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Research Paper

Fate of *Listeria monocytogenes,* Pathogenic *Yersinia enterocolitica,* and *Escherichia coli* O157:H7 *gfp*$^+$ in Ready-to-Eat Salad during Cold Storage: What Is the Risk to Consumers?

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ABSTRACT

In this study, we investigated the fate of *Listeria monocytogenes,* pathogenic *Yersinia enterocolitica,* and *Escherichia coli* O157:H7 *gfp*$^+$ inoculated in low numbers into ready-to-eat baby spinach and mixed-ingredient salad (baby spinach with chicken meat). Samples were stored at recommended maximum refrigerator temperature (8°C in Sweden) or at an abuse temperature (15°C) for up to 7 days. Mixed-ingredient salad supported considerable growth when stored at 15°C during shelf life (3 days), with populations of *L. monocytogenes,* pathogenic *Y. enterocolitica,* and *E. coli* O157:H7 *gfp*$^+$ increasing from less than 2.0 log CFU/g on day 0 to 7.0, 4.0, and 5.6 log CFU/g, respectively. However, when mixed-ingredient salad was stored at 8°C during shelf life, only *L. monocytogenes* increased significantly, reaching 3.0 log CFU/g within 3 days. In plain baby spinach, only pathogenic *Y. enterocolitica* populations increased significantly during storage for 7 days, and this was exclusively at an abuse temperature (15°C). Thus, mixing ready-to-eat leafy vegetables with chicken meat strongly influenced levels of inoculated strains during storage. To explore the food safety implications of these findings, bacterial numbers were translated into risks of infection by modeling. The risk of listeriosis (measured as probability of infection) was 16 times higher when consuming a mixed-ingredient salad stored at 8°C at the end of shelf life, or 200,000 times higher when stored at 15°C, compared with when consuming it on the day of inoculation. This indicates that efforts should focus on preventing temperature abuse during storage to mitigate the risk of listeriosis. The storage conditions recommended for mixed-ingredient salads in Sweden (maximum 8°C for 3 days) did not prevent growth of *L. monocytogenes* in baby spinach mixed with chicken meat. Manufacturers preparing these salads should be aware of this, and recommended storage temperature should be revised downwards to reduce the risk of foodborne disease.

Key words: Baby spinach; Deli salad; Growth potential; Leafy vegetables; Mixed-ingredient salad; Temperature abuse

Consumption of healthy and convenient foods such as ready-to-eat (RTE) fresh-cut vegetables or mixed-ingredient salads has increased in recent years *(16, 43)*. Mixed-ingredient salads typically consist of raw vegetables, e.g., leafy vegetables, and a protein source, e.g., poultry meat. They may also include carbohydrate sources, such as pasta. Mixed-ingredient salads may be prepared by a manufacturer, in a catering facility, or in-store, and are often distributed in single-serve containers *(22)*. Some of the ingredients in mixed salads, for example, poultry meat and ham, are heat treated before preparation; however, they may be contaminated after this treatment, e.g., when diced or sliced, and then may provide an excellent protein-rich substrate for bacterial growth in the final product. Other ingredients, like leafy vegetables, rely on good agricultural practices, good manufacturing practice, a washing step, and cold storage to minimize microbial risk. However, there are several opportunities for pathogens to contaminate during the production of RTE leafy vegetables, e.g., via fecally contaminated irrigation water *(3, 20)*. Chlorine is often used as a sanitizing agent during washing of fresh produce, resulting in a reduction in microorganism levels of 1 to 2 log CFU/g *(8),* but use of sanitizers is not permitted in some countries, e.g., Sweden *(30)*. Use of potable water for washing of leafy vegetables reduces the number of bacteria by only 0.1 to 1 log unit *(8)*. Consequently, several different food sources, such as meat and raw vegetables, may play an important role in determining the microbiological quality of the final mixed-ingredient salad product.

Shiga toxin–producing *Escherichia coli* and *Salmonella* are the most common bacterial sources of foodborne outbreaks linked to leafy vegetables *(7, 11)*. One well-known *E. coli* O157:H7 outbreak in the United States was associated with bagged spinach, with 205 confirmed cases of illness and three deaths *(52)*. Another *E. coli* O157:H7

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outbreak in Sweden, with 135 cases of illness, was associated with iceberg lettuce *(47)*. Other examples of pathogens associated with outbreaks caused by RTE leafy green vegetables are pathogenic *Yersinia enterocolitica* O:9 *(33)* and *Listeria monocytogenes (54)*. Because both of these are psychrotrophs and, thus, are able to grow at refrigerator temperature, they are of particular concern for refrigerated RTE food during its shelf life *(55)*. *L. monocytogenes* is of special interest, because it is ubiquitous and may, therefore, be present in raw foods. Moreover, this pathogen has the ability to form biofilms in food processing plants, which may be one cause of cross-contamination during processing *(19)*.

