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ß-phenylethylamine as a novel nutrient treatment to reduce bacterial contamination due to *Escherichia coli* O157:H7 on beef meat

Ty Lynnes, S.M. Horne, B.M. Prüß *

Department of Veterinary and Microbiological Sciences, North Dakota State University, Fargo, ND 58108, United States

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ABSTRACT

Bacterial infection by *Escherichia coli* O157:H7 through the consumption of beef meat or meat products is an ongoing problem, in part because bacteria develop resistances towards chemicals aimed at killing them. In an approach that uses bacterial nutrients to manipulate bacteria into behaviors or cellular phenotypes less harmful to humans, we screened a library of 95 carbon and 95 nitrogen sources for their effect on *E. coli* growth, cell division, and biofilm formation. In the initial screening experiment using the Phenotype MicroArray™ technology from BioLog (Hayward, CA), we narrowed the 190 starting nutrients down to eight which were consecutively tested as supplements in liquid beef broth medium. Acetoacetic acid (AAA) and ß-phenylethylamine (PEA) performed best in this experiment. On beef meat pieces, PEA reduced the bacterial cell count by 90% after incubation of the PEA treated and *E. coli* contaminated meat pieces at 10 °C for one week.

1. Introduction

Escherichia coli is a common Gram-negative bacterium that populates the majority of animal GI tracts. While most *E. coli* are harmless, some are considered human pathogens. The most commonly known pathogenic *E. coli* is the serotype O157:H7, which was described as a novel pathogen in 1982 (41) and has since emerged as a major food safety concern (Rangel, Sparling, Crowe, Griffin, & Swerdlow, 2005). In cattle, colonization by *E. coli* O157:H7 does not cause disease. However, cattle can serve as a reservoir for human infection via the consumption of contaminated meat (Hussein & Sakuma, 2005). Such contamination occurs primarily at the dehiding stage of slaughtering because the bacteria are able to adhere to the extracellular matrix of the skeletal muscle (Chagnot et al., 2013). In humans, *E. coli* O157:H7 infection is associated with bloody loose bowel movements, but it can also cause other complications, such as hemolytic uremic syndrome (HUS), accompanied by kidney failure (Kaper, Nataro, & Mobley, 2004).

Food regulatory agencies have a zero tolerance policy for *E. coli* O157:H7 on beef meat. This requires food processing companies to subject their food products to additional treatments, as well as test the final products for *E. coli* O157:H7 and the other six shiga toxin producing E. *coli* (STEC) serotypes that the USDA considers adulterants (Big 6). Among the treatments used to increase the safety of beef meat products, spraying the hot carcasses with organic acids and other food-grade antimicrobials is partially successful (Buncic et al., 2013; Carpenter, Smith, & Broadbent, 2011; Yoder et al., 2012). These treatments may also increase the shelf life of the products, which has become increasingly important because of the globalization of the food industry, which often transports food long distances before consumption (Outtara, Simard, Holley, Piette, & Begin, 1997). Over time however, bacteria have the tendency to become resistant to standard antimicrobial treatments (Castanie-Cornet, Penfound, Smith, Elliott, & Foster, 1999). In the case of acid resistance, these bacteria can then survive the acid barrier of the stomach (Mead et al., 1999).

Current research on the development of novel treatments for *E. coli* infections is aimed at bacterial signal transduction pathways with the ultimate goal of manipulating the bacteria into phenotypes that are less harmful to humans (Giaouris et al., 2013). Among the signal transduction pathways that are targeted in this way are quorum sensing (Bassler, 2010; Njoroge & Sperandio, 2009; Raina et al., 2009) and two-component signaling (Kostakioti et al., 2012; Rasko et al., 2008). As one specific example, we were able to control the cell division rate of a harmless *E. coli* K-12 laboratory strain by changing the concentration of serine in the bacterial growth medium (Prüß & Matsumura, 1996). This increase in bacterial cell numbers occurred without an effect on the bacterial biomass, bacteria simply divided more rapidly and became smaller. The signal transduction cascade from serine to cell division involved one of the two-component response regulators, OmpR (Shin & Park, 1995), and another global regulator, FlhD/FlhC.

