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Evaluating the growth potential of *Listeria monocytogenes* in Ready to Eat Vegetables

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ABSTRACT: There has been a growing increase in the consumption of Ready To Eat (RTE) vegetables among people owing to the health benefits of a diet rich in vegetables, because of hectic life styles, the use of Ready To Eat vegetables has gained popularity in recent years. RTE vegetables are subject to minimal processing and are consumed without being heat treated, they may, thus, contain human food borne pathogens. Listeria monocytogenes is one of the pathogens that has been widely reported in different outbreaks associated with the consumption of RTE vegetables. In this study the growth potential of L. monocytogenes in different types of RTE pre-cut vegetables stored at reasonably foreseen temperatures in the distribution chain of fresh produce was evaluated. Total aerobic microorganisms, lactic acid bacteria, pH and Aw were measured to characterise the vegetables. The results showed that some of the tested products (grapes, tomato, white cabbage and red cabbage) had a growth potential (δ) higher than 0.5 Log10 (CFU/g) during their shelf life and can therefore, support the growth of Listeria monocytogenes. By way of conclusion, conducting different challenge tests in the factories producing these convivence products seems of great importance.

Keywords: Growth Potential, Listeria monocytogenes, RTE Vegetables.

Introduction

In recent years there has been a considerable increase in the consumption of ready to eat (RTE) vegetables due to the growing awareness of the role of vegetables in a healthy diet and an increasing trend of using convenience food products (Grassi *et al*., 2013; Issa-zacharia *et al*., 2011). RTE vegetables (e.g. prepared salad mixes) are subject to minimal processing which can include peeling, cutting, washing, centrifugation and packaging (Caponigro *et al.*, 2010; Abaza, 2017). Many of these products tend to have an increasingly long shelf life of up to ten days and are consumed without being heat treated.

Processed fruits and vegetables may harbor human food borne pathogens (Sagoo *et al.*, 2003; Castro-Rosas *et al.*, 2012). *Listeria monocytogenes* is one of the pathogens that has been the cause of

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outbreaks associated with chopped celery, mixed salads, mung bean and whole cantaloupes (Laksanalamai *et al.*, 2012; Garner and Kathariou, 2016; Zhu, Gooneratne and Hussain, 2017). *L. monocytogenes* is a foodborne pathogen that is of great importance in refrigerated RTE foods because of its ubiquitous and psychotropic nature, ability to grow at refrigerated temperatures and potential to cause serious infection in susceptible people (Carrasco *et al*., 2008). *L. monocytogenes* is naturally found in the environment and may be present in animal manure and in feces from wild animals, and can, thus, contaminate raw vegetables or the soil adhering to them (FARBER *et al*., 1998). Fresh produce products do not receive a treatment during processing that eliminate pathogens or inhibit their growth potential (Fröder *et al.*, 2007). However, washing with chlorine to reduce overall microbial load, is extensively used in many countries e.g. in Western Europe. It is uncertain how effective this disinfection is as it may depend on the vegetable type and its microbial load. Thus, there is a risk that *L. monocytogenes*is present on the final products either due to its presence on the raw product or contamination during processing. If present, *L. monocytogenes* may multiplies during shelf life although less is known about its ability to grow on such products. There have been some studies about evaluating the growth potential of *L. monocytogenes* in ready to eat vegretables and salads (Culliney & Schmalenberger, 2020; Marras *et al*., 2019; Ziegler *et al*., 2019)

The aim of this study was to provide information about the growth potential of *L. monocytogenes* in different types of RTE pre-cut vegetables stored at reasonably foreseen temperatures in the distribution chain of fresh produce.Total aerobic microorganisms, lactic acid bacteria, pH and a_w were measured to characterise the vegetables.

Materials and Methods

Overall, a microbiological challenge test assessing grwoth potential in RTE fruit and vegetables was conducted. TheEURL Lm technical guidance document for conducting shelf-life studies on *Listeria monocytogenes* in ready-to-eat foods (Version 3 – 6 June 2014) was followed with a few exceptions as described below.

- *L. monocytogenes strains and preperation of inoculum*

Five strains of *L. monocytogenes* previously isolated from leeks, parsley, RTE kale salad, iceberg salad and honeydew melon, respectively were used. They were all able to grow at 5 °C in Brain heart infusion broth (BHI, Difco). Stock cultures were stored at -80°C in BHI with 15% glycerol. For each experiment, frozen cultures were streaked on BHI agar plates and after incubation for 24 h at 37° Cused for inoculating 5 ml of BHI. After incubation at 37°C for 18 h these early stationary phase cultures were diluted 1:1000 into 10ml BHI and incubated at 7°C for five days to obtain cold enriched cultures. Thereafter, a pool of strains was made by mixing one ml of each cold adapted strain and this pool was diluted to 10^4 CFU/ml in saline solution (0.9%).

