Evaluation of growth potential and growth dynamics of *Listeria monocytogenes* on ready-to-eat fresh fruit

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Abstract

The consumption of fresh or RTE fruits is increasing every year and *Listeria monocytogenes* has been identified on raw or minimally processed fruits. A food product can become contaminated with *L. monocytogenes* anywhere along the pathway of food production during planting, harvesting, packaging, distribution and serving. The aim of this work was to assess the microbiological risks associated with consumption of ready- to- eat fruit such as melon, pineapple, coconut and fruit salad. The presence of *Escherichia coli*, *Salmonella spp.* and *L. monocytogenes* was also evaluated. Microbiological challenge tests were carried out for the evaluation of the *L. monocytogenes* growth potential in RTE fruit stored at 4 and 8°C. *E. coli* counts resulted under the detection limit of 10 CFU g^{-1}, *Salmonella* and *L. monocytogenes* were not detected (absence in 25g). The growth potential values in coconut and melon (δ>0.5) showed the growth capacity of *Listeria* at the temperatures considered. A low initial load, also derived from good hygiene practices, and correct storage temperatures are essential to reduce bacterial growth in RTE fruit. The challenge test showed how each type of RTE fruit has a different commercial life based on its specific growth potential and that food should be stored at temperatures not higher than 4°C for a short period.

Introduction

L. monocytogenes is a ubiquitous and invasive (Kocks *et al.*, 1992; Dussurget, Pizarro-Cerda, & Cossart, 2004) food-borne pathogen, responsible for listeriosis in humans.

This microorganism causes many diseases, ranging from mild gastroenteritis to severe blood and central nervous system infections, and can result in a high fatality rate in immune-compromised populations and the elderly. In pregnant women, infection can lead to miscarriage, premature birth or infection of the newborn (Drevets & Bronze, 2008). The incubation period of listeriosis occurs generally within 28 days (Angelo *et al.*, 2016). In 2018, 28 European countries reported 2549 confirmed cases of listeriosis. Almost all (42.4%) listeriosis cases were hospitalised in 2018 and 229 were fatal (ECDC, 2018).

More than 90% of invasive listeriosis is supposed to be caused by the ingestion of RTE food, and one-third of cases are due to growth in the marketing phase (Ricci, Allende, Bolton, Chemaly & Davies, 2018).

L. monocytogenes is broadly distributed in natural environments and has a marked capability to survive under stress conditions over food-processing and produce-packaging settings and equipment (Taormina & Beuchat, 2002). A food product can become contaminated with *L. monocytogenes* anywhere along the food production pathway (planting, harvesting, packaging, distribution, serving) (Silva, Teixeira, Oliveira, & Azeredo, 2008) because of strong adaptive capacity and due to inappropriate hygiene conditions (Ricci, Allende, Bolton, Chemaly, & Davies, 2018).

RTE foods and cold-stored products (principally meat and dairy) are commonly considered high-risk foods for *L. monocytogenes* infections (Zhu, Gooneratne, & Hussain, 2017). RTE foods are processed so that they are RTE without any additional handling steps (Bencardino, Vitali, & Petrelli, 2018). Nevertheless, RTE vegetables and fruits might represent a potential danger for human health due to the high risk of growth of undesirable microorganisms (Beuchat, 1996; Salazar *et al.*, 2017).

Several outbreaks were reported involving raw and processed vegetables and RTE foods due to *L. monocytogenes* contamination in many countries (Zhu, Gooneratne, & Hussain, 2017; Buchanan, Gorris, Hayman, Jackson, & Whiting, 2017). The 2011 *cantaloupe* outbreak determined 143 hospitalisations and 33 deaths (McCollum *et al.*, 2013). The 2014-2015 multistate *L. monocytogenes* outbreak related to caramel apples caused 35 illnesses in 12 states including 7 deaths (Angelo *et al.*, 2017). Moreover, there were several *L. monocytogenes* recalls related to fresh apples, sliced apples, and stone fruits including whole peaches, nectarines, plums, and pluots (Sheng, Edwards, Tsai, Hanrahan, & Zhu, 2017).

In particular, the consumption of RTE fruits is increasing every year, and *L. monocytogenes* has also been detected on raw or minimally processed fruits (Bae, Seo, Zhang, &Wang, 2013).

This work explored the growth potential and growth dynamics related to *L. monocytogenes* in fresh-cut minimally processed fruit (melon, pineapple, coconut and fruit salad). We also characterised the microbiological profiles (*E. coli*, *Salmonella* and *L. monocytogenes*) in order to verify compliance with the legal limits (Table 1).

