THE MICROBIOLOGICAL STATUS OF (READY TO EAT) LETTUCE BEFORE AND AFTER STANDARD WASHING

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Lettuce is a vegetable that is always consumed raw. The number and types of microorganisms that may be found on the lettuce leaves depend on growing, harvesting, and particularly on handling practices, like proper washing and preparing lettuce before eating. The aim of this study was to investigate the effect that washing under running water has on the microbiological status of lettuce leaves purchased from the local market place in Banja Luka. Smears were taken from the obverse and reverse sides of lettuce leaves, and the total count of aerobic mesophilic bacteria, yeasts and molds was determined as well as the presence of sulphite-reducing clostridia, the coagulase positive staphylococci, Salmonella species, and Escherichia coli. The analysis showed that the washing reduced the total count of aerobic mesophilic bacteria by approximately 10 times, and the total count of yeasts and molds by approximately 8 times. Of the lettuce samples examined, 66,67% contained sulphite-reducing clostridia and 13,33% Salmonella species, before the washing. The number of samples with sulphite-reducing clostridia was reduced by five times after the washing, but the number of samples contaminated with Salmonella species remained the same.

Key words: *microbiological status, lettuce, washing*

INTRODUCTION

Lettuce (*Lactuca sativa* L.) is a vegetable available during the whole year, and is always consumed minimally processed. Due to its low calorie content and pleasant, refreshing taste, lettuce is recommended as a healthy food and is often the main ingredient in many diets. The content of vitamins, minerals, fibers and other valuable biological components which may be unstable and depend on the types of lettuce and growing conditions (1-4), makes this vegetable highly valuable food choice despite the fact that lettuce is consumed without thermal processing in order to have its thermolabile nutritive components preserved. Lettuce is believed to have a soothing effect, to facilitate expectoration, and to increase appetite and urination (5). Recent research has confirmed that lettuce leaves contain a huge number of substances that exhibit antioxidant (3,6-7), antiproliferative, and anti-inflammatory properties as well as protect from cardiovascular diseases (7-13). Many studies have also confirmed that lettuce leaves and seed have a tranquilizing and anti-anxiety effect (14-17). Phenolic substances, with their antioxidant properties, also play a role in antimicrobial activity of ethanol and methanol extract of lettuce leaves (18-19).

Alongside its verified nutritional and medicinal properties, lettuce is considered one of the produce most contaminated by pathogenic microorganisms (20-23).

Human pathogens may be present during the entire production process, as well as at the moment of consumption (23-24). Due to an increased consumption of fresh and minimally processed fruits and vegetables, there have been a large number of studies conducted on different types of infections, transfer, adherence and survival of pathogenic microorganisms on contaminated foodstuffs (25). Regulations on microbiological criteria for foodstuffs in the Republic of Srpska (26) and Bosnia and Hercegovina (27), only allow for the presence of *Escherichia coli* in minimally processed fresh fruits and vegetables. The aim of this study was to establish the microbiological status of lettuce purchased from the local market place in Banja Luka, before and after washing, by determining the total count of aerobic mesophilic bacteria and the count of yeasts and molds, as well as to detect the presence of pathogenic and potentially pathogenic microorganisms.

MATERIALS AND METHODS

Samples used in this study were purchased from the local market place in Banja Luka in March and April, when the lettuce season generally commences. The average daily temperatures for those two months were 6,2°C in March and 11,5 °C in April respectively. Thirty samples were tested in two independent repeats, before and after washing, and the lettuce samples were washed thoroughly for 10 minutes in warm water. Only undamaged inner leaves of the lettuce were taken, and the swabs were taken before and after the washing of the lettuce leaves. The samples' weight and planimetric measurement were then determined (28). The standard testing method was used for the experiment. For

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the primary dilution (10^{-1}), the swab sample method was used and swabbing was performed on each lettuce leaf, both the obverse and reverse sides. The cotton swab was then put back into the test tube filled with 10mL of saline which was fairly homogenized. To determine the total count of aerobic mesophilic bacteria, as well as the total count of yeasts and molds, 1mL of particular dilution was plated onto nutrient agar and Sabouraud dextrose agar.

