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Growth potential of *Listeria monocytogenes* in twelve different types of RTE salads: Impact of food matrix, storage temperature and storage time

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ABSTRACT

Listeriosis is a food borne disease associated with high hospitalization and fatality rates; in 2014, EU member states reported 2194 cases with 98.9% hospitalization rates and 210 fatalities. Proper risk analysis and the development of effective food safety strategies critically depend on the knowledge of the growth characteristics of *L. monocytogenes* on the product in question. Ready-to-eat (RTE) salads present a challenge in this context due to the absence of a heat treatment step before consumption. This study provides challenge-test based data of the growth characteristics of *L. monocytogenes* on twelve RTE salads. The food matrix, storage time and storage temperature were factors with a significant impact on the growth of *L. monocytogenes*. While most tested salads permitted a significant increase of *L. monocytogenes* in at least one of the tested conditions, no growth was observed on celeriac, carrot and corn salad products. There was a considerable increase in growth at 8 °C compared to 5 °C. Our data indicate that the reduction of the storage temperature at retail level to 5 °C and product shelf life could help mitigate the risk of *L. monocytogenes* in RTE salads.

1. Introduction

Listeriosis remains one of the most severe food borne diseases. The relatively low incidence (0.46 cases/100,000 in the EU in 2015 with an ongoing, significant increase since 2008) (European Food Safety Authority, European Centre for Disease Prevention and Control, 2016) is offset by the a high mortality rate (15–30 deaths/100 cases) (Barton Behravesh et al., 2011; de Valk et al., 2005; Popovic et al., 2014; Werber et al., 2012), mostly due to severe central nervous system infections, septicemia and abortions/neonatal infections (Allerberger and Wagner, 2010). Food borne outbreaks of Listeriosis have been associated in the past with dairy products, fish and seafood, meat products, fresh fruit and vegetables, and ready-to-eat (RTE) products (Datta et al., 2013; European Food Safety Authority, European Centre for Disease Prevention and Control, 2016). Within the RTE food category, raw products without a heat-treatment step before consumption (e.g. RTE-salads, fruits, vegetables or dairy products) have an inherently increased health risk for the consumer. *Listeria monocytogenes* is a challenging problem in this context and a priority for food producers due to its frequent presence in the environment and the ability to grow at refrigeration temperatures. A current literature review of studies on the contamination levels of RTE salads with mixed ingredients (defined as raw salads combined with processed foods such as ham, chicken, salmon or pasta) concludes that within the EU, about 2–10% of products were contaminated with *L. monocytogenes* (Söderqvist, 2017). In 2015 the EU member states reported 0.04% of tested RTE salad products to exceed the legal limit of *L. monocytogenes* of 100 colony forming units (CFU)/g (European Food Safety Authority, European Centre for Disease Prevention and Control, 2016). In Switzerland, leafy green RTE salads caused an outbreak in 2013–2014 (Stephan et al., 2015). Producers of RTE salads are faced with two fundamental and contradictory requirements: (i) to provide food at the highest possible safety standards while (ii) meeting the demand from retailers and consumers for food with an increasingly long shelf life. The EU food safety regulations limit *L. monocytogenes* to < 100 CFU/g at the end of the shelf life of a food product. Accordingly, different food safety criteria are applicable based on whether a food supports the growth of *L. monocytogenes*: producers must test for absence in five samples of 25 g product before it leaves the immediate control of the food business operator, when he is not able to demonstrate, to the satisfaction of the competent authority, that the product will not exceed the limit of 100 CFU/g throughout the shelf-life. A food safety criterion of < 100 CFU/g applies if the manufacturer is able to demonstrate, to the satisfaction of the competent authority, that the product will not exceed the limit 100 CFU/g throughout the shelf-life (EC regulation No 2073/2005). To assess the risk associated with extending the shelf life, it is

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Table 1
L. monocytogenes strains used in this study.

Species	Strain designation	Serotype	ST	CC	Lineage
Listeria monocytogenes	N16-1125	1/2a	ST91	CC14	II
Listeria monocytogenes	N16-0716	1/2b	ST517	CC517	I
Listeria monocytogenes	N16-0855	4b	ST6	CC6	I

ST: sequence type. CC: clonal complex.

therefore crucial to determine the growth potential of *L. monocytogenes* in the respective food product.

The aim of this study was to determine the growth potential of *L. monocytogenes* on twelve RTE salad products, under packaging and storage conditions that mirror retail and home conditions.

2. Materials and methods

All experiments were carried out in three independent replicates.

2.1. Bacterial strains, growth conditions and subtyping

The three strains of *L. monocytogenes* used in this study were isolated from a RTE salad production facility (Table 1). Stock cultures of *L. monocytogenes* were maintained at −80 °C in brain heart infusion (BHI; Oxoid, Basel, Switzerland) 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 (9.6 ± 0.1 log CFU/ml). This culture was then incubated at 5 °C for 20 h for cold adaptation. Strain pools were obtained by combining equal quantities of the cold adapted stationary phase cultures.

The three strains used in this study were serotyped using Listeria antisera from Denka Seiken (Pharma Consulting, Burgdorf, Switzerland) according to the manufacturer's protocol. They were then further characterized using multi locus sequence typing (MLST) based on seven housekeeping genes as described by Ragon et al. (2008) with minor modifications. DNA was extracted using the DNeasy Blood& Tissue Kit (Qiagen, Hilden, Germany) according to manufacturer's protocol. The PCRs were performed using a HotStarTaq master mix (Qiagen, Hilden, Germany) and the following conditions: for *bglA, cat* and *ldh*: 95 °C for 15 min, followed by 41 cycles of 95 °C for 30 s, 45 °C for 30 s and 72 °C for 30 s; for *abcZ, dapE, dat*, and *lhkA*: 95 °C for 15 min followed by 35 cycles of 95 °C for 30 s, 52 °C for 30 s, and 72 °C for 30 s. The final extension was at 72 °C for 10 min for all amplifications.