Materials and Methods

Microbiological profiles RTE fruits

L. monocytogenes growth was investigated in different vegetal RTE matrices: coconut (*Cocos nucifera*), fruit salad (grapes, kiwi, melon and pineapple), pineapple (*Ananas sativus*), melon (*Cucumis melo*var. *piel de sapo*) and melon (*Cucumis melo*var. *cantalupo*) which were purchased from local supermarkets. The

fresh-cut fruit was packed in transparent plastic-sealed tray packaging, stored in air (not in a modified atmosphere or under vacuum). The shelf-life indicated by the producer was 4 days by storing at displayed temperature under 8°C. The samples, of various shapes and sizes, presented a net weight between 120g and 300g.

The study was conducted on 42 RTE fruits (melon n. 6 packs, coconut n. 6 packs, fruit salad n. 15 packs, pineapple n. 15 packs) collected from local retail shops during year 2019 in south Sardinia. Samples were randomly selected from three different batches. The RTE fruit samples were packed in flexible packaging, transported to the laboratory and stored at 4±2°C until the experiment was performed. Experimental samples were defined as the RTE fruit samples artificially contaminated with *L. monocytogenes*. Control samples were defined as the non-inoculated units and used to evaluate the natural presence of *L. monocytogenes* in RTE fruit samples from the batches used in our experiment. During the work, the testing times (T) were defined as T0, which was 6 h after inoculation, and T1, T2, T3, T4 and T5 which were, respectively, the analysis points every 2 days for a total of 10 days after inoculation.

Following EC Reg. 2073/2005 microbial limits for fruit RTE, the presence of *E. coli*, *Salmonella spp.* and *L. monocytogenes* was evaluated. *L. monocytogenes* was detected according to UNI EN ISO 11290-1:2017 and the UNI EN ISO 11290-2:2017 method was used for enumeration, while enumeration of beta-glucuronidase-positive *E. coli* and *Salmonella spp.* was carried out following UNI ISO 16649-2:2010 and UNI EN ISO 6579-1:2017 respectively (Table 2).

Water activity (a$_w$) and pH

Water activity (aw) and the pH of the samples were measured using an aw Hygrometer Dew Point Water activity meter 4TE (AcquaLab) and a digital pH meter with a glass electrode pH 510 Eutech Instruments (Cyberscan), following the manufacturer's instructions.

Challenge test

The study was performed according to the Technical Guidance Document prepared by the EU Community Reference Laboratory (CRL) for *L. monocytogenes* (Guidelines EURL 2004). A mixture of three *L. monocytogenes* strains was used to challenge RTE fruit units (Coroneo *et al.*, 2016; Marras *et al.*, 2019). The inoculum was composed of *L. monocytogenes* reference strain ATCC 35152 obtained from the American Type Culture Collection and two wild type strains previously isolated from the RTE fruit samples. All the strains were stored at -80°C in Brain Heart Infusion (BHI) broth (Oxoid, Basingstoke, UK) with glycerol (15%, v/v). Separate trials were conducted to determine the growth conditions necessary to standardise the level of inoculum to approximately 10-100 CFU/g. Cultures were then adapted at refrigeration temperature by storing at 4±2°C for ten days. Prior to starting the experiment, a bead of each strain was surface plated onto a Petri dish with Trypticase Soy Agar (TSA, Microbiol Diagnostici, Uta, Cagliari, Italy) and incubated at 37°C for 24 h. Then, a loopful of one isolated cell was transferred aseptically into 10 mL of BHI and incubated at 37°C overnight. To determine the initial concentration of each working cocktail, a suspension of approximately 1.5×10^8 CFU/mL of 0.5 McFarland (McF) was prepared. Each working cocktail was diluted and mixed to obtain two "Challenge Working Culture" (CWC) of the three *L. monocytogenes* strains, approximately 0.9 log10/mL and 2 log10/mL at the stationary phase. Colony counts were confirmed by plate count on TSA. Samples of 10g RTE fruit were inoculated both with 100 μL of CWC containing 2 log10 CFU/ml and 0,9 log10 CFU/ml of *L. monocytogenes* suspension homogenised with a stomacher to simulate two different origins of contamination: one natural and the other associated with low levels of GMP by food sector operators even if from an anlitic point of view the first aspect examined is associated with a higher level of uncertainty attributable to lower counts (Bartholome, 2005; François, 2007; Hwang, 2007). Subsequently, 3 samples for each type of inoculated fruit samples were stored at two different temperatures, 4°C, 8°C.

