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Microbiological Quality of Fresh Iceberg Lettuce Harvested in Tunisia

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The elaboration of fresh-cut vegetables requires hygienic requirements at all levels of processing as well as raw material of good quality. The aim of this study was to assess the microbiological quality of lettuce in Tunisia, in particular the prevalence of selected pathogens. 150 samples were tested for the presence of aerobic mesophilic, psychrotrophic microorganisms, lactic acid bacteria, coliforms, yeasts and moulds, *Escherichia coli* β-glucuronidase positive, *Salmonella spp.* and *Listeria monocytogenes*. The mean aerobic mesophilic counts were 7.0 $\log_{10}$ CFU g^{-1} for Gafsa and 7.1 $\log_{10}$ CFU g^{-1} for Bizerte and Cap Bon. Lactic acid bacteria were present and the highest percentage of the samples was found at values between 5.0 and 6.0 $\log_{10}$ CFU g^{-1}. The mean counts of coliforms were ranging from 4.8 to 5.3 $\log_{10}$ CFU g^{-1}. Yeasts and molds were present. While no pathogenic bacteria were found in the lettuces analyzed, imply that effective control measures must be carried out to improve the microbiological quality of this fresh vegetable.

Key words: Microbiological quality; Fresh lettuce; *Listeria monocytogenes*; *Salmonella*; *Escherichia coli.*

The diet rich in green leafy vegetables offer many health benefits; there is a direct relationship between the consumption of these vegetables and the reduction of chronic diseases, such as hypertension, diabetes, atherosclerosis and cancer[1]. Lettuce vegetable is considered as a source of fiber, minerals and the vitamins A, B1, B2, B6 and C, in addition to possessing laxative, diuretic and lenitive properties as well as a pleasant and refreshing taste[2].

In Tunisia, among leafy vegetables, iceberg lettuce (*Lactuca sativa L.*) is the most frequently consumed, meaning that its production is spread throughout the year. This production of lettuce has been increased in recent years from 14,000 tonnes in 2000 to 64,000 tonnes in 2012[3]. It is however subject to important losses[4] mainly due to cropping techniques. To reduce these losses, lettuce could be transformed into fresh-cut vegetable.

Fresh-cut or minimally processed vegetable (peeled, cored and sliced) are a growing segment among food products because it is convenient and has a fresh-like quality[5]. Consumers judge the quality of fresh-cut vegetable on the basis of appearance and freshness at the time of purchase[6]. The disadvantage of fresh-cut vegetables is that shelf-life may be greatly reduced during handling, transport and storage. The elaboration of this type of product therefore requires hygienic requirements at all levels of processing (cutting, washing, and packaging) as well as raw material of good microbiological quality[7].

Lettuce is susceptible to microbial attack after harvest due to the loss of natural resistance and their high water and nutrient content[8].

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Knowledge of the native microflora of fresh lettuce is of fundamental importance when this product is traded as a fresh commodity in which microorganisms play a primary role in keeping quality[9]. A number of outbreaks associated with consumption of vegetables demonstrated that pathogens, such as *Listeria monocytogenes*[10,11] and *Escherichia coli* O157:H7[12] may be present on lettuce. In a previous study, Oliveira *et al.* (2010) proved that *Escherichia coli* O157:H7, *Salmonella* and *Listeria monocytogenes* could grow in fresh-cut lettuce under certain conditions[30]. To maintain quality and safety of fresh lettuce for human consumption, the control of pathogenic microorganisms plays an important role. Therefore, the aim of this study is to evaluate the microbiological quality of lettuce in Tunisia.

MATERIALS AND METHODS

Origin of samples

Lettuce samples of the iceberg variety (*Lactuca sativa L.*) were harvested at optimal maturity from three regions in Tunisia each characterized by specific climatic conditions (Cap Bon, Bizerte and Gafsa). The average temperature and annual precipitation of these regions are 19.2 °C, from 467.6 mm / year; 22.8 °C, 300 to 800 mm / year and 19.8 °C, 48.9 mm respectively. The sampling unit established for this analysis was one head of lettuce, regardless of weight or size, and 50 samples per region. Samples were taken at the following steps, transported to the laboratory (during one hour for the region of Cap Bon and Bizerte and three hours for the region of Gafsa) under refrigeration conditions (+ 4 °C) and stored during 24 hours at + 4 °C before treatment.

