HAPPY BEEF: THE DEVELOPMENT OF ß-PHENYLETHYLAMINE AS A NOVEL NUTRIENT TREATMENT REDUCING BACTERIAL CELL COUNT BY *ESCHERICHIA COLI* O157:H7 ON BEEF MEAT

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Happy beef: the development of ß-phenylethylamine as a novel nutrient treatment reducing bacterial cell count by Escherichia coli O157:H7 on beef meat

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ABSTRACT

This research looks at the use of nutrients as a novel way to control *E. coli* O157:H7. In this research Phenotype MicroArray™ technology from BioLog (Hayward, CA) technology were used to screen nutrient sources ability to reduce respiration, biofilm amounts and cell number. Top performing nutrients were screened a second time to look at the effects of concentration on their ability to reduce biomass, biofilm amounts and cell number. This screening reduced the number of chemicals from eight to two chemicals, acetoacetic acid and ß-phenylethylamine. In a final experiment, these two chemicals were used as treatment on beef, which was then inoculated with *E. coli* O157:H7. ß-phenylethylamine, a natural trace amine, reduced the bacterial cell counts of *E. coli* O157:H7 over the seven day incubation period by >74%, showing a nutrient can be used to control phenotypic traits in *E. coli* O157:H7 in a preventative manner.

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LIST OF ABBREVIATIONS

2CSTS ..... Two component signal transduction system

AI ..... Auto-inducer

ANOVA ..... Analysis of variance

ATP ..... Adenosine triphosphate

ATR..... Acid Tolerance Response

CFU..... Colony forming units

DMSO ..... Dimethyl sulfoxide

DNA..... Deoxyribonucleic acid

EPS..... Extrapolymeric substance

FDA..... Federal Drug Administration

FEMA ..... Flavor and Extract Manufacturing Association

GRAS ..... Generally regarded as safe

HC ..... hemorrhagic colitis

HUS..... Hemolytic uremic syndrome

IC_{50} ..... Inhibitory concentration 50%

IS ..... Insertion Sequence

LB ..... Luria Bertani

PBS ..... Phosphate buffered saline

PM..... Phenotypic MicroArray

QS ..... Quorum sensing

SMAC ..... Sorbitol MacConkey agar

STEC..... Shiga like toxin producing E. coli

TSB ..... Tryptic Soy Broth

LITERATURE REVIEW

Escherichia coli, a common harmless gram-negative bacteria, populate the majority of warm blooded animal GI tracts. While most are harmless, some serotypes are extremely pathogenic with serious pathological consequences (Park *et al.* 2013; Tzipori *et al.* 1987). *E. coli* O157:H7, one such serotype, has become a major food safety concern since its emergence in the 1980's (Riley *et al.* 1983; Remis *et al.* 1984). This pathogen is often associated with beef meat because cattle act as a reservoir (Hussein & Sakuma 2005). This association with beef in comparison to other meats may be the result of preference for lower beef cooking in comparison to other meat like pork. *E. coli* O157:H7 is a major concern in part because it is a Shiga-like toxin, Stx,(producing *E. coli* (STEC)) that has effects on human health. STEC infections often lead to bloody diarrhea and other pathogenic sequelae (Puttamreddy and Minion 2011). Current theories state that *E. coli* O157:H7's ability to produce both Stx1 and Stx2 toxins is the result of an evolutionary event, involving the transfer of the genes from another pathogen to STEC via different bacteriophages (Allison 2007). An *E. coli* O157:H7 infection is most known for causing bloody loose bowel movements which are symptoms of a disease called hemorrhagic colitis (HC), but due to the Stx it can also cause other complications such as hemolytic uremic syndrome (HUS). HUS is the major cause of post-gastroenteritis acute and chronic renal failure in children; which may result in kidney function loss and/or death (Kaper and Karmali 2008). Estimates show more than 63,000 domestic cases of foodborne STEC infections have been reported in a single year, of those more than 2000 will result in hospitalization and 20 will result in death (Scallan *et al.* 2011). One of the reasons that STEC is such a successful pathogen is that its genome is plastic in nature with many mobile elements. Many of these mobile elements are virulence factors that can move between strains (Imamovic *et al.* 2010) allowing for the

development of resistances (Castanie-Cornet *et al.* 1999). Because of its effects on human health and its variable genome, E. *coli* O157:H7 is difficult for food manufacturers to control. The purpose of our study is to develop a new treatment to use on beef to reduce *E. coli* O157:H7 growth.

