Food Control 73 (2017) 117–126

Contents lists available at ScienceDirect

Food Control

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

Impact of spiking techniques on the survival of *Staphylococcus aureus* in artificially contaminated condiments

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ARTICLE INFO

Article history:
Received 31 March 2016
Received in revised form
3 October 2016
Accepted 5 October 2016
Available online 6 October 2016

Keywords:
Food safety
Microorganism
Tenacity
Inoculation technique
Herb
Spice

ABSTRACT

Tenacity studies are of relevance for microbial food safety risk assessment. Investigations on the survival of microorganisms in food matrices often require an artificial contamination step prior to microbial testing. Artificial contamination of low-moisture foods, like dried culinary herbs and spices, is challenging. In order to investigate the effect of several spiking techniques on the survival of *Staphylococcus* (*S.*) *aureus* in condiments, dried and ground paprika, pepper, and oregano were spiked with *S. aureus* using four different techniques: (1) aqueous bacteria suspensions were air-dried directly on the condiments; (2) aqueous bacteria suspensions were air-dried on sand, and then added to the condiments; (3) aqueous bacteria suspensions, or (4) bacteria suspended in liquid nutrient media with lyoprotectant were freeze-dried, crushed to powder, and added to the condiments. For each technique-condiment combination, and point in time, three distinct spiked samples (in three flasks) were prepared and stored in a dark environment at 24 ± 2 °C for up to 25 weeks. Cell counts directly after spiking and over storage time were analyzed. For the latter approach, mathematic models were used to fit the survival curve of each technique-condiment combination and to estimate their D-values. Results indicated that the reduction of the initial *S. aureus* cell counts depended on the applied spiking techniques and the used matrix. For instance, the initial cell counts in oregano were reduced by 0.8 ± 0.1 $\log_{10}$ cfu/g with technique 2 and by 6.1 ± 0.5 $\log_{10}$ cfu/g with technique 1. Technique 1 at the same time reduced the initial cell counts in paprika by only 0.4 ± 0.1 $\log_{10}$ cfu/g. The calculated D-values ranged from 1.3 ± 0.1 days (technique 3) to 120.1 ± 33.4 days (technique 4). The lowest as well as the highest D-value were observed for paprika samples, which emphasized the effect of the spiking technique. Thus, the behavior of bacteria can be strongly influenced by the selection of the spiking technique used for tenacity studies. Therefore, at least two spiking techniques should be considered, if the survival of microorganisms in low moisture food has to be investigated. In favor of high cell counts after drying, techniques 2 and 4 should be considered, as they performed best in our study.

1. Introduction

Natural products like dried culinary herbs and spices (condiments) have been used as flavorings and seasonings since antiquity. They are added to various foods, including ready-to-eat products; however, due to contaminations with pathogenic microorganisms, they have shown to be a potential source of foodborne illness outbreaks (Van Doren et al., 2013; Zweifel & Stephan, 2012).

Among the detected microorganisms in condiments, *Salmonella* spp., *Bacillus cereus*, and *Clostridium perfringens* are prominent pathogens, and *Staphylococcus* (*S.*) *aureus* has been detected as well (Banerjee & Sarkar, 2003; McKee, 1995; Sagoo et al., 2009; Sospedra, Soriano, & Mañes, 2010). Microbial loads can depend on the geographical source, pre- and post-harvest processes, decontamination treatments as well as age and type of condiments (Atungulu & Pan, 2012; Schweigert, Carle, & Schieber, 2007). The drying process – as one of the post-harvest processes – can reduce the available water (a_w) value of the condiments. In so-called low-moisture food with a_w values less than 0.85 (FAO/WHO, 2014), most microorganisms are not able to grow (Finn, Condell, McClure, Amézquita, & Fanning, 2013). Nevertheless, microbiological quality testing revealed that condiments can show contamination levels of up to 10^8 colony forming units (cfu)/g (Banerjee & Sarkar, 2003; McKee, 1995). Despite such high microbial loads, only limited data

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http://dx.doi.org/10.1016/j.foodcont.2016.10.021
0956-7135/

on the tenacity of microorganisms in dried condiments are available so far.

Staphylococcus spp. are gram-positive, non-spore formers and can produce heat-stable enterotoxins, which can cause food poisoning (Hennekinne, De Buyser, & Dragacci, 2012). Food poisoning caused by ingestion of staphylococcal enterotoxins is among the leading causes of foodborne outbreaks in the European Union (EFSA, 2015; Hennekinne et al., 2012). Compared to *Bacillus cereus* and *Salmonella*, *S. aureus* has been rarely detected in condiments (Banerjee & Sarkar, 2003; FAO/WHO, 2014; Kara, Gokmen, & Akkaya, 2015; Sospedra et al., 2010). Nevertheless, *S. aureus* has the potential to survive for months in low-moisture foods due to its tolerance to low a_w values (Scott, 1958). In addition, some *S. aureus* strains require only a minimum a_w value of approximately 0.83–0.86 for growth and 0.86 for toxin production (Brown, 1976; Ewald & Notermans, 1988; Medved'ová, Valík, & Studeničová, 2009; Schelin et al., 2011), whereas the minimum a_w value for growth of most microorganisms is in the range of 0.88–0.91 (Farkas, Doyle, & Beuchat, 2007). Contaminated condiments might act as a vehicle to transfer *S. aureus* to foods with optimal conditions for growth and toxin production. Therefore, understanding the survival properties of *S. aureus* in dried condiments is important for microbial food safety risk assessment; but hitherto, little research has been done.

