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Growth potential of *Salmonella* spp. and *Listeria monocytogenes* in nine types of ready-to-eat vegetables stored at variable temperature conditions during shelf-life

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ABSTRACT

Growth potential (δ) is defined as the difference between the population of a microorganism at the end of shelf-life of specific food and its initial population. The determination of δ of *Salmonella* and *Listeria monocytogenes* in RTE vegetables can be very useful to determine likely threats to food safety. However, little is known on the behavior of these microorganisms in several RTE vegetables. Therefore, the aim of this study was to determine the δ of both pathogens in nine different types of RTE vegetables (escarole, collard green, spinach, watercress, arugula, grated carrot, green salad, and mix for yakisoba) stored at refrigeration (7 °C) and abuse temperature (15 °C). The population of aerobic microorganisms and lactic acid bacteria, including those showing antimicrobial activity has been also determined. Results indicated that *L. monocytogenes* was able to grow ($\delta \geq 0.5$ $\log_{10}$) in more storage conditions and vegetables than *Salmonella*. Both microorganisms were inhibited in carrots, although a more pronounced effect has been observed against *L. monocytogenes*. The highest δ values were obtained when the RTE vegetables were stored 15 °C/6 days in collard greens ($\delta = 3.3$) and arugula ($\delta = 3.2$) (*L. monocytogenes*) and arugula ($\delta = 4.1$) and escarole ($\delta = 2.8$) (*Salmonella*). In most vegetables and storage conditions studied, the counts of total aerobic microorganisms raised significantly independent of the temperature of storage ($p < 0.05$). Counts of lactic acid bacteria were higher in vegetables partially or fully stored at abuse temperature with recovery of isolates showing antimicrobial activity. In conclusion, the results of this study show that *Salmonella* and *L. monocytogenes* may grow and reach high populations in RTE vegetables depending on storage conditions and the definition of effective intervention strategies are needed to control their growth in these products.

1. Introduction

Surveillance data have shown that vegetables have been implicated in foodborne disease outbreaks caused by a variety of pathogenic microorganisms (Sivapalasingam et al., 2004; Anon, 2008a, 2010). As a result, numerous studies have been performed to determine the occurrence of microorganisms such as *Salmonella* (Giusti et al., 2010; Gorski et al., 2011; Sant'Ana et al., 2011), pathogenic *Escherichia coli* (Bohaychuk et al., 2009; Rúgeles et al., 2010) and *Listeria monocytogenes* (Little et al., 2007; Oliveira et al., 2010; Sant'Ana et al., 2012) in different types of vegetables.

The contamination of vegetables by *Salmonella* spp. and *L. monocytogenes* might occur either in the field or during handling or processing (Ailes et al., 2008; Caponigro et al., 2010) as *Salmonella* is present in the intestinal tract of humans and animals (Adley et al., 2011; Rostagno and Callaway, 2011), and *L. monocytogenes* is a markedly ubiquitous microorganism being isolated from diverse sources (Gandhi and Chikindas, 2007). Despite presence in the vegetables, the capability of these microorganisms to survive, grow and cause disease will depend upon plant–microbe and microbe–microbe interactions (Brandl, 2006) and their responses to unfavorable conditions during minimal processing and storage (Capozzi et al., 2009).

Regardless the low prevalence and counts of *L. monocytogenes* and *Salmonella* in RTE vegetables reported in several surveys (Little et al., 2007; Oliveira et al., 2010; Sant'Ana et al., 2011, 2012), populations as high as 10^6 CFU/g may be reached depending on storage conditions (Koseki and Isobe, 2005a,b). Although the infectivity of *Salmonella* is highly variable (Mintz et al., 1994; Musher and Musher, 2004), the infection by *L. monocytogenes* seems to be mostly associated with ingestion of high doses of this pathogen in healthy individuals (>8 log CFU) or with low doses in susceptible individuals (2–3 log CFU) (Takeuchi et al., 2006; Williams et al., 2007; Warriner and Namvar, 2009). Given this, the knowledge of growth potential (δ) in RTE vegetables can be very useful to identify critical storage conditions to be respected to prevent the growth of pathogens in these products to unacceptable levels. In addition, this knowledge may help to determine the likely threats these microorganisms may pose to food safety.

