Microbiological assessment of lettuce salads and antimicrobial resistance of Staphylococcus spp

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Abstract

Introduction: self-service restaurants in which food is served ready to be consumed are liable to have some products contaminated by pathogenic microorganisms causing food-transmitted diseases.

Aim: evaluates the microbiological quality of lettuce salads in Pelotas RS Brazil by counts of thermo-tolerant coliforms, E. coli, Staphylococcus spp., and detection of Salmonella spp. Antimicrobial resistance of Staphylococcus spp. isolates are also assessed.

Methods: thirty-six samples of lettuce salads were collected from nine restaurants and thermo-tolerant coliforms, Escherichia coli and Staphylococcus spp. were quantified, coupled to a research on Salmonella spp., following methodology by the Bacteriological Analytical Manual. Staphylococcus spp. isolates underwent antimicrobial resistance test by the disc-diffusion method.

Results and discussion: results showed that 61.1% of the salad samples contained more thermo-tolerant coliforms than allowed by Brazilian legislation and E. coli was confirmed in 5.6% of the samples. Positive and negative coagulase Staphylococcus occurred respectively in 5.6% and 77.8% of isolates, but no sample had Salmonella spp. Further, 56.7% of the thirty isolates of Staphylococcus spp. tested were resistant to penicillin; 46.7% to oxacillin; 26.7% to erythromycin and 23.3% were multi-resistant.

Conclusion: inadequate quality of the salad was due to pathogenic microorganisms, while Staphylococcus spp. isolates had a high percentage of antimicrobial resistance.

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Key words: Microorganism markers. Restaurants. Antibiotics.

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EVALUACIÓN MICROBIOLOGICA DE ENSALADAS DE LECHUGA Y PERFIL DE RESISTENCIA ANTİMICROBIANA DE STAPHYLOCOCCUS SPP

Resumen

Introducción: la procura por establecimientos que ofrecen alimentos prontos para consumo ha aumentado, sin embargo, los alimentos disponibles en estos locales pueden estar contaminados con microorganismos patogénicos, pudiendo causar enfermedades transmitidas por alimentos.

Objetivos: evaluar la calidad microbiológica de las ensaladas de lechuga en los restaurantes de Pelotas RS Brasil, a través de los recuentos de coliformes termotolerantes, Escherichia coli, Staphylococcus spp. y la detección de Salmonella spp. Resistencia a los antimicrobianos de Staphylococcus spp. también se evalúan.

Métodos: fueron colectadas 36 muestras de ensaladas de lechuga en nueve restaurantes y realizada la cuantificación de coliformes termotolerantes, Escherichia coli y Staphylococcus spp. e investigación de Salmonella spp., siguiendo la metodología del Bacteriological Analytical Manual. Los aislados de Staphylococcus spp. fueron sometidos al examen de resistencia a antimicrobianos por el método de difusión con disco.

Resultados y discusión: de las 36 muestras de ensalada de lechuga, 61,1% presentaron cuantificación de coliformes termotolerantes superiores a lo permitido por la legislación brasileña, y hubo confirmación de E. coli en 5,6% de las muestras. La cuantificación de Staphylococcus coagulasa positiva representó 5,6% de los aislados y Staphylococcus coagulasa negativa representó 77,8%. Todas las muestras presentaron ausencia de Salmonella spp. De los 30 aislados de Staphylococcus spp. examinados, 56,7% fueron resistentes a penicilina, 46,7% a oxacilina, 26,7% a eritromicina y 23,3% fueron multirresistentes.

Conclusión: la calidad microbiológica de las ensaladas de lechuga se mostró inadecuada debido a la presencia de microorganismos patogénicos, y los aislados de Staphylococcus spp. presentaron elevado porcentaje de resistencia antimicrobiana.

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Palabras clave: Microorganismos indicadores. Restaurantes. Antibióticos.
Introduction
Self-service restaurants in which food is served ready to be consumed are liable to have some products contaminated by pathogenic microorganisms causing foodborne diseases (FD)\(^1\). Thermotolerant coliforms and *Escherichia coli* are microorganisms indicators of fecal contamination; *Staphylococcus* spp. indicates poor hygiene conditions during food processing; and *Salmonella* spp. is a pathogenic microorganism which may cause infections by food\(^2\). Epidemiological data in Brazil show that *Salmonella* spp., positive coagulase *Staphylococcus* and *Escherichia coli* are the main agents in FD outbreaks\(^3\).

