Monitoring of *Listeria spp* and *L. monocytogenes* in the deep-frozen vegetable industry

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Abstract

Listeriosis is a foodborne illness, caused by the pathogen *Listeria monocytogenes*, with a mortality rate around 30%. An outbreak with origin in frozen vegetables imposed an update of the HACCP of *Listeria spp*. and *monocytogenes* in vegetable processing industries. The latter tended to ensure food safety and quality. To control contamination occurrence, equipment was swabbed and product samples were taken. About 50% of the sanitation sessions left *Listeria spp*. in the production lines. EQP07 probability of contamination after sanitation rounds 70%, the highest verified. EQP01 and EQP18 results showed that the procedure applied is somehow efficient. It permitted to obtain *Listeria* free lines regularly by introducing mechanical action and an acid disinfectant in the procedures, exception made for EQP20. Nonetheless, *Listeria spp*. was constantly detected in the lines during production. EQP01 was the only equipment free of contamination in one of the lines but, at the same time, its swab results were 100% positive for the other. Nevertheless, products leaving those lines were generally *Listeria* free and even when *Listeria monocytogenes* was detected its count was always <10 CFU/g. Foodstuffs with higher calcium content showed higher incidence of contamination. Thus, sanitizers with EDTA could be considered to mitigate the problem.

Listeriosis

Modern society impels a rhythm in people's lives that is pushing food industry to increasingly provide easy to prepare and ready-to-eat products. In the last few years, the concern for healthy life styles is restricting produce treatments, and frozen crops industry can answer to these demands. Thus, dealing with perishable food products and environmental contaminants increases the challenge faced by food industry to guarantee the standards of food safety and quality.

Listeriosis is a foodborne gastric illness, mostly affecting individuals in risk groups, as pregnant or immunosuppressed. As the consequence of a *Listeria monocytogenes* foodborne outbreak with origin in frozen vegetables, which led to a few illnesses and even deaths, products and industrial environments *Listeria spp.* free are now a food quality requisite.

Listeria is a small, Gram-positive, non-spore-forming, rod-shaped bacteria [1]. It is aerobic and facultatively anaerobic, catalase-positive except for a few rare strains, oxidase negative and hydrolyses esculin [2]. *Listeria spp*. is taken as an indicator of *L. monocytogenes* presence, so HACCP (Hazard Analysis and Critical Control Point) demands regular sampling of the machinery and produce, to assure this contaminant is absent in the transformation areas or under 10 CFU/g when present.

Eliminating *Listeria spp*. from the production surroundings is extremely hard since *Listeria spp*. is considered an environmental contaminant. *Listeria monocytogenes***Erro! A origem da referência não foi encontrada.** can be found on visually clean surfaces but it is most frequently found at wet and soiled places where the bacterium is able to grow and persist. Thus, one should follow the evolution of emergence during production along the line, to understand if the duration of the batches is allowing the microorganisms to proliferate and contact with the produce still during transformation. While the factory is running becomes difficult to preclude the microorganism spreading since water is a pleasant environment to *Listeria spp*. development.

Finally, the goal of the two previous points is to ensure the innocuity of the crops that reach to the consumer. Hence, *Listeria spp*. and *L. monocytogenes* incidence in the products is systematically monitored. To eliminate produce contamination, one should study *Listeria spp*. capacity to adapt to its surroundings using whatever strategies are available, like nutrients and pH variation. This knowledge may work as an indicator of options to disinfect not only the produce, but also production lines while working.

Materials and Methods

Hygiene plan

The composition of the chemical products used in the sanitation procedures (Table 1) allows one to understand their functionality.

Production lines

ROWϕ: The product leaves the blancher into a vibrating plate, which allows it to follow its right path. When cutting is needed, it leaves the blancher to a cutter machine and only afterwards proceeds to the vibrator. From here, it follows to a water separator apparatus, to decrease the amount of water that follows with the product through the line. The product follows to the tunnel belt, where it will run the freezing tunnel and be deep-frozen. It will exit the tunnel by the tunnel exit belt and fall into the grid of the air cleaner. From here, it reaches the Genius™ belt, where it will be selected and rejected in case it presents any imperfections. When needed, it is sorted by size in a calibrator, and finally falls in the hoppers that lead it to the octabins where the product stays, while preserved in the cold chamber. Only equipment that will intervene in the following batch are tested since not all of them were used whenever the line is producing. Thus, testing procedures may exclude a few of the equipment, depending on the production period.

ROWλ: The short path of this line is identical to ROWϕ. Fragile products, after leaving the freezing

tunnel, were sent to a recirculation belt, perpendicular to the line's route, leading it to the glazing line. The glazer pours a fine layer of cold water (<0.5% w/w) over the just frost product, and a conveyor leads it back to the entrance of the freezing tunnel, at the blancher room. A belt conduces the product into a vibrating plate that will spread it over a second belt that runs inside the freezing tunnel. When exits the tunnel, drops to the exit tunnel belt, is selected by the Genius™, and a conveyor leads it to an upper level, passing in calibrating drums. Every size-range fraction follows its sub-line into the respective hopper and to be stored in octabins.

Gross residues are removed with shovels or by hand. Residues covering the line are removed with pressurized water, at 25bar. Once residues are completely removed, alkaline detergent CHE01 is applied as a 3% concentrated foam, acting for 15-30 minutes. Detergent is rinsed and the neutral disinfectant CHE02 is applied as a 1% concentrated foam, acting for 15-30 minutes, and then rinsed.

