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RESEARCH ARTICLE

EVALUATION OF THE SURVIVAL OF *LISTERIA MONOCYTOGENES* AND LACTIC ACID BACTERIA IN MANGO, CUSTARD APPLE AND BLACKBERRY FRUIT JUICE CONCENTRATES FROM COSTA RICA

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ABSTRACT

In the last years, the consumption of natural products has increased. Fruit concentrates and derivates are part of these new products of high demand. Although these products present high acidity, they have been linked to the transmission of different pathogenic microorganisms. The aim of this study was to determine the potential survival of three different concentrations of *Listeria monocytogenes* inoculated into three different fruit juice concentrates of high demand in Costa Rica, including custard apple, black berry and mango, through its shelf life at refrigeration temperature. At the same time, lactic acid bacteria count was determined in order to describe the potential spoilage of these products. Blackberry concentrate did not allow the survival of the different inoculum of *L. monocytogenes* added as well as lactic acid bacteria. Custard apple concentrate allowed a slight survival of *L. monocytogenes* and lactic acid bacteria. Mango concentrates allowed, however, the survival of both *L. monocytogenes* and lactic acid bacteria during the storage period. Although juice concentrates have many intrinsic and extrinsic properties that represent a barrier for the survival and growth of microorganisms, *L monocytogenes* may overcome them, representing a risk for human health.

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INTRODUCTION

In the last years, there has been a collective increase in the awareness of a healthful nutrition, resulting in a higher demand of products with no or very little chemical and preserving agents added, and being rich source of minerals and vitamins. Fruits concentrates and derivates are part of these new products of high demand. Fruit concentrates are obtained from the disintegration of edible parts of fruits and usually preserved through pasteurization. These products have a considerable commercial potential, because they hold most of the nutritional properties of fruits and can be used as juices, marmalades, conserves and similar products (Fernandes, 2007). Consumption of these kinds of products has been linked to a lower risk of developing cancer appearance, a lower mortality and to lower indexes of coronary disease. Spoilage bacteria associated to fruit juice concentrates include lactic acid bacteria [LAB], yeasts and molds. These microorganisms can produce undesirable organoleptic changes, including strange tastes, smells and color variations of the product (Awojobi *et al.,* 2016). Also, most of them are psycrotrophs and might survive pasteurization (Ray and Bhunia). Although the acidity of fruit juices has been thought as an important barrier against the survival and growth of food borne pathogens, fruit juice concentrates have also been linked to the transmission of different pathogenic microorganisms (Vojdani *et al.,* 2008). Pathogenic bacteria mostly associated with fruit pulps include *Salmonella* enterica and *E coli* O157:H7. *Listeria monocytogenes* has not been directly linked to outbreaks associated with fruit juice concentrates, however, it can be isolated from different fruits and also can survive in acidic foods for long periods of time (Oyarzabal *et al.,* 2003). Also, *L. monocytogenes* can survive and reproduce at refrigeration temperatures (Abarzúa and Solavi, 2009), and, at high concentrations might survive thermic and sanitary processes used in food industry (Vitas *et al.,* 2004). The potential transmission of pathogens through fruit juice concentrates represents a growing risk for human health. In response, the Food and Drug Administration (FDA) regulated, in 2002, the application of hazard analysis and critical control points (HACCP) principles for fruit and vegetable juices processors, requiring for a 5-log pathogen reduction step before packaging for retail distribution (Food and Drug Administration, 2001). Costa Rica has a growing fruit juice concentrate market. Exportation to different countries has increased significantly in the last years, nevertheless, the potential survival of *L. monocytogenes* in these has not been evaluated yet. The aim of this study was to determine the potential survival of *Listeria monocytogenes* inoculated into three different fruit juice concentrates of high demand in Costa Rica, including custard

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Table 1. Survival of a high (10^7CFU/g), medium (10^5 CFU/g)and low (10^3 CFU/g) concentration of *L. monocytogenes* inoculated to fruit juice concentrates through different storage times at refrigeration temperature

