Hygienic conditions of minimally-processed watercress, lettuce and cabbage, and fresh-cut lettuce

Condições higiênico-sanitárias de agrião, alface e repolho minimamente processados e alface *in natura*

RIALA6/1193

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Recebido: 02.09.2008 – Aceito para publicação: 10.12.2008

ABSTRACT

With the purpose of assessing the hygienic conditions of package ready-to-eat vegetables sold in the ABC region - SP, 20 samples of each type of minimally-processed vegetables, watercress, lettuce and cabbage were analyzed, in addition to 25 fresh-cut lettuce samples. Coliforms and *Escherichia coli* were analyzed by means of the most probable number (MPN) technique, and *Salmonella*, *Listeria monocytogenes* and *Yersinia enterocolitica* by the conventional culture technique. Centrifugal-flotation technique was carried out for enteroparasites detection. Fecal coliforms MNP values higher than 10^2/g were observed in 60.0% of cabbage, 50.0% of watercress, and 20.0% of lettuce minimally-processed samples. In 48.0% of fresh-cut lettuce samples, the MPN values of fecal coliforms was higher than $2x10^2$/g. *Escherichia coli* was evidenced in 45.0%, 30.0% and 20.0% of watercress, cabbage and fresh-cut lettuce samples, respectively. *Salmonella* was isolated from 5.0% and 4.0% of watercress and fresh-cut lettuce samples, respectively, and *Yersinia enterocolitica* was found on 5.0% of cabbage samples. Enteroparasites were detected on 7.1% of watercress and on 20.0% of fresh-cut lettuce samples. Good manufacturing practices implementation on whole vegetable-produce chain is relevant, in order to assure the availability of safe products to population

Key words. vegetables, minimally processed, hygienic conditions, pathogenic bacteria, enteroparasites.

RESUMO

Com o intuito de avaliar as condições higiênicas das hortaliças empacotadas e prontas para o consumo, foram analisadas 20 amostras de cada tipo dessas hortaliças - agrião, alface e repolho minimamente processadas e, também, 25 amostras de alface *in natura*. A determinação de coliformes e *Escherichia coli* foi realizada por meio de técnica do número mais provável (NMP). Foi utilizada a técnica convencional de cultura para efetuar a pesquisa de *Salmonella*, *Listeria monocytogenes* e *Yersinia enterocolitica*, e a análise parasitológica pela técnica de centrífugo-flutuação. O valor de NMP de coliforme fecal acima de 10^2/g foi encontrado em 60,0% das amostras de repolho, em 50,0% de agrião e em 20,0% de alface minimamente processadas. Em 48,0% das amostras de alface *in natura*, o valor de NMP de coliformes fecais foi acima de $2x10^2$/g. *Escherichia coli* foi evidenciada em amostras de agrião, repolho minimamente processados e em alface *in natura*, respectivamente na proporção de 45,0%, 30,0% e 20,0%. *Salmonella* foi isolada em 5,0% de amostras de agrião e em 4,0% de alface *in natura*; e em 5,0% de amostras de repolho foi detectada *Yersinia enterocolitica*. Os enteroparasitas foram detectados em 7,1% de amostras de agrião e em 20,0% de alface *in natura*. Os resultados do presente estudo mostram a necessidade de implantar as boas práticas de manufatura de hortaliças em todas as etapas de produção, visando obter produtos mais seguros para saúde pública.

Palavras-chave. hortaliças, minimamente processadas, condições higiênicas, bactérias patogênicas, enteroparasitas.

INTRODUCTION

The packaged minimally-processed vegetables are those undergone to minimally- processing operations and to be offered to the consumers in a practical and attractive formats. The minimally-processing procedures involve selection, classification, cleaning, washing, sanitation, peeling, cutting, packing, and storage[1]. Due to the fact of being highly perishable, it is recommended to store the minimally-processed vegetables at below 5ºC after being packed in a specific plastic film and sealed, in order to maintain O_2 absorption and CO_2 production balance. The permeability to the water vapor produced by condensation inside the package provides optimal conditions for inducing growth of microorganisms[2].