- *RTE vegetables*

The RTE vegetables used in this study were yellow pepper, red pepper, green pepper, pineapple, grapes, white cabbage (Brassica oleracea capitata), red cabbage (Brassica oleracea capitata rubra), frisee lettuce, radicchio, lollorossa,tomato, sliced beetroot and kohlrabi. The vegetables were processed, cut in 20mm pieces, packed and sent refriegerated (5 °C) to the laboratory.

There was at least one month between each batch analysed.

- ***Inoculation of vegetables and storage conditions***

In order to avoid sampling effects due to uneven distribution of *L. monocytogenes* within a package, the whole content of a bag was used for each analysis. 10g of each product was allocated in small plastic bags made of the same material as used at the company and spot inoculated with 0.5% V/w of the cold adapted pool to reach a final concentration in the products of 100 CFU/g. Three bags of 10 g were inoculated per product. The bags were sealed with a headspace volume mimicking the commercial food products as closely as possible and stored at 5°C for two days followed by 10 °C until a few days after shelf life.

- ***Microbiological analyses***

L. monocytogenes was enumerated in the inoculated products at day 0, at the end of shelf life and two days after shelf life, all in triplicate order. Total aerobic count and lactic acid bacteria count were also determined in the inoculated samples. The whole content of each bag (10 g) was transferred to a stomacher bag, 90 ml of BPW was added and then mixed in a stomacher for 1 minute. Decimal serial dilutions were made in 10 ml of maximum recovery diluent. To enumerate Listeria the samples were plated on ALOA and incubated at 37°C for 48h- To increase the detection limit, 1 ml of the initial dilution was plated on 2 pre-dried agar plates. Total aerobic count and lactic acid bactria count were determined following ISO 4833 and ISO 15214 respectively with appropriate dilutions plated on Plate Count Agar (PCA) and MRS-agar respectively and incubated at 25 °C for 72 hours before counting colonies.

Non-inoculated samples of each product were included to test for the presence of L. monocytogenes in 10g following ISO 11290:1with the two-step enrichment in Half Fraser broth and Fraser Broth (OXOID) followed by plating on ALOA. This was done at day 0 and at end of shelf life.

- ***Measurement of pH and aw***

Surface and homogenized pH were determined. Surface pH is the average of pH values measured three different places on the surface of the products using a combined pH electrode (InLab® Surface sensor). pH was measured on the surface on day zero and at end of shelf life.

To measure pH of a homogenized sample, 20 ml of milli Q water was added to 80g of non-inoculated sample before mixing in a blender. The sample were left at room temperature for a few minutes before pH was measured using a glass electrode. Homogenized pH was measured (NMKL 179). aw was measured as well using water activity meter analyzer.

- ***Calculation of the growth potential***

The growth potential (δ) of L. monocytogenes was calculated according to the EURL Lm technical guidance document using the median of the replicates for each sampling point. The growth potential is the difference in $\log_{10}$ (CFU/g) between the median of the replicates at the end of (or two days after) shelf life and the median of results at day zero. The growth potential δ was defined as the highest value obtained among three replicates. Results presented are mean of two independent biological replicas each performed as described above.

- ***Statistical analysis***

Statistical analysis using SPSS 16 software and one way anoval test was

applied.

Results and Discussion

Qualitative analysis of L. *monocytogenes* in non-inoculated products showed that none of the products were contaminated with L. *monocytogenes*. The water activity a_w was 0.99-1.00 in all products. The pH-value was measured on the surface of the vegetables and in homogenized samples and the differences between batches was negligible. In general, there was agreement between the measurements of pH on the surface and in the homogenized sample. However, for beetroot, kohlrabi, frisee, radicchio and lollarossa the pH values were 1-1.5 unit higher in the homogenized sample compared to the surface measurements.

The growth potential (δ) of a pool of 5 strains of L. *monocytogenes* in RTE vegetables and fruits inoculated with 100 CFU/g are shown in Table 1 together with the corresponding pH values of the products.

