Arch Lebensmittelhyg 69,
115–122 (2018)
DOI 10.2376/0003-925X-69-115

ISSN 0003-925X

Korrespondenzadresse:
riikka.keto-timonen@helsinki.fi

[1]) Department of Food Hygiene and Environmental Health, Faculty of Veterinary Medicine, University of Helsinki, P.O. Box 66, FI-00014 University of Helsinki, Finland; [2]) Apetit Ruoka Ltd, Kivikonlaita 25, FI-00940 Helsinki, Finland; [3]) Service de Parasitologie/Mycologie, Groupe Hospitalier Pitié-Salpêtrière, AP-HP, Paris, France

Growth of *Listeria monocytogenes, Yersinia pseudotuberculosis* and yeasts on fresh vegetables packaged under modified atmospheres at 6 °C

Wachstum von Listeria monocytogenes, Yersinia pseudotuberculosis und Hefen auf frischen Gemüse verpackt unter Schutzatmosphären bei 6 °C

Juho Koskinen[1]), Sonja Takalo[1]), Anssi Vuorinen[2]), Anne-Cecile Normand[3]), Hannu Korkeala[1]), Riikka Keto-Timonen[1])

Summary

Growth and survival of *Listeria monocytogenes* on shredded cabbage and cut onions, *Yersinia pseudotuberculosis* on cubed and shredded carrots along with yeasts on cubed bell peppers and cubed potatoes were studied during a nine-day storage period at 6 °C. Fresh vegetables were packaged in retail packages under three different initial atmospheres including (A) air, and two atmospheres with reduced oxygen (O_2) and elevated carbon dioxide (CO_2) concentrations (B) 5 % O_2, 5 % CO_2, and (C) 2 % O_2, 15 % CO_2, all balanced with nitrogen (N_2). Shredded carrots were the only exception, as they were packaged only under air. Vegetables were inoculated with a mixture of three *L. monocytogenes,* three *Y. pseudotuberculosis* or five *Candida sake* strains depending on the study. Headspace gas compositions and microbial counts were studied from three replicate packages from each vegetable and atmosphere combination after inoculation (day 0) and after three and nine days of cold storage. All the three microbes were able to grow on the vegetables with the exception that *L. monocytogenes* failed to grow on cut onions. While modified atmospheres may be used to improve product quality and shelf life, the safety of ready-to-eat vegetables must rely on other factors, such as good hygienic and manufacturing practices along with strict temperature control and shelf lives short enough.

Keywords: cold storage, food safety, minimal processing

Zusammenfassung

Das Wachstum und das Überleben von *Listeria monocytogenes* in zerkleinertem Kohl und geschnittenen Zwiebeln, *Yersinia pseudotuberculosis* in gewürfelten und geraspelten Karotten sowie Hefen in gewürfelten Paprikaschoten und gewürfelten Kartoffeln wurden während einer Lagerzeit von neun Tagen bei 6 °C untersucht. Frisches Gemüse wurde in Einzelhandelsverpackungen unter drei verschiedenen ursprünglichen Schutzatmosphären verpackt: (A) Luft und zwei Atmosphären mit reduziertem Sauerstoffgehalt (O_2) und erhöhten Kohlendioxidkonzentrationen (CO_2) (B) 5 % O_2, 5% CO_2, und (C) 2 % O_2, 15 % CO_2, alle mit Stickstoff (N_2) ausgeglichen. Als einzige Ausnahme wurden die geraspelten Karotten nur unter Luft verpackt. Das Gemüse wurde je nach Studie mit einer Mischung aus drei *L. monocytogenes-,* drei *Y. pseudotuberculosis*- oder fünf *Candida sake*-Stämmen inokuliert. Die Zusammensetzung der Schutzgase und die Keimzahlen wurden aus drei Replikatpackungen aus jeder Kombination von Gemüse und Atmosphäre nach der Inokulation (Tag 0) und nach drei und neun Tagen Kühllagerung bestimmt. Alle drei Mikroben konnten in dem Gemüse wachsen, mit der Ausnahme, dass *L. monocytogenes* nicht in geschnittenen Zwiebeln wachsen konnte. Während modifizierte Atmosphären verwendet werden können, um die Produktqualität und Haltbarkeit zu verbessern, muss die Sicherheit von essfertigem Gemüse auf anderen Faktoren beruhen, wie guten Hygiene- und Herstellungspraktiken zusammen mit einer strengen Temperaturkontrolle und genügend kurzen Lagerzeiten.

Schlüsselwörter: Kühllagerung, Lebensmittelsicherheit, minimale Verarbeitung

Journal of Food Safety and Food Quality 69, Heft 4 (2018), Seiten 105–130

Introduction

The consumption of fresh produce, such as minimally processed ready-to-eat vegetables, has increased during past decades. Concurrently, notable number of food poisoning outbreaks associated with vegetable products have occurred in Europe (EFSA, 2013) and North America (Erickson, 2010). Primary production, processing stages e. g. washing, peeling and cutting along with packaging and storage may cause risk for pathogen contamination and growth on vegetables (Zagory, 1999; Francis et al., 2012). The microbial quality of fresh vegetables on the market has raised safety concerns (Cardamone et al., 2015; Nousiainen et al., 2016). Detailed knowledge is needed to better understand microbial growth on fresh produce in various storage conditions.

L. monocytogenes and *Y. pseudotuberculosis* are psychrotrophic facultative anaerobic bacteria widely spread in nature, capable of growing in various conditions and potential sources of food-borne diseases. *L. monocytogenes* may cause invasive disease forms, such as abortion, meningoencephalitis or septicaemia, especially among risk groups (Swaminathan and Gerner-Smidt, 2007), and mortality rates of listeriosis may be high (de Noordhout et al., 2010). Vegetables have been widely known to be potential *L. monocytogenes* vehicles (EFSA, 2013) since the listeriosis outbreak caused by contaminated coleslaw in the early 1980s (Schlech et al., 1983). Yersiniosis usually presents as gastrointestinal symptoms, but may also cause long-lasting complications, e. g. erythema nodosum or reactive arthritis (Fredriksson-Ahomaa et al., 2010). *Y. pseudotuberculosis* outbreaks have mainly been reported in the Northern Hemisphere including Canada (Press et al., 2001), Finland (Tertti et al., 1984; Jalava et al., 2004; Nuorti et al., 2004; Jalava et al., 2006; Kangas et al., 2008; Rimhanen-Finne et al., 2009; Pärn et al., 2014), Japan (Inoue et al., 1988; Nakano et al., 1989) and Russia (Tseneva et al., 2012). Numerous outbreaks have been linked to vegetables such as iceberg lettuce (Nuorti et al., 2004) and carrots (Jalava et al., 2006; Kangas et al., 2008; Rimhanen-Finne et al., 2009).