There is little information about outbreaks of foodborne pathogens associated with RTE mixed-ingredient salads. There have been recalls because food companies found *Salmonella,* Shiga toxin–producing *E. coli,* and *L. monocytogenes* in their self-monitoring of these products *(18, 26, 49)*. However, the prevalence and level of pathogens in RTE mixed-ingredient salads have not been well investigated. *L. monocytogenes* has been the focus in a few studies and has been reported to be present in 1 to 5% of RTE mixed-ingredient salads *(5, 27, 46)*.

As reviewed by Oliveira et al. *(41),* there are numerous studies examining the growth of pathogens in leafy vegetables under different storage conditions. However, to the best of our knowledge, there are few studies investigating the fate of pathogens contaminating RTE mixed-ingredient salads. One such study investigated growth of *Salmonella* in lettuce mixed with cheese or chicken meat *(10)* and found that contaminated lettuce in contact with cooked chicken meat stimulated rapid *Salmonella enterica* growth. Lokerse et al. *(28)* found that different salad ingredients, e.g., iceberg lettuce, tomato, carrot, or pasta, differed in their ability to support growth of *L. monocytogenes,* but these ingredients were never mixed during those trials.

Temperature control and maintenance of adequate cold chain conditions are critical to food safety, because temperature is the single most important factor contributing to bacterial growth and survival *(56)*. The recommended refrigeration temperature varies among different countries. In the United States, consumers are advised by the U.S. Food and Drug Administration to keep refrigerator temperature at or below 4.4°C (40°F) *(53),* whereas in Sweden, the recommended maximum refrigerator temperature is often indicated to be 8°C *(37)*. Regardless of the recommended storage temperature, many studies show abuse temperatures in domestic refrigerators *(17)*. In a Swedish survey from 2011 *(34)* that included almost 2,000 Swedish schoolchildren, it was concluded that more than half (58%) of home refrigerators had an air temperature that was higher than 8°C in at least one spot *(14)*.

The main objective of the present study was to examine the fate of *L. monocytogenes,* pathogenic *Y. enterocolitica,* and *E. coli* O157:H7 *gfp*$^+$ inoculated in low numbers into RTE leafy vegetables (baby spinach) and mixed-ingredient salad (baby spinach with cooked chicken meat). The inoculated samples were stored at 8°C, which is the recommended maximum refrigerator temperature in Sweden, and at 15°C, which is an abuse temperature. Another objective was to explore the food safety implications of these RTE food products for healthy adults and risk groups.

MATERIALS AND METHODS

Bacterial strains and preparation of inoculates. The *L. monocytogenes* and pathogenic *Y. enterocolitica* strains were chosen based on the following criteria: the strains should be available from the Culture Collection University of Göteborg, Sweden (CCUG), the strains should have been characterized and shown to have been stable over time, and the strains should originally have been isolated from food. The *L. monocytogenes* strain SLV-444 (CCUG 69007) used in this study was originally isolated from hamburger meat, and the pathogenic *Y. enterocolitica* strain SLV-408 bioserotype 4/O:3 (CCUG 45643) was originally found in raw pork. Both strains were cultured on tryptic soy agar (TSA) plates with increasing concentrations of rifampin (Duchefa Biochemie, Haarlem, The Netherlands). The strains were finally resistant to 200 μg/ml rifampin.

The *E. coli* strain O157:H7 (registry no. E81186) used in the study, which was verotoxin 1- and 2-negative and *eae*-positive, was obtained from the Public Health Agency of Sweden (formerly Swedish Institute for Communicable Disease Control), Solna. It was made resistant to ampicillin (100 μg/ml) and was labeled with green fluorescent protein (GFP), which was induced to fluoresce in UV light when grown on a specific agar medium *(2)* (see below).

The inoculated bacterial concentration was 50 to 100 CFU/g for all strains. Tubes with the appropriate concentration (checked by plate counting on 5% bovine blood agar, National Veterinary Institute, Uppsala, Sweden) were stored at −70°C in brain heart infusion broth with 17% glycerol. One tube of each pathogenic strain was thawed before each trial and was centrifuged at 10,000 × *g* (Eppendorf centrifuge 5424, Eppendorf, Hamburg, Germany) for 2 to 3 min; the pellet obtained was washed with sterile 0.085% NaCl, a concentration used to avoid leaf tissue damage owing to high salt concentration. Supernatants were discharged, and pellets were washed and centrifuged three times before inoculation.

Preparation of samples and inoculation of strains. Bags with RTE baby spinach (*Spinacea oleracea* L.) from one Swedish processing plant were used in all trials. All bags within each trial were from the same batch. Because all experiments were performed during winter and spring, which are not part of the growing season in Sweden, the baby spinach originated from Spain but was washed and packaged in Sweden. For each trial, packages of RTE baby spinach were collected on delivery to the supermarket, before entering the refrigerated cabinets. They were then transported chilled to the laboratory within 30 min. The experiments were performed immediately upon arrival at the laboratory.