	High inoculum [CFU/g]			Medium inoculum [CFU/g]			Low inoculum [CFU/g]		
Days of storarge	***Blackberry***	***Custardapple***	***Mango***	***Blackberry***	***Custardapple***	***Mango***	***Blackberry***	***Custardapple***	***Mango***
0	$1.0x10^7$	$3.0x10^7$	$2.0x10^7$	$1.6x10^5$	$1.0x10^5$	$1.1x10^5$	$2.0x10^3$	$1.5x10^3$	$1.0x10^3$
3	<10	$8.3x10^4$	$2.7x10^4$	<10	$2,0x10^2$	$2.1x10^4$	<10	<10	$2.0x10^2$
6	<10	<10	$7.0x10^3$	<10	<10	$7.0x10^3$	<10	<10	$1.6x10^1$
9	<10	<10	$4.3x10^2$	<10	<10	$3.7x10^2$	<10	<10	$7.0x10^1$
12	<10	<10	$3.6x10^2$	<10	<10	$7.0x10^1$	<10	<10	<10
15	<10	<10	$1.0x10^1$	<10	<10	$1.1x10^1$	<10	<10	<10

Table 2. Survival Lactic Acid Bacteria (LAB) on fruit juice concentrates through different storage times at refrigeration temperature

	High inoculum inoculation			Medium inoculum inoculation			Low inoculum inoculation		
Days of storarge	***Black-berry***	***Custardapple***	***Mango***	***Blackberry***	***Custardapple***	***Mango***	***Blackberry***	***Custardapple***	***Mango***
0	$2,0x10^2$	$4.0x10^3$	$2,8x10^3$	$2,0x10^2$	$4.0x10^3$	$2,8x10^3$	$2,0x10^2$	$4.0x10^3$	$2,8x10^3$
3	<10	$3,0x10^4$	$3,9x10^4$	<10	$1,0x10^4$	$9,0x10^4$	<10	<10	$2.0x10^4$
6	<10	<10	$3.0x10^3$	<10	<10	$3,3x10^2$	<10	<10	$1.0x10^2$
9	<10	<10	$1,0x10^2$	<10	<10	$1,0x10^2$	<10	<10	$1,0x10^1$
12	<10	<10	<10	<10	<10	<10	<10	<10	<10
15	<10	<10	<10	<10	<10	<10	<10	<10	<10

apple, black berry and mango, through its shelf life at refrigeration temperature. At the same time, LAB count was determined in order to compare the behavior of spoilage with bacteria with the pathogen inoculated.

MATERIALS AND METHODS

Juice concentrates: Three different juice concentrates were selected according to their pH. These included blackberry, custard apple and mango. Three samples of 1 L of each concentrate were acquired, on different dates, from a local distributor in San José, Costa Rica, between the months of September and October 2017. Samples wereimmediately transported to the Microbiology Food Lab, Microbiology Faculty, Universidad de Costa Rica, and kept at 2-5°C from the time of purchase to the onset of experiments.

Inoculum preparation. A *Listeria monocytogenes* pool was prepared from ATCC 19116 strain kindly supplied by the Medical Bacteriology Lab, Microbiology School and other 5 different strains previously obtained and characterized from food samples. Stock cultures were maintained at −70 ºC on tryptic soy agar slants [Oxoid] and were activated in tryptic soy broth (pH 7.0) at 35 ºC. Culture was transfered by loop inocula twice at 24 h intervals to 100 ml of tryptic soy broth in 250 ml Erlenmeyer flasks. Appropriate dilutions were performed in peptone water 0, 1 % in order to obtain high inoculum concentration(10^7 CFU/ml), medium inoculum concentration (10^5 CFU/ml) and low inoculum concentration(10^3 CFU/ml) populations.