PCR products were purified using the GenElute PCR Clean-Up Kit (Sigma, Steinheim, Germany) according to manufacturer's protocol and were sequenced using primers oR and oF (Ragon et al., 2008). The clonal complex (CC) and sequence type (ST) were determined using the *Listeria* MLST database hosted at the Institute Pasteur (http://bigsdb.pasteur.fr).

2.2. Cold growth in rich medium

Growth at cold storage temperatures was determined for the three *L. monocytogenes* strains used in this study individually in a control experiment. Early stationary phase cultures were obtained and cold adapted as described above. This culture was then diluted 1:1000 into 10 ml fresh BHI broth and incubated at 5 °C and 8 °C for 11 days. CFU/ml were determined by plate counting 10 µl aliquots from each culture at t = 0 and after 1, 4, 5, 6, 7, 8, and 11 days. Aliquots were serially diluted in Maximum Recovery Diluent (MRD; Oxoid, Basel, Switzerland) and 10 µl of the serial dilutions were spotted on BHI agar plates and run over by tilting as described by Kühbacher et al., 2014. Plates were incubated at 37 °C for 24 h. Final results were expressed as log CFU/ml.

2.3. Salad products

The range of salads was limited to plant based products without added ingredients of animal origin, and were chosen to represent commonly sold RTE salad products. A total of 12 different RTE salad products from a producer in Switzerland were used for the challenge tests: Iceberg lettuce (*Lactuca sativa*, cut to 6 mm, 100%), corn salad (*Valerianella locusta,* entire rosettes 100%), grated carrots (*Daucus carota* subsp. *sativus*, peeled, grated 100%), carrot julienne (*Daucus carota subsp. sativus,* peeled, julienne, 1.6 mm 100%), mixed salad 1 (iceberg lettuce *Lactuca sativa* 35%, endive *Cichorium endivia* 14%, sugarloaf lettuce *Cichorium intybus* var. *foliosum* 20%, lollo rosso lettuce *Lactuca sativa* var. *crispa* 15%, frisee lettuce *Cichorium endivia* L. var. *crispum Lam.*16%, all cut 20 mm), mixed salad 2 (endive *Cichorium endivia* 25%, multileaf lettuce 20%, rosso 15%, lollo rosso salad *Lactuca sativa* var. *Crispa* 10%, head lettuce *Lactuca sativa* var. *Capitata* 5% [all cut 40-50 mm], arugula *Eruca sativa* [entire leaves] 5%, carrot *Daucus carota* subsp. *sativus* [peeled, chips] 10%, garden radish *Raphanus sativus* var. *Sativus* [slices] 10%), parsley (*Petroselinum crispum,* minced 100%), garden radish (*Raphanus sativus* var. *Sativus,* julienne, 3 mm, 100%), beetroot (*Beta vulgaris,* spaghetti-style, 100%), arugula (*Eruca sativa* entire leaves, 100%), celeriac (*Apium graveolens* julienne, 1.6 mm dipped in an ascorbic acid/citric acid bath [0.875 g/l and 16.25 g/l] for 20–30 min, 100%), white cabbage (*Brassica oleracea* convar. *Capitata* var. *alba*, julienne, 1 mm 100%).

Three RTE salad products were packaged under modified atmosphere (Table 2). To avoid sampling effects due to uneven distribution of *L. monocytogenes* within a package, the whole content of a bag was used for analysis. For this purpose, 30 g salad portions (parsley: 20 g portions) were produced specifically for this study, shipped to our facility under preservation of the cold chain at 5 °C and inoculated 12–24 h after production. The packaging foil and, where appropriate, the modified atmosphere was identical to the larger packages that were produced for retail.

2.4. Inoculation of the food matrix

To determine the growth of all three strains used in this study individually on salad products, a first experiment was performed with the three *L. monocytogenes* strains inoculated individually on two RTE salad products (iceberg lettuce and arugula). Based on the results of these preliminary experiments, the other ten RTE salad products were inoculated with an equalized pool of the three strains.

To achieve a final bacterial load of 5 log CFU/g in the products, the cold adapted stationary phase culture was serially diluted in 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. Unlike in other products that include a microbial kill step during processing, the normal microbiota in RTE salad products is in the range of 5–6 log CFU/g and the administered inoculum is therefore only a fraction of the total bacteria present in the samples. 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 modified atmosphere. Negative control samples were inoculated in the same way with 1 ml MRD, and for RTE salad products packaged under modified atmosphere, uninoculated bags of product served as controls for the modified atmosphere. After inoculation, all samples were shaken for 1 min in a standardized manner to optimize the distribution of the inocula. To preserve the cold chain, the bags of salad and the bacteria were kept on ice at all times during these procedures.

Table 2
Growth potential of *L. monocytogenes* in different RTE salad products.

