Microbiological evaluation of ready-to-eat iceberg lettuce during shelf-life and effectiveness of household washing methods

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Abstract

The aim of this study was to assess the microbiological quality of ready-to-eat (RTE) iceberg lettuce. Our investigation was based on the consumption tendency of university students considered a target market for this product. A total of 78 RTE samples were collected from chain supermarkets and analysed for the enumeration of aerobic mesophilic count (AMC), *Escherichia coli* and the detection of *Salmonella* spp. and *Listeria monocytogenes*. All samples were negative for the presence of pathogens. The mean value of AMC at the beginning, in the middle and after the expiration date was: 6.88, 8.51 and 8.72 log CFU g^{-1}, respectively. The same investigation was performed on 12 samples of fresh iceberg lettuce samples. No pathogens were found and the mean value of AMC was lower than the RTE category (5.73 log CFU g^{-1}; $P<0.05$). The effectiveness of 5 washing methods was determined on 15 samples of both fresh and RTE iceberg lettuce. Samples were washed for 15' and 30' in tap water (500 mL), tap water with NaCl (4 g/500 mL), tap water with bicarbonate (8 g/500 mL), tap water with vinegar (10 mL/500 mL) and tap water with chlorine-based disinfectant (10 mL/500 mL). A significant bacterial load reduction was recorded for vinegar and disinfectant after 30' and 15', respectively. Overall, these results showed that RTE iceberg lettuce is more contaminated than the fresh product. Also, the consumption in the first few days of packaging and after washing with disinfectants reduces the risk for health consumers.

Introduction

A ready-to-eat (RTE) food is a product intended for the human consumption without cooking or other processing (Regulation EC No 2073/2005, European Commission). Consumer tendencies towards convenient foods increased the demand for RTE vegetables, especially leafy green salads (Abadias *et al.*, 2008; de Oliveira *et al.*, 2011). Notwithstanding their healthy and convenient aspects, the global growing consumption of RTE salads is dramatically related to several outbreaks of food infections (Mercanoglu and Halkman, 2011; Jung *et al.*, 2014). Fresh produce can become contaminated at any point of chain production. Animals, soil, irrigation water and manure are the main sources of contamination in the field. The hydrophobicity of surface leave reduces the effectiveness of washing procedures resulting unable to reach microorganisms that colonise internal vegetable tissues. Moreover, nutrients released by leaf surface cells after cutting and slicing facilitate the microbial growth (Qadri *et al.*, 2015). Cross-contamination during human and mechanical handling is an important risk factor affecting the final microbiological quality of the product. In light of this, the minimal processing not only is unable to eliminate the indigenous microflora of fresh vegetable but also increase it and provides many opportunities to bring in human pathogens (Jeddi *et al.*, 2014). The cold-chain is an essential measure to ensure the quality and safety of RTE salads because refrigerated temperatures decrease the growth of some pathogens (Khalil and Frank, 2010). For this reason, the Regulation No 3746/2014 (Italian Regulation, 2014) specifies the temperature of production (T<14°C) and the temperature of raw material storage (T<8°C). Among the wide range of microbial hazards associated with RTE salads outbreaks, bacteria such as *Escherichia coli* (*E. coli*), *Salmonella* spp., *Shigella* spp., *Listeria monocytogenes* (*L. monocytogenes*) and *Campylobacter jejuni* are largely reported (Jung *et al.*, 2014; Mercanoglu and Halkman, 2011). The overall aim of this study was to assess the microbiological quality of RTE iceberg lettuce, one of the most commonly consumed RTE salads in Italy. Iceberg lettuce belongs to *Lactuca sativa* family and it grows in spherical head. External crisp leaves, mainly exposed to contamination, are removed during post-harvest operations increasing the risk of cross-contamination. Our objectives were to investigate the degree of contamination during shelf life and the reduction of bacterial load after domestic washing methods. To our knowledge, this is the first study evaluating the microbiological aspects of RTE iceberg lettuce commercialised in Italy.