Growth of bacteria in vegetables may depend on many factors such as plant variety and maturity, processing including degree of cutting, packaging conditions and foil and storage temperature. Furthermore, the specific microbial composition of the product may vary due to variations in the raw product and the processing/washing environment and this will also influence the growth of L. monocytogenes. According to the EFSA guidance document (Beaufort et al., 2014) a food product is unable to support the growth of L. monocytogenes if δ is lower or equal to 0.5 Log_{10} (CFU/g). Contrary, if δ is higher than 0.5 Log_{10}(CFU/g) the food is able to support the growth. Based on this definition L. monocytogenes is unable to grow in sliced products of kohlrabi, green, red and yellow peppers, pineapple, beetroot, frisee, lolla rosa, and radicchio salads incubated at 5 °C for 2 days followed by 10 °C for 4-6 days (Table 1). The growth potential in grapes, white cabbage, red cabbage and tomato were higher (1.5-2.8 Log_{10} CFU/g) (Table 1) showing that L. monocytogenes is multiplying in these products from the start level of 100 CFU/g to ~5-10.000 CFU/g in 6-7 days.

Table 1. Growth potential of L. monocytogenes in RTE fruit and vegetables. The products were inoculated with 2 Log_{10} (CFU/g) and incubated for 2 days at 5 °C followed by 10 °C until 2 days after shelf life

Product	pH (Surface/mixed product) day 0	pH (Surface/mixed product) day end	L. *monocytogenes* average cell count on inoculated samples (log CFU/g ± SD) End of shelf life	δ(Log_{10} CFU/g) end of shelf life	L. *monocytogenes* average cell count on inoculated samples (log CFU/g ± SD) 2 days after shelf life	Δ (Log_{10} CFU/g) 2 days after shelf life	Shelf life (days)
Pineapple	3.3/3.3	2.7/3.2	2.0 ± 0.08	0.05	1 ± 0.00	0.95	7
Grapes	4.1/3.8	3.8/4.0	4.2 ± 0.64	2.77	4.9 ± 0.15	3.12	7
Tomato	4.1/4.4	4.2/4.2	4.0 ± 0.46	1.78	4.1 ± 0.98	2.22	6
Yellow pepper	4.8/4.9	4/4	2.0 ± 0.00	-0.18	1.7 ±0.70	-0.40	7
Red pepper	4.7/4.9	3.9/3.8	2.0 ± 0.00	-.020	1.6 ± 0.49	-0.36	7
Green pepper	5.2/5.8	3.8/3.8	2.4 ± 0.17	0.00	2.1 ± .17	-0.70	7
Sliced beetroot	4.7/6.1	4.2/4.2	2.3 ± 0.24	0.12	2 ± 0.00	-0.18	8
Kohlrabi	4.8/6.3	4.3/4.1	2.4 ± 0.10	0.48	2.3 ± 0.28	0.48	8
Frisee lettuce	4.7/5.9	4.3/5.7	2.7 ± 0.10	0.49	2.6 ± 0.24	0.33	7
Radicchio	4.3/6.0	4.3/5.9	2.5 ± 0.15	0.25	3.0 ± 0.52	0.71	7
Lolla Rossa	4.4/6.0	6.2/6.5	2.6 ± 0.21	0.44	2.9 ± 0.62	1.00	7
White Cabbage	6.3/6.3	4.5/6.8	4.1 ± 0.26	1.96	4.2 ± 0.15	2.26	7
Red Cabbage	5.2/6.2	4.5/6.2	3.6 ± 0.10	1.55	4.0 ± 0.58	1.60	7

To our knowledge the growth potential of L. *monocytogenes* in kohlrabi, radicchio, yellow pepper and frisee lettuce has not been reported previously. The inability of pineapple, red pepper and lollarossa to support the growth of L. *monocytogenes* is supported by previous data where a limited growth of L. *monocytogenes* in these products is described, the reason for this was supposed to be related to the low pH-value and the competitive microbiota (Lokerse *et al*., 2016). Recently Ziegler et al. (2018) reported the growth potential of twelve RTE salads including iceberg, corn salad, beetroot, white cabbage and celeriac which was also analyzed in this study. Contrary to our results they reported growth of L. monocytogenes in beetroots at both 5 and 8 °C (Ziegler *et al*., 2018). This may be due to the factors described above including the level of perforation in the packaging foil as this influences the diffusion of oxygen and carbon dioxide. Also the start concentration of L. *monocytogenes* may explain the difference as Ziegler et al used 5 Log (CFU/g) compared to 2 Log (CFU/g) used here as also recommended by the EU guideline for challenge test to determine growth potential. The relatively high growth potential in white cabbage of 1-2 Log units has been found previously (Ziegler *et al*., 2018; Wang *et al*., 2013).

The a_w and pH values of the majority of products were in range for growth of L. *monocytogenes* except pineapple, grapes and tomatoes. The pH values of these were below 4.4 which are regarded as the lower limit for growth of L. *monocytogenes*. According to the EU food safety criteria (EC 2073/2005) RTE food is regarded as stabilized against L. *monocytogenes* if pH is below 4.4. Surprisingly, L. *monocytogenes* had a growth potential above 1.8 in both tomatoes and grapes. One explanation could be the presence of local spots with a higher pH value due to growth of yeast or molds.