RTE salad	Modified atmosphere packaging (MAP)	Challenge test results summary				
			5 °C		8 °C	
		contrast[c]	δ[a]	p-value[b]	δ[a]	p-value[b]
Iceberg	O_2 1–7%, CO_2 9–15%	0–4	0.82	*	1.44	*
		0–5	0.85	*	1.73	*
		0–6	0.81	*	1.93	*
		0–7	1.00	*	1.71	*
		0–8	0.90	*	2.23	*
Corn salad	n/a[d]	0–4	−0.83	*	−0.61	*
		0–5	−0.87	*	−0.71	*
		0–6	−1.06	*	−0.81	*
		0–7	−1.23	*	−0.76	*
		0–8	−1.13	*	−0.58	*
Grated carrots	n/a[d]	0–4	−2.62	*	−2.15	*
		0–5	−2.76	*	−2.06	*
		0–6	−3.10	*	−2.53	*
		0–7	−2.76	*	−2.63	*
		0–8	−3.10	*	−3.10	*
Julienne carrots	n/a[d]	0–4	−2.15	*	−2.58	*
		0–5	−2.73	*	−2.36	*
		0–6	−2.51	*	−2.36	*
		0–7	−2.73	*	−2.51	*
		0–8	−2.91	*	−2.58	*
Mixed salad 1	O_2 4–10%, CO_2 8–14%	0–4	0.56	ns	0.93	*
		0–5	0.49	ns	0.95	*
		0–6	0.54	ns	0.91	*
		0–7	0.69	ns	1.00	*
		0–8	0.62	ns	0.77	*
Mixed salad 2	O_2 5–11%, CO_2 9–15%	0–4	0.09	ns	0.82	*
		0–5	0.33	ns	1.50	*
		0–6	0.42	ns	1.37	*
		0–7	0.53	ns	1.46	*
		0–8	0.64	ns	1.14	*
Parsley	n/a[d]	0–4	0.66	ns	2.14	*
		0–5	0.73	ns	2.37	*
		0–6	1.14	ns	2.52	*
		0–7	1.27	ns	2.46	*
		0–8	1.25	ns	2.84	*
Garden radish	n/a[d]	0–4	0.20	ns	1.23	*
		0–5	0.41	ns	1.34	*
		0–6	0.38	ns	1.50	*
		0–7	0.32	ns	1.28	*
		0–8	0.56	ns	1.31	*
Beetroot	n/a[d]	0–4	0.53	ns	1.59	*
		0–5	0.53	ns	1.31	*
		0–6	0.96	ns	1.59	*
		0–7	0.64	ns	1.42	*
		0–8	0.58	ns	1.58	*
Arugula	n/a[d]	0–4	0.23	ns	1.06	ns
		0–5	0.42	ns	1.03	ns
		0–6	0.21	ns	0.89	ns
		0–7	0.28	ns	0.89	ns
		0–8	0.39	ns	1.05	ns
Celeriac	n/a[d]	0–4	−0.30	*	−0.78	*
		0–5	−0.48	*	−1.17	*
		0–6	−0.56	*	−1.38	*
		0–7	−0.81	*	−1.68	*
		0–8	−1.01	*	−1.75	*
White cabbage	n/a[d]	0–4	0.70	ns	1.84	*
		0–5	0.92	ns	1.76	*
		0–6	0.91	ns	1.67	*
		0–7	0.74	ns	1.51	*
		0–8	0.89	ns	1.48	*

[a] Growth potential defined as the maximal delta between t = 0 and t = x in three independent replicates.

[b] * denotes p-values for the contrast between t = 0 and t = x that are significant ($p < 0.05$). ns denotes non-significant p-values.

[c] Contrasts define the time interval in days that was measured, e.g. between t = 0 and t = 4.

[d] Not applicable, these products were not packaged under modified atmosphere.

2.5. Storage conditions

The RTE salad products were stored at 5 °C and 8 °C, for 4, 5, 6, 7 and 8 days. Temperature in both cold rooms was continuously controlled and recorded with temperature loggers (EasyLog, Lascar Electronics, Pennsylvania, USA).

2.6. Microbiological analyses

In addition to *L. monocytogenes*, total viable counts (TVC) and total *Enterobacteriaceae* were determined to assess the level of the background microbiota and its change over time.

Enumeration of *L. monocytogenes*, *Enterobacteriaceae* and the TVC were performed according to ISO 11290-2:1998, ISO 21528-2:2004 and ISO 4833-2:2013 respectively with minor modifications.

L. monocytogenes and TVC were determined immediately after inoculation (t = 0) and 4, 5, 6, 7 and 8 days after inoculation. The counts of *Enterobacteriaceae* were determined at t = 0 and 8 days after inoculation. At each time point, one inoculated sample and one negative (uninoculated) control sample per temperature were analyzed. The whole content of a unit was transferred into sterile stomacher bags, diluted 1:10 with MRD and homogenized for 30 s in a Stomacher® 400 Circulator (Seward, Worthing, United Kingdom). In products that were packaged under modified atmosphere, the gas composition was measured at each time point in all samples and the uninoculated negative controls using a "CheckPoint O_2/CO_2" sensor (Dansensor, Denmark) according to the manufacturers guidelines (Supplementary Table 1). Serial dilutions in 10 ml 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* (aerobic incubation for 24 h at 37 °C); plate count (PC; Oxoid, Basel, Switzerland) for TVC (aerobic incubation for 48 h at 37 °C); violet red bile glucose (VRBG; BD, Allschwil Switzerland and Merck, Darmstadt Germany) for *Enterobacteriaceae* (anaerobic incubation for 48 h at 37 °C). The average of the duplicate plates was calculated and expressed as log CFU/g. The limit of detection was 2 log CFU/g.

2.7. 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 salad 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 salad product was classified as "unable to support the growth of *L. monocytogenes*".

2.8. Statistical analysis

Statistical analysis and graphics were performed in R (Version 3.4.0) (R Core Team, 2015) using R studio (Version Version 1.0.143) (RStudio Team, 2015). A linear mixed effects model was calculated using lmer in LmerTest (Kuznetsova et al., 2017) with up to three-way interactions between time, salad and temperature as random effects and replicate as fixed effect. Pairwise contrasts were calculated using lsmeans (Lenth, 2016). The ggplot2 package (Wickham, 2009) was used for visualization. Holm-Bonferroni adjusted *p*-values were calculated for multiple comparisons within one type of salad. p-values < 0.05 were considered significant.

To determine if the three *L. monocytogenes* strains differed from each other in their growth on salad, linear mixed effects models were calculated; model selection was AIC based. An ANOVA was used to determine if the factor "strain" had an effect on the outcome "log CFU/ml". For the final analysis, the results from the individual strains on

Table 3
Combase growth predictions for *L. monocytogenes* growth on selected RTE salad products.

			Combase prediction					
			5 °C			8 °C		
RTE salad	aw-value	pH	max log CFU[1]	doubling time[2]	μ max[3]	max log CFU[1]	doubling time[2]	μ max[3]
Grated carrots	0.999	6.43	7.05 (1.6)	16.0	0.019	8.52 (1.6)	8.86	0.034
Corn salad	0.995	6.84	7.53 (3.6)	14.2	0.021	8.52 (4.1)	7.82	0.038
Iceberg	0.999	6.72	7.52 (6.2)	18.0	0.017	8.52 (6.8)	9.92	0.030
Parsley	0.999	6.82	7.39 (5.8)	14.7	0.021	8.52 (7.4)	8.11	0.040

[1] maximal log CFU after 8 days. Values in brackets represent the log CFU that were experimentally measured at day 8 for comparison [2] in hours [3] maximal growth rate in log CFU/h.
[2] in hours
[3] maximal growth rate in log CFU/h.

iceberg lettuce and arugula were combined to be comparable to the results from the experiments with the pooled strains. The R scripts can be downloaded from (Supplementary R_scripts_and_data).

