Behaviour of *Escherichia coli* O157:H7 and Listeria Monocyto-Genes in Normal and DFD Beef of an Autochthonous Portuguese Breed

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Article

Title Behaviour of *Escherichia coli* O157:H7 and *Listeria monocytogenes* in Normal and DFD Beef of an Autochthonous Portuguese Breed

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Abstract: This study was carried out to identify the behaviour of *Escherichia coli* O157:H7 and of *Listeria monocytogenes* inoculated in Maronesa breed beef with different ultimate pH (pHu) (Normal and DFD), and stored at two different temperatures (4 and 9 ºC), during 28 days *post mortem (pm)*. The main objective was to illustrate the problematic feature of dealing with beef showing high pHu and stored at mild abusive temperatures (9 ºC). Beef steaks (ms. *longissimus dorsi*) were inoculated with low levels (2-3 log CFU/g) of those both pathogens and packed in air, vacuum and three gaseous mixtures with decreasing O_2 and increasing CO_2 concentrations (MAP70/20, MAP50/40 and MAP30/60). At 4 ºC, the growth of *E. coli* O157:H7 presented the same pattern on Normal and DFD meat. On contrary, the growth of *L. monocytogenes* was higher on DFD meat, revealing the effect of the pHu and its psychotropic character. At abusive temperature, both pathogens grew, achieving high levels on DFD meat. In these cases, the MAP with the highest CO_2 concentration (60%) revealed to be more effective against the development of *E. coli* O157:H7, therefore, not exceeding levels of 5 log CFU/g at the end of storage, while in *L. monocytogenes* it reaches 8 log CFU/g under the same condictions.

Keywords: autochthonous breed; beef; DFD meat; *Listeria monocytogenes*; *Escherichia coli* O157:H7; ultimate pH

1. Introduction

Beef from the Maronesa cattle has high commercial value due to its autochthonous breed, environmentally sustainable production system and it has Protected Designation of Origin **(**PDO) [1,2]. However, in order to exploit the full potential of meat products it is important to ensure that products reach the consumer in the best condition [3]. The quality of the meat can be affected by intrinsic and extrinsic parameters, such as age, sex, breed [4], feeding [5], enzyme activity [6], meat modified atmospheres packaging (MAP) [7], or meat contamination [8]. The hygienic quality, which is a result of beef processing and handling, is particularly important because it has direct implications on the consumer's confidence [9]. Contamination of beef occurs throughout slaughtering, deboning, cutting, packaging, and storage, during which, time, temperature and hygiene are important parameters affecting the shelf-life of fresh meat for industrial use or retail [10,11].

Microbial pathogens are usually associated with the most serious meat safety issues in product recalls and foodborne disease [12–14]. Nowadays, besides the considerable knowledge about the microbiota responsible for foodborne diseases, a high number of outbreaks and incidents keep occurring, many of them associated with meat and meat products' which in 2021 represented 11.9%

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of positive units reported by MS of EU [15]. Beef, in particular, has been associated with foodborne diseases, causing tragic outcomes, involving death in some cases. An aiding fact of foodborne infections due to beef consumption may be related to its preparation methods, in which these meats are many times only subjected to mild heat treatments, commonly referred to as undercooked or rare [16]. Enterohemorrhagic *E. coli* O157:H7 is a highly pathogenic subset of Shiga toxin-producing *E. coli* [17]. *E. coli* belongs to the enteric microflora of many healthy animals being cattle the main reservoirs of *E. coli* O157:H7 [18,19]. *E. coli* O157:H7 has emerged as an important foodborne pathogen that causes diarrhea, hemorrhagic colitis, and hemolytic-uremic syndrome in humans [17] and, in severe cases, can cause death [20]. On the other hand, *L. monocytogenes* has an ubiquitous distribution and a great ability to grow in a wide range of conditions such as refrigeration [21,22] and has been regarded as the pathogen of concern in ready-to-eat (RTE) meat [23]. *L. monocytogenes* is also one of the most serious agent of foodborne diseases under EU surveillance and, infection in humans still high, some of them with death [15]. Unsafe practices including high storage durations and abusive temperatures have a potential impact on the human listeriosis risk [24]. The temperature of domestic refrigerators was shown to be very variable through a review of survey studies from 1991 to 2016. It was observed that mean temperatures ranged from <5 to 8.1°C, the minimum temperatures from -7.9 to 3.8°C and maximum from 11.4 to 20.7°C [24].