Bacteriological analysis

Aerobic mesophilic plate count and psychrotrophic plate count

Three external leaves were removed by hand (using steriles gloves) and the other parts of the lettuce were shredded in pieces of approximately 1 cm^2, using a sterile blade. Twenty five g of each sample were placed in a sterile stomacher bag and homogenized using a stomacher with 225 ml of sterile 0.1% peptone water for 2 min. Each whole sample was then agitated and rubbed by hand in the stomacher bag for 2 min to suspend surface microbes[13, 14]. Each homogenized portion was designated as the initial dilution (10^{-1}), from which serial decimal dilutions were performed (10^{-2} - 10^{-7}) in the same diluent.

Samples were prepared as described above. One ml of appropriate dilutions was placed in duplicate sterile Petri dishes and molten Plate Count Agar (PCA) (Merck, Darmstadt, Germany) was added via the pour plate technique. For mesophilic plate count, the plates were placed in an incubator for 24 h at 30 °C[15]. For psychrotrophic plate count, the PCA plates were placed in an incubator at 6 °C for 5-7 days[16, 17, 18]. After incubation, plates with at least 20 colonies were enumerated using an automated plate counter (aCOLyte, Microbiology International, California, US). Each analysis was carried out in triplicate. Results were expressed as $\log_{10}$ CFU g^{-1}.

Enumeration of lactic acid bacteria

Samples were prepared as described above. Lactic acid bacteria were estimated following incubation in duplicate Man, Rogosa and Sharpe agar (MRS) (Merck) under anaerobic condition for 48 h at 37 °C[15].

Enumeration of coliforms

Samples were prepared as described above. Desoxycholate 1‰ (Merck, Darmstadt, Germany) was used for the enumeration of coliforms which were incubated at 37 °C for 24 h[15].

Enumeration of yeasts and moulds

Samples were prepared as described above. Yeast and mould were estimated following incubation on Sabouraud Chloramphenicol agar (Merck, Darmstadt, Germany) containing 0.1% chloramphenicol culture plates for 3-5 days at 25 °C[13]. For counts, 0.1 ml of appropriate dilutions were plated out in duplicate and incubated.

Isolation of *Escherichia coli* â-glucuronidase positive

Samples were prepared as described above. *Escherichia coli* colonies were observed by pour plating on TBX selective agar (Oxoid, UK) media for 18–24 h of incubation at 44 °C. The presence of stressed cells was solved by pre-incubating the plates for 4 h at 37 °C[19].

Isolation of *Salmonella spp*.

Twenty five g of each sample were transferred into 225 ml of sterile buffered peptone water (BPW) (Oxoid) for 2 min. Each whole sample was then agitated and rubbed by hand in the stomacher bag for 2 min. The homogenate was

incubated for 24 h at 37 °C for pre-enrichment. Selective enrichment was then done by transferring 0.1 ml of pre-enrichment to 10 ml of Rappaport Vassiliadis (RVS) broth(Oxoid) and 1 ml to 9 ml of tetrathionate of sodium and bright green (MKTTn) broth (Oxoid), followed by incubating of RVS and MKTTn for 24 h at 42 °C and 37 °C respectively. After incubation, one loopful was taken from each broth and streaked on XLD (Xylose –lysine – désoxycholate) agar (Oxoid) and BGA (Bright green and neutral red) agar (Oxoid) and incubated for 24 h at 37 °C. The presumptive colonies was inoculated on the surface of nutrient agar previously dried.

For the conûrmation of presumptive colonies, the biochemical tests were carried out and API 20E (bioMerieux, Inc., Marcy-l'Etoile, France) was used[20].

Isolation of *Listeria monocytogenes*.