There was no correlation between the pH values and the growth potential showing that other factors are of importance for the growth of L. *monocytogenes*. This may be other inhibitory factors not measured during the present study including the presence of natural antimicrobial compounds (Aziz & Karboune, 2018; Ponce *et al*., 2008), bacteriocins (Yu *et al*., 2013) or weak organic acidsproduced by LAB (Amezquita & Brashears, 2002; Piard & Desmazeaud, 1991). For example some vegetables like celeriac and spinach contain nitrite in levels that may be antimicrobial (King *et al*., 2016; Walker, 1990). And in carrots the presence of phytoalexins has been shown to inhibit growth of gram positive organisms and fungi (Kurosaki & Nishi, 1983). Citric and malic acid are the dominating acids in both pineapples and tomatoes (Beullens *et al*., 2006; Cámara *et al*., 1994; GANCEDO & Luh, 1986; Ketnawa *et al*., 2012), although many other acids are present in tomatoes such as oxalic, succinic, glycolic, tartaric acids (Morvai & Molnár-Perl, 1992). According to the literature, the antimicrobial effect of weak organic acids are pH dependent (Hirshfield *et al*., 2003). pK_a values of citric acid and malic acid are 3.13 and 3.40, respectively, when the pH is lower than the pK_a, acids become weak (Pintado *et al*., 2009) so these two organic acids are expected to be the most effective against Listeria monocytogenes when the pH is around 3, this may probably explain why pineapple was unable to support the growth of Listeria monocytogenes in our study. The relative amount of different acids varies widely with the variety, degree of ripeness and seasonal influences (Gautier *et al*., 2008). Citric acid has

different modes of action and can be both protective and bactericidal against L. monocytogenes (Buchanan & Golden, 1994).

Listeria is also known to grow poorly in competition with other microorganisms (Amezquita & Brashears, 2002) and therefore the levels of TVC and LAB were determined at day 0 and at end of shelf life. The initial TVC ranged from 3.0 to 6.4 Log_{10} CFU/g lowest in grapes, tomatoes, cabbage and leafy salad and highest in beetroot (Table 2). This high initial level of other bacteria and mainly LAB in beetroot may explain the absence of growth of *L. monocytogenes.*

Tomatoes, white cabbage and grapes also had a low concentration of LAB (3.7-4.7 Log_{10} CFU/g) whereas the counts for the other products ranged from 5.3-7.5 Log_{10} CFU/g. Overall, LAB is the dominating group of bacteria at day 0 and in addition a high growth potential for LAB is seen in all products except beetroot and kohlrabi. However, beetroot and kohlrabi had a high initial concentration of LAB which can explain the limited increase in these products. In peppers and leafy salad products the concentration of LAB is higher than 8.5 Log_{10}CFU/g at the end of shelf like indicating a partly fermented product supported by the decrease in pH. The products were packed at normal atmosphere, but due to respiration of vegetables, oxygen concentration will be decreased in the package and CO_2 increase which explain the ability of LAB to grow to high levels. The increase in TVC was less except for peppers and pineapple where an increase of 3.5 Log units were seen (Table 2). Similar variations in the initial levels of bacteria in fresh-cut vegetables have been reported previously (Abadias *et al*., 2008; Sant'Ana *et al*., 2012). Interestingly, tomato, grapes, white and red cabbagehad the lowest concentration of TVC and LAB, and these four products are the only products which can support the growth of *L. monocytogenes*, indicating that the background microbiota play a role in controlling growth of *L. monocytogenes*. Similar associations between the role of the background microbiota on growth/survival of *L. monocytogenes* has

Table 2. Total bacterial count and lactic acid bacteria count of RTE vegetables on day 0 and day end