The gaseous composition of the packaging and pHu values are parameters that can affect the growth of microorganisms in meat [25,26]. In red meat products, one of the most common methods of packaging is the use of MAP, usually through mixtures of CO_2, O_2 and N_2, each one with a specific function. The MAP generally inhibits microbial spoilage of fresh meat, minimizing the loss of products and maintaining a higher quality in perishable food during its normal shelf-life or extending it [27]. Nevertheless, MAP should be associated with strict temperature control to achieve maximum microbial inhibition [11,28]. The pHu of meat higher than the normal values (pHu: 5.5-5.8), as in dark, firm and dry (DFD) (pHu: >6.1) or moderate DFD (pHu: 5.9-6.1) meat, can be responsible for the meat spoilage [29,30]. The production system of Maronesa beef is known for the occurrence of DFD condition [32], consequently reducing the product's shelf-life therefore less acceptable to the consumer [32]. According to Silva el al. [32], during two years of measurements of pHu, it was noticed that in *longissimus dorsi* about 25% of the cases had a final pH equal or superior to 6.2 and were classified as DFD. Thus, the results revealed that the occurrence of high pH meat is dependent on the muscle. Other muscles as *gracillis* (8%) and *psoas major* (≤2%) muscles presented less percentage of DFD condition. DFD meat is related with animal stress and transport with multifactorial origin and has been reported in many countries, with variable occurrence rates of 1.3% in Canada, 3.2% in USA, 4.5% in Brazil or 13.45% in Mexico [29,33–35]. The incidence of DFD cases was also dependent on the sex of the animal, with the higher occurrence of DFD cases observed in males [32].

Considering the lack of studies related to the quality of autochthonous breeds meat associated with the occurrence of DFD beef in Maronesa breed, the aim of this study was to evaluate the influence of pHu (Normal and High) and meat packaging (air, vacuum and three MAP with gas) on the behaviour of *E. coli* O157:H7 and *L. monocytogenes* inoculated on beef of Maronesa breed and stored at 4±0.5ºC and 9±0.5ºC, during 28 days of storage.

2. Materials and Methods

2.1. Experimental Design

2.1.1. Sampling

Beef *longissimus dorsi* muscle was obtained from eight Portuguese autochthonous Maronesa, 9 to 11 month old bulls whose carcass weighed from 90 to 150 kg. *Longissimus dorsi* was excised from the carcasses between the 6th thoracic and the 2th lumbar vertebra at 24h *post mortem* (*pm*). Based on pHu measured at 24h *pm* directly in the muscle using a combined glass electrode with a pH-meter Crison 2002, muscles were divided into two pH groups: Normal (pH≤5.8, n=4) and DFD (pH≥6.2, n=4). After that, muscles were cut into pieces of approximately 200g, packed in vacuum and stored

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at -80 °C until the beginning of the experiment. In the day 1 of the experiment, cuts of muscles were kept at 2ºC during 2h. After this time, approximately 1 cm of external surface of meat was aseptically removed and cuts were sliced. At the end, pieces of meat (0.5 cm thick, surface 2x2.5cm, ≈5g) were obtained. Immediately after this preparation, meat samples were analysed (24h *pm)*, for meat characterisation and discarding *L. monocytoge*nes and *E. coli* O157:H7 contamination, prior to inoculation. Experiments were performed using four animals in each experimental unit per each pH group and a control sample was prepared in all conditions of the experimental design.

2.1.2. Microorganisms and Growth Conditions

Pieces of meat were inoculated with 20 µl of a suspension of *E. coli* O157:H7 (NCTC 12900) and *L. monocytogenes* (ATCC 7973), for an inoculation level of 2-3 log (CFU/g) per strain and package.

Inoculates used in this study were prepared at growth suspension of *L. monocytogenes* (30ºC, 24h) and *E. coli* O157:H7 (37ºC, 24h) in brain heart infusion broth (Oxoid CM225). Cells were centrifugated (5000xg, 15 min, 4ºC) and washed three times in 0.85% sterile physiologic saline and compared to a 0.5 McFarland turbidity standard. Serial (10-fold) dilutions were performed to yield, approximately $1x10^3$ cells/cm^2. To verify the number of viable *L. monocytogenes* and *E. coli* O157:H7 in the suspension, dilutions were spread on Compass *L. monocytogenes* Agar (Biokar BM06508) and CT-SMAC (Biokar BK147+BS037), respectively.

2.1.3. Packaging

Inoculated and control samples were packed in five different types of packaging namely: air; vacuum; 70%O_2/20%CO_2/10%N_2 (MAP70/20); 50%O_2/40%CO_2/10%N_2 (MAP50/40); and 30%O_2/60%CO_2/10%N_2 (MAP30/60).