The blancher performs 3 cycles of CIP (Clean in Place), first with water, second with alkaline solution CHE06 and finally with acid solution CHE07. Exteriorly, CHE04 is applied as a 3% concentrated foam, between CHE01 and CHE02 applications to remove calcification and rust deposits, acting for 15-30 minutes, and rinsed.

Packing lines

The product arrives in the packing area already frozen in octabins, contacts with the line for short periods of time, being released in a bunker. From here falls in a vibrating plate that spreads the crops through a short selection belt. Two of the lines have human verification and selection of the product, as the other two have electronic selection. In one line the product is selected by the Genius™ and in the other by the Sortex®, and then verified by an X-Ray machine. From here falls in scales where is weighed and bagged immediately.

Residues are removed with pressurised water. CHE01 is applied as a 3% concentrated foam, acting for 15-30 minutes, and then rinsed. CHE08 is applied as a 3% concentrated foam, acting for 15-30 minutes and then rinsed to remove greases from the line when fried or grilled crops are processed. If the latter does not work, disinfectant CHE02 is applied immediately after the detergent, acting for 15-30 minutes and, at last, rinsed with unpressurised water.

Transwab: MW570 LISTERIA ISOLATION TRANSWAB® is a test, ISO 9001 certified, and can be applied anywhere where the presence of *Listeria spp.* would be critical. This Transwab works on an enhanced esculin media formulation. The hydrolysis of esculin results in a dark/black product distinguishable from the fresh medium. Inhibitors are present to obstruct the growth of microorganisms not included in the genus *Listeria spp.*

The swab should rub a surface of about 100cm^2, when practicable. The culture is incubated at 37^{o}C for up to 48 hours. The moister level on the sampled surface may influence the results, whereby wet surfaces may be more suitable to obtain accurate results.

Table 1- Chemical products used on sanitation procedures: description and composition

Description	Main Components
CHE01 Detergent pH 13 14	NaOH 2.5 – 5.0 %
	NaClO 2.5 – 5.0 %
CHE02 Disinfectant pH 7.5 – 8.0	$C_{18}H_{41}N_3$ 3.0 – 5.0 %
	CH_3COOH 1.0 – 2.5 %
	Alcohols 1.0 – 2.5 %
CHE03 Disinfectant pH 1.0 – 1.5	CH_3COOH 10 – 25 %
	H_2O_2 5.0 – 8.0 %
CHE04 Detergent pH 1.5 – 2.0	H_3PO_4 30 – 50 %
CHE05 Disinfectant pH 6 - 9	VOC 59.4 %
CHE06 CIP solution pH 13 - 14	NaOH 35 – 50 %
CHE07 CIP solution pH ≈1	HNO_3 30 – 50 %
	H_3PO_4 30 – 50 %
CHE08 Degreaser pH 13 - 14	$C_6H_{14}O_2$ 10 – 20 %
	$(CH_3)_2C_6H_3SO_3Na$ 5.0-10%
	KOH 2.5 – 5.0 %

MW570 LISTERIA ISOLATION TRANSWAB® was tested against a battery of organisms[3], *Listeria spp.* and non-*Listeria spp.*, to define the detection range and the accuracy of the growth medium. On the one hand, no change was detected when organisms like *Staphylococcus aureus*, *Bacillus subtilis*, *Escherichia coli*, *Salmonella typhimurium*, *Pseudomonas aeruginosa*, among other pathogens, were tested, even with initial sample concentrations over 10^6 CFU. On the other hand, the medium changed to black when tested to several *Listeria* species, at different microorganisms' concentrations.

Biotox: 200/300 g of final product is sterile collected every 24 hours, per line, and stored at -20^{o}C

Listeria spp. presence is verified in 25g of produce, in VIDAS medium, according to the standard AFNOR BIO 12/2 – 06/94, through ELISA method

The presence of *L. monocytogenes* is accounted through the detection method permissible by the standard AFNOR BRD 07/16 – 01/19

The colony count method that follows ISO-11290-2 is applied when one or both methods confirm the pathogen presence in the sample, returning results in CFU/g

Listeria spp. environment adaption: ALOA agar plates were prepared according to the *Listeria* agar acc. OTTAVIANI and AGOSTI (VWR) indications.

To isolate a single strain of *Listeria spp.*, a positive Transwab from the "cleanest" site of the factory was sampled, and plated. A detached colony was plated in a fresh plate 48 hours later. To decrease the results variability dependent on the adaptive characteristics that different strains would have, isolates of colonies of this same sample were used through all the following experiments.

To study the ability of *Listeria spp.* to take advantage on the available calcium in the food matrices and its pH resistance, this ALOA basic medium was altered accordingly.

