Exposure to minimally processed pear and melon during shelf life could modify the pathogenic potential of *Listeria monocytogenes*

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Abstract

Survival and virulence of foodborne pathogens can be influenced by environmental factors such as the intrinsic properties of food as well as the extrinsic properties that contribute to food shelf life (e.g., temperature and gas atmosphere). The direct contribution of food matrix characteristics on the survival of *L. monocytogenes* during fresh-cut fruit shelf life is not very well understood. In addition, the gastrointestinal tract is the primary route of listeriosis infection and penetration of the intestinal epithelial cell barrier is the first step in the infection process. Hence, the pathogenic potential of *L. monocytogenes*, measured as the capability for the organism to survive a simulated gastrointestinal tract and the proportion of cells able to subsequently adhere to and invade differentiated Caco-2 cells, subjected to fresh-cut pear and melon shelf life, was investigated. Samples were inoculated, stored at 10 °C for 7 days and evaluated after inoculation and again after 2 and 7 days of storage. A decrease in *L. monocytogenes'* capacity to survive a simulated gastrointestinal tract was observed with increasing storage time, regardless of the fruit matrix evaluated. Furthermore, *L. monocytogenes* placed on fresh-cut pear and melon was subjected to an attachment and invasion assay after crossing the simulated gastrointestinal tract. After inoculation, pathogen on fresh-cut pear showed 5-fold more capacity to adhere to Caco-2 cells than pathogen on fresh-cut melon. After 2 days of storage, *L. monocytogenes* grown on fresh-cut melon showed similar adhesive capacity (1.11%) than cells grown on pear (1.83%), but cells grown on melon had the higher invasive capacity (0.0093%). We can conclude that minimally processed melon could represent a more important hazard than pear under the studied shelf life.

Keywords: fresh-cut fruit, simulated gastrointestinal tract, adhesion, invasion, virulence.

1 Introduction

Listeria monocytogenes is a foodborne pathogen that can cause listeriosis. It has a high mortality rates among infected neonates, elderly, and immunocompromised persons (Walls and Buchanan, 2005). Changes in consumer lifestyles, specifically with significant expansion of the shelf life of foods under refrigerated conditions alongside increased consumer demand for ready-to-eat food, have revealed that *L. monocytogenes* is an important foodborne pathogen causing severe disease (Rantsiou et al., 2012). In recent years, several listeriosis outbreaks have been linked to the consumption of fresh or processed foods such as soft cheeses, ice cream, caramel apples, soy sprouts, dairy products and cantaloupe. The largest listeriosis outbreak in the United States was associated with consumption of cantaloupe, where 147 illnesses, 33 deaths, and 1 miscarriage occurred in 2011 (CDC, 2011). In 2012, economic studies in the USA concluded that fresh-cut cantaloupe had the fifth position in the ranking of minimally processed fruit sales and accounted for 5.4% (from 431.8 million dollars of total sales) while fresh-cut pear did not appear in the ranking (UCDavis, 2015) and has not been linked with any outbreak. Produce outbreaks seem frequently associated with processed produce and often involved storage under suboptimal conditions or environmental cross-contamination after processing (Hoelzer et al., 2012). Human pathogen survival and growth on fresh-cut produce is affected by many factors, including temperature, interaction with the indigenous microbiota, nutrient availability, and use of controlled or modified atmospheres for storage and/or packaging (Sapers et al., 2009). To survive adverse conditions (food processing, gastrointestinal tract, e.g.), bacteria must sense the changes and then respond with appropriate alterations in gene expression and protein activity (Boor, 2006).

Epidemiological evidence shows that the gastrointestinal tract is the primary route of infection and that penetration of the intestinal epithelial cell barrier is the first step in the infection process (Jaradat and Bhunia, 2003; Lecuit and Cossart, 2001). Thus, the serotype, the immune status of the host, the contamination level of the food, and the virulence capacity of the strain all play an important role in the ability to develop listeriosis (Werbrouck et al., 2009). To assess the food safety hazard associated with *L. monocytogenes*, some steps in the infection process, such as gastrointestinal survival or invasiveness, can be measured with an *in vitro* bioassay using a simulated gastrointestinal tract (static or dynamic system) and the intestinal epithelial cell line Caco-2. With these tools, some researchers have been focused on assessing the behaviour of *L. monocytogenes* subjected to stressful environmental conditions to study whether its virulence capacity could be affected. It has been previously reported that environmental conditions can modulate *in vitro* virulence characteristics such as invasiveness (Garner et al., 2006). Moreover, the ability of *L. monocytogenes* to invade Caco-2 cells is affected by the presence of NaCl, organic acids, pH, growth temperature, and oxygen restriction as well as interactions between these variables (Conte et al., 2000; Garner et al., 2006; Pricope-Ciolacu et al., 2013; Rieu et al., 2009; Werbrouck et al., 2009).

The aim of this work was to study the *in vitro* virulence of *L. monocytogenes* inoculated on two minimally processed fruits. Minimally processed 'Piel de sapo' melon has a pH approximately 6 while minimally processed 'Conference' pear has a pH approximately 5. To mimic a real-life scenario, samples were stored under abuse temperature conditions that resemble some commercial and household practices (10 °C) for 7 days (Marklinder et al., 2004). At each sampling point, the population of *L. monocytogenes* was enumerated and pathogen survival under simulated gastrointestinal tract was studied. Finally, the pathogenic potential of *L. monocytogenes*, measured as the capability for the organism to survive a simulated gastrointestinal tract and the proportion of cells able to subsequently adhere to and invade differentiated Caco-2 cells, subjected to fresh-cut pear and melon shelf life, was investigated.