Modified atmosphere packaging (MAP), which utilises atmospheres other than air, can be used to prolong the shelf lives of fresh produce, and the packaging technology including its microbiological risks, has been reviewed extensively (Zagory, 1999; Farber et al., 2003; Francis et al., 2012; Caleb et al., 2013). MAP may enable psychrotrophic facultative anaerobic pathogens to reach high numbers, which can be a food safety risk (Zagory, 1999; Farber et al., 2003; Francis et al., 2012; Caleb et al., 2013). Product type and storage conditions affect the ability of *L. monocytogenes* to grow on MAP vegetables (Hoelzer et al., 2012). In contrast, *Y. pseudotuberculosis* growth on MAP vegetables has not been studied. Furthermore, yeasts cause economic losses for the vegetable industry by deteriorating the sensorial quality of produce and because CO_2 production packages may expand or even collapse (Fleet 2011). Yeasts have been shown to grow on vegetables packaged in polymeric films and packaging may even enhance their growth compared to unpacked vegetables (Brackett, 1988, 1989, 1990). However, the effect of modified atmospheres on yeast growth appear to be relatively limited (Beuchat and Brackett, 1990a; Babic et al., 1992). More information concerning yeast growth on various commodities as well as under different atmospheres within the same packaging system is needed to improve product quality.

The aim of our study was to assess the survival and growth of *L. monocytogenes, Y. pseudotuberculosis* and yeasts on fresh vegetables packaged under O_2 and CO_2 atmospheres balanced with N_2 during a nine-day cold storage period at 6 °C.

Material and methods

Microbial strains and preparation of inoculums

L. monocytogenes and *Y. pseudotuberculosis* strains (Tab. 1.) used in this study were selected from the culture collection of the Department of Food Hygiene and Environmental Health (University of Helsinki, Helsinki, FI) and had been isolated from vegetables, or a warehouse used for storing vegetables. In addition, five yeast strains were isolated from cubed MAP bell peppers and potatoes, and the isolates were identified as *Candida sake* strains using MALDI-TOF Mass Spectrometry method (Microflex, Bruker Daltonics GmbH, Leipzig, DE). Protein extraction was performed using formic acid and acetonitrile (Cassagne et al., 2012), and the obtained spectra were compared to an online reference database using the MSI application as described by Normand et al. (2017). Prior to inoculation, *L. monocytogenes* strains were cultured in brain heart infusion (BHI) broth (Oxoid, Basingstoke, Hampshire, UK) at 37 °C for 16 h, *Y. pseudotuberculosis* strains in tryptic soy (TS) broth (Oxoid) at 28 °C for 16 h and *C. sake* strains in wort broth (Scharlau, Scharlab, Barcelona, ES) at 25 °C for 48 h. The final inoculum consisted of equal counts of three *L. monocytogenes,* three *Y. pseudotuberculosis* or five *C. sake* strains diluted in peptone water.

MAP vegetables and inoculation

Shredded white cabbage (*Brassica oleracea* var. *capitata*), cut red onions *(Allium cepa),* cubed (20 mm) carrots (*Daucus carota* subsp. *sativus*), shredded carrots, cubed (20 mm) red bell peppers *(Capsicum annuum)* and cubed (20 mm) potatoes *(Solanum tuberosum)* were packaged and delivered by a local food company one day prior to inoculation (day –1) and stored at 6 °C. Each vegetable product, except the shredded carrots, had been packaged under three different atmospheres (A–C) initially containing (A) air (21 % O_2, 0 % CO_2), or reduced O_2 and elevated CO_2 concentrations (B) 5 % O_2, 5 % CO_2, or (C) 2 % O_2, 15 % CO_2, while N_2 was used as a balance gas. The shredded carrots were packaged only under atmosphere A (air), to

TABLE 1: *Strains used for inoculation of fresh vegetables.*

Strain	Serotype	Origin	Year	Country
Listeria monocytogenes				
LM109	4b	Frozen broccoli	1999	Finland
LM116	1/2b	Frozen onion product	1999	Finland
LT30E	1/2a	Bell pepper	2000	Finland
Yersinia pseudotuberculosis				
1435/8/2004	1b	Carrot	2004	Finland
2161/13/2006	1b	Floor surface[a]	2006	Finland
2484/2006	1b	Potato waste[a]	2006	Finland
Candida sake				
CS-KP-pap-1		Bell pepper	2016	Finland
CS-KP-pap-3		Bell pepper	2016	Finland
CS-KP-per-1		Potato	2016	Finland
CS-KP-per-3		Potato	2016	Finland
CS-KP-per-4		Potato	2016	Finland

[a] Sampled from storage room floor.

study whether processing type affects the growth of *Y. pseudotuberculosis.* Impermeable films were used to seal the packages.

Shredded cabbage and cut onions were both inoculated with 3.3 log CFU/g of *L. monocytogenes* inoculum, while the cubed carrots and shredded carrots were inoculated with 4.4 log CFU/g and 3.3 log CFU/g of *Y. pseudotuberculosis* inoculum, respectively. The *C. sake* inoculation level used for cubed bell peppers and cubed potatoes was 3.2 log CFU/g. Inoculation was performed by injecting through a septum (PBI Dansensor, Ringsted, DK) inserted on the packaging film to retain impermeability. The injection volume (1 ml per 50 g of vegetables) was evenly directed on the vegetables and further spread by rotating the packages using a Roto-shake Genie system (Scientific Industries, Bohemia, New York, US) for 60 s. Headspace gas analyses and microbial enumerations were performed immediately after inoculation (day 0) and after three and nine days of storage at 6 °C. Three replicate packages were examined from each vegetable, atmosphere and storage time combination.

Analysis of headspace gas

Headspace gas compositions (O_2, CO_2) were tested with an Oxybaby gas analyser (Witt-Gasetechnik Gmbh & Co KG, Witten, DE) at the processing plant during packaging of the vegetables one day before inoculation (day –1) to ensure initial gas compositions. Gas compositions were measured from each of the packages using a Checkpoint gas analyser (PBI Dansensor) before opening the packages for microbial enumerations on days 0, 3 and 9.

Enumeration of *L. monocytogenes*

L. monocytogenes counts were enumerated according to the ISO method 11290-2/2004 (ISO, 2004b) with slight modifications. Presumptive *L. monocytogenes* colonies were confirmed using the multiplex PCR method that have specific 16S rRNA gene sequence primers (Border et al., 1990) for *Listeria* spp. and listeriolysin O virulence gene sequence primers (Bansal, 1996) for *L. monocytogenes.*