According to one of our previous studies, FlhD/FlhC was also involved in controlling biofilm amounts in addition to the cell division

* Corresponding author at: Department of Veterinary and Microbiological Sciences, North Dakota State University, PO Box 7690, United States. Tel.: +1 701 231 7848; fax: +1 701 232 9692.
E-mail address: Birgit.Pruess@ndsu.edu (B.M. Prüß).

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rate when *E. coli* O157:H7 was grown on beef meat (Sule, Horne, Logue, & Prüß, 2011). In this study, we took advantage of this observation and used the signal transduction concept for the development of a novel prevention technique for *E. coli* contamination of beef meat. In three experiments, we narrowed down 95 carbon and 95 nitrogen sources to one nutrient that was most successful at reducing the bacterial cell count of an *E. coli* O157:H7 strain on beef meat. This nutrient was ß-phenylethylamine (PEA).

2. Materials and methods

2.1. Bacterial strain and storage conditions

Escherichia coli O157:H7 (American Type Culture Collection, ATCC 43894) was kindly provided by Scott A. Minnich (University of Idaho, Moscow). The strain had been made resistant towards streptomycin sulfate and nalidixic acid previously (Sule et al., 2011). The strain was stored in dimethyl sulfoxide (DMSO) at −80°C. Before use, the strain was plated onto agar plates made from lysogeny broth (LB; 10 g/l tryptone, 5 g/l yeast extract, 5 g/l NaCl, 15 g/l agar) and incubated overnight at 37 °C.

2.2. Screening of 95 carbon and 95 nitrogen sources for their effect on E. coli O157 respiration/growth, bacterial cell counts, and biofilm amounts

The BioLog Phenotype MicroArray (PM) technology has been developed for the determination of bacterial growth phenotypes (Bochner, 2009; Bochner, Gadzinski, & Panomitros, 2001; Bochner, Giovannetti, & Viti, 2008). The PM technology permits the testing of more than 2000 bacterial phenotypes in a 96 well format, where individual nutrients or chemicals are dried to the base of each well. When used with the tetrazolium dye that is provided by the manufacturer, the bacterial phenotype that is measured is respiration, which is indicative of growth. This original protocol has been used in many previous studies (Baba et al., 2006; Zhou, Lei, Bochner, & Wanner, 2003). Omitting the dye, the technology can be used to determine another bacterial phenotype, biofilm amounts. For this determination, PM technology is used in combination with the ATP assay (Sule et al., 2009), using BacTiter Glo™ from Promega (Madison, WI). For this study, we used PM technology with the dye for the determination of respiration/growth and without the dye to determine bacterial cell counts and biofilm amounts. Four replicate plates were inoculated for the determination of each of the three different bacterial phenotypes.

Bacteria of the strains *E. coli* O157:H7 and its isogenic *flhC* mutant were removed from the LB plates with a nylon flocked swap and suspended in sterile beef broth (3% beef extract, 5% peptone) to an OD_{600} of 0.1. 100 µl of this solution was used to inoculate each well of PM1 (carbon sources) and PM3b (nitrogen sources) plates. All plates were then incubated at 10 °C for seven days. The temperature was selected to simulate beef storage. Each experiment was done in four replicates. For the determination of respiration/growth, the OD_{600} was determined from the plates that had been inoculated with the 1% Dye MixA, using an EL808 Ultra Microplate reader (Bio-Tek Instruments, Winooski, VT). To determine bacterial cell counts, the liquid beef broth from each well (of plates without the dye) was serially diluted in phosphate buffered saline (PBS), plated onto LB, and incubated overnight at 37 °C. Bacterial cell counts were expressed in Colony Forming Units (CFU) per ml of original beef broth. For the determination of biofilm amounts, the liquid beef broth was removed from the wells (of plates without the dye). Biofilms were gently rinsed with 100 µl PBS. After rinsing 100 µl of BacTiter-Glo™ reagent (Promega, Madison WI) was added to each well. The bacteria were homogenized by carefully pipetting the reagent up and down. Relative luminescence was then measured using a TD 20/20 luminometer (Turner Designs, Sunnyvale, CA).