Heat-treated chicken meat, hereafter referred to as chicken meat, was used in the mixed-ingredient salad models. Packages of frozen RTE chicken ("grilled diced chicken," Guldfågeln AB, Mörbylånga, Sweden) were obtained from a supermarket, and the same batch was used for all experiments. According to the package label, this product also included salt, glucose syrup (derived from maize), spices (paprika, sugar, onion, rosemary, black pepper, garlic, and cumin), potato starch, and stabilizers E451 and E450. The chicken meat was thawed in a water bath directly before preparation of samples.

In samples with baby spinach, 10 g of baby spinach was transferred to separate bags. In samples with mixed salad, 5 g of baby spinach and 5 g of chicken meat were mixed in each bag.

The bags consisted of biaxially oriented polypropylene, a material with water vapor and oxygen permeability that is often used in the fresh-cut industry. The film used in this study had an original oxygen transmission rate of 1,100 ml/m^2/24 h at 23°C (film thickness, 30 μm) and was perforated (perforations, 80 μm; distance between pores, 16.25 mm). A roll of the film was kindly provided by Svenskt Pacsystem AB, Helsingborg, Sweden, and we were informed that it was similar to films supplied by that company to the fresh-cut industry. A piece of the film was cut aseptically for each sample, folded once, and then sealed with Auto-seal 102 (Nitech AB, Hestra, Sweden) on two sides, creating a bag with an opening.

To reach a final concentration of 50 to 100 CFU/g of each inoculant in the baby spinach or mixed salad, a concentration of 500 to 1,000 CFU mixed with 100 μl of 0.085% NaCl was inoculated into the bags containing 10 g of matrix. Separate samples were used for each inoculated strain. In control samples, 100 μl of 0.085% NaCl was inoculated into the bags. The volume was administered with a pipette. The bags were then sealed with Auto-seal 102, and the final size of the bags was approximately 20 by 20 cm.

Study design and bacterial analysis. For each of three trials, the experiment was set up as a three-factorial study with repeated measures. The fixed factors were (i) inoculant (*L. monocytogenes,* pathogenic *Y. enterocolitica, E. coli* O157:H7 *gfp*$^+$, and control), (ii) chicken meat (with or without), and (iii) storage temperature (8 ± 1°C or 15 ± 1°C). The normal shelf life for RTE baby spinach in Sweden is approximately 7 days, whereas it is 3 days for a mixed-ingredient salad. Therefore, samples were analyzed directly after inoculation (day 0) and after 3 and 7 days, for each inoculant and trial. From each sample with inoculated strain, plating was performed in triplicate.

At the time of analysis, the 10-g samples were transferred to stomacher bags and were homogenized with 90 ml of peptone water for 1 min in a stomacher (easyMIX Lab Blender, AES-Chemunex, Weber Scientific, Hamilton, NJ).

From each of the *L. monocytogenes* and pathogenic *Y. enterocolitica* homogenates (dilution 1/10), 1 ml was spread onto three TSA agar plates (full strength; Oxoid, Basingstoke, UK), respectively, containing 200 μg/ml rifampin (Sigma-Aldrich, Steinheim, Germany). On days 3 and 7, 0.1-ml aliquots from suitable dilutions of samples in which we expected growth of the inoculant (>100 CFU per plate, based on results of a prestudy) were spread onto separate agar plates. The plates were incubated at 37 ± 1°C for *L. monocytogenes* or at 30 ± 1°C for pathogenic *Y. enterocolitica,* and colonies were enumerated after 48 h.

Aliquots (1 ml) of the *E. coli* O157:H7 *gfp*$^+$ homogenate (dilution 1/10) were spread onto three Luria-Bertani agar plates (Honeywell, Morristown, NJ; BD, Franklin Lakes, NJ) supplemented with 100 μg/ml ampicillin (Sigma-Aldrich) and 0.1% L-arabinose (Merck, Darmstadt, Germany) *(2)*. As with the other inoculants, on days 3 and 7, 0.1-ml aliquots from suitable dilutions of samples in which we expected growth of the inoculant (>100 CFU per plate based on results of a prestudy) were spread onto separate agar plates. The plates were incubated at 37 ± 1°C overnight and then were placed in a cabinet with UV light (wavelength 365 nm; model CM-10A, Spectroline, Westbury, NY), where fluorescent colonies were counted.

Total counts (aerobic viable bacteria) were enumerated in control samples on day 0 and in all samples on day 7. Nordic Committee on Food Analysis method 86 *(40)* was used, and standard plate count agar plates (Oxoid) were incubated at 30 ± 1°C for 3 days before enumeration of all colonies present.

To analyze presumptive *E. coli* from the matrices, Chromo-Cult plates (Merck) were incubated at 37 ± 1°C for 24 h.

pH, water activity, and gas atmosphere. The pH values were determined by a pH meter (InoLab pH Level 1, Wissenschaftlich-Technische Werkstätten GmbH, Weilheim, Germany), using a glass electrode (Mettler Toledo, Greifensee, Switzerland) calibrated with buffers with pH 4.00 ± 0.02 and pH 7.00 ± 0.02. The pH was measured at room temperature on days 0, 3, and 7, in the first dilution of noninoculated samples of baby spinach and baby spinach mixed with chicken meat.