The total counts of aerobic mesophilic bacteria and yeasts and molds can be obtained per unit of the surface area. The surface area of lettuce leaves was calculated from the following formula:

$$A\ (cm^2) = \frac{m \times P}{m_1} \quad (28) \qquad (1)$$

m – weight of the area covered by the leaf outline (g)
P – surface area of paper square (5x5cm)
m_1 – weight of the paper square (g)

To detect the presence of *Escherichia coli,* 1mL of primary dilution was plated on Andrade lactose peptone broth, with an inverted Durham tube, and then incubated at 37°C for 24 hours. After incubation, with a calibrated inoculating loop EMB agar was inoculated with the material from the test tubes containing gas. To isolate coagulase-positive staphylococci, Chapman and Baird-Parker ETGP agars were plated after 24-hour incubation on nutrient agar (salt broth). To prove the presence of *Salmonella,* smears were taken by dipping a cotton swab in a selective enrichment media in double concentration (Selenite broth). The cotton swab was then dipped in 10mL of Selenite broth and incubated at 37°C for 18-24 hours. After incubation, the SS agar and WB agar were plated with a calibrated inoculating loop. Anaerobic sporogenic sulphite-reducing clostridia were isolated by heating 1mL of primary dilution at 80°C for 10 minutes, after which the previously melted (at 45°C) and cooled sulphite agar was poured into test tubes. All plates were incubated at 37°C for 24-48 hours, except Sabouraud dextrose agar, which was incubated at 25°C for 3-5 days and sulphite agar which was incubated at 37°C for 72 hours. The colonies grown on selected media were Gram stained and subjected to a different biochemical tests such as catalase, sugar fermentation, IMViC test (Indole test, Methyl red and Voges Proskauer tests and citrate), urease test and Phenylalanine test. The tests showed that Gram-negative bacteria, which were positive for phenylalanine and citrate tests, did not produce H_2S and did not swarm, were also tested in their ability to use mannose, and were identified as *Providencia* sp. (29). To prove the presence of pectinovor species, 1 ml of the primary dilution was plated onto CVP agar and incubated at 28°C for 48 hours. The bacterial colonies that took on a characteristic morphology were plated with a calibrated inoculating loop onto King B agar, and incubated again for 48 hours at 28 °C. Creamy immuno-fluorescent colonies were identified as *Erwinia* species, whereas the colonies that produced fluorescent pigment were identified as *Pseudomonas* species (30). All nutrient media used for standard and biochemical tests were obtained from Torlak, Belgrade, except CVP and King B agar which were obtained from HiMedia, India.

RESULTS AND DISCUSSION

The total count of aerobic mesophilic bacteria on lettuce leaves before washing varied from 45 cfu/cm^2 to 18.57 cfu/cm^2. Due to higher temperatures in April, particularly in the second half of the month, the number of aerobic mesophilic bacteria increased significantly (Table 1). Besides temperature, other environmental factors such as wind, rainfall, or sunshine may have an impact on large variations in counts of mesophilic epiphytic microflora during the growing season, protection and selling (31) as well as during the harvest season, storage and transport. The samples used for testing were purchased from the local market place, and there was no information available as to their cultivation and treatment, from harvesting to selling. Research have shown that cutivation practices, the quality of water during irrigation and growing season can affect the total count and prevalence of certain groups of epiphytic microorganisms (32). These experiments, in which DNA analysis and other modern methods were used, showed that the diversity of bacterial microflora was higher on the lettuce leaves that were irrigated by spraying than by soaking (drop by drop). Water spray lifts soil particles that contain microorganisms and transfers them from the soil onto the leaves. The quality of water is also an important factor that can affect the structure of epiphytic microflora, and increase the risk of the presence of human pathogens (33). Lettuce age and position of leaves, their surface and weight can also affect the variety of epiphytic microflora (31).

Qualitative differences were observed in lettuce grown in spring in terms of higher concentration of gram-positive bacteria of the phylum Firmicutes, such as lactic acid bacteria (*Leuconostoc, Streptococcus* and *Lactococcus*) and *Bacillaceae* bacteria. However, the bacteria of the phylum Proteobacteria, that belong mainly to the Enterobacteriaceae and Pseudomonadacea families, were dominant later in the season. The total count of bacteria include human pathogenic or potentially pathogenic bacteria that may be found in lettuce such as: *Salmonella*, *Escherichia coli*, *Listeria monocytogenes*, *Yersinia enterolitica,*and *Aeromonas hydrophila* (25). From the traditional growing methods tiny differences in qualitative composition of epiphytic microflora cannot be detected. It is estimated that only 1-10% of total number of bacteria can be grown on a nutrient medium (32), which may be yet another reason for the difference in numbers of mesophilic bacteria of analyzed samples. The total count of bacteria in examined samples was lower than the average quality score varying from 10^4 cfu/g to 10^6 cfu/g (34-35). There is even a possibility that the lettuce samples used for testing were washed immediately after picking or shortly after picking. This practise is quite common and is used to remove dirt from the leaves, which (depending on the washing procedure) helps reduce the number of bacterial loads to less than 2 $\log_{10}$ cfu/g (25). Duration and storage conditions of lettuce may contribute to the increased number of epiphytic microflora, and the number of aerobic mesophilic bacteria. Washing under tap water for 10 minutes reduces additionaly the number of aerobic mesophilic bacteria by 1.12 log_{10} cfu/g.

