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Control of the Growth of *Listeria monocytogenes* in Cooked Ham through Combinations of Natural Ingredients

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Abstract: In the food industry, *Listeria* is a standard control microorganism in ready-to-eat food since it may be pathogenic and cause a disease called listeriosis. The objective of the present study was to carry out a challenge test to verify the efficiency of different combinations of natural antimicrobial ingredients, against *Listeria monocytogenes*, to be used in ready-to-eat foods. Six different formulations of cooked ham were elaborated: a control, and five different formulations. An initial inoculation of 2 log cycles was used in the different products and the growth of *Listeria* was monitored at different temperatures and times (4° C for 17 w; 7ºC for 12 w). Control samples showed a progressive growth, reaching 5-6 log after 3 or 4 weeks. The rest of samples showed constant counts of *Listeria* during the all study. Only samples containing: 100 ppm nitrite + 250 PPM ascorbic acid+0,7% PRS DV-5 did not control the growth of Listeria at 7 ° C after 7 w of storage. The results obtained allowed to classified the cooked ham elaborated using any natural ingredients combinations as a "Ready-to-eat food unable to support the growth of *L. monocytogenes* other than those intended for infants and for special medical purposes".

Keywords: challenge test; *Listeria monocytogenes*; natural ingredients combinations; food safety

1. Introduction

Meat products such as cooked ham are an important category of processed foods consumed throughout the world. These products are highly susceptible to contamination by pathogenic microorganisms during the production chain due to their physico-chemical characteristics and the multiple stages of preparation and handling [1]. This situation is of great concern to food industries because it is associated with large economic losses and consumer safety. The ingestion of this type of food of animal origin does not require additional cooking before consumption, they are at higher risk of contamination and of becoming sources of contaminated food-borne diseases, being a frequent public health problem worldwide [2]. Therefore, any *L. monocytogenes* (*L. monocytogenes*) or other pathogens are considered as adulterants [3]. Despite improved hygiene and production techniques, ready-to-eat (RTE) meat products are still associated with outbreaks of foodborne diseases worldwide [4].

In recent years, outbreaks of foodborne illness related to RTE products reported by the Centers for Disease Control and Prevention (CDC) of the United States have been due to infection with *L. monocytogenes* or *Salmonella* (CDC) [5]. *Listeria* is a standard control micro-organism in the food industry as it can be pathogenic and cause a disease called listeriosis which can affect humans and animals. Cases of listeriosis were linked to deli ham in 2018, deli meats and sliced cheeses in 2019, deli meats (possibly salami, mortadella and prosciutto) in 2020 and sausages (mortadella, salami and ham) and sliced cheeses in 2023 [6]. *L. monocytogenes* has a higher mortality rate [7]. Due to its

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facultative anaerobic metabolism, its psychrotrophic properties and its ability to survive in environmental conditions, this micro-organism can not only persist in contaminated foodstuffs, but also persists in various industrial environments and food contact surfaces (e.g. cutting boards) [8]. In cooked ham, *L. monocytogenes* emerges as the most dominant pathogen, being associated with contamination and cross-contamination throughout the production and processing process [9,10]. The food industry should to elaborate safe food at reasonable prices using techniques, treatments or ingredients which assure the innocuousness for consumers. The food industry must produce safe food at reasonable prices using techniques, treatments or ingredients that ensure safety for consumers.

Food additives are increasingly being used as an antimicrobial compound for the development of safe food, and are used not only for the concern of consumer health but also for the tendency towards natural food additives, the so-called "clean labelling," which has prompted them to be used to replace synthetic additives [11–13]. Among natural antimicrobial compounds, a number of plant-derived extracts have been extensively studied [14,15]. The extracts of spices and herbs commonly used in food receive great attention. In meat and meat products, the plant extract can be used alone or combined with other extracts or with a minimum process for a synergistic result [13].

The main objective of the present study is to carry out a challenge test according to the "EURL Lm technical guidance document for carrying out shelf-life studies of *L. mono-cytogenes* in ready-to-eat foods", to verify the efficiency of different combinations of natural antimicrobial ingredients, against *L. monocytogenes*, to be used in ready-to-eat foods, in order to classify these cooked hams as a ready-to-eat food capable or not of supporting the growth of *L. monocytogenes*.