Data was analyzed as follows: reductions of OD_{600}, RLU, or CFU/ml by each respective nutrient were determined as ratios, dividing the value for the respective carbon source by that of the negative control that contained un-supplemented liquid beef broth medium. Low ratios were indicative of large reductions (of growth, biofilm amounts, or bacterial cell counts) and high inhibitory effectiveness (of the respective nutrient). Nutrients were ranked by increasing ratios (decreasing effectiveness) and the 10 nutrients that yielded the largest reduction in OD_{600}, RLU, or CFU/ml were presented and discussed. Rankings were done separately for carbon and nitrogen sources, as well as the three bacterial phenotypes (resulting in a total of six panels of data). As a final criterium to determine top performing nutrients, we obtained information for selected nutrients, such as cost and toxicity levels for humans. A list of eight top performing nutrients was obtained after this analysis.

2.3. Determination of the IC_{50} values for eight nutrients

The eight chemicals that the initial screening identified as top performing were purchased and suspended in ddH_2O to the concentrations listed in Table 1. Concentrations were selected based upon the solubility of the respective nutrient in water. The bacterial inoculum was prepared in 2× beef broth to an OD_{600} of 0.2. Each well of a 96-well plate was loaded with 100 µl of nutrient solution and inoculum. Plates were incubated at 10 °C for four days. Bacterial cell counts were measured as CFU/ml in three replicate experiments and biofilm amounts were determined as RLU in four replicate experiments, using a Synergy H1 Hybrid Reader (BioTek Instruments, Inc., Winooski, VT).

Data for each nutrient and concentration were divided by those of the un-supplemented liquid beef broth negative control. Average and standard deviations were calculated across the replicate experiments and plotted against the concentration of the respective nutrient. Curve fitting was performed as five parameter logistic (Gottschalk & Dunn, 2005) with Master Plex® Reader Fit analysis software (Hitachi

Table 1
Concentrations[a] of nutrients, companies[b] that nutrients were purchased from, and water solubility (Sol) for the liquid beef broth experiment.

Nutrient	Concentrations[c]					Company	Sol. in H_2O
	1	2	3	4	5		
Acetoacetic acid (lithium salt)	1.5	7	15	70	150	Research Organics 101107-864	1000 mg/ml
L-lyxose	0.4	2	10	50	250	Alfa Aesar AAB21583-03	555 mg/ml
D,L-α-glycerol phosphate (magnesium salt)	0.4	2	10	50	250	Sigma 17766-50 g	1000 mg/ml
D,L-α-amino-caprylic acid	0.016	0.08	0.4	2	10	Acros Organics 200018-918	2 mg/ml
ß-phenylethylamine (hydrochloride)	1.5	7	15	70	150	TCI America TCP0086-25 g	100 mg/ml
D-mannosamine (hydrochloride)	0.5	2.5	13	65	330	Toronto Research Chemicals 100573-506	1000 mg/ml
Thymine	0.016	0.08	0.4	2	10	TCI America A65100A	15 mg/ml
D-asparagine	0.016	0.08	0.4	2	10	Amresco 94341-100 g	17 mg/ml

[a] Concentrations for are all given in mg/ml. A negative control, containing un-supplemented liquid beef broth medium was performed for each experiment.
[b] Companies that chemicals were purchased are indicated, together with the ordering number.
[c] Note that these are the concentrations of the nutrient solution before the inoculum was added. The final concentration in the experiment is half that.