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Occurrence and genetic characterization of *Listeria* spp. in minimally processed vegetables commercialized in Porto Alegre, Brazil*

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ABSTRACT

Minimally processed vegetables go through many steps before they are refrigerated, selection, washing, peeling, cutting, disinfection and finally packaging. However, if no care is taken at the origin of the raw materials and in the processing stages, there is a chance of finding pathogenic bacteria, such as *Listeria monocytogenes*, which are able to grow at low temperatures. The aim of this research was to verify the occurrence of *Listeria* sp. in minimally processed vegetables sold in Porto Alegre, Brazil, and the genetic relationship among the isolates. Minimally processed salads were sampled monthly from local supermarkets and analyzed by inoculation on Listeria Enrichment Broth and subsequent seeding on two selective media, Palcam and Modified Oxford Agar. The typical colonies were identified to species level and their intergenic region 16S-23S rDNA were amplified in order to verify the genetic variability. Species of *Listeria* were found in 23 of the 52 processed salad samples analyzed and *L. monocytogenes* was found in seven. The presence of *L. monocytogenes* in the samples is a health concern, as these salads are eaten without further treatment by the consumer. The amplification of the intergenic region 16S-23S rDNA, showed a great genetic diversity among the isolates, with 43 different patterns, proving the usefulness of this technique in epidemiologic studies.

Key words: *Listeria,* minimally processed, vegetables, 16S-23S rDNAS, spacer region.

RESUMO

Hortaliças minimamente processadas passam por etapas como: lavagem, seleção, descasque, corte, sanificação e, depois de embaladas, são armazenadas sob refrigeração. Todavia, se cuidados sobre a origem do produto bruto ou durante as fases de processamento não são respeitados, existe a possibilidade de se encontrar bactérias patogênicas, como *Listeria monocytogenes*, a qual pode crescer em temperaturas baixas. Este trabalho teve por objetivo verificar a ocorrência de *Listeria* sp. em hortaliças folhosas minimamente processadas comercializadas em Porto Alegre – RS e verificar a relação genética entre os isolados. Saladas folhosas minimamente processadas foram coletadas mensalmente em supermercados locais, semeadas em caldo de enriquecimento para *Listeria* e subsequentemente em dois meios seletivos, ágar PALCAM e ágar Oxford Modificado. Foi realizado isolamento e identificação das espécies das colônias características e a amplificação da região intergênica 16S-23S do rDNA para verificar a variabilidade genética. Espécies de *Listeria* foram encontradas em 23 das 52 amostras de saladas processadas analisadas, sendo *L. monocytogenes* encontrada em sete amostras. A presença de *L. monocytogenes* é preocupante, pois estes produtos são consumidos sem nenhum tratamento pelo consumidor. Através da amplificação da região intergênica 16S-23S do rDNA de *Listeria* sp. observou-se grande diversidade entre os isolados, gerando 43 padrões, comprovando a utilidade desta técnica em estudos epidemiológicos.

Descritores: *Listeria*, minimamente processados, vegetais, 16S-23S rDNA, região espaçadora.

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INTRODUCTION

The increasing demand for fresh fruit and vegetables, has encouraged the consumption of minimally processed foods [22,23]. However, if these ready-to-eat food products meet the claims for practicality of modern society, they may also represent hazard to consumers, once they do not systematically carry out any sanitization process before such products are eaten. When present in minimally processed vegetables, *Listeria* isolates may be able to survive if no inhibition mechanism is prompted during storage [12], since this bacteria grows at ordinary refrigeration temperatures and in modified atmosphere [5,21]. Since these ready-to-eat products are quite popular, the investigation of the occurrence of *L. monocytogenes* in leafy vegetables, especially of the kind used in salad preparations, is particularly important.

The contamination of vegetables may be of diverse sources and take place at different stages of a food processing plant. It may take place in the processing environment as a whole [8,17], either before or even after the food has been sanitized [10,20]. In this scenario, molecular methods have proved themselves useful in the establishment of contamination sources throughout a production line [13,14,18,19]. With this intent, the amplification of the 16S – 23S rDNA spacer regions, whether cleaved or not with restriction enzymes, has been used in many studies [1,3,6,26].

This study aimed (i) to establish the occurrence of *Listeria* sp. in minimally processed leafy vegetables, (ii) to characterize the isolates by amplification of the 16S - 23S rDNA spacer regions., and (iii) to correlate the data obtained with the origin of the product.

