiment, it was decided to study the antimicrobial effect of thyme essential oil against the growth of *L. monocytogenes* Scott A and *E. coli* ATCC 25922 in fresh cheese only at 10°C, since this temperature is close to the temperature at which the fresh cheese is commonly stored.

The systems prepared to study the effectiveness of the thyme essential oil are the following:

- System 1: cheese + inoculum
- System 2: cheese + inoculum + thyme essential oil at the MIC
- System 3: cheese + inoculum + thyme essential oil at twice the MIC (2MIC)

It was decided that the cheeses should be stored for 14 days, as that is the average storage time of this type of cheese in a home. It was also decided that the cheeses should be analyzed every 3 or 4 days, in such a way that they were analyzed 5 times during the 14 days of assay. So, in order to not take out the cheese of each system every analysis day, and avoid the risk of contamination and temperature fluctuations, it was decided to make 5 little cheeses for each studied system, in such a way that each day of the analysis only one of the cheeses was taken out of the refrigerated-incubator (Thermo Fisher Scientific, Precision, Mexico City, Mexico) and the others remained stored. Thus, a large cheese was made in order to obtain from it 15 rectangles of cheese of approximately 6 cm in length, 3 cm wide and 0.5 cm thick.

As Han et al. (2014) did in their study, a mixture of broths (cocktail) of *L. monocytogenes* Scott A and *E. coli* ATCC 25922, at the same proportion, was prepared in order to inoculate each of the cheese rectangles with both microorganisms at the same time. The quantity of both microorganisms in this mixture was determined by triplicate in three EMB and three Oxford agar plates.

The MIC and 2MIC solutions were prepared in such a way that it was possible to lay 125 µl of these solutions in the corresponding cheese rectangles, applying the following equation:

$$C_1V_1 = C_2V_2 \qquad (Eq.5)$$

Where C stands for concentration and V stands for volume. C_1 is the concentration of the thyme essential oil solution used for finding the MIC (49%), V_1 is the volume corresponding to the MIC or 2MIC (depending on the case) divided by the grams of agar in the culture mediums used and multiplied by the grams of cheese in the cheese rectangles at the corresponding temperature, C_2 is the concentration one wants to know to prepare the solutions, and V_2 is 125 µl.

First, 125 µl of the corresponding thyme essential oil solution, according to the system, were spread on both sides of the cheese rectangles. Afterwards, 250 µl of the 10^{-4} dilution of the inoculum mixture were spread the same way. After spreading the inoculum in each side, the cheese was allowed to dry for 15 min, the same was done after spreading the essential oil. This whole procedure was made under sterile conditions in a laminar flow hood.

Each cheese rectangle was stored in a perfectly closed Whirl-Pak bag (Nasco, California, United States) (see figure 1) inside the refrigerated-incubator (Thermo Fisher Scientific, Precision, Mexico City, Mexico) at 10°C and every 3-4 days (for 14 days) a cheese of each system was taken for analysis. The analysis consisted in making dilutions of the cheese and inoculating a sample of them in two EMB agar plates and two Oxford agar plates, in order to observe if there was growth of *E. coli* and/or *L. monocytogenes*, namely to observe if these microorganisms grew on the cheese. These agar plates were later incubated at 37±1°C for 24-72 h. After that time, the colonies in each agar were counted using a bacterial colony counter. In this manner, it was possible to know the amount of *E. coli* and *L. monocytogenes* that were in the cheeses through the 14 days and so it was observed if the thyme essential oil inhibited the growth of these microorganisms, at any of the two tested concentrations (MIC or 2MIC) of the oil.

Figure 1. Cheese rectangles in Whirl-Pak bags for their storage at 10°C