Lettuce (*Lactuca sativa*) is consumed worldwide and has an important role on the Brazilian market. In fact, this leafy vegetable has beneficial qualities for health due to its fiber rates and antioxidant properties\(^4,5,6\). Since the lettuce is consumed raw, adequate hygiene procedures should be undertaken to eliminate pathogen microorganisms\(^7,8,9,10\). In fact, the lettuce has been associated with contamination by certain pathogenic microorganisms such as *Salmonella* spp., *Escherichia coli* and *Listeria monocytogenes*\(^11\).

*Staphylococcus* spp., especially positive coagulase *Staphylococcus*, may occur in food intoxication outbreaks\(^9,10,11,12\). Further, since it acquires antimicrobial resistance and bacterial multiresistance, it becomes the main problem in the treatment of infections\(^11\).

Antimicrobial resistance is actually a growing health issue in animal breeding and public health. Several bacteria in the environment are resistant and may contaminate water and food with serious consequence to health\(^13,14\). Due to their capacity in acquiring antimicrobial resistance, *Staphylococcus* infections are on the increase, with a greater number of multi-resistant strains and rising difficulties in their treatment\(^15,16,17,18\).

Current analysis evaluates the microbiological quality of lettuce salads in restaurants in Pelotas, RS, Brazil by counts of thermo-tolerant coliforms, *E. coli*, *Staphylococcus* spp. and detection of *Salmonella* spp. Antimicrobial resistance of *Staphylococcus* spp. isolates are also assessed.

Methods

**Experimental design and sampling**

A transversal analysis was conducted in self-service restaurants in Pelotas, RS, Brazil. Information on the number and place of the restaurants was retrieved from the sanitary and food vigilance sector of the Municipal Health Office for the experimental design. Sampled population comprised 10% of the number (91) of self-service restaurants in the town, with the random selection of 9 restaurants.

**Collection of samples**

A sample of lettuce salad was retrieved from each restaurant (n=9), once a week, during four weeks, with a total of 36 samples. Samples were retrieved as if one were buying lettuces; collection occurred at the distribution section and the lettuces were packed in disposable thermal packages available at the restaurant. The package was closed, identified and taken immediately to the laboratory for analysis.

**Microbiological analyses**

Thermo-tolerant coliforms, *Escherichia coli* and *Staphylococcus* spp were counted and search for *Salmonella* spp was undertaken, following requirements by Brazilian legislation\(^17\). Microbiological analyses followed methodology by the Bacteriological Analytical Manual\(^18\). Counts of thermo-tolerant coliforms followed the Most Probable Number (MPN) method in a Lauril Sodium Sulfate broth test (LST, Merck\(^19\)) and a confirmation test in *Escherichia coli* broth (EC, Merck\(^20\)). Confirmation analysis for *E. coli* was undertaken where the confirmed culture was streaked by Eosin-Methylene Blue Agar Plate Protocol (L-BEM, Merck\(^21\)), incubated at 36 °C for 24 h. Typical colonies were transferred to Count Standard Agar plates (PCA, Merck\(^22\)) and incubated at 36 °C for 24 h. Colonies were Gram stained and pure cultures underwent biochemical tests.

For the analysis for *Salmonella* spp., pre-heating was done in Buffered Peptone Water (Merck\(^23\)), at 37 °C for 24 h. After incubation, 1 mL was transferred to Tetramionate Broth (Merck\(^24\)) to which 0.1 mL of brilliant green and 0.2 mL iodine were added; 0.1 mL was transferred to Rappaport-Vassiliadis broth (Merck\(^25\)) and incubated at 42 °C for 24 h. Seeding in Hektoen Agar (HE, Merck\(^26\)) and agar Xylose-lysine-deoxycholate (XLD, Merck\(^27\)) was performed after the above selective enrichment; it was then incubated at 37 °C for 24 h. Characteristic colonies underwent biochemical and serum tests.

Three decimal dilutions were performed for the isolation of *Staphylococcus* spp. and 1 mL of each was divided into three Agar Baird Parker plates (BP, Merck\(^28\)) enriched with egg yolk emulsion and potassium tellurite 1% by surface spreading, and incubated at 37 °C for 48 h. Further, typical and atypical presumptive colonies were counted and given in Colony-Forming Units per gram of food (CFU/g). Isolated colonies were Gram-stained and the characteristic and pure ones were...
selected. A loopful of each selected colony was inoculated in Brain Heart Infusion broth (BHI, Merck®) and incubated at 37 °C for 24 h. The coagulase test was performed and colonies with coagulates were considered positive; otherwise they were considered negative.

