International Journal of Food Microbiology xxx (xxxx) xxx–xxx

Contents lists available at ScienceDirect

International Journal of Food Microbiology

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

The preservation of *Listeria*-critical foods by a combination of endolysin and high hydrostatic pressure

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ARTICLE INFO

Keywords:
Bacteriophage lysin
Synergy
Cell wall hydrolase
Enzymes
Foodborne pathogen

ABSTRACT

The aim of this work was to examine the combination of endolysin PlyP825 and high hydrostatic pressure (HHP) processing against a cocktail of stationary phase *Listeria monocytogenes* cells in several *Listeria*-critical food products (i.e. milk, mozzarella and smoked salmon). In order to determine the efficacy of the combined application, both challenge-lethality tests and storage tests were performed. In milk and mozzarella, we could demonstrate that the application of PlyP825 prior to HHP processing allowed for a synergistic inactivation of cells, a reduction in the pressure level with equal antimicrobial efficacy and an enhanced eradication of *L. monocytogenes* during storage at abuse temperatures. For smoked salmon, no such effects were detected. Although the efficacy of the method was highly dependent on the food vehicle and parameters applied, we hereby demonstrated the potential of the combined endolysin-HHP application for complete eradication of *L. monocytogenes* from foods at milder processing conditions.

1. Introduction

Listeria monocytogenes is the causative agent of listeriosis. Although the number of people that get infected by *Listeria* is relatively small, this bacterium is one of the leading causes of death from foodborne illness in Europe (EFSA, 2015). The bacterium is ubiquitous present in the environment and is notable for its persistence in food-manufacturing environments (Chen, 2012). Outbreaks of listeriosis are frequently associated with the consumption of raw or minimally processed ready-to-eat foods (e.g. soft and semi-soft cheese or smoked fish products), which are consumed without further processing. The production processess of these food products can however not guarantee for the complete elimination of *L. monocytogenes*, which poses a major threat, especially because this bacterium is relatively salt-tolerant and able to grow at refrigerator temperature. This makes the organism particularly problematic to the food industry and prompts the development of preservation techniques that can eradicate *L. monocytogenes,* but at the same time does not deteriorate food quality and nutritional value.

High hydrostatic pressure (HHP) is an effective and promising alternative to conventional thermal processes to produce microbiologically stable, safe and high quality products. It has been shown in several studies that HHP can reduce *L. monocytogenes* in both buffer systems (Alpas et al., 2000) and food products (Huang et al., 2015; Montiel et al., 2015; Patterson et al., 2011; Valdramidis et al., 2015). However, due to tailing effects and sublethal damage of cells, pressure treatment alone is usually not sufficient to completely eradicate *Listeria* from food (Jofré et al., 2010; Stratakos et al., 2015; Tomasula et al., 2014). Since this is required from both a food safety and in many cases also legislative perspective (USDA FSIS, 2014), the elimination of foodborne pathogens by HHP processing has been extensively studied in combination with various other preservation methods (Koseki et al., 2008; Ogihara et al., 2009; Somolinos et al., 2008).

The increasing consumer demand for minimally processed, additive-free and high-quality food products has led to a higher interest in natural antimicrobials such as lysozyme or bacteriocins. These bactericidal compounds can be synergistically used with non-thermal processing methods to provide food safety and shelf-life prolongation (Corbo et al., 2009; Montiel et al., 2015). Among them, bacteriophages as well as their lysins have been described as biocontrol agents in foods (Bai et al., 2016; Guenther et al., 2009; Nelson et al., 2012; Schmelcher and Loessner, 2016; Tabla et al., 2012). Bacteriophage lysins, or endolysins, are produced by phages in the final stage of their lytic cycle and specifically hydrolyse peptidoglycan of the bacterial cell wall, which results in cell lysis and death. Although the combination of endolysin and HHP provides a promising approach for the synergistic inactivation of both Gram-positive (van Nassau et al., 2017) and -negative bacterial pathogens (Briers et al., 2008), this combination has only been investigated in buffers but never in actual foods.

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http://dx.doi.org/10.1016/j.ijfoodmicro.2017.10.004
Received 21 March 2017; Received in revised form 31 August 2017; Accepted 1 October 2017

For the validation of a process lethality step, both the reduction in microbial numbers as well as the product stability over time is of interest (Beuchat et al., 2003). Herein, the combination of endolysin PlyP825 and HHP processing for the inactivation of *L. monocytogenes* in several *Listeria*-critical food products (i.e. milk, mozzarella and smoked salmon) was investigated by both challenge lethality as well as challenge storage tests.

2. Materials and methods

2.1. Bacterial strains and culture conditions

A total of five *L. monocytogenes* strains from the TMW internal strain collection were used; TMW 2.594 (WSLC 11017, serovar 1/2b), 2.595 (WSLC 11021, serovar 1/2a), 2.597 (WSLC 11043, serovar 4b), 2.601 (WSLC 1361, serovar 1/2c) and 2.1512 (ATCC 15313, serovar 1/2a). The strains were maintained as stock culture in a 1:1 mixture of 80% glycerol and tryptic soy broth supplemented with yeast extract (TSBYE; 17 g/L casein peptone, 3 g/L soy peptone, 2.5 g/L glucose, 5 g/L NaCl, 2.5 g/L KH_2PO_4, 6 g/L yeast extract, pH ~7.3) and stored at −80 °C. All experiments were done using a cocktail of five strains harvested in their stationary growth phase. Therefore, each strain was cultured separately by inoculation of TSBYE from the stock culture and incubating aerobically at 37 °C. After 24 h, fresh TSBYE was inoculated with the overnight culture and harvested by centrifugation (7000 × *g*, 10 min, 25 °C) after another 24 h. The number of stationary phase cells per mL culture was determined for each strain individually, which allowed for the preparation of a five-strain cocktail with an equal amount of cells per strain.