4°C represents the correct temperature at which these foods should be maintained (or collected), whereas "8°C" should simu-

Table 1. Legal food safety criteria and process hygiene criteria for microbial limits in all ready-to-eat fruits.

Parameter	Reference limits	Application of criteria	Reference
E. coli	10^2CFU/g	Manufacturing process. (Process hygiene criteria)	EC N.2073-2005/ DM n.3746-2014
Salmonella spp.	Absence in 25g	Products placed on the market during their shelf-life. (Food safety criteria)	EC N.2073-2005/ DM n.3746-2014
L. monocytogenes	10^2CFU/g	Products placed on the market during their shelf-life. (Food safety criteria)	EC N.2073-2005/ DM n.3746-2014
	Absence in 25g	Before the food has left the immediate control of the food business operator who has produced it (Food safety criteria)	EC N.2073-2005/ DM n.3746-2014

Table 2. Assessment of growth potential test (δ).

Fruit	Growth Potential (δ)			
	Storage temperature 4°C		Storage temperature 8°C	
	Growth Potential δ	Growth potential considered during at various times δ 1	δ	δ 1
Pineapple	-0.52	-0.49 (T1)	-0.55	-0.44 (T1)
Fruit salad	-0.59	-0.46 (T1)	-0.69	-0.37 (T1)
Coconut	0.02	0.09 (T3)	0.41	0.78 (T5)
Honeydew melon	0.58	1.04 (T5)	1.50	1.53 (T1)
Cantaloupe melon	0.13	0.60 (T4)/0.66 (T5)	0.97	1.57 (T4)

late the uncontrolled temperatures at which these products are often stored (*i.e.* during the cold chain or more often in household fridges, especially in the hot season when they are normally consumed). Negative control (sterile physiological water) were considered. The data are the mean values of the duplicate analysis of triplicate batches.

The challenge was carried out in independent trials for each batch performed one week apart. *L. monocytogenes* enumeration was conducted according to International Standard methods UNI EN ISO 11290-2:2017. For the *L. monocytogenes* enumeration, the samples were subjected to a 1:10 dilution in Fraser Broth base (Microbiol Diagnostici, Uta, Cagliari, Italy) and maintained at 20±2°C for 1 h ± 5 min. A 1 mL aliquot from initial suspension was directly streaked on three ALOA (Agar Listeria Ottaviani & Agosti) and incubated at 37°C for 24 and 48 h. After the incubation period, samples were taken using the typical colony count. For each time point, the results of the samples analysed were aggregated and reported as the median concentration of microorganisms expressed in log10 CFU/g.

Challenge tests assessing growth potential (δ) of *L. monocytogenes* were used (Gnanou Besse, Beaufort, Rudelle, Denis, & Lombard, 2008). A measure for the potential risk posed to consumers by *L. monocytogenes* in a certain product is the growth potential δ, defined as the difference between the log^{10} CFU/g at the beginning and end of the products shelf life (Beaufort, Bergis, Lardeux & Lombard, 2014). δ depends on the physical properties of the product, the storage temperature and the shelf life. The food samples were considered as supporting *L. monocytogenes* growth when the growth potential value was higher than 0.5 log10. Any value lower than 0.5 log10was considered as not supporting growth. δ 1 is the maximum Growth Potential estimated during the whole period considered (ten days). To characterize the growth trend of *L. monocytogenes* during the shelf life, a growth potential was also evaluated between the various analytical times. Starting from time 0 (beginning of the incubation), the inoculated samples were analysed every 2 days for 4 days for δ evaluation (T0, T1, T2) and for 10 days for growth dynamics studies (from T0 to T5). This condition was considered in order to simulate a hypothetical consumption by consumers beyond the expiration date.

Statistical data analysis

All experiments were performed twice and repeated in triplicate. Therefore, the reported data represent the means of six values. Means and standard deviations (SD)

were calculated in replicates within the experiments, and analyses were carried out using Microsoft Excel XP 2010.

Results and Discussion

pH and water activity were measured in the various types of RTE fruit. Fresh-cut pineapple showed average values of pH equal to 3.6±0.015, while melon showed a pH equal to 5.58±0.018. Average pH values of 3.74±0.015 were found for fruit salad, while values of 6.27±0.006 were found for coconut at the end of the shelf-life phase (Figure 1).