2.9. Measurement of pH and aw-values, modelling of microbial growth

The aw-value and pH for four of the RTE-salad products included in this study (iceberg, parsley, grated carrots, corn salad) were determined. For pH measurements, the products were homogenized using a kitchen appliance homogenizer (Braun, Germany), the pH of the homogenate was measured with a SevenCompact pH meter (Mettler Toledo, Greifensee, Switzerland) two times and the average of the two values was reported (Table 3).

To determine a_w values, an AQUALAB water activity meter 3TE (METER Food, Munich, Germany) was used according to the manufacturer's instructions. These values were used as an input into the growth predictor module within Combase (https://www.combase.cc) with the "physical state" value at default.

3. Results and discussion

3.1. Strain characterization and results from the proof of concept experiments

The three strains used in this study belonged to serotype 1/2a, 1/2b and 4b, CC 14, 517 and 6 and ST 91, 517 and 6 respectively (Table 1). This diversity between strains that were isolated from the same production facility eliminates a single source of contamination and suggest multiple introduction events of *L. monocytogenes* into the facility.

All three *L. monocytogenes* strains were able to grow at the 5 °C and 8 °C in rich medium and reached early stationary phase after eleven days at 5 °C vs. after five days at 8 °C. There was no difference in cold growth between the three strains (Supplementary Fig. 1).

The results of the first experiments (on iceberg lettuce and arugula samples) that were performed with each strain individually showed no significant effect of "strain" on "log CFU/ml" in the outcome ($F = 2.57$, $p = 0.08$).

The temperature was logged hourly for the 5 °C storage unit (mean 4.9 °C, SD 1.13, maximum 8 °C) and the 8 °C storage unit (mean 8.11 °C, SD 0.53, maximum 13 °C) over the complete duration of the experiments (152 days).

For those salads packaged under modified atmosphere, the gas composition was monitored. In mixed salad 1, none of the values were outside the O_2/CO_2 value range specified by the producer (Supplementary Table 1), while in the iceberg lettuce, some of the units stored at 8 °C were out of range. Many of the mixed salad 2 units were out of range, including some of the uninoculated controls (Supplementary Table 1). This may indicate a partial failure of this specific packaging/produce combination to reach a steady state between plant respiration and diffusion of gases through the packaging foil. The airtightness of the scotch tape used to seal the inoculation hole was tested by puncturing three bags and applying the seal before storing them for 72 h with 2.2 kg weight on top of them. None of the bags deflated (data not shown).

3.2. Growth of L. monocytogenes on RTE salad products

We found that storage temperature, storage duration and the food matrix had a significant impact on the growth potential of *L. monocytogenes* under the tested conditions.

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

At 5 °C, the growth potential of *L. monocytogenes* exceeded 0.5 log CFU in both mixed salads, parsley, iceberg lettuce, garden radish, beetroot and white cabbage at least on one time point (Fig. 1, Table 2). This was either at very late time points (e.g. at t = 8 for garden radish) and/or not by a large margin, e.g. the growth potential was between 0.5 and 1 log CFU. Also, the increase in CFU/g between t = 0 and the later time points was only statistically significant in iceberg lettuce (Table 2).

At 8 °C, the situation was drastically different. With the exception of corn salad, celeriac and carrots, *L. monocytogenes* exhibited a growth potential over or very close to 1.0 log CFU. This increase in CFU/g between t = 0 and the later time points was statistically significant in all salads except arugula (Table 2).

The clearly higher growth potential of *L. monocytogenes* at 8 °C compared to 5 °C may challenge the common practice to store RTE products at 8 °C at retail level. Our data suggest that in some cases, growth of *L. monocytogenes* to > 100 CFU/g during shelf life could be mitigated by a stricter temperature management at retail level to limit storage temperature of RTE salad products to a maximum of 5 °C.

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

As expected, time was a crucial factor in the outcome of total counts (CFU/g) of *L. monocytogenes*. In many of the salad products that supported the growth of *L. monocytogenes* (e.g. garden radish and white cabbage at 8 °C and iceberg lettuce at 5 °C and 8 °C), *L. monocytogenes* increased significantly between t = 0 and t = 4, after which a steady state was reached where no more significant increase occurred (levels between 10^5 and 10^7 CFU/g depending on the product). This steady state was not observed in the total viable counts. A potential explanation for this plateau might be the interaction with the plant microbiota associated with individual plant species that limits further growth. In contrast, parsley at 8 °C permitted a significant increase in CFU/g also between t = 4 and the later time points. This suggests that minced parsley is a product that supports substantial growth of *L. monocytogenes*.