2.2. Endolysin

Endolysins PlyP825 (34.2 kDa) as described in WO patent 2012/159774 (Grallert et al., 2012) was provided by *Hyglos GmbH* (Bernried am Starnberger See, Germany) in a stock concentration of 3.5 mg/mL and stored at −21 °C. For experimental assays, the endolysin stock solution was thawed on ice and diluted with either milk, mozzarella liquid or a lactic acid buffer with similar pH and NaCl characteristics as the smoked salmon product (10 mM lactic acid/sodium lactate, pH 6.0 – 6.3, 30 g/L salt; Table 1).

2.3. HHP

The pressure unit TMW-RB (Knam Schneidetechnik GmbH, Langenargen, Germany) consists out of two parallel linked 7 mL pressure vessels equipped with thermostating jackets and temperature-regulated by a recirculating thermostat (FC 600; JULABO Labortechnik GmbH, Germany). A mixture of 70% polyethylene glycol 400 (Roth, Karlsruhe, Germany) and 30% deionised water was used as pressure-transmitting fluid. The compression and decompression rates were kept constant at 200 MPa/min. The pressure holding time was kept constantly at 10 min throughout all experiments, whereas the pressure level varied from 200 to 500 MPa (described individually for each assay in the results section).

Table 1
Food products. Nutritional information from the food label plus measured water activity and pH.

Nutritional facts (per 100 g)	UHT-milk (g)	Mozzarella (g)		Smoked salmon (g)
Fat	3.5	19.0		12.0
– Of which saturated fatty acids	2.3	12.0		2.4
Carbohydrates	4.8	1.0		0.0
– Of which sugars	4.8	1.0		0.0
Protein	3.3	18.0		22.0
Salt	0.1	0.5		4.0
Intrinsic characteristics		Cheese	Liquid	
a_w	0.997	0.789	0.952	0.893
pH	6.67	6.04	6.17	6.08

2.4. Sample preparation and inoculation procedure

The inactivation of *L. monocytogenes* was investigated in three different food products (i.e. milk, mozzarella cheese and smoked salmon) bought in a local supermarket (Tengelmann, Freising, Germany). Specific product information as described on the food label as well as measured pH and water activity are depicted in Table 1. For all products, both challenge lethality and storage tests at mild abuse temperature of 10 °C were performed (Hudson and Mott, 1993; Szabo and Cahill, 1999; Soni and Nannapaneni, 2010). Since these products have different consistencies (liquid vs. solid in liquid, vs. solid), different inoculation and sample preparation procedures were used.

2.4.1. Milk

The harvested cells were taken up and diluted in milk to the desired concentration of cells per mL. The endolysin was diluted with milk to 10 × the desired end concentration before it was added 1:10 to the cell suspension (e.g. endolysin was diluted with milk to 34 µg/mL, after which it was added to the cell suspension reaching a final concentration of 3.4 µg/mL). The cell suspension with endolysin (or milk as control) was subsequently filled into 0.5 mL cryotubes with an internal thread (Thermo Fisher Scientific, Germany).

2.4.2. Mozzarella

Mozzarella cheeses were sliced into stripes of ~0.7 g, kept in mozzarella liquid at −21 °C and thawed overnight at 4 °C before use. The harvested cells were taken up in mozzarella liquid and diluted to the desired concentration. Thawed mozzarella stripes (i.e. without liquid) were inoculated with 10 µL of the cell suspension which was spread over the surface and allowed to dry under the laminar flow for 15 min. Mozzarella liquid (without or with endolysin) was added to the mozzarella stripes in 1.8 mL cryotubes with an internal thread (Thermo Fisher Scientific, Germany).

2.4.3. Smoked salmon

Smoked salmon samples were sliced into pieces of ~0.85 g, kept in 1.5 mL microcentrifuge tubes (Sarstedt AG & Co, Germany) at −21 °C and thawed overnight at 4 °C before use. The harvested cells were taken up in lactic acid buffer (see Section 2.2) and diluted to the desired concentration. Thawed slices were inoculated at one spot with 10 µL of the working culture and allowed to dry under the laminar flow for 15 min (Soni and Nannapaneni, 2010). Next, 50 µL lactic acid buffer (without or with endolysin) was applied to the exact same inoculation spot and samples were vacuum sealed.

2.5. Experimental set-up

After the addition of endolysin to the food product, samples were incubated at 25 °C (milk and mozzarella samples were additionally rotated overhead). After 3 h of coincubation with endolysin, samples were placed into the high pressure vessel preheated to 25 °C and the pressure ramp was started (reference samples without HHP treatment were simultaneously incubated at 25 °C). After HHP processing, samples were kept at room temperature until a total endolysin coincubation time of 4 h. Next, samples were either microbiologically analysed (challenge lethality tests and t0 of challenge storage tests) or further incubated at an abuse temperature of 10 °C (challenge storage tests t1 to t27).