In air packaging, meat pieces were tray-packaged in air overwrapped with polyetilen film and in vacuum they were individually vacuum packaged in COMBITHERM bags (WIPAK Walsrode, HAFRI) which have an oxygen transmission rate (OTR) of 63 cm^3 $m^{-2}d^{-1}atm^{-1}$ at 23ºC, 0% RH and water vapor transmission (WVT) of 1g $m^{-2}d^{-1}$ at 23ºC, 85% RH. For MAP, pieces of meat were individually placed in COMBITHERM XX bags (WIPAK Walsrode, HAFRI) 0.115 mm thick and OTR of 1 cm^3 $m^{-2}d^{-1}atm^{-1}$ at 23ºC, 0% RH and WVT of 1g $m^{-2}d^{-1}$ at 23ºC, 85% RH. The atmosphere in the MAP was first removed and then flushed with the appropriate gas mixture (Praxair, Portugal) using a SAMMIC V-420 SGA. The final ratio between gas and meat was approximately of 3:1.

Samples were stored at 4±0.5ºC and 9±0.5ºC and examined for microbiological counts on days 1 (2h after packaging), 3, 7, 10, 14 *pm*. For air packaging, microbiological counts were not carried out at 14 days *pm*. On the contrary, on vacuum packaging microbiological counts were also carried out at 21 and 28 days *pm*.

2.2. Microbial Analysis

Meat pieces were aseptically collected at each interval. Samples were homogenized with sterile buffered peptone water for 90s in a Stomacher (IUL, Barcelona, Spain). Serial decimal dilutions were prepared in the same solution and were plated on CT-SMAC (Biokar BK147+BS037) for *E. coli* O157:H7 counts (37ºC for 24h) and Compass L. mono agar (Biokar BM06508) in case of *L. monocytogenes* (37ºC for 24-48h). After incubation, typical colonies were counted and results were expressed in log CFU/g.

2.3. Data Analysis

One-way analysis of variance (ANOVA) was conducted to test the effect of pHu (Normal and DFD) and of temperature of storage (4±0.5ºC and 9±0.5ºC), for each day of microbiological counts (1, 3, 7, 10, 14, 21, 28 days *pm*) using the Systat programme 10.2 (Systat Software Inc., 2002) at 5% level of probability.

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3. Results

3.1. Behaviour of Escherichia coli O157:H7

The results of counts of *E. coli* O157:H7 in inoculated beef samples, according to pHu (Normal and DFD) and type of packaging for each storage temperature are presented in Table 1. Two hours after inoculation the Normal and DFD samples had a similar count for *E. coli* O157:H7. These counts were slightly greater than the programmed level of inoculation, though this was a very slight excess, never attained a logarithmic unit. The final pH of the meat was not decisive on the growth of *E. coli* O157:H7 in beef stored at 4±0.5°C. The highest *E. coli* O157:H7 counts for DFD meat and temperature of 9°C were observed in vacuum packaging for all storage times (4.39±0.65 log CFU/g at 3 days *pm*; 6.57±0.26 log CFU/g at 7 days *pm*; 7.77±0.32 log CFU/g at 10 days *pm* and 8.35±0.21 log CFU/g at 21 days *pm*), however no significant differences were observed between Normal and DFD pHu at 3 days *pm*. At 10 days *pm* the largest difference between the DFD and Normal meat were observed on vacuum atmosphere (4.05 log CFU/g; P <0.001) followed by air atmosphere (3.61 log CFU/g; P <0.01). In the MAP70/20, differences between the DFD and Normal meat reached 3.04 log CFU/g (P <0.001). The MAP packaging with more concentration of CO_2, namely MAP30/60 showed the lowest counts with no significant differences between pH groups at 10 and 14 days *pm*.

Table 1. Counts of *E. coli* O157:H7 (log CFU/g) in inoculated beef samples according to pH groups (Normal and DFD), in five different packaging and stored at two temperatures (4±0.5ºC and 9±0.5ºC), at day 1, 3, 7, 10, 14, 21 and 28 *pm*.