MATERIALS AND METHODS

Ready-to-eat salad samples were randomly collected in supermarkets of Porto Alegre (RS, Brazil) from August 2004 to August 2005. Four samples were collected per month, totaling 52 samples that varied for salad mix types and commercially available brands (A, B and C). Each sample was composed by three 200g salad packages of the same brand and salad mix, to make up a 600g analysis unit. Twenty-five grams of each sample were homogenized in 225 mL *Listeria* enrichment broth (LEB) and incubated at 30°C for 48 hours, the agar plates were inspected for typical colonies for the following 7 days. Afterwards, *Listeria* colonies were isolated from Modified Oxford Agar (MOX) and Palcam agar (PAL) and seeded in Triptone Soy Agar supplemented with yeast extract (TSA – YE 0.6%) for purification. The following biochemical assays were used to establish genus and identify species: Gram test; catalase; specific motility at 28°C; Triple-Sugar-Iron; methyl red; Voges-Proskauer; nitrate reduction; fermentation of xylose, rhamnose, and manitol; hemolysis and CAMP-test.

Chromosomal DNA was extracted [4] and the amplification of the 16S – 23S rDNA spacer region was carried out using primers designed based on the sequences for different *Listeria* sp. found in GenBank: 16S LisF 5' GCTGGATCACCTCCTTTCTA 3', and 23S LisR 5' GCGCCCTTYCTAACYTAACC 3'. The reactions were carried out in 10mM Tris/HCl, pH 8.3; 50mM KCl; 2 mM $MgCl_2$; 0.2 mM dNTPs; 0.38 μM primer 16S LisF; 0.5 μM primer 23S LisR; 1 U Taq DNA polymerase and 100 ng of template DNA, to a final volume of 25 μL. Amplification reactions were conducted in a thermal-cycler (Mastercycler Personal Eppendorf) as follows: an initial denaturation step at 94°C for 2 min followed by 30 cycles at 94°C for 1 min, at 53°C for 1 min, at 72°C for 1 min, and a final extension step at 72°C for 10 min. For each set of PCR analyses, positive controls were prepared with *L. monocytogenes* ATCC 15315 and *L. innocua* ATCC 33090, these reference strains behaved unvaryingly throughout the experiments. The PCR products were electrophoresed in 8% polyacrylamide gels (10 V/cm for 2 h 30 min.) stained with ethidium bromide (0.5 μg/mL), analyzed under UV light and photographed with a digital camera Kodak 1D (version 3.5.2). The molecular marker λ *Eco*RI / *Hind*III / *Cla*I was used as a standard for inspecting fragment size.

RESULTS

In the samples analyzed in the present study *Listeria* species were isolated in 23 of the 52 samples of minimally processed salad products analyzed. As for the commercial brands chosen, *Listeria* sp. were detected in 16 samples of brand A (n=24), 5 samples of brand B (n=10), and in 2 samples of brand C (n=18). *L. seeligeri* was detected in 11 samples, *L. monocytogenes* in 7, *L. welshimeri* in 7, *L. grayi* in 6, *L. innocua* in 4, and *L. ivanovii* in 3 samples. No correlation between the presence of *Listeria* species and the composition of the salad mixes was observed. (Table 1). The highest occurrence of *Listeria* was between January and April 2005, when *Listeria* were isolated in 3 samples per collection. The most common species

throughout the year were *L. seeligeri* and *L. welshimeri*, detected in almost all brands analyzed. *L. seeligeri* prevailed in the samples as a whole, whereas *L. monocytogenes* prevailed in warm weather samples.

At the molecular analysis, 160 strains of the 175 *Listeria* isolates were characterized, since 15 strains did not recover for DNA extraction (6 *L. welshmimeri*, 3 *L. innocua*, 2 *L. ivanovii*, 2 *L. seeligeri*, 1 *L. monocytogenes*, and 1 *L. grayi* subspecies *grayi*). A total of 43 different patterns with 15 fragments were observed. The most frequent pattern was pattern 41, detected in 33 isolates (Table 1). *L. monocytogenes* and *L. seeligeri* presented the highest number of patterns in common, patterns 18, 35, and 38. Fourteen patterns were found in more than one species, and 7 patterns were detected in one species exclusively. As for the commercial brands chosen for investigation, more specifically, Brands A and B shared different patterns for *L. grayi* (pattern 13) and *L. seeligeri* (patterns 18, 24, 35, and 39). Brands A and C shared pattern 35 for *L. monocytogenes*, whereas Brands B and C did not share any patterns. Recurring strains were observed, as follows: *L. monocytogenes* pattern 29, detected in brand A at different summer collections; *L. seeligeri* pattern 41, identified in brand A in (autumn and winter 2004 and 2005); *L. seeligeri* pattern 23, detected in brand A at different winter collections; *L. seeligeri* pattern 24, detected in brand A in winter 2004 and 2005 and in brand B in spring; *L. seeligeri* pattern 18, detected in brand B in spring, and in brand A in autumn and winter; *L. seeligeri* pattern 39, detected in brand B in spring, and in brand A in winter. These last 3 strains recurred in samples of identical origin (Table 1). The pattern found for the isolate *L. grayi* subspecies *murrayi* was the same as observed for the 15 isolates of *L. grayi* subspecies *grayi* (pattern 28).

Table 1. Distribution of *Listeria* species isolated from minimally processed vegetables commercialized in Porto Alegre, Brazil, and their molecular patterns. The sampling period was from August 2004 to August 2005.