The first step in *E. coli* O157:H7 pathogenesis is ingestion of the bacteria through contaminated food and or water by the host, thus food safety practices are very important, especially during food processing. *E. coli* O157:H7 has the ability to survive in many different and often hostile environments (House *et al.* 2009). Environmental survival requires the STEC to have the ability to cope with environmental stresses. Coping with environmental change is achieved by changing metabolic processes, which can allow them to live on many different energy sources and expend that energy on processes that provide advantages in relation to the environment they inhabit (Carter *et al.* 2011). This means that one microorganism might be found in multiple environments and their metabolic processes are dependent on that environment. This increases the difficulties of food processors to control *E. coli* O157:H7. Metabolism changes allow them to survive. The environments may be manipulated to reduce the hazard STEC's pose.

Biofilm

One of the reasons *E. coli* O157:H7 copes so well with sanitation efforts and is hard to control in food processing areas is that it has the ability to form biofilm (Puttamreddy and Minion 2011). Biofilm is a community of microscopic organisms, usually bacteria that have produced an extracellular matrix to attach to each other and a surface structure. This encapsulating self-produced matrix is often referred to as the ExtraPolymeric Substance or EPS and is made up primarily of polysaccharides, proteins and DNA (Sambanthamoorthy 2011). The 3D shape is variable and complex, with the complexity increasing as the environment and

microbial communities' complexity increases (Klapper and Szomolay 2011). Living as a biofilm allows microorganisms to cope with environmental stresses in a variety of ways including: increased resistance to antimicrobials, predation, and host defenses (Lewis 2007); improved attachment to substrate to overcome shear forces; exchange of plasmids, genetic information, especially at liquid air interface because of increased cell density and increased oxygen (Krol *et al.* 2011); and cell to cell communications or quorum sensing (Cogan *et al.* 2011). The ability of a biofilm to overcome these potentially selective forces suggests biofilm is the way for most bacteria to exist regardless of its metabolic cost (Shafai and Vafai 2011). Since its discovery the importance of biofilm has been the focus of much research.

The majority of the research being done on biofilm centers on the formation, protection or degradation of biofilm. One reason for doing this research is to increase understanding of how we can manipulate microorganisms to stop biofilm formation of pathogenically important microbiological colonies or communities responsible for biofouling. *E. coli* O157:H7 is not the only pathogen that produces biofilm. Microorganisms that produce biofilm are responsible for more than 14 million infections with more than 350,000 deaths a year (Wolcott *et al.* 2010). Studies have shown that pathogenic microorganisms that produce biofilm show a substantial increase in antibiotic resistance along with decreasing the host healing and should be treated like cancer because biofilm infection result in uncontrolled cell division that overwhelms host immunity (Wolcott *et al.* 2010). The complexity of pathogenesis increases as the number of microorganism species increases and can result in chronic infections of the sinuses or tonsils (Wolcott *et al.* 2010). Formation of biofilm on food processing equipment by *E. coli* O157:H7 can lead to an outbreak of potentially lethal food poisoning and production of a biofilm increases its resistance to sanitation. This is particularly true in cases where *Acinetobacter calcoaceticus* forms biofilm in multispecies culture which increases the colonization of *E. coli* O157:H7 by up

to 400 fold (Habimana *et al.* 2010) New pharmaceuticals or sanitation chemicals targeting pathogens abitlity for biofilm formation are being investigated for treatment of biofilm infections, because these drugs do not kill the organisms and therefor produce little selective pressure on the microorganisms in comparison to traditional antibiotics (Sambanthamoorthy *et al.* 2011). In food processing industries, the formation of biofilm on food processing equipment by STEC can lead to an outbreak of lethal food poisoning and production of biofilm exacerbates challenges involved with sanitation of equipment.