Days	Temp.	Air			Vacuum			MAP70/20			MAP50/40			MAP30/60		
pm	(ºC)	N**	DFD	*Sig.*	N	DFD	*Sig.*	N	DFD	*Sig.*	N	DFD	*Sig.*	N	DFD	*Sig.*
1	**A.I.***	3.35±0.26	3.41±0.32	ns	3.15±0.52	3.31±0.37	ns	3.30±0.20	3.32±0.25	ns	3.36±0.37	3.33±0.37	ns	3.33±0.29	3.37±0.33	ns
	4±0.5	3.23±0.78	3.20±0.42	ns	3.48±0.27	3.30±0.33	ns	3.60±0.18	3.23±0.27	ns	3.39±0.46	3.25±0.27	ns	3.34±0.26	3.33±0.26	ns
3	**9±0.5**	3.55±0.45	4.16±1.15	ns	3.51±0.48	4.39±0.65	ns	3.35±0.43	3.84±0.29	ns	3.58±0.27	3.88±0.56	ns	3.36±0.27	3.56±0.30	ns
	Sig.	ns	ns		ns	*		ns	*		ns	ns		ns	ns	
	4±0.5	3.33±0.21	3.68±0.76	ns	3.28±0.29	3.04±0.30	ns	3.51±0.26	3.18±0.21	ns	3.28±0.19	2.99±0.37	ns	3.28±0.16	2.84±0.23	*
7	**9±0.5**	3.43±0.73	6.31±1.26	**	3.36±0.42	6.57±0.26	***	3.24±0.30	5.61±0.12	***	3.27±0.35	5.30±0.49	***	3.20±0.35	4.52±0.78	*
	Sig.	ns	*		ns	***		ns	***		ns	***		ns	**	
	4±0.5	3.36±0.25	3.09±0.60	ns	3.28±0.36	2.95±0.43	ns	3.27±0.24	3.17±0.42	ns	3.23±0.19	2.98±0.51	ns	3.19±0.26	2.97±0.34	ns
10	**9±0.5**	3.44±0.92	7.05±1.42	**	3.72±0.76	7.77±0.32	***	3.26±0.39	6.30±0.52	***	3.24±0.36	6.25±0.37	***	3.39±0.42	4.65±1.22	ns
	Sig.	ns	**		ns	***		ns	***		ns	***		ns	*	
	4±0.5	-	-	-	3.65±0.12	2.66±0.54	*	3.27±0.40	3.50±0.62	ns	2.97±0.42	3.04±0.37	ns	3.12±0.40	3.19±0.39	ns
14	**9±0.5**	-	-	-	3.67±0.56	7.84±0.15	***	3.24±0.54	6.59±0.77	***	3.31±0.33	6.65±0.38	***	3.28±0.33	4.90±1.48	ns
	Sig.	-	-	-	ns	***		ns	***		ns	***		ns	ns	
	4±0.5	-	-	-	3.17±0.16	2.60±0.60	ns	-	-	-	-	-	-	-	-	-
21	**9±0.5**	-	-	-	3.74±0.68	8.35±0.21	***	-	-	-	-	-	-	-	-	-
	Sig.	-	-	-	ns	***		-	-	-	-	-	-	-	-	-
	4±0.5	-	-	-	2.91±0.22	2.81±0.28	ns	-	-	-	-	-	-	-	-	-
28	**9±0.5**	-	-	-	3.61±0.59	8.76±0.64	***	-	-	-	-	-	-	-	-	-
	Sig.	-	-	-	ns	***		-	-	-	-	-	-	-	-	-

* A.I. – after inoculation; **N – Normal (pH); Sig. – Significance: ns - not significant; * p <0.05, **p<0.01 and ***p <0.001.

The counts of *E. coli* O157:H7 on Normal and DFD beef were similar at temperature of 4 ºC during all the storage period, maintaining the initial levels. However, at 9 ºC were achieved growth levels for DFD meat of ~8 log CFU/g in vacuum from the day 10 *pm*, reaching at day 28 *pm* the highest value of 8.76±0.64 log CFU/g. In the MAP with the high CO_2 concentration (MAP30/60) counts were not higher than 5 log CFU/g, which appears to reveal the inhibitory effect of CO_2 in the development of *E. coli* O157:H7 (Figure 1). The pathogen *E. coli* O157:H7 packed in MAP70/40 and MAP30/60 and stored at mild abusive temperature (9ºC) followed the same pattern and showed similar counts over time.

