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Inhibition of food-spoilage and foodborne pathogenic bacteria by a nisin Z-producing *Lactococcus lactis* subsp. *lactis* KT2W2L

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ABSTRACT

The growth of food-spoilage and foodborne pathogenic bacteria was inhibited by a nisin Z-producing *Lactococcus lactis* subsp. *lactis* KT2W2L as determined by the agar spot test and agar well diffusion assay. The growth of *Brochothrix thermosphacta* DSMZ 20171^T and *Staphylococcus aureus* CIP 76.25 was inhibited when co-cultivated with *L. lactis* subsp. *lactis* KT2W2L whereas the growth of *Escherichia coli* CIP 76.24 and *Salmonella* Enteritidis CIP 81.3 was not. However, the growth level of *E. coli* CIP 76.24 and *S.* Enteritidis CIP 81.3 co-cultivated with *L. lactis* subsp. *lactis* KT2W2L was diminished compared to cultivation without *L. lactis* subsp. *lactis* KT2W2L. The neutralized cell-free supernatant (NCFS) collected from the culture of *L. lactis* subsp. *lactis* KT2W2L, cultivated with and without indicator strain, exhibited inhibition zones against the indicator strains as determined by the agar well diffusion assay. The results reported in this study indicate that a nisin Z-producing *L. lactis* subsp. *lactis* KT2W2L may find application as a bio-preservative for reducing food-spoilage and foodborne pathogens in food products.

1. Introduction

Lactic acid bacteria (LAB) are generally recognized as safe (GRAS microorganisms) (Juodeikiene, Zadeike, Bartkiene, & Klupsaite, 2016; Varsha & Nampoothiri, 2016) and play an important role in food and feed fermentation and preservation, either as the natural microflora or as starter cultures added under controlled conditions. The preservative effect exerted by LAB is mainly due to the production of organic acids (such as lactic acid), which results in lowered pH. LAB also produce antimicrobial compounds including hydrogen peroxide (H_2O_2), CO_2, diacetyl, acetaldehyde, D-isomers of amino acids, reuterin and bacteriocins (Yang, Fan, Jiang, Doucette, & Fillmore, 2012).

L. lactis subsp. *lactis* KT2W2L was isolated from brackish water obtained from the mangrove forest in Kantang district, Trang province, southern Thailand. *L. lactis* is a nonpathogenic Gram-positive bacterium. It is one of the best-characterized LAB and considered by the European Food Safety Authority to be suitable for the Qualified Presumption of Safety (QPS) approach (Hwanhlem et al., 2013). *L. lactis* subsp. lactis KT2W2L produces nisin Z, as already reported by Hwanhlem et al. (2013, 2015).

Nisin is the most important and only commercially available antimicrobial peptide, which belongs to the lantibiotic class of bacteriocins (Class I) (Zacharof & Lovitt, 2012). It is employed as a safe and natural antibacterial food preservative in over 50 countries (Campion et al., 2013; Field et al., 2012; Piper, Hill, Cotter, & Ross, 2011). Delves-Broughton, Blackburn, Evans, and Hugenholtz (1996) highlighted that nisin can be used as a preservative in processed cheese, various pasteurized dairy products and canned vegetables. More recent applications of nisin are as a preservative in high moisture, hot baked flour products (crumpets) and pasteurized liquid eggs. The use of nisin to control spoilage LAB has also been reported in beer, wine, alcohol production and low pH foods such as salad dressings. The nisin propeptide contains 57

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amino acids, of which the first 23 amino terminal residues form the leader peptide (Reunanen, 2007). Nisin A is the originally isolated form of nisin. Its five natural variants have been described as differing by up to ten amino acids (of a total of 34 in nisin A). Nisins A, Z, F and Q comprise 34 amino acids produced by *L. lactis*. Each of their mature molecules contains one lanthionine, four methyllanthionines, two didehydroalanines and one didehydrobutyrine. Nisins U and U2 are produced by *Streptococcus* sp. They are composed of 31 amino acids and have only one didehydroalanine but two didehydrobutyrines (Campion et al., 2013; Field et al., 2012; Kaletta & Entian, 1989; Kwaadsteniet, Doeschate, & Dicks, 2008; Piper et al., 2011; Reunanen, 2007; Rouse et al., 2012). Nisin Z differs from nisin A by just one amino acid in the final active peptide. It contains an asparagine in position 27 instead of a histidine present in nisin A (De Vos, Mulders, Siezen, Hugenholtz, & Kuipers, 1993; Hwanhlem et al., 2013, 2015; Schneider, Werkmeister, & Pischetsrieder, 2011). It has been reported that *L. lactis* subsp. *lactis* produces nisin with an antimicrobial activity against closely related Gram-positive bacterial strains, food-spoilage and foodborne pathogens such as *Bacillus cereus, B. thermosphacta*, *Clostridium botulinum*, *S. aureus*, *Listeria innocua* and *Listeria monocytogenes* (Hwanhlem et al., 2013).

This study was performed to evaluate the inhibition potential of a nisin Z-producing *L. lactis* subsp. *lactis* KT2W2L against related LAB, food-spoilage organisms and foodborne pathogens for its use as a potential bio-control agent and starter culture in Thai fermented chicken sausage.