Enumeration of *Y. pseudotuberculosis*

Y. pseudotuberculosis counts were enumerated using the most probable number (MPN) method of five parallel tubes followed by multiplex PCR detection. Carrot samples (25 g) were homogenised and diluted in peptone water (1:10). Ten-fold dilutions were inoculated into five TS broth (Oxoid) tubes and incubated at 28 °C for 24 h. A 200 µl sample from each culture was centrifuged (16 000 × g, 10 min) and the supernatant removed. Cells were lysed and DNA extracted in 100 µl of DyNAzyme PCR buffer (Thermo Fisher Scientific, Waltham, Massachusetts, US) containing 0.2 mg/ml of proteinase K (Thermo Fisher Scientific) by incubating the samples at 37 °C for 1 h and further heating at 97 °C for 10 min. Samples were stored at –20 °C until PCR detection, which was based on primers for *virF* (Kaneko et al., 1995) and *inv* (Nakajima et al., 1992) genes. Samples were centrifuged (16 000 × g, 5 min) and 1 µl of supernatant was added into 24 µl of mastermix, which contained 1 x PCR buffer (DyNAzyme buffer, Thermo Fisher Scientific), 200 µM of 4dNTP mix (Thermo Fisher Scientific), 0.5 µM of each *virF* and *inv* primers (Metabion Oligomer, Helsinki, FI) and 1 U DyNAzyme II DNA polymerase (Thermo Fisher Scientific). PCR parameters were as follows: initial denaturation at 94 °C for 60 s, 30 cycles consisting of denaturation at 94 °C for 30 s, annealing at 55 °C for 60 s and extension at 72 °C for 30 s followed by final extension at 72 °C for 5 min.

Enumeration of yeasts

Yeast counts were determined according to the ISO method 21527-1/2008 (ISO, 2008) with slight modifications. Samples were spread on OGYE (Oxoid) plates and colonies counted after three, five and seven days of incubation at 25°C. Typical colonies were confirmed by gram staining.

Examination of uninoculated vegetables and pH

Before inoculation, each batch of vegetables was sampled to test for the presence of *L. monocytogenes, Y. pseudotuberculosis* or yeasts depending on the study. The presence of *L. monocytogenes* was studied in two parallel packages according to the ISO detection method 11290-1/2004 (ISO, 2004a) with slight modifications. LMBA (Lab M, Heywood, Lancashire, UK) and ALOA (Lab M) were used for plating, and identification of typical colonies was performed using multiplex PCR as described earlier. The presence of *Y. pseudotuberculosis* was studied in two parallel packages using a detection method described by Virtanen et al. (2012) with minimal modifications. Temperature for the cold enrichment was 5 °C, 0.5 % KOH solution was used for the alkali treatment, and typical colonies on CIN agar (Lab M) were identified using the multiplex PCR method for *Y. pseudotuberculosis* as described earlier. The presence of yeasts was studied in two parallel packages on days 0 and nine using the yeast enumeration method as described earlier to determine the amount of uninoculated yeasts at the beginning and end of the nine-day storage period. After the microbial determinations on days 0, three and nine, pH was measured from one randomly selected package of each vegetable and atmosphere combination using an inoLab pH 720 pH meter (WTW, Weilheim, DE).

Statistical analysis

The experiments were performed in three replicates. Arithmetic means and standard deviations were calculated for the headspace gas compositions. Microbial counts were transformed to base-10 logarithmic values and reported as arithmetic means and standard deviations. To compare microbial counts, the one-way analysis of variance (ANOVA) and post-hoc Tukey's test were performed using IBM SPSS Statistics 24 software (IBM, Armonk, New York, US). Statistical significance was set at $P < 0.05$.

Results

Changes in headspace gas compositions and pH

Metabolism of fresh vegetables and microbes changed the gas compositions of the vegetable packages during storage (Fig. 1. and 2.). On day nine no O_2 or less than 1 % O_2 was detected in all packages, whereas CO_2 concentrations had increased to levels between 16 % and 34 %. Changes in gas compositions within the packages of shredded carrots were similar to those for the cubed carrots under atmosphere A (results not shown). The pH values decreased towards the end of the cold storage (Tab. 2.).

L. monocytogenes counts on cabbage and onion

L. monocytogenes counts increased on shredded cabbage towards the end of the storage ($P < 0.05$) under every

Journal of Food Safety and Food Quality 69, Heft 4 (2018), Seiten 105–130

atmosphere examined (Fig. 3.). On day nine, the mean counts varied between 6.1 and 7.6 log CFU/g, and the counts were statistically significantly lowest ($P < 0.05$) under atmosphere A, and highest ($P < 0.05$) under atmosphere B. On day three, the counts under atmospheres A and B were lower than under atmosphere C ($P < 0.05$). *L. monocytogenes* was unable to grow on cut onions, and by day nine the counts had decreased below the inoculation level under each atmosphere studied ($P < 0.05$) when the mean counts varied between 2.7 and 2.8 log CFU/g (Fig. 3.). No *Listeria* spp. were detected in uninoculated shredded cabbage or cut onions.

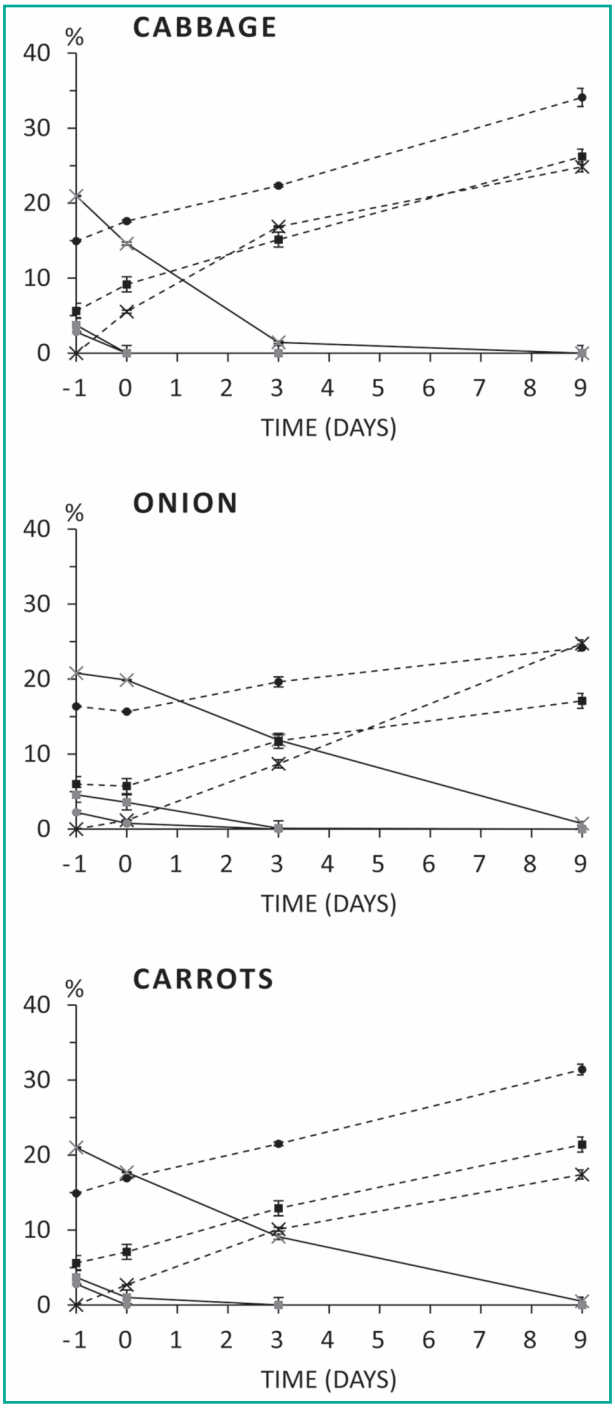

FIGURE 1: *Headspace gas compositions within packages of shredded cabbage, cut onions, and cubed carrots by atmosphere during the nine-day cold storage. (×) atmosphere A (initially 21 % O_2, 0 % CO_2), (■) atmosphere B (initially 5 % O_2, 5 % CO_2) and (●) atmosphere C (initially 2 % O_2, 15 % CO_2) all balanced with N_2. Grey symbols represent O_2 concentrations fitted with solid lines and black symbols represent CO_2 concentrations fitted with dashed lines. Values are means calculated from three packages, and error bars show standard deviation. Day –1 represents initial gas compositions measured at the processing plant, and inoculation was performed on day 0.*