Biofilm formation, a complex process, relies on the regulation of multiple gene pathways controlled by external signals, both environmental (Sule *et al.* 2011) and biological (Gupta *et al.* 2013). In *E. coli,* biofilm production is generally dependent on nutrient composition and temperature in the environment (Prüβ *et al.* 2010). Other chemicals in the environment can trigger responses, allowing the bacteria to cope with a variety of environmental stresses. Both *csg* and *flhD* operons, which consist of multiple well characterized genes, have significant impacts on biofilm formation. In STEC, *flh*C is the second gene in the *flhD* operon that encodes the master regulator of motility. FlhDC of *E. coli* O157:H7 regulates 287 genes involved in various aspects of cell physiology, including metabolic processes, cell division, and virulence (Sule *et al.* 2011). Some of these genes are related to flagella protein production as well as assembly, including *fli* and *flg* operons (Ogasawara *et al.* 2011). The protein CsgD, the master regulator of biofilm formation in STEC, promotes not only the *csg* operon which codes for curli (a type of aggregative fimbriae that are often associated with virulence) production but also other genes involved in changing mode from planktonic to biofilm (Ogasawara *et al.* 2011, Uhlich *et al.* 2002). CsgD regulated genes that promote biofilm formation include *adr*A, which controls the production of cyclic-di-GMP, that modulates cell physiology changes in the bacteria (Wolfe and Visick 2008). CsgD simultaneously up regulates genes that promote biofilm formation

while at the same time down regulates genes used for motility (Ogasawara *et al.* 2011). The DNA spacer sequence between *fli*E and *fli*F has multiple binding sites for CsgD one of these sites overlaps that of FlhDC, so when CsgD is bound it represses expression of FliF and FliE (Ogasawara *et al.* 2011). This competitive binding of two master regulators acts like a genetic switch. If the environment is conducive of biofilm formation, CsgD will bind to spacer sequence interrupting the binding of FlhDC repressing *fliE* and the *fliF* operon needed for planktonic existence. Because of the role of CsgD and FlhDC as master regulators of the planktonic/biofilm phenotype change, the regulation of both must be under tight control to prevent the formation of biofilm in unfavorable environments or motility in favorable environments. Multiple other regulators not discussed here also play into the control of this molecular switch; for a review of some of these related with the control of CsgD please see Ogasawara *et al.* (2010). The complexity of these molecular switches is increased when looking across stains, because not all strains have shown dependence on the same molecular switch. For example strains have a range of dependence on *csg*A, some strains depend on *csgA* while others do not (Carter *et al.* 2011). Because of its complexity, biofilm formation has led to multiple debates about which genes/proteins are important in both spatial and temporal models for example: in the case of *Pseudomonanas aeruginosa*, one of the major genes that play a role in biofilm formation is *motA. I*f *motA* is inhibited then formation of biofilm is also inhibited (Hu *et al.* 2011). In *Enterococcus faecalis*, an organism that is common in various nosocomial infections, inhibiting the gene *bgs*B, which codes for a glycolsytransferase, will also inhibit biofilm formation (Theilacker *et al.* 2011). Overall, no biofilm specific genes have been identified leading to the understanding that biofilm formation is a result of many metabolic and regulatory networks. Control of these networks could be used to reduce biofilm.

Microorganisms' control of phenotypic expression level is not limited to variation of signal transduction, but rather involves a complex system of controlling transcription itself. Phenotype can also be changed by changing the DNA sequence, via mutation, leading to increased fitness and evolution in an organism over a long period of time. Another way that DNA sequences can be changed or added is the use of movable DNA segments called Insertion Sequences (IS) that influence gene activity by moving in an action called transposition (Wang and Wood 2011). Insertion sequences can increase survival of microorganisms in harsh environments by enhancing adaptations (Zhang and Saier 2009), Koonin and Wolf hypothesized mutations can be dependent on environment (2009). In environments that favor motility, IS5 is inserted in front of *fhl*D which causes a hereditary increase in motility by promoting the production of FlhDC in *E. coli* (Wang and Wood 2011). Further study into these mechanisms can be used to develop new techniques for food processing to help eliminate or reduce biofilms reducing the risk of many food borne pathogens and reducing the number of outbreaks they are associated with.

Current control efforts

Food regulatory agencies have a zero tolerance policy for *E. coli* O157:H7 and require extensive testing by the manufactures to help protect the community. In response to this zero tolerance policy, food processing companies have developed various processes to help them insure the quality and safety of their product. These processes involve treating the meat with various chemicals, the identity of which is dependent on the food type: examples include chlorine for vegetables or nitrates and organic acids for meat. By creating environmental hurdles for the bacteria to overcome these treatments can along with storage and packaging technologies, increase food safety as well as the shelf life of the product by reducing bacterial loads. The increase in shelf life has become increasingly important because of the globalization of the food

industry, which often involves the transport of food over long distances before consumption (Ouattara *et al.*1997).

