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Incorporating fermented by-products of *Lactobacillus diolivorans* in food grade coatings designed for inhibition of *Tyrophagus putrescentiae* on dry-cured hams

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ABSTRACT

Distillate solutions that were derived from concentrated ferment of *Lactobacillus diolivorans* were incorporated into either a carrageenan (CG) and propylene glycol alginate (PGA) gum blend or into a CG, PGA, and xanthan gum (XG) blend. Fermented distillate was produced in a model system to mimic fermentation in food grade coatings to produce 1, 3 propanediol, acetic acid, and lactic acid. Distillate treatments were compared against a 10% propylene glycol treatment, a gum only control, and negative control on $2.5 \times 2.5 \times 2.5$ cm cubes ($n = 50$) that were dipped into solutions prior to conducting mite bioassays. Coated and control ham cubes were inoculated with 20 adult *T. putrescentiae* (Schrank), and incubated for 2 and 3 weeks. The distilled treatments with CG + PGA + XG had a 20 fold or greater reduction ($P < 0.05$) in mite populations than all other treatments with the exception of the 10% PG coated treatments. In addition, there were no differences between treatments with respect to sensory texture, flavor, and moistness. This indicates that fermentation within food grade coatings could be evaluated on the surface of dry-cured ham to determine if the success in a model system could be reproduced in practice.

1. Introduction

The initial production of dry-cured ham in the United States dates back to the settlement of Jamestown during the 1600's (Stradley, 2004). During the 3 months to 3 years of aging process, hams develop their distinct flavor from lipolysis and proteolysis (Toldra, 1998). *Tyrophagus putrescentiae* (Schrank), also known as the ham, mold, or cheese mite, infests grains and stored food products, and has an affinity towards high fat and protein containing food items (Abbar et al., 2016). Dry-cured ham is most susceptible to mite infestation after 4–6 months of aging due to its high fat and protein content, mold growth, and water activity (Rentfrow et al., 2008). Currently, methyl bromide fumigation is the principal means of pest control in ham houses. With the signing of the Montreal Protocol in 1987, the United States joined 196 other countries in an effort towards gradually eliminating the use of ozone depleting substances, thus limiting access to methyl bromide for use in ham houses (US Environmental Protection Agency, 2016). The United States Department of Agriculture (USDA) considers the presence of one or more ham mites on a dry cured ham an adulterant, and mandates that producers make corrective actions before the sale of affected products (United States Department of Agriculture, 2014; United States Department of Agriculture, 2011a; United States Department of Agriculture, 2011b). Therefore, potential alternatives to methyl bromide need to be evaluated for their cost efficacy and ability to control mites on dry-cured ham since there are no current commercial methods that are used as a direct replacement for methyl bromide.

In previous research on the application of food safe compounds to the surface of hams, both 1,3 propanediol and 1,2 propanediol,

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most commonly known as propylene glycol, controlled mites with 99% inhibition when used at concentrations of 50 and 100% in benchtop studies on 2.54 cm × 2.54 cm × 2.54 cm cubes (Abbar et al., 2016; Zhao et al., 2016). Propylene glycol was also effective at inhibiting the growth and reproduction of the ham mite on dry-cured hams during trials performed on the benchtop when incorporated into food grade coatings that contained carrageenan and propylene glycol alginate or xanthan gum. Use of propylene glycol, though effective at inhibiting mite reproduction, may not be feasible on a commercial scale, due to its relatively high cost of $0.82 or greater per ham when implemented in coating formulations (Campbell et al., 2017).

Though commonly synthesized through the chemical alteration of acrolein or ethylene oxide, several species of bacteria demonstrate the ability to commercially produce 1,3 propanediol through the metabolism of glycerol (Ainala et al., 2013; Lee et al., 2015; Pflügl et al., 2012). The use of lactic acid bacteria in the genera *Lactobacillaceae*, specifically heterofermentative strains such as *L. reutteri, L. buchneri, L. brevis,* and *L. diolivorans* can produce 1,3 propanediol for use in products that are intended for human consumption (Pflügl et al., 2014; Schütz and Radler, 1984; Stevens et al., 2011). *L. diolivorans* produces a greater concentration of 1, 3 propanediol than other species of lactobacilli. Pflügl et al. (2012) reported that *L. diolivorans* produced 41.7 g/L of 1, 3 propanediol in MRS broth when supplemented with a 7% (w/v) concentration of glycerol using a batch cultivation method in a stirred tank bioreactor. Anaerobic fermentation using *L. diolivorans* also produces lactic acid, acetic acid, and ethanol as primary by-products. Incorporating fermented by-products of lactic acid bacteria may provide benefits beyond mite inhibition, as production of antifungal metabolites in tandem with acetic acid may contribute to mold inhibition. Ham mites feed on food sources that are high in fat and protein, but also feed on various mold species. *Penicillium nordicum*, a strain of mold commonly found in fermented meat products, is a potential food source for *T. putrescentiae*. In contrast, several aspergillus species can produce aflatoxins that are detrimental to mite reproduction (Rodriguez et al., 1980; Smrž and Čatská, 1987). However, many species of Fusarium fungi can grow and produce mycotoxins on dry-cured ham and also serve as a food source for *T. putrescentiae* (Nesvorna et al., 2012). Previous research indicated that antifungal metabolites that were produced by *Lactobacillus planterum* in the presence of acetic acid, with a pH of 4.0 were effective at preventing the growth of *P. nordicum* (Schillinger and Villarreal, 2010). Similar inhibitory effects on mold growth may potentially be exhibited from acetic acid and anti-fungal metabolites that are produced by *L. diolivorans*, which in turn would prevent mites from utilizing mold growth as a food source during aging. Since natural fermentation with *L. diolivorans* could occur within a food grade coating. The objective of this study was to determine if fermentation by-products created by *L. diolivorans*, primarily 1,3 propanediol, lactic acid, and acetic acid were effective at controlling *T. putrescentiae* growth when incorporated into food grade coatings that contained xanthan gum, propylene glycol alginate and carrageenan.