Y. pseudotuberculosis counts on carrots

Y. pseudotuberculosis counts increased on cubed carrots towards the end of the storage ($P < 0.05$) under every atmosphere examined (Fig. 4.). However, after day three statistically significant increases ($P < 0.05$) in the counts between days three and nine were observed under atmospheres B and C only. Mean counts varied between 6.4 and 7.7 log CFU/g at the end of the storage, and there was a statistical difference ($P < 0.05$) in counts between atmospheres A and C. The counts for shredded carrots under atmosphere A increased ($P < 0.05$) during the first three days, but after day

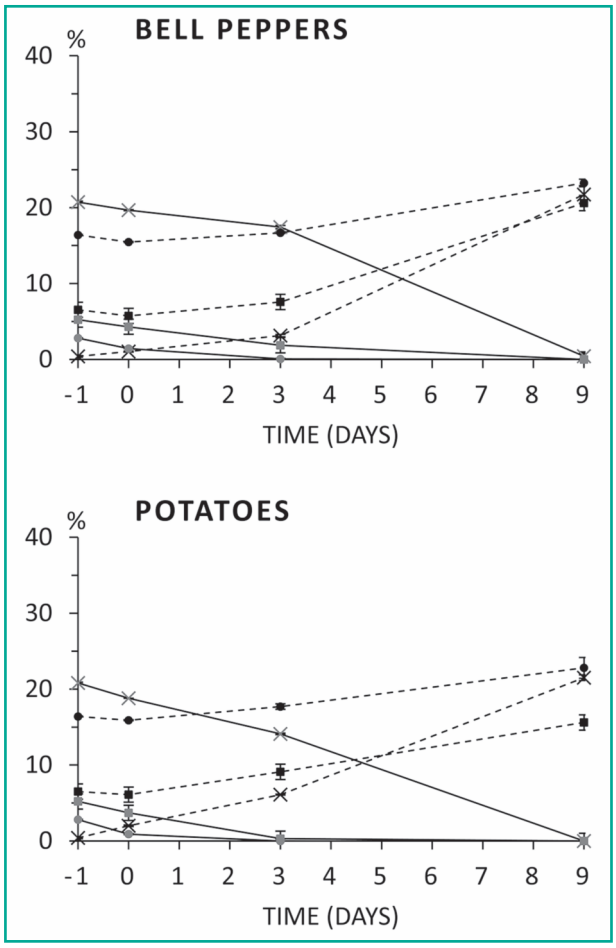

FIGURE 2: *Headspace gas compositions within packages of cubed bell peppers and cubed potatoes by atmosphere during the nine-day cold storage. (×) atmosphere A (initially 21 % O_2, 0 % CO_2), (■) atmosphere B (initially 5 % O_2, 5 % CO_2), and (●) atmosphere C (initially 2 % O_2, 15 % CO_2) all balanced with N_2. Grey symbols represent O_2 concentrations fitted with solid lines and black symbols represent CO_2 concentrations fitted with dashed lines. Values are means calculated from three packages, and error bars show standard deviation. Day –1 represents initial gas compositions measured at the processing plant and inoculation was performed on day 0.*

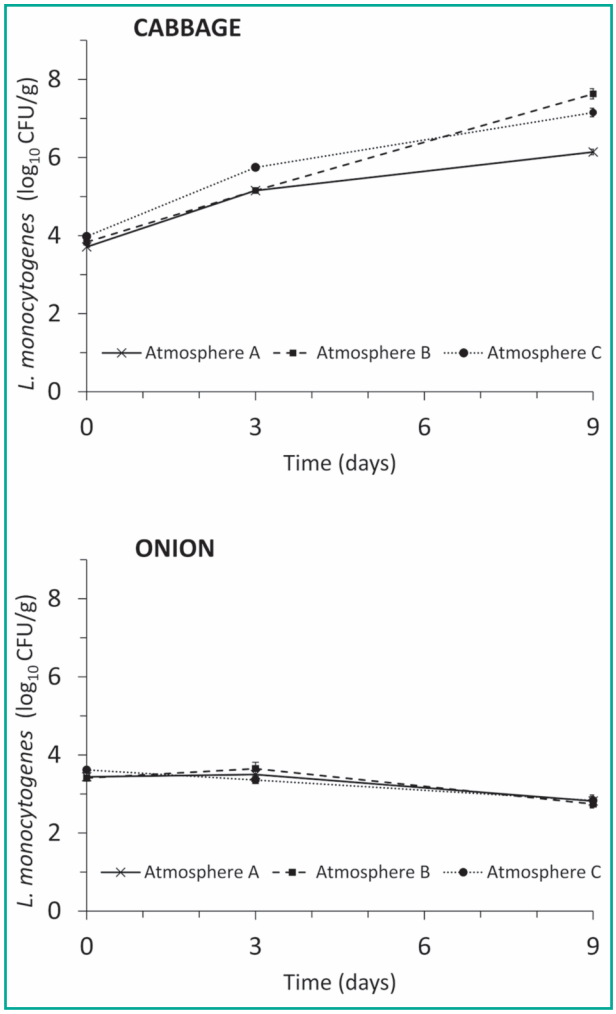

FIGURE 3: *Growth and survival of Listeria monocytogenes on shredded cabbage and cut onions under atmospheres A–C stored at 6 °C for nine days. Symbols represent the mean logarithmic counts of three samples, and error bars show standard deviation. Initial gas compositions of the atmospheres were (A) 21 % O_2, 0 % CO_2, (B) 5 % O_2, 5 % CO_2, and (C) 2 % O_2, 15 % CO_2 all balanced with N_2. For changes in gas compositions during storage, see Fig. 1.*

three the increase was not statistically significant ($P > 0.05$). On day three under atmosphere A, the counts for cubed produce were similar to those of shredded produce. However, the increase was higher on shredded than cubed produce ($P < 0.05$), as a lower inoculation level was used for shredded carrots (Fig. 4.). No *Yersinia* spp. were detected in uninoculated cubed or shredded carrots.