2 Material and methods

2.1. Fruit

'Conference' pears (*Pyrus communis*) were obtained from local packing-houses in Lleida (Catalonia, Spain). 'Piel de Sapo' melons (Cucumis *melo* L.) were purchased in local supermarkets the day before each experiment. Pears were used in their optimal ripeness stage for processing (44 ± 3.2 N) according to Soliva-Fortuny et al. (2004). Pears were stored at 20 °C until they reached the desired firmness. Firmness of whole pears was measured on opposite sides of each fruit with a penetrometer (Effegi, Mila, Italy) equipped with a probe 8 mm in diameter. When values of ripeness fell within the selected range, pears were subjected to processing. Prior to processing, the fruits were washed with water, their surfaces were disinfected with 70% ethanol, and then they were left to dry at room temperature. Pears were peeled and cut into ten slices using a manual fruit slicer/corer. Melons were cut transversally in 14- to 16-mm slices, seeds and rind were removed, and each slice was cut into trapezoidal pieces.

2.2. Fruit quality parameters

Quality analysis of fresh-cut fruits (pH, soluble solid contents and titratable acidity) were performed before each experiment. Fruit flesh pH was measured using a pH meter (Model GLP22, Crison Instruments S.A.) with a penetration electrode (5231 Crison). Soluble solid contents (SSC) were measured at 20 °C with a handheld refractometer (Atago Co. Ltd.) in juice extracted by crushing fruit pieces in a blender. The results were expressed as °Brix. To measure titratable acidity (TA), 10 mL of fruit juice plus 2 drops of phenolphthalein solution 1% RV (Panreac, Barcelona, Spain) were diluted with 10 mL of deionized water and titrated with 0.1 N NaOH until the pH indicator changed colour. The results were calculated as g of citric acid/L for melon and g of malic acid/L for pear. There were three determinations of each parameter per fruit.

2.3. Bacterial strain and growth conditions

The *L. monocytogenes* serovar 1/2a strain used in this study was previously isolated from ready-to-eat iceberg lettuce (Abadias et al., 2008). To prepare inoculum for assays, the strain was streaked onto Tryptic Soy Agar (TSA, Biokar Diagnostics) supplemented with 0.6% w/v Yeast Extract (YE, Biokar Diagnostics) (TSAYE) plates and incubated at 37 °C for 24 h.

Subsequently, a single colony was inoculated into 50 mL of Tryptic Soy Broth (TSB, Biokar Diagnostics) supplemented with 0.6% w/v YE (TSBYE) and incubated with shaking at 150 rpm for 18-20 h at 37 °C. Cells were harvested by centrifugation at 9800X g for 10 min at 10 °C (Sorvall Legend XTR centrifuge, Thermo Scientific) and re-suspended in 25 mL of saline solution (SS; 8.5 g/L NaCl) to obtain an approximately 10^9 CFU/mL suspension.

For the inoculum preparation, a volume of the bacterial concentrated suspension was added to deionized water to obtain approximately 10^7 CFU/mL. Inoculum concentration was checked by plating appropriate dilutions onto Palcam agar (Palcam Agar Base with selective supplement, Biokar Diagnostics), followed by incubation at 37 °C for 48 h.

2.4. Inoculation procedures

Separately, pear and melon wedges were dipped (1:2 w/v) for 2 min at 150 rpm in the inoculation suspension and then were allowed to dry in a laminar flow biosafety cabinet. Each type of fruit wedge was packaged (100 ± 5 g) in polypropylene trays (375-mL) sealed with a non-peel-able polypropylene plastic film (PP-110, ILPRA, Italy). Nine holes of 400 µm were made in the sealed film using a needle to maintain air conditions. Samples were stored at 10 °C.

2.5. Enumeration of *L. monocytogenes* in fruit samples

Samples were examined on the day of inoculation and after 2 and 7 days of storage. *L. monocytogenes* population was determined in three sample trays for each food matrix at each sampling point. For pathogen population enumeration, 10 g of pear or melon from each tray was mixed with 90 mL of buffered peptone water (BPW, Biokar Diagnostics) in a sterile bag (BagPage 400 mL, Interscience BagSystem) and homogenized in a blender for 2 min at high speed (Bagmixer 100, Minimix, Interscience). Additionally, ten-fold dilutions were made with saline peptone (SP; 8.5 g/L NaCl and 1 g/L peptone) and plated, as described previously. These enumerations were used as initial counts in the simulated gastrointestinal tract experiment.