A possible method to investigate survival properties is the conduction of tenacity studies, which often require an artificial contamination (spiking) step. Spiking of low-moisture foods is challenging. Firstly, the spiking technique should somehow reflect natural contamination routes; yet, there are many potential ways in which herbs and spices might become contaminated along the whole production chain, for example, during cultivation as well as at harvest, drying, processing, and storage. Thus, one spiking technique cannot mimic all contamination ways. Secondly, the process of drying bacteria for spiking purposes requires careful methodic considerations to prevent high initial losses of cells and further effects that might influence the survival capacity of the microorganisms after drying. Consequently, multiple tenacity studies with several spiking techniques will be needed to mimic the various contamination pathways.

In general, there are two approaches to spike low-moisture foods, either using wet or dry inocula. In this study, the wet-spiking technique implies that the liquid inoculum is directly added to the test matrix, followed by air drying. This technique is commonly used in laboratory studies (Beuchat & Mann, 2010; Blessington, Mitcham, & Harris, 2012; Danyluk, Uesugi, & Harris, 2005) and might mimic contamination by contaminated water. For dry spiking, the test matrix is directly spiked with a dry inoculum, for example, by vacuum-dried or freeze-dried bacteria (Farakos, Frank, & Schaffner, 2013; Flowers, Mozola, Curiale, Gabis, & Silliker, 1987; Riemann, 1968), or indirectly using an inert carrier like sand (Blessington, Theofel, & Harris, 2013; Bowman, Waterman, Williams, & Ponder, 2015; Shrestha & Nummer, 2016) or chalk (Hoffmans & Fung, 1993). Like the wet spiking technique, the dry spiking technique includes a drying process of the bacteria; however, the reduction of the a_w value takes place before spiking the test matrices. Freeze-dried bacteria are produced by vacuum drying of a liquid suspension at low temperatures (Berner & Viernstein, 2006). Some condiments are also processed by freeze drying. As microorganisms are able to survive the freeze-drying process, using freeze-dried bacteria for tenacity studies might mimic such a contamination scenario. The dry spiking technique using a carrier substance implies that a liquid inoculum is added on a solid carrier, followed by air drying and spiking of the test matrices. This technique might mimic contamination of condiments by dust or sand.

Our study compared four different spiking techniques in order to cover various potential contamination ways of dried herbs and spices. The aim of the present study was firstly, to evaluate the applicability of the spiking techniques for tenacity studies regarding reduction rate of the initial cell counts directly after spiking, and secondly, to investigate the effect of the four spiking techniques on the survival of *S. aureus* in dried herb and spice matrices during long-term storage.

2. Material and methods

2.1. Spice, herb, and sand matrices

Dried and ground culinary paprika (*Capsicum annuum*), pepper (*Piper nigrum*), and oregano (*Origanum vulgare*) were provided by FUCHS Gewürze GmbH (Dissen, Germany). The matrices were sealed in aroma-tight food packaging bags in portions of 500 g. The bags were stored at room temperature (24 ± 2 °C) and stayed sealed until usage. Water activity of the dried condiments and pH values of the condiment suspensions (1:20, w/v) were measured in triplicates, according to the manufacture's manual (Aqualab LITE; Decagon Devices Inc., Pullman, WA, USA; pH-Meter 765; Knick, Berlin, Germany). The level of total aerobic mesophilic bacteria was determined according to ISO 4833, and the limit of detection (LOD) was 20 cfu/g. Physicochemical and microbial characteristics of the condiments are summarized in Table 1.

Washed and sterilized (U15; Memmert, Schwabach, Germany) sea sand with granulation of 0.1–0.3 mm (Dinkelberg analytics, Gablingen, Germany) was used as control and carrier matrix.

2.2. Bacterial stock culture

In this study, we used an enterotoxin A producing *S. aureus* strain, BfR-ST345, from the National Reference Laboratory for coagulase-positive Staphylococci including *Staphylococcus aureus* (Federal Institute for Risk Assessment, Berlin, Germany). This strain was obtained from a foodborne illness incident, caused by contaminated potato salad. The strain was maintained frozen as a glycerol stock at −80 °C. In order to obtain single colonies, the frozen isolate was transferred to Mueller Hinton Agar (Oxoid, Basingstoke, UK) with 5% (v/v) sheep blood. After 24 h growth at 37 °C, agar plates were kept at 4 °C during the experiment set up.

2.3. Bacterial suspension

A single *S. aureus* colony was transferred into 5 ml 0.1% (w/v) peptone water and incubated at 37 °C statically for 20–24 h. From the overnight culture, 100 µl were added to 100 ml brain heart infusion (BHI) broth and grown at 37 °C while shaking at 200 rpm for 20 h. The BHI-overnight culture was harvested at 3200 × *g* and resuspended in a liquid carrier, either in distilled water (aqueous *S. aureus* suspension) or in a nutrient lyophilization medium, containing sucrose as the lyoprotectant compound.

To obtain a liquid inoculum of about 9 $\log_{10}$ cfu/ml, bacteria suspensions were adjusted to an OD_{598} of 0.2 (measured with ten-fold diluted suspensions; LP2W Digital Photometer Messsystem, Bruno Lange GmbH, Berlin, Germany). In addition, cell counting was conducted by plate counting, resulting in final concentrations of 9.1–9.4 $\log_{10}$ cfu/ml.

Lyophilization glass flasks (5 ml) containing 0.5 ml *S. aureus* suspension were prepared and bacteria suspensions were freeze dried for 24 h (Lyovac GT 4; Finn-Aqua, Tuusula, Finnland). All freeze-dried bacteria used in this study were produced 1 week before the experiment started on the same day and were stored at