	Average Total Bacterial Count ±SD (log10 CFU/g)		Lactic Acid Bacteria Count (log10 CFU/g)	
RTE Vegetables	Day 0	Day end	Day 0	Day end
Pineapple	3.16 ± 2.03	7.66 ±0.11	4.59±0.11	7.63±0.11
Grapes	3.66 ±0.94	6.89 ± 0.10	3.0 ± 0	5.60 ± 0.26
Tomato	3.74 ± 0.10	7.28 ±0.35	3.0±0	3.70±0.33
Yellow pepper	6.29 ±0.10	9.17±0.10	5.47±0.10	8.97 ±0.14
Red pepper	6.48 ±0.05	8.94 ±0.27	4.89±0	8.22±0.12
Green pepper	6.54± 0.02	8.20±.026	4.50±0.09	7.82 ±0.05
Sliced beetroot	7.52±0.11	8.91 ±0.10	6.43 ±0.12	7.97 ± 0.2
Kohlrabi	7.12 ±0.12	8.75 ± 0.25	3.0 ± 0	3.53 ±0.16
Frisee lettuce	6.70±0.08	8.82 ± 0.12	3.0 ± 0	3.10 ±.014
Radicchio	6.61 ±0.20	8.75 ±0.20	3.0 ± 0	3.26 ±0.36
Lolla Rossa	6.59 ± 0.04	8.66 ±0.43	3.10 ± 0.14	3.16 ±0.22
White cabbage	3.96 ±0.18	6.65 ± 0.24	3.0 ± 0	3.94±0.34
Red cabbage	5.24±0.19	8.50 ± 0.14	3.0 ± 0	4.77 ±0.38

been found in shredded organic lettuce and mushrooms ((Oliveira *et al*., 2012) and specifically the presence of LAB for the inhibition of growth in white cabbage (Lokerse *et al*., 2016). Wash with chlorine is used in many countries to reduce the load of pathogens including *L. monocytogenes* on fresh RTE produce products. However, one challenge arise if pathogens are not fully eliminated, or the final product is re-contaminated and the natural competitiveness of indigenous microbiota is reduced as this may give better opportunities for *L. monocytogenes* to initiate growth.

The EU food safety criteria (EU2073/2005) define the acceptable concentrations of *L. monocytogenes* in RTE food products. According to these, food business operators shall conduct studies to evaluate whether their foodstuff is in compliance with the criteria through shelf life. In addition to challenge test or durability studies, predictive mathematical modeling can be used to assess if the food product in question is able to support growth. Here, Combase growth predictor (www.combase.cc) was used to predict the growth of *L. monocytogenes* in the tested products for comparison with the data obtained by the challenge test. However, it is known that these models are not as accurate for raw products as for e.g. processed meat and dairy products as the indigenous microbiota plays a minor role for the growth potential of *L. monocytogenes* in these products likely compared to vegetables (Ziegler *et al*., 2018). In combase only one pH value can be used and therefore we calculated the growth potential both for the mean and the highest pH value, there is deviations between the predictions and the growth potential obtained in this study. Some of the results were similar to our data however Combase could not predict the growth of *L. monocytogenes* in tomato and grapes. Combase also predicted growth in sliced beetroot, kohlrabi and lollorosaa which is not in agreement with our data.

According to the EU legislation, the limit of *L. monocytogenes* is 100 CFU/g (n=5, c=0) (products for infants or medical purposes not included) if the producer is able to demonstrate that the product will not exceed this limit throughout shelf life or the product is stabilized against growth of *L. monocytogenes*. A RTE food product is regarded as stabilized if a) pH $\leq$ 4.4 or aw$\leq$0.92, b) pH $\leq$5.0 and aw$\leq$ 0.94, c) shelf life less than 5 days d) frozen or e) based on scientific justifications (EU 2073). In case the producers are unable to demonstrate this to the satisfaction of the competent authority, *L. monocytogenes* must be absent in 25 g (n=5, c=0) before the food has left the immediate control of the food business operator. In case of noncompliance with the food safety criteria the food item must be recalled. It is therefore of great importance that the food business operator knows which of the produced products that are unable to support the growth of *L. monocytogenes* as no recall of food placed on the market is needed if *L. monocytogenes*<100 CFU7g is detected in the food products as part of the companies sampling as part of their own inspection program.

Conclusion

Producers of RTE food products with the risk of *L. monocytogenes* must have a risk based sampling plan for testing for compliance with the food safety criteria and for the control of the production environment. Knowledge about risk for both presence of *L. monocytogenes* in raw products and the growth potential is very important in the design of the sampling plan. In this study it was found that *L. monocytogenes* is unable to grow in

processed products of kohlrabi, green, red and yellow peppers, pineapple, beetroot, frisee, lollarosa, and radicchio salads incubated at 5 °C for 2 days followed by 10 °C for 4-6 days. In contrast, growth was detected in white cabbage, red cabbage and tomatoes. Growth of *L. monocytogenes* in these products was influenced by other factors than pH and a_w but these factors were not identified. However, the data indicates that the indigenous microbiota plays an important role to prevent growth of *L. monocytogenes*. Interestingly we found tomatoes and grapes to support the growth of *L. monocytogenes*despite the products have a pH value below 4.4 which is regarded as the lower limit for growth of *L. monocytogenes.*

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