2.6. Survival of *L. monocytogenes* in a simulated gastrointestinal tract

L. monocytogenes from pear and melon samples stored at 10 °C were evaluated for their survival after exposure to a simulated gastrointestinal stress at each sampling time (day of inoculation and after 2 and 7 days). The experimental design is shown in Fig. 1. Simulated salivary fluid (SSF), simulated gastric fluid (SGF), and simulated intestinal fluid (SIF, composed of duodenal and bile solution) were prepared according to Oomen et al. (2003) and Oliveira et al. (2011) with some modifications (Table 1). To simulate mastication, 10 g of each sample was placed into a sterile plastic bag (BagPage 80 mL, Interscience BagSystem) and 9 mL of SSF tempered at 37 °C were added. The mixture was then homogenized in a blender for 2 min at high speed (Bagmixer 100, Minimix, Interscience) and incubated at 37 °C for 5 min. Afterwards, pH was measured and an aliquot (1 mL) was taken out to enumerate *L. monocytogenes*. These enumerations were then used as the post-saliva population in the simulated gastrointestinal tract experiment. The remaining sample was mixed with 13.5 mL of SGF (pH 2.0 adjusted with HCl 0.1 N). Subsequently, the pH was measured. Due to the different buffering effects of pears and melons, the pH of mixture increased differently between fruits. To avoid these differences, sample pH was normalized to a pH of 3.5 with hydrochloric acid (0.1 N) and incubated at 37 °C for 1 h. Then, the pH was measured and an aliquot (1 mL) was taken out to enumerate *L. monocytogenes*. These enumerations were then used as the post-gastric population counts in the simulated gastrointestinal tract experiment. The remaining sample was mixed with 36 mL of SIF which was composed of 27 mL of duodenal solution (pH 7.8) and 9 mL of bile solution (pH 8.0). The pH of this mixture was measured and incubated at 37 °C for 2 h. Finally, the pH was measured and a last aliquot (1 mL) was taken out to enumerate *L. monocytogenes*. These enumerations were used as the post-intestinal population counts in the simulated gastrointestinal tract experiment. For *L. monocytogenes* enumeration, appropriate dilutions of aliquots were placed onto Palcam agar and plates were incubated at 37 °C for 48 h. Three samples were analysed for each fruit and sampling time and the experiment was carried out in triplicate.

2.7. Attachment and invasion assay

Human intestinal epithelial Caco-2 cells were cultivated in DMEM (Dulbecco's Modified Eagle Medium, Gibco) supplemented with 20% heat-inactivated FBS (foetal bovine serum, Gibco) and 1% Penicillin/Streptomycin (10,000 units/mL penicillin and 10,000 µg/mL streptomycin, Gibco) in 12-well tissue culture plates (Costar, Corning). The cells were seeded at 2.0 X 10^5 cells per well and incubated until they reached confluence.

Attachment and invasion assays were performed as previously described by Oliveira et al. (2011) with minimal modifications. Briefly, prior to the assay, confluent Caco-2 cells were washed twice with pre-warmed sterile phosphate-buffered saline (PBS) to remove traces of antibiotic. After the final washing, 1 mL of pre-warmed DMEM was added to each well. At each sampling point (the day of inoculation and after 2 and 7 days of storage), the experiment was performed with *L. monocytogenes* exposed to the aforementioned simulated gastrointestinal tract. An aliquot (50 mL) of *L. monocytogenes* sample (obtained from the endpoint specimen of the simulated gastrointestinal tract) was removed and centrifuged (9800X g for 10 min at 10 °C) and then was re-suspended in 3 mL of DMEM. This was carried out to obtain high enough levels of *L. monocytogenes* cells to perform the invasiveness study. Bacterial suspension concentration was checked on Palcam agar plates. These enumerations were used as the initial bacterial count in the attachment and invasion assay. Afterwards, the plates were inoculated with 40 µL of this bacterial suspension per well. The plates were incubated at 37 °C in a 5% CO_2 humidified atmosphere for 1 h for the attachment assay. After incubation, the medium was aspirated and the monolayers were rinsed three times with PBS to remove non-adhered and loosely adhered bacteria. Cells were lysed (to liberate the bacteria) with using 1 mL of 0.1% (v/v) Triton-X100 (Sigma) in PBS for 5 min at room temperature. Triton lysates from three wells were combined and used for determining the number of *L. monocytogenes* that adhered to the Caco-2 cells.

For the invasion assay, non-adherent bacteria were removed via washing as above and then the Caco-2 cells were treated with DMEM supplemented with 150 µg of gentamicin/mL (50 mg/L, Gibco) to quantify invasive bacteria. The plates were incubated for 3 h at 37 °C in 5% CO_2. After incubation, the cells were rinsed three times with PBS to remove excess antibiotic and lysed with Triton-X100 as described above to liberate invaded bacteria. Triton lysate from three wells was combined and used for determining the number of *L. monocytogenes* that invaded the Caco-2 cells. For *L. monocytogenes* enumeration, appropriate dilutions of aliquots were placed onto Palcam agar and plates were incubated at 37 °C for 48 h. The results were expressed as CFU/mL. The experiment was performed with three independent biological replicates with three technical replicates for each biological replicate.

2.8. Data analysis

All of the data were collected from three independent experiments. To evaluate the survival capacity of *L. monocytogenes* against the gastrointestinal simulation, microbial counts were transformed to logarithmic reduction using the equation: log (N/N_0), where N is the microbial cell density at the particular sampling time (N_{SGF}, after the gastric step; N_{SIF}, after the intestinal step) and N_0 is the initial cell density. The pathogen capability to adhere to Caco-2 cells (adhesion index) was reported as the number of *L. monocytogenes* (CFU/mL) recovered after 1 h of contact with Caco-2 cells from each well following Caco-2 cell lysis divided by the number of bacteria (CFU/mL) that had been used for inoculation, expressed as a percentage. The pathogen invasion capabilities in relation to Caco-2 cells (invasion index) was calculated as the number of bacteria (CFU/mL) recovered after 3 h treatment of the Caco-2 cells with 150 µg/mL gentamicin divided by the total number of inoculated bacteria (CFU/mL), expressed as a percentage. The data are expressed as the average of three biological replicates with three technical replicates per biological replicate. Each matrix and sampling point was analysed using a one-way analysis of variance (ANOVA) using JMP8 (SAS software). When one-way ANOVA was significant, the Tukey's test was used to locate significant differences.