Yeast counts on bell peppers and potatoes

Yeasts grew on cubed bell peppers and cubed potatoes under each of the three atmospheres during storage ($P < 0.05$), and reached levels from 7.0 to 8.1 log CFU/g and from 6.4 to 7.9 log CFU/g, respectively, by the end of the storage period (Fig. 5.). Higher O_2 levels allowed more yeast growth on both vegetables, as the counts were the highest ($P < 0.05$) under atmosphere A and the lowest ($P < 0.05$) under atmosphere C (Fig. 5.). Uninoculated material contained yeasts below the detection limit (≤ 100 CFU/g) on day 0, whereas the counts were 3.8 log CFU/g and 3.2 log CFU/g on day nine for uninoculated cubed bell peppers and cubed potatoes, respectively.

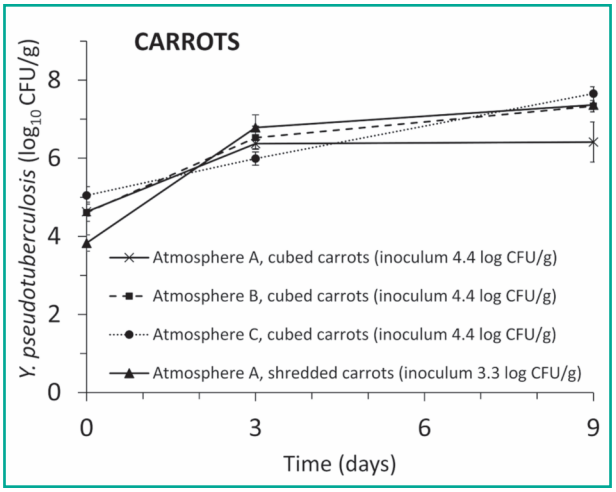

FIGURE 4: *Growth and survival of Yersinia pseudotuberculosis on cubed carrots under atmospheres A–C and shredded carrots under atmosphere A stored at 6 °C for nine days. Symbols represent the mean logarithmic MPN estimate counts of three samples, and error bars show standard deviation. Initial gas compositions of the atmospheres were (A) 21 % O_2, 0 % CO_2, (B) 5 % O_2, 5 % CO_2, and (C) 2 % O_2, 15 % CO_2 all balanced with N_2. For changes in gas compositions during storage, see Fig. 1.*

Discussion

L. monocytogenes was able to grow on shredded white cabbage under each of the three atmospheres studied. Previous studies have shown that *L. monocytogenes* is able to grow on raw shredded cabbage stored aerobically at 5 °C and on white cabbage stored at 4 °C in plastic bags (Beuchat et al., 1986; Breer and Baumgartner, 1992). *L. monocytogenes* has also been shown to persist on intact cabbage tissue during a 28-day storage period at 5 °C, but cut tissue is more likely to favour *L. monocytogenes* growth (Ells and Truelstrup Hansen, 2010). Cabbage appears to be a potential growth medium for *L. monocytogenes.* Careful handling and processing are needed, as *L. monocytogenes*

TABLE 2: *The pH values of the inoculated vegetable products during the nine-day storage at 6 °C.*

Product	Atmosphere[a]	pH value[b]		
		Day 0	Day 3	Day 9
Shredded cabbage	A	ND[c]	6.1	5.7
	B	5.8	6.2	4.8
	C	5.8	6.0	4.8
Cut onions	A	4.8	4.3	4.7
	B	5.6	4.7	4.4
	C	5.6	4.8	4.3
Cubed carrots	A	6.0	4.0	5.2
	B	5.8	4.9	5.2
	C	ND[c]	5.3	4.7
Shredded carrots	A	5.7	4.3	4.6
Cubed bell peppers	A	5.7	5.4	4.0
	B	5.3	4.9	4.5
	C	5.1	4.9	4.5
Cubed potatoes	A	6.6	5.4	5.3
	B	6.8	5.6	5.1
	C	6.9	6.3	5.1

[a] Initial gas compositions: (A) 21 % O_2, 0 % CO_2, (B) 5 % O_2, 5 % CO_2, and (C) 2 % O_2, 15 % CO_2 all balanced with N_2. [b] One randomly selected package of each atmosphere and vegetable combination was studied. [c] Not determined.

Journal of Food Safety and Food Quality 69, Heft 4 (2018), Seiten 105–130

is difficult to eliminate from vegetable leaves by washing (Hellström et al., 2006).

The effect of modified atmospheres on the growth of *L. monocytogenes* on vegetables has been marginal or nonexistent (Berrang et al., 1989; Beuchat and Brackett, 1990a, Kallander et al., 1991; Bennik et al., 1996), which is supported by our results. However, the growth was slightly delayed when more O_2 was present. This is probably due to indirect factors, such as competition for nutrients or production of inhibitory compounds by other microbial populations (Carlin et al., 1996; Francis and O'Beirne, 1998), or changes in conditions or morphology of the vegetable tissues (Zagory et al., 1999; Francis et al., 2012) rather than direct effects by the atmospheres. *L. monocytogenes* tolerates very high CO_2 concentrations (Farber et al., 1996) and should grow faster under aerobic than anaerobic conditions in a pure laboratory culture (Al-Qadiri et al., 2015). In the present study, the highest counts were reached under atmosphere B, with low initial O_2 and CO_2 levels, as it may have allowed a competitive advantage for *L. monocytogenes* without marked inhibitory effects directly by CO_2. Possible reduction of other microbes may enhance *L. monocytogenes* growth as shown in disinfection studies on endives (Bennik et al., 1996; Carlin et al., 1996).

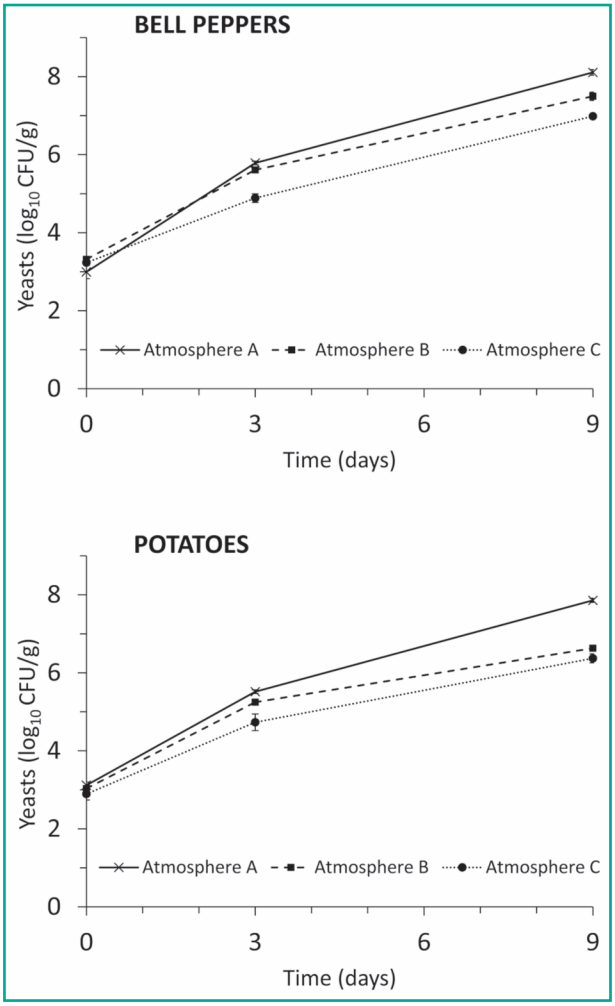

FIGURE 5: *Growth of yeasts on cubed bell peppers and cubed potatoes under atmospheres A–C stored at 6 °C for nine days. Symbols represent the mean logarithmic counts of three samples, and error bars show standard deviation. Initial gas compositions of the atmospheres were (A) 21 % O_2, 0 % CO_2, (B) 5 % O_2, 5 % CO_2, and (C) 2 % O_2, 15 % CO_2 all balanced with N_2. For changes in gas compositions during storage, see Fig. 2.*

