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The inhibition of cell-free supernatant of *Lactobacillus plantarum* on production of putrescine and cadaverine by four amine-positive bacteria *in vitro*

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ABSTRACT

Putrescine and cadaverine are common diamines in fermented food and represent a toxicological risk for consumer health. This work aimed to investigate the inhibitory effects of cell-free supernatant (CFS) and water bathed (100 °C, 10 min) cell-free supernatant (WCFS) from *Lactobacillus plantarum* on cell growth, diamine production, and the related decarboxylase activities of four amine-positive bacteria, namely, *Enterobacter aerogenes*, *Enterobacter cloacae*, *Enterococcus faecium*, and *Enterococcus faecalis*. Results showed that compared with Man Rogosa Sharpe (MRS) broth (control), both CFS and WCFS treatment significantly reduced the cell growth and diamine production of the four amine-positive bacteria, but only caused limited inhibition (less than 15%) on related decarboxylase activities from *E. aerogenes* and *E. cloacae*. Meanwhile, cell growth and related decarboxylase activities significantly declined when initial pH value decreased from 6.5 to 4.5. Furthermore, a significant positive relationship was found between diamine production and cell number of the four amine-positive bacteria. However, no significant relationship was observed between diamine production and related decarboxylase activities (except for *E. aerogenes* and *E. cloacae* in pH 6.5). These results indicate that the reduction in diamine accumulation by *L. plantarum* may mainly result from the inhibition of cell growth, but not from the inhibition of relevant decarboxylases.

1. Introduction

Biogenic amines (BAs) are organic bases with low molecular weight that widely exist in fermented food and beverages (Halasz, Barath, Simonsarkadi, & Holzapfel, 1994). BAs are mainly formed by amino acid decarboxylating properties of natural microbiota or the enzymes of raw material (Silla Santos, 1996). Excessive consumption of food containing high levels of BAs may affect the nervous and vascular systems and lead to various types of food-borne diseases, such as edemas, rashes, hypotension, headache, diarrhea, vomiting, and death in very severe cases (Papavergou, Savvaidis, & Ambrosiadis, 2012; Shalaby, 1996). Relatively high amounts of BAs have been reported in fermented meat products in many countries because of abundant free amino acids and the factors affecting decarboxylase activity and bacterial growth (Gonzalez-Tenorio et al., 2013; Latorre-Moratalla et al., 2008; Lu et al., 2010). In meat products, the principal BAs are putrescine (PUT) and cadaverine (CAD) (Bover-Cid, Hugas, Izquierdo-Pulido, & Vidal-Carou, 2001; De Filippis et al., 2013). These two amines are generally not considered toxic, but they can enhance the acute reaction of aromatic BAs, such as histamine and tyramine, because they can interact with amino oxidases and decrease their activity to detoxify BAs (Bover-Cid et al., 2009). When a certain amount of nitrites are present in the meat products, these amines may also form nitrosoamines (Ruiz-Capillas & Jimenez-Colmenero, 2004) which have been shown to induce tumors in liver, lung, esophagus, bladder and pancreas in various experimental animal species (Lijinsky, 1999).

The presence of BAs in fermented food reflects a highly complex equilibrium dependent upon the balance of amino oxidases and decarboxylases in food (Alvarez & Moreno-Arribas, 2014). The decarboxylases responsible for BA formation are widespread among Gram-negative and Gram-positive bacteria (Suzzi & Gardini, 2003). Although the capacity to produce BAs is a strain-specific characteristic within microbial groups, such capacity is more widely

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distributed among certain genera or species (Linares, Martin, Ladero, Alvarez, & Fernandez, 2011). High amounts of PUT and CAD have mainly been related to Gram-negative bacteria, especially in the family Enterobacteriaceae (Bover-Cid, Hernandez-Jover, Miguelez-Arrizado, & Vidal-Carou, 2003; Durlu-Ozkaya, Ayhan, & Vural, 2001). However, some Gram-positive bacteria, such as *Enterococcus* sp., have also been described to be capable of producing CAD and PUT in a substrate-rich medium (Pleva et al., 2012).

Inoculation of starter cultures is one of the most important methods that affects the accumulation of BAs during meat fermentation (Latorre-Moratalla, Bover-Cid, Veciana-Nogues, & Vidal-Carou, 2012). Zhang et al. (Zhang, Lin, & Nie, 2013) found that adding an amine-negative *Lactobacillus plantarum* as starter culture can reduce the accumulation of PUT and CAD because of the rapid acidification and inhibition of undesirable bacteria, such as Pseudomonas and Enterobacteriaceae. Mangia et al. (Mangia et al., 2013) and Xie et al. (Xie et al., 2015) also found that BAs formation in sausage is reduced by adding *L. plantarum.*

Most studies have focused on the isolation and utilization of *L. plantarum* as a starter culture to inhibit the accumulation of PUT and CAD. However, no information is available on the inhibiting effect of cell-free supernatant (CFS) from *L. plantarum* on PUT and CAD production by amine-positive bacteria *in vitro*, except that of Toy et al. (Toy, Ozogul, & Ozogul, 2015), who reported inhibitory effects of CFS from lactic acid bacteria (LAB) on tyramine and other BA production by different food borne-pathogens in tyrosine decarboxylase broth. In the present study, the effects of CFS from *L. plantarum* on the cell growth, related decarboxylase activities, and diamine production of four amine-positive bacteria in three pH levels (4.5, 5.5, and 6.5) were investigated. Furthermore, Pearson correlation was performed to study the mechanism by which CFS from *L. plantarum* inhibited PUT and CAD production by four amine-positive bacteria.