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Hence, the purpose of this study was to determine the growth potential (δ) of *Salmonella* and *L. monocytogenes* in nine types of RTE vegetables stored at different temperature conditions. In addition, the populations of aerobic microorganisms and lactic acid bacteria, including those showing antimicrobial activity were also investigated as they may influence the survival and growth of the pathogens.

2. Material and methods

2.1. Microorganisms and preparation of cell suspensions

Five strains of *L. monocytogenes* and five strains of *Salmonella* spp. were used in the study. The *L. monocytogenes* strains were isolated from RTE vegetables marketed in Sao Paulo, Brazil (Sant'Ana et al., 2012), and belonged serotypes 4b (strains 349, 564, 586 and 480) and 1/2b (strain 221) and ribotypes DUP 1038 (strains 349, 564 and 586), DUP 19191 (strain 480) and DUP 19175 (strain 221). Three *Salmonella* spp. strains were also isolated from RTE vegetables marketed in Sao Paulo, Brazil (Sant'Ana et al., 2011) and belonged to serovars *S.* Typhimurium (strain 227), *S.* Typhi (strain 386) and *S. enterica* subsp. *enterica* O:47:z4,z23:−(strain 994). Two *Salmonella* spp. strains (strains 2494 and 5711), from human and animal origin, kindly provided by Dr. Ernesto Hofer from Oswaldo Cruz Institute (Rio de Janeiro, Brazil), belonged to serovars *S.* Infantis IOC 2494 and *S.* Concord IOC 5711.

The strains of *Salmonella* spp. and *L. monocytogenes* were separately grown in 10 mL of tryptic soy broth (TSB) and TSB supplemented with 0.6% of yeast extract (TSB-YE), respectively, at 37 °C for 24 h under static conditions. One mL aliquots were transferred to fresh broths for incubation for extra 24 h, twice. The cultures were centrifuged at 8 °C for 10 min at 2810 ×*g* (Mikro 22R, Hettich Zentrifugen, Germany), supernatants were discharged and pellets were washed three times with phosphate buffered saline pH 6.0. Pools of each pathogen were prepared mixing equal volumes of each washed suspension, to achieve an optical density at 630 nm equal to 0.5, corresponding to 10^8 CFU/mL, as checked by plate counting on TSA and TSA-YE.

2.2. Vegetables

Packages of RTE vegetables with no more than one day of storage after processing were acquired from supermarkets in the city of Sao Paulo, Brazil. Nine types of vegetables were selected either based on their consumption in Brazil (IBGE, 2011), prevalence of *Salmonella* spp. (Sant'Ana et al., 2011) and *L. monocytogenes* (Sant'Ana et al., 2012). All the RTE vegetables used (escarole, collard green, spinach, watercress, arugula, grated carrot, green salad (crisp, romaine and butter lettuces), and mix for yakisoba (broccoli, cabbage, cauliflower, leek, carrots and chard) were packaged under modified atmosphere, *i.e.*, contained a mixture of gases such as O_2, CO_2 and N_2. The packages were transported to the laboratory in isothermal boxes and kept under refrigeration until the experiments were performed. The experiments were performed in the same day of the purchase of the RTE vegetables.

2.3. Inoculation of vegetables, packaging and storage conditions

Portions of 25 g of each RTE vegetable were placed in plastic bags (62 μm thickness, O_2 permeability of 1.375 $m^3\ m^{-2}\ day^{-1}$ at 23 °C and water steam permeability of 3.5 g water $m^{-2}\ day^{-1}$ at 38 °C and 90% relative humidity) and spot inoculated with 0.5 mL of the pools of *Salmonella* and *L. monocytogenes* properly diluted in 0.1% peptone water to achieve a final concentration of 10^3 CFU/g in the products. The inoculation method and final concentration were according to the guidelines for challenge tests described in Anon (2003) and Anon (2008c). The bags containing the inoculated samples were sealed in a vacuum sealing machine AP 500 (Tecmaq, Sao Paulo, Brazil) under modified atmosphere containing 5% O_2, 15% CO_2 and 80% N_2 (White Martins, Osasco, Brazil), which corresponds the gaseous composition commonly used by RTE vegetables processors in Sao Paulo.