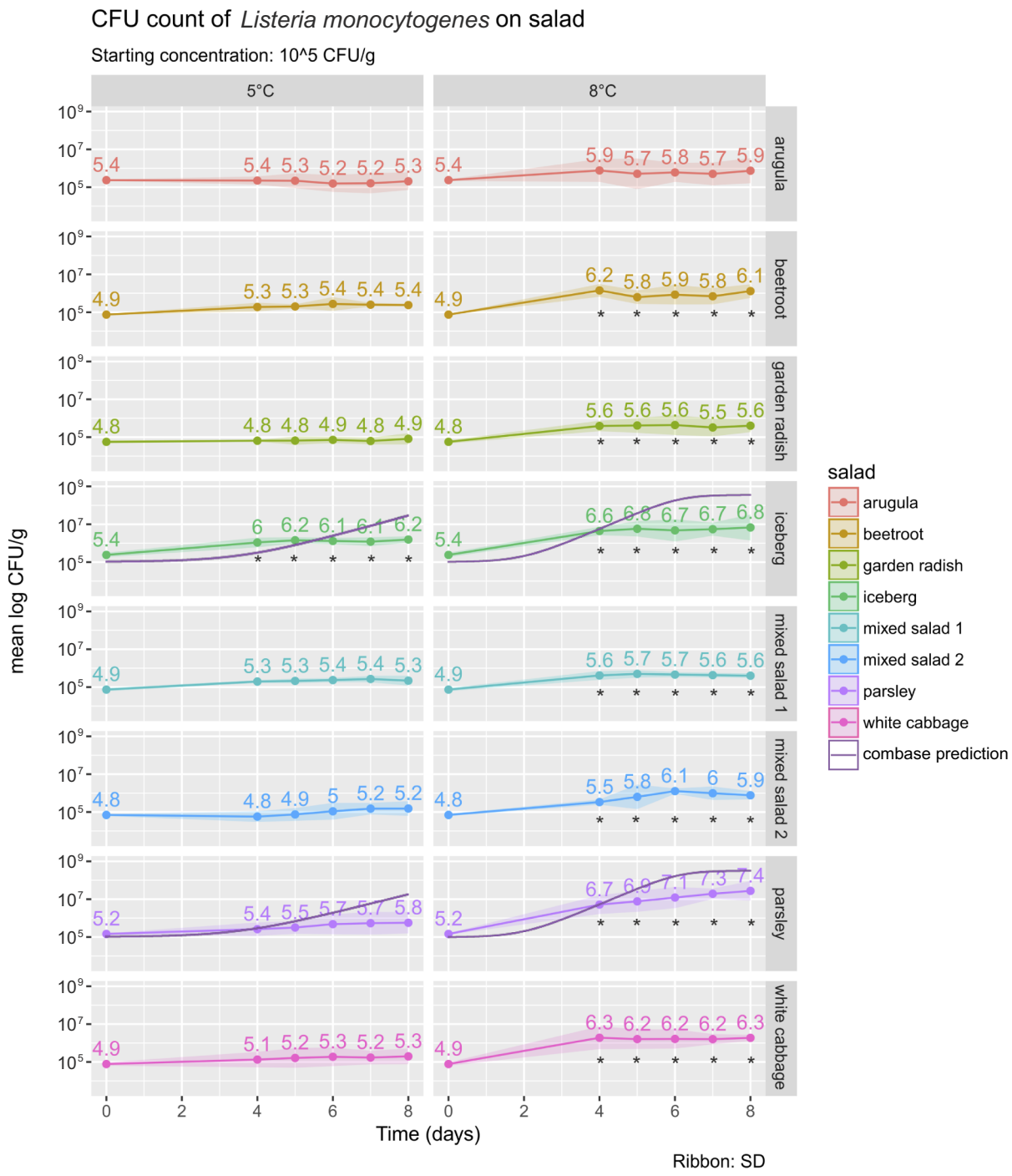

Fig. 1. *L. monocytogenes* on RTE salads that supported growth. The numbers above time points reflect mean log CFU/g. Asterisks denote time points where the log CFU/g were significantly different from $t = 0$. The ribbon around the line represents the standard deviation. For comparison, Combase predictions for a selection of products were added to the graph.

3.2.3. Impact of the food matrix and its associated microbiota on the growth potential of L. monocytogenes

The food matrix had a large impact on the growth potential of *L. monocytogenes*. Four of the twelve RTE salad products tested in this study did not support the growth of *L. monocytogenes* in a significant way under the tested conditions, while the other eight products supported growth of *L. monocytogenes* at varying levels (Table 2). Potential reasons for these vast differences may lie in the normal microbiota of the plant, the composition of the product, the level of processing of the plant matrix (e.g. availability of plant juice depending on the level of cutting vs. whole leaves), and in the natural defense mechanisms of the plants in question.

The product group that supported growth of *L. monocytogenes* comprised iceberg lettuce, parsley, arugula, beetroot, garden radish, white cabbage and the mixed salads. The maximal growth potential of 2.8 log CFU/g was observed in parsley (t = 8 days, 8 °C) (Fig. 1).

Listeria has previously been shown to grow on iceberg lettuce (at 3, 5 and 10 °C) (Beuchat and Brackett, 1990b; Koseki and Isobe, 2005), arugula (6 days at 7 °C) (Sant'Ana et al., 2012) and "green salad" (6 days at 7 °C) (Sant'Ana et al., 2012). Conflicting data exist on raw cabbage: one study on white cabbage found considerable growth (15 days at 4 °C, 7.5 days at 10 °C) (Wang et al., 2013), while another study on RTE