2. Materials and Methods

2.1. Cooked ham elaboration

Six different cooked ham formulations were manufactured using Good Manufacturing Practices in the Pilot Plant of PROSUR SAU.

Ingredients included: pork ham meat (80 %), potato starch (15g/kg); tripolyphosphate (5 g/kg); carageenan (3 g/kg); salt (2% in the final product); and the preservatives composition indicated in Table 1.

Table 1. Description of preservatives included in the different cooked ham and their identification.

Sample	Preservative content
P1	Negative Control – no preservatives
P2	Celery (100 ppm Nitrite); 250 ppm ascorbic acid+0,7% PRS DV-5
P3	1% NATPRE T-10 DV HS + 0.5% PRS-DV-5
P4	1% NATPRE T-10 DV LS + 0.5% PRS-DV-5 LS - 1.3% NaCl + 0.35-0.40% KCl
P5	1% NATPRE T-10 EML + 0.5% PRS-DV-5
P6	1% NATPRE T-10 EML + 0.75% PRS-DV-5

[1] NATPRE: a combination of extracts from lemon, orange and grapefruit with extract of pepper and coriander spices; HS: high solubility; LS: Low salt content; EML: emulsion; DV: dry vinegar.

The cooked ham was elaborated according to the following steps: mince the meat using a 16 mm plate; dissolve the dried ingredients into the water for preparing the brine and mix it with the minced meat under vacuum conditions for 1.5 h; stuff the meat in a plastic casing and cook until inner temperature reach 74 °C; slicing, pack under vacuum conditions and storage at refrigerated temperature.

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2.2. Prosur ingredients description

Prosur ingredients combinations tested in this study are a combination between different dried ingredients: apple cider vinegar, citrus extract (lemon, orange and grapefruit) and spice extracts (pepper and ground coriander). Dried vinegar has been used as a preservative in combination with plant extracts, including those that improving its sensory attributes. The primary role of dried vinegar in this combination is to provide antimicrobial properties and help inhibit the growth of microorganisms that cause food spoilage. Meanwhile, the citrus and spices extracts can serve multiple purposes, including enhancing flavor, masking off-flavors, and potentially providing additional antimicrobial properties. This combination can help to prevent the growth of bacteria like *L. monocytogenes* and other spoilage bacteria without any impact in the organoleptic properties of the final product.

2.3. Microbiological analysis

2.3.1. Choice of strains and preparation of the inoculum

A cocktail of five strains were selected and acquired in the Spanish Type Culture Collection. *Listeria innocua* (*L. innocua*) CECT 8848, CETC 910T, CECT 4030, CECT 5377 and CECT 5378. *L. innocua* strains were used as a surrogate for *L. monocytogenes*, as mentioned the EURL Guide. The preparation of inoculum was carried out according to the protocol cited in the EURL guide [16]. Firstly, a pure culture of each strain was inoculated individually in Tryptone Soy Broth (TSB, Pronadisa, Madrid, Spain) at 37 °C and 24 h. This first subculture is mainly aimed at getting the cells in the stationary phase. Secondly, 100 µL of overnight culture was transferred to a new TSB tube and incubated at a temperature close to the storage temperature of the product (7 °C-7 days, 10°C-3 days), in order to adapt the strain to the storage conditions of the product. Thirdly, each second subculture was combined in equal quantity. From the mixed culture, successive dilutions were prepared in buffer peptone water to obtain an inoculum at the expected concentration. The inoculum was used immediately. The targeted inoculum level was checked by enumeration on a selective agar.

2.3.2. Preparation and inoculation of the test units

Cooked ham was sliced under sterile conditions. Non-inoculated vacuum packages were prepared for microbiology analysis, containing three slices per package, and were analyzed initially, in the middle and at the end of study. Total aerobic count, lactic bacteria and *Listeria* were determined in order to evaluated the initial good practices during the slicing process and a possible *Listeria* contamination. Triplicate non inoculated samples were analyzed in each analysis time.

