Growth potential of *Listeria monocytogenes* in six different RTE fruit products: impact of food matrix, storage temperature and shelf life

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Abstract

We tested the growth potential of *Listeria monocytogenes* on six RTE fruit products at low (4°C at the factory followed by 8°C retail/home storage) and abusive (4°C followed by 12°C) storage temperatures. Sliced coconut and fresh cut cantaloupe, as well as a fruit mix containing diced pineapple, cantaloupe, apples and grapes supported the growth of *L. monocytogenes* with a growth potential δ>0.5 log CFU/g over six days. Mangoes, a mix of diced kiwi, cantaloupe and pineapple as well as a mix of diced pineapple, mango, grapefruit, kiwi and pomegranate did not support a growth potential that exceeded 0.5 log CFU/g over six days. The growth potential of *L. monocytogenes* correlated significantly with the pH; no product with a pH below 4 showed a significant growth potential of *L. monocytogenes*. Time after inoculation was also a significant predictor of the growth potential, while the fruit type and storage temperature were not.

Introduction

Lifestyle choices and the demand for healthier food in its natural state result in an upwards trend in sale of minimally processed raw fruit or vegetables products (EFSA, 2017). These products do not typically undergo any processing step intended to kill pathogens and are consumed as ready to eat (RTE) products. This raises a significant challenge to food safety, and various outbreaks due to food borne pathogens on ready to eat fruit and vegetable products have been reported. Typical examples are outbreaks with *E. coli* O157:H7 (Sharapov *et al.*, 2016), *Salmonella* (Sivapalasingam *et al.*, 2003; Beatty *et al.*, 2004; FDA, 2014; Vestrheim *et al.*, 2016), *L. monocytogenes* (McCollum *et al.*, 2013; Chen *et al.*, 2016) and norovirus (Harris *et al.*, 2003; Bassett and McClure, 2008; Hall *et al.*, 2012). A British study in 2016 found that

5.4% of RTE precut fruit products were contaminated with *L. monocytogenes* and other *Listeria* species (Willis *et al.*, 2016). The process of cutting fruit pieces as well as the mixing of different fruits are assumed to increase the risk of contamination with *L. monocytogenes* at the production stage. 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 *et al.*, 2014). δ depends on the physical properties of the product, the storage temperature and the shelf life. To adequately assess the risk associated with raw RTE products and to take effective preventive measures, it is therefore crucial to determine the growth potential of *L. monocytogenes* on the food matrix under storage conditions that reflect the production, retail and consumer home environment. The risk of contamination of food and subsequent human infection with *Listeria monocytogenes* is exacerbated by its frequent presence in the environment and growth potential at refrigeration temperatures (Chan and Wiedmann, 2009). In this context, temperature conditions in challenge studies should account for the fact that while production facilities tend to have closely monitored temperature management systems, refrigerated display cases at the retail level and home refrigerators are often found at abusive temperatures (James *et al.*, 2016; Jouhara *et al.*, 2017).

We have recently published data on the growth potential of *L. monocytogenes* on different RTE salad products (Ziegler *et al.*, submitted). Apart from salads, several outbreaks of listeriosis in the past were linked to fruit; examples are stone fruit (Chen *et al.*, 2016), caramel apples (Angelo *et al.*, 2017), and cantaloupe (McCollum *et al.*, 2013). The aim of the present study was to determine the growth potential of *L. monocytogenes* on RTE fruit products. All products included in this study were precut fruit, with both mono-products as well as mixed fruit products represented. Since some fruits and vegetables produce antimicrobials that are active against *L. monocytogenes* (Beuchat and Brackett, 1990; Cvetnić and Vladimir-Knezević, 2004; Babic *et al.*, 2008; Hayrapetyan *et al.*, 2012), a relatively high inoculum was used to allow accurate measurements of growth of *L. monocytogenes* as well as antimicrobial plant effects.

Materials and Methods

All experiments were carried out in three independent replicates.