Water activity was measured for the chicken meat used in this study (three replicates) and for three different batches of RTE baby spinach. Water activity was measured at room temperature (17.5 to 19.8°C) with AquaLab (Pullman, WA).

Gas levels in packages were measured at SP Food and Bioscience in Gothenburg, Sweden, for the different sample types and conditions during one trial. A total of 15 samples of baby spinach and 15 samples of baby spinach mixed with chicken (all without inoculants) were transported chilled to Gothenburg, where gas levels were measured in triplicate at arrival (day 0) and after 3 and 7 days at 8 or 15°C, using a Check Mate II (PBI Dansensor, Ringsted, Denmark).

Growth potential. The growth potential (δ) of the *L. monocytogenes* strain in RTE baby spinach and in baby spinach mixed with chicken meat (mixed-ingredient salad) was determined by the difference between the concentration (log CFU per gram) at the end of shelf life and the concentration at the beginning of the test (corresponding to the end of the processing), as defined in Beaufort *(6)*. We followed the protocol in the European Union Reference Laboratory technical guidance document for conducting shelf-life studies on *L. monocytogenes* in RTE foods *(1),* with modifications. In this study, one single strain of *L. monocytogenes* was inoculated in the samples, and we analyzed one replicate sample for each batch (calculating the mean concentration for three subsamples of each replicate). Baby spinach has a longer shelf life than mixed-ingredient salad, according to the producers. Pathogen counts at the end of shelf life were, therefore, used as the endpoint of the challenge tests for baby spinach (day 7) and mixed-ingredient salad (day 3). These food products were considered to support growth of the inoculated bacterial strain when the δ-value was higher than 0.5 log CFU/g *(1)*.

Risk assessment modeling for *L. monocytogenes* and *E. coli* O157:H7. We illustrated the consumer risk (measured as probability of infection) presented by our findings for the inoculants with the applicable dose-response models available, i.e., for *L. monocytogenes* and *E. coli* O157:H7.

To capture variability and uncertainty, a lognormal-Poisson dose-response model developed by Pouillot et al. *(42)* was used to evaluate the risk of acquiring invasive listeriosis when a certain dose (*d*) of bacterial cells was ingested. The model was an exponential model: $P = 1 - e^{(-rd)}$. Various estimates of *r* were used for three different population subgroups: healthy adults (<65 years old), elderly (≥65 years of age), and persons with solid organ transplants *(42)*.

An exponential dose-response model was also used for *E. coli* O157:H7, to illustrate the consumer risk represented by our findings of *E. coli* O157:H7 *gfp*$^+$. The same equation as above was used for estimating the risk of illness in adults, with $r = 0.00113$ *(48)*.

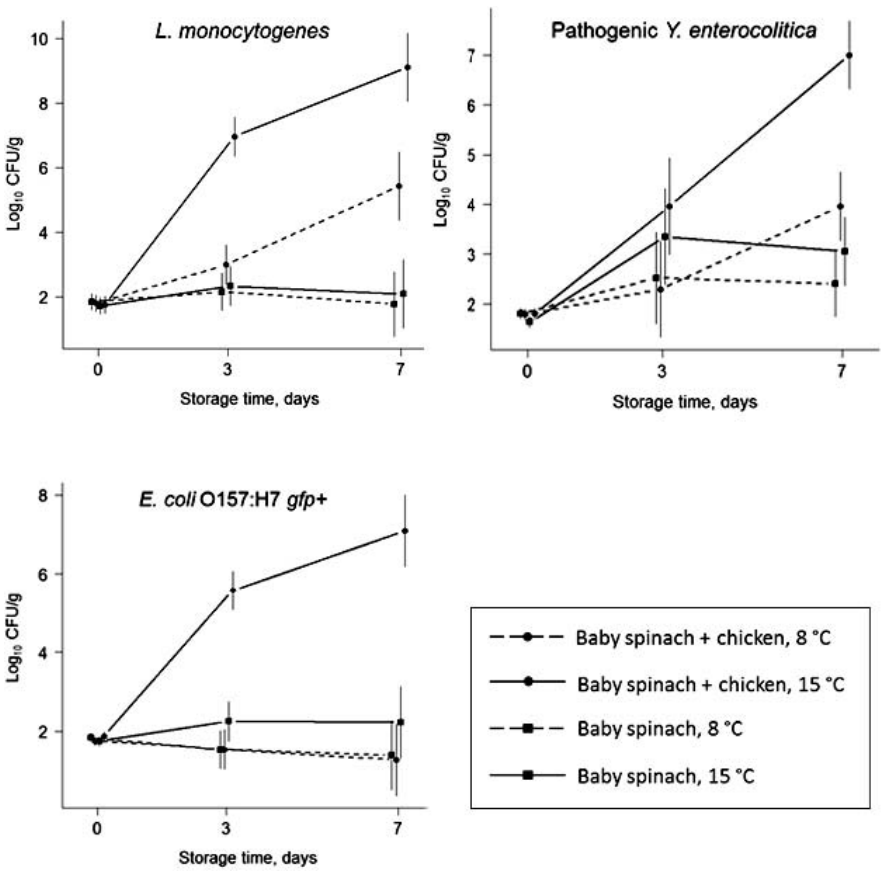

FIGURE 1. *Populations of* Listeria monocytogenes, *pathogenic* Yersinia enterocolitica, *and* Escherichia coli *O157:H7* gfp$^+$ *on ready-to-eat baby spinach or baby spinach mixed with chicken meat stored at 8 or 15°C for 3 and 7 days. Each data point is the average of three trials. Vertical bars display 95% confidence limits for each average.*