With regard to the average a_W values, 0.989±0.0001 values were recorded for-pineapple, 0.991±0.0004 for melon, while fruit salad and coconut showed a_W values of 0.998±0.0003 and 0.990±0.0008, respectively (Figure 2).The data shown for melon represent the mean of results detected for the two different samples (var. *piel de sapo* and var. *cantaloupe*).

L. monocytogenes growth results agree with the association of favourable factors, referred to ecological parameters such as water activity and hydrogenionic concentration. High water activity measured in RTE products always resulted favourable for microbial growth in the different types of

products examined. On the contrary, the lower hydrogenionic concentration values determined in more acid fruits, particularly RTE pineapple and fruit salad, inhibited *L. monocytogenes* growth, confirming the results reported by Abadias and Sinigallia (Abadias, Usall, Anguera, Solsona, & Viñas, 2008; Sinigallia, Bevilacqua, Campaniello, D'Amato, & Corbo, 2006).

E. coli counts resulted below the detection limit of 10 CFU g^{-1}, compared to the counts observed by other authors (Mukherjee, Speh, Dyck, &Diez-Gonzalez, 2004; Loncarevic, Johannessen, & Rørvik, 2005; Mukherjee, Speh, Dyck, & Diez-Gonzalez, 2007; Bohaychuk *et al.*, 2009).

Salmonella and *L. monocytogenes* were not detected (absence in 25g) according to Sagoo (Sagoo, Little, & Mitchell, 2001; Sagoo, Little, Ward, Gillespie, & Mitchell, 2003) and McMahon (McMahon &Wilson, 2001) in organic vegetables, while other authors have detected these microorganisms in fruit (Sagoo, Little & Mitchell, 2004).

As for the Challenge test results, a negative δ was obtained for pineapple and fruit salad (-0.52 and -0.59 at 4°C and -0.55 and -0.69 at 8°C respectively), therefore considered as not supporting *L. monocytogenes* growth. Only melon (both varieties analysed) was shown to support *L. monocytogenes* growth (0.58 and 0.13 at 4°C and 1.50 and 0.97 at 8°C in honeydew and *can-*
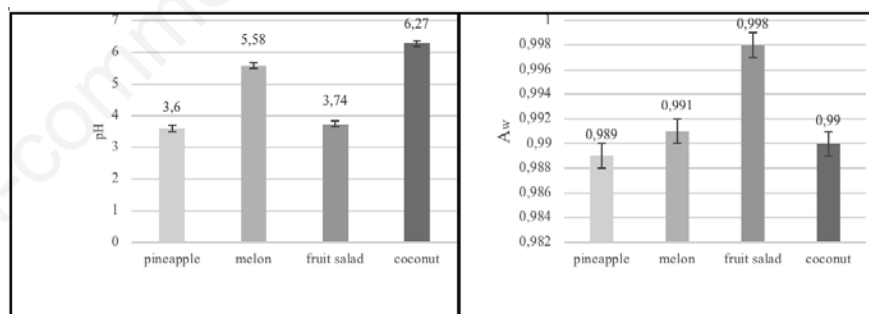

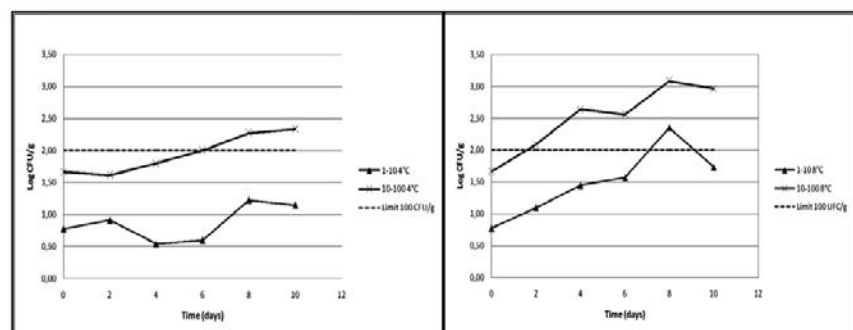**Figure 1. pH and Water Activity (Aw) on RTE fruits.**

Figure 2. Growth dynamics of *L. monocytogenes* on cantaloupe melon at 4 and 8°C

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taloupe melons respectively (Table 2).

Moreover, the *cantaloupe* melon showed values >0.5 log10 (0.97) when stored at temperatures of 8°C, while δ remains below this limit (0.13/4°C) when the melon is maintained at the correct refrigeration temperatures (Figure 3). Particular attention is required with respect to good manufacturing practices, transport and refrigeration of the product on the part of both producer and consumer.