The minimally-processed vegetables were introduced in Brazil in the 90s, and they become well-received products due to the advantages of the way they are offered to consumers as cleaned and packed products, besides of convenience in saving time in meals preparation and in minimizing waste[3].

Microbiota in fresh-cut vegetables is diverse, but it usually does not include pathogenic microorganisms to man. Nonetheless, at some points at the vegetable produce chain some incorrect procedures have been put into practice, such as the use of human or animal feces as fertilizer, and the employment of improper irrigation water from rivers and creeks that receive untreated domestic sewer, which can contaminate the vegetables. And these practices increase the risk to diseases caused by pathogenic bacteria, parasites and viruses[4]. Also, pathogens can be transmitted to the products handled by infected worker, in addition to the used vehicles, insects and domestic animals which are crucial sources of contamination[5]. Therefore, the fresh-cut vegetables that are eaten raw have been considered as a potential source in transmitting pathogenic agents related to food-borne disease.

Concurrently to the rise of popularity in consuming minimally-processed vegetables, there is an increase of the risk for microorganisms contamination derived from inadequate handling, inappropriately cleaned or sanitized equipments, which contribute to produce high counts of spoilage and/or pathogenic microorganisms[6]. According to Beuchat[7], the food-borne disease outbreaks associated with raw fruits and vegetables consumption were very common highly prevalent in the USA during the last decade. These outbreaks occurred on account of the *per capita* growth in consuming raw and minimally-processed fruits and vegetables, in the international trade expansion, and also owing to the increasing number of immunosuppressed consumers.

Since the minimally-processed vegetables consumption has also been increasing in Brazil, and because of the lack of information on the quality of these products, the present study evaluated the hygienic conditions of minimally-processed watercress, lettuce and cabbage, and fresh-cut lettuce, searching for the total and fecal coliforms, *Escherichia coli*, enteroparasites, and pathogenic bacteria, such as *Salmonella*, *Listeria monocytogenes* and *Yersinia enterocolitica*.

MATERIAL AND METHODS

▪ Samples

A total of 60 samples, being 20 samples of each type of the minimally-processed vegetables - watercress, lettuce and cabbage were collected in the ABC region, SP. The minimally-processed vegetables were ready-to-eat, packed in a plastic film, and stored at refrigerated temperatures. Sampling units were made up of two 350g-package of each vegetable. Twenty-five conventional fresh-cut lettuce samples were also collected and analyzed, being the sampling unit one lettuce head, no matter the weight or size.

The vegetables samples were collected from July 2005 to October 2006 from supermarkets and vegetable dealers in the ABC region, SP. The vegetables samples were taken to the laboratory in their original packages, and they were kept under refrigeration until the moment of analysis, which occurred in a maximum of 24 hours after being collected.

▪ Samples processing

Twenty-five g of each sample were homogenized in 225 mL of 0.1% peptone water, to get a 10^{-1} dilution, which was used to prepare the serial and sequential ten-fold dilutions with the same diluent solution.

▪ Total and fecal coliforms, and *E. coli* counts

The microorganisms counts were carried out using the most probable number procedure (MPN), following the methodology described by Hitchins et al.,1992[8]. Presumptive test for total coliforms was carried out on lauryl sulphate broth (BD-Sparks, USA), and the confirmatory testing was performed on brilliant green bile

broth (BD-Sparks, USA), both incubated at 35ºC for 24-48h. *Escherichia coli* broth (BD-Sparks, USA) was used to determine the fecal coliforms and examined after 24-48h at 45ºC. *E. coli* was detected by culturing the positive-fecal coliform tubes onto Levine agar (BD-Sparks, USA) and incubated at 35ºC for 24h. Five nucleated colonies, with or without metallic sheen, were subcultured on tryptic soy agar (Oxoid-Basingstone, England) during 24h at 35ºC. The presence of *E. coli* was confirmed by means of Gram staining, lactose broth**,** and IMViC tests (indole, methyl red, Voges Proskauer and citrate). The following criteria determined the positive *E. coli* strain: Gram-negativity, lactose-fermenting bacilli, IMViC patterns (++--) and (-+--).

