Study of Biological Hazards Present on the Surfaces of Selected Fruits and Vegetables

Lúcia Noronha1*, Ana Castro1, Vânia Ferreira1, Rui Magalhães1, Gonçalo Almeida1, Cristina Mena1, Joana Silva1, and Paula Teixeira1*

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Abstract

This study evaluated the microbial load on the surface of fruits with rough and very pronounced textured peels, namely pineapples and melons (cantaloupe), and investigated the presence of foodborne pathogens in these products since they are usually eaten raw. Similarly, lettuce is one of the most common salad vegetables consumed raw in Portugal, it therefore being important to study the microbial status of lettuce leaves. Enumerations of aerobic mesophilic counts, Enterobacteriaceae, Escherichia coli and coagulase-positive staphylococci, as well as detection of Listeria monocytogenes and Salmonella spp., were performed for all samples. Only in melon samples were E. coli and coagulase-positive staphylococci not detected. Contamination with L. monocytogenes varied from 2.5% and 15% in pineapple/melon and lettuce samples, respectively. Salmonella spp. were not detected. All coagulase-positive staphylococci had similar characteristics and none exhibited multidrug resistance. L. monocytogenes isolates belonged to serogroups II (1/2c or 3c), or to serogroup IV (4b, 4d and 4e). None were found to be resistant to antibiotics commonly used in therapy of listeriosis.

Keywords: Lettuce; Pineapple; Cantaloupe; L. monocytogenes; Coagulase-positive staphylococci; Salmonella

1. Introduction

Fruits and vegetables are part of a human diet worldwide, but when consumed raw they become potential vehicles of food-borne disease since no antimicrobial treatments, e.g. thermal, are applied (De Roever, 1998; Viswanathan and Kaur, 2001). Many of the contaminant microbiota are part of the production environment, including soil and water. These two sources of contamination have a very rich and diverse microflora, including several species of fungi, viruses and ubiquitous bacteria, such as Listeria monocytogenes. Wild or domestic animals and farmers may also be important

*Corresponding e-mail: ppteixeira@porto.ucp.pt
1 CBQF – Centro de Biotecnologia e Química Fina – Laboratório Associado, Escola Superior de Biotecnologia, Universidade Católica Portuguesa/Porto, Rua Arquiteto Lobão Vital, 4202-401 Porto, Portugal
vehicles of contamination. The use of animal manure as fertilizer and untreated water for irrigation or washing, are also major factors which might are likely to increase the microbial load on fruit and vegetables. Contamination with pathogens may also occur during harvesting, transportation, processing, storage, marketing, or even in a consumer’s home (De Roever, 1998; Beuchat and WHO, 1998). Lettuce (Lactuca sativa L.) is one of the most commonly consumed raw salad vegetables in Portugal. This vegetable is grown in contact with soil and exposed to intensive handling from harvesting until consumption, which can increase the probability of presence of pathogens in the leaves and, being eaten raw, may thereby cause foodborne disease (Lin et al., 1996; Beuchat and WHO, 1998; Barry-Ryan et al., 2007). The presence of L. monocytogenes, Salmonella spp. and Staphylococcus aureus has been reported in ready-to-eat lettuce and mixed salads (Soriano et al., 2001; Sagoo et al., 2003; Abadias et al., 2008; Carrasco et al., 2008). Escherichia coli O157:H7 has also been associated with outbreaks of foodborne disease from consumption of this vegetable (Altekruse et al., 1997; Delaquis et al., 2007). Some authors have shown that these bacteria are able to survive for long periods in the soil and manure (Franz et al., 2005; Scott et al., 2006), which increases the probability of contamination of crops. Fruits have a physical barrier, the peel or skin, which protects the internal flesh from microbial contamination and proliferation. However, microorganisms may be present in the peel, and if this biological structure suffers any damage, either during harvesting, transportation or storage, entry is facilitated, allowing microbial multiplication in the internal part of the fruit or during the processing (De Roever, 1998). Some authors reported the growth of L. innocua in peel of melons (Behrsing et al., 2003). Many fruits possess rough peels and very pronounced textures that can contribute to a significant increase in microbial load transported, and therefore can result in contamination of the interior of the fruit during peeling. Melons (Cucumis melo L.) and pineapples (Ananas comosus L. Merrill) become special cases for this study because they have rough peels with very pronounced textures, grow near the soil, are exposed to intensive handling from harvesting until consumption, are usually consumed raw and are not usually washed before peeling. In 2011, an outbreak of L. monocytogenes occurred in cantaloupe melons from a Colorado farm and caused at least 72 illnesses and up to 16 deaths [http://foodpoisoning.pritzkerlaw.com/archives/cat-cantaloupe-listeria-lawyer.html]. The aim of this study was to evaluate the microbial load on the surface of fruits with different texture peels, namely pineapples and melons, as well as leaves of Portuguese lettuce.