Different serving sizes were used for RTE baby spinach (30 g) and mixed-ingredient salad (300 g), representing the current consumption patterns. This is based on the assumption that 30 g of RTE baby spinach would cover 40 to 50% of the plate, which is the part of a healthy meal that should consist of vegetables, according to nutrition recommendations *(38)*. Mixed-ingredient salad is, however, intended to be consumed as a full meal. Packages of mixed-ingredient salad may range in weight from 310 to 550 g *(46),* but because they rarely consist of only baby spinach and chicken meat, a combination selected for practical reasons in this study, we opted for a slightly smaller serving size.

The risk was presented as number of illnesses per 1,000,000 servings when the risk was low and in percentage when the risk was high. This was estimated using the Monte Carlo simulation software @Risk (Palisade Corporation, Newfield, NY). The variability of the bacterial numbers per serving was expressed as a triangular distribution, with the minimum, median, and maximum results of the bacteriological analyses for *L. monocytogenes* and *E. coli* O157:H7 *gfp*$^+$. Each simulated scenario for *L. monocytogenes* and *E. coli* O157:H7 *gfp*$^+$ was run using 10,000 iterations. Furthermore, the increase in risk during the shelf life, i.e., the relative risk ratio (the risk of illness at the end of shelf life/the risk of illness on the production day), was also estimated.

Statistical analysis. The mean number (log CFU per gram) of inoculated bacteria was calculated for samples on days 0, 3, and 7 in the three trials. The number was transformed to log CFU per gram to normalize data before statistical analysis. An analysis of variance (ANOVA) was performed using a mixed model, taking into account the repeated measures, using R software *(44),* and differences between treatments were assessed using least-square means ($P < 0.05$). Pairwise comparisons between time points were made using Tukey's method.

RESULTS AND DISCUSSION

Populations of *L. monocytogenes*, pathogenic *Y. enterocolitica,* and *E. coli* O157:H7 *gfp*$^+$ during storage. Populations of the inoculated strains in both matrices at different temperatures on days 0, 3, and 7 are presented in Figure 1 (ANOVA model estimates available online at https://doi.org/10.4315/0362-028X.JFP-16-308.s1). In baby spinach mixed with chicken meat, the populations of all inoculated strains increased significantly ($P < 0.001$) when stored at an abuse temperature (15°C) for 3 or 7 days (Table 1). After a normal shelf life of 3 days, populations of *L. monocytogenes,* pathogenic *Y. enterocolitica,* and *E. coli* O157:H7 *gfp*$^+$ increased from less than 2.0 log CFU/g to 7.0 (CI, 6.4 to 7.6), 4.0 (CI, 3.0 to 4.9), and 5.6 (CI, 5.1 to 6.1) log CFU/g, respectively (Fig. 1). Moreover, populations of *L. monocytogenes* also increased significantly ($P \leq 0.0001$) in baby spinach mixed with chicken after 3 days at 8°C (Table 1), i.e., the recommended storage conditions for mixed-ingredient salads in Sweden. In plain baby spinach stored at 15°C, there was significant growth of *L. monocytogenes* during the first 3 days of storage ($P < 0.005$) and of pathogenic *Y. enterocolitica* during the whole shelf life of 7 days ($P < 0.004$). There was no significant growth in baby spinach at 8°C. Growth in plain baby spinach was, however, limited compared with growth in samples in which baby spinach was mixed with chicken. Similar results have been reported by Bovo et al. *(10),* who

TABLE 1. *Pairwise comparisons between number of inoculated bacteria in ready-to-eat baby spinach or baby spinach mixed with chicken meat at 8 or 15°C on days 0, 3, and 7*[a]