▪ ***Salmonella.***

Salmonella contamination was investigated according to Andrews et al,1992[9] . Twenty-five g of samples were pre-enriched in 1% buffered peptone water and incubated at 35ºC for 24h. Selective enrichment was carried out on tetrathionate and Rappaport Vassiliadis broths (Oxoid-Basingstone, England), and incubated during 24-48h at 43ºC; and the isolation was done on bismuth sulfite agar (Oxoid-Basingstone, England) and brilliant green agar (Oxoid-Basingstone, England) at 35ºC for 24h. The presumptive identification was carried out triple sugar iron agar (Oxoid-Basingstone, England) and lysine iron agar (Oxoid-Basingstone, England). *Salmonella*-suggestive strains were investigated by biochemical tests using API 20E (BioMérieux Marcy l'Etoile, France)**,** and subtyped by seroagglutination assay by using polyvalent flagellar and somatic antisera. *Salmonella* serotyping was performed at Bacteriology Department of Instituto Adolfo Lutz - Central Laboratory in São Paulo, SP.

▪ ***Listeria monocytogenes***

A 25g-sample aliquot was homogenized in buffered Listeria enrichment broth (Oxoid-Basingstone, England) and incubated for 24-48h at 30ºC. Bacteria isolation was carried out on Listeria selective agar - Oxford formulation (Oxoid-Basingstone, England) incubated at 35ºC for 48h; and the typical colonies were isolated on tryptic soy agar with 0.6% yeast extract incubating at 30ºC for 24h. Biochemical characterization was based on the following assays: catalase production, ß-hemolysis production on 4% horse blood agar, motility on semi-solid agar**,** and API for *Listeria* (BioMérieux Marcy l'Etoile, France). *Listeria monocytogenes* contamination on analyzed vegetables was investigated according to Hitchins, 2003[10]**.**

▪ ***Yersinia enterocolitica***

Yersinia enterocolitica contamination on analyzed vegetables was determined according to Weagan et al., 2003[11]**.**

A 25g-sample aliquot was homogenized in peptone sorbitol bile broth and then divided into two equal parts: one was enriched at 7ºC during the period from 10 to 21 days, and the second portion at 25ºC from 2 to 7 days. After incubation, 1 mL of each enriched portion was decontaminated by adding 9mL of 0.5% KOH in 0.5% saline. The bacteria isolation was carried out on MacConkey agar (Oxoid-Basingstone, England) at 25ºC for 48h**,** and on Yersinia selective agar (Oxoid-Basingstone, England) at 30ºC during 24h. Five typical colonies were cultured onto triple sugar iron agar, bile esculin agar, Christensen urea agar**,** and tryptic soy agar, incubating for 48h at 25ºC. H_2S or other gas non-producing-strains on triple sugar iron agar and also either positive or negative saccharose, positive urease, and positive or negative esculin were analyzed by biochemical testing using API 20E.

▪ **Enteroparasites**

Each vegetable sample was washed down with a brush using 300mL of neutral detergent (10mL Extran MA diluted in 2,000 mL of saline)**.** The washing liquid was filtered in gauze, transferred to a conical glass and left to rest for 24 hours. Then, the supernatant was discarded and the sediment was homogenized. A part of it (≈10ml) was poured in centrifuge tubes for processing centrifugal-flotation technique using zinc sulfate; the remaining portion was employed for sedimentation technique. Enteroparasites contamination on analyzed vegetables was investigated following the methodology described by Oliveira & Germano, 1992[12]**.**

RESULTS AND DISCUSSION

Of 20 minimally-processed watercress and cabbage samples**,** seven (35.0%) presented total coliform counts higher than 10^6/g **,** and 10.0% (2/20) of minimally-processed lettuce samples showed MPN of total coliforms ranging from 10^5 to 10^6/g (Table 1). Although the Brazilian regulation[13] for food products does not include the total coliform counts in ready-to-eat vegetables, Caruso and Camargo[14] showed that MPN of total coliforms higher than 10^6/g, turning the vegetables inadequate for human consumption due to organoleptic changes, loss in nutritional value and risks to consumers health.