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Materials and Methods

Experimental design

The investigation was based on consumption data collected from a survey targeting university students as a market segment for convenient foods. Considering the answers collected by interview, the microbiological analysis was performed at the beginning, in the middle and after the expiration date. Later, the effectiveness of household washing methods indicated by responders was evaluated through the determination of bacterial load reduction. The same investigation was also carried out on a representative number of fresh samples to get a global view of the microbiological quality of the two categories.

Survey administration

The survey was conducted to determine the consumption of RTE salads among 100 students of the University of Camerino, Italy. A random sample of 40 males and 60 females between the ages of 19 and 30 was interviewed.

Sample collection

Iceberg lettuce bags in modified atmosphere packaging (MAP) were collected from two chain supermarkets between May and July 2016 in Camerino. Three companies were considered and two bags for each one of them were collected (n=30 bags;

c=60 units) at 5 different collection times. As indicated by the Regulation EC No 2073/2005 (European Commission, 2005), the number of units were reduced considering satisfactory the procedure based on Hazard Analysis and Critical Control Point (HACCP) system applied by companies. All samples were collected at the beginning of their shelf-life, kept under refrigerated conditions and transported to the laboratory for the analysis. The second group of 30 samples was collected in the middle of their shelf-life and analysed. Instead, 9 samples (n=9; c=18 units) were collected at 3 different collection times and analysed after two days of the expiration date. Finally, 12 samples (c=24 units) of fresh iceberg lettuce were collected from three companies at 4 different times and analysed as RTE samples.

Microbiological analysis

Ten grams of each sample was aseptically weighed in sterile bags, diluted in 90 mL of Maximum Recovery Diluent (MRD) and homogenised for 2 minutes using a stomacher (Model 400 circulator, Seward, UK). To determine the AMC, aliquots of decimal dilutions were analysed according to the standard culture method (UNI EN ISO 4833:2004). The acceptability of results was established in accordance with the limits set by the Regulation (EC) No 2073/2005 (European Commission, 2005). The detection of *E. coli* beta glucuronidase positive (UNI EN ISO 16649-2:2001), *Salmonella* spp. (UNI EN ISO 6579:2008) and *L. monocytogenes* (UNI EN ISO 11290-1:2005) was carried out in according to their ISO methods.

Washing treatment and bacterial load reduction

On the basis of data collected through our survey and those found in previous studies (de Oliveira *et al.*, 2012; Nascimento *et al.*, 2003; López-Gálvez *et al.*, 2010), samples were washed through the immersion in tap water (500 mL); tap water (500 mL) with the addition of bicarbonate (8 g); tap water (500 mL) with NaCl (4 g); tap water (500 mL) with vinegar (10 mL); tap water (500 mL) with a conventional chlorine-based disinfectant (10 mL).

A total of 15 samples (c=30 units) of RTE iceberg lettuce from three companies and 15 samples (c=30 units) of the fresh category were collected. All were kept under refrigerated conditions, transported to the laboratory, washed and analysed. Fresh samples were cut into triangles sections including the external, central and internal part of each head iceberg.

At first, the AMC of each sample and that of tap water used for each washing method were performed in order to calculate the potential bacterial load reduction. Twenty-five grams of all samples were washed for different immersion times (t_0=0 s; t_1=15 min; t_2=30 min). After each washing time, the AMC was performed. For the t_0 step, the sample was immediately rinsed after the immersion.

Statistical analysis

All samples were tested in duplicate and the bacterial counts were expressed as log CFU g^{-1}. Assuming a log-normal distribution, standard deviations and means were calculated. Independent t-test and one-way analysis of variance (ANOVA) were used to determine any statistically significant difference ($P<0.05$) among two or more than of two groups of data, respectively.