For primary enrichment, ten grams of lettuce were macerated in 90 ml of Half-Fraser broth in a Stomacher and incubated for 24 h at 30 °C. Secondary enrichment was carried out by transferring 0.1 ml of primary enrichment to 10 ml of Fraser broth, followed by incubating for 48 h at 37 °C. Cultures are then plated on to selective/ differential agar plates (agar Palcam and agar ALOA: Agar Listeria according to Ottaviani and Agosti Medium) for isolation of presumptive colonies of *Listeria monocytogenes*. Five typical *Listeria monocytogenes* colonies are then transferred from PALCAM and agar ALOA to Trypticase soy agar with yeast extract (TSAYE) and incubated for 18 h at 37 °C. Typical colony are tested for catalase, gram coloration and mobility. *Listeria* species are catalase-positive, short, Gram-positive rods and mobile. Purified isolates may be rapidly identified by using API Listeria (bioMerieux, Marcy-l'Etoile, France) which does not require an additional CAMP test[21].

Statistical analysis

The mean values obtained from the microbiological evaluation of lettuce were analyzed by one one-way ANOVA procedure of SPSS® 17.0. Duncan's multiple range test were used to determine any significant difference between mean values and evaluations were based on a significance level of $p < 0.05$.

RESULTS AND DISCUSSION

The present study was intended to provide some assessment on the microbiological quality of lettuce in Tunisia.

Mesophilic microorganisms

Aerobic mesophilic count in the samples analyzed is demonstrated in table 1. The mean aerobic mesophilic counts were 7.0 $\log_{10}$ CFU g^{-1} for Gafsa and 7.1 $\log_{10}$ CFU g^{-1} for Bizerte and Cap Bon, indicating that all samples were acceptable for consumption because the aerobic bacterial count for lettuce fresh is less than 7.7 $\log_{10}$ CFU g^{-1}[7]. No significant differences were found in the initial microbial counts in the three regions. The mean aerobic bacterial count of lettuce obtained in this study was similar with that of a recent study conducted by Aguero *et al.* (2011) in lettuce fresch from Argentina with values in the range of 7.0-7.4 $\log_{10}$ CFU g^{-1}[22]. Similarly, the average count was similar to results obtained by Wießner *et al.* (2009) for lettuce grown in an organic cropping system in Germany[23]. In Likewise, an analysis in Brazile had reported that most lettuce samples analyzed had a count ranging from 6.0 to 7.0 $\log_{10}$ CFU g^{-1}[24]. Generally, for indicating the shelf-life duration and microbial quality of foods, mesophilic aerobic

Table 1. Results of aerobic mesophilic count in the samples analyzed

Name of region	No. of sample units	Percentage (%) of samples in the indicated interval		Mean [a]
		6-7 [a]	>7	
Cap Bon	50	34	56	7.1 ± 7.0 [A]
Bizerte	50	52	48	7.1 ± 6.9 [A]
Gafsa	50	66	34	7.0 ± 6.9 [A]

Means having same letters are not significantly different ($P > 0.05$)
[a] The unit of number is $\log_{10}$ CFU g^{-1}.

counts are useful[25]. Since these vegetables are farmed on soil and exposed to all kinds of environmental conditions, they reûect the conditions in which they were farmed, and their counts can be as high as 7.0 $\log_{10}$ CFU g^{-1}[26]. Thus, attention must be focus on hand hygiene especially the aspects of practices among the food handlers from primary product[27].

Psychrotrophic microorganisms

Table 2 shows the counts of psychrotrophic microorganisms of lettuce samples. The mean psychrotrophic counts of the three regions were compared to verify whether they differed signiûcantly ($p > 0.05$). The psychrotrophic microorganisms present a special problem as they can multiply during retail, notably when packaged produce are not stored at the correct temperature as recommended by producers (usually 1-5 °C)[28]. Psychrotrophic microorganism counts were highly comparable to those of mesophilic microorganisms[28, 29]. Such a trend was not observed in this present study, where mesophilic counts were considerably higher than psychrotrophic counts.