	Baby spinach (log CFU/g)				Baby spinach with chicken (log CFU/g)			
	8°C		15°C		8°C		15°C	
Comparisons	Estimate	*P* value	Estimate	*P* value	Estimate	*P* value	Estimate	*P* value
L. monocytogenes								
Days 0–3	0.30	0.19	0.62	**0.0048**	1.19	**<0.0001**	5.21	**<0.0001**
Days 0–7	−0.08	0.98	0.36	0.63	3.64	**<0.0001**	7.37	**<0.0001**
Days 3–7	−0.38	0.55	−0.25	0.77	2.44	**<0.0001**	2.15	**0.0001**
Pathogenic *Y. enterocolitica*								
Days 0–3	0.71	0.29	1.72	**0.0044**	0.51	0.52	2.16	**0.0006**
Days 0–7	0.59	0.19	1.42	**0.0012**	2.17	**<0.0001**	5.21	**<0.0001**
Days 3–7	−0.12	0.91	−0.30	0.56	1.67	**0.0001**	3.05	**<0.0001**
E. coli O157:H7 *gfp*$^+$								
Days 0–3	−0.30	0.38	0.50	0.08	−0.22	0.59	3.70	**<0.0001**
Days 0–7	−0.44	0.52	0.48	0.46	−0.48	0.46	5.22	**<0.0001**
Days 3–7	−0.14	0.81	−0.02	1.00	−0.27	0.50	1.52	**<0.0001**

[a] Tukey's method was used for *P* value adjustment of multiple comparisons. Statistically significant differences ($P < 0.05$) are shown in boldface. Positive values indicate bacterial growth, and negative values bacterial death.

demonstrated no growth of *S. enterica* in romaine lettuce at 6 or 14°C but considerable growth after addition of chicken meat and storage at an abuse temperature (14°C). *E. coli* O157:H7 *gfp*$^+$ did not grow during storage at 8°C, either in baby spinach or in baby spinach mixed with chicken. Luo et al. *(31)* reported slight growth of *E. coli* O157:H7 during storage of RTE baby spinach at 8°C, but a reduction in bacterial numbers at ≤5°C.

pH, water activity, and growth potential of *L. monocytogenes*. Growth potential (δ) depends on many factors *(1),* including extrinsic (e.g., time-temperature profile) and intrinsic (e.g., pH and water activity) properties of foods. Growth potential of the inoculated strain of *L. monocytogenes* and pH ranges for the different sample types are shown in Table 2. The water activity was 0.96 ± 0.01 for both the cooked chicken meat and RTE baby spinach used in these trials. According to European Union legislation, food business operators are obliged to evaluate growth potential of *L. monocytogenes* that may be present in a RTE product during its shelf life, not only under recommended but also under realistic abuse conditions (Annex II *(1)*). We found that the growth potential of *L. monocytogenes* in baby spinach at 8°C slightly exceeded the limit of 0.5 log CFU/g for one of the batches; however, a decrease in bacterial concentration was observed in the other batches. Similar results for baby spinach have been reported by others, with numbers of *L. monocytogenes* slightly exceeding 0.5 log CFU/g in one study *(45)* and declining numbers during shelf life in another *(28)*. The mean growth potential of *L. monocytogenes* in baby spinach mixed with chicken meat was 1.2 log CFU/g at 8°C for 3 days, i.e., storage conditions within the recommended shelf life and temperature limits for mixed-ingredient salad retailed in Sweden. This is a particular concern because *L. monocytogenes* has been found in 1 to 5% of mixed-ingredient salads *(5, 27, 46)*. The results on growth potential of baby spinach and baby spinach mixed with chicken need to be validated

TABLE 2. *Growth potential (δ) of* Listeria monocytogenes *in baby spinach and baby spinach mixed with chicken meat (mixed-ingredient salad)*[a]

		Growth potential (δ) for *L. monocytogenes* (log CFU/g)			
Storage condition	pH range	Batch 1	Batch 2	Batch 3	Batches 1–3, mean (±SD)
Baby spinach					
8°C, 7 days	6.1–6.5	−0.33	0.64	−0.41	−0.03 (±0.59)
15°C, 7 days	6.1–7.1	1.08	0.60	−0.40	0.43 (±0.75)
Baby spinach with chicken					
8°C, 3 days	6.5–6.8	1.13	1.43	1.17	1.25 (±0.16)
15°C, 3 days	6.5–7.3	5.43	5.34	4.98	5.25 (±0.24)

[a] Negative values indicate a decrease in bacterial concentration during shelf life. Because baby spinach has a longer shelf life than mixed-ingredient salad, pathogen count on day 7 was used as the endpoint for baby spinach and count on day 3 as the endpoint for mixed-ingredient salad. Standard deviation is shown for the growth potential of the three batches for each sample type and condition. The pH levels for control samples of baby spinach and baby spinach mixed with chicken meat are presented in ranges for each storage condition.