2. Materials & Methods

2.1. Sampling

Between June 2009 and June 2010, 120 samples (40 lettuces, 40 pineapples and 40 melons) were collected in several distribution sites in northern Portugal. Some lettuces were taken directly from the growing crop in the field. The fruits were peeled without a pre-wash and the peels analyzed after storage at 4 °C overnight. Leaves of lettuces were analyzed directly without any treatment.

2.2. Microbiological analyses

Twenty-five grams of each sample (leaves of lettuce or peel of the selected fruit) were added aseptically to 225 mL of sterile buffered peptone water (Biokar Diagnostics, France) and 225 mL to Half Frazer Broth (Biokar Diagnostics) and then homogenized in a stomacher (Interscience, Saint Nom la Bretèche, France) for 1 min. Appropriate decimal dilutions were prepared in Ringer's
solution (Biokar Diagnostics) for microbial enumeration: mesophilic aerobic counts on Plate Count Agar (Pronadisa, Spain) incubated at 30 °C for 72 h, according to the International Standard ISO 4833:2003 (ISO, 2003); *Enterobacteriaceae* on Violet Red Bile Glucose Agar (Merck, Germany) incubated at 37 °C for 24 h, according to ISO 21528-2:2004 (ISO, 2004); *E. coli* on Tryptone Bile X-glucuronide Agar (BioRad, USA) incubated at 44 °C for 24 h, according to ISO 16649-2:2001 (ISO, 2001); coagulase-positive staphylococci on Baird-Parker Agar (Pronadisa) with egg yolk and potassium tellurite (Sigma Aldrich, Germany) incubated at 37 °C for 48 h. Characteristic colonies were tested for the presence of the enzyme coagulase (Biokar Diagnostics), based on ISO 6888-1:1999 (ISO, 1999). The coagulase positive isolates were transferred to Mannitol Salt Agar (Merk) and then tested for Gram staining, oxidase and catalase reactions. The detection of *Salmonella* spp. was performed for all samples according to ISO 6579:2002 (ISO, 2002). Detection of *L. monocytogenes* was performed using the VIDAS method according to AFNOR Bio 12/9-07/02 (2002) and positive results were confirmed according to ISO 11290-1:2004 (ISO, 2004). All isolates were preserved at -20 ºC in Tryptic Soy 101 Broth (Pronadisa) supplemented with 30 % of glycerol (v/v) (Panreac).

2.3. Characterization of coagulase-positive staphylococci isolates

2.3.1. DNA extraction and Multiplex PCR

DNA extraction was done according to the method described by Aires de Sousa et al. (1996), based on the lysis of the cell wall by the action of lysostaphin (200 μg/mL, Sigma Aldrich), bursting cells by Guanidine Isothiocyanate (4 M, Sigma Aldrich) and DNA purification with a suspension of celite (0.2 g/mL, Fluka, USA). A Multiplex PCR was performed to evaluate the presence of genes 16S rRNA (*Staphylococcus* genus specific), *nuc* (*S. aureus* species specific), and *mecA* (determinant of methicillin resistance). The method was done according to Zhang et al. (2004) with minor modifications: an aliquot of 5 μL of DNA extraction product was added to a 25 μL PCR mixture containing 50 mM KCl (Fermentas Life Science, Germany), 2.5 mM MgCl2 (Fermentas Life Science), 0.2 mM of each deoxynucleoside triphosphate (dATP, dUTP, dGTP, and dCTP, Bioron, Germany), 0.2 μM each primer (Stab Vida, Portugal) and 1.0 U/μL of Taq DNA polymerase (Fermentas). As controls the strains *S. aureus* ATCC 29213 (16S rRNA and *nuc*), *S. aureus* DSM 11729 (16S rRNA, *nuc*, *mecA*) and *S. epidermidis* DSM 20044 (16S rRNA) were used.