2. Materials and methods

2.1. Strains and growth conditions

The *Enterococcus faecium* and *Enterococcus faecalis* along with the *L. plantarum* used in this work were isolated from traditional Chinese sausage obtained from the Meat Processing and Quality Control Center of China. *Enterobacter aerogenes* (ATCC 13048) and *Enterobacter cloacae* (ATCC 13047) were purchased from the China Center of Industrial Culture Collection. *L. plantarum* was grown on Man Rogosa Sharpe (MRS) broth, and the other four bacteria were incubated in nutrient broth at 37 °C.

2.2. Preparation of CFS and water bathed CFS (WCFS) from L. plantarum

L. plantarum was cultured in 500 mL of MRS at 37 °C for 24 h. The culture was then centrifuged at 8000 g for 10 min (4 °C), and the supernatant was sterilized by passing through 0.22 μm filters. The supernatant was then divided into two equal portions. One part was used as CFS, and another part was maintained in a water bath at 100 °C for 10 min as WCFS. The pH values of the CFS and WCFS were 3.80. After the water bath, protein ingredients in CFS were denatured, so that compared of results of group CFS and group WCFS can determine whether the reduction of diamines was due to protein ingredients such as amino oxidases or enzymes.

2.3. Preparation of crude decarboxylases from four amine-positive bacteria

For enzyme collection, the bacteria were inoculated in nutrient broth and incubated at 37 °C for 24 h. Cells were harvested by centrifugation at 12,000 g for 10 min at 4 °C and suspended in potassium phosphate buffer (pH 5.8). The suspended cells were then cooled in an ice bath and subjected to ultrasonication for about 30 min so that cells were broken. The suspensions were then centrifuged at 12,000 g for 20 min at 4 °C, and supernatant solutions were used as the crude decarboxylases after being sterilized by passing through a 0.22 μm filter.

2.4. Effects of CFS and WCFS on cell growth and amine generation of Enterobacteria

Three different groups were established according to the added solution: CFS (S), WCFS (W), and MRS broth used as control (C). Each tube containing 3 mL of nutrient broth was added with 3 mL of CFS/WCFS/MRS broth, and every batch had three initial pH levels (4.5/5.5/6.5). The pH value of each tube was adjusted using sterile solutions of 1 N NaOH or 1 N HCl after the mix of nutrient broth and CFS/WCFS/MRS broth. After that, tubes were injected with 0.1 mL of suspension (10^7 CFU/mL) of amine-positive bacterium then incubated at 37 °C. After 24 h, 2 mL of suspensions from each tube was extracted after vibration, and 1 mL of suspension was diluted to appropriate dilutions and spirally plated on PCA plates using an automatic spiral plater (EasySpiral, Interscience, France). The plates were incubated at 37 °C for 48 h, and the colonies were then counted using an automatic colony counter (Scan500, Interscience, France). Another 1 mL of suspension was used to determine the concentrations of PUT and CAD. Four repetitions were done in every batch.

2.5. Effects of CFS and WCFS on related decarboxylase activities

Three different groups were established according to the added solution: CFS (S), WCFS (W), and MRS broth used as control (C). CFS/WCFS/MRS broth (5 mL) containing ornithine (1 mg/mL), lysine (1 mg/mL), arginine (1 mg/mL), and pyridoxal-5′-phosphate (0.25 μg/mL) was added into tubes, and the pH value of each group was adjusted using sterile solutions of 1 N NaOH or 1 N HCl to three initial pH levels (4.5/5.5/6.5). The reactions were started by adding 1 mL of crude decarboxylases and stopped by adding 0.1 mL of 0.4 N perchloric acid. Thereafter, 1 mL of the mixture was extracted to determine the PUT and CAD concentrations.

2.6. Determination of amine contents of samples

The BA contents in samples were measured by HPLC as previously described (Liu et al., 2014), with slight modifications. The suspension was centrifuged at 12,000 g for 5 min, and the supernatants were collected. BAs were derived as follows: 0.2 mL of 2 N NaOH, 0.3 mL of saturated $NaHCO_3$, and 2 mL of dansyl chloride solution (10 mg/mL in acetone) were added to 1 mL of supernatant solution and incubated at 40 °C for 45 min in the dark. Subsequently, 0.1 mL of ammonia water was added to the mixture to remove the unbound dansyl chloride. The volume of the solution was adjusted to 5 mL by adding acetonitrile, and the mixture was filtered through a 0.22 μm size nylon membrane filter. The BA contents were determined by HPLC techniques (Waters Alliance 2695). Separation was performed on a C18 column (Agilent ZORBAX SB-C18 4.6*250 mm^2, 5 μm) at 30 °C, and the peaks were detected at 254 nm. A gradient elution program was used with a mixture of ultrapure water as solvent A and with acetonitrile as solvent B. The gradient elution procedure was 35% A + 65% B at 0 min, 30% A + 70% B at 5 min, 0% A + 100% B at 20 min, 0% A + 100% B at 24 min, 35% A + 65% B at 25 min, and 35% A + 65% B at 30 min. A standard solution of mixed PUT and CAD was prepared in a