Procedure for preparing and inoculating fruit juice concentrates: Each sample was divided in three portions (333, 3 mL each), placed in sterile Erlenmeyer flasks. Immediately, the pH was determined with a Sartorius Pb-11 pH meter. Same time, water activity (a_w) was determined using the Aqua Lab 4TE Dew point water activity meter. One of these portions was inoculated with 7 mL of a high inoculum concentration of *L. monocytogenes* pool and pummeled in Stomacher for 2 min. Thereafter, an initial *L. monocytogenes* count was performed, and samples were incubated at 4 ºC for 15 days. The same procedure was done for portions inoculated with medium and low concentrations of the bacteria.

Three independent trials were performed for each food analyzed. For each trial, a non-inoculated control was included.

Enumeration of *Listeria monocytogenes:* The *L. monocytogenes* count was done according to the methodology described in the Bacteriological Analytical Manual (American Public Health Association, 2017) on days 0, 3, 6, 9, 12 and 15 of storage at refrigeration temperature. Immediately after each incubation time, 25 gof the samples were taken and mixed with 225ml of sterile peptonated water 0.1 % in sterile polyethylene bag and pummeled with a Stomacher for 2 min. This initial solution was serially (1: 10) diluted and surface plated (0.1 ml) on duplicate Oxford agar (Oxoid). Plates were incubated at 35 ºC for 24h before colonies were counted. The same procedure was used for control samples (American Public Health Association, 2017).

Enumeration of lactic acid bacteria: LAB were enumerated from each portion of the different fruit juice concentrates analyzed, during the same periods described above according to the methodology described on Compendium of Methods for the Microbiological Examination of Foods (American Public Health Association, 2015). Briefly, 0, 1 mL of the dilutions previously prepared were inoculated onto MRS agar and incubated for 48h at 35°C and under capnophilic atmosphere. Confirmation of LAB included Gram staining and oxidase and catalase tests.

RESULTS

The averaged pHs found for the fruit juice concentrates ranged between 2, 0-2,4 for blackberry, 3,9-4,2 for custard apple and 5,0-5,2 for mango. These different levels of acidity from fruit juice concentrates result ideal for studying the behavior of *L. monocytogenes* under different environmental conditions. The averaged a_w for fruit juice concentrates tested ranged between 0,87 and 0,90. Results obtained for each of the different inoculums of *L. monocytogenes* added to each juice concentrate are shown on Table 1. All concentrates were negative *for L. monocytogenes* before inoculation. Results obtained for LAB on each juice concentrate sample are shown on Table 2. Blackberry concentrate inhibited completely the survival of the different inoculums of *L. monocytogenes* and

LAB. Custard apple concentrate showed a slight survival of *L. monocytogenes* when high and medium inoculums were added, however, it did not last longer than 6 days. LAB showed a similar behavior with a maximum growth of 10^4 CFU/g at day 3. Mango juice concentrate allowed the survival of *L. monocytogenes* and LAB during all the storage period and for both high and medium inoculums. The maximum concentration of LAB found was also 10^4 CFU/g at day 3. Survival was not detected on days 12 and 15 of storage when low inoculum was added to this concentrate.

DISCUSSION

Food industries, especially those associated with fruits, have to deal with many challenges in order to prevent the contamination of raw material with pathogenic microorganisms and its spoilage (Von Breymann *et al.,* 2013). Several outbreaks of illness caused by bacteria and parasites have been linked to juices and ciders for a long time (Vojdani *et al.,* 2008). The contamination source of these outbreaks has been difficult to define; nevertheless, contamination is believed to occur mostly during final steps of the production process (Vojdani *et al.,* 2008). Juice concentrate industries have introduced different conservative techniques; including the thermic process in order to eliminate spoilage and potential pathogenic microorganisms (Silveira *et al.,* 2013). However, if these techniques are not effective, the inocuity of the product might not be ensured. One of the most important pathogenic microorganisms related to juice concentrate industries is *L. monocytogenes.* It is a widely distributed microorganism, thus it is difficult to avoid its potential cross contamination with products, such as fruit concentrates. This bacterium can grow at different pH, ranging from 4,4 to 9,4 (Schobitz *et al.,* 2009), and can also grow at refrigeration temperatures. In addition, it has the ability to form biofilms that tightly adhere to places difficult to clean (Von Breymann *et al.,* 2013). Results obtained in the present work showed that blackberry concentrates inhibited the survival of both *L. monocytogenes* and LAB. This might be due mainly because of the low pH of this product (i.e. average pH 2.2).

