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Inactivation of *Salmonella enterica* and spoilage microorganisms in orange juice treated with dimethyl dicarbonate (DMDC)

Rebecca M. Cheng, John J. Churey, Randy W. Worobo*

Department of Food Science, Cornell University, Ithaca, NY 14853, United States

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ABSTRACT

Salmonella enterica is the pertinent pathogen associated with orange juice products that have resulted in numerous foodborne outbreaks. Although fresh orange juice typically has a pH below 4.0, which inhibits most pathogen growth, *S. enterica* can survive at low pH for extended periods. Additionally, fresh juice contains spoilage microorganisms such as natural yeasts and molds, which can grow at low pH, and may cause fermentation and product spoilage if left untreated. Numerous *Salmonella* outbreaks linked to fresh orange juice, as well as the burden of product spoilage, have generated increased demand for alternative, non-thermal treatments that can ensure pathogen- and spoilage-free products. In this study, the effect of dimethyl dicarbonate (DMDC) on pathogen and spoilage microorganism inactivation in orange juice has been investigated with two experiments. First, pasteurized orange juice was inoculated with approximately 10^6–10^7 CFU/ml of five serotypes of *S. enterica* per ml and treated with DMDC to test the effectiveness of inactivation against *Salmonella*. For the fungal spoilage microorganism study, fresh orange juice was held at room temperature to increase natural yeast and mold count to roughly 10^5–10^6 CFU/ml, followed with treatment with DMDC. DMDC at two concentrations (172 and 200 ppm) was used, and the tests were carried out at ambient (21 °C ± 3 °C) and refrigeration (4 °C) temperatures. There was a > 5-log reduction of *Salmonella* at 4 °C after 24 h at both 172 and 200 ppm of DMDC. For the treatment of fungal spoilage microorganisms, a nearly 5 and 4 log reduction of yeasts and molds was observed at ambient temperature and 4 °C, respectively. These results suggest that DMDC is most effective for use under the 4 °C holding conditions to inactivate *S. enterica*, and should be coupled with an additional preservative system for fungal spoilage control to produce safe orange juice that retains fresh quality.

1. Introduction

Orange juice is the most consumed juice in the United States, with an average annual consumption rate of 2.7 gal per person (USFDA, 2017). Traditional orange juice sales have been slowly decreasing due to increasing consumer demand for functional benefits and healthy products, such as vitamin and mineral added, fiber-rich beverages (Mintel, 2017a). Most commercial juices are pasteurized using heat treatment to inactivate pathogens and spoilage microorganisms (Yeom et al., 2000). However, heat treatment of juices can lead to thermal degradation of nutrients, particularly vitamins and antioxidants, and loss of flavor (Jia et al., 1998; Polydera et al., 2004; Vikram et al., 2005). These traits are undesirable and do not keep up with the current trend in the juice industry, where minimally processed or unprocessed products that retain a fresh quality with large nutritional benefits are actively pursued (Mintel, 2017b). However, fresh or unpasteurized juice is highly susceptible to fungal spoilage and may be contaminated with pathogenic bacteria, particularly under conditions without proper processing steps or treatment for controlling microbial growth.

Salmonella is a rod-shaped, Gram-negative bacterium that has been associated with over 1 million foodborne illnesses in the United States, with 19,000 hospitalizations and an estimated 380 deaths annually (CDC, 2012). The genus *Salmonella* is comprised of two species, *Salmonella enterica* and *Salmonella bongori* (Reeves et al., 1989). *S. enterica* is divided into seven subspecies, including *Salmonella enterica enterica*, the subspecies responsible for most cases of nontyphoidal salmonellosis in humans (Beltran et al., 1988; Uzzau et al., 2000; Winfield and Groisman, 2004). Salmonellosis is an infection caused by the consumption of contaminated water or food, characterized by gastroenteritis, nausea, vomiting, abdominal pain, headaches, elevated body temperature, and non-bloody diarrhea (Chen et al., 2013; Sharma et al., 2001). There have been several outbreaks associated with fresh, unpasteurized orange juice in the past several years, most of which are associated with nontyphoidal *Salmonella* (Butler, 2000; CDC, 1995,

* Corresponding author.
E-mail address: rww8@cornell.edu (R.W. Worobo).

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1999; Jain et al., 2009; Krause et al., 2001). To reinforce food safety, the U.S. Food and Drug Administration (FDA) has regulated that juice manufacturers must treat their products to achieve a minimum 5-log reduction for the most pertinent or resistant pathogen of public health concern, or provide a warning label (USFDA, 2001a).

Fresh juice also contains non-pathogenic microorganisms, such as yeasts and molds, which are the primary juice spoilage microorganisms. Unlike pathogens, spoilage microorganisms do not cause harmful effects such as sickness or disease, but produce unwanted characteristics that make the product undesirable for consumption, such as fermentation and off flavors (intVeld, 1996). Microbial spoilage is usually controlled using thermal or nonthermal processing or with food additives as a preservation method (Gabriel, 2015).

Dimethyl dicarbonate (DMDC) is a microbial control agent that has been used primarily in wine preservation, as it inactivates yeast (Delfini et al., 2002; Bartowsky, 2009). DMDC, a colorless liquid, controls microbial growth by inactivating enzymes through protein modification via methoxycarbonylation of enzymes, and thus cell death (Bartowsky, 2009). The FDA approved its use in wines in 1988, with the maximum level permitted set at 250 ppm (USFDA, 2001b). Studies have shown that DMDC is useful as an alternative processing treatment in fruit juices, such as apple and citrus, to inactivate pathogenic microorganisms (Assatarakul, 2017; Basaran-Akgul et al., 2009; Whitney et al., 2008; Williams et al., 2005). However, its use as the sole pathogen inactivating and fungal spoilage reducing agent in fresh orange juice has not been extensively studied.