The total count of yeasts and molds was lower when compared to the total count of aerobic mesophilic bacteria, and varied from 1 cfu/cm^2 to 91 cfu/cm^2. However, the total count of yeasts and molds did not change significantly in March and April (Table 1). Typically, the number of yeasts and molds is usually lower in vegetables than in fruits due to higher pH values, as it was observed in lettuce (34). Washing procedure further reduces that total count. When lettuce is chopped and set aside for a while, the number of yeasts and molds will increase. Among the most common yeast contaminants that cause rot are: *Bremia lactucae*, *Botrytis cinerea* and *Sclerotonia sclerotiorum.* Further examination has shown that possibly pathogenic yeasts may also be found on lettuce leaves (36).

Table 1. Mean microbial load observed in lettuce samples from Banja Luka market place (Bosnia and Herzegovina)

Month	Treatment	APC[a] (cfu/cm^2)	FC[b] (cfu/cm^2)
March	[c]BW	2,54 x 10^2	31,71
	BW (log)	2,40	1,50
	[d]AW	88	5
	AW (log)	1,94	0,70
April	BW	7,91 x 10^2	34,57
	BW (log)	2,90	1,54
	AW	82	3
	AW (log)	1,91	0,48

[a]aerobic plate count; [b]fungi count; [c]before washing; [d]after washing

Table 2. Pathogenic and potentially pathogenic microorganisms detected in lettuce samples from Banja Luka market place (Bosnia and Herzegovina)

Microorganisms	[c]BW	[d]AW(X)
Escherichia coli (%)	ND	ND
Citrobacter spp. (%)	6,66	ND
Clostridium spp. (%)	66,67	13,33
Staphylococcus spp. (%)	ND	ND
Salmonella spp. (%)	13,33	13,33
Pseudomonas spp. (%)	13,33	6,66
Providencia spp. (%)	6,66	3,33
Erwinia spp. (%)	6,66	3,33
Not identified (NI)	6,66	6,66

[c]before washing; [d]after washing ND - not detected; NI – not identified

The present results indicate that the presence of pathogenic and potentially pathogenic microorganisms detected in lettuce samples did not change significantly in March and April (Table 2). Most of the lettuce samples tested positive for sulphite-reducing clostridia (66,67%). Sulphite-reducing clostridia are anaerobic, sporogenic bacteria, mostly present in the soil. During the growing process, soil particles inevitably appear on the leaves and particularly in the root zone, and they can be removed simply by washing. But a definite risk to human health is the presence of *C. botulinum*, a bacterium which in the form of a vegetative cell, under certain conditions, (temperatures higher than 5^{o}C, pH over 4, and an absence of oxygen) produces a dangerous toxin (34), as found in a specific type of packaging, e.g. hermetically sealed containers. The mechanism of spore adhesion is still unknown, but they can be removed to a greater extent by careful washing.