2.3.2. Antimicrobial susceptibility

The minimal inhibitory concentrations (MICs) of eleven different antimicrobial agents: ampicillin, penicillin G, oxacillin, vancomycin, chloramphenicol, ciprofloxacin, erythromycin, gentamicin, nitrofurantoin, rifampicin and tetracycline (Sigma Aldrich) were determined by the agar dilution method as recommended by the Clinical Laboratory Standards Institute (CLSI, 2003), with concentrations in the range of 0.125 to 512 μg/mL. *Staphylococcus aureus* ATCC 29213 and *Enterococcus faecalis* ATCC 29212 were used as control strains. For each antibiotic susceptibility determination, at least duplicate experiments were performed. The breakpoints used were those defined by the CLSI for *S. aureus*. 
2.4. Characterization of L. monocytogenes isolates

2.4.1. Geno-serotyping

DNA extraction and Multiplex PCR were performed according to Doumith et al. (Doumith et al., 2004) to identify the serogroups of the isolates present in the samples.

2.4.2. Molecular characterization by PFGE

The DNA macrorestriction was performed with the restriction enzymes AscI (New England BioLabs, USA) and ApaI (MBI Fermentas, Canada), and electrophoretic separation of the major bands, following the PFGE protocol described by Graves and Swaminathan (2001).

2.4.3. Antimicrobial susceptibility

The MICs of eleven different antimicrobial agents were determined as described in section 2.3.2 but using Muller-Hinton agar (bioMérieux, France) supplemented with 3 % of lysed horse blood (Oxoid, UK). Concentrations ranged from 0.062 to 512 μg/mL. The MICs of trimethoprim-sulfamethoxazole were evaluated by Etest® (AB Biodisk, bioMérieux, Sweden), in duplicate, according to the manufacturers instructions. E. coli ATCC 25922 was used as control. Apart from penicillin and ampicillin, for which specific breakpoints for Listeria susceptibility testing are defined by the CLSI, in the present study, breakpoints used for the agar dilution method were those recommended by the CLSI criteria for staphylococci.

3. Results and Discussion

3.1. Microbiological evaluation of lettuces

In lettuces (n=40) the number of aerobic mesophilic organisms was around 7.4 ± 0.5 log CFU/g (< 8 log CFU/g for pre-washed and sliced vegetables). These findings were in agreement with Oliveira et al. (2010) and Abadias et al., (2008); they reported in unwashed lettuce values of 5.7 ± 0.8 log CFU/g and in pre-washed and cut ttuce values of 6.3 log CFU/g, respectively. Concerning Enterobacteriaceae, 4.8 ± 1.8 log CFU/g, were detected; values of 4.4 log CFU/g (in pre-washed lettuce) and 3.8 ± 1.5 log CFU/g (in unwashed lettuce) have been previously reported (Anonymous, 2006; Carrasco et al., 2008). Escherichia coli ranged from 2 to 3 log CFU/g. These values are within the levels permitted in Europe for washed and cut vegetables (Regulation (EC) No 2073/2005) and are similar to the results obtained by other authors (Oliveira et al., 2010). At least 3 samples were positive for coagulase-positive staphylococci (7.5 %; ± 3 log CFU/g), not higher than the maximum values recommended for S. aureus in washed and cut vegetables (Anonymous, 2008). Listeria monocytogenes was found in 15 % of the samples. Cordano and Jacquet (2009) reported that in Chile, 10.2% of ready-to-eat lettuces, prepared daily at supermarkets, contained L. monocytogenes. In Spain lower values have been reported (Carrasco et al., 2008).

3.2. Microbiological evaluation of Pineapples

The pineapple is the most important tropical fruit in terms of global production. The fruits do not
grow in contact with soil; the plant is about 1 m tall and pineapple grows on the top. Pineapple is usually consumed raw and does not have any treatment after harvest (Bartholomew et al., 2003). Forty samples were analysed during this study. Concerning the aerobic mesophilic counts, the values ranged around 6.3 ± 0.6 log CFU/g and Enterobacteriaceae, 3.0 ± 1.1 log CFU/g. Of the samples, 5% were positive for E. coli. The values for E. coli were around the 3 log CFU/g, acceptable values according to the European legislation (Regulation (EC) No 2073/2005), and 2.5% of the samples were positive for coagulase-positive staphylococci and L. monocytogenes. Salmonella spp. was not found in pineapple peels.