Lactic acid bacteria enumeration

Lactic acid bacteria (LAB) were also present (Table 3). No significant differences were detected in lactic acid bacteria counts among regions. The highest percentage of the samples (66, 58, and 50% from Cap Bon, Bizerte and Gafsa regions, respectively) was found at values between 5.0-6.0 $\log_{10}$ CFU g^{-1}. These results agree with those obtained by *et al*. (2011) in lettuce fresh[22]. Values considerably lower than those found in this study for lactic acid bacteria in lettuce have already been reported[28, 30].

Lactic acid bacteria are broadly distributed in nature. There are a diverse group of microorganisms that generate lactic acid as the primary end-product of the fermentation of carbohydrates[31]. There are widely distributed in nature. Lactic acid bacteria could be considered antimicrobial candidate against both microbial spoilage and enteric infections caused by pathogenic microorganisms[32].

Coliforms enumeration

Total coliform counts of lettuce samples are presented in table 4. In the present study, no significant differences were recorded in the coliforms counts between the three regions. The mean coliform counts were 5.3 $\log_{10}$ CFU g^{-1} for Cap Bon and 4.8 $\log_{10}$ CFU g^{-1} for Bizerte and Gafsa. These results were similar with another investigation[33], where most of vegetables samples

Table 2. Results of aerobic psychrotrophic count in the samples analyzed

Name of region	No. of sample units	Percentage (%) of samples in the indicated interval			Mean [a]
		4-5 [a]	5-6	6-7	
Cap Bon	50	42	46	12	5.6 ± 5.7 [A]
Bizerte	50	32	52	16	5.8 ± 5.9 [A]
Gafsa	50	34	54	12	5.7 ± 5.9 [A]

Means having same letters are not significantly different ($P > 0.05$)
[a] The unit of number is $\log_{10}$ CFU g^{-1}.

Table 3. Results of lactic acid bacteria enumeration in the samples analyzed

Name of region	No. of sample units	Percentage (%) of samples in the indicated interval				Mean [a]
		3 -4 [a]	4-5	5-6	6-7	
Cap Bon	50	14	12	66	8	5.6 ± 5.6 [A]
Bizerte	50	10	28	58	4	5.6 ± 5.6 [A]
Gafsa	50	12	30	50	8	5.6 ± 5.6 [A]

Means having same letters are not significantly different ($P > 0.05$)
[a] The unit of number is $\log_{10}$ CFU g^{-1}.

showed coliform counts from 2.1 to 5.7 $\log_{10}$ CFU g^{-1}.

To account for the lettuce commodity's relatively high coliform counts, there are a few possible explanations. Lettuce leaves have a large surfaces and folds areas. This makes the vegetables more susceptible to bacterial contamination and adhesions[34]. Their open leaves may also be in contact with soil and irrigation water, trapping dirt in the folds[28].

Total coliform are widely spread in nature and commonly found in raw vegetables; therefore, they are not linked with fecal contamination[26].

Yeasts and moulds enumeration

Table 5 shows the counts of yeasts and molds in lettuce samples of three regions. The mean yeasts and moulds counts were 6.1 $\log_{10}$ CFU g^{-1} for Bizerte and 6.2 $\log_{10}$ CFU g^{-1} for Cap Bon and Gafsa. No significant differences were recorded in yeast and moulds count between the three lettuce regions ($P > 0.05$). Other studies found similar results for samples of lettuce, with yeasts and molds present in smaller counts than bacteria[24, 30].

High counts of yeasts and moulds can contribute to spoilage of fermented vegetable products and the development of soft rot[35]. Spoilage as a result of mould growth is not a major issue in fresh-cut salads, as far as limited research has shown[36]. However, some authors[37] referred the possible health problems associated with the presence of moulds in vegetables, as some are capable of producing toxic secondary metabolites (mycotoxins) while growing in fresh-cut salads and, consequently, pose a threat to human health. Therefore, the occurrence and levels of such organisms in foods should be monitored.