Additionally the acidity of blackberries, the fruit contains a high concentration of vitamin A, a factor that has shown a protective effect in experimental models of *L. monocytogenes* infection (Castillo *et al.,* 2016). Molds, yeast and LAB are predominant in the spoilage of fruits and sub products, thus their absence in blackberry concentrate suggests a longer shelf life of this product when stored under appropriate refrigeration temperatures (Silveira *et al.,* 2013). Custard apple concentrate is less acid than blackberry, a characteristic that allows the survival of some microorganisms. High and medium inoculums of *L. monocytogenes* survived between days 0 – 6, and low inoculum survival was not detected. LAB showed the same behavior. The survival of both groups of microorganisms may be due to a lower acidity compared with the blackberry concentrate and to the fact that this concentrate has a high concentration of fiber and non-homogenized material, which may allow the accumulation of bacteria and even exert a protective action during first days of storage. Both *L.monocytogenes* and LAB are inhibited after day 6 of storage. This might be due to a lower pH associated with normal metabolism of the bacteria and to the high osmolarity of this fruit, which limits the amount of water available for growth (a_w 0,87) (Márquez, 2009). The juice concentrate tested with higher pH was mango. In this product, both groups of bacteria showed survival during all the storage period (except for low inoculum of *L. monocytogenes* after day 12). This survival is associated with a pH that allows bacterial multiplication and with the presence of a high concentration of fiber that acts as protective agent. Although microorganisms are metabolically active, the pH drop is not enough to cause inhibition. Several researches have reported the survival and growth capacity of *L. monocytogenes* in fruit juice concentrates (Uchima *et al.,* 2008). This organism can tolerate low water activity (aw) (Rossi *et al.,* 2008), their carbohydrate content in juice concentrates is an adequate substrate for its growth (Ray and Bhunia) and it is considered an acid tolerant bacteria. This bacterium can resist low pH because of the presence of glutamate decarboxylase, an enzyme that allows the bacteria to maintain an adequate intracellular pH despite of an hostile external pH (Cotter and Hill, 2003). This acid tolerance also allows bacteria to have a stronger resistance to high temperatures, as has been demonstrated before (Caggia *et al.,* 2009). The characteristics described above, added to the fact that this bacterium can grow and survive at refrigeration temperatures (Leverentz *et al.,* 2013; Penteado and Leitao, 2004) promote its development under conditions that are inappropriate for other pathogens, which is the reason why this bacterium is so important for public health[18].Because of this, the Food and Drug Agency (FDA) and the European Union have established a zero tolerance for *L. monocytogenes* on ready to eat food (Leverentz *et al.,* 2013). These are aspects to consider for the exportation of Costa Rican fruit juice concentrates to other countries.

Lactic acid bacteria are normal flora of fruits. Analyzing the behavior they presented on the samples evaluated, and although they were stored for two weeks at refrigeration temperature, their number did not show a marked increase through time. This may be due to the fact that all products tested had, as preservant, sodium benzoate 0,01% and were pasteurized. Von Breyman *et al.* have reported similar results in a previous work in Costa Rica (Von Breymann *et al.,* 2013). This behavior is associated with a longer shelf life without organoleptic changes. The data presented in this work shows that although juice concentrates have many intrinsic and extrinsic properties that represent a barrier for the survival and growth of microorganisms, *L monocytogenes* may overcome them, representing a risk for human health. Producers should have special attention to prevent the potential contamination of the processing plant or the final product with this pathogen.

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