As an example, chlorine treatment, a common sanitation treatment used for vegetables does reduce the risk, but it is not perfect and can lead to phenotypic changes in the bacteria. This treatment has been shown to up regulate the gene *ycfR* in *E. coli* O157:H7 (Deng *et al.* 2011). This gene is responsible for production of the YcfR membrane protein that results in changes in membrane hydrophobicity in *E. coli* K-12 (Zhang *et al.* 2007). The same protein in *E. coli* O157:H7 has an unknown function and does not change the hydrophobicity but is linked to chlorine resistance, which shows *E. coli* O157:H7 can change its phenotype to help it overcome environmental stress (Deng *et al.* 2011). So, new treatments must be developed to reduce the *E. coli* O157:H7 in vegetables.

E. coli O157:H7 has been associated with vegetables. Even though it has more historically been associated with meat and meat products. One of the oldest processes in meat manufacturing is treating it with salt (curing) which reduces water activity and inhibits bacterial growth (Parthasarathy and Bryan 2012). Most curing uses nitrites or nitrates which are easily converted into nitrites by bacteria (Binkerd and Kolari, 1975). Curing with nitrites not only reduces bacterial loads but also stabilizes the fat in the muscle preventing the meat from going rancid (Reddy et al 1983) along with providing a visual cue (brown color) for spoilage or improper storage (Parthasarathy and Bryan 2012). While curing meat does have some advantages it has recently come under scrutiny.

This is especially true in many different processed food sectors including both organic foods as well as nitrate/nitrite free foods. Sodium nitrite, the most commonly used curing agent, is classified as a chemical additive and cannot be used in organic food processes (Mead *et al.* 2000). Organic food production requires the use of natural curing agents. One example of these

is the use of celery powder, which with is high in nitrates and through fermentation can produce nitrites and give the processed foods many of the same characteristics of food cured in a traditional sense (Sindelar 2007). While this treatment uses a lowered input of nitrite, it may increase the risk of food borne pathogens and spoilage microorganisms requiring the use of other processes like antimicrobials to reduce these risks (Sullivan *et al.* 2012). The reason that many food processors have lowered the amount of nitrate/nitrite they add to meat is related to N-nitrosamines.

In the 1970's, N-nitrosamine from cigarette smoke was identified as being carcinogenic in animals (Bryan et al 2013). With respect to foods, nitrites can convert into N-nitrosamines through microbial metabolism in the stomach (Archer 1989), a process that has been shown by epidemiological studies to be associated with certain cancers (Larsson *et al.* 2006). These findings forced food processing companies to find alternative processing and ingredients to decrease the formation of N-nitrosamines (Assembly of Life Sciences (US) Committee on Nitrite and Alternative Curing Agents in Food 1981). For a comprehensive review on the debate that followed the initial observation, please see Bryan *et al.* 2012. This article outlines the cause for the nitrite debate is inconsistent findings in research which leads to the overall conclusion, current evidence shows the risk of stomach cancer is not linked to normal dietary nitrate/nitrite intake. Despite these findings, a large need to identify other chemicals to use in the curing of meat still exists to increase food safety.

Curing is not the only process meat processers use to reduce the risk of food borne illness and stabilize food quality. Another safety precaution meat often goes though is treatment with chemicals post-harvest. A common group of chemicals used in treating meat post-harvest is weak acids, most commonly organic acids, to help reduce bacterial loads (Carpenter *et al.* 2011; Yoder et el. 2012).. Organic acids are used because they are Generally Regarded As Safe

