Food Microbiology 51 (2015) 179–185

Contents lists available at ScienceDirect

Food Microbiology

journal homepage: www.elsevier.com/locate/fm

Evaluation of microbial quality and yeast diversity in fresh-cut apple

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ARTICLE INFO

Article history:
Received 14 February 2015
Received in revised form
7 June 2015
Accepted 10 June 2015
Available online 11 June 2015

Keywords:
Fresh-cut apple
Microbial quality
Coliforms
Staphylococci
Spoilage
Yeasts

ABSTRACT

The present work's aim was to study the microbial quality of minimally processed apples commercialized in Portugal. Sixty eight samples of fresh-cut apple were analyzed before their best-before date in 2011 and 2012 for aerobic mesophilic and psychrotrophic microorganisms, total coliforms, lactic-acid bacteria (LAB), coagulase-positive staphylococci and fungi. The parameters of food safety studied were *Cronobacter sakazakii*, *Salmonella* spp. and *Listeria* sp. Samples were analyzed according to standard methodologies and using Chromocult Agar for coliforms and *Escherichia coli*. The yeasts were identified by restriction analysis of the ITS-5.8S rDNA-region and 26S rDNA partial sequencing.

The mesophilic and psychrotrophic microorganisms ranged from 3.3 to 8.9 and from 4.9 to 8.4 log CFU/g, respectively. Coliforms were detected in all the samples and staphylococci in 5.8% of them. LAB numbers varied from 2.8 to 8.7 and fungi (yeast and molds) from 3.6 to 7.1 log CFU/g. The most common yeasts were *Candida sake* and *Pichia fermentans* followed by *Hanseniaspora* spp., *Candida* spp., *Meyerozyma guilliermondii*, *Metschnikowia pulcherrima*, *Cryptococcus* spp. and the psychrotrophic *Cystofilobasidium infirmominiatum*. Foodborne bacteria and opportunistic pathogenic yeasts were not detected in the apples studied. The results obtained respected the European Commission regulation regarding criteria of food hygiene and safety.

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1. Introduction

Fresh products, like fruits and vegetables, are important components of a balanced diet and a healthy life style and their consumption is being encouraged by several government health authorities all over the world. These trends stimulated the growing demand for "quick" and convenient fresh food products and the rapid growth of the fresh-cut fruits and vegetables' industry. Minimally processed foods offer many advantages, as they reduce the meal preparation time and increase access to food that retains high nutritional and sensory quality.

Minimally processed fruits and vegetables are fresh, raw fruits or vegetables processed in order to supply a ready-to-eat or ready-to-use food product. The processing of the foods may include cutting, peeling, slicing, shredding, trimming, washing and disinfecting. Products are packed and refrigerated becoming ready-to-eat items that are very attractive to consumers looking for healthy and convenient meals. This type of food is characterized by the presence of non-sterile cut surfaces with damaged tissues where active metabolism may occur (Berger et al., 2010; Francis et al., 2012; Olaimat and Holley, 2012). Fresh-cut fruits are susceptible to microbial contamination in any phase of the production or distribution, due to destruction of natural protective barriers and their high water and nutrient contents. Additionally, they are neither heat treated nor contain added preservatives. As a result, they may be a vehicle for microbial pathogens causing health problems. Fruits may contain various microorganisms that are naturally present in nature or acquired/gained during harvest, processing operations or even during the handling by workers or consumers. If the initial load of microorganisms is high and/or the preparing operations are inadequate, some microorganisms will survive and subsequently will grow and cause spoilage and possibly illness if

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http://dx.doi.org/10.1016/j.fm.2015.06.003
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the surviving microorganisms are virulent (Beuchat, 2002).

Although an extensive range of minimally processed vegetables have been offered to consumers for many years, the market for minimally processed fruits is economically less important, even though it has an interesting potential for growth, as fruits are relevant sources of vitamins, minerals, sugars and organic acids. In fact, fresh-cut fruit is being offered as an alternative to whole fruits in restaurants, supermarkets and in caterings (e.g. airline travel). Fresh-cut apples were the first kind of fresh-cut fruit to appear in the Portuguese market and presently are available in restaurants and supermarkets, thus contributing to a healthier dessert alternative to sweets, especially for children.

Teixidó et al. (1999) studied the microbial aspects of "Golden delicious" apples in the orchard and found that the main microbiota were fungi (*Cladosporium*, *Alternaria* and yeasts). After this work, Abadias et al. (2006) analyzed apples of the same variety throughout the production and shelf-life regarding their contamination with bacteria of the family *Enterobateriaceae* and did not find *Salmonella* or pathogenic *Escherichia coli*. However, low levels of other enterobacteria (*Pantoea*, *Citrobacter*, *Enterobacter*, *Klebsiella* and *Escherichia*) were identified. Numerous studies (e.g. Abadias et al., 2009; Alegre et al., 2010a,b) have shown that different pathogenic bacteria, like *E. coli*, *Salmonella* spp., and *Listeria monocytogenes* can survive and grow in fresh fruit tissues, such as apples and peaches, stored with no refrigeration or at temperatures of 10 °C or higher. Under refrigeration conditions (5 °C) *E. coli* was able to survive in apple wounds, flesh, peel and juice (Abadias et al., 2009).