The initial load (at T0, both 1-10 and 10-100 CFU/g concentrations) of inoculated *L. monocytogenes* decreases over time for pineapple (Figure 3) and for fruit salad (Figure 4). Honeydew melon was found to be an excellent growth substrate for *L. monocytogenes* even at low temperatures (Figure 5). *Cantaloupe* melon has proved to be a supportive food if maintained for longer times at 4°C (Figure 2). It reached δ=0.66 (T5/10-100) on the eighth day and even δ=1.57 (T4/1-10) and δ=1.57 (T4/10-100) if refrigerated at 8°C (Table 2).

Concerning fresh-cut *cantaloupe*, refrigeration temperatures are fundamental for the development of *L. monocytogenes*. At 4°C in fact its growth is limited if the recommended consumption times are respected. On the other hand, if the temperature is higher (8°C) or if the product is consumed after 4 days, the risk is increased.

Fresh-cut coconut has a growth potential of 0.02 at 4°C and 0.41 at 8°C showing that the coconut stored at 8°C (Table 2) could support growth, while at lower temperatures it allowed survival but did not support *L. monocytogenes* growth.

Fresh-cut coconut (Table 2) showed very similar δ values both at the end of the shelf-life period and several days after inoculation (0.02 *vs.* 0.09 at T3/1-10) if stored at the correct temperature of 4°C. This data show that coconut is not a great substrate for *L. monocytogenes,* but it is able to keep the bacterium alive for several days. Instead, at higher temperatures (8°C*),* the *L. monocytogenes* present in the coconut were easily able to replicate up to and exceeding the permitted limits (Figure 6). *L. monocytogenes* reaches δ = 0.78 log10 CFU/g on the tenth day (T5/10-100) at 8°C (Table 2).

In pineapple and fruit salad, the initial load (at T0) of inoculated *L. monocytogenes* decreases over time, both at 4 and 8°C.

In outbreaks related to food consumption, as reported by Gaul *et al*., 2013, a low level of *L. monocytogenes* contamination is required to affect individuals, predominantly the elderly and immunosuppressed persons. So, as confirmed by our data, starting from a very low concentration (inoculum 1-10), *L. monocytogenes* is capable of rapidly repl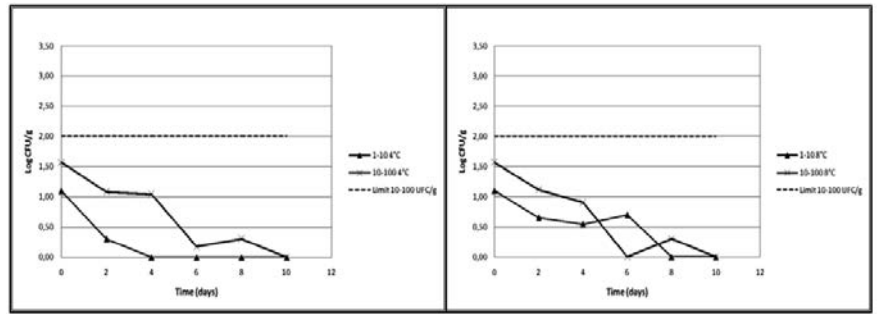icating at low temperatures. For these reasons, RTE food matrices like coconut or melon could be a health risk even at a low

Figure 3. Growth dynamics of *L. monocytogenes* on pineapple at 4 and 8°C.
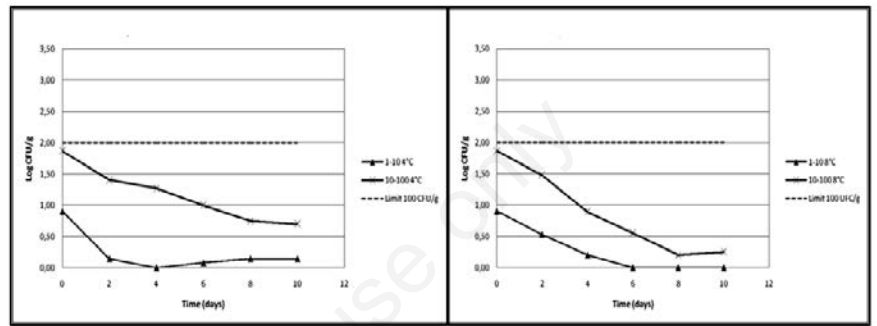
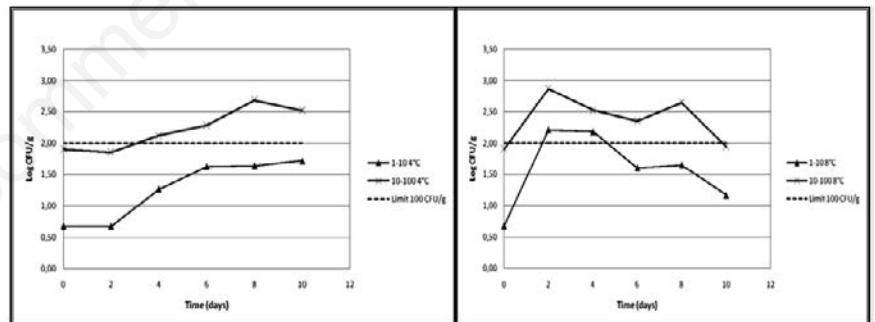**Figure 4. Growth dynamics of *L. monocytogenes* on fruit salad at 4 and 8°C**