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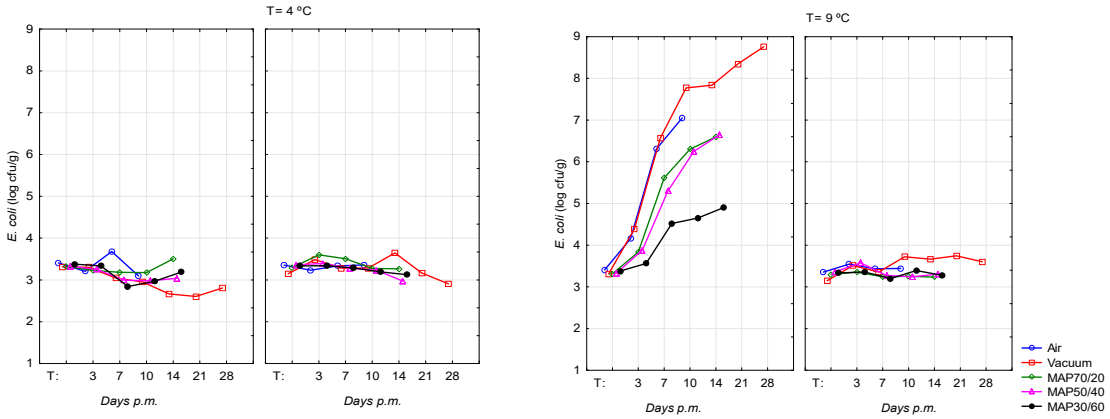

Figure 1. Evolution of *E. coli* O157:H7 levels (log UFC/g) on DFD (**left**) and Normal (**right**) beef stored at 4ºC and at 9ºC, packed in air, vacuum and MAPs -O_2/CO_2/N_2- 70/20/10 (MAP70/20), 50/40/10 (MAP50/40), 30/60/10 (MAP30/60), during time of storage.

3.2. Behaviour of Listeria monocytogenes

The results of counts of *L. monocytogenes* in inoculated beef samples, according to pHu (Normal and DFD) and type of packaging for each storage temperature are presented in Table 2. At 1 day *pm*, in the five packaging, DFD and Normal samples had a similar count for *L. monocytogenes* with a score closer to that desired in all packaging. Two more days after storage, the consequences of temperature started to show that *L. monocytogenes* increased considerably in DFD compared to Normal meat and, these differences, were more pronounced particularly in MAP70/20 compared to the other two MAP. *L. monocytogenes* achieved at 3 days *pm* and 7 days *pm* average counts of 3.41±0.92 log CFU/g and 6.49±0.83 log CFU/g in DFD meat and 2.61±0.65 log CFU/g and 1.74±0.76 log CFU/g in Normal meat which generally maintained the same pattern during the remaining storage time.

In Normal pH, the effect of abusive storage temperature was not noticed in the development of *L. monocytogenes*, contrarily to DFD meat which presented very significant differences in most situations in which the meat was in abusive temperature.

The MAP30/60 with the higher content of CO_2 seems to inhibit a little more this microorganism, showing inferior counts for all storage times when compared with MAP70/20 and MAP50/40.

Table 2. Counts of *L. monocytogenes* (log CFU/g) in inoculated beef samples according to pH groups (Normal and DFD), in five different packaging and stored at two temperatures (4±0.5ºC and 9±0.5ºC), at day 1, 3, 7, 10, 14, 21 and 28 *pm*.

Days	Temp.	Air			Vacuum			MAP70/20			MAP50/40			MAP30/60		
pm	(ºC)	N**	DFD	*Sig.*	N	DFD	*Sig.*	N	DFD	*Sig.*	N	DFD	*Sig.*	N	DFD	*Sig.*
1	**A.I.***	3.10±0.88	3.02±1.12	ns	2.90±0.64	3.13±0.81	ns	2.96±0.94	3.02±0.75	ns	2.91±0.96	3.06±0.82	ns	2..46±1.07	2.97±0.99	ns
	4±0.5	2.78±1.19	2.91±1.15	ns	2.80±1.21	3.20±0.86	ns	2.64±0.96	2.64±0.89	ns	2.56±0.89	2.60±0.98	ns	2.34±1.29	2.55±1.21	ns
3	**9±0.5**	2.81±0.93	3.39±1.05	ns	2.95±1.04	3.74±1.17	ns	2.61±0.65	3.41±0.92	ns	2.61±0.79	3.17±1.00	ns	2.74±0.66	3.19±0.92	ns
	Sig.	ns	ns		ns	ns		ns	ns		ns	ns		ns	ns	
	4±0.5	2.51±0.90	3.76±0.90	ns	2.71±0.92	3.86±0.92	ns	2.21±0.93	3.06±0.69	ns	2.08±0.87	2.92±0.81	ns	2.25±0.66	2.64±0.99	ns
7	**9±0.5**	1.83±0.67	7.25±0.68	***	2.68±0.83	7.11±0.61	***	1.74±0.76	6.49±0.83	***	1.79±0.91	6.17±0.91	***	1.99±0.77	5.55±0.62	***
	Sig.	ns	***		ns	**		ns	***		ns	**		ns	**	
	4±0.5	2.48±0.62	4.47±0.91	*	2.55±1.10	4.55±0.90	*	1.59±0.88	3.89±0.90	*	1.63±1.08	3.21±0.79	ns	1.49±0.85	2.63±0.98	ns
10	**9±0.5**	2.07±0.75	8.25±0.45	***	2.54±0.81	8.24±0.20	***	1.69±1.01	7.91±0.57	***	1.37±0.74	7.79±0.20	***	1.42±0.72	7.01±0.37	***
	Sig.	ns	***		ns	***		ns	***		ns	***		ns	***	
	4±0.5	-	-	-	2.04±1.04	5.71±0.81	**	1.35±0.79	4.82±1.05	**	1.32±0.72	4.07±1.29	**	1.68±1.15	3.33±1.27	ns
14	**9±0.5**	-	-	-	2.66±0.53	8.33±0.13	***	2.50±0.78	8.55±0.32	***	2.54±0.74	8.53±0.30	***	2.45±0.75	7.91±1.09	***
	Sig.	-	-	-	ns	***		ns	***		ns	***		ns	**	
21	**4±0.5**	-	-	-	2.08±0.64	6.78±0.69	***	-	-	-	-	-	-	-	-	-
	9±0.5	-	-	-	2.23±0.72	8.29±0.28	***	-	-	-	-	-	-	-	-	-