Inoculated samples were prepared with 4 slices per packages. An initial microbial concentration of 10^2 cfu/g was inoculated per slice. Slices were surface-inoculated with the cocktail of L*isteria* and the inoculum distributed over one surface of each slice, and then stacked such that the inoculum was between the slices. Inoculated products were vacuum packaged in gas-impermeable pouches and stored at the appropriate incubation temperature (4 °C or 7 °C). Triplicate inoculated samples were assayed for *Listeria* populations.

2.3.3. Microbiology analysis and storage conditions

In the non-inoculated samples, total aerobic counts and lactic bacteria were assessed via a spread plating method on specific selective agars, Plate count Agar (PCA) and Man, Rogosa and Sharpe Agar (MRS Agar), respectively. Plates were incubated at 30 °C between 48-72 h, before counting. *Listeria* was determined by PCR. Samples were analyzed by triplicated the day 0, in the middle and at the end of study.

The inoculated samples were analyzed initially and after 4, 6, 8, 10, 11, 12, 13, 14, 15, 16 and 17 weeks for samples storage at 4 °C; or after 0, 3, 4, 5, 6, 7, 8, 10, 12 weeks for samples storage at 7 °C. The counts of *Listeria* were assessed via a spread plating method on AL Agar, specific Rapid Cromogenic Media. Three independent samples of each temperature were analyzed for each analysis

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time. Ten gram of cooked ham were homogenized with buffer peptone water in a stomacher for 60 s. 1 ml and 100 µl of each solution were spread on the specific agar. Plates were incubated 24 h at 37 °C before counting.

2.3.4. Growth potential parameter

In the present challenge test, based on the "EURL Lm Technical Guidance Document for conducting shelf-life studies on *L. monocytogenes* in ready-to-eat foods", the growth potential parameter "δ" has been calculated and used for the different cooked ham samples, in order to classify these products such as:

- When δ > 0.5 log 10 cfu/g, the food is classified into "Ready-to-eat food able to support the growth of *L. monocytogenes* other than those intended for infants and for special medical purposes".
- When δ < 0.5 log 10 cfu/g, the food is classified into "Ready-to-eat food unable to support the growth of *L. monocytogenes* other than those intended for infants and for special medical purposes".

Being $\delta = \log_{10} N - \log_{10} N_0$; where N is the number of cells in the sample after the different times of storage and N_0 is the initial number of cells just after inoculation. This parameter has been calculated for all the analyzed times, and the most unfavorable data should to be used in order to can classify the cooked ham according the previous description.

2.3. Statistical analyses

For microbiological analysis, the results of three slices of each cooked ham were averaged and analyzed for each formulation and sampling day. All means were compared using a one-way analysis of variance (ANOVA) with a 95% confidence level and a Tukey's multiple comparison test for significantly different means (p= 0.05) in SPSS software (vs. 28.0, IBM, Armonk, Armonk, USA).

3. Results

The results obtained in the present challenge test are divided in two sections, non-inoculated samples and inoculated samples.

3.1. Non-inoculated samples

Microbiological analysis of non-inoculated samples was determined for all the cooked ham samples (P1-P6), but only the results obtained for P1 (control sample), without preservatives, are showed in Table 2. The rest cooked ham samples did not show counts in non-inoculated samples for any of the analysis time selected, even the analysis of Listeria by PCR. For this reason, the results for non-inoculated samples from P2 to P6 are not shown.

The counts of all the parameters analyzed at time 0 for all the samples were <10 cfu/g, which indicates a good manufacturing practice used during all the slicing process. Despite of that, a minimal count is normal to have in the sliced samples, which increased during the all study. This increase was higher in samples storage at 7 °C compared to samples storage at 4 °C, in which, the counts at the end of study were < 1 log cycle. No *Listeria* was detected by PCR, with negative result in all cases, this suggest a high efficiency of the heat treatment during the production of the cooked ham, an adequate aseptic condition during the slicing process, and that there was no cross contamination of the inoculated samples.

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Table 2. Microbial counts of non-inoculated samples for P1 cooked ham (Negative Control – no preservatives) storage at 4 and 7 °C, initially, in the middle and at the end of study.