3 Results and discussion

3.1. Population of *L. monocytogenes* on fresh-cut pear and melon throughout shelf life

The population of *L. monocytogenes* on fresh-cut pear and melon after inoculation was 5.38 and 5.37 log CFU/g, respectively (Fig. 2). *L. monocytogenes* grew in fresh-cut pear and melon at 10 °C, reaching a final population of 7.43 and 9.25 log CFU/g after 7 days of storage, respectively. These results agree with previous studies on fresh-cut pear and melon, which assessed the behaviour of *L. monocytogenes* on minimally processed fruits (Abadias et al., 2014; Colás-Medà et al., 2015; Oliveira et al., 2014).

Initial quality parameters of the fresh-cut pear and melon used in our studies were determined before inoculation. The flesh of pear had a pH 4.99 ± 0.27 while the pH of melon was significantly higher (6.13 ± 0.19). Pear flesh showed a higher SSC (15.1 ± 1.1 °Brix) than melon flesh (11.9 ± 1.0 °Brix). Slight differences were found on titratable acidity between matrices; pear presented 1.59 ± 0.11 g of malic acid/mL of pear juice while melon had 1.23 ± 0.18 g of citric acid/mL of melon juice. The major acid present in melon is citric acid whereas in pear flesh it is malic acid. *L. monocytogenes* growth was not inhibited by the citric acid in the melon samples. Nevertheless, other studies carried out with other bacteria such as enterobacteriaceae (Deng et al., 1999) found more of an inhibitory effect by citric acid than malic acid against them. In the current study, the inhibitory effect of citric acid was not observed, which could be due to the low levels of citric acid in the melon flesh. On the other hand, the flesh of pears had higher soluble solid contents than melon and lower *L. monocytogenes* populations were reached on the pear, probably due to its lower pH.

3.2. Survival of *L. monocytogenes* in a simulated gastrointestinal tract

The *L. monocytogenes* population values that were obtained along the digestive simulation are shown in Fig. 3 (SGF) and 4 (SIF). On the day of inoculation, the same quantity of *L. monocytogenes* entered the simulated gastrointestinal tract regardless of the fresh-cut fruit evaluated. Challenge in SGF revealed that there were no significant differences between ‘pearadapted' (pH 4.9, mainly malic acid) and 'melon-adapted' (pH 6.1, mainly citric acid) bacteria in both fruit matrices throughout the storage period (Fig. 3). When *L. monocytogenes* on fresh-cut melon grew at 10 °C during 7 days, the log reduction was higher than at inoculation day.

At inoculation day*, L. monocytogenes* on fresh-cut pear was able to survive the exposure to the gastric fluid and survive in intestinal fluid, whereas it survived gastric fluid exposure and grew during intestinal step on fresh-cut melon. Similar results were observed after 2 h adaptation in an artificial cheese medium (Melo et al., 2013). Furthermore, at inoculation day and after 2 days of storage at 10 °C, *L. monocytogenes* grown on fresh-cut melon better overcame intestinal step (including bile fluid and high osmolality) than that grown on fresh-cut pear and the final population increased about 0.4 log units (Fig. 4). Peterson et al (2007) found that listerial cells grown on turkey meat were significantly more resitant to SGF than listerial cells grown in brain heart infusion broth (Peterson et al., 2007). Barbosa et al. (2012) reported that the osmotic and acidic sub-lethal exposure (modified Buffered Peptone Water) did not confer resistance to the simulated gastrointestinal tract conditions. Nevertheless, they noticed that the resistance of *L. monocytogenes* in a food matrix would be much higher due to the protection conferred by food components.

Based on these results, minimally processed melons could represent the more important hazard at inoculation day and after 2 days of storage as compared to pears under the studied shelf life (7 days at 10 °C), because listerial cells better survived and even grew to the exposure to SIF. Moreover, cells survival decreased with storage time, regardless of the fruit matrix evaluated.

After the whole simulated gastrointestinal tract, *L. monocytogenes* on fresh-cut pear reached 5.52 ± 0.23, 7.08 ± 0.32 and 7.17 ± 0.36 log CFU/g at inoculation day and after 2 and 7 days of storage, respectively. While *L. monocytogenes* on fresh-cut melon reached 5.77 ± 0.11, 8.00 ± 0.15 and 8.99 ± 0.38 log CFU/g at inoculation day and after 2 and 7 days of storage, respectively (data not shown).