(GRAS) and are effective against many food spoilage organisms, though the effects of the acid is largely dependent on the organisms it is used on and work on either a linear or threshold type action (Ouattara *et al.*1997). Organic acids inhibit microbial growth by both uncoupling energy production and regulation and by toxic accumulation of disassociated acid anion (Taylor *et al.*2012). Acid treatment effectiveness can be altered by the environment and have proven ineffective in preventing *E. coli* O157:H7 with only a very slight inhibition that quickly wears off (Brackett *et al.*1994). For example, organic acids are effective against *Campylobacter jejuni* in broths but have a greatly reduced effect when used on meat (Birk *et al.* 2009). While the acid treatments can be effective against many spoilage and some pathogenic microorganisms, they may also induce an acid tolerance response (ATR), a complex phenomenon (Álvarez-Ordóñez *et al.* 2009) leading to an increased resistance to acid (Foster and Hall, 1990). This increase in tolerance can be seen in multiple food borne pathogens including *Listeria monocytogenes* (Gahan and Hill 1999), *Salmonella typhimurium* (Álvarez-Ordóñez *et al.* 2009), and *E. coli* O157:H7 (Leenanon and Drake 2001). ATR as a result of treating meat with organic acids may have unforeseen consequences of increasing pathogens resistance to the high acid environment of the digestive system, increasing the chance of food borne illness (Álvarez-Ordóñez *et al.* 2009). This concern is justified considering citric, acetic, and lactic acids are not only the most commonly used in meat processing for carcass decontamination (Smulders and Greer 1998) and these acids have been shown to cause the highest ATR in *S. typhimurium* (Álvarez-Ordóñez *et al.* 2009). The use of organic acids for decontamination, while effective, may also have negative side effects, as an example increasing the ATR, so new food processing practices must be developed to increase food safety.

Current research in control efforts

Some newer treatments that are being investigated are based on understanding complex signal transduction pathways in bacteria, including Quorum Sensing (QS) and Two Component Signal Transduction Systems (2CSTS). These signal pathways may be able to be manipulated to control *E. coli* O157:H7 phenotypic properties.

Qourum sensing (QS) is a signaling process that is used by a bacterial community to control gene regulation on a population level allowing for the microbiological community to interact and to coordinate shifts in gene regulation as a community. QS is a fundamental part of many pathological processes like biofilm formation and toxin production (Dobretsov *et al.* 2009) and can be considered a virulence factor (Lim *et al.* 2012). This makes manipulation of QS a way to control bacterial phenotypes (Bassler 2010; Njoroge & Sperandio 2009; Raina *et al.* 2009). Biofilms in turn have been shown to increase QS responses(Cogan *et al.* 2011) and increase host immune evasion (Dobretsov *et al.* 2009). QS can be considered a virulence factor, in that it allows for phenotype changes on a population level QS takes place between microorganisms not only of the same species but between multiple species, but also between different kingdoms (Atkinson and Williams 2009). QS takes place by microbes using Auto-Inducer (AI) molecules, produced by the organism. As the population of microorganisms increase so does the concentration of the AI. At low concentrations it diffuses out of the cells but as the concentration increases it passes a threshold and diffuses back into the cells, signaling for phenotypic changes on a population level. This allows the community of microorganisms to monitor their numbers, both on an interspecies and intraspecies level. Many pathogens use this type of signaling to control pathogenicity level to evade host immune responses until the population is large enough to overcome the host immunity (Dobretsov *et al.* 2009). Interrupting QS would slow or prevent virulence factors including, biofilm formation and toxin production,

but not killing the organisms and reducing the selective force applied to the microorganism in a community level (Ren *et al.* 2004). One example of this is the inhibition of *E. coli* O157:H7 biofilm formation and virulence by a *Bifidobacterium longum* protein ATCC 15707 which interrupts the action of AI-2 molecules (Kim *et al.* 2012). These same principles could be applied to food manufacturing practices to help limit pathogenic bacterial growth on food.

Two-component signal transduction systems (2CSTS), another type of signaling, have also been shown to regulate various genes involved in virulence, providing another way to control the bacterial phenotype (Kostakioti *et al.* 2009). This type of signaling is different from QS in a way that QS signals are produced by the microorganisms. In 2CSTS, the signals come from the environment. Before an environmental signal can cause a change in phenotype, information on the environment must be collected and then transmitted inside the cell though various physiological pathways in a process called signal transduction. The process of signal transduction is dependent on the species of microorganism though many similarities in genes and pathways can be seen in between different species. One of the most common systems of signal transduction is the use of a 2CSTS which, as its name implies, relies on two separate components. These components include a histidine kinase, generally a membrane protein, and a response regulator that regulates gene expression on a transcriptional level (Chakrabority *et al.* 2011). When stimulated by the environment, the kinase engages in an auto-phosphorylation event and then transfers the phosphate to the response regulator, causing a conformational change. Once activated by a phosphorylation event, the regulator molecule has the ability to act in many different ways: it can bind to DNA to affect the cells transcription process or it can bind to the flagellar switch and reverse the rotation of the flagellum. Ultimately, this will result in a change in phenotype (Fredrick *et al.* 2011).