Storage T[a]	Analysis Time	PCA[1] (cfu/g)	MRS[2] (cfu/g)	*Listeria*
7	0	<10	<10	Negative / 25 g
	7	2,35E+03	3,16E+02	Negative / 25 g
	12	2,00E+06	2,43E+04	Negative / 25 g
4	0	<10	<10	Negative / 25 g
	9	6,67E+01	3,17E+02	Negative / 25 g
	17	<10	<10	Negative / 25 g

[1] PCA: count of total aerobic count; [2]MRS: count of lactic bacteria.

3.2. Inoculated samples

3.2.1. Microbial growth during storage at different temperatures

Table 3 shows the log cfu/g of *L. innocua* for the six different cooked ham samples at the different temperatures of study. The good results obtained in the non-inoculated samples, regarding the *Listeria* results, indicate that the observed growth in the inoculated samples is due to the inoculation using the bacterial cocktail.

From Table 3 it can be deduced that the initial microbial inoculation in all the samples was the same, there were not significant differences ($p<0.05$) between the total cells of *Listeria* inoculated in samples, except for P2, in which the level was slightly lower. The initial inoculation level is important to can compare the evolution in the same conditions.

Sample P1 (no preservatives) showed a progressive increase in the growth of *Listeria* at both temperatures. The counts of *Listeria* obtained in the different analysis times were significantly higher ($p<0.05$) compared to results of the rest of samples during the all study and reached a level of 7.35 log cycle after 17 weeks at 4 °C and 7.76 log cycles after 12 weeks at 7 °C. The statistical analysis showed that the microbial growth obtained after 12 weeks at 4 °C, was significantly lower ($p<0.05$) than those obtained at 7 °C. This fact confirms the importance to maintain a correct temperature during the shelf-life of foods.

Regarding the results obtained in the rest of samples, the evolution at 4 °C was very similar in all the samples for the different analysis time. Increasing microbial growth of *Listeria* was not detected in any of the cooked ham samples. In case of samples storage at 7 °C, the evolution of microbial growth was similar to those observed at 4 °C for all the different cooked ham products, except in case of P2 (100 ppm Nitrite; 250 ppm ascorbic acid+0,7% PRS DV-5). Microbial counts of *Listeria* in P2 samples were increasing progressively, from 1.89 log cycles at time 0, to 3.38 log cycles after 12 weeks.

A comparison between temperatures has been evaluated for all the samples in the time 12 weeks. Table 3 shows as only P1 (as was explained previously) and P2 showed significant higher data of microbial growth ($p<0.05$) in case of 7 °C compared to 4 °C. However, in case of P3, P4, P5 and P6, samples containing the natural ingredients combinations developed by PROSUR, no significant differences were obtained, therefore, it can be deduced that the used of natural ingredients combinations such as preservatives, can help to maintain the microbial growth of a pathogen, such as *Listeria*, even at 7 °C. These results are important to stablish the maximum shelf-life of a product and the maximum temperature of storage allowed, depending on the type of preservatives used in the elaboration of different types of cooked products.

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Table 3. *Listeria* microbial counts of inoculated samples for the different cooked ham samples (P1-P6) storage at 4 and 7 °C, at different analysis time.