3.3. Attachment and invasion assay

L. monocytogenes was grown on two different support matrices (fresh-cut pear and melon) under the same storage conditions and were subjected to a simulated gastrointestinal tract before subsequently testing for their capacity to adhere to and invade Caco-2 cells. This testing was performed on inoculation day and after 2 and 7 days of storage at 10 °C. On inoculation day, *L. monocytogenes* grown on pear showed the greatest adhesive capacity (6.5%), while it was only 1.4% with pathogen grown on melon (Fig. 5). In spite of the higher adhesive capacity of pathogen grown on pear, these cells exhibit similar invasive capacity (0.0015%) than cells on melon (0.0047%). After 2 days of storage, similar pathogen adhesive capacity was observed for pathogen grown on both matrices (1.83% vs 1.11% for pear and melon matrices, respectively). Nonetheless, the invasive capacity of pathogen grown on melon (0.0093%) was significantly different (3-fold higher) than pathogen grown on pear (0.0033%). *L. monocytogenes'* ability to adhere to Caco-2 cells showed a weak reduction with increasing storage time in both matrices (0.3% vs 0.6% for pear and melon matrices after 7 days, respectively). Additionally, a reduction in pathogen invasive capacity was observed in both matrices after 7 days (0.0001% vs 0.0007% for pear and melon, respectively). The capacity of *L. monocytogenes* to invade Caco-2 cells was below 1% in all evaluated times. This is in the same, or slightly lower, range than in comparable studies carried out in other food matrices (Lorentzen et al., 2011; Rieu et al., 2009).

A general overview of the results obtained, demonstrates that just after processing, pathogen grown on fresh-cut pear was 5-fold more adhesive to Caco-2 cells than pathogen grown on fresh-cut melon. Although after 2 days of storage, *L. monocytogenes* showed similar adhesive capacity on both matrices, pathogen grown on melon had the highest invasive capacity. If our contaminated fresh-cut fruits had been consumed after 2 days of storage (when the same initial load of pathogen in both matrices was observed), the fresh-cut melon could potentially cause a higher number of human infections than the fresh-cut pear. The last sampling point at 7 days post-inoculation demonstrated that pathogen grown on both fresh-cut pear and melon had lower capacity to overcome the simulated gastrointestinal tract and lower capacity to adhere to and invade Caco-2 cells compared to earlier sampling points. It is known that the environmental conditions to which *L. monocytogenes* is exposed prior to ingestion have an influence on the subsequent *in vivo* pathogenic potential. Unfortunately, the majority of researchers that have evaluated this effect on foodborne pathogens, although having studied both gastrointestinal survival and invasion capacity, have always done it separately. However, in the real infection process *L. monocytogenes* is subjected first to the gastrointestinal tract, followed by subsequent contact to the epithelial cells of the host. In this sense, Oliveira et al. (2011) first examined the pathogenic potential of *Salmonella* Thyphimurium, measured as the capability for it to survive a simulated gastrointestinal tract system and the proportion of cells adhering to and invading differentiated Caco-2 cells, after sequential incubations simulating the various production stages of pre-cut, ready-to-eat lettuce. They observed that the sequential incubation of *S*. Thyphimurium in soil and lettuce slightly increased the capability for surviving the simulated gastric fluid and increased the capability to grow in the simulated intestinal fluid, but decreased the capability of epithelial attachment and invasion and decreased the overall probability of surviving the gastrointestinal tract system. In addition, Conte et al. (2000) demonstrated that *L. monocytogenes* exposed to a sub-lethal acidic pH (BHI adjusted with lactic acid up to pH 5.1) showed increased invasion of intestinal epithelial Caco-2 cells relative to non-exposed bacteria. Previously, they determined that all of their exposed *L. monocytogenes* were able to readily develop acid tolerance. However, Conte et al. (2000) subjected acid-adapted *L. monocytogenes* cells to adhesion and invasion assays, without gastrointestinal tract simulation. To evaluate the effect of some organic acids and temperature on invasiveness, Garner et al. (2006) performed an invasion experiment with *L. monocytogenes* grown until stationary phase at 7 or 37 °C. For both temperatures, *L. monocytogenes* cells grown at pH 7.4 were also more invasive than bacteria grown in BHI broth adjusted to pH 5.5 with different combinations of organic acids. We observed that the invasive capacity of

L. monocytogenes significantly increased from day 0 to day 2 in both matrices, with this increase being more noteworthy on cells grown on melon than on pear. Thus, we could not attribute this behaviour to the difference in pH between the two food matrices.

In the current study, an increase in *L. monocytogenes* population was observed on both matrices during the experimental shelving time. Furthermore, a significant decrease in the percentage of bacteria associated with the epithelial cells (counts of adherent bacteria plus counts of intracellular bacteria), as well as reduced *L. monocytogenes* invasive capacity, were noted with increasing storage time. Similarly, Pricope-Ciolacu et al. (2013) noticed that the period of storage of milk samples, which increased *L. monocytogenes* cell numbers in the food matrix, decreased *in vitro* virulence. Walecka et al. (2011) demonstrated that increased density of bacterial culture is accompanied by a stepwise reduction in invasiveness in all of the tested strains. However, Garner et al. (2006) explored whether the number of added bacteria affected the relative invasion efficiencies, and no significant correlation was found. Thus, in our studies the reduction of *L. monocytogenes* invasive capacity with increasing storage can be not only caused by the higher load of pathogen in the longer-stored inoculums.

Moreover, in *in vitro* assays Andersen at al. (2007) noticed that *L. monocytogenes* cultivated under oxygen-restricted conditions were approximately 100-fold more invasive than similar cultures grown without oxygen restriction. Packaging under modified atmosphere conditions is widely established to improve the quality, shelf life as well as some safety aspects of minimally processed fruit. Thus, it could be suggested that *L. monocytogenes* subjected to minimally processed pear or melon stored under modified atmosphere packaging could increase their invasive capacity due to the low oxygen levels presents inside the package, but more research is still required to prove this hypothesis.