Isolation of *Escherichia coli* β-glucuronidase positive, *Salmonella spp* and *Listeria monocytogenes*

Escherichia coli β-glucuronidase positive, *Salmonella spp*. and *Listeria monocytogenes* were not detected in any lettuce samples analyzed in this study (Table 6). Several studies are consistent with our results.

United States (US), *Salmonella*, and *Escherichia coli O157* were not detected in 1028 domestic produce sampled[38]. Johnston *et al.* (2006) reported no *Salmonella* or *Escherichia coli* O157:H7 contamination in 466 fresh produce on both of US and Mexican origins(including leafy green, herbs, melons and vegetables)[39]. Likewise, in a study conducted on organically grown lettuce

Table 4. Results of coliforms enumeration in the samples analyzed

Name of region	No. of sample units	Percentage (%) of samples in the indicated interval				Mean [a]
		2 -3 [a]	3-4	4-5	5-6	
Cap Bon	50	22	24	30	24	5.3 ± 5.5 [A]
Bizerte	50	20	46	8	26	4.8 ± 5.2 [A]
Gafsa	50	12	30	44	14	4.8 ± 5.2 [A]

Means having same letters are not significantly different ($P > 0.05$)
[a] The unit of number is $\log_{10}$ CFU g^{-1}.

Table 5. Results of yeasts and moulds enumeration in samples analyzed

Name of region	No. of sample units	Percentage (%) of samples in the indicated interval		Mean [a]
		5-6 [a]	6-7	
Cap Bon	50	68	32	6.2 ± 6.2 [A]
Bizerte	50	70	30	6.1 ± 6.2 [A]
Gafsa	50	60	40	6.2 ± 6.2 [A]

Means having same letters are not significantly different ($P > 0.05$)
[a] The unit of number is $\log_{10}$ CFU g^{-1}.

Table 6. Results of incidence of pathogens in the samples analysed

Name of region	No. of sample units	Percentage (%) of samples with presumptive colonies			Percentage (%) of confirmed positive samples		
		E.coli positive β-glucuronidase	*Salmonella spp*	*Listeria monocytogenes*	*E.coli* positive β-glucuronidase	*Salmonella spp*	*Listeria monocytogenes*
Cap Bon	50	ND[a]	8	8	ND	ND	ND
Bizerte	50	ND	6	ND	ND	ND	ND
Gafsa	50	ND	8	8	ND	ND	ND

[a] ND: not detected

of Norway, where 179 samples were examined, no *Salmonella* and *Escherichia coli* O157:H7 was isolated[40]. Mukherjee *et al.* (2004) also found no *Escherichia* coli O157:H7 contamination in any of the organic and conventional produce analyzed[41]; though, *Salmonella* was secluded in one lettuce sample. In Spain, range of vegetables had *Salmonella* species contamination[42] but in other recent studies, no *Salmonella* have been isolated from a variety of organic fresh produce samples in United Kingdom producers were free of[43]. Authors concluded that the use of contaminated irrigation water or animal manure as fertilizer can be an important factor in such outbreaks.

In samples from the three regions analyzed in this study, the presence of *Listeria monocytogenes* was not detected. The absence of this pathogen from lettuce was also reported in previous studies[43, 11]. These results are comparable to that found by Oliveira *et al.* (2010) in fresh lettuce grown in organic and conventional farms in Spain [30]. However, in Thrissur (India) 8.3 % of vegetable samples collected from retail markets contained *Listeria spp* [44]. *Listeria* is environmental microorganism that is found in soil and water. Thus, vegetables can be readily contaminated by these bacteria.

CONCLUSION

The results of this study show that lettuce harvested in the Cap Bon, Bizerte and Gafsa not contain *Escherichia coli* â-glucuronidase positive, *Salmonella spp*. and *Listeria monocytogenes*. However, samples contain considerable levels of the other microorganisms analyzed, indicating the need of hygiene practices during farming of lettuce, as well as of making the population aware of the importance of vegetable sanitization before consumption to enhance the microbiological quality of vegetables in Tunisia.

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