Temperature	Week	P1	P2	P3	P4	P5	P6
4 °C	0	2.04 ± 0.10 [a]	1.89 ± 0,11 [b]	2.28 ± 0.21 [a]	2.24 ± 0.18 [a]	2.24 ± 0.28 [a]	2.57 ± 0.28 [a]
	4	5.69 ± 0.17 [a]	1.68 ± 0.29 [b]	1.77 ± 0.28 [b]	1.71 ± 0.18 [b]	1.74 ± 0.31 [b]	1.93 ± 0.20 [b]
	5	5.05 ± 0.49 [a]	1.53 ± 0.21 [b]	1.74 ± 0.28 [b]	1.80 ± 0.17 [b]	2.03 ± 0.38 [b]	2.26 ± 0.23 [b]
	7	5.60 ± 0.27 [a]	1.90 ± 0.10 [b]	1.82 ± 0.11 [b]	1.84 ± 0.10 [b]	1.96 ± 0.23 [b]	2.11 ± 0.10 [b]
	8	5.96 ± 0.61 [a]	1.72 ± 0.24 [b]	1.92 ± 0.15 [b c]	1.89 ± 0.30 [b c]	2.14 ± 0.30 [b c]	2.36 ± 0.13 [c]
	10	6.11 ± 0.29 [a]	1.75 ± 0.18 [b]	2.08 ± 0.13 [b c]	1.97 ± 0.07 [b c]	2.11 ± 0.15 [b c,]	2.34 ± 0.10 [c]
	12	5.53 ± 0.15 [a, 1]	2.03 ± 0.14 [b, 1]	1.93 ± 0.08 [b, 1]	2.01 ± 0.09 [b, 1]	2.04 ± 0.04 [b c, 1]	2.40 ± 0.22 [c, 1]
	13	5.39 ± 0.09 [a]	1.77 ± 0.07 [c]	1.63 ± 0.13 [c]	1.86 ± 0.07 [c]	2.32 ± 0.02 [b]	2.25 ± 0.25 [b]
	14	6.00 ± 0.38 [a]	1.72 ± 0.16 [b]	1.70 ± 0.12 [b]	1.84 ± 0.13 [b]	2.06 ± 0.21 [b]	2.25 ± 0.25 [c]
	15	7.60 ± 0.83 [a]	1.78 ± 0.27 [b]	1.80 ± 0.21 [b]	1.78 ± 0.18 [b]	2.15 ± 0.14 [b]	2.37 ± 0.28 [b]
	16	7.36 ± 0.70 [a]	1.60 ± 0.35 [b]	1.94 ± 0.42 [b]	1.73 ± 0.45 [b]	1.98 ± 0.20 [b]	2.10 ± 0.15 [b]
	17	7.35 ± 0.57 [a]	1.50 ± 0.32 [b]	1.76 ± 0.33 [b]	1.81 ± 0.20 [b]	2.14 ± 0.25 [b]	2.44 ± 0.11 [b]
7 °C	0	2.04 ± 0,10 [a]	1.89 ± 0,11 [b]	2.28 ± 0.21 [a]	2.24 ± 0.18 [a]	2.24 ± 0.28 [a]	2.57 ± 0.28 [a]
	3	6.48 ± 0.04 [a]	2.17 ± 0.30 [b]	1.93 ± 0.10 [c]	1.93 ± 0.20 [c]	2.29 ± 0.22 [b]	2.46 ± 0.11 [b]
	4	6.64 ± 0.45 [a]	2.10 ± 0.19 [b]	1.85 ± 0.20 [b]	1.94 ± 0.30 [b]	2.01 ± 0.52 [b]	2.16 ± 0.30 [b]
	5	7.03 ± 0.21 [a]	2.23 ± 0.24 [b]	1.98 ± 0.09 [c]	1.92 ± 0.20 [c]	2.28 ± 0.09 [b c]	2.45 ± 0.12 [b]
	7	7.01 ± 0.14 [a]	2.48 ± 0.46 [b]	1.84 ± 0.18 [b]	2.05 ± 0.02 [b]	2.02 ± 0.17 [b]	2.19 ± 0.22 [b]
	8	7.28 ± 0.32 [a]	2.70 ± 0.10 [b]	1.78 ± 0.16 [c]	1.90 ± 0.13 [c]	1.98 ± 0.03 [c]	2.17 ± 0.26 [c]
	9	6.65 ± 0.43 [a]	3.16 ± 0.05 [b]	1.83 ± 0.07 [c]	2.13 ± 0.13 [c]	1.98 ± 0.19 [c]	2.14 ± 0.25 [c]
	10	7.60 ± 0.35 [a]	2.98 ± 0.09 [b]	1.82 ± 0.07 [c]	1.87 ± 0.07 [c]	2.04 ± 0.04 [c d]	2.48 ± 0.10 [d]
	12	7.76 ± 0.19 [a, 2]	3.38 ± 0.31 [b, 2]	1.76 ± 0.13 [c, 1]	1.57 ± 0.18 [c, 1]	1.94 ± 0.47 [c, 1]	2.18 ± 0.22 [c, 1]