L. monocytogenes was unable to grow on cut onions. This could be due to the low pH and antimicrobial properties of onions. In our present study, pH values for the onion products had decreased below 5 after three days of storage, being lower than the corresponding pH values for cabbage (Tab. 2). When temperature is lowered, *L. monocytogenes* is not so likely to grow in low pH environments (Buchanan et al., 1989; Farber et al., 1996; Tienungoon et al., 2000). Farber et al. (1998) observed that *L. monocytogenes* grew over 1 log unit on onion slices stored at 10 °C but not at 4 °C, where the counts had slightly reduced during the nine-day storage. Crude onion extracts and onion flavonols quercetin and kaempferol as well as green onion extract have been shown to have inhibitory properties against *L. monocytogenes* (Santas et al., 2010; Yan et al., 2011). Being possibly quite limited on their own, antimicrobial properties might still explain why the surface of an onion is not so likely to support *L. monocytogenes* growth at low temperatures although *L. monocytogenes* was able to survive during storage.

Y. pseudotuberculosis was able to grow on cubed carrots under each of the three atmospheres studied, which is consistent with its psychrotrophic facultative anaerobic nature. Modified atmospheres used for vegetables appear to not inhibit its growth. Although the differences in counts between the three atmospheres were slight, our findings imply that modified atmospheres might even favour the growth of *Y. pseudotuberculosis,* which can be a food safety risk. Carrots, especially grated produce, have been linked to several *Y. pseudotuberculosis* outbreaks in epidemiological studies (Jalava et al., 2006; Kangas et al., 2008; Rimhanen-Finne et al., 2009). The increase in *Y. pseudotuberculosis* counts was higher on shredded than cubed produce during the first three days of storage, which may implicate that cutting carrots into smaller pieces may enhance *Y. pseudotuberculosis* growth. This might be due to increased surface area along with better adhesion for bacteria and a release of nutrients caused by increased tissue damage (Zagory, 1999; Francis et al., 2012). In contrast, raw carrots have been shown to possess inhibitory activity against *L. monocytogenes* (Beuchat and Brackett, 1990b; Nguyen-the and Lund, 1991).

Yeasts were able to grow on both cubed bell peppers and cubed potatoes under each of the atmospheres studied. The highest counts were observed on vegetables packaged under atmosphere A. The presence of O_2 support yeast growth, despite many of the species potentially growing anaerobically (Visser et al., 1990). Yeasts are also relatively tolerant of CO_2 (Jones and Greenfield, 1982), which may explain why they were able to grow under atmospheres B and C with limited O_2 amount already by day three. Yeasts are present on different fruits and vegetables in various numbers ranging from below 2 to 8 log CFU/g, and salads generally contain higher counts of yeasts than single vegetables, which may be explained by the handling and cutting of salad items during processing (Tournas, 2005). Albeit modified atmospheres does not appear to have distinct effects on yeast growth on vegetables (Beuchat and Brackett, 1990a; Babic et al., 1992), yeasts appear to be able to grow under modified atmospheres and a higher O_2 level may enhance the growth. However, if very high O_2 rates (e. g. > 70 %) are used, microbial growth may be delayed probably due to reactive oxygen species. An O_2 level of

80 % in combination with a 20 % CO_2 level delayed the growth of *C. sake* and *Candida guilliermondii* (Amanatidou et al., 1999), whereas O_2 rates over 70 % were retardant for *Candida lambica* (Jacxsens et al., 2001).

In conclusion, modified atmospheres used for packaging fresh vegetables appear to have only limited effects on the growth of *L. monocytogenes* and *Y. pseudotuberculosis* on fresh vegetables. Thus, control of those psychrotrophic facultative anaerobic pathogens must rely on good agricultural and processing practices along with strict temperature control and shelf lives short enough to prevent pathogens from reaching infectious counts. Low O_2 concentrations may be used to delay yeast growth on fresh vegetables.

Acknowledgements

This study was partly funded by Apetit Ruoka Ltd. We acknowledge Mikael Skurnik for providing the *Y. pseudotuberculosis* strains, Thomas Grönthal and Merja Rantala for their contribution to the identification of yeast strains, Kirsi Ristkari and Anu Seppänen for technical assistance and Marie Verkola for assistance with the German translation.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

.

References

Al-Qadiri H, Sablani SS, Ovissipour M, Al-Alami N, Govindan B, Rasco B (2015): Effect of oxygen stress on growth and survival of *Clostridium perfringens, Campylobacter jejuni* and *Listeria monocytogenes* under different storage conditions. J Food Prot 78: 691–697.

Amanatidou A, Smid EJ, Gorris LGM (1999): Effect of elevated oxygen and carbon dioxide on the surface growth of vegetable-associated micro-organisms. J Appl Microbiol 86: 429–438.

Babic I, Hilbert G, Nguyen-the C, Guiraud J (1992): The yeast flora of stored ready-to-use carrots and their role in spoilage. Int J Food Sci Tech 27: 473–484.

Bansal NS (1996): Development of a polymerase chain reaction assay for the detection of *Listeria monocytogenes* in foods. Lett Appl Microbiol 22: 353–356.

Bennik MHJ, Peppelenbos HW, Nguyen-the C, Carlin F, Smid EJ, Gorris LGM (1996): Microbiology of minimally processed, modified-atmosphere packaged chicory endive. Postharvest Biol Technol 9: 209–221.

Berrang ME, Brackett RE, Beuchat LR (1989): Growth of *Listeria monocytogenes* on fresh vegetables stored under controlled atmosphere. J Food Prot 52: 702–705.

Beuchat LR, Brackett RE, Hao DY-Y, Conner DE (1986): Growth and thermal inactivation of *Listeria monocytogenes* in cabbage and cabbage juice. Can J Microbiol 32: 791–795.

Beuchat LR, Brackett RE (1990a): Survival and growth of *Listeria monocytogenes* on lettuce as influenced by shredding, chlorine treatment, modified atmosphere packaging and temperature. J Food Sci 55: 755–758.