4 mL of a $CaCl_2$ solution with concentrations of 2.0, 3.0, 5.0, 7.0 and 9.0 g/L were added to 100 mL of basic ALOA medium to obtain growth media with 100, 150, 250, 350 and 450 ppm of Ca^{2+}. 0.5, 1.0, 1.5, 2.0, 3.0 and 4.0 mL of 1 M HCl were added to 100 mL of basic ALOA medium to obtain growth media with pH 6.5, 6.0, 5.5, 5.0, 4.5 and 4.0, respectively

Modified *Listeria* Enrichment Broth was prepared, adding 3.8 g of Tryptic Soy Broth (Liofilchem, Italy), and 0.75 g of Yeast Extract (OXOID, England), to 125 mL of deionized water, where a loop full of the isolated strain was inoculated

Successive dilutions of that were plated. Duplicates were incubated for 48 hours at 37ºC. Duplicates were kept at 4ºC for 120 hours and then incubated at 37ºC for 48 hours

Results and Discussion

To evaluate the presence and incidence of *Listeria spp.* on the production and packing lines, four zones of contact were established. Zone 1 was defined as the equipment that contacts directly with the product, immediately after hygienic actions, downstream the blancher. Zone 2 was defined as the infrastructures of those same equipment, during production. Zone 3 corresponds to the factory infrastructures, not contacting directly with the product, then influencing the factory environment inoquity. Finally, Zone 4 represents the surroundings of the factory, like offices, bathrooms or the cantina, that affect the operators' cleanliness and will later influence the remaining zones.

Encrypted areas as the tunnel exit, the preparation room, the feedstock area, the freezing tunnel and the blancher room are named as ROOXX. ROWX is then the designation of specific transformation or packing lines. The code EQPXX includes transformation apparatuses and pieces of equipment. Among these are belts, the blancher, vibrator plates, receiving platforms, calibrators, cutters, the Genius™, the tunnel freezing belt, drums, blades and another specimen of this kind. Infrastructures as ceilings, walls, floors and sewages are codified as INFXX. The utensils and accessories as boots, uniforms and spreaders are grouped in the UTIXX cluster. Produce transformed or packed in the factory, as well as raw material in general, as tomato, capsicum, onion, peas, broad beans, zucchini, and aubergine were coded as PROXX. The stage of transformation of produce, as glazed, blanched, washed, frozen or decontaminated, are signed as STAXX. Last, the chemical products used to sanitize the factory are encoded by CHEXX.

Swab results were grouped in two production periods. The first period contains the study of historical information collected by the company between June 2016 and February 2017. The second set covers the swab results collected between April 2017 and August 2017. Along the second period hygiene interventions were attended. Different cleaning systems were executed with implementation of mechanical action and stronger acidic disinfectants were applied more frequently after training was provided to operators and site managers.

Sanitation efficiency against *Listeria spp*.

Positive results represent high risk of contamination, which may compromise the entire production batch run afterwards. Positive results also mean that inefficient or inadequate sanitation procedures were being applied. One works with the purpose to obtain exclusively negative swab results, or, at least, improve general results obtaining less positive results while changes and progresses are sited.

First testing period

Only 50% of the registered hygiene sessions in the ROWϕ showed the equipment completely free of *Listeria spp*. during the first swabbing period. For the same period, 58% of ROWλ cleaning sessions resulted in a line free of *Listeria spp.* Despite being a lower number than intended, it suggests that when the actual cleaning plan was applied thoroughly, it was possible to obtain satisfying hygiene results. This also indicates that one or more apparatuses were left dirty when the line was washed, which may be resulting in contamination sites.

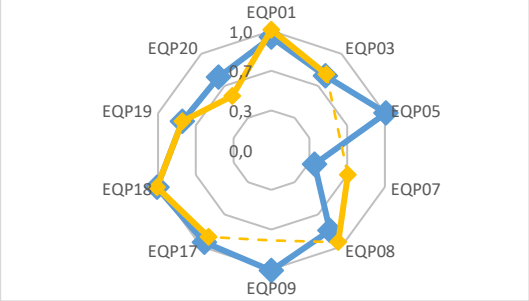

Figure 1 - Swab relative results for Zone 1, in the first period of testing, between June 2016 and February 2017. Yellow line represents ROWϕ and blue line represents ROWλ negative swab results.

Equipment EQP18, as shown in Figure 1, was the only one free of *Listeria spp*. 100% of the times that was swabbed immediately after hygiene procedures were performed for both production lines. EQP18 receives the product from ROO04 already STA02, and transports it through the line. It is not a complex equipment and, despite being plastic and extremely articulated, was replaced relatively recently and its surface was widely exposed, which attributes should contribute to its easy cleanse.

EQP01 receives the product STA02 that falls into its vibrating EQP10 allowing the product to dissociate in small pieces when clumps formed during freezing. The engine STA03 at a certain flow rate, allowing the product to spread before preceding to the next equipment. Zone 1 of this gear was usually swabbed at its EQP10 or at the surface of its vibrating EQP13, where the product contacts directly with. However, the engines should be tested too from now on, once the STA03 may contaminate the product when starts functioning. Considering the slightly worse results obtained for ROWλ, where 6% of the swabs resulted in positive presence of *Listeria spp*., residues were probably not being properly removed from EQP10 part of EQP01. Some products processed in this

production line leave a significant amount of minute residues that get neglected when washing leading to a contaminated equipment.

EQP08 swabs present significantly better results for ROWϕ, for which only 6% positive *Listeria spp.* results were obtained, while ROWλ showed 3 times this value. This equipment is by the end of the line in ROO05 and receives the product STA02. Usually its EQP02 was the swabbed compartment, being that it carries the product along the line and contacts with it for longer than the previous. This is due to its retarding role to allow EQP08 to do the product sorting. This EQP02 is a plain plastic continuous apparatus, completely exposed to the washer except where it merged with the EQP21. Hence, this may be the EQP08 compartment that was compromising its cleaning. The disparity between lines, is probably since ROWλ has more gears to wash in this area. Nonetheless, the responsible operator had the same means to proceed. Thus, the cleaning procedure might have been compromised.