In conclusion, these findings suggested that fresh-cut melon is more likely to cause listeriosis if the pathogen has been introduced just before packaging than fresh-cut pear stored under the same conditions. This is supported by the high load of *L. monocytogenes* observed on fresh-cut melon that is a direct consequence of its pH, which is higher than pear pH, allowing for a higher *L. monocytogenes* population, even at 10 °C. In addition, when *L. monocytogenes* grown on fresh-cut melon was subjected to a simulated gastrointestinal tract, it was able to overcome the gastric step and was able to grow during intestinal step on processing day and after 2 days of storage. Finally, an enhancement in invasive capacity of *L. monocytogenes* was observed in this matrix after 2 days of storage at 10 °C. Molecular analyses could be useful to elucidate the genes that might be affected and cause the increase in invasive capacity seen after 2 days of contact with minimally processed pear and melon.

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Highlights

- *Listeria monocytogenes* grew on fresh-cut pear and melon with shelf storage at 10 °C.
- A decrease in *L. monocytogenes* capacity to survive a simulated gastrointestinal tract was observed with increasing storage time.
- On inoculation day, *L. monocytogenes* grown on fresh-cut pear showed the highest capacity to adhere to Caco-2 cells (6.5%).
- After 2 days of storage, *L. monocytogenes* showed an increased invasion capacity than on inoculation day.
- Artificially contaminated melon could potentially cause a high number of human infections than fresh-cut pear.

Table 1

Composition of synthetic juices of the *in vitro* gastrointestinal simulation

	Synthetic saliva fluid (SSF)	Synthetic gastric fluid (SGF)	Synthetic intestinal fluid (SIF)	
			Duodenal solution	Bile solution
Inorganic solutions	0.90 g KCl/L 0.20 g KSCN/L 1.15 g $NaH_2PO_4 \cdot 2H_2O$/L 0.57 g Na_2SO_4/L 0.30 g NaCl/L 0.07 g NaOH/L	0.82 g KCl/L 0.35 g $NaH_2PO_4 \cdot 2H_2O$/L 2.75 g NaCl/L 0.40 g $CaCl_2 \cdot 2H_2O$/L 0.31 g NH_4Cl/L	0.56 g KCl/L 7.00 g NaCl/L 3.39 g $NaHCO_3$/L 0.08 g KH_2PO_4/L 0.05 g $MgCl_2$/L 0.20 g $CaCl_2 \cdot 2H_2O$/L	0.38 g KCl/L 5.26 g NaCl/L 5.79 g $NaHCO_3$/L 0.22 g $CaCl_2 \cdot 2H_2O$ /L
Organic solutions	0.20 g urea/L	0.09 g urea /L 0.65 g glucose/L 0.02 g glucuronic acid/L 0.33 g glucosamine hydrochloride/L	0.10 g urea/L	0.25 g urea /L
Add to mixture organic + inorganic solutions	145 mg α-amylase/L 15 mg uric acid /L 50 mg mucin/L	1.00 g bovine serum albumin fraction V (BSA)/L 1.00 g pepsin/L 3.00 g mucin/L	1.00 g BSA/L 3.00 g pancreatin/L 0.50 g lipase/L	1.80 g BSA/L 6.00 g bile/L
pH	6.5 ± 0.1	2.0 ± 0.1	7.8 ± 0.1	8.0 ± 0.1

Figure caption

Figure 1 Schematic overview of the experimental design.

Figure 2 Population (log CFU g^{-1} or ml^{-1}) of *L. monocytogenes* inoculated onto fresh-cut pear (diamonds) and melon (squares) under storage at 10 °C. Results are the means of three biological replicates each with three technical replicates (n=9), and vertical bars indicate the standard deviation of the mean.

Figure 3 Logarithmic variation (log N_{SGF}/N_0) obtained after the exposure to synthetic saliva fluid (pH 6.5) for 2 min and to synthetic gastric fluid (pH 3.5) for 1 h of *Listeria monocytogenes* inoculated onto fresh-cut pear and melon along of storage at 10 °C. The values are the average of triplicate samples from three independent experiments (n=9). Different lowercase letters (a, b and c) in fresh-cut pear samples indicate significant differences ($P < 0.05$) between reductions along the storage. Different uppercase letters (A, B and C) in fresh-cut melon samples indicate significant differences ($P < 0.05$) between reductions along the storage. * Indicates significant differences between matrices at each sampling point.

Figure 4 Logarithmic variation (log N_{SIF}/N_{SGF}) obtained after the exposure to synthetic intestinal fluid for 2 h of *Listeria monocytogenes* inoculated onto fresh-cut pear and melon along of storage at 10 °C. The values are the average of triplicate samples from three independent experiments (n=9). Different lowercase letters (a, b and c) in fresh-cut pear samples indicate significant differences ($P < 0.05$) between reductions along the storage. Different uppercase letters (A, B and C) in fresh-cut melon samples indicate significant differences ($P < 0.05$) between reductions along the storage. * Indicates significant differences between matrices at each sampling point.

Figure 5 The adhesion index (the number of bacteria recovered from lysed Caco-2 cells after 1 h of contact divided by the number of bacteria inoculated x 100) to Caco-2 cells of *L. monocytogenes* on fresh-cut pear and melon after the gastrointestinal simulation, along the storage at 10 °C. Different lowercase letters (a, b and c) in fresh-cut pear samples indicate significant differences ($P < 0.05$) between reductions along the storage. Different uppercase letters (A, B and C) in fresh-cut melon samples indicate significant differences ($P < 0.05$) between reductions along the storage. * Indicates significant differences between matrices at each sampling point.