In this work, we have investigated the non-thermal treatment of orange juice by DMDC and measured the effectiveness of the agent to achieve a 5-log reduction of pathogens in unpasteurized juice. To assess the efficiency of the process, the microbial tests were carried out at both ambient (21 °C ± 3 °C) and cold (4 °C) temperatures. Five strains of *S. enterica* isolated from juice were used for determining the efficacy of DMDC. We also investigated the effect of DMDC on natural fungal spoilage microorganisms under similar conditions. This study was conducted to determine the effects of DMDC as a viable measure for controlling microbial growth.

2. Materials and methods

2.1. Orange juice and bacterial strains

Two types of orange juice were used for this study. Pasteurized and unpasteurized orange juices were purchased from the local supermarket (Wegmans, Ithaca, NY). Both were divided into 50 ml aliquots and frozen at −20 °C. Frozen samples were brought to the targeted temperature (4 °C or 21 °C) before use, depending on the conditions specified in the study. Orange juices were evaluated for pH and °Brix values. The pH values ranged from 3.65–3.79 and the °Brix values ranged from 10.07–11.47.

Five strains of *S. enterica* ATCC 8324, ATCC 10717, Hartford H0778, ATCC 14028, ATCC 8387 of serotypes Gaminara, Rubislaw, Hartford, Typhimurium, and Montevideo, respectively, were employed for this work (obtained from M.E. Parish of the University of Florida). These strains were isolated from juices and maintained in frozen culture at −80 °C. Each serotype was streaked out on Brain Heart Infusion (BHI) Agar (Sigma-Aldrich, St. Louis, MO) from the frozen stocks and maintained by restreaking on fresh agar monthly.

2.2. Preparation of DMDC

DMDC (Velcorin™, 99.8%; LANXESS, Pittsburgh, PA) solution was prepared by a 1:4 dilution in 100% ethyl alcohol to yield a stock solution with a concentration of 312.5×10^3 parts per million (ppm). Specifically, 200 µl of fresh DMDC were added to 600 µl of ethyl alcohol to achieve a 1:4 dilution. Aliquots of 28 µl and 32 µl of DMDC stock solution were added to 50 ml orange juice to reach final concentrations 172 ppm and 200 ppm, respectively. The DMDC treatments of 172 and 200 ppm were selected as levels that are more commonly employed by the juice and beverage industry, and recommended by the commercial manufacturer of DMDC to ensure that levels of DMDC are not higher than regulatory limits due to the variability of the DMDC dosing apparatus.

2.3. Orange juice preparation and Salmonella inoculation

Salmonella serotypes were streaked out and grown on Brain Heart Infusion (BHI) plates (Difco, Detroit, MI). Single colonies were used to inoculate 5 ml BHI liquid media and were grown overnight at 37 °C, tilted and shaking at 150 rpm for 10–12 h. 500 µl of each serotype was mixed to form a cocktail mixture of five *Salmonella* serotypes. 500 µl of the cocktail mixture was added to 50 ml of pasteurized orange juice to achieve a starting bacterial concentration of 10^7 CFU/ml. Pasteurized orange juice was used to study the effects of DMDC on *Salmonella* without competing microorganisms.

DMDC was added to achieve concentrations of 172 ppm and 200 ppm. Orange juice samples containing *Salmonella* without DMDC were used as controls. Samples were incubated at both 4 °C and 21 ± 3 °C. Samples were collected at 0, 1, 2, 4, 6, 24, 48, 72, and 96 h. Samples were then serial diluted in phosphate buffered saline (PBS). 100 µl of serial dilutions were spread plated on Standard Plate Count (SPC) and Xylose Lysine Deoxycholate (XLD) agar plates (Sigma-Aldrich, St. Louis, MO). The plates were incubated at 37 °C for 48 h, and then enumerated to determine the CFU/ml at each time point. Each of the enumerations for the respective media were averaged and converted into log numbers. The *Salmonella* inoculation and DMDC experiments were conducted in triplicate.

2.4. Orange juice preparation and fungal spoilage growth

Fresh, unpasteurized orange juice was left at an ambient temperature of 21 °C overnight (12–16 h) to simulate temperature abuse and to achieve natural microbiota growth of 10^5–10^6 CFU/ml on acidified potato dextrose agar (aPDA) (Sigma-Aldrich, St. Louis, MO) and Standard Plate Count Agar (SPC) (Sigma-Aldrich, St. Louis, MO). DMDC was added to achieve the concentrations of 172 ppm and 200 ppm. Temperature abused orange juice without DMDC were used as controls. Samples were incubated at both 4 °C and 21 ± 3 °C. Samples were collected at 0, 1, 2, 4, 6, 24, 48, 72, and 96 h. Samples were then serial diluted in PBS. 100 µl of serial dilutions were spread plated on SPC and aPDA. The plates were incubated at 30 °C for 48 h, and then enumerated to determine the CFU/ml at each time point. Each of the enumerations for the respective media were averaged and converted into log numbers. Fungal spoilage and DMDC experiments were conducted in triplicate.

2.5. Statistical analysis

The statistical software R (R Core Team, Vienna, Austria) and package lme4 were used to fit linear mixed effects regression models. Means and post-hoc comparisons were estimated from the model using the lsmeans package. Significant relationships and analysis were determined based on initial populations and at time points where the DMDC reaction had been fully exhausted. CFU/ml per time point were converted to log CFU/ml and averaged with the standard deviation. Due to the method of plating, the lowest observable counts are recorded at 10 CFU/ml.

3. Results

3.1. Reduction of Salmonella in orange juice

Salmonella strains were grown and inoculated into orange juice to