EQP17 presented only 5% and 11% positive swab results for ROWλ and ROWϕ, respectively. This was, from all the results, the most surprising for being so low because this equipment was extremely long, very articulated and composed exclusively of metallic rings, so, punctured from edge to edge. This allowed residues to stick to its irregularities and to accumulate in its structure. Another considerable disadvantage of this equipment was that it was only possible to start its cleaning once ROO04 had already completely defrost. The previously referred operation takes usually about 2 hours, depending on how long it had been working non-stop. Usually, two unspecialized operators are allocated here to remove residues, using water exclusively, for over 5 hours. The engine that moved EQP17 was continuously running, enabling the washers to reach the totally of its surface. However, due to its nature, residues got trapped in the reverse of the EQP17 once its surface was being washed.

Listeria spp. was absent immediately after sanitation 78% of the times EQP03 and EQP19 were swabbed (76% in EQP03 for ROWλ). Both these apparatuses were allocated at ROO01, an area that presented a higher average temperature. This was also an extremely moist space, where water was vaporized while the line was running. This, for itself, gave microorganisms better conditions to proliferate. Despite this, EQP19 was a plain stainless steel EQP13 completely exposed, without wrinkles and dumps. Thus, both residues removal as well as chemical application were extremely easy. Consequently, immediately after hygienic implemented procedures, it should be *Listeria spp.* free. On the one hand, EQP19 was the first gear that contacts with the product once it was STA01, and if it was contaminated, it may compromise the entire production batch. On the other hand, EQP03 was a big, hollow, irregular equipment, that had a punctured plastic EQP02 continuously circulating, that was exposed to the exterior only in some fractions of EQP03. The extremity of this equipment was a plastic fall, so overworked that scratches were visible in its full length. This set of factors makes this an extremely difficult to wash apparatus. Hence, it was not surprising that the absence of *Listeria spp.* is far from total. The fact that EQP03 purpose is to STA04 the product is what turns this result unexpected and even more unacceptable. A minor presence of microorganisms should be present in EQP03 by the end of the batch production, otherwise its operation may be compromised. If bacteria are still present after completion of hygiene procedures, it means that those procedures were inefficient. Moreover, that before those hygiene procedures, bacteria were already present in the gear. As a result, this contaminated equipment was responsible for the STA04 of the product at the start of the run. Being unable to fight a contamination, the product will most probably end the run also contaminated.

Both EQP17 and EQP20 are critically located, receiving the product immediately after STA01 but before inflowing ROO04. This means that the inefficient cleaning verified in both these apparatuses may be compromising the line downstream. EQP20 is either swabbed in its EQP13 or EQP10 and surprisingly the results are usually as bad in its plane side. The EQP10 is detachable, but extremely heavy and thus not always removed when washing. Worse results were expected to ROWλ since it is bigger and heavier than its similar. However, the residues that were trapped in EQP10 have higher influence in its contamination, once none of the EQP10 was scrubbed. Meanwhile, only 56% of the swabbing results is negative for ROWϕ, while 75% was observed for ROWλ, contradicting the expectations. The inaccessibility to one of ROWϕ flanks may be the reason to this.

Last, the unacceptable results obtained for EQP07 in ROWλ may have to do with the fact that it was used, and so swabbed, only for half of the first period testing time, since not all the equipment is necessary according to the product that is being transformed. Even, 62% of the times EQP07 was swabbed after cleaning presented *Listeria spp*. contamination. The probability of contamination was almost half for the other line, where 67% of the swabs were negative in ROWϕ. This means that its EQP23 trap residues and allow the creation of an environment where bacteria proliferate, and EQP23 are extremely difficult to wash since it must be caged for operators' safety.

Second testing period

While the hygiene procedure was equivalent to the one in the first period of testing, 55% of the swabs were negative for *Listeria spp*. presence immediately after sanitation, for both lines. In average, this shows that the procedures were taken with the same thorough and had the same efficiency. It is though important to refer that this period of testing is significantly shorter than the previous, and that the products that run in the lines were of different nature. Following the tendency for good results after sanitation, EQP01, EQP08 and EQP18 were correctly washed and absent of *Listeria spp*. immediately after hygiene procedures during this period.

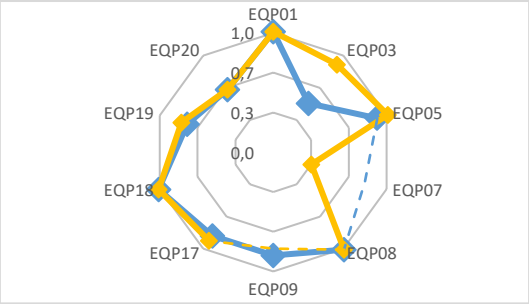

Figure 2 - Swab relative results for Zone 1, in the second period of testing, between April 2017 and June 2017. Yellow line represents ROWϕ and blue line represents ROWλ negative swab results.

EQP08 improvement may be related to the products run in the lines that leave different kinds of residues, being easier removed with water ejection, than the previous.

EQP05 tendency was maintained. The hygiene procedures were 100% efficient in ROWϕ, while 91% of the times eliminated *Listeria spp*. in ROWλ. EQP05 in ROWϕ are composed of EQP10 that receives the product with the help of an EQP19, while in the other line EQP05 consists of large moving EQP22. As a result, these gears have the same function but have different features. Considering the equipment design features, washing ROWϕ's EQP05 was easier, and so better results were expectable and justified.