Bacterial strains, growth conditions and subtyping

The three strains of *L. monocytogenes* used in this study were all isolated from an RTE salads or RTE fruit mixes (Table 1). The growth properties of strain N16-0716 have been previously characterized (Ziegler *et al.*, submitted) and its genome was published (Ziegler *et al.*, 2018). Strains N16-2670 and N16-1278 were isolated from a RTE fruit production plant and are therefore inherently likely to be found on RTE fruit products. Stock cultures of *L. monocytogenes* were maintained at -80°C in brain heart infusion (BHI; Oxoid, Basel, Switzerland) broth with 15% glycerol. To prepare the inocula, stock cultures were streaked on BHI agar plates and incubated overnight. A single colony was inoculated into 5 mL BHI broth and incubated overnight (37°C, 200 rpm), subcultured in the morning 1:100 into 5 mL fresh BHI broth, and incubated for 6 h (37°C, 200 rpm) to obtain an early-stationary-phase culture (9.5±0.8 log CFU/mL). This culture was then incubated at 5°C for 20 h to simulate the storage conditions *L. monocytogenes* would undergo during the production of RTE fruit products at room temperature followed by cold storage. Strain pools were obtained by combining equal quantities of the cold adapted stationary phase cultures.

Fruit products

The six different RTE fruit products were obtained from a manufacturer who produced 30 g portions specifically for this study (Table 2). This small unit size was chosen to allow processing of the total content of each package for microbial analysis, thus avoiding sampling bias. The fruit pieces contained within each package were identical in size and shape to the pieces contained in the larger packaging units destined for retail; the edges of the fruit cubes were approximately 1-2 cm long. The products were shipped to the lab under preservation of the cold chain at 5°C and inoculated 12-24 h after production. The packaging was identical to the larger packages that were produced for retail, and the products were packaged in the same production line as the commercially sold products. None of the products were packaged under modified atmosphere. To achieve three independent replicates, products from three different lot

numbers were shipped to our facility on three different days.

Inoculation of the RTE fruit products

Maximum Recovery Diluent (MRD; Oxoid, Basel, Switzerland) was chosen as a diluent for the inoculum as well as for the serial dilutions for colony counting because it is recommended in ISO6887-1 to achieve maximal recovery of microbes, while the low peptone concentration of 1% prevents immediate multiplication of microorganisms to allow for an accurate enumeration. To achieve a final bacterial load of 4 log CFU/g in the products, the cold adapted stationary phase culture was serially diluted in 10 mL MRD, and 1 mL of the appropriate dilution was homogeneously distributed over the product. This relatively high concentration was chosen to be able to accurately quantify not only an increase, but also a decrease in CFU/g. The inoculum was administered through a septum of scotch

tape using a syringe and a gauge 22 needle. Immediately after inoculation, the syringe hole was sealed with a second scotch tape to maintain the integrity of the packaging. Negative control samples were inoculated in the same way with 1 mL MRD. After inoculation, all samples were shaken for 1 min in a standardized manner to optimize the distribution of the inocula. This shaking had no visible effect on the size or integrity of the fruit pieces. To preserve the cold chain, the packaged fruit and the bacteria were kept on ice during all procedures.

Storage conditions

The products were stored at two different storage conditions for 3, 4, 5 and 6 days. Storage condition 1 mimics compliance with reasonable storage temperatures (4°C at the factory, 8°C at retail/at the consumer), while the storage condition 2 mimics abusive temperatures (4°C at the factory, 12°C

Table 1. Bacterial strains.

Bacteria	Source	Serotype	Sequence type	Clonal	Complex Lineage	Internal identification number
Listeria monocytogenes	Fruit production company	1/2a	ST403	CC403	II	N17-2670
Listeria monocytogenes	Salad production company	1/2b	ST517	CC517	I	N16-0716
Listeria monocytogenes	Fruit production company	4b	ST388	CC388	I	N17-1278

RTE product	Ingredients	Additives	pH	Challenge test	results summary	
				Contrast*	4C + 8C Growth potential**	4C + 12C Growth potential**
Fruit mix 1	Mix of diced pineapple (50.1%), mango (17.6%), grapefruit (17.6%), kiwi (11.8%) and pomegranate (2.9%) *Ananas comosus, Mangifera indica, Citrus paradisi, Cucumis melo, Punica granatum*	E302, E330 (mango)	3.4	0-3 0-4 0-5 0-6	0.06 0.11 0.14 0.10	0.10 0.09 0.04 0.18
Fruit mix 2	Mix of diced pineapple (36.6%), cantaloupe (26.7%), apple (26.6%) and grapes (10%) *Ananas comosus, Cucumis melo, Malus domestica, Vitis vinifera*	E302, E330 (apple)	4.2	0-3 0-4 0-5 0-6	0.14 0.40 0.52 0.92	0-15 0.95 1.03 1.07
Cantaloupe	Cantaloupe (diced) 100% *Cucumis melo*	n/a	5.6	0-3 0-4 0-5 0-6	1.23 2.04 2.65 2.56	1.29 2.73 2.79 2.60
Fruit mix 3	Mix of diced kiwi (20%), cantaloupe (50%) and pineapple (30%) *Actinidia deliciosa, Cucumis melo, Ananas comosus*	n/a	3.9	0-3 0-4 0-5 0-6	0.19 0.15 0.15 0.16	0.12 0.21 0.46 0.42
Coconut	Diced coconut pieces 100% *Cocus nucifera*	n/a	6.4	0-3 0-4 0-5 0-6	1.26 3.00 3.04 3.63	1.39 2.69 4.19 4.80
Mango	Peeled, diced mango pieces (100%) *Mangifera indica*	E302, E330 (mango)	3.6	0-3 0-4 0-5 0-6	0.14 0.11 0 0.13	0.08 0.09 0.01 0.03