according to the full European Union Reference Laboratory guidance protocol *(1)*. When interpreting the microbiological criteria in European Commission Regulation EU 2073/2005 *(15)*, there are stricter regulations for foodstuffs able to support growth of *L. monocytogenes* than for foodstuffs unable to support growth. For both categories, *L. monocytogenes* must not exceed a level of 100 CFU/g during its shelf life, whereas in food with growth potential, the bacteria must be absent (in five 25-g samples) before the product leaves the food business operator *(15)*. RTE products with a shelf life of fewer than 5 days, for example mixed-ingredient salads, are classified in the category of RTE foods unable to support growth of *L. monocytogenes*. Our results indicated that mixed-ingredient salad stored at 8°C actually supports growth of *L. monocytogenes* and should, therefore, be categorized as able to support, with stricter controls, despite the short shelf life. These results emphasize the importance of appropriate storage temperatures to reduce the risk of pathogen growth and the fact that 8°C may increase the risk of foodborne disease. In Sweden, there is no regulation on storage temperature, and it is up to the food producers to decide storage temperature of a particular food product *(37)*. The recommended storage temperature for RTE baby spinach in Sweden is on the package label; it is often indicated as a maximum of 4°C, although it may be indicated as a maximum of 5 or 8°C on packages from some producers *(21)*. However, compliance may be poor at both the retail and customer level. In an Irish study of mixed-ingredient salads, almost one in four samples were retailed at temperatures exceeding 5°C, the recommended temperature for refrigerated food in Ireland *(5)*. In a Swedish study concerning consumer handling of refrigerated food, the mean storage temperature of RTE salad was 7.4°C, whereas one of five salads were stored at temperatures above 10°C *(35)*. Even if the recommended storage temperature for refrigerated food is lower (e.g., 4 or 5°C) in many other countries than it is in Sweden, it is clear that many refrigerators throughout the world are running at higher temperatures *(23)*. Approximately 5% of domestic refrigerators included in European surveys had temperatures above 10°C *(17)*.

Risk modeling of *L. monocytogenes* and *E. coli* O157:H7 *gfp*$^+$. For plain baby spinach, the estimated risks of listeriosis were <1 per 1,000,000 servings for all included population groups (healthy adults [<65 years], elderly [≥65 years], and persons with solid organ transplants) at all storage scenarios. The median risk of acquiring invasive listeriosis per 1,000,000 servings of mixed-ingredient salad (baby spinach mixed with chicken meat) based on the levels of *L. monocytogenes* in this study are shown in Table 3. After storage at 15°C, the risk varied considerably among the three different population groups included. The median risk of acquiring invasive listeriosis for healthy adults consuming a mixed-ingredient salad (with an initial level of 50 to 100 CFU/g) stored at 15°C during its shelf life and reaching high levels of *L. monocytogenes* (approximately log 7 CFU/g), was estimated to be 28 per 1,000,000 servings. However, for individuals who had undergone solid organ transplantation, the corresponding risk was 11,000 per 1,000,000 servings. Even if initial *L. monocytogenes* levels of 50 to 100 CFU/g are below the legal European Union limit (<100 CFU/g), these levels are rarely found in RTE food at retail *(25)*, and mixed-ingredient salads generally do not consist solely of baby spinach and chicken meat. To reduce uncertainties associated with absolute risk estimations, relative risk was also estimated. For all three subpopulations, the risk of listeriosis was 16 times higher when consuming a mixed-ingredient salad stored at 8°C at the end of shelf life and was approximately 200,000 times higher when stored at 15°C, compared with when consuming it on the day of inoculation. This indicates that efforts should focus on preventing temperature abuse during storage to mitigate risk of listeria infection.

TABLE 3. *Median risk of acquiring invasive listeriosis*[a]

	8°C		15°C	
Subpopulation	Day 0	Day 3	Day 0	Day 3
Healthy <65 years	<1	<1	<1	28
Healthy ≥65 years	<1	<1	<1	530
Organ transplant	<1	1	<1	11,000

[a] Risk measured as number of infections predicted per 1,000,000 servings of baby spinach mixed with chicken meat (300 g) following inoculation of *Listeria monocytogenes* (50 to 100 CFU/g) and consumption in the beginning (day 0) and end of shelf life (day 3) for different subpopulations (values >1 rounded to two significant figures).

The risk of *E. coli* O157:H7 infection, illustrated by our findings of *E. coli* O157:H7 *gfp*$^+$, was high also at initial levels (50 to 100 CFU/g), because even a few bacterial cells (fewer than 100) may cause human illness *(50)*. After consuming inoculated mixed-ingredient salad, the risk of acquiring disease was close to 100%, both before storage and after storage at 8 or 15°C. For plain baby spinach, the corresponding risks of *E. coli* O157:H7 infection ranged from approximately 60 to 100%. The risk of *E. coli* O157:H7 infection was 1.7 times higher from consumption of baby spinach stored at 15°C for 7 days compared with that stored at 8°C. Hence, the results emphasized that, for *E. coli* O157:H7, control of the raw material is crucial, because disease in humans may develop even without prior multiplication of this bacterium in the food product.