Antimicrobial resistance

Antimicrobial resistance by disc diffusion technique was verified from isolates of positive coagulase Staphylococcus (PCS) and negative coagulase Staphylococcus (NCS), according to the Clinical and Laboratory Standards Institute (CLSI)21. Isolates were first cultivated in BHI broth (Merck®) at 36 °C and inoculated in a saline solution 0.85 % (Merck®) till Mac Farland scale 0.5. The culture was then spread on a plate containing Agar Muller Hinton (MH, Merck®) and the antimicrobial discs (Invitrogen®); they were similar spread and incubated at 37 °C for 24 h for Gram positive bacteria. Halo was measured in centimeters from one extremity to the other and result was obtained by comparing tables for CLSI resistance standard22. The 12 antimicrobial agents tested composed the antimicrobial disc: ampicillin (10 µg), penicillin (10 units), oxacillin (1 µg), clindamycin (2 µg), sulfamethoxazole/trimethoprim (23.75/1.25 µg), chloramphenicol (30 µg), erythromycin (15 µg), gentamicin (10 µg), tetracycline (30 µg), vancomycin (30 µg), ciprofloxacin (5 µg), cefepine (30 µg), rifampicin (5 µg) (CLSI, 2007).

Statistical analysis

Statistical analyses were performed with SPSS® (Chicago, v. 17.0, 2008) for antimicrobial resistance test.

Results and discussion

Twenty-two (61.1%) of the 36 lettuce salad samples analyzed contained thermotolerant coliforms above the limit allowed by Brazilian legislation, or rather, up to 10² NMP/g21 (Table I).

Table I

Amount of thermotolerant coliforms in samples of lettuce salads. Pelotas, 2015. (n=36)

<table>
<thead>
<tr>
<th>Samples</th>
<th>Counts (MPN/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thermotolerant coliforms</td>
<td>15 (41.7) 1.1x10⁰ to 4.6x10⁴</td>
</tr>
<tr>
<td></td>
<td>7 (19.4) 1.6x10⁴ to 4.6x10⁴</td>
</tr>
<tr>
<td></td>
<td>10 (27.8) 3.6 to 93</td>
</tr>
<tr>
<td></td>
<td>4 (11.1) &lt; 3</td>
</tr>
</tbody>
</table>

MPN/g – Most Probable Number per gram of food.

Brandão et al.24 evaluated 30 samples of lettuce salad in self-service restaurants in Rio de Janeiro, RJ, Brazil and detected 22 samples (73.3%) contaminated by thermotolerant coliforms, with 6 samples (19.9%) above the limit allowed by Brazilian legislation. Another study evaluated the microbiological quality of lettuce salads in 10 self-service restaurants in Gurupi, TO, Brazil and reported 12 samples (60%) contaminated by thermotolerant coliforms above the minimum rate25.

Moreover, two samples (5.6%) out of the 36 samples in current analysis were confirmed for E. coli, with 3.6 MPN/g in each sample. Several other studies register E. coli in lettuces ready for consumption, with percentages ranging between 3.5 and 30% contamination24,26,27,28.

The amount of thermotolerant coliforms at percentages higher than those permitted by the legislation in ready foods, and the confirmation of E. coli indicate either improper prime matter conditions, or unsatisfactory food processing conditions or inadequate hygiene–sanitary conditions21,24,29,30,31. Contamination in current analysis may be related to the fact that lettuce salads were served raw and either there were inadequate hygiene procedures during food handling or contamination occurred in the post-processing period24,27,28.

No Salmonella spp. was reported in any of the analyzed samples (25 g of food) and thus Brazilian legislation was complied with (BRASIL, 2001). Results were similar to other studies that assessed Salmonella spp. in lettuce salad24,26,27,28,32.

Two (5.6%) out of the 36 samples were reported with PCS, whereas 28 (77.8%) were reported with NCS (Table II).

Whereas a study in São Bernardo do Campo, SP, Brazil evaluated 30 samples of raw green vegetables in self-service restaurants and detected one sample (3.3%) with PCS33. Another study in Porto Alegre, RS,
Brazil evaluated 26 samples of different types of food, including raw and boiled salads, and reported 15 samples (57.7%) contaminated with NCS. The same study assessed the hands of 21 food handlers and discovered 9 people with Staphylococcus spp.