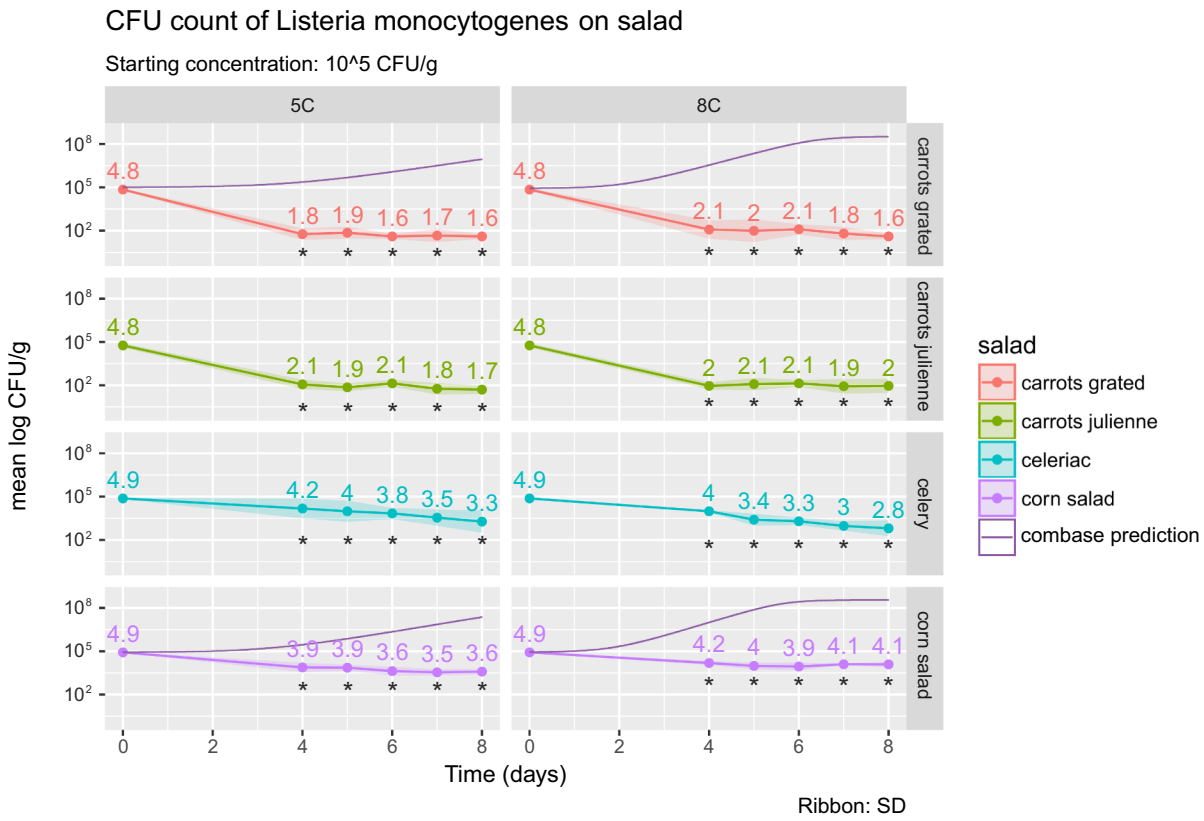

Fig. 2. *L. monocytogenes* on RTE salads that did not support growth. The numbers above time points reflect mean log CFU/g. Asterisks denote time points where the log CFU/g were significantly different from t = 0. The ribbon around the line represents the standard deviation. For comparison, Combase predictions for a selection of products were added to the graph.

cabbage found a reduction in *L. monocytogenes* numbers (6 days at 7 °C = −0.21 (SD 0.9) log CFU/g) (Sant'Ana et al., 2012). However, it is unclear if the cabbage in this latter study was processed or raw. Juice from fermented cabbage might have some antimicrobial properties against Gram negative organisms (Gogo et al., 2010).

Celeriac, carrots and corn salad did not support the growth of *L. monocytogenes*. At no point in time did the growth potential of *L. monocytogenes* exceed 0.5 log CFU in these products. In fact, the CFU/g *L. monocytogenes* dropped significantly between t = 0 and the later time points at both 5 and 8 °C (Fig. 2). To our knowledge, this is the first report on the fate of *L. monocytogenes* on corn salad and celeriac root. Other authors have found temperature-dependent effects on growth or survival of *L. monocytogenes* on celery stalks: at 4 °C a decrease in CFU/g was observed over 7 days (Vandamm et al., 2013), while at 10 °C growth or a minimal increase was found over 7 days (Kaminski et al., 2014; Vandamm et al., 2013). Also, celery stalks have been implied in an outbreak of listeriosis (Gaul et al., 2013). The relatively high content of nitrite in celery in combination with the fine cut of the celeriac julienne analyzed in the present study might provide a possible explanation for our observations; in fact celery powder is used as a natural source of nitrite in meat curing processes for its antimicrobial activity and coloring properties (Buchanan et al., 1989; Junttila et al., 2016; McClure et al., 1991). The anti-listerial effect of carrots has been attributed to phytoalexins produced by carrots in response to fungal infections and other types of stress (Abdul-Rauf et al., 1993; Babic et al., 2008; Beuchat and Brackett, 1990a; Kurosaki and Nishi, 1983). It is conceivable that harvesting might result in increased concentrations of phytoalexins in carrots due to microlesions in the plants. The activity of phytoalexins is mainly directed against fungi and Gram+ organisms (Kurosaki and Nishi, 1983). This would spare the mostly Gram- natural microbiota of the plants, which is reflected in the fact that the total viable count increased over time in all salads except celeriac at 5 °C.

3.3. Total viable count on RTE salad products

The initial TVC ranged from 4.9 log CFU/g (celeriac (SD = 0.09), white cabbage (SD = 0.14)) to 6.7–6.8 log CFU/g (parsley (SD = 0.44), arugula (SD = 0.71), garden radish (SD = 0.24)). At 8 °C, all products showed a significant increase in TVC at all time points, reaching as much as 9 log CFU/g (parsley). At 5 °C, celeriac (all time points) and arugula (all time points except at t = 8) showed no significant increase in TVC. In all other products, there was a significant increase in TVC (Fig. 3). While other authors found similar counts for the TVC on iceberg lettuce (5.61 ± 0.41 log CFU/g) (Koseki and Isobe, 2005) and carrots (5.79 ± 0.04 log CFU/g) (Sant'Ana et al., 2012), studies found higher numbers for cabbage (7.67 ± 0.09 log CFU/g) (Sant'Ana et al., 2012), arugula (8.11 ± 0.16 log CFU/g) (Sant'Ana et al., 2012) and corn salad (6.63–6.85 log CFU/g) (Wei et al., 2005). However, the validity of comparisons between studies is at least questionable, as many factors like plant variety and maturity, the processing conditions, packaging and gas composition, or storage temperature will influence the outcome. Additionally, the specific microbiota of the plant may vary regionally and will engage in complex interactions with pathogens like *L. monocytogenes* (Brandl, 2006).