Packages of RTE vegetables were exposed to three different storage conditions: I (100% of shelf-life at 7 °C ± 1 °C), II (30% at 7 °C ± 1 °C and 70% at 15 °C ± 1 °C) and III (100% at 15 °C ± 1 °C). These storage scenarios were chosen with the purpose to understand the behavior of *Salmonella* and *L. monocytogenes* under optimal storage conditions (≤7 °C) and under partial or full abuse temperature during shelf-life (15 °C). The shelf-life established by processors of RTE vegetables sold in Sao Paulo (6 days) was set as the maximum storage time. A total of 288 bags of RTE vegetables were prepared per replicate that consisted of: *i)* bags to be contaminated with both pathogens (n = 216), *ii)* control samples of the nine RTE vegetables (n = 36) and *iii)* samples of the nine RTE vegetables for total plate count and lactic acid bacteria enumeration (n = 36). The amount of packages prepared per condition *i*, considered the storage conditions tested (n = 3), types of vegetables (n = 9), number of packages analyzed (n = 2 at the beginning and n = 2 at the end of the shelf-life) and pathogens tested (n = 2). For calculation of number of bags needed *ii* and *iii*, the following bases were considered: types of vegetables (n = 9), number of packages analyzed at the end of the shelf-life (n = 1), and storage conditions tested (n = 3 at the end of shelf-life). As at the beginning of the experiments all the vegetables were from the same lot, fewer bags were needed. Thus, for microbiological examinations at the beginning and at the end of shelf-life, 9 and 27 bags were needed (n = 36), respectively, for each of *ii* and *iii*. These experiments were carried out twice. Control samples comprehended vegetables spot inoculated with 0.5 mL of sterile distilled water. The pH values of the vegetables were determined using a pH meter (Láctea, LCP-210, Brazil) according to Scott et al. (2001).

2.4. Enumeration and detection of Salmonella spp. and L. monocytogenes

Salmonella spp. and *L. monocytogenes* were enumerated in two packages of RTE vegetables at time "0" (after inoculation) and at the end of shelf-life (day "6"). In addition, two packages were submitted to tests for presence–absence of both pathogens before inoculation and the end of shelf-life. Enumerations of *Salmonella* spp. and *L. monocytogenes* were performed by homogenizing 25 g of samples with 225 mL of 0.1% peptone water, following decimal dilutions and inoculation in duplicate plates of Mannitol Lysine Crystal Violet Brilliant Green (MLCB) agar (*Salmonella* spp.) and Oxford (OXA) agar added of selective supplement (SR0206) (*L. monocytogenes*). MLCB and OXA plates were incubated at 37 ± 1 °C for 24 h and 48 h, respectively. Mauve colonies with black centers in MLCB agar were counted as *Salmonella*, while brown colored colonies with aesculin hydrolysis in OXA were enumerated as *L. monocytogenes*. Up to 3 colonies per sample were selected for further confirmation of *Salmonella* spp. and *L. monocytogenes* by polyvalent serotyping and biochemical tests, respectively (Anon, 1996, 2002). The final results were expressed as $\log_{10}$ CFU/g. Samples of vegetables used for enumeration were kept frozen at −20 °C until the results were obtained. When no colonies were recovered by the quantification method (below the detection limit of 10^1 CFU/g), the samples were submitted to tests for presence–absence of *Salmonella* spp. and *L. monocytogenes* using ISO 6579 (Anon, 2002) and ISO 11290–1 (Anon, 1996) methods, respectively.

2.5. Determination of growth potential (δ) of Salmonella spp. and L. monocytogenes

The growth potential (δ) of *Salmonella* spp. and *Listeria monocytogenes* in each type of RTE vegetable was determined by the