Results

Results of survey

Fifty-nine out of 100 students stated that they ate RTE salads. Of them, the 69% reported a consumption of RTE salad once a week. Concerning the washing methods, 26 (44%) respondents revealed that they always wash the product before consumption. Specifically, 25 stated that they usually wash it just with water while one respondent indicated the addition of bicarbonate to water. None practised washing treatment for more of 10 minutes and the leaves are washed only once by 22 respondents, twice by 3 and more than twice by a single respondent. Twenty-four students (41%) consumed the salad in the same day, 24 (41%) within two days and the remaining 11 (18%) in more than two days. A large portion of respondents (77%) revealed the tendency to consume the product after the expiration date.

Microbiological analysis of RTE iceberg leaves.

Table 1 summarises the results obtained from our analysis indicating the number of samples for each range of AMC value detected. The AMC of samples analysed at the beginning of their shelf-life ranged from 6.03 to 8.43 log CFU g^{-1}, with an average of 6.88 log CFU g^{-1}. On the other hand, the samples analysed in the middle of their shelf-life had higher bacteria levels ranging from 6.04 to 9.26 log CFU g^{-1}, with an average of 8.51 log CFU g^{-1}. Counts recorded in the middle of shelf-life compared with those at the beginning of shelf-life increased by an average of 25%. This difference was statistically significant ($P<0.05$). Comparing the means of microbial loads among the three companies, no significant differences were found (ANOVA, P=0.74). The AMC values of samples analysed after the expiration date ranged from 6 to 10 log CFU g^{-1}, with an average of 8.72 log CFU g^{-1} (Table 1). All samples, with one exception, were recorded with a very high bacterial load (>8 log CFU g^{-1}) from 1 to 3 log higher than the allowed AMC. All RTE iceberg lettuce samples were negative for the presence of *L. monocytogenes* and *Salmonella* spp. whereas *E. coli* was under the detection limit of 10 CFU g^{-1}.

Microbiological analysis of fresh iceberg lettuce samples

Also in fresh iceberg samples, no presence of *L. monocytogenes*, *Salmonella* spp. and *E. coli* (<10 CFU g^{-1}) was observed. AMC ranged from the minimum value of 4.7 log CFU g^{-1} to the maximum of 7.5 log

Table 1. Aerobic mesophilic count of ready-to-eat iceberg samples during the shelf-life period.

Range[a]	At the beginning of shelf-life	In the middle of shelf-life	After the expiration date
10^5-10^6	2	ND	ND
10^6-10^7	13	5	1
10^7-10^8	11	7	ND[b]
10^8-10^9	4	17	4
>10^9	ND	1	4

[a]Range in log CFU g^{-1} of products; ND, Not detected.

CFU g^{-1}, with the medium of 5.73 log CFU g^{-1}. In the majority of the samples, the counts ranged between 5 and 6 log CFU g^{-1}, only one sample reported a count of 7 log CFU g^{-1}. The bacterial load of fresh samples was lower of 1.2 logs than RTE samples.

Effectiveness of washing treatment in RTE salad leaves

Figure 1 summarizes the results obtained from the washing treatments used for RTE salad leaves. For each one, the AMC of unwashed leaves was indicated as 100%. Only in the case of water, no bacterial load reduction was registered before 30 minutes of washing and the percentage of reduction was very low (2%). A similar percentage was obtained by washing the leaves in water with NaCl and in water with bicarbonate (91% and 94%, respectively). However, the reduction of bacterial load after 15 minutes of washing in water and bicarbonate was higher than in water and NaCl (3% and 1%, respectively), but this difference was not significant (P>0.05). The washing treatment based on water and vinegar was more efficient than the treatments considered above. Finally, the samples washed in water with the addition of chlorine-based disinfectant showed a reduced load (81%) already after a quick washing cycle (t_0). A consistent reduction (85%) was obtained between 15 and 30 minutes of washing. Hence, the washing treatments based on vinegar and disinfectant significantly reduced the AMC of RTE samples.