Not all histidine kinases are membrane proteins. One such exception is CheA which is involved with chemotaxis and found in the cytoplasm (Wang *et al.* 2012). As one other example of a 2CSTS, one of *S. typhimurium's* kinases, KdpD, is activated by low K+ concentrations to allow the bacteria to cope with osmotic and oxidative stresses (Alegado *et al.* 2011As one example of interrupting *E. coli* O157:H7 2CSTS to control virulence factors; LED209, a small organic molecule, can be used to bind to the histidine kinase QseC preventing it from up regulating virulence factors (Rasko *et al.* 2008).

Rationale

As previously presented in this review, both QS and 2CSTS have been used to control virulence factors of *E. coli* O157:H7. With more research, these mechanisms may be used to control virulence factors of *E. coli* O157:H7 in both preventative and curative ways. Previous work from our lab using Phenotypic MicroArrays has shown that externally added nutrients can influence biofilm amounts. For example, many 6 carbon sugars increased biofilm amounts, whereas the 5 carbon sugar D-ribose decreased biofilm amounts (Prüβ *et al.*, 2010). Though not all nutrients could be connected to specific 2CSTSs, we were able to show that biofilm amounts formed on C4-dicarboxylic acids were reduced in a *dcuR* mutant (Prüβ, *et al.*, 2010). DcuSR had previously been shown to be involved in metabolism of C(4)-dicarboxylic acids (Golby *et al.*1999).

This research is an attempt at controlling *E. coli* O157:H7 phenotypic properties using supplemental nutrients (carbon and nitrogen), the specific 2CSTS that is involved in their signaling is not being tested at this time. We propose using supplemental nutrients to stimulate the bacteria to divide more slowly and produce less biofilm. Because we are looking at nutrients as a control mechanism we suspect that this control mechanism would produce little selective pressure in comparison to treatments focused on killing bacteria. We also believe that these

treatments may also affect other microorganisms not tested, including other serotypes of *E. coli* and possibly other bacterial species, because the nutrient used would affect metabolism.