In addition, the occurrence of total coliforms is valuable indicators for processing conditions**.** The high counts of these microorganisms suggest an inadequate food processing, and/or recontamination after being processed. The most frequent causes of microorganisms contamination on minimally-processed vegetables are the lack of quality of raw material, the inaccurate cleaning and sanitization of used equipment and utensils, and the inadequate product handling [15].

Table 1 also indicates that 60.0% of the cabbage samples, 50.0% of the watercress samples and 20.0% of the minimally-processed lettuce samples demonstrated MPN of fecal coliforms higher than 10^2/g**.** The Resolution RDC nº12[13] determines a tolerance of up to 10^2/g of the product for fecal coliforms; therefore, according to the observed results a clear nonconformity with the regulation was evidenced. The results found in the present study were lower than those reported by Fröder et al.[16], in São Paulo**.** These investigators analyzed different types of minimally-processed vegetables, and observed that 84.6% of the watercress samples and 61.0% of the lettuce samples showed higher than 10^2 fecal coliforms/g. López et al.[17], in Chile, reported the adequate hygienic conditions in 100% of analyzed minimally-processed cabbage samples, being MPN of fecal coliforms lower than 3/g.

According to Brackett et al.[18], the occurrence of fecal coliforms is not considered to be an indicator of pathogenic bacteria derived from gastrointestinal tract. Some species, particularly *Klebsiella*, belonging to the fecal coliforms group**,** take part in the microbiota on fresh-cut vegetables. The presence of these microorganisms may produce positive results for fecal coliforms. *E. coli* is the unique valid indicator for fecal contamination. *E. coli* was isolated from 30.0% and 45.0% of the cabbage and watercress samples, respectively, although it occurrence was not detected on minimally-processed lettuce (Table

Table 1. Class intervals/g for the most probable number of total and fecal coliforms on minimally-processed cabbage, watercress and lettuce, and on fresh-cut lettuce samples collected from 2005 to 2006 in the ABC region, SP

Class intervals/g	mp cabbage			mp watercress			mp lettuce			fresh-cut lettuce		
	TC Nº (%)	FC Nº (%)	*E.coli* Nº (%)	TC Nº (%)	FC Nº (%)	*E.coli* Nº (%)	TC Nº (%)	FC Nº (%)	*E.coli* Nº (%)	TC Nº (%)	FC Nº (%)	*E.coli* Nº (%)
0 – 10	-	7 (35.0)	14 (70.0)	-	10 (50.0)	12 (60.0)	2 (10.0)	16 (80.0)	20 (100.0)	1 (4.0)	9 (36.0)	20 (80.0)
10 - 10^2	-	1 (5.0)	-	-	-	1 (5.0)	3 (15.0)	-	-	-	4 (16.0)	-
10^2 - 10^3	1 (5.0)	4 (20.0)	2 (10.0)	1 (5.0)	6 (30.0)	6 (30.0)	5 (25.0)	3 (15.0)	-	2 (8.0)	2 (8.0)	2 (8.0)
10^3 - 10^4	4 (20.0)	2 (10.0)	1 (5.0)	4 (20.0)	1 (5.0)	-	4 (20.0)	1 (5.0)	-	5 (20.0)	2 (8.0)	-
10^4 - 10^5	5 (25.0)	5 (25.0)	2 (10.0)	5 (25.0)	3 (15.0)	2 (10.0)	4 (20.0)	-	-	12 (48.0)	6 (24.0)	2 (8.0)
10^5 - 10^6	3 (15.0)	-	-	3 (15.0)	-	-	2 (10.0)	-	-	3 (12.0)	2 (8.0)	1 (4.0)
> 10^6	7 (35.0)	1 (5.0)	1 (5.0)	7 (35.0)	-	-	-	-	-	2 (8.0)	-	-

mp = minimally-processed, TC = total coliforms, FC = fecal coliforms, cabbage = 20 samples, watercress = 20 samples, lettuce = 20 samples; fresh-cut lettuce = 25 samples.

1). The maximum value of most probable number of *E. coli* observed on cabbage and watercress samples were >1.1x10^{6}g and 9.3x10^4/g, respectively (data not shown).