	Sig.	-	-	-	ns	**	-	-	-	-	-	-	-	-
	4±0.5	-	-	-	1.71±1.20	7.57±0.41	***	-	-	-	-	-	-	-	-
28	**9±0.5**	-	-	-	1.86±0.69	8.14±0.41	***	-	-	-	-	-	-	-	-
	Sig.	-	-	-	ns	ns		-	-	-	-	-	-	-	-

* A.I. – after inoculation; **N – Normal (pH); Sig. – Significance: ns - not significant; * p <0.05, **p<0.01 and ***p <0.001.

At temperature of storage of 4ºC, the growth of *L. monocytogenes* was not observed for Normal meat. On DFD meat, the counts achieved levels of 6 log CFU/g, 7 log CFU/g and 8 log CFU/g in vacuum at day 14 *pm*, 21 *pm* and 28 *pm*, respectively. The lowest counts were obtained on meat packaged in the MAP30/60 along time.

At abusive temperature (9ºC) and Normal pHu *L. monocytogenes* presented similar counts during the storage period. On contrary, for DFD meat *L. monocytogenes* developed very fast after day 3 *pm* achieving values higher than 8 log CFU/g in air and vacuum at day 10 *pm*. These levels were attained in MAPs later at day 14 *pm*.

4. Discussion

The pHu and storage temperature influenced significantly the growth of both *E. coli* O157:H7 and *L. monocytogenes.*

At 4ºC of storage, the counts of *L. monocytogenes* for all packages in DFD beef were below 5 log CFU/g (10 day *pm*) with the higher counts observed in air (4.47 log CFU/g; *P* <0.05) and vacuum (4.55 log CFU/g; *P* <0.05) packages (Table 2.). On contrary, at 4ºC the counts of *E. coli* H157:H7 were about 3 log CFU/g during all times of storage (3, 7, 10, 14, 21 and 28 days *pm*) in all types of packaging showing no significant differences (Table 1). Thus, the low storage temperature (4ºC) associated to the DFD condition was not enough to produce an extensive inhibition of *L. monocytogenes* as observed in *E. coli* O157:H7. These results can be justified by the fact that *L. monocytogenes* being a psychrotrophic bacterium, capable of survive and multiply at low temperatures, both under aerobic and anaerobic conditions, and adhere to various surfaces [36]. Moreover, *L. monocytogenes* has the ability of grow at pH of 6 or higher, as observed in DFD meat [37] and has a high tolerance to low pH and high salt concentration [38]. According to Nissen et al. [39] at 4ºC, *L. monocytogenes* and *Y. enterocolitica* are considered the most serious pathogens in meat. Low temperatures induce enzymes such as RNA helicase which improves the activity of *L. monocytogenes*, as well as replication at low temperatures. Moreover, the capacity to produce biofilms enhances *L. monocytogenes* ability to survive harsh environments and also use flagella at lower temperatures which enables the ability to propel itself [42]. Elevated CO_2 and reduced O_2 levels are commonly used to extend shelf-life of food products through inhibition of microbial growth and oxidative changes [42]. Use of O_2-free atmospheres in packages has been suggested for different meat products [42]. In the present study, MAPs inhibited, compared to the air and vacuum atmospheres, the development of *L. monocytogenes* mainly in the MAP with the highest concentration of CO_2. The lower OTR (1.0 $cm^3m^{-2}day^{-1}$) of the packaging film used in this experiment for MAP, in comparison to films of greater O_2 permeability (OTR=4.5 $cm^3m^2day^{-1}$) used in the study conducted by Tsigarida et al. [43] can justify these results. Saraiva et al. [41] using the same packaging film of the present study found that *L. monocytogenes* in beef may be reduced by ~1.0 log in vacuum packaging and by ~1.5 log on average in the MAPs. Generally, under anaerobic modified atmosphere the LAB compete with the support microflora and have shown to be effective in inhibiting the growth of pathogenic bacteria such as *L. monocytogenes* in meat products [42]. The combination of selected LAB strains with antimicrobial compounds, for instance, acid and sodium lactate or the use of active packaging could be the next step strategy for eliminating risk of *L. monocytogenes* in meat and dairy-ripened products [42]. Many food-spoiling LAB are facultative aerobic and quite resistant to CO_2, this contributes to the fact that LAB can be found as main spoilers on high oxygen packaged meat [41]. Therefore, *L. monocytogenes* can represent a risk when stored at temperatures higher than 0°C and the efficacy of thermal treatments is limited by the ability to survive and actively replicate at temperatures between -0.4 and 45ºC [42]. Air and

