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Protective role of *Lactobacillus fermentum* R6 against *Clostridium perfringens in vitro* and in chicken breast meat under temperature abuse conditions

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ABSTRACT

Six bacterial species were evaluated to determine their inhibitory effects on *Clostridium perfringens in vitro* (brain heart infusion broth) and *in situ* (chicken breast meat) under temperature abuse conditions (4 ± 1 °C for 12 h, followed by 7 h at 28 ± 1 °C and then 4 ± 1 °C for 53 h). During abusive storage, rapid growth of *C. perfringens* from vegetative cell and spore inocula was observed, exhibiting a 2.68–3.37 log CFU/mL (or g) increase in bacterial counts. In the presence of *Pediococcus pentosaceus* P1 or *Lactobacillus fermentum* R6, the counts of *C. perfringens* remained unchanged in the samples containing vegetative cells at the end of storage ($P < 0.05$); for those containing spores, the germination and outgrowth were also effectively inhibited, decreasing in bacterial counts of >1.9 log CFU/mL (or g) compared to those of the control ($P < 0.05$). The pH of chicken meat was slightly declined by 0.09 in the presence of *L. fermentum* ($P > 0.05$), and the inhibitory effect against *C. perfringens* was ascribed to non-acid antimicrobial substances. These results indicate a potential solution for bio-protecting chicken meat from *C. perfringens* growth.

Industrial relevance: Clostridium perfringens is a common pathogen that contaminates meat and meat products, but the organism cannot multiply under cold chain conditions at 4 °C. However, it was reported that temperature abuses commonly occurred during the transportation, storage or retail display of the food chill chain. During the abusive storage, *C. perfringens* could grow rapidly, which may lead to food poisoning. It is a serious problem for food safety.

In this study, *Lactobacillus fermentum* R6 was found to show effective inhibition on both the growth of *C. perfringens* vegetative cells and the germination and outgrowth of its spores in chicken meat ($P < 0.05$) under temperature abuse conditions, and also it had a minimal effect on the pH of the meat ($P > 0.05$). The results reveal a potential technology for bio-protecting chicken meat from *C. perfringens* growth.

1. Introduction

Clostridium perfringens is a gram-positive, spore-forming rod-shaped anaerobic bacterium that is widespread in the environment, such as in soil, sewage and the intestinal tracts of animals (Angela, Alan, Amanda, & Buys, 2008; Steele & Wright, 2001). It can be classified into five types (A–E) based on the production of four major toxins (α, β, ε, and ι toxins), among which *C. perfringens* type A is highly related to food poisoning due to the production of another important toxin, *C. perfringens* enterotoxin. *C. perfringens* is among the most frequently reported pathogens leading to food-borne illness (Bennett, Walsh, & L Hannah, 2013; Scallan et al., 2011). Outbreaks of *C. perfringens* illness are commonly associated with the consumption of contaminated meat and poultry products (Grass, Gould, & Mahon, 2013). The symptoms of food poisoning caused by *C. perfringens* include drastic abdominal pain, nausea, and diarrhoea following the ingestion of 10^8 or more vegetative cells, some of which survive under the acid conditions of the stomach, and then form spores producing enterotoxin in the intestinal tract (Garde, Gómez-Torres, Hernández, & Ávila, 2014).

Meat is an excellent substrate for microbial growth (Miki, Miyamoto, Kaneko-Hirano, Fujiuchi, & Akimoto, 2008). *C. perfringens* is one of the common pathogens contaminating meat, especially chicken meat (Grass et al., 2013; Mor-Mur & Yuste, 2010). Numerous studies have shown that *C. perfringens* contamination of chicken meat has a high natural occurrence of >60% (Guran & Oksuztepe, 2013; Nowell et al., 2010) or even 100% (Miki et al., 2008). Contamination may occur either as a result of the systemic spread of the bacteria in the carcasses of infected animals or as a result of soiling the meat with intestinal contents during the slaughtering process (Khan, Nazir, Anjum, Nawaz, & Shabbir, 2014), as *C. perfringens* can also be hosted in the digestive tract of healthy animals (Chalmers et al., 2008).

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C. perfringens cannot multiply under the cold chain conditions of meat processing (4 °C), since a minimal temperature of 10 °C is required for its growth (Vijayk, Harry, & Harshavardhanh, 2010). Thus, temperature is one of the critical control points for food safety. However, temperature abuse commonly occurs during the transportation, storage, or retail display of the food chill chain (Giannakourou, Koutsoumanis, Nychas, & Taoukis, 2001). *C. perfringens* has been reported to grow rapidly when products are kept at improper storage temperatures (Juneja, Call, Marmer, & Miller, 1994). Although a stable cold chain is required, careful application is not always expected and temperature abuse may lead to *C. perfringens* growth on chicken meat, increasing risk on food safety. Therefore, it is essential to study how to control the growth of *C. perfringens* under temperature abuse conditions.

Cultures with a long history of safe use, once added to meat products, which can inhibit pathogens and/or prolong the shelf life while only minimally changing the sensory properties are referred to as protective cultures (Maragkoudakis et al., 2009; Vermeiren, Devlieghere, & Debevere, 2004). Indeed, some cultures have already been added to meat and successfully inhibited various psychrotolerant pathogens during chill storage, including *Listeria monocytogenes*, *Staphylococcus aureus*, and *Escherichia coli* (Akbar & Anal, 2014; Bredholt, Nesbakken, & Holck, 1999; Chen & Hoover, 2003; Maragkoudakis et al., 2009). Since *C. perfringens* cannot grow during chill storage, there have been no reports regarding the inhibitory effect of protective cultures on the growth of *C. perfringens* or the germination and outgrowth of its spores *in vitro* (test tubes) and *in situ* (meat). However, *C. perfringens* and its spores are supposed to grow or germinate under temperature abuse conditions. Therefore, it is necessary to evaluate the inhibitory effects of beneficial microbes, particularly meat-borne bacteria, on the growth of *C. perfringens* under temperature abuse conditions.