EQP17 sanitation did not seem to be related to the product processed in the line, since it presented, in average, the same results as in the first period. Presenting only 9% positive results for ROWϕ and 14% for ROWλ, the cleaning procedure applied so far may have reached its maximum efficiency for EQP17. Due to its nature, this equipment should be disinfected with stronger chemicals. Residues can get trapped in its interior and it is completely unviable to disassemble it during production.

The emergence increased in positive results on EQP03, especially in ROWλ where it showed 50% contamination probability after sanitation. This equipment for itself should be the less contaminated, due to its purpose. During PRO05 and PRO06 production, especially while anti-foam products were not allowed, the line got covered in a brown clayish protein that was extremely difficult to remove. Inside of the EQP03, probably due to the high temperatures it was submitted to, this substance was strongly adhered to all the inner surfaces of the equipment, and even deposited on the bottom as an extremely dense mud like residue. Even after CIP cycles, acid application, and scrubbing with sodium hypochlorite solutions, there were still deposits that almost certainly contributed to these unwanted results. There is also the possibility that the scraper of the EQP02 of EQP03 and even the EQP02 itself are getting damaged with time, and biofilms are forming in its scratches and roughness.

EQP07 was not used during this second period in ROWλ, but its sanitation was as compromised for ROWϕ during this period as it was for the other line during the first period. Only 33% of the testing revealed the equipment was free of *Listeria* after hygiene procedures. It was mentioned above that EQP07 is unreachable during sanitation, so chemical alternatives should be considered to clean it more efficiently.

EQP09 shows a slight worsening in hygiene results. Only 86% of the results were negative. Considering the abovementioned description, these results may have occurred due to neglected hygiene sessions that followed long batches and thus with enhanced the microbiological growth that allowed the development of strongly adhered biofilms.

Apparatuses EQP19 and EQP20 kept their average results. EQP19 was *Listeria spp.* free immediately after sanitation 80% and 75% of the times for ROWϕ and ROWλ, respectively, while EQP20 was correctly sanitized 64% of the times. Concerning the EQP20, it was possible that biofilms were forming here, unable the elimination of this contamination focus at least if disinfectant is not applied together with mechanical action. The equipment is made of smooth stainless material which misleads the operators who assume it is easy to clean. Perhaps, as the EQP19 that precedes it. In fact, there is a chance that the problem of this equipment is its structure. During production, a considerable quantity of residues got trapped immediately under the grids of EQP20. Since the temperature in this area is almost constant during production and the environment is extremely humid, it would not take long for biofilms to form here. *Listeria spp.* forms strong biofilms and have the best conditions at this particular point.

Coaching period

To understand if the current hygiene plan could be optimized without major alterations, all the operators and site managers somehow involved in sanitation procedures received specialized training in eliminating *Listeria spp.* during the washing processes. The importance of sustaining the adequate actuation time of the chemical products, to guarantee its efficiency was emphasized, as well as mechanical action application while detergent is working. The concept of biofilms and microorganisms were introduced to the working teams, so that annihilation of these contamination focuses would start to be a real concern. Until this point, the factory working force was exclusively concern in eliminating visible residues.

To establish a starting point, the main EQP02 and structures were disassembled, CHE03 and CHE04 were applied thoroughly along the line, and both the interior and the exterior structures of the apparatuses were scrubbed and brushed, to eliminate all the residues and break the biofilms covering the equipment. Then, at this point, every site that was tested was confirmed both *Listeria spp.* and total coliforms free.

These coaching sessions occurred mid-June 2017 and from this point on to the end of the second period, scrubbing and acid disinfectant application were permanent instead of periodical, which revealed

results much closer to the ideals than before, presented below (Figure 3).

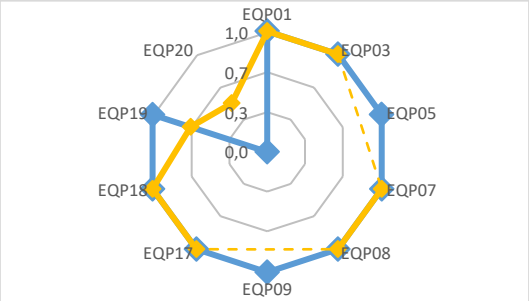

Figure 3 - Swab relative results for Zone 1, in the third period of testing, between June 2017 and August 2017. Yellow line represents ROWϕ and blue line represents ROWλ negative swab results.

Successive minor alterations were needed for a long way in improving hygiene outcomes. It was possible to eliminate and keep the lines almost free of contamination during a period of intense production. EQP05 of ROWϕ was not used during this period and consequently not tested, while EQP09 is exclusive to ROWλ, therefore there are no data relatively to these two points in the yellow line**.**

The EQP20 is still the major problem in ROWϕ, being *Listeria spp.* free only 50% of the times it was tested immediately after sanitation. As previously referred, this equipment consists in a plain smooth EQP13, followed by an EQP10 that permits water to fall and the product to proceed in the production line, succeeding to the EQP17. There is still no explanation for these unwanted results. However, the formation of strong biofilms is still the most plausible one. EQP19 was still one of the most problematic pieces of equipment. Nonetheless, the team could clean it properly about 67% of the times.

Considering ROWλ, one must keep in mind that this line is longer, upstream and downstream the EQP03. Hence, the cleaning team needs more time and to do a greater effort to wash and hygiene this line properly. Still, the improvement of results was as accentuated as in the other line. EQP20 was always contaminated. Thus, the hygiene procedure as to be differently adapted to this gear. Furthermore, it was the only piece of equipment that stayed contaminated after the training sessions and thus, while sanitation was perform carefully.