Inoculation levels and selection of inoculated strains. In this study, we aimed for inoculation levels that mimicked realistic contamination conditions but that were still sufficiently high for accurate quantification. Natural contamination often occurs at very low levels, e.g., between 0.1 and 1 CFU/100 g, as estimated for *L. monocytogenes* on fresh produce *(12)*. We applied inoculation levels of 1.5 to 2 log CFU/g, which is higher than the estimated contamination level but slightly lower than the recommended inoculation level (2 to 3 log CFU/g), according to different guidelines *(1, 4)*. The levels used in this study were also lower than those (4 to 5 log CFU/g) often used in similar studies *(10, 24)*. Labeling of inoculated strains is necessary to evaluate growth in foodstuffs with high levels of background

microbiota. In this study, we used a GFP-labeled *E. coli* O157:H7 strain that was available at the Swedish University of Agricultural Sciences and that has been used in other studies *(2, 9, 13)*. GFP labeling of *E. coli* O157:H7 has been reported to be a stable surrogate for safety-related studies, and the plasmid has been shown to have an insignificant effect on bacterial growth *(32)*. GFP-labeled bacterial cells have also been found to remain fluorescent following stress and have been shown to be detectable in all growth phases *(29, 51)*. We used rifampin-resistant strains of *L. monocytogenes* and pathogenic *Y. enterocolitica* in this study for practical reasons. These strains have shown growth rates essentially equal to those of the parent strains (data not shown). Similar results have been shown by others using rifampin-resistant strains *(36, 39)*.

Total aerobic counts and *E. coli* as a hygiene indicator. Total aerobic counts are shown in Table 4. The initial count for RTE baby spinach was 7.3 log CFU/g, which represented the microbial load for this product when it was introduced to the consumers at the retail level, with 7 days of shelf life still ahead. The total count increased significantly during storage for 7 days, by approximately 1 log CFU/g at 8°C, which is similar to results reported by Sant'Ana et al. *(45),* and by 2 log CFU/g at 15°C. Baby spinach mixed with chicken had initial total aerobic counts similar to those in plain baby spinach, but counts increased to approximately 10 log CFU/g during storage for 7 days at an abuse temperature. High aerobic counts (9.1 log CFU/g) were also observed in a previous study, in which lettuce mixed with chicken meat was stored at 14°C, but after 3 days instead of 7 *(10)*. In this study, we did not evaluate organoleptic alterations, which may have been significant in samples representing mixed-ingredient salad stored too long at inappropriate temperature, with total counts of approximately 10 log CFU/g. However, total aerobic counts of 7 to 8 log CFU/g seem to be normal for RTE spinach when it is consumed, at least when the leaves have only been washed in potable water during processing. Luo et al. *(31)* showed that visual quality of spinach leaves was fully acceptable after 6 days at 12°C, despite significant levels of both *E. coli* O157:H7 (approximately 5.5 log CFU/g) and aerobic mesophilic counts (close to 7 log CFU/g). This suggests that acceptance by consumers based on visual, flavor, and textural sensory quality factors *(43)* may not be sufficient to ensure food safety of RTE leafy vegetables. No *E. coli* was isolated from any control sample, which indicates that samples were not fecally contaminated.

Atmospheric conditions in sample bags. Gas levels in our sample packages did not deviate greatly from aerobic conditions. The O_2 levels slightly declined from normal air concentration (approximately 21%) on day 0 to 17.2 to 19.7% on day 7. The lowest concentrations were found in bags with baby spinach mixed with chicken meat stored at 15°C, and the highest were found in bags with baby spinach stored at 8°C. The CO_2 concentrations increased from 0.1% on day 0 to 0.5 to 2.8% on day 7; the lowest concentrations were found in baby spinach stored at 8°C and the highest in bags with baby spinach mixed with chicken meat stored at 15°C. These levels are similar to those in commercial packages of RTE leafy vegetables in Sweden (data not shown).

TABLE 4. *Total aerobic counts in control samples at days 0 and 7*[a]

	Before storage	Stored for 7 days, 8°C	Stored for 7 days, 15°C
Baby spinach	7.30 ± 0.39	8.43 ± 0.19	9.24 ± 0.34
Spinach with chicken	6.88 ± 1.14	9.12 ± 0.60	10.15 ± 0.11

[a] Values (log CFU per gram) are means ± standard deviations of the three experiments for ready-to-eat baby spinach and baby spinach mixed with chicken meat.

In conclusion, this study demonstrated that mixing fresh-cut leafy vegetables with ingredients that support rapid microbial growth (e.g., chicken meat) influenced the growth of pathogens during storage of these mixed-ingredient salads. The results emphasized that temperature control and maintenance of adequate cold chain conditions is critical to maintain food safety. In Sweden, storing mixed-ingredient salad at 8°C for 3 days complies with current recommendations. However, these conditions support growth of the psychrotroph *L. monocytogenes*. Moreover, growth of pathogens may emerge before the spoilage flora affect sensory attributes. Therefore, accepting food based on its good appearance, flavor, and lack of odor may not be sufficient for assuring food safety. Hence, manufacturers preparing mixed-ingredient salads should be aware of this potential threat to food safety. The recommended storage temperature for mixed-ingredient salads should be set to a level that limits growth of any contaminant during the intended shelf life. Manufacturers should also focus on using raw ingredients (e.g., leafy vegetables) produced under good hygienic conditions and on minimizing the risk of cross-contamination during preparation. Consumers need guidance on how to store RTE leafy vegetables and mixed-ingredient salads to prevent growth of potential contaminants.

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SUPPLEMENTAL MATERIAL

Supplemental material associated with this article can be found online at: https://doi.org/10.4315/0362-028X.JFP-16-308.s1.

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