Therefore, fresh-cut fruit can be a vehicle for the transmission of foodborne pathogens such as *E. coli*, *Salmonella* spp. and *L. monocytogenes* (Sivapalasingam et al., 2004). For example, non-pasteurized apple cider (Beuchat, 2002) and strawberries (FDA, 2011) were responsible for outbreaks of a foodborne illness due to *E. coli* O157:H7; cantaloupe (CDC 2011) was implicated in outbreaks due to *L. monocytogenes*; fruit salad, cantaloupe, papaya (CDC, 2011) and watermelon (Beuchat, 2002) were responsible for salmonellosis outbreaks.

Fungi, especially yeasts, are another major concern in fresh-cut fruits as they can be responsible for spoilage by changing color, producing gas, off-flavors and souring (Loureiro and Querol, 1999; Tournas et al., 2006). The surface of whole fruits carries yeasts populations (Teixidó et al., 1999) that can cross-contaminate the fresh-cut fruit during processing. Several genera of yeasts such as *Candida*, *Cryptococcus*, *Debaryomyces*, *Kloeckera*, *Kluyveromyces*, *Pichia*, *Rhodotorula*, *Saccharomyces* and *Zygosaccharomyces* have being referred to be present in fresh fruits (Ruiz-Cruz et al., 2010; Tournas et al., 2006).

Despite the microbiological problems (safety and spoilage) associated with fresh-cut produce, little data exists on evaluating the microbial quality of fresh-cut fruit available in the Portuguese commerce. To this end, the main aim of the present work was to study the microbiological quality of minimally-processed apples commercialized in the south of Portugal, including the detection of some bacterial foodborne pathogens (*Salmonella* spp., *Cronobacter sakazakii* and *L. monocytogenes*). Additionally, changes in the microbiota were studied in packages sampled prior to and after their best-before date. Finally, another objective of this work was to characterize the yeast population present in the fresh-cut apples studied due to the importance of these microorganisms as spoilers in low pH food.

2. Methods

2.1. Samples

During 2011 (21 samples) and 2012 (47 samples), a total of 68 samples of fresh-cut apples were purchased in restaurants and supermarkets in southern Portugal before their best before date. Samples were obtained throughout the year and consisted of individual packs of 70 g–100 g of apple slices that were transported to the laboratory in a cooler box and analyzed on the day of purchase. The 2011 samples were collected from mid-September until the end of November. The 2012 samples were bought in two periods; the first period, from May to the end of July (23 samples) and the second period, from mid-September till the beginning of December (24 samples). Two samples were studied per week. Data related to the samples were recorded, including date and place of purchase, lot number and expiry date.

2.2. Microbial analysis

Samples were analyzed, respecting the aseptic manipulation, according to standard (ISO) methodologies, summarized in Supplemental Table S1, and using Chromocult Agar (Merck, Darmstadt, Germany) for total coliform and *E. coli* (González et al., 2003) and Brilliance *Enterobacter sakazakii* chromogenic DFI (Druggan-Forsythe-Iversen, Oxoid, Basingstoke, England) for *C. sakazakii* (Chap et al., 2009).

Twenty five grams of each sample were diluted in 225 mL of buffered peptone water (BPW, Oxoid) and homogenized in a Stomacher (Model 400 Circulator, Seward, Norfolk, England) for 2 min at normal speed. From this suspension the enumeration of mesophilic (AM) and psychrotrophic microorganisms (PM), lactic acid bacteria (LAB), total coliform (TC) and *E. coli*, yeasts and molds (YM) and staphylococci (STAPH) was performed. The remaining suspension was incubated at 37 °C for 18 h for the detection of *Salmonella* spp. and *C. sakazakii*. The detection of total coliform and *E. coli* was performed by spread plating aliquots of the serial dilutions in Chromocult agar (Merck). To detect *C. sakazakii*, 10 mL of the pre-enrichment suspension on buffered peptone water was inoculated in EEbroth (Scharlau, Barcelona, Spain). After a 24 h incubation at 37 °C, an aliquot was inoculated on Brilliance *E. sakazakii* chromogenic DFI (Oxoid). According to the methodology, another portion of 25 g of the sample was homogenized in 225 mL Half-Fraser broth (Oxoid) and incubated at 30 °C for 24 h for the detection of *L. monocytogenes*.

In an extra set of 12 samples of fresh-cut apples, in addition to the 68 analyzed, six were analyzed 5–6 days prior to their best-before date for mesophilic and psychrotrophic microorganisms, total coliforms, LAB and fungi. The remaining samples were stored at 4 °C and analyzed 10–15 days after their expiration date, when visible spoilage/alterations were detectable for the same microbiological parameters.

2.3. Foodborne pathogens confirmation

Salmonella spp., *L. monocytogenes* and *C. sakazakii* presumptive isolates, obtained after the selective plating, were streaked onto tryptic soy agar TSA (Scharlau, Barcelona, Spain) until purification of the colonies. Single colonies were Gram stained and tested for cytochrome c oxidase activity and catalase activity. The confirmation of the pathogenic bacteria was performed using PCR with specific genus and species primers. Pure colonies of presumptive *Salmonella* spp. and *C. sakazakii* were sub-cultured in Brain Heart Infusion (Scharlau) and *L. monocytogenes* in TSB (Biokar Diagnostics, Beauvais, France) with yeast extract (0.6%) for further DNA extraction. Gram positive bacteria DNA extraction was performed using the guanidine thiocyanate method (adapted from Pitcher et al., 1989) and Gram negative bacteria DNA extraction was performed with the boiling method (Sambrook et al., 1989). All the PCR reactions were performed in volumes of 70 μl containing

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