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vacuum atmospheres had similar counts for this pathogen (Figure 2) which could be related to the O_2 permeability rate of the packaging film used for vacuum atmosphere (OTR=63cm^{3}m^{-2}day^{-1}), not so efficient in inhibiting the *L. monocytogenes* [41].

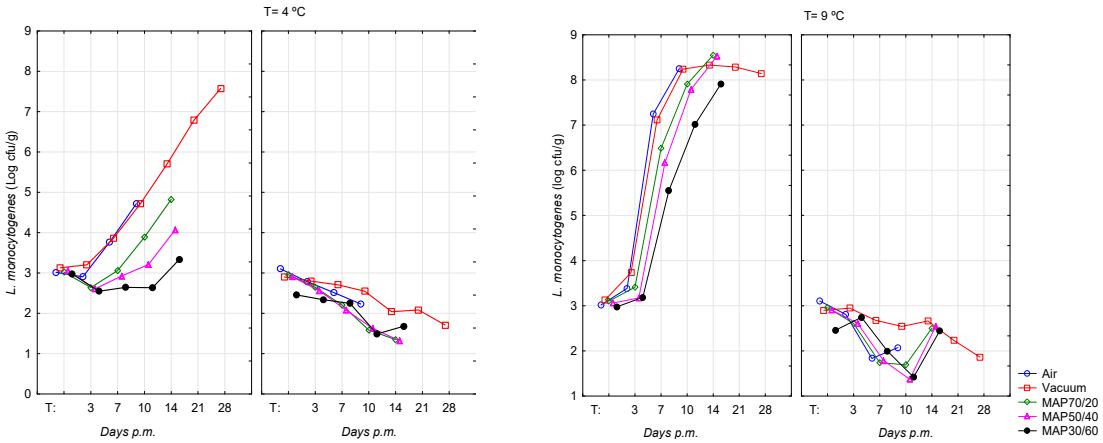

Figure 2. Evolution of *L. monocytogenes* levels (log UFC/g) on DFD (**left**) and Normal (**right**) beef Scheme 4. C and at 9ºC, packed in air, vacuum and MAPs -O_2/CO_2/N_2- 70/20/10 (MAP70/20), 50/40/10 (MAP50/40), 30/60/10 (MAP30/60), during time of storage.

A higher proportion of CO_2 in MAP50/40 and even more in MAP30/60 have led to a lower growth of *L. monocytogenes* compared to other types of packaging. This result is consistent with other researches which results confirmed that the growth of *L. monocytogenes* was inhibited with increasing concentrations of CO_2 [42]. According to Saraiva et al. [41] *L. monocytogenes* requires CO_2 levels of 40% *v/v* or higher for an effective inhibition. In view to maintain the cytoplasmic pH within a range that is consistent with growth and survival the decarboxylation reaction need to occur helping maintain the cytoplasmic pH, though high concentration of CO_2 can inhibit the decarboxylation reaction by which CO_2 is released through feedback mechanisms [42].

At temperature of storage of 4ºC, the counts of *E. coli* O157:H7 on Normal and DFD, as well, for abusive temperature on Normal beef, were similar during all the storage period, maintaining the initial levels (Figure 1). Regarding DFD meat and temperature of storage of 9ºC, the *E. coli* O157:H7 achieved the highest growth levels in vacuum atmosphere (~8 log CFU/g), showing statist differences from Normal meat ($P< 0.001$) and from temperature of storage of 4ºC ($P< 0.001$) at 7, 10, 14, 21 and 28 days *pm*.