Figure 6 The invasion index (the number of bacteria recovered from lysed Caco-2 cells after 3 h of contact divided by the number of bacteria inoculated x 100) to Caco-2 cells of *L. monocytogenes* on fresh-cut pear and melon after the gastrointestinal simulation, along the storage at 10 °C. Different lowercase letters (a, b and c) in fresh-cut pear samples indicate significant differences ($P < 0.05$) between reductions along the storage. Different uppercase letters (A, B and C) in fresh-cut melon samples indicate significant differences ($P < 0.05$) between reductions along the storage. * Indicates significant differences between matrices at each sampling point.

Figure 7 Overview of pathogenic potential of *L. monocytogenes* with fresh-cut fruit storage. The invasion index (the number of bacteria recovered from lysed Caco-2 cells after 3 h of contact divided by the number of bacteria inoculated x 100) are indicated on the x-axis. The adhesion index (the number of bacteria recovered from lysed Caco-2 cells after 1 h of contact divided by the number of bacteria inoculated x 100) are indicated on the y-axis. The values are the average of triplicate samples from three independent experiments (n = 9).

Fig. 1

Fig. 2

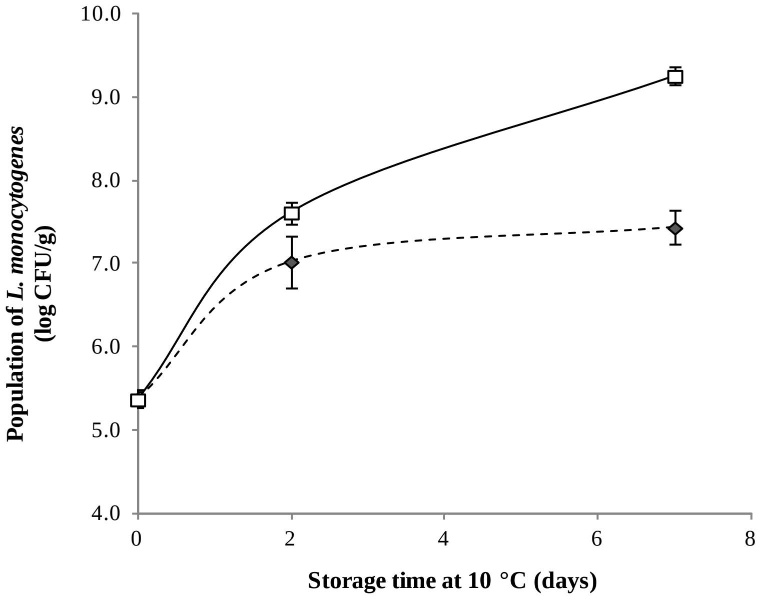

Population of L. monocytogenes (log CFU/g)
10.0
9.0
8.0
7.0
6.0
5.0
4.0
0
2
4
6
8
Storage time at 10 °C (days)

Fig. 3

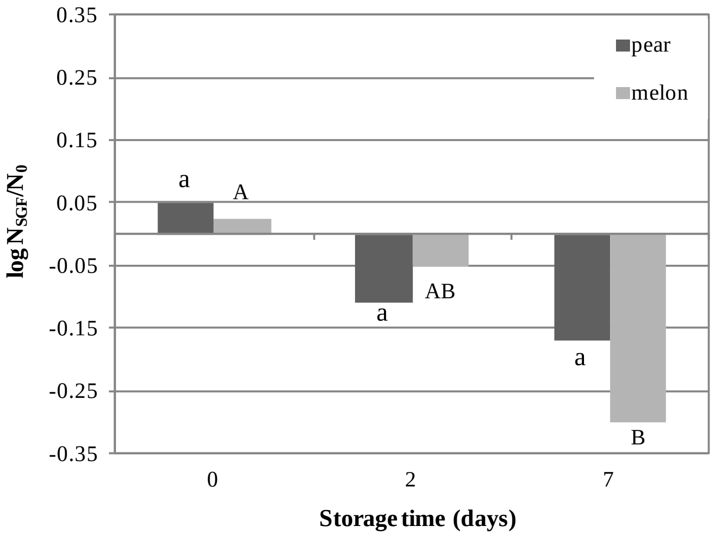
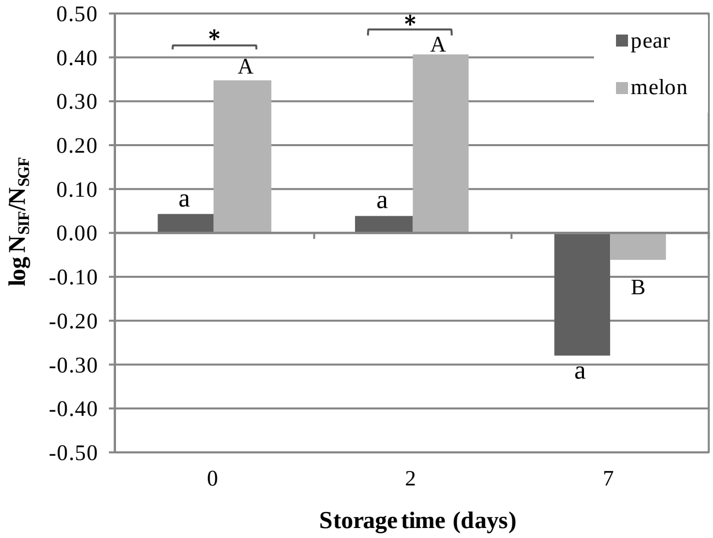
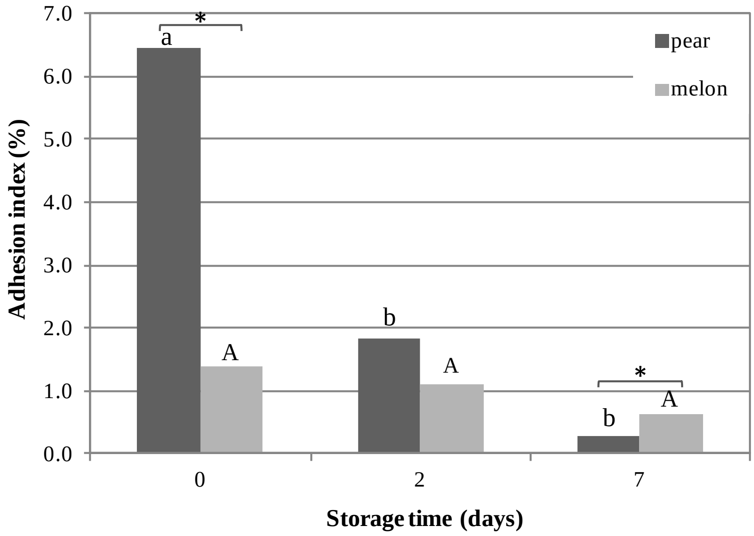