at retail/at the consumer and an additional 2h of sitting without cooling prior to consumption). Therefore, storage condition 1 comprised storage at 4°C for 72 h, then at 8°C for the remainder of the protocol. Storage condition 2 comprised storage at 4 C for 72 h, then at 12°C. Prior to processing the condition 2 samples, they were kept at 20°C for 2h. Temperature in all cold storage facilities was continuously controlled and recorded with temperature loggers (EasyLog, Lascar Electronics, Pennsylvania, USA).

Microbiological analyses

L. monocytogenes and total viable count (TVC) were determined immediately after inoculation (t=0) and 3, 4, 5 and 6 days after inoculation. At each time point, one inoculated sample and one negative (uninoculated) control sample per temperature were analyzed, representing one replicate. The whole content of a unit was transferred into sterile stomacher bags, diluted 1:5 with MRD and homogenized for 30 s in a Stomacher® 400 Circulator (Seward, Worthing, United Kingdom). Serial dilutions in MRD were prepared and 0.1 mL was spread-plated on the following agar plates in duplicate: PALCAM (Merck, Darmstadt, Germany) for the enumeration of *L. monocytogenes* and Plate Count Agar (PC; Oxoid, Basel, Switzerland) for TVC. While the low pH of the fruit may have negatively influenced the recovery of stressed cells on PALCAM agar, the dilution steps in MRD would mitigate most of this effect. The average of the duplicate plates was calculated and expressed as log CFU/g. The limit of detection was 2 log CFU/g. The pH of the negative control samples was determined at each time point using an Orion Versa Star pH meter (Thermo Fisher, Switzerland) equipped with a solid matter probe. In products containing only one type of fruit, two individual pieces of fruit and the accumulating juice in the container were measured. For products comprised of more than one type of fruit, the pH of all individual constituents of the product and the accumulating juice were measured. Since it was impossible to determine on which pieces of fruit bacteria grew to what extent, the average pH was calculated as the mean from these values to approximate the overall acidity of the product. The pieces of coconut in our samples were too small to allow accurate pH measurement and we therefore used the mean of 6.4 from different published values (Sinigaglia *et al.,* 2006a). The pH of pomegranade seeds was not measured due to their small size.

Calculation of the growth potential δ

For each time point at each temperature,

the difference between the log CFU/g at the evaluation point and the log CFU/g at the beginning of the challenge test was calculated for each of the three independent replicates. The growth potential δ was defined as the highest value obtained among three replicates. When δ was higher than 0.5 log CFU/g the RTE fruit product was classified as able to support the growth of *L. monocytogenes* at the corresponding temperature. If δ was ≤0.5 log CFU/g the RTE fruit product was classified as unable to support the growth of *L. monocytogenes*.

Statistical analysis

Statistical analysis and graphics were performed in R (Version 3.4.0) using R studio (Version 1.0.143) (RStudio Team 2015). Generalized linear models without interactions were fitted (glm in R stats package version 3.4.2) to model the growth potential

of *L. monocytogenes* and the log CFU TVC in each fruit product as a function of product, temperature, pH and time. The ggplot2 package (Wickham, 2009) was used for visualization. Please see online Appendix for R scripts.