Description of cooked ham samples: P1: negative Control – no preservatives; P2: 100 ppm Nitrite + 250 ppm ascorbic acid + 0,7 % PRS DV-5; P3: 1% NATPRE T-10 DV HS + 0.5% PRS-DV-5; P4: 1% NATPRE T-10 DV LS + 0.5% PRS-DV-5 LS - 1.3% NaCl + 0.35-0.40% KCl; P5: 1% NATPRE T-10 EML + 0.5% PRS-DV-5; P6: 1% NATPRE T-10 EML + 0.75% PRS-DV-5. Statistical analysis: letters a-d: Different letters within the same row indicate

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significant differences between samples in the same analysis time ($p < 0.05$); Numbers 1-2: Different numbers within the same column in the 12th week between temperatures ($p < 0.05$).

3.2.2. Growth potential parameter

The growth potential parameter (δ) has been calculated for all the samples and for all the analysis time using the equation $\delta = \log10\ N - \log10\ N_0$ (Table 4). Table 4 indicates the data for each type of cooked ham. After to know the data for any time, the most unfavorable value (and safest for consumers) was selected in order to classify the different products, as indicates the EURL Lm Technical Guidance Document for conducting shelf-life studies on *Listeria monocytogenes* in ready-to-eat foods.

As it can be observed, for any of the temperatures studied, 4 °C and 7°C, the cooked ham products elaborated using any of the natural ingredients combinations developed at PROSUR, allowed to obtain a growth potential parameter lower than 0.5, therefore, all of them, P3, P4, P5 and P6, can be classified as a "Ready-to-eat food **unable to support** the growth of *L. monocytogenes* other than those intended for infants and for special medical purposes". In case of cooked ham elaborated using nitrates and ascorbic acid (P2), the classification depends on the temperature, and in case of control sample (P1), the result is clearly a food able to support the growth of *L. monocytogenes*.

Table 4. Growth potential parameter obtained after different storage time and temperatures for the different cooked ham samples (P1-P6).

Tª	Week	P1	P2	P3	P4	P5	P6
4	4	3,65	-0,22	-0,51	-0,46	-0,50	-0,64
	5	3,00	-0,36	-0,54	-0,37	-0,21	-0,31
	7	3,56	0,01	-0,47	-0,33	-0,28	-0,46
	8	3,92	-0,18	-0,37	-0,28	-0,10	-0,21
	10	4,07	-0,14	-0,21	-0,21	-0,13	-0,23
	12	3,49	0,13	-0,35	-0,16	-0,20	-0,17
	13	3,35	-0,12	-0,66	-0,31	0,08	-0,32
	14	3,96	-0,17	-0,58	-0,33	-0,18	-0,31
	15	5,56	-0,12	-0,49	-0,39	-0,09	-0,20
	16	5,32	-0,30	-0,34	-0,44	-0,26	-0,47
	17	5,31	-0,39	-0,52	-0,36	-0,10	-0,13
	δ maximum	**5,56**	**0,13**	**-0,21**	**-0,16**	**0,08**	**-0,13**
7	3	4,43	0,28	-0,35	-0,24	0,05	-0,11
	4	4,60	0,21	-0,43	-0,23	-0,24	-0,41
	5	4,98	0,33	-0,30	-0,25	0,04	-0,12
	7	4,97	0,59	-0,44	-0,12	-0,22	-0,38
	8	5,23	0,81	-0,50	-0,27	-0,26	-0,40
	9	4,61	1,27	-0,45	-0,04	-0,26	-0,43
	10	5,56	1,09	-0,46	-0,30	-0,20	-0,09
	12	5,72	1,48	-0,52	-0,60	-0,30	-0,39
	δ maximum	**5,72**	**1,48**	**-0,21**	**-0,04**	**0,08**	**-0,09**

Description of cooked ham samples: P1: negative Control – no preservatives; P2: 100 ppm Nitrite + 250 ppm ascorbic acid + 0,7 % PRS DV-5; P3: 1% NATPRE T-10 DV HS + 0.5% PRS-DV-5; P4: 1% NATPRE T-10 DV LS + 0.5% PRS-DV-5 LS - 1.3% NaCl + 0.35-0.40% KCl; P5: 1% NATPRE T-10 EML+ 0.5% PRS-DV-5; P6: 1% NATPRE T-10 EML + 0.75% PRS-DV-5.