As for fresh-cut lettuce, 48.0% (12/25) of samples showed fecal coliform counts higher than 2x10^2/g. These results indicate the inadequacy in the vegetable hygienic condition, according to the Decree 12.486[19]. The values detected in the present study were higher than those reported by Takayanagui et al.[20], in Ribeirão Preto, and by Simões et al.[21], in Campinas, wherein the most probable number of fecal coliforms above 2x10^2/g was found on 17.0% and 19.0% of analyzed fresh-cut vegetable samples, respectively. The occurrence of *E. coli* was observed on 20.0% (5/25) of fresh-cut lettuce samples (Table 1), and the maximum counts were 1.1x10^5/g of the product (data not shown).

None of the minimally-processed vegetables or fresh-cut lettuce harbored *L. monocytogenes* (Table 2), although other species were isolated, such as: *L. innocua* from one watercress sample, and *L. seeligeri* and *L. innocua* from some samples of fresh-cut lettuce. *L. innocua* is not considered to be pathogenic to human, and listeriosis caused by *L. seeligeri* is extremely rare in humans[10]. Similar data were reported in United Kingdom by Sagoo et al.[22], these investigators did not detect *L. monocytogenes*, but isolated other *Listeria* species from 0.2% of analyzed minimally-processed vegetables samples: *L. innocua* from watercress and *L. seeligeri* from raddish. In Brazil, Fröder et al[16], isolated *L. monocytogenes* and *Listeria spp* from 0.6% and 1.7% of minimally-processed vegetables, respectively, whereas De Curtis et al.[23], in Venezuela, detected *L. monocytogenes* from 30.0% of minimally-processed vegetables samples.

Yersinia enterocolitica was isolated from 5.0% (1/20) of cabbage samples (Table 2), and other *Yersinia* species were detected on three varieties of minimally-processed vegetables samples: *Y. frederiksenii* (from minimally-processed cabbage and lettuce), *Y. intermedia* (from minimally- processed lettuce), and *Y. kristensenii* (from watercress). Among the isolated species, *Y. enterocolitica* is the only one considered to be potentially pathogenic to humans, and yersinosis outbreaks associated with the consumption of foodstuffs contaminated with this bacterium were reported elsewhere[24,25]. Pingulkar et al.[26] did not detect *Y. enterocolitica* in India, but *Yersinia sp* was isolated from 20.0% of ready-to-eat vegetables samples.

Investigation on both *Yersinia enterocolitica* and *L. monocytogenes* are required for foodstuffs stored under refrigeration due to the psychrotrophic characteristics of these organisms[27].

Salmonella was isolated from 5.0% (1/20) of watercress samples and 4.0% (1/25) of fresh-cut lettuce samples, which showed lower than 10 fecal coliforms/g (Table 2). Therefore, these vegetables are considered inadequate for consumption by reason of *Salmonella* detection on 25g of the product, according to the current regulation. These results were similar to those reported by Fröder et al.[16] and Takayanagui et al.[20], wherein *Salmonella* sp was isolated from ≈3.0% of minimally-processed

Table 2. Occurrence of Listeria monocytogenes, Yersinia enterocolitica e Salmonella on minimally-processed cabbage, watercress and lettuce, and on fresh-cut lettuce samples collected from 2005 to 2006 in the ABC region, SP

Type of vegetable	Nº (%) of positive samples for			
	Total nº of samples	*L.monocytogenes* in 25g	*Y.enterocolitica* in 25g	*Salmonella* in 25g
mp cabbage	20	-	1 (5.0)	-
mp watercress	20	-	-	1 (5.0)
mp lettuce	20	-	-	-
Fresh-cut lettuce	25	-	-	1 (4.0)

mp = minimally-processed.

vegetables and fresh-cut vegetables samples, respectively. On the other hand, in the study conducted in Chile by López et al.[17], none of the analyzed minimally-processed vegetables samples harbored *Salmonella*.

Salmonella serotypes identified in the present study were *S. enteritidis*, isolated from watercress and *S. panama* from fresh-cut lettuce samples. The bacteria genus *Salmonella* continue to be the main etiological agents of food-borne disease outbreaks. In 2005, *Salmonella* was responsible for 39.0% of food-borne disease outbreaks, being *S.* Enteritidis as the second mostly prevalent serotype in 10 States of the Unites States of America [28].