Results

Growth potential of *L. monocytogenes* on RTE fruit products

We measured the growth potential for each RTE fruit product at t= 3, 4, 5 and 6 days (Table 2). Any products that show a growth potential δ>0.5 log CFU are considered permissive to the growth of *L. monocytogenes* under EU regulations (Beaufort *et al.,* 2014). Among the products tested here,
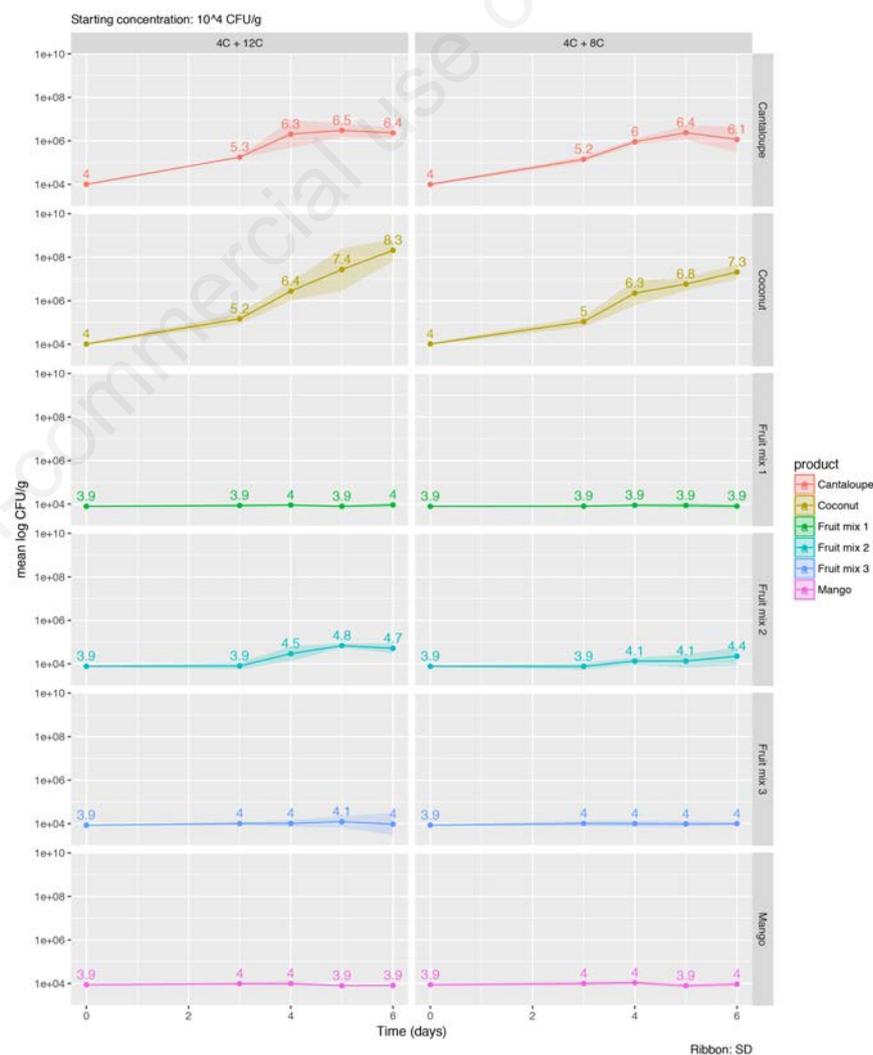
Figure 1. CFU count of Listeria monocytogenes on salad. Growth curves of L. monocytogenes on RTE fruit products. The numbers above the lines represent the mean CFU/g at this time point, the ribbon around the line the SD of each value.

the highest growth potential was observed in RTE coconut after 8 days at the abusive storage condition 2. δ exceeded 0.5 log CFU at most time points and under both storage conditions in coconut, fruit salad 2 and cantaloupe. The lowest growth potential trending towards zero was observed in mango. In mango, fruit mix 1 and fruit mix 3 δ was <0.5 log CFU at all time points at both storage conditions (Table 2).

Impact of the pH and fruit product on the growth potential of L. monocytogenes

pH had a significant impact on the growth potential of *L. monocytogenes* (p < 0.001), while the fruit type did not (p = 0.86). A significant growth potential for *L. monocytogenes* was only observed in products with relatively high pH: coconut, cantaloupe and fruit mix 2 (Table 2). The highest number of *L. monocytogenes* after six days was observed in coconut stored under condition 2 (δ = 4.8, resulting in 8.3 (SD=0.5) log CFU/g) (Figure 1, Table 2).

In fruit mix 1, fruit mix 3 and mango, the pH was lower (Table 2) and the growth potential of *L. monocytogenes* did not exceed 0.5 log CFU over the whole experimental period. The lowest number of *L. monocytogenes* after six day was observed in mango stored under condition 2 (δ = 0.03, resulting in 3.9 (SD=0.04) log CFU/g) (Figure 1, Table 2).