MATERIALS AND METHODS

Bacterial strains and growth conditions

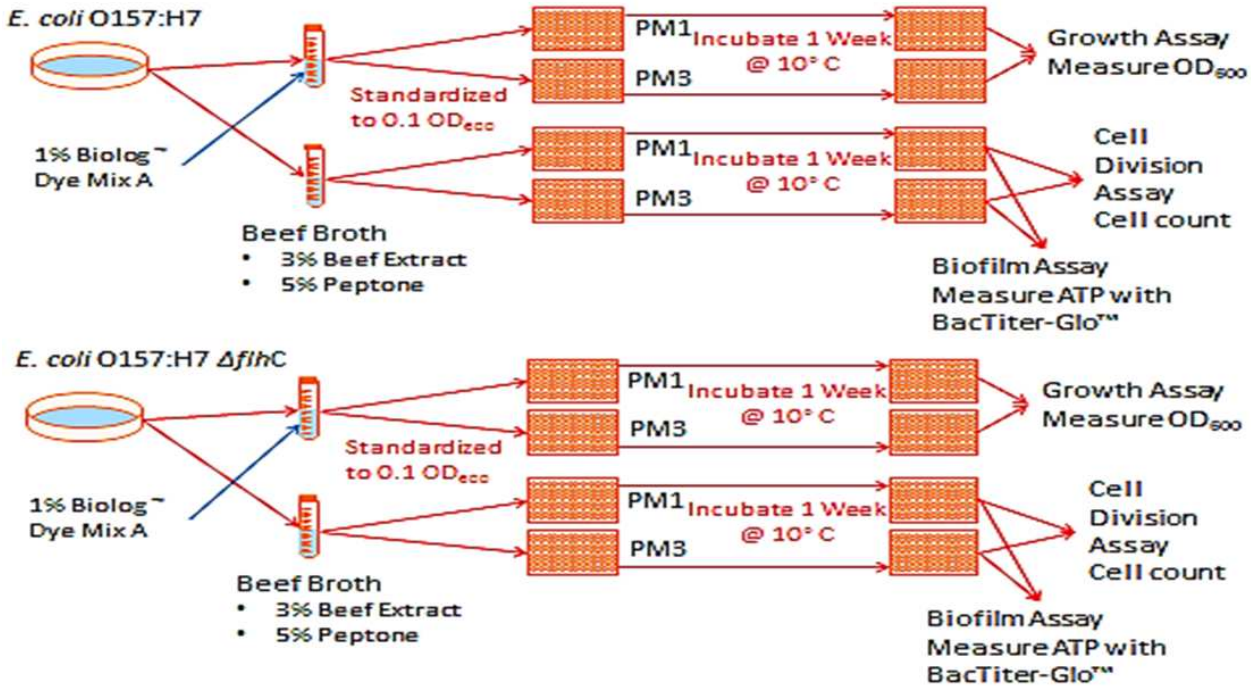
Escherichia coli O157:H7 (American Type Culture Collection, ATCC 43894) and its isogenic *flhC* mutant (Dobbin *et al.*, 2006) were obtained from Scott A. Minnich (University of Idaho, Moscow). Previously, these strains had been made resistant to streptomycin sulfate (Sigma-Aldrich, MO), and the parent strain is alsoresistant to nalidixic acid (Sigma-Aldrich, MO) (Sule *et al.*, 2011). These strains were stored in dimethyl sulfoxide (DMSO) at -80°C. Before use, all strains were streaked out for isolation on Luria-Bertani (LB) plates (10 g tryptone, 5 g yeast extract, 10 g NaCl, 15 g agar, 1 l ddH_2O) and incubated overnight at 37°C.

Phenotypic MicroArray screening of carbon and nitrogen sources

BioLog Phenotypic MicroArrays (PM) (Hayward CA) are a technology used to determine bacterial growth (Bochner *et al.* 2001; Bochner 2003; Bochner *et al.* 2008; Bochner 2009). This technology consists of 96 well plates with 95 different chemicals dried in 95 of the wells, with one well as a control. These plates in conjunction with a company provided tetrazolium dye can be used to measure respiration (indicative of growth) of the bacteria under the effects of different chemicals depending on the type of PM plate used (Zhou *et al.* 2003). The PM technology can also be used to measure biofilm amounts if used in combination with crystal violet or an ATP assay instead of the tetrazolium dye (Sule *et al.* 2009). PM1 plates having 95 different carbon sources and PM3 plates having 95 nitrogen sources were used. Figure one shows the layout of the experiment. Both *E. coli* strains were standardized to 0.1 OD_{600} in sterile beef broth (3% beef extract, 5% peptone) to provide two inocula. To determine respiration/growth, 1% Biolog Dye Mix A tetrazolium dye was added to each inoculum, this dye was left out of the inocula for the bacterial cell count and biofilm amount determinations. PM1

and PM3 plates were inoculated with 100 µl of prepared inoculum. This was done separately for the two strains. All plates were then incubated at 10°C, to simulate meat storage, for seven days.

Figure 1. Experimental flow for Phenotypic MicroArray experiments



Experimental design used for Phenotypic MicroArray experiments. Design uses both strains on both PM1 and PM3 plates to give data for OD_{600} representative of growth/respiration, ATP representative of biofilm amounts, and CFU/ml representative of cell number.

In order to determine respiration/growth, the OD_{600} was measured from the plates containing Dye mix A using an EL808 Ultra Microplate reader (Bio-Tek instruments, Winooski, VT). To determine bacterial cell counts, bacterial growth media from the wells were serially diluted in phosphate buffered saline (PBS), plated onto LB agar plates, and incubated overnight at 37°C. Bacterial cell counts are expressed in Colony Forming Units (CFU) per ml of original growth medium. For the determination of biofilm amounts, wells were drained of growth medium. Biofilms were gently rinsed with 100 µl PBS. 100 µl of BacTiter-Glo™ reagent (Promega, Madison WI) was added to each well. The bacteria were homogenized in the reagent by carefully pipetting up and down. The reagent was then transferred to a 1.5 ml centrifuge tube

and relative luminescence was then