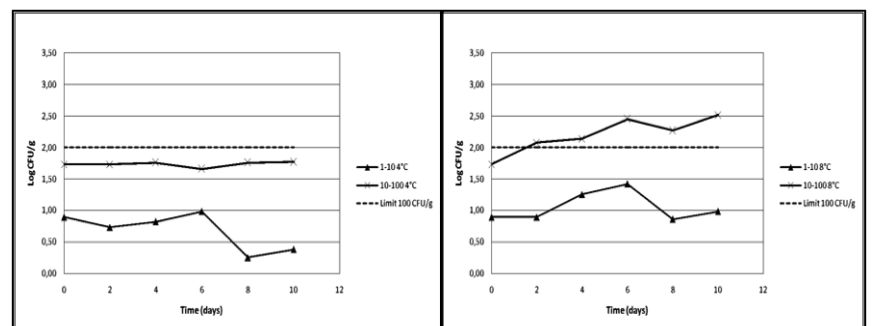**Figure 5. Growth dynamics of *L. monocytogenes* on honeydew melon at 4 and 8 °C.**

Figure 6. Growth dynamics of *L. monocytogenes* on coconut at 4 and 8°C.

infective dose *of L. monocytogenes*. However, criteria for growth and no-growth of *L. monocytogenes* in RTE foods for risk assessment is would be limited. It is necessary in fact, to consider this risk as dependent on the expiration date of the product including storage conditions and spoilage.

These findings are valuable if we consider the average temperatures of household refrigerators, which maintain values often closer to 8-10°C than 4°C. Some authors (Roccato, Uyttendaele, & Membré, 2017; Chang & Hsu, 2008) have discussed storage temperature by consumers as being one of the critical points along the food chain. The consumer often does not respect the manufacturer's instructions for consumption for different reasons (distraction, economic reasons, tendency not to waste food and others).

Conclusions

Our study highlighted the potential risk of contamination by *L. monocytogenes* in particular categories of foods which are increasingly consumed nowadays. Some fruit allow the bacteria to replicate very easily, while others totally block its growth.

The microbiological quality of the fruit was overall satisfactory, probably related to the use of high- quality raw materials, respect for the cold chain and good hygiene practices.

From our findings it is possible to state that RTE products such as fruit salad or pineapple can be safer in terms of the risk associated with the presence of *L. monocytogenes*, while fresh-cut coconut and especially fresh-cut melon (both our types) should be considered as risk products.

A low initial load also derived from good hygiene practices and correct storage temperatures are essential to reduce bacterial growth in RTE fruit, in particular referring to consumer behaviour, a domestic level with refrigeration temperatures closer to 8°C instead of the desirable 4°C, it could to further minimize the risk associated with growth of *L. monocytogenes*.

On the other hand, if one considers the structural specificity and high availability in terms of nutrients that melon is able to offer as a substrate for microbial growth, the different producers of fresh-cut fruit should package melons together with acid fruits (*i.e.* melon with pineapple or kiwi, etc.). Such a combination would result in products whose water activity value (a_w) is very high, but in which the pH value obtained is low enough to prevent the development of *L. monocytogenes*, thereby reducing the risk to the consumer.

Each kind of RTE fruit has a different commercial life based on its specific Growth Potential (Ziegler, Rüegg, Stephan & Guldimann, 2018) and food should be stored at temperature no higher than 4 degrees for a short period within up production time and shelf life.

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