0.35
0.25
0.15
0.05
-0.05
-0.15
-0.25
-0.35
log NSGF/N0
pear
melon
a
A
a
AB
a
B
0
2
7
Storage time (days)

Fig. 4



0.50
0.40
0.30
0.20
0.10
0.00
-0.10
-0.20
-0.30
-0.40
-0.50
log N_{SIF}/N_{SGF}
*
A
a
B
pear
melon
0
2
7
Storage time (days)

Fig. 5



7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
Adhesion index (%)
*
a
b
A
pear
melon
0
2
7
Storage time (days)

Fig. 6

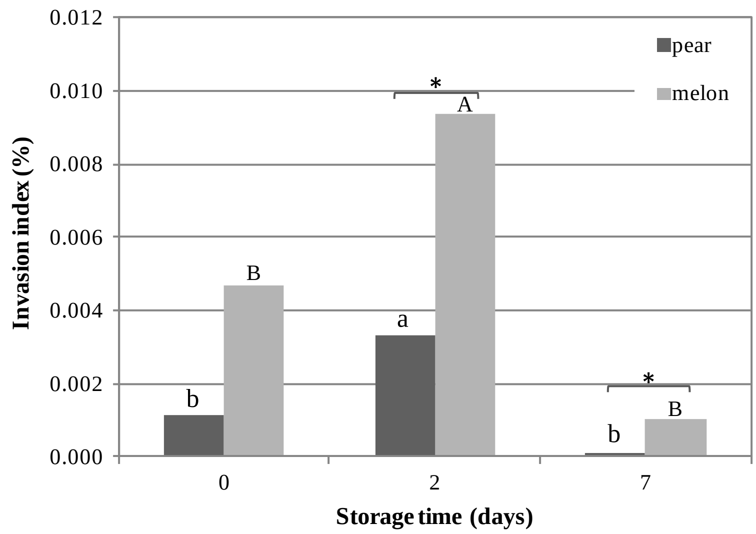
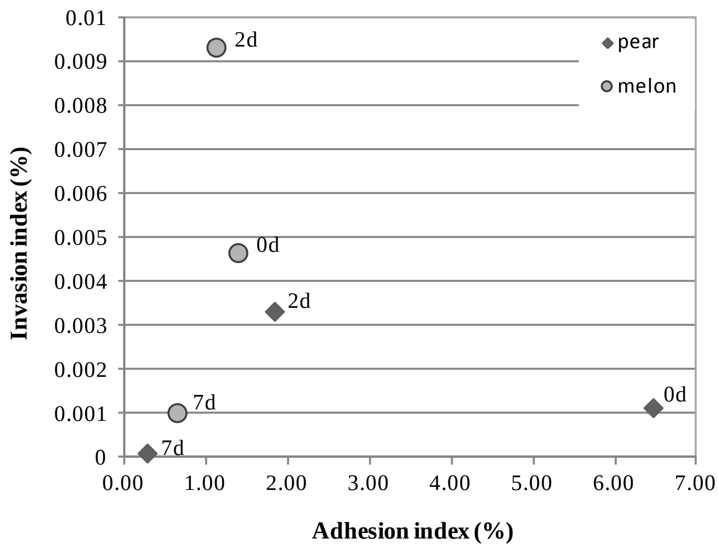

0.012
0.010
0.008
0.006
0.004
0.002
0.000
Invasion index (%)
pear
melon
*
A
B
a
b
*
B
b
0
2
7
Storage time (days)

Fig. 7


0.01
0.009
0.008
0.007
0.006
0.005
0.004
0.003
0.002
0.001
0
Invasion index (%)
pear
melon
2d
0d
2d
7d
0d
7d
0.00
1.00
2.00
3.00
4.00
5.00
6.00
7.00
Adhesion index (%)