Food Chemistry 271 (2019) 174–181

Contents lists available at ScienceDirect

Food Chemistry

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

Effects of inoculating autochthonous starter cultures on *N*-nitrosodimethylamine and its precursors formation during fermentation of Chinese traditional fermented fish

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ARTICLE INFO

Chemical compounds studied in this article:
Trimethylamine-*N*-oxide (PubChem CID: 1145)
Trimethylamine (PubChem CID: 1146)
Dimethylamine (PubChem CID: 674)
Formaldehyde (PubChem CID: 712)
Sodium nitrite (PubChem CID: 23668193)
N-nitrosodimethylamine (PubChem CID: 6124)

Keywords:
Autochthonous strains
Fermented fish
Dimethylamine
Nitrite
N-nitrosodimethylamine

ABSTRACT

This study investigated the effects of *Lactobacillus plantarum* 120, *Saccharomyces cerevisiae* 2018 and *Staphylococcus xylosus* 135 inoculation on *N*-nitrosodimethylamine (NDMA) and its precursors formation, and on microbiological characteristics of Chinese traditional fermented fish products (CTFPs). The results indicated that three strains could directly degrade NDMA in culture broth, and the highest degradation rate was observed in *L. plantarum* 120. The lactic acid bacteria counts in samples inoculated with *L. plantarum* 120 and mixed starter cultures were significantly ($P < 0.05$) higher than the others during the initial and middle fermentation stages (≤ 3 weeks). The final contents of total volatile base nitrogen, trimethylamine, dimethylamine, nitrite and NDMA in inoculated samples were significantly ($P < 0.05$) lower than those in spontaneous fermentation samples. According to these results, the inoculation with autochthonous starter cultures was a promising method to inhibit the NDMA and its precursors accumulation in CTFPs during fermentation process.

1. Introduction

Volatile *N*-nitrosamines (VNAs), including *N*-nitrosodimethylamine (NDMA), *N*-nitrosodiethylamine (NDEA), *N*-nitrosodipropylamine (NDPA), *N*-nitrosodibutylamine (NDBA), *N*-nitrosopiperidine (NPIP), *N*-nitrosopyrrolidine (NPYR), *N*-nitrosomorpholine (NMOR) and *N*-nitrosodiphenylamine (NDPheA), are classified as a subgroup of the *N*-nitroso compounds, which were formed by the nitrosation of secondary amines (Honikel, 2008). Meanwhile, they are a class of potent carcinogens, and high-dose or long-term intake can both cause various kinds of cancers (De Mey et al., 2014). In previous researches, VNAs was universally detected in meat products, especially in fermented meat products (Seel et al., 1994; Sun, Kong, Chen, Han, & Diao, 2017), including NDMA, NDEA and NPYR in Chinese Rugao ham (Wei et al., 2009), NDMA and NPIP in heated cured pork meat (Drabik-Markiewicz et al., 2011), NDMA, NMOR and NPIP in fermented sausages collected in Belgium market (De Mey et al., 2014), and NDMA and NPYR in pepperoni and salami sausages (Byun et al., 2004). Among them, NDMA has been most commonly detected in food products, and nitrite and dimethylamine (DMA) are the immediate precursors (Choi, Chung, Lee, Shin, & Sung, 2007). Otherwise, the reaction of nitrite and DMA could be promoted significantly by the acidic conditions of the stomach (Kim, Kang et al., 2017).

Trimethylamine-*N*-oxide (TMAO) is widely found in aquatic products, plays an important role as an osmoprotectant (Gou, Lee, & Ahn, 2010). Extensive studies have demonstrated that there are two possible degradation pathways of TMAO. One is that the TMAO degrades into equimolar DMA and formaldehyde (FA) by enzymatic reaction catalysed by TMAO demethylase (TMAOase) (Gou et al., 2010; Lee & Park, 2016), and the other pathway is a microbial spoilage reaction, in which TMAO degrades to trimethylamine (TMA) (Fu et al., 2008; Gou et al., 2010). Therefore, the presence of TMAO, TMA, DMA, nitrate and nitrite might be associated with the generation of VNAs.