Beuchat LR, Brackett RE (1990b): Inhibitory effects of raw carrots on *Listeria monocytogenes.* Appl Environ Microbiol 56: 1734–1742.

Border PM, Howard JJ, Plastow GS, and Siggens KW (1990): Detection of *Listeria* species and *Listeria monocytogenes* using polymerase chain reaction. Lett Appl Microbiol 11: 158–162.

Brackett RE (1988): Changes in the microflora of packaged fresh tomatoes. J Food Qual 11: 89–105.

Brackett RE (1989): Changes in the microflora of packaged fresh broccoli. J Food Qual 12: 169–181.

Brackett RE (1990): Influence of modified atmosphere packaging on the microflora and quality of fresh bell peppers. J Food Prot 53: 255–261.

Breer C, Baumgartner A (1992): Vorkommen und Verhalten von *Listeria monocytogenes* auf salaten und Gemüsen sowie in frischgepreßten Gemüsesäften. Arch Lebensmittel 43: 108–110.

Buchanan RL, Stahl HG, Whiting RC (1989): Effects and interactions of temperature, pH, atmosphere, sodium chloride, and sodium nitrite on the growth of *Listeria monocytogenes.* J Food Prot 52: 844–851.

Caleb OJ, Mahajan PV, Al-Said FA-J, Opara UL (2013): Modified atmosphere packaging technology of fresh and fresh-cut produce and the microbial consequences – A review. Food Bioprocess Technol 6: 303–329.

Cardamone C, Aleo A, Mammina C, Oliveri G, Di Noto AM (2015): Assessment of the microbiological quality of fresh produce on sale in Sicily, Italy: preliminary results. J Biol Res (Thessalon) 22: 3. doi: 10.1186/s40709-015-0026-3.

Carlin F, Nguyen-the C, Morris CE (1996): Influence of background microflora on *Listeria monocytogenes* on minimally processed fresh broad-leaved endive (*Cichorium endivia* var. *latifolia*). J Food Prot 59: 698–703.

Cassagne C, Cella A-L, Suchon P, Normand A-C, Rangue S, Piarroux R (2012): Evaluation of four pretreatment procedures for MALDI-TOF MS yeast identification in the routine clinical laboratory. Med Mycol 51: 371–377. doi: 10.3109/13693786.2012.720720.

de Noordhout CM, Devleesschauwer B, Angulo FJ, Verbeke G, Haagsma J, Kirk M, Havelaar A, Speybroeck N (2010): The global burden of listeriosis: a systematic review and meta-analysis. Lancet Infect Dis 14: 1073–1082.

EFSA Panel on Biological Hazards (BIOHAZ) (2013): Scientific Opinion on the risk posed by pathogens in food of non-animal origin. Part 1 (outbreak data analysis and risk ranking of food/pathogen combinations). EFSA J 11: 3025. [138 pp.] doi:10.2903/j.efsa.2013.3025.

Ells TC, Truelstrup Hansen L (2010): Growth of *Listeria* spp. in shredded cabbage is enhanced by a mild heat treatment. J Food Prot 73: 425–433.

Erickson MC (2010): Microbial Risks Associated with cabbage, carrots, celery, onions, and deli salads made with these produce items. Compr Rev in Food Sci Food Saf 9: 602–619. doi:10.1111/j.1541-4337.2010.00129.x.

Farber JM, Cai Y, Ross WH (1996): Predictive modeling of the growth of *Listeria monocytogenes* in CO_2 environments. Int J Food Microbiol 32: 133–144.

Farber JM, Wang SL, Cai Y, Zhang S (1998): Changes in populations of *Listeria monocytogenes* inoculated on packaged fresh-cut vegetables. J Food Prot 61: 192–195.

Farber JN, Harris LJ, Parish ME, Beuchat LR, Suslow TV, Gorney JR, Garrett EH, Busta FF (2003): Microbiological safety of controlled and modified atmosphere packaging of fresh and fresh-cut produce. Compr Rev Food Sci Food Saf 2: 142–160.

Fleet GH (2011): Yeast Spoilage of Foods and Beverages. In: Kurtzman CP, Fell JW, Boekhout T (ed.), The yeast: A taxonomic study. Elsevier, London, UK, 53–63.

Francis GA, O'Beirne D (1998): Effects of storage atmosphere on *Listeria monocytogenes* and competing microflora using a surface model system. Int J Food Sci Tech 33: 465–476.

Francis GA, Gallone A, Nychas GJ, Sofos JN, Colelli G, Amodio ML, Spano G (2012): Factors affecting quality and safety of fresh-cut produce. Crit Rev Food Sci Nutr 52: 595–610.

Fredriksson-Ahomaa M, Lindström M, Korkeala H (2010): *Yersinia enterocolitica* and *Yersinia pseudotuberculosis.* In: Juneva

Journal of Food Safety and Food Quality 69, Heft 4 (2018), Seiten 105–130

VK, Sofos JN (ed.), Pathogens and toxins in foods: Challenges and interventions. ASM Press, Washington, DC, 164–180.

Hellström S, Kervinen R, Lyly M, Ahvenainen-Rantala R, Korkeala H (2006): Efficacy of disinfectants to reduce *Listeria monocytogenes* on precut iceberg lettuce. J Food Prot 69: 1565–1570.

Hoelzer K, Pouillot R, Dennis S (2012): *Listeria monocytogenes* growth dynamics on produce: A review of the available data for predictive modelling. Foodborne Pathog Dis 9: 661–673.

Inoue M, Nakajima H, Ishida T, Tsubokura M (1988): Three outbreaks of *Yersinia pseudotuberculosis* infection. Zentralbl Bakteriol Mikrobiol Hyg B 186: 504–511.

International Organization for Standardization (ISO) (2004a): Microbiology and animal feeding stuffs – Horisontal method for the detection and enumeration of *Listeria monocytogenes:* Part 1. Detection method. ISO standard 11290-1:1996 and Amd. 1:2004. ISO, Geneva.

International Organization for Standardization (ISO) (2004b): Microbiology and animal feeding stuffs – Horisontal method for the detection and enumeration of *Listeria monocytogenes:* Part 2. Enumeration method. ISO Standard 11290-2:1998 and Amd. 1:2004. ISO, Geneva.

International Organization for Standardization (ISO) (2008): Microbiology and animal feeding stuffs – Horisontal method for the detection and enumeration of yeasts and moulds: Part 1. Colony count technique in products with water activity greater than 0.95. ISO standard 21527-1:2008. ISO, Geneva.

Jacxsens L, Devlieghere F, Van der Steen C, Debevere J (2001): Effect of high oxygen modified atmosphere packaging on microbial growth and sensorial qualities of fresh-cut produce. Int J Food Microbiol 71: 197–210.

Jalava K, Hallanvuo S, Nakari U-M, Ruutu P, Kela E, Heinäsmäki T, Siitonen A, Nuorti JP (2004): Multiple outbreaks of *Yersinia pseudotuberculosis* infections in Finland. J Clin Microbiol 42: 2789–2791.