Sampling month	Brand of salad mix analysed#	16S-23S rDNA intergenic region pattern observed	No. of isolates for each brand and salad mix							
			L. monocytogenes	*L. innocua*	*L. seeligeri*	*L. welshimeri*	*L. ivanovii*	*L. grayi* subsp. *grayi*	*L. grayi* subsp. *murrayi*	*Listeria* sp.
August 2004	A1**	24,29,41	-	-	3	1*	-	-	-	-
	A2	16,27,13	3	-	-	-	-	1	-	-
October 2004	B1**	16,13,28	-	-	1	1*	-	18 (1*)	-	-
November 2004	B2	4,6,9,18,19,24, 30,34,35,39,9	-	-	13	-	-	1	-	-
December 2004	A1	29	-	-	-	2*	-	1	-	-
	A3	1,5, 11, 14, 15, 37,29,28	-	3	1	3 (1*)	-	4	1	-
January 2005	A1	29	1	-	-	-	-	-	-	-
	A2		-	1*	-	-	-	-	-	-
	C1**	1	-	-	1*	-	-	1	-	-
February 2005	A1	17	-	1	-	-	-	-	-	-
	A2	29,33,35	4 (1*)	2*	-	-	-	-	-	-
	B1	8	-	-	-	-	1	-	-	-
March 2005	A2	31,41,9	-	-	2	-	1	-	-	-
	B1	37	1	-	-	-	-	-	-	-
	B3	28	-	-	-	1	-	-	-	-
April 2005	A2	2,18,32	3	-	-	-	-	-	-	-
	A3	38, 3,10,18,20, 22,23,25,37, 38,41,43	2	-	19	-	-	-	-	-
	C1**	21,35,36,41,42	6	-	-	-	-	-	-	-
May 2005	A1	-	-	-	1*	-	-	-	-	-
June 2005	A3	7,17,18,23,26, 35,39,41,26	-	-	38	1	-	-	-	-
July 2005	A1	23,24,39,40,41	-	-	5	1*	-	-	-	1
	A3	39,40,41	-	-	6	-	-	-	-	-
August 2005	A2	5,12,23	-	-	-	-	18 (2*)	-	-	-
Total of samples		23	7	4	11	7	3	6	1	1
Total of isolates			20	7	90	10	20	26	1	1

#Capital letters correspond to the brand and numbers, to the salad mix type. *and (n*) correspond to isolates that didn't recovered for DNA extraction. **Salad brand A and B, original from Porto Alegre, Rio Grande do Sul, salad brand C, original from São José dos Pinhais, Paraná

DISCUSSION

In vegetables, the occurrence of *Listeria* genus varies greatly in product range, especially in ready-to-eat foods [7,9,16,26]. The genus *Listeria* can be found in different environments and *L. monocytogenes* is the species most associated with food borne diseases. However, the presence of other *Listeria* species indicates that environmental conditions are ideal to microbial growth. The presence of *L. monocytogenes* in ready-to-eat produce raises health concerns, as these food products are extensively consumed without further treatment. The amplification of the 16S – 23S rDNA spacer regions in *Listeria* isolates has proved its genetic diversity, with the detection of 43 patterns. Such high number of patterns may occur due to the quantity of operons in the 16S – 23S rDNA spacer regions present in the *Listeria* genome [11]. The genetic diversity among strains, as detected in the present study, may be explained by several aspects involved in the contamination route, such as the conditions of soils, manure, and of the water destined to irrigation and to washing. These aspects may vary considerably between food processing units, between farms, and even between seasons, which makes contamination difficult to track down for a specific point in the production chain.

Recurring strains were observed in the samples investigated, proving that these strains may be present in these food processing units or, at least, that they recur in the vegetable raw materials therein processed. This last hypothesis is plausible in the light of the fact that these recurring strains are detected in different commercial brands that process raw food material of identical origin. Recurring *Listeria* strains have also been identified by other authors [2,15,24,27], who suggested that the main contamination source is ultimately the processing unit itself. Interestingly, the occurrence of the same genotypic profile in isolates of distinct origin evinces the role played by one same strain in the contamination of different products. Nevertheless, it remains to be established whether this given strain has recently been dispersed or is a common strain adapted to the food processing environment.

CONCLUSIONS

All six *Listeria* species were detected and isolated from the salad samples analyzed, roughly throughout the entire sampling period. These findings may reveal the degree of extensive distribution and resistance of this microorganism during the processing and storing procedures adopted for this kind of food product. Though simple, the molecular method used in the present study afforded the discrimination between isolates of the same species. The method described may be equally useful in the investigation of disease outbreaks, in the identification of contamination sources, and in the characterization of inter- and intraspecies genetic diversity. The mapping of isolate dispersal, both in food processing environments and in food items themselves, will assist the outlining of hygiene measures against *Listeria* sp. in the production of safe foods.

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