Currently, inoculation of starter cultures is considered as a potential solution for NDMA inhibition, and some studies had begun during the past period. Nowak, Kuberski, and Libudzisz (2014) investigated the effect of four *Lactobacillus* strains on NDMA in model system, and found that the NDMA could be degraded directly by these strains. A similar result was reported by Kim, Kang et al. (2017) who found that the NDMA inhibition could be attributed to the direct degradation by *L. sakei*, *L. curvatus* and *L. brevis* and the suppression of its precursors in kimchi inoculated with these strains. Meanwhile, Kim, Kim et al. (2017)

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https://doi.org/10.1016/j.foodchem.2018.07.186
Received 23 March 2018; Received in revised form 24 July 2018; Accepted 25 July 2018
Available online 26 July 2018

also found that *Le. carnosum*, *Le. mesenteroides*, *L. plantarum*, and *L. sakei* had an obvious inhibitory effect on NDMA in culture broth and LAB-fortified kimchi. Besides, although addition of plant polyphenols has been used as an other effective method to inhibit the NDMA accumulation in dry-cured bacon (Wang et al., 2015), it may be unsuitable for fermented products due to the broad-spectrum antibacterial activities of many plant polyphenols (Wang et al., 2015), which might have a negative impact on fermentation process.

Chinese traditional fermented fish products (CTFPs) are low-salt fermented whole fish snack with characteristic flavor, which are widely consumed in south of China and commonly manufactured by spontaneous fermentation (Gao, Wang, Jiang, Xu, & Xia, 2016). According to previous investigation results on commercial CTFPs in Chinese market (our unpublished data), the contents of NDMA in 50% samples exceeded the limit (≤ 4 μg/kg) established by the Department of health of China (Qiu et al., 2017). However, there are few reports focused on the NDMA inhibition in fermented fish products. Therefore, this study aimed to investigate the influence of three previously isolated dominant autochthonous strains, namely *Lactobacillus plantarum* 120, *Saccharomyces cerevisiae* 2018 and *Staphylococcus xylosus* 135, on NDMA degradation in culture broth and CTFPs during fermentation process, as well as microbiological characteristics and changes of NDMA precursors were also evaluated.

2. Materials and methods

2.1. Starter cultures

Strains of *L. plantarum* 120, *S. cerevisiae* 2018 and *S. xylosus* 135 were chosen as starter cultures because of their technological and antimicrobial characteristics, which were previously isolated and identified from Chinese traditional fermented fish (Zeng, Xia, Jiang, & Yang, 2013). *L. plantarum* 120 was subcultured twice in Man Rogosa and Sharpe (MRS) broth at 37 °C statically for 48 h. *S. cerevisiae* 2018 was subcultured twice in Yeast Extract Peptone Dextrose (YPD) broth at 37 °C statically for 24 h. *S. xylosus* 135 was subcultured twice in Manitol Salt Agar (MSA) broth at 37 °C statically for 72 h. After the incubation, all the cell pellets were harvested by a high-speed cryogenic centrifuge (Model 4 K15, Sigma Laborzentrifugen, Osterode, Germany) at 10,000 × *g* for 15 min at 4 °C. After washed twice with saline water (0.9% NaCl, w/v), the pellets were re-suspended with 10 mL of saline water. Finally, the number of bacterial cells in the suspension was adjusted to 10^7-10^8 cfu/mL by measured optical density at 600 nm with a spectrophotometer (UV 1000, Techcomp Scientific Instruments Co. Ltd., Shanghai, China).