Jalava K, Häkkinen M, Valkonen M, Nakari U-M, Palo T, Hallanvuo S, Ollgren J, Siitonen A, Nuorti JP (2006): An outbreak of gastrointestinal illness and erythema nodosum from grated carrots contaminated with *Yersinia pseudotuberculosis.* J Infect Dis 194: 1209–1216.

Jones RP, Greenfield PF (1982): Effect of carbon dioxide on yeast growth and fermentation. Enz Microbiol Tech 4: 210–223.

Kallander KD, Hitchins AD, Lancette GA, Schmieg JA, Garcia GR, Solomon HM, Sofos JN (1991): Fate of *Listeria monocytogenes* in shredded cabbage stored at 5 and 25 °C under a modified atmosphere. J Food Prot 54: 302–304.

Kaneko S, Ishizaki N, Kokubo Y (1995): Detection of pathogenic *Yersinia enterocolitica* and *Yersinia pseudotuberculosis* from pork using the polymerase chain reaction. Contrib Microbiol Immunol 13: 153–155.

Kangas S, Takkinen J, Häkkinen M, Nakari U-M, Johansson T, Henttonen H, Virtaluoto L, Siitonen A, Ollgren J, Kuusi M (2008): *Yersinia pseudotuberculosis* O:1 traced to raw carrots, Finland. Emerg Infect Dis 14: 1959–1961.

Nakajima H, Inoue M, Mori T, Itoh K-I, Arakawa E, Watanabe H (1992): Detection and identification of *Yersinia pseudotuberculosis* and pathogenic *Yersinia enterocolitica* by an improved polymerase chain reaction method. J Clin Microbiol 30: 2484–2486.

Nakano T, Kawaguchi H, Nakao K, Maryama T, Kamiya H, Sakurai M (1989): Two outbreaks of *Yersinia pseudotuberculosis* 5a infection in Japan. Scand J Infect Dis 21: 175–179.

Nguyen-the C, Lund BM (1991): The lethal effect of carrot on *Listeria* species. J Appl Bacteriol 70: 479–488.

Normand A-C, Becker PT, Gabriel F, Cassagne C, Accoceberry I, Gari-Toussaint M, Hasseine L, De Geyter D, Piérard D, Surmont I, Djenad F, Donnadieu J-L, Piarroux M, Ranque S, Hendrickx M, Piarroux R (2017): Online identification of fungi using MALDI-TOF mass spectrometry: validation of a new web application. J Clin Microbiol 55: 2661–2670. doi:10.1128/JCM.00263-17.

Nousiainen L-L, Joutsen S, Lundén J, Hänninen M-L, Fredriksson-Ahomaa M (2016): Bacterial quality and safety of packaged fresh leafy vegetables at the retail level in Finland. Int J Food Microbiol 232: 73–79.

Nuorti JP, Niskanen T, Hallanvuo S, Mikkola J, Kela E, Hatakka M, Fredriksson-Ahomaa M, Lyytikainen O, Siitonen A, Korkeala H, and Ruutu P (2004): A widespread outbreak of *Yersinia pseudotuberculosis* O:3 infection from iceberg lettuce. J Infect Dis 189: 766–774.

Press N, Fyfe M, Bowie W, and Kelly M (2001): Clinical and microbiological follow-up of an outbreak of *Yersinia pseudotuberculosis* serotype 1b. Scand J Infect Dis 33: 523–526.

Pärn T, Hallanvuo S, Salmenlinna S, Pihlajasaari A, Heikkinen S, Telkki-Nykänen H, Hakkinen M, Ollgren J, Huusko S, Rimhanen-Finne R (2015): Outbreak of *Yersinia pseudotuberculosis* O:1 infection associated with raw milk consumption, Finland, spring 2014. Euro Surveill 20: pii=30033. DOI: http://dx.doi.org/10.2807/1560-7917.ES.2015.20.40.30033.

Rimhanen-Finne R, Niskanen T, Hallanvuo S, Makary P, Haukka K, Pajunen S, Siitonen A, Ristolainen R, Pöyry H, Ollgren J, Kuusi M (2009): *Yersinia pseudotuberculosis* causing a large outbreak associated with carrots in Finland, 2006. Epidemiol Infect 137: 342–347.

Santas J, Almajano MP, Carbo R (2010): Antimicrobial and antioxidant activity of crude onion (*Allium cepa,* L.) extracts. Int J Food Sci Tech 45: 403–409.

Schlech WF3rd, Lavigne PM, Bortolussi RA, Allen AC, Haldane EV, Wort AJ, Hightower AW, Johnson SE, King SH, Nicholls ES, Broome CV (1983): Epidemic listeriosis – evidence for transmission by food. N Engl J Med 308: 203–206.

Swaminathan B, Gerner-Smidt P (2007): The epidemiology of human listeriosis. Microbes Infect 9: 1236–1243.

Tertti R, Granfors K, Lehtonen O-P, Mertsola J, Mäkelä A-L, Välimäki I, Hänninen P, Toivanen A (1984): An outbreak of *Yersinia pseudotuberculosis* infection. J Infect Dis 149: 245–250.

Tienungoon S, Ratkowski DA, McMeekin TA, Ross T (2000): Growth limits of *Listeria monocytogenes* as a function of temperature, pH, NaCl, and lactic acid. Appl Environ Microbiol 66: 4979–4987.

Tournas VH (2005): Moulds and yeasts in fresh and minimally processed vegetables, and sprouts. Int J Food Microbiol 99: 71–77.

Tseneva GY, Chesnokova MV, Timofeevich KV, Aleksandrovna VE, Burgasova OA, Sayapina LV, Aleksandrovna TK, Karimova TV (2012): Pseudotuberculosis in the Russian federation. Adv Exp Med Biol 954:63-68. doi: 10.1007/978-1-4614-3561-7_9.

Virtanen S, Salonen L, Laukkanen-Ninios R, Fredriksson-Ahomaa M, Korkeala H (2012): Piglets are a source of pathogenic *Yersinia enterocolitica* on fattening-pig farms. Appl Environ Microbiol 78: 3000–3003.

Visser W, Scheffers WA, Batenburg-van der Vegte WH, van Dijken JP (1990): Oxygen requirements of yeasts. Appl Environ Microbiol 56: 3785–3792.

Yan M, Li C-W, Hsieh Y-H (2011): Synergistic inhibition of *Listeria monocytogenes* by nisin and green onion extract. J Food Safety 31: 505–510.

Zagory D (1999): Effects of post-processing handling and packaging on microbial populations. Postharvest Biol Technol 15: 313–321.

Address of corresponding author:
Riikka Keto-Timonen, DVM, PhD
Department of Food Hygiene and Environmental Health
Faculty of Veterinary Medicine, University of Helsinki
P.O. Box 66
FI-00014 University of Helsinki
Finland
riikka.keto-timonen@helsinki.fi