Effectiveness of washing treatment in fresh salad leaves

For the leaves washed in water, a weak reduction (4%) of the bacterial load was detected and only after 30 minutes (Figure 2). Comparing the AMC of leaves washed in water with NaCl and that of leaves washed in water with bicarbonate, similar results were obtained. During the first 15 minutes of washing, no reduction was detected in both treatments. Between 15 and 30 minutes of washing, the total count decreased by 2% (from 96% to 94%) in water with NaCl and by 4% (from 96% to 92%) in water with bicarbonate. The washing in water with vinegar showed a higher reduction if compared to the treatments with water plus bicarbonate and water alone. Consistently, the leaves reported a 22% reduction in the initial total count after 30 minutes of washing. The AMC of the fresh iceberg was significantly reduced washing them in water with chlorine-based disinfectant.

Discussion

Nowadays several studies on microbiological contamination of RTE vegetables are carried out (Jeddi *et al.*, 2014; Erkan and Vural, 2008). This is the first work focused on RTE iceberg lettuce commercialised in Italy. Our survey, targeted to university students, confirmed the high consumers' demand of RTE salads by young people. The increased consumption of RTE foods, including salads, is strongly associated with the reduction of time spent by modern society for meal preparation (Buckley *et al.*, 2007).

In 2013, the European Food Safety Authority (EFSA, 2013) examined the risk from leafy green salads observing that many outbreaks were caused by their microbial contamination. The guidelines provided by the Public Health Laboratory Service (2000) indicates that no mandatory unacceptable AMC reference limits are defined for the RTE vegetables. However, the poor hygienic level of chain production is indicated by the exceeding of the unsatisfactory limit defined as $\geq 10^7$ (PHLS, 2000). Our data confirmed the results of other studies (Chen and Godwin, 2012; Jeddi *et al.*, 2014; Erkan and Vural, 2008), where all collected lettuce samples were contaminated by a high bacterial load. No significant difference in terms of contamination was found among the commercial brands highlighting the same hygienic level. Noteworthy, RTE samples showed higher AMC values than fresh category pointing to the additional contamination of product during the processing. Concerning this issue, De Giusti *et al.* (2010) provided the evidence that preventive strategies such as the observation of both good agricultural and manufacturing practices (GAPs; GMPs) could improve the microbiological quality of RTE vegetables. Generally, the improvement of good hygienic practices during processing could be fundamental not only to increase the microbiological quality of the products but also to extend their shelf-life.

In compliance with the Regulation EC No 2073/2005 (European Commission, 2005), no *Salmonella* spp. and *L. monocytogenes* were detected in any samples (RTE and fresh iceberg leaves). This was a positive result as the presence of these pathogens is an unacceptable criterion for food consumption. Our data are consistent with those of previous studies (Almualla *et al.*, 2010; De Giusti *et al.*, 2010) where *Salmonella* spp. and *L. monocytogenes* were not detected in analysed samples. Conversely, these results are conflicting with the high rate of *Salmonella* spp. (46.7%) reported by Bruno *et al.* (2005) in Brazil as well as with the high rate (22.5%) of *L. monocytogenes* reported by Ponniah *et al.* (2010) in Malaysia.

Surprisingly, no occurrence of *E. coli* was detected in our investigation. In spite of this result, *E.coli* has been observed with a high percentage in other studies: 30% by Prado *et al.* (2008), 53.1% by de Oliveira *et*

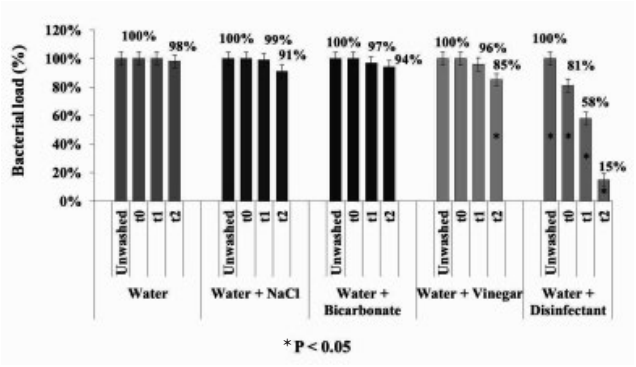

Figure 1. Effect of different washing procedures on the reduction of bacterial load in RTE iceberg lettuce.