3.4. Total Enterobacteriaceae on RTE salad products

As most of the epiphytic microbiota of plants belongs to either *Pseudomonas* or the *Enterobacteriaceae* (Lund, 2008), the *Enterobacteriaceae* count was determined in all RTE salad products. The initial count for *Enterobacteriaceae* ranged from 2.5 log CFU/g (celeriac, SD = 0.61) to 5.5 log CFU/g (corn salad, SD = 0.69). With the exception of corn salad, the CFU count for *Enterobacteriaceae* increased between t = 0 and t = 8 at 5 °C and at 8 °C. The sharpest increase in *Enterobacteriaceae* was observed in iceberg lettuce (3.5 and 3.6 log CFU/g

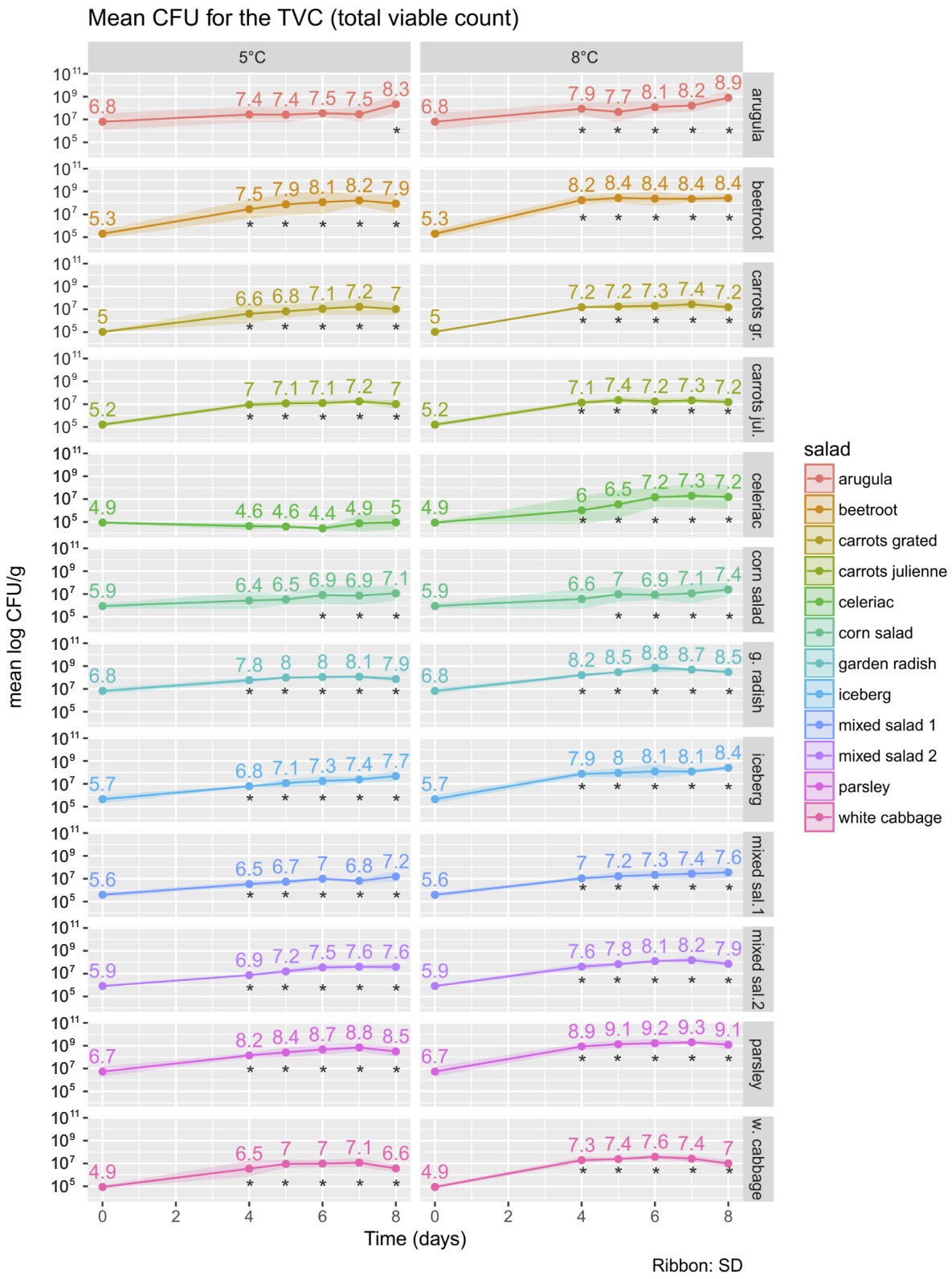

Fig. 3. Total count of viable bacteria at 37 °C in RTE salad products. The numbers above time points reflect mean log CFU/g. Asterisks denote time points where the log CFU/g were significantly different from t = 0. The ribbon around the line represents the standard deviation. gr. grated; jul. julienne; g. garden; sal. Salad; w. white.