The pathogen *E. coli* O157:H7 in air packaging multiplied rapidly; however, MAP30/60 showed the lower growth levels during all the storage period. In this study, this was the atmosphere with high CO_2 content (60%) and *E. coli* O157:H7 counts do not attained 5 log CFU/g at 14 days *pm*. These results can be due to the inhibitory effect of CO_2. In previous experiments, *E. coli* O157:H7 in atmospheres with high concentrations of CO_2 (ratio of 30%CO_2:70% O_2 or 0.4%CO:60%CO_2:39.6%N_2) have been reported as being inhibitory to the multiplication *of E. coli* O157:H7 at an abusive storage temperature of 10°C [39]. As mention above for *L. monocytogenes*, *E. coli* uses the same mechanism of decarboxylation systems to protect the cell from a precipitous drop in pH. These systems depend on the activity of cytoplasmic pyridoxal-5′-phosphate (PLP)-containing amino acid decarboxylases which consume one proton and release one CO_2 for every molecule of substrate amino acid, thus helping maintain the cytoplasmic pH [42]. At refrigeration temperatures from 0 to 2°C, high concentrations of CO_2 (ratio of 20%CO_2:80%O_2 or 0.4%CO:30%CO_2:69.6%N_2) can led to a reduction of one logarithmic unit in the levels from *E. coli* O157:H7 [50]. In accordance with the present study Nissen el al. [39] reported that *E. coli* O157:H7 could be inhibited at 10 ºC at high CO_2 concentration and pHu inferior of 6. Moreover, even in MAP with high CO_2 and low CO mixture meat acquires a stable color and the shelf-life can be extended.

The behaviour of *L. monocytogenes* in abusive temperatures differed between DFD and Normal pHu meats, for all types of packaging after 7 days *pm*. For instance, at 10 day *pm*, Normal and DFD

meat showed, respectively counts of 2.07 log CFU/g and 8.25 log CFU/g in air ($P< 0.001$); 2.54 log CFU/g and 8.24 log CFU/g ($P< 0.001$) on Vacuum; 1.69 log CFU/g and 7.91 log CFU/g in MAP70/20 ($P< 0.001$); 1.37 log CFU/g and 7.79 log CFU/g in MAP50/40 ($P< 0.001$); and 1.42 log CFU/g and 7.01 log CFU/g in MAP30/60 ($P< 0.001$). However, regarding the stored temperatures, for instance at 14 day *pm*, count obtained at 4ºC and 9ºC of storage for DFD meat were respectively 5.71 log CFU/g and 8.33 log CFU/g ($P<0.001$) in Vacuum; 4.82 log CFU/g and 8.55 log CFU/g in MAP70/20 ($P<0.001$); and 4.07 log CFU/g and 8.53 log CFU/g in MAP50/40 ($P< 0.001$). Thus, even with meat stored at temperatures within recommended limits, the development of *L. monocytogenes* occurred markedly in meat with a high pHu, though when comparing both temperatures, the effect was not so intense as was the effect of pHu. However, unlike *E. coli* O157:H7 where the effect of pH was not felt when storage was carried out at the appropriate temperature, also in this pathogen, at 9±0.5ºC of storage; a significantly greater growth was noted in the DFD meat samples.

5. Conclusions

The present study highlights the importance of maintaining the cold chain under strict surveillance conditions is confirmed to avoid abuses, which can have consequences, even more serious in the case of DFD meat. As referred, the occurrence of DFD condition was more frequently observed in *L. dorsi* muscle of Maronesa breed, mainly in males. The results revealed that this condition associated to abusive storage temperatures allowed the growth of *E. coli* O157:H7 at higher levels, specially more evident in air and vacuum packages. For *L. monocytogenes* the low storage temperature (4ºC) associated to the DFD condition was not enough to inhibit the growth of *L. monocytogenes* as observed in *E. coli* O157:H7. This is due to the character psychotropic of *L. monocytogenes*.

The use of special packing conditions such as vacuum or MAP in order to increase the shelf-life of meat, or for technological and sensorial reasons, can have consequences in terms of development of some of the pathogens present in meat. Overall, the MAP were the most effective in controlling the development of *E. coli* O157:H7 and *L. monocytogenes*. In MAP, the effect of the CO_2 level was observed, being even more noticeable in pH that better supported microbial growth.

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