***Listeria spp*. incidence in the product**

Both lines worked in batch and ideally the runs stopped when cleaning was needed or when product feedstock was unavailable. Breakdowns along the line or any damaged piece during production also led to the line stoppage. Nevertheless, usually this did not allow the sanitation of the complete line, because urgent works are priority. When fresh product was available in reasonable quantities, the procedure was continuous for a 3-day periods (72 hours) in average. However, while excessive product was contained as raw-material, the run takes longer to stop, being the longest batch registered during the second period of testing was of 12 shifts, that is, approximately 5 complete days or about 120 hours.

The product was tested for *Listeria spp*. and *L. monocytogenes* independently on hygiene procedures applied on the line. Once a day (every three shifts), a sample was taken per line. When required, for example when baby food is produced, or when the receiving country legislation obliges so, a sample was taken in every producer's lot, being that the line did not stop between loads.

The amount of processed product varied with its characteristics, being that in average 50 tons of PRO03 were processed per shift, and so a sample of about 200g was taken for every 150 tons.

The verification of both *Listeria spp*. and *L. monocytogenes* in the product was under the responsibility of an external certified laboratory, according to the method described above. To understand how the final product was being compromised by *Listeria spp*. emergence, results will not be associated with the line of production from now on.

First testing period

Products showing higher incidence of *Listeria spp*., independently of contamination verified in the line, were PRO10, PRO06, PRO01 and PRO02, with positive results of about 29%, 25% and 23% for the last two, of the collected samples, respectively.

The remaining products leave the production site normally contaminated more than 6% of the times. Samples of PRO05 were *Listeria spp*. positive 17% of the times, as PRO04 only 7%. The least probable to be contaminated products are PRO09 and PRO07 with 5% and PRO03 4% of positive results. *Listeria spp*. contamination in the product increased significantly in the second testing period, to 23% in average. PRO01 and PRO10 incidence of *Listeria spp*. augmented to 47% and 57%, keeping these two products in the top of the most probable to be contaminated.

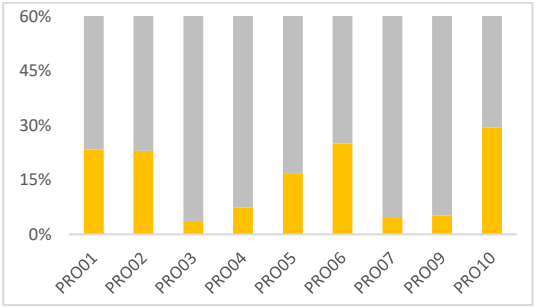

Figure 4 – *Listeria spp*. incidence in the product, during the first period of testing. Grey bars represent absence of contamination and yellow bars represent *Listeria spp*. including *L. monocytogenes* presence.

Second testing period

The increasing of *Listeria spp*. presence in PRO04 was vast, being that odds were that 50% of the samples are contaminated.

Lower than average, but still expressively more contaminated than before, were the lasting products. From the collected samples, 7% of the PRO05, 12% of the PRO07, 13% of the PRO03 and PRO06 and more than 15% PRO02 showed *Listeria spp*. presence.

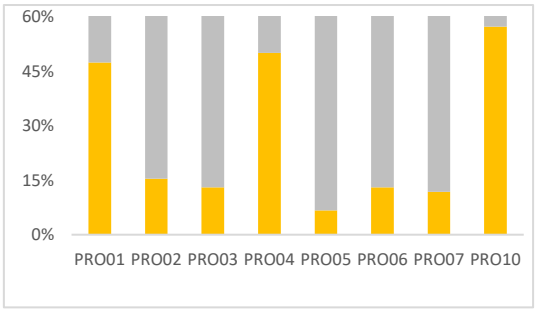

Figure 5 – *Listeria spp*. incidence in the product, during the second period of testing. Grey bars represent absence of contamination and yellow bars represent *Listeria spp*. including *L. monocytogenes* presence.

Listeria monocytogenes incidence in the product

Based on the Biotox results, it was possible to relate *L. monocytogenes* incidence with *Listeria spp*. occurrence in product samples (Figure 6). *Listeria spp*. presence may specify a product as of inferior quality or contaminated by filth, but *Listeria monocytogenes* indicates that that same product represents a risk to the consumer security. Thus, positive results are scrutinized to understand if this pathogen was somehow compromising produce.

PRO01 and PRO10 were not only the ones presenting more contamination, but also the ones carrying *L. monocytogenes* more often among the contaminated. In the first testing period, almost 86% of the contaminated samples of PRO01, were positive for *L. monocytogenes* presence, while in the second period this potentially decreased to 35%. Count of *L. monocytogenes* was possible in 50% of the contaminated samples of PRO10, for both testing periods. Hence, the probability of pathogenicity decreased in PRO01, but remained constant in PRO10.

PRO02 and PRO03 were *L. monocytogenes* positive 29% and 20% of the times tested in the first period. However, in the second period no *L. monocytogenes* was observed in these products. Contrariwise, PRO06 and PRO07 were *L. monocytogenes* free in the first period, but left the production line compromised 33% and 50% of the times, respectively, during the second period. One should notice, even though *L. monocytogenes* presence should be alarming, none of the results showed counts higher or equal to 10 CFU/g of sample.