The presence of *Salmonella* was found in a large number of the lettuce samples tested unwashed, but then, not even washed samples showed reduced bacterial population. For the experiment, two leaves of each lettuce sample were swabbed on both sides, but other leaves from the same lettuce samples were also used after washing. This, however, was of no importance because different strains of *Salmonella* adhere differently to varous parts of the same leaf, both on the adverse and reverse sides (25), due to microenvironmental modifications. Comparing the number of samples tested for the presence of *Salmonella*, it was surprising to discover that the presence of *E. coli* was not found, considering that it is the most common bacteria of faecal origin. Adherence of bacteria to the leaf surface is a prerequisite for contamination. Both *E. coli* and *Salmonella* have several mechanisms for adhesion onto the leaf surface (32). Some strains of *Salmonella* are more successful in the adhesion process than the ones of *E. coli* O157:H7 (38). Even certain serovars of *S. enterica* adhere differently to the leaves, which is connected with their ability to form biofilm (38). This ability influences the survival of *Salmonella* in the environment, its resistance to washing and sanitizing treatments as well as antibiotics (39). Recent research has found that *Salmonella*, more than any other human pathogens, penetrates into the cells of leaf parenchyma which are rich in sugars and other nutrients. After entering the leaf parenchyma via stomata and, stimulated by positive chemotaxis, *Salmonella* moves toward photosynthetically active cells through the apoplast and the xylem. Furthermore, *Salmonella typhimurium* has the ability to suppress the defense mechanisms of plants and can use plants as alternative hosts (38). Different types of *E. coli* also have mechanisms for adhesion and survival on the plant surfaces. It is important to point out that the samples tested for *Salmonella* were swabbed and dipped into nutrient medium with double concentration, and that the primary dilution (10^{-1}) for *E.coli* was made in saline. This means that our data demonstrate a low cell density of *Salmonella*, whereas *E.coli* could not be detected at all.

The presence of *Pseudomonas*, *Erwinia*, *Providencia*, *Citrobacter,* and other species may affect the relative prevalance of *Salmonella* and *E.coli*. Experiments have shown that the presence of *Erwinia* can affect negatively *E.coli* O157:H7 inoculum on lettuce leaves (32), and this is explained as competition for food, where better adapted species suppress competition. It was determined in previous experiments that *Enterobacter* adversely affect the adherence and colonization of *Erwinia* species in lettuce (31). However, some recent research have confirmed the opposite, as in the case of pectinovorum species, which makes lesions on the surfaces as additional sites for pathogenic microorganisms to adhere and penetrate their hosts more easily (40).

CONCLUSIONS

The total count of aerobic mesophilic bacteria in 30 samples of lettuce purchased from the local market place in Banja Luka was found to be higher in April than in March. One of the factors that may have contributed to the difference in the microbial counts is an increase in daily temperatures, but also other contributing factors should be taken into account, such as farming practices during the cultivation, harvesting and handling of lettuce, or storage. In the same period of time, yeasts and molds showed no substantial change in their number.

With a simple washing method, the total count of aerobic mesophilic bacteria decreased to values less than 1 $\log_{10}$ cfu/cm^2, whereas the yeasts and molds counts were lower by approximately 1 $\log_{10}$ cfu/cm^2. In the samples tested the presence of pathogenic and potentially pathogenic microorganisms was observed, and they were only partially removed by washing. *Clostridium* species were easiest to remove, whereas *Salmonella* species were hardest to remove by washing. But retention of *Salmonella* species on lettuce, which is always eaten raw, is of special concern since recent studies have confirmed the ability of *Salmonella* species to attach to lettuce leaves more strongly than other pathogenic and potentially pathogenic bacteria.

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MIKROBIOLOŠKI STATUS ZELENE SALATE PRIJE I NAKON STANDARDNOG PRANJA

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Zelena salata je povrće koje se uvijek konzumira sirovo. Broj i vrsta mikroorganizama koji se mogu naći na listovima salate zavise od načina uzgoja, branja, a posebno od kvaliteta pranja i pripreme salate za konzumaciju. Cilj ovog rada je bio da se ispita uticaj pranja pod mlazom vode na mikrobiološki status listova zelene salate kupljene na pijaci u Banjoj Luci. Za mikrobiološke analize su uzimani brisevi lica i naličja lista i određivan je ukupan broj aerobnih mezofilnih bakterija, ukupan broj kvasaca i plijesni, te prisustvo sulfitoredukjućih klostridija, koagulaza pozitivnih stafilikoka, Salmonella vrsta i Escherichia coli. Analizama je utvrđeno da se ukupan broj aerobnih mezofilnih bakterija pranjem redukuje za oko 10 puta, a ukupan broj kvasaca i plijesni oko 8 puta. Od ukupnog broja analiziranih uzoraka zelene salate prije pranja 66.67 % je sadržavalo sulfitoredukujuće klostridije i 13.33 % Salmonella vrsta. Pranjem se broj uzoraka sa pozitivnim nalazom sulfitoredukujućih klostridija redukovao pet puta, a broj uzoraka kontaminiranih Salmonella vrstama je ostao isti.

Ključne riječi: *mikrobiološki status, zelena salata, pranje*

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