Although staphylococcus contamination occurs by inadequate hygiene of hands, the production of toxins occurs in food with Staphylococcus spp. counts over $10^5$ CFU/g.

Moreover, Staphylococcus spp. is resistant to several antimicrobial classes and may cause illness and even death owing to infections. The microbial resistance test (n=30) was undertaken from Staphylococcus spp. isolates. Results revealed that isolates had a higher resistance percentage to penicillin (56.7%), oxacillin (46.7%) and erythromycin (26.7%). Only one isolate (3.3%) had an intermediate resistance to the antimicrobial agent erythromycin. All Staphylococcus spp. isolates under analysis were sensitive to the antimicrobial agent gentamycin (Table III).

Other studies that evaluated the antimicrobial resistance of S. aureus, a PCS isolated from food and humans, detected 31 (58.5%) of samples resistant to penicillin; 133 (47.6%) resistant to oxacillin; 29 (22.7%) resistant to erythromycin and 8 (100%) samples sensitive to gentamicin.

Penicillin and oxacillin belong to the antimicrobial β-lactam class and antimicrobial resistance occurs through the interference in the synthesis and remodeling of bacterial peptidoglycans. However, erythromycin belongs to the macrolide class and its antimicrobial resistance occurs by the inhibition of protein synthesis of the susceptible bacterial cells in the 50S ribosomal subunit. β-lactam resistance in positive Gram bacteria mainly occurs on the alteration of the target protein, with enzyme degradation.

Seven (23.3%) among the 30 Staphylococcus spp. isolates evaluated for antimicrobial resistance were multi-resistant, of which one isolate was resistant to six antimicrobial agents (clindamycin, vancomycin, erythromycin, rifampicin, oxacillin and penicillin). Similar studies on antimicrobial resistance of Staphylococcus isolates also reported multi-resistance to the same anti-microbial agents.

Several mechanisms may trigger bacterial multi-resistance: either by intercellular decrease of the antimicrobial agent by an alteration in the permeability of the external membrane and decrease transport through the internal membrane, or by active efflux through enzyme mutation or modification and the deviation of the drug to the target. Multi-resistant isolates may increase the number of highly persistent infections, limit the use of available antimicrobial agents and, consequently, make difficult clinical treatment, increase the dissemination of infections, hospital costs and mortality rates.

**Conclusion**

The microbiological quality of lettuce salads is very poor. Although Salmonella spp. was not detected, more than half the samples contained thermotolerant coliforms above the limit permitted by legislation. Important pathogens in food, such as E. coli and Staphylococcus spp. were reported, coupled to a high percentage of antimicrobial resistance of isolated microorganisms.

**Acknowledgement**

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<table>
<thead>
<tr>
<th>Antimicrobial agents</th>
<th>Sensitive (%)</th>
<th>Intermediate (%)</th>
<th>Resistant (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vancomycin</td>
<td>96.7</td>
<td>0</td>
<td>3.3</td>
</tr>
<tr>
<td>Erythromycin</td>
<td>70</td>
<td>3.3</td>
<td>26.7</td>
</tr>
<tr>
<td>Chloramphenicol</td>
<td>93.3</td>
<td>0</td>
<td>6.7</td>
</tr>
<tr>
<td>Rifampicin</td>
<td>96.7</td>
<td>0</td>
<td>3.3</td>
</tr>
<tr>
<td>Cefepime</td>
<td>93.3</td>
<td>0</td>
<td>6.7</td>
</tr>
<tr>
<td>Oxacillin</td>
<td>53.3</td>
<td>0</td>
<td>46.7</td>
</tr>
<tr>
<td>Penicillin</td>
<td>43.3</td>
<td>0</td>
<td>56.7</td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td>90</td>
<td>0</td>
<td>10</td>
</tr>
<tr>
<td>Gentamicin</td>
<td>100</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Tetracycline</td>
<td>96.7</td>
<td>0</td>
<td>3.3</td>
</tr>
<tr>
<td>Sulfamethoxazole/trimethoprim</td>
<td>96.7</td>
<td>0</td>
<td>3.3</td>
</tr>
</tbody>
</table>

Microbiological assessment of lettuce salads and antimicrobial resistance of Staphylococcus spp.
References


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