Impact of time on the growth potential of *L. monocytogenes*

Not surprisingly, time had a significant impact on the growth potential of *L. monocytogenes* on fruit products (P<0.001). Where the product was permissive for the growth of *L. monocytogenes*, the growth potential was higher at later time points compared to earlier time points, which is also reflected in the log CFU/g over time (Figure 1).

Impact of temperature on the growth potential of *L. monocytogenes*

The storage temperatures were chosen to reflect likely scenarios during the shelf life of RTE fruit products. Correct storage at 4°C at the production facility was assumed for both temperature regimens, followed by either correct (condition 1, 8°C) or abusive (condition 2, 12°C) storage during retail/at home. The two different storage conditions did not have a significant impact on the growth potential in this study (P=0.36). Accordingly, in products that did not permit the growth of *L. monocytogenes*, we observed steady numbers of log CFU/g under both temperature regimens.

However, where the product permitted the growth of *L. monocytogenes*, a trend towards higher numbers of CFU/g was observed in samples stored under condition 2 compared to condition 1 (Figure 1). For example, in coconut at t=6 we observed 7.3 (SD=0.34) log CFU/g under storage condition 1 which follows regulations vs 8.3 (SD=0.5) log CFU/g under the abusive storage condition 2. Given the overlap of the SD these differences were not statistically significant.

Total viable count on RTE fruit products

All fruit products had initial TVC counts around 4 log CFU/g (Figure 2). The highest final TVC count was 8.8 (SD=0.5) log CFU/g in coconut stored under condition 2. The lowest final TVC count was 5

log CFU/g (SD=0.7) in fruit mix 3 stored under condition 1.

For the TVC, time and storage temperature had significant impact on the outcome CFU/g, while pH did not. Fruit mix 3 supported significantly less growth compared to the other fruit products.

Discussion and Conclusions

The pH of the RTE fruit products was a significant determinant of the *L. monocytogenes* growth potential, while it did not significantly influence the TVC counts. This may be explained by the fact that the TVC counts reflect a large number of species, some of them adapted to the specific fruit
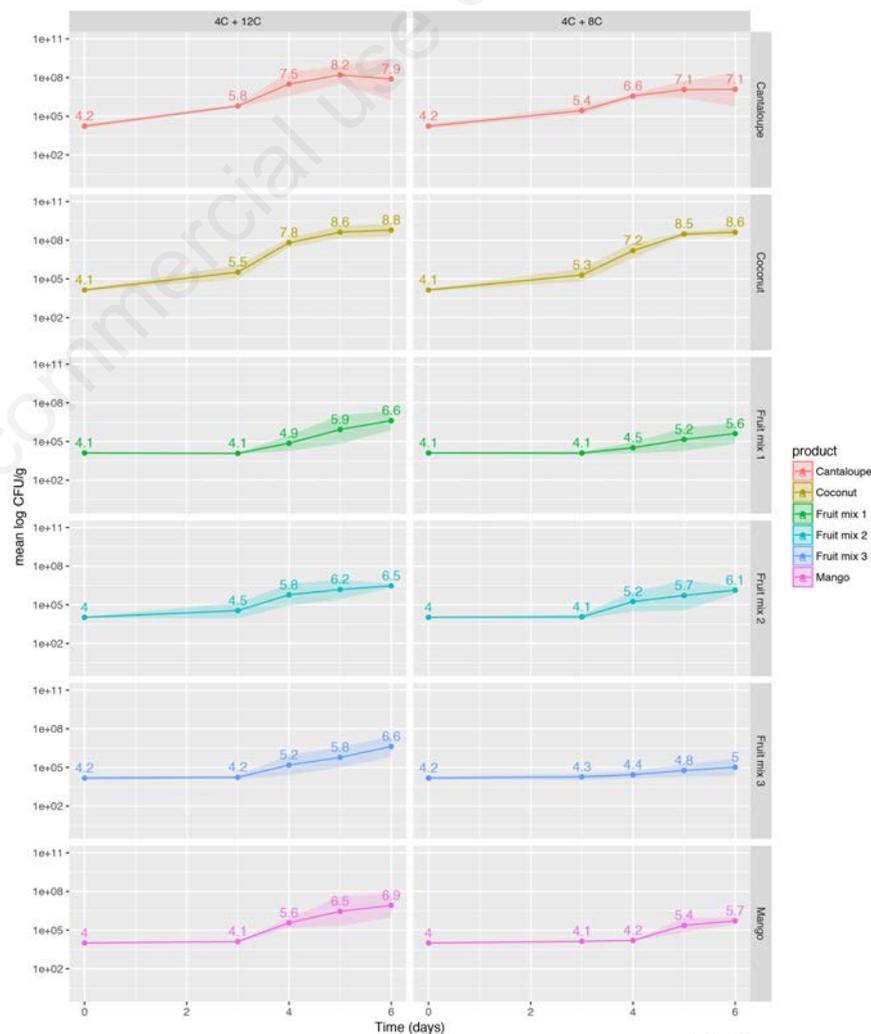