3.3. Microbiological evaluation of melon

Forty melons were analysed. Aerobic mesophilic counts were around 6.2 ± 0.8 log CFU/g. Enterobacteriaceae ranged around 4.5 ± 0.9 log CFU/g. Listeria monocytogenes was found in 2.5% of the samples and no E. coli, Salmonella spp. nor coagulase-positive staphylococci were detected. Listeria monocytogenes has already been reported in melon and it can grow in moderately acid food like melon (pH 6.7) (Beuchat et al., 1998; Bartholomew, 2003).

3.4. Characterization of coagulase-positive staphylococci isolates

Most of the gastroenteritis caused by Staphylococcus spp. are attributed to coagulase-positive, enterotoxigenic strains, like S. aureus (Bartholomew, 2003). From the 120 samples analyzed, seven coagulase-positive staphylococci isolates were recovered. The isolates were confirmed to belong to the Staphylococcus genus (16S RNA gene present in all), but none of the isolates belonged to S. aureus species, and did not possess the mecA gene.

The isolates were tested against those antibiotics commonly used to treat infections caused by Staphylococcus spp. Two isolates were resistant to ampicillin (28.6 %) and three to penicillin G (42.9 %). None of the seven isolates showed resistance to oxacillin. This antibiotic is active against some β-lactamase producing staphylococci and is used as the replacement of methicillin in the laboratory. Vancomycin has been used as a treatment of choice for infections caused by multi-resistant strains, but there are already some resistant strains reported (Richardson, 1992). Three isolates (42.9 %) were classified as intermediate susceptible, but none showed resistance. Ciprofloxacin has limited action against Gram positive bacteria, but it has been reported as effective against some multi-resistant strains (Palavecino, 2007). Only two isolates (28.6 %) presented intermediate susceptibility, but none were resistant. The seven isolates were considered as sensitive to the rest of the antibiotics tested.

3.5. Characterization of L. monocytogenes isolates

Of the 120 samples, eight were positive for L. monocytogenes and 21 isolates were recovered for further characterization. Strains of serogroup II (includes serotypes 1/2c and 3c) and serogroup group IV (serotypes 4b, 4d and 4e) were detected. These serogroups are often found in food products (Sousa, 2005). PFGE was performed for one isolate from each sample. Fig. 1 presents the dendrogram generated by the combination of Ascl and Apal restriction patterns of eight isolates of L. monocytogenes. PFGE revealed a total of 2 Ascl and 3Apal macrorestriction types among the isolates, distinguished by one or more band differences. Combined analyses of Ascl and Apal PFGE
data yielded a total of 3 PFGE pulsotypes. Similar PFGE profile (pulsotype 0203; Fig. 1) was obtained for *L. monocytogenes* strains collected from different food products. One of the isolates shared the same pulsotype as isolate 3 (0019; Fig. 1) that had been collected between 2004 and 2006 from six unrelated cases of clinical cases of human listeriosis.

![Fig. 1. Dendrogram generated by the combination of Ascl and Apal restriction patterns of eight isolates of *L. monocytogenes* from the eight samples positive for this pathogen. Isolate represented with number 7 is from one pineapple and the isolate with number 8 from one melon, the rest are from lettuces. From each sample were analyzed three isolates, except in case of the fruits: two isolates analyzed for the pineapple and one for the melon.](image)

### 3.5.1 Antimicrobial susceptibility

All the isolates demonstrated intermediate susceptibility to chloramphenicol, erythromycin and nitrofurantoin and were susceptible to all the other antibiotics investigated.

### 3. Conclusions

Not only spoilage microorganisms, but also pathogens were found on the surfaces of the fruits and lettuce. Despite the count levels of the various microorganisms analysed being acceptable, some pathogens were detected. Whilst these pathogens did not exhibit resistance to antibiotics, rigorous farming practices are still recommended, to ensure the protection of irrigation water quality and organic fertilizers used (mainly manure) during growing, during harvesting, postharvest handling, or during distribution. In addition, it is necessary for the consumer to wash such products, before consuming, in particular fruits with rough and very pronounced textured peels. They are not usually washed and during the peeling process contamination of the edible part can occur by pathogens potentially present in their peels. If consumption is not immediate, even being stored for a certain period of time at chill temperatures, can lead to microbial growth, increasing levels of psychrotrophic pathogens, (e.g. *L. monocytogenes*), and presenting a risk to the health of consumers.

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Conflict of Interest Statement

There are NO conflicts of interest in the subject matter or materials discussed in this manuscript.

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