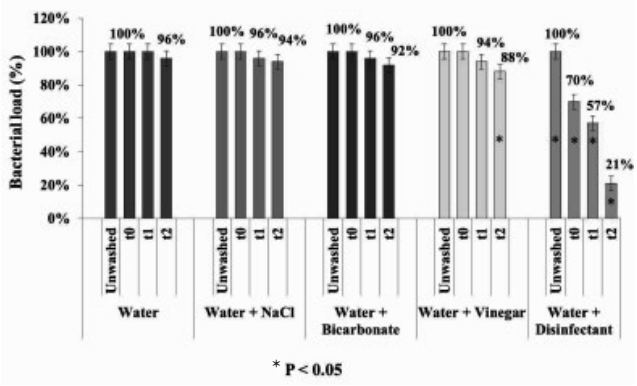

Figure 2. Effect of different washing procedures on the reduction of bacterial load in fresh iceberg lettuce.

al. (2011). Many outbreaks resulted from the consumption of leafy vegetables contaminated by the presence of *E. coli* in manure and soil (Wendel *et al.*, 2009). Cracks and intercellular spaces of vegetables are colonised by many pathogens (Mercanoglou and Halkman, 2011; Jung *et al.*, 2014) and prevention measures to reduce microbial contamination are available.

Washing and sanitising efficiencies can be influenced by various factors such as production surface, water quality, sanitizer used and contact time (De Giusti *et al.*, 2010; Parish *et al.*, 2003). According to literature, warm water is not able to reduce bacterial load and it could facilitate the growth of pathogens such as *L. monocytogenes* (Li *et al.*, 2002). Our data revealed that the effectiveness of washing varied according to the method and to the immersion time. The most common wash based only on tap water resulted in being unable to detach bacteria from leaves as well as those with the addition of NaCl and bicarbonate. This result is consistent with that reported by a Brazilian work analysing washing procedures in some restaurants (de Oliveira *et al.,* 2012) and also with that of Neto *et al.* (2012) analysing the microbial quality of lettuce obtained from different cropping systems. On the other hand, the wash treatment based on vinegar seems to be more efficient than others, but only after a long time of immersion. In spite of its ability to reduce bacterial load, vinegar may also be responsible of the browning effect on leaves reducing the appeal of the product (de Oliveira *et al.*, 2012). The combination of disinfectant and time of immersion showed an increased effect on the total bacterial load reduction. Nascimento *et al.* (2003) and López-Gálvez *et al.* (2010) reported a similar great reduction of bacterial load after disinfectant use. In the present work, the differences in bacterial load comparing the washing times were not statistically significant ($P \geq 0.05$) irrespective of the washing method, the only exception represented by the use of disinfectant. As expected, a relevant decline in bacterial counts was obtained by washing with disinfectant for a long time (30 minutes).

Conclusions

This study presented a general assessment of the microbiological quality of RTE iceberg lettuce commercialised in Italy. The research clearly evidenced that the RTE product has higher contamination level than the fresh produce. Hence, industrial processing is an important additional source of microbial contamination. Thus, the improvement of Good Agricultural and Manufacturing Practices (GAPs; GMPs) by producers is necessary to ensure food safety. The consumption of RTE iceberg lettuce during the first period of shelf-life and after washing with disinfectants minimise the risk for human health. Further studies analysing these microbial aspects can provide consumers with useful information about the use of RTE iceberg lettuce at its highest quality.

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