The aim of this study was to investigate the effects of six strains belonging to meat-borne bacterial species on *C. perfringens* growth, germination, and outgrowth of its spores under temperature abuse conditions.

2. Material and methods

2.1. Bacterial strains and growth conditions

C. perfringens ATCC 13124 (type A) was obtained from the China Center of Industrial Culture Collection. The six bacterial strains tested in this study, *Lactobacillus plantarum* R2, *Lactobacillus pentosus* R3, *Lactobacillus fermentum* R6, *Lactobacillus sakei* R7, *Pediococcus pentosaceus* P1 and *Staphylococcus xylosus* S8, were all isolated from dry sausage, a traditional type of naturally fermented Chinese-style meat product. MRS (Guangdong Huankai Microbial Sci. & Tech. Co., Ltd., Guangzhou, China) and mannitol high salt (MS; Hopebio, Qingdao, China) broths were used to cultivate the lactic acid bacteria (LAB) and *S. xylosus* strains, respectively. *C. perfringens* ATCC 13124 was cultivated in brain heart infusion (BHI; Hopebio) broth at 37 °C for 24 h under anaerobic conditions.

2.2. Preparation of C. perfringens spores

The *C. perfringens* ATCC 13124 spores were prepared as described by Li et al. (2012), with minor modifications. After *C. perfringens* was grown in BHI broth at 37 °C for 18 h, a 0.3-mL aliquot of the culture was transferred into 8 mL of modified Duncan Strong (DS) medium, and then incubated for 24 h at 37 °C under anaerobic conditions. The DS medium was formulated by 15 g/L proteose peptone, 4 g/L yeast extract, 10 g/L disodium hydrogen phosphate, 4 g/L starch, and 1 g/L sodium thioglycollate, with a final pH of 7.5. The cells were harvested by centrifugation at 7012 ×*g* for 20 min at 4 °C and washed twice using 50 mL of sterile distilled water. The spores were stored at 4 °C until use.

2.3. Temperature abuse conditions

Temperature abuse was performed using a biochemical incubator system BSD-250 (Boxun Medical Biological Instrument Corp., Shanghai, China) with a fluctuation range of 4–28 °C as described by Rajkowski and Marmer (1995) to simulate extreme disruption of the cold chain. The process consisted of 12 h at 4 ± 1 °C, followed by 7 h at 28 ± 1 °C and then 4 ± 1 °C for the remaining time until the end of storage (72 h).

2.4. Inhibition of C. perfringens in vitro under temperature abuse conditions

Six samples of BHI broths were inoculated with *C. perfringens* vegetative cells or spores and one of the six tested strains at 10^4 and 10^7 colony-forming units (CFU)/mL, respectively. Control samples were inoculated with *C. perfringens* vegetative cells or spores at 10^4 CFU/mL. The culture media were then shaken by a lab dancer S25 (IKA Works, Staufen, Germany) to distribute the inocula. All samples were incubated anaerobically under the temperature abuse conditions for 72 h.

2.5. Inhibition of C. perfringens in chicken meat under temperature abuse conditions

Chicken breast meat was purchased from a local supermarket and shipped to the lab in an ice cooler. The surface of the raw meat was trimmed of connective tissue in a cold room (4 °C). Fourteen groups of inoculated meat were prepared, among which twelve treatments were each inoculated with *C. perfringens* vegetative cells or spores and one of the tested strains at 10^4 and 10^7 CFU/g, respectively. Control samples were mixed with *C. perfringens* vegetative cells or spores at 10^4 CFU/g, without the tested strains. All inoculated samples and a non-inoculated sample were vacuum-packed and stored under the temperature abuse conditions for 72 h.

2.6. Microbiological analysis

All samples were subjected to microbiological analysis after 0 (after inoculation treatments), 12, 19, and 72 h of storage. For test tubes, 1 mL of the culture fluid was mixed with 9 mL of sterile saline solution. For meat samples, 10-g meat were mixed with 90 mL of sterile saline solution and blended in a stomacher for 2 min. The resulting solutions were serially diluted by 10-fold and then plated onto tryptose–sulfite–cycloserine agar (TSC; Hopebio), MRS double layer agar or MS agar for enumeration of *C. perfringens*, LAB, and *Staphylococcus* spp., respectively. TSC agar plates were incubated anaerobically at 37 °C for 24 h, whereas MRS and MS agar plates were placed at 30 °C for 48 h. The results are expressed in CFU/mL for test tubes and CFU/g for meat samples.

2.7. pH measurements

The pH of 5 mL of culture liquid in test tubes was determined directly using a FE20 pH meter (Mettler Toledo, Columbus, OH, USA). For meat samples, 5-g meat were homogenized with 45-mL deionized distilled water using an Ultra Turrax T18 basic homogenizer (IKA Works), and then tested using the pH meter.

2.8. Preparation of cell-free supernatants

The five LAB strains were grown in MRS broths with an inocula of 10^7 CFU/mL. Cultures were centrifuged (10,000 ×*g*, 5 min) at 4 °C after 72 h of abusive incubation and the cell-free supernatants (CFS) were collected. The CFS were divided into two aliquots, one of which was adjusted to pH 5.6, which was the initial pH of the MRS broth. CFS without and with pH adjustment (CFS-pH) were then sterilized by filtration (0.22-μm pore size; Acrodisc syringe filter, Pall, Port Washington, NY, USA) and used to determine inhibitive activity.