2. Materials and methods

2.1. Culture preparation

A stock culture of *L. diolivorans* (DSM 14421) (DSMZ, Braunschweig, Germany) was rehydrated using 0.5 ml of de Man, Rogosa, Sharpe (MRS) medium (Sigma-Aldrich. Milwaukee, WI) for 30 min under a vacuum hood (Labconco, Kansas City, MO) at 20° C. An aliquot of 0.25 ml hydrated inoculant was transferred to 5 ml of MRS medium while the remaining 0.25 ml of solution was streaked onto a plate containing MRS agar per the manufacturer's instructions for reconstitution. The plate and tube were subsequently placed in a Gas-Pak™ 150 anaerobic chamber (Becton Dickinson and Co. East Rutherford, NJ) with added AnaeroPack-Anaero sachets (Mitsubishi Gas Chemical Co. Inc. Tokyo, Japan) and incubated at 30 °C for 48 h. Cells were supplemented with a 50% (w/v) glycerol solution and then stored at −80 °C. MRS broth (Sigma-Aldrich, Milwaukee, WI) and MRS agar (Remel, Hampshire, United Kingdom) were prepared for the enumeration of Lactobacilli according to the manufacturer's instructions and autoclaved at 121 °C for 15 min (Sterilmatic STM-E, Market-Forge Industries, Everett, MA). For Bioreactor trials, a modified MRS media was prepared. The composition of modified MRS (per liter deionized water) contained 10 g of casein peptone (pancreatic digest) (Becton, Dickinson and Co. Franklin Lakes, NJ), 10 g meat extract (Sigma-Aldrich. Milwaukee, WI), 5 g yeast extract (Himedia, West Chester, PA), 33 g glucose × H_2O (Modernist Pantry, Eliot, ME), 5 g Na-acetate (Eisen-Golden Laboratories, CA), 1 g polysorbate 80 (Modernist Pantry, Eliot, ME), 2 g K_2HPO_4 (Fischer Scientific, Waltham, MA) 2.6 g Na3-citrate x 2H2O (Sigma-Aldrich. Milwaukee, WI), 1.17 g $(NH_4)_2HPO_4$ (Sigma-Aldrich. Milwaukee, WI), 0.2 g $MgSO_4 \times 7H_2O$ (Eisen-Golden Laboratories, CA), and 0.05 g $MnSO_4 \times H_2O$ (Eisen-Golden Laboratories, CA). Media was supplemented with 7% (w/v) glycerol (Bulk Apothecary, Aurora, OH) for all cultivations. Twenty g of casein peptone (pancreatic digest), 20 g meat extract, 10 g yeast extract, 10 g Na-acetate, 2 g Tween 80, 4 g K_2HPO_4, 5.2 g Na_3-citrate × $2H_2O$, 2.34 g $(NH_4)_2HPO_4$, 0.4 g $MgSO_4 \times 7H_2O$ and 0.10 g $MnSO_4 \times H_2O$ were combined into 1 L of distilled water, and autoclaved for 15 min at 121 °C inside the vessel compartment of the bioreactor system. Sixty-six g glucose × H_2O and 140 g USP grade vegetable glycerin were combined in 960 ml of distilled water and prepared using a 500 ml bottle top sterile filtration unit with a 0.45 μm membrane (Nalgene, Rochester, NY) and incorporated into the protein and mineral solution using a feed pump 24 h prior to inoculation.

A stock culture of *L. diolivorans* (DSM 14421) (DSMZ, Braunschweig, Germany) was removed from storage at −80 °C and thawed for 1 h under a sterilized fume hood (Labconco, Kansas City, MO). One ml of inoculant was pipetted into 40 ml of sterile MRS media in a 100 ml conical flask and subsequently placed in a Gas-Pak™ 150 anaerobic chamber (Becton Dickinson and Co. East Rutherford, NJ) with AnaeroPack-Anaero sachets (Mitsubishi Gas Chemical Co. Inc. Tokyo, Japan) and incubated at 35 °C for 28 h. Cultured media was removed under a fume hood and aseptically transferred into a sterile 60 ml syringe (Becton, Dickinson and Co. Franklin Lakes, NJ) and injected into the modified MRS media inside the pre-sterilized vessel. MRS media was sparged to create an anoxic environment and stabilized to a temperature of 35 °C prior to injecting the inoculant. The injected inoculant amounted to 2% of the total working volume.

2.2. Vessel preparation

A Celligen/BioFlo 310 bioreactor system (Eppendorf, Hamburg, Germany) was utilized to conduct 2 L batch fermentation studies. Media components were combined and then stabilized to 35 °C and sparged using 10 standard L per minute (SLPM) of industrial grade nitrogen (Airgas, Radnor, PA). Dissolved oxygen concentration was monitored using an internal probe, with media maintaining a 3% ± 2 oxygen concentration throughout the duration of the experiment. Initial pH of the media was 6.2 ± 0.1 and was maintained at 5.5 using a 5M KOH solution. Stir rate was maintained at 150 rpm for the duration of the study. A 10% (w/v) antifoam solution was prepared using antifoam 204 solution (Sigma-Aldrich. Milwaukee, WI) and distilled water. Temperature, pH, dissolved