2.2. Sample preparation of CTFPs

The CTFPs were prepared according to the method used by Gao et al. (2016) with some modification. Briefly, fresh live grass carps (weight: 3.0 ± 0.2 kg; length: 60.0 ± 3.0 cm) were purchased from a local market (Wuxi, Jiangsu, China). After scaled, decapitated and eviscerated, they were cut in 3–5 cm pieces, followed by washing with tap water. According to the traditional techniques, the grass carp pieces (95%, w/w) were mixed with salt (3%, w/w) and sucrose (2%, w/w). After curing at 4 °C for 2 days, they were dried for 3 h at 50 °C and 65% RH. Then, above dried pieces (75%, w/w) were mixed sufficiently with roasted corn meal (25%, w/w) by hand. All pieces and corn meal were randomly divided into five groups and prepared with different starter cultures that suspended in saline water (0.9% NaCl, w/v). Then, the grass carp pieces, roasted corn meal and starter cultures (1%, v/w) were fully mixed and placed in pickle jars with water sealing. The group without inoculation was identified as natural fermentation group (NS). Prepared starter cultures *L. plantarum* 120, *S. cerevisiae* 2018 and *S. xylosus* 135 were respectively used to inoculate samples, which were named as group LP 120, SC 2018 and SX 135. In another group, above three starter cultures were mixed with equal quantity (*L. plantarum* 120, *S. cerevisiae* 2018 and *S. xylosus* 135 [1:1:1]) as starter culture, and this group was named as MS. Each group of sample was randomly collected every week, packed with vacuum and stored at -50 °C (MDF-U53V, SANYO Electric Co., Ltd., Osaka, Japan) for further determination within two weeks.

2.3. NDMA degradation ability of autochthonous strains

The NDMA degradation ability of *L. plantarum* 120, *S. cerevisiae* 2018 and *S. xylosus* 135 were studied in a model system similar to that previously described by Kim, Kang et al. (2017). The broth consisted of MRS, YPD or MSA added with 2 μg/mL of NDMA. Separated strains were inoculated at a level of 2% (v/v) and incubated at 37 °C for 48 h. After centrifuged at 10,000 × *g* for 15 min at 4 °C, the supernatant of broth was used to NDMA determination. The control was culture broth containing 2 μg/mL of NDMA without inoculation.

2.4. TMAO degradation ability of autochthonous strains

The TMAO degradation ability of *L. plantarum* 120, *S. cerevisiae* 2018 and *S. xylosus* 135 were studied in culture broth with 2 mg/mL TMAO concentration. Separated strains were inoculated at a level of 2% (v/v) and incubated at 37 °C for 24 h. The control was culture broth containing 2 mg/mL TMAO without inoculation. The broth was centrifuged at 10,000 × *g* for 15 min at 4 °C, and the supernatant was used for TMAO, TMA and DMA determination.

2.5. Microbiological analysis

Samples (5 g) were aseptically mixed with 45 mL of normal saline (0.9% NaCl, w/v) in plastic pouches. The mixtures were blended in a bag mixer (BM-400P, Truelab Laboratory Equipment co. LTD., Shanghai, China) for 120 s. A series of 10-fold dilutions of homogenate were prepared for microbiological analysis. Each dilution (0.1 mL) was inoculated in corresponding growth media to determinate the microbial counts: LAB on MRS agar in anaerobic conditions at 37 °C statically for 48 h; Yeast on YPD agar in anaerobic conditions at 37 °C statically for 24 h; *Staphylococcus* on MSA plates at 37 °C statically for 72 h. The colonies number was expressed as colony-forming units per gram (log cfu/g).

2.6. Determination of pH and titratable acid

Each 2 g sample was homogenized (Ultra-Turrax T18D, IKA, Staufen, Germany) with 18 mL distiled water at 10,000 rpm for 1 min, and the pH was measured by pH meter (FE20, Mettler-Toledo International Inc., Switzerland). Titratable acid was determined according to the method of AOAC (2002), and the results were expressed as a percentage of lactic acid.

2.7. Determination of total volatile basic nitrogen (TVB-N)

The TVB-N content of the CTFPs sample was measured according to the procedure of Huang et al. (2010) with slight modification. Ground samples (2 g) were homogenized with 18 mL of 6% cold trichloroacetic acid (TCA) solution (w/v) and centrifuged at 10,000 × *g* for 10 min at 4 °C (4K15, Sigma Laborzentrifugen, Osterode, Germany). The supernatant (5 mL) was mixed with 5 mL of 10g/L MgO solution, and stream distillation (KDN-103F, Xianjian Instruments Co. Ltd., Shanghai, China) was carried out for 5 min on the TVB-N extract. The TVB-N was absorbed by 20g/L boric acid solution and then titrated with 0.01 M HCl. The results were expressed as mg/100g sample.