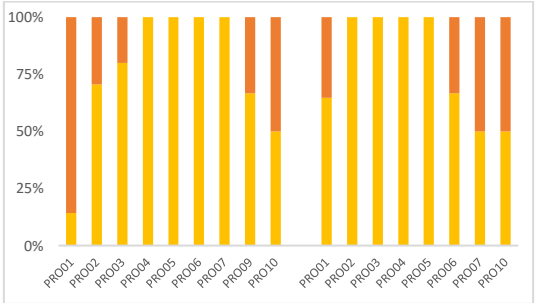

Figure 6 – Confrontation of *Listeria spp*. and *L. monocytogenes* in the product, in all the emergences of the two tested periods. From the contamination validation, orange bars represent *Listeria spp*. identification, while red bars represent *L. monocytogenes* identification.

Obliteration of *Listeria spp*.

Introducing mechanical action as brushing and scrubbing to break or weaken biofilms may be a crucial step in hygiene, conducting to results as the close to ideal as achieved during the coaching period, since biofilms of *Listeria* have been shown to be more resistant to stress and to sanitizing agents than planktonic cells. It has been shown that *L. monocytogenes* has the ability to form biofilms on abiotic surfaces [4] and that nutritionally poor media favours the growth of *L. monocytogenes* in biofilm state [4]. Even though this pathogen does not form thick biofilms, they presented a firm and highly organized structures, with cells grown under diverse physical conditions, which can offer selective advantage for bacterial survival under suboptimal circumstances [4]. Biofilms formed by *L. monocytogenes* also have the capacity to resist to sanitizing agents [5], or develop that resistance when exposed repeatedly to the same sanitizers [5]. More, there are many different types of organisms in commercial food processing facilities that can form biofilms with *L. monocytogenes* [5].

Then, to eliminate *Listeria spp*. from the lines, CHE02 should be replaced by CHE03 in the disinfecting step. Peroxides have been reported to be effective for the removal of bacterial biofilms and are widely used in the food industry [5]. Furthermore, the pH of CHE03 is low enough to inactive *Listeria spp*. [6] guarantying better results. Despite being more aggressive to the equipment, it will be more efficient has a sanitizer. One should also consider that the resistance of biofilms to a sanitizer is greater on Teflon and on polyester substrates than on stainless steel substrate [5]. Thus, replacing some of the main plastic equipment with stainless still substitutes may permit to annul more effectively the pathogens, without compromising the longevity of the equipment.

Based on the results obtained for packing lines, one should also routinely sanitize the surroundings systematically with care, while the line is being sanitized, to achieve a cleaner result and decrease the chances of cross contaminating the line.

To follow the evolution of results one should consider adopt quantitative analysis in the equipment. Particularly because problematic equipment like EQP20 may be contaminated by its surroundings, and one would be able to locate the contamination focus when searching under more precise examination.

Efficient sanitation procedures will diminish *Listeria spp*. presence in the factory, but will not be a permanent solution to product contamination. From the moment the run starts, the line will be in contact with produce, water and operators, and so “Zone 2” testing will continue to present large signs of contaminations. Thus, disinfecting the line while running and the products are necessary steps. Answers to eliminate this problem must present solutions that may touch the food products without compromising its quality neither its consumer health. It is more disadvantageous to test the products, rather than the transformation lines, since positive results in product give no information on the mode of

contamination or how to prevent further occurrences [7]. To obtain accurate feedback on procedures efficacy, hard to reach places such as holes or crevices in fibrous, porous, rusting and hollow materials, poorly cleanable equipment should be sampled [8]. Keeping continuous track of the lines is also important, since parallel lines located a few feet apart may present different testing results.

Unblanched produce have been washed with chlorinated water, that has little antimicrobial effect and is known to react with organic matter subsequently creating carcinogenic halogenated by-products [9] thus alternatives are highly suggested.

Natural compounds with phenolic groups have proved to have inhibitory activity against Gram-positive bacteria [10] and could work as a disinfectant for the foodstuffs. For example, citron essential oil doubled the time needed for the wild microflora to reach concentrations able to produce a perceivable spoilage showed a strong inhibition against *L. monocytogenes* [10].

Organic acids are considered GRAS for use as food ingredients and have a potential application as sanitizers for organic fresh produce. Solutions of 1% and 2% of acetic, lactic and citric acids exhibit significant antibacterial effects and, despite *Listeria monocytogenes* capacity to resist in low pH ambiences, acid treatment was somehow effective [9]. Changes in sample colour subjected to organic acids treatment were not significant during storage [9]. Hence, direct application in the foodstuff would not compromise its quality.

Hydrogen peroxide solutions work has effective disinfectants in foodstuffs, as 5% solutions provide a significant reduction in microbial loads [11] and possible residues are non-hazardous to consumers. A more diluted solution of 0.5% is enough to decrease pathogenic population to below detectable levels [12]. Peroxyacetic acid is one of the main components of CHE03 used to sanitize the line. Furthermore, it has been studied as a solution to disinfect foodstuffs, mixed with hydrogen peroxide, aiming to kill *Listeria monocytogenes* [13].