Salmonellae, as Gram negative bacteria, are easily eliminated by using low concentrations of free chlorine. Nonetheless, on some occasions these microorganisms are unsuccessfully eliminated from pre-contaminated vegetables. Chlorine efficacy as a disinfectant depends on the concentration, pH, temperature, time of exposure, and the growth phase of the pre-existent pathogen[29]. Other factors also abrogate the lethal effect of hypochlorite on microorganisms, as the hydrophobic nature of fat droplets covering the vegetable surface[30], and the vegetable structure, which may prevent the contact between the sanitizing agent and the pathogens[7].

Parasitological analyses were carried out in 70.0% of each minimally-processed vegetables variety, and in 100% of fresh-cut lettuce samples.

Results of these analyses are shown in Table 3**;** 7.1% (1/14) of watercress samples were contaminated with enteroparasite eggs, as from *Ascaris lumbricoides*, but no enteroparasite was detected on two other minimally-processed vegetables varieties. On 14.3% (2/14) of watercress samples and on 7.1% (1/14) of cabbage samples, contaminants as mites and nematode larvae were detected. Hence, 21.4% of watercress samples and 7.1% of cabbage samples did not comply with Decree SVS 326/97[31], considering the presence of unusual biological matters, either harmful or harmless to human health on the processed foodstuffs. Only the lettuce sample was negative for both enteroparasites and biological contaminants, among the three minimally-processed vegetables varieties analyzed in the present study. This fact might be in part correlated to the vegetable structure, as the large and smooth leaves of lettuce could be favored by the cleaning procedure and by the action of the sanitizing agent on the vegetable.

Twenty percent (5/25) of fresh-cut lettuce samples were contaminated with enteroparasites as *Strongyloides stercoralis*, *Ascaris lumbricoides* eggs, *Giardia lambria* and *Entamoeba coli* cystis (Table 3), which indicate the occurrence of fecal contamination from human and/or animal origin, and these findings are important for public health, excepting *Entamoeba coli*, for being considered as no pathogenic for man[32]. Even so, the presence of this

Table 3. Number and percentage of positive samples for enteroparasites and contaminating agents on minimally-processed cabbage, watercress and lettuce, and on fresh-cut lettuce collected from 2005 to 2006 in the ABC region, SP

Type of vegetable	Nº (%) of positive samples			
	Total nº of samples	Enteroparasites	Biological contaminants	Total nº of positive samples
mp cabbage	14	-	1 (7.1)	1 (7.1)
mp watercress	14	1 (7.1)	2 (14.3)	3 (21.4)
mp lettuce	14	-	-	-
Fresh-cut lettuce	25	5 (20.0)	4 (16.0)	9 (36.0)

mp = minimally-processed, enteroparasites= *Strongyloides stercoralis*, *Ascaris lumbricoides eggs*, *Giardia lambria and Entamoeba coli cysts*, and biological contaminants = mites, nematode larvae.

protozoan is relevant when the fecal coliform counts are high, as observed in the present study. Silva et al.[33] and Soares et al.[34] have recently reported the high rates of enteroparasites on analyzed fresh-cut lettuces samples.

The results found in the present study show the poor hygienic conditions of the minimally-processed vegetables, evidenced by high rate of fecal and total coliform populations, and also by detecting *Escherichia coli*, *Salmonella, Yersinia enterocolitica,* pathogenic parasites and biological contaminants. For that reason, the implementation on good manufacturing practices is crucial, from the vegetable cultivation to each stage of minimally processing, including packing and refrigeration, in order to prevent cross-contamination and multiplication of microorganisms during production and storage.

As for fresh-cut vegetables, especially those receiving no heat treatment before consumption, the quality will be improved if some measures are assumed by means of educational programs directed to agriculturists**,** combined with periodically monitoring the water used for irrigation procedures.

Good manufacturing practices implementation on the whole vegetable-produce chain is relevant, in order to identify and control the factors for preventing contamination and restraining microorganisms growth, and to assure the availability of safe products to population. In addition, hygienic status, appropriate storage and transportation conditions are crucial factors for extending the vegetables shelf life and safety.

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