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4. Discussion

According to the results obtained for the different cooked ham samples, the antimicrobial properties of the natural ingredients combinations used, developed by PROSUR, has been demonstrated their capacity to inhibit the microbial growth of *Listeria*. As previously has been described, the natural ingredients are combinations of extracts from citrus, spices such as coriander and pepper, and also dehydrated vinegar. Essential oils from spices have been studied such as antimicrobial ingredients previously. Lastra-Vargas et al. (2023) [17] which studied the antimicrobial capacity of oregano essential oil for the control of *Listeria monocytogenes* in Turkey mortadella. The study demonstrated that the oregano essential oil could help to control de growth of *Listeria*, but the study was carried out only for 13 days. The challenge test evaluated in the present study was until 12 or 17 weeks of storage, which indicates a higher capacity to control the growth of *Listeria* in case of natural ingredients combination developed by Prosur.

Regarding the effect of citrus on the microbial growth of *Listeria*, Kanmani and Rhim (2014) [18] investigated grapefruit seed extract such as antimicrobial in packaging film on *Listeria monocytogenes*, *Bacillus cereusand* and *Escherichia coli*. These authors found a distinctive antimicrobial activity against *Listeria monocytogenes,* which suggested that the agar containing the grapefruit seed extract can be used in an active food packaging systems for maintaining food safety and extending the shelf-life of the packaged food. In this case, the final objective was similar to those followed in the present study, to extend shelf-life of food and to have safe product for consumers. Along the same lines of including antimicrobial ingredients in packaging films, Zhao et al. (2019) [19] investigated the effect of including bioactives, gallic acid, chitosan, and carvacrol in packaging films to control competitive microbiota and *L. monocytoges* in ready-to-eat ham products. These authors observed that starch films with gallic acid had the least effect on the antimicrobial activity of ham; however, starch films with chitosan and carvacrol completely inhibited the growth of *L. monocytogenes* during the 4 weeks of storage. The results are in the same line as those obtained in the present study, but it should be noted that the prepared challenge test lasted up to 17 weeks, longer than the time analyzed by Zhao et al. (2019) [19].

Regarding the use of citrus, Saleem [20] studied the antimicrobial properties of extracts obtained from waste fruit peels of orange and yellow lemon. They used six gram negative bacteria: *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Serratia marcescens*, *Escherichia coli*, *Proteus vulgaris*, *Salmonella typhi* and six gram positive bacteria: *Staphylococcus aureus*, *Enterococcus faecalis*, *Aeromonas hydrophila*, *Streptococcus pyogenes*, *Listeria monocytogenes*, and *Lactobacillus casei.* The extracts were obtained with different solvents: methanol, ethyl acetate, ethanol anddistilled water. The highest inhibitions areas were obtained using distilled water such as solvent, 22 and 28 mm of inhibition in case of *L. monocytogenes*, using orange and yellow lemon extract, respectively, measured by agar well diffusion method.

The antimicrobial effect of extracts from citrus, spices and dehydrated vinegar has been investigated by several authors, but in this paper, the antimicrobial effect has been tested using a real challenge test, using real commercial products, developed and investigated at Prosur.

5. Conclusions

The current study has demonstrated that using different combinations of natural ingredients developed at Prosur, such as NATPRE T-10 and PRS-DV-5, in their different options (high solubility, low salt, emulsion), in the formulation of cooked ham, allows that these products can be classified such as Ready-to-eat food unable to support the growth of *L. monocytogenes* other than those intended for infants and for special medical purposes. The use of these natural ingredients combinations offers advantages to consumers and to food industry. Their utilization gives the possibility to obtain safer products, with a longer shelf-life, reducing the food waste and the economic losses. Additionally, using these natural ingredients combinations avoid the use food synthetic antimicrobial ingredients, allowing a clean labelling in the final product.

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