2. Materials and methods

2.1. Bacterial strains and growth conditions

L. lactis subsp. *lactis* KT2W2L, a nisin Z producer, was isolated from brackish water from a mangrove forest in southern Thailand (Hwanhlem et al., 2013; Hwanhlem, Chobert, & H-Kittikun, 2014) and was maintained frozen at −80 °C in a cryoprotective medium containing 30% glycerol. For all experiments, the strain was subcultured twice at 30 °C in M17 medium (Biokar Diagnostics, France) for 24 h.

All indicator strains were selected from the culture collections of the National College of Veterinary Medicine, Food Science and Engineering (Oniris) Nantes, France (Table 1). Strains were maintained as frozen stocks at −80 °C in a cryoprotective medium containing 30% glycerol. For all the experiments, strains were subcultured twice in each growth medium and growth condition specific to each strain.

2.2. Preparation of cell-free supernatant (CFS) and neutralized CFS (NCFS)

CFS was obtained by centrifugation at 10,000 rpm for 10 min at 4 °C to separate bacterial cells and supernatant. The neutralized CFS (NCFS) was prepared by adjusting the pH of CFS to pH 7.0 with 12 N NaOH to exclude the antimicrobial effects of organic acids. Inhibitory activity due to hydrogen peroxide (H_2O_2) was eliminated by the addition of 1 mg/mL of catalase. Samples were heated at 100 °C for 10 min to inhibit enzyme activity. The CFS and NCFS were stored at −20 °C until their use for screening the sensitivity of related LAB, food-spoilage and foodborne pathogenic bacteria by the agar well diffusion assay as described below.

2.3. Determination of sensitivity of related LAB, food-spoilage and foodborne pathogenic bacteria to L. lactis subsp. lactis KT2W2L

The sensitivity of related LAB, food-spoilage and foodborne pathogenic bacteria to *L. lactis* subsp. *lactis* KT2W2L was determined by the agar spot test and agar well diffusion assay.

2.3.1. Agar spot test

An agar spot test was used to determine the sensitivity of related LAB, food-spoilage and foodborne pathogenic bacteria (Table 1). One microliter of overnight culture (approximately 10^6 CFU/mL) of *L. lactis* subsp. *lactis* KT2W2L was spotted onto the surface of MRS agar plates (1.5% agar) and left to dry for 15 min at room temperature under sterile conditions. The plates were then overlaid with 10 mL of BHI or MRS soft agar (1% agar) seeded with 10^6 CFU/mL of related LAB, food-spoilage or foodborne pathogenic bacteria obtained from Oniris. Plates were then incubated for 24 h at the optimum temperature of each indicator strain and checked for inhibition zone diameters.

2.3.2. Agar well diffusion assay

BHI or MRS soft agar (1% agar, w/v) was seeded with 10^6 CFU/mL of related LAB, food-spoilage and foodborne pathogenic bacteria (Table 1), mixed and poured into sterile Petri dishes. After setting, agar wells of 5 mm in diameter were made. Twofold serial dilutions of CFS or NCFS produced by *L. lactis* subsp. *lactis* KT2W2L were made in 50 mM potassium phosphate buffer (50 mM K_2HPO_4, 50 mM KH_2PO_4, pH 7) and aliquots of 50 μL from each dilution were placed in wells in agar plates seeded with related LAB, food-spoilage or foodborne pathogenic bacteria. Plates were incubated overnight at the optimum temperature of each indicator strain; the inhibition zones were then observed and recorded.

2.4. Inhibitory effect in co-cultivation

The efficacy of *L. lactis* subsp. *lactis* KT2W2L to inhibit the growth of *B. thermosphacta* DSMZ 20171^T, *E. coli* CIP 76.24, *S. aureus* CIP 76.25 and *S.* Enteritidis CIP 81.3 was further studied in a co-cultivation in mixed BHI-M17 broth (initial pH 7). Double concentrations (2×conc.) of BHI and M17 were prepared according to the supplier's recommendations and then autoclaved. Mixed BHI-M17

Table 1
Growth media and growth conditions of related LAB, food-spoilage and foodborne pathogenic bacteria used as indicators.

Indicator strains	Growth medium	Growth conditions (°C/h)	Anaerobic condition
Brochothrix thermosphacta DSMZ 20171^T	BHI	25 °C/24 h	No
Carnobacterium maltaromaticum NCDO 2762	BHI	30 °C/24 h	Yes
Enterococcus faecalis CIP 103015	MRS	37 °C/24 h	No
Escherichia coli CIP 76.24	BHI	37 °C/24 h	No
Lactobacillus sakei subsp. *sakei*	MRS	37 °C/24 h	No
Listeria innocua CIP 80.11^T	BHI	30 °C/24 h	No
Listeria ivanovii DSMZ 20750^T	BHI	30 °C/24 h	No
Salmonella Enteritidis CIP 81.3	BHI	37 °C/24 h	No
Salmonella Typhimurium CIP 58.58	BHI	37 °C/24 h	No
Staphylococcus aureus CIP 76.25	BHI	37 °C/24 h	No