Figure 2. Mean CFU for the Total Viable Count in the inoculated samples. Growth curves of total viable counts on RTE fruit products. The numbers above the lines represent the mean CFU/g at this time point, the ribbon around the line the SD of each value.

they were isolated from. In contrast, *L. monocytogenes* can survive a pH as low as 2.5 for two hours but growth is impaired with sinking pH and absent below pH 4.0 (Smith *et al.,* 2013). EU regulations assume no growth potential for *L. monocytogenes* in products with a pH below 4.4, (Codex Alimentarius, EC Regulation No 2073/2005). The fruit type did not significantly influence the growth potential. While there is an obvious connection between fruit type and pH, fruit from different lots may vary in acidity depending on their ripeness, and in fruit mixtures the pH of individual pieces of fruit may be influenced by the mixture as a whole. Our pH measurements for one fruit type over different experiments had standard deviations in the range of pH 0.1-0.6, which offers an explanation why pH was a more accurate predictor of the growth potential compared to fruit type.

Among the single ingredient products included in this study, mangoes did not support the growth of *L. monocytogenes*. A study analyzing the growth of *L. monocytogenes* on fresh cut mango slices also found no growth at 3±2°C (Rangel-Vargas *et al.,* 2018). This is contrary to other authors who found a significant increase of *L. monocytogenes* on fresh cut mango pieces over time: a 4 log CFU increase of *L. monocytogenes* Scott A was observed after 200h at 10°C in sterilized mango pulp (Penteado *et al.,* 2014). Potential explanations for this discrepancy are the missing plant microbiota and the higher pH (5.16) of the mango pulp used by these authors compared to the mango pieces in our study with an average pH of 3.9. In another study, the inoculation of fresh cut mango cubes (pH 4.2) with a mixture of six strains of *L. monocytogenes* resulted in 2 and 5.4 log CFU increases over 6 days at 5°C and 13°C, respectively (Feng *et al.,* 2015).

We observed significant growth of *L. monocytogenes* on cantaloupe and coconut pieces, which represent the two low-acidity fruits in our sample setup. In agreement with our data, other authors also found that *L. monocytogenes* grew on coconut slices packed under air and under modified atmosphere (Sinigaglia *et al.,* 2006b). Growth of *L. monocytogenes* on cut cantaloupe or cantaloupe pulp has also been demonstrated before (Ukuku and Fett, 2002; Penteado and Leitão, 2004; Martinez *et al.,* 2016; Nyarko *et al.,* 2016).

Neither fruit mix 1 or fruit mix 3 permitted growth of *L. monocytogenes* under the tested conditions. Interestingly, individual components of these fruit mixtures by themselves have been shown to permit growth of *L. monocytogenes* (or *L. innocua* as a surrogate), such as sliced apples

(Alegre *et al.,* 2010) and cantaloupes (Ukuku and Fett, 2002; Penteado and Leitão, 2004; Nyarko *et al.,* 2016) (and this study), while pineapples have proven unsuitable to support the growth of *L. monocytogenes* (Penteado *et al.,* 2014), and compounds in grapefruit and pomegranade possess antimicrobial activity (Cvetnić and Vladimir-Knezević, 2004; Hayrapetyan *et al.,* 2012). Fruit mix 2 did not allow significant growth of *L. monocytogenes* under condition 1, while under the abusive storage condition 2 there was growth potential that exceeded 1 log CFU. This is despite the fact that both fruit mix 2 and 3 contained pieces of cantaloupe with a mean pH of 5.4. Mixing of different fruits affects the overall pH, combines their individual microbiota and creates a new environment that has to be carefully evaluated in terms of its suitability for microbial growth. To conclusively assess the growth potential of *L. monocytogenes* in RTE fresh fruit mixtures, our data suggests that it is crucial to perform individual challenge tests for each new product. Since pH and time were significant predictors of the growth potential, particular diligence is advisable for products with longer shelf life and if the product pH exceeds 4.0.

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