Extracts based on ethanol have shown to be efficient has sterilizers against *L. monocytogenes* [14][15][16], and reach yields of microbial inhibition of 100% when used in 60mg/L solutions [15]. Furthermore, the use of bacteriocin-producing lactic acid bacteria or purified bacteriocins has been received bigger attention. Bacteriocins are small bacterial peptides that have shown antimicrobial activity against closely related bacteria. Nisin is a GRAS polypeptide produced by *Lactococcus lactis spp*. particularly effective against heat-resistant bacteria, as *L. monocytogenes* case [10]. Pore formation by nisin molecules is thought to be responsible for its bactericidal action. Those pores exist for a matter of milliseconds but increasing the membrane fluidity or reducing the hydrophobic reactions between phospholipid acyl chains may encourage the formation of nisin induced pores and perhaps increase their duration, debilitating the pathogenic cells [16]. The sensitivity of *L. monocytogenes* to nisin is however enhanced by the presence of ethanol. When ethanol was used in conjunction with nisin a greater drop in the number of viable cells was seen for different concentrations of nisin in the solution [16]. Its bactericidal activity increases at pH levels below 5, due to its greater solubility at low pH, which makes nisin suitable for use on vegetables with pH levels ranged between 3 and 6 [17].

Another option to enhance nisin effectiveness is using it in conjunction with EDTA [10], particularly to fight *Listeria*. Its usefulness ascends of its capacity as a chelating agent, turning ions as Ca^{2+} and Fe^{2+} unavailable [18]. Considering the relation established between *Listeria spp*. growth and available [Ca^{2+}] (data not shown) and that PRO01 presents the higher contamination occurrences, during its production a solution containing nisin and EDTA could be applied to sterilize the line and the product, not compromising its quality, and decreasing product corruption.

Conclusion and Further Work

Listeriosis in a rare foodborne disease that affects less than 1% of the population, but its mortality rate is close to 30%. Thus, it becomes vital to eliminate its originator, *Listeria monocytogenes*, from food transformation zones.

As an indicator of *L. monocytogenes* presence, *Listeria spp.* presence is sought, testing equipment with swabs, and collecting product samples. HACCP relatively to *Listeria spp*. establishes that the industry should target its contamination levels less than 10 CFU/g, and that crops should be discarded when counts are over 100 CFU/g.

First, the historical incidence of *Listeria spp*. was studied, to find problematic areas and understand which wrong procedures could be adjusted. The factory owns two production lines that are regularly swabbed downstream the blancher.

Immediately after sanitation sessions, several apparatuses that will contact with the product were swabbed to validate its hygiene. On the one hand, the most problematic points were EQP07 of ROWλ with only 38% of negative results, and EQP20 of ROWϕ contaminated 44% of the times after cleaning during the first testing period. On the other hand, those same pieces of equipment stayed among the most contaminated, and EQP03 in ROWλ negative results decreased to 50% during the second testing period.

A slightly modified hygiene plan was established: the lines are washed to remove residues; CHE01 is applied, and mechanical action is applied. The detergent is rinsed, and instead of neutral, an acid disinfectant, CHE03, is applied, and then rinsed. Swab results, in eight out of the ten tested equipment, were 100% free of *Listeria spp*. after the modification of the application of this novel hygiene procedures. In ROWϕ EQP19 and EQP20 were still positive for *Listeria spp*. in 33% and 50% of the times, while in ROWλ the only equipment that was ever contaminated was EQP20, with 100% of positive swab results. EQP20 sanitation should be redesigned in the future, once the actual hygiene procedure is effective against *Listeria spp*.

While the factory is operating (data not shown), equipment structures are swabbed and product samples are collected. Swab results demonstrate that lines frames always have *Listeria spp.* presence. Not surprisingly, since its sanitation was one of the hardest to accomplish, EQP07 was contaminated in 100% of the inspections for both lines during the first testing period and in ROWϕ during the second period. The average probability of obtaining positive swab results increased from around 43% to 54% in ROWϕ, and 52% to 70% in ROWλ. In ROWϕ, EQP07 was the only equipment constantly contaminated. On the other hand, swabs of EQP01, EQP08, EQP12 and EQP20 were *Listeria spp*. positive 100% of the times.

So far, there are no cleaning procedures applied while the lines are running, making these results not so surprising. There are solutions to eliminate *Listeria spp*. without compromising food safety and quality, and considering the ability of *Listeria spp*. to form resistant biofilms, different sanitizers should be used if possible. Solutions like ethanol based extracts, organic acids or essential oils have been shown as 'listericidal'.

Building infrastructures and factory surroundings are highly compromised, considering swab results. A mixture of peroxyacetic acid and hydrogen peroxide (CHE03) is a sufficiently strong sterilizer, as the results obtained during the third period show, that could be applied in every zone of interest.

Listeria spp. and *L. monocytogenes* presence was tested in the crops. Produce with higher contents in Ca^{2+} presented the higher probability of contamination during the first period. Among those are PRO01, PRO05 and PRO06. In general, the probability of product contamination was higher in the second testing period, and PRO01 and PRO04 were the most problematic. PRO10 is a by-product of PRO01, whereby its contamination incidence increases when PRO01 contamination probability rises. Out of the first testing period, more than 23% of PRO01 samples were contaminated and 86% of those were identified as *Listeria monocytogenes*. In the second period, *Listeria spp*. occurrence in PRO01 increased to 47%, but *Listeria monocytogenes* incidence decreased to 35%. The decrease of *L. monocytogenes* occurrence was indeed from 20% to 17% out of the total samples.

Tests were performed to relate produce contaminations with its intrinsic characteristics, like pH or nutrients available. A direct relation between $[Ca^{2+}]$ and *Listeria spp*. counts was established as increasing $[Ca^{2+}]$ favours contamination of produce. To counteract, a sanitizer additivated with EDTA should be considered, to make Ca^{2+} unavailable and weaken *Listeria* growth.

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