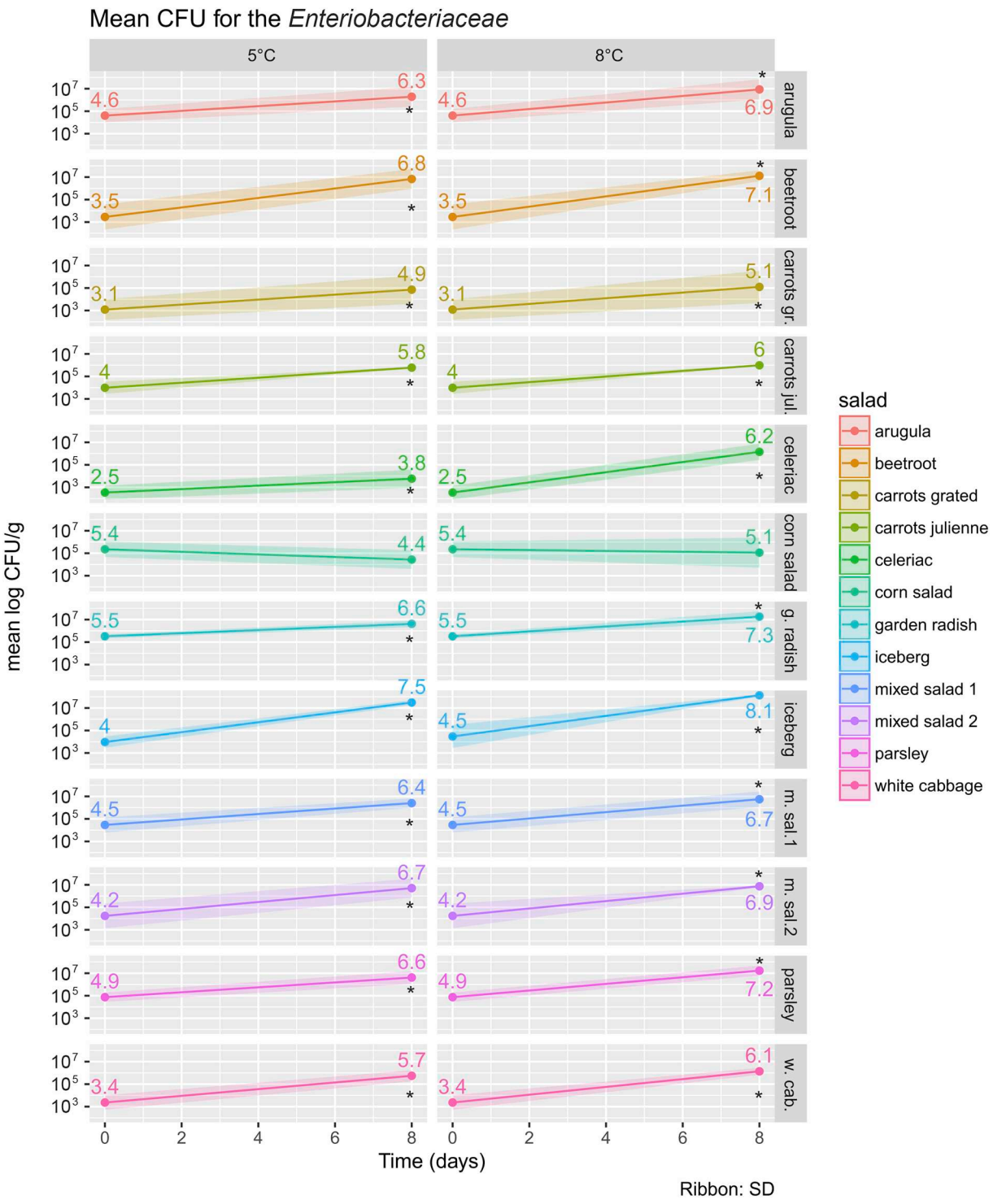

Fig. 4. Total count of *Enterobacteriaceae* in RTE salad products. The numbers above time points reflect mean log CFU/g. Asterisks denote time points where the log CFU/g at t = 8 were significantly different from t = 0. The ribbon around the line represents the standard deviation. gr. grated; jul. julienne; g. garden; m. mixed; sal. salad; w. white; cab. cabbage.

difference between t = 0 and t = 8 at 5 °C and 8 °C, respectively, $p < 0.001$). In corn salad, a decreasing trend in *Enterobacteriaceae* was observed at both temperatures (−0.9 and − 0.3 log CFU/g difference between t = 0 and t = 8 at 5 °C and 8 °C, respectively, not statistically significant) (Fig. 4). These numbers we found are within the expected range of 4–8 log CFU/g (European Commission, Scientific Committee on Food, 2002).

3.5. Available microbial models

EC no 2073/2005 regulates the microbial criteria for foodstuffs and mandates that the food production officer shall “conduct studies (…) to investigate the compliance with the criteria during shelf-life”. Apart from challenge tests, which are labor intensive and require advanced research capacities, annex II of the EC no 2073/2005 allows the use of

"predictive mathematical modelling established for the food in question" to assess the growth potential of *L. monocytogenes* in food. To assess the feasibility of predictive mathematical modelling for the growth of *L. monocytogenes* on products included in this study, predictions for two RTE salads that showed a large growth potential in our experiments (iceberg, parsley) and two products with no growth potential (grated carrots, corn salad) were obtained using the Combase growth predictor (https://www.combase.cc). These calculations resulted in very similar predictions between all four RTE-salads (Table 3, Figs. 1 and 2) that were in the range of one log higher than the results of the challenge test. In the challenge test situation, factors other than pH and aw value had an influence on the growth of *L. monocytogenes* in iceberg, parsley, grated carrots and corn salad. While existing modelling tools use physicochemical properties such as temperature, water phase salt, pH, CO2, smoke intensity, nitrite and organic acids to predict growth of microorganisms, the plant microbiota as well as the specific defense mechanisms inherent to individual plant species are not part of the models. They are likely factors to contribute to the large differences in growth potential that were observed experimentally. Other authors also found large discrepancies when comparing observed growth of *L. monocytogenes* in a cheese food matrix with predictions obtained from the Combase Modelling Toolbox in 2011, and the authors caution against applying data obtained in laboratory media to model growth in food systems (Schvartzman et al., 2011). Therefore, while conservative estimates of the growth potential can be obtained using Combase, challenge tests in individual products provide a much more accurate representation of the microbial growth potential. Other available models for *L. monocytogenes* are either geared towards meat/seafood ("food spoilage and safety predictor FSSP" http://fssp.food.dtu.dk, "listeria meat model" http://www.cpmf2.be/software.php, "DMRI predictive models for meat" http://dmripredict.dk) or dairy products ("dairy products safety predictor" https://aqr.maisondulait.fr). It remains a fact that the growth of pathogens on food matrix, and especially raw products, is notoriously difficult to model due to interactions with their microbiota. The linear mixed effects model calculated on the data in this study (Supplementary R_scripts_and_data) is perfectly valid for the dataset established in this study. However, extrapolations to other salad products would have to be done very carefully. The results are likely affected by changes in the microbiota and composition of the salad matrix due to differences in soil composition, farming practices and seasonal fluctuations in temperature and humidity. The same is true for extrapolations from public databases on microbial growth in food such as Combase (https://www.combase.cc). Therefore, even though the EU-legislation allows for the use of microbial modelling tools to assess the growth potential of *L. monocytogenes* in food, the generation of solid wet lab data in challenge tests is crucial for complex food matrices such as RTE salads composed of different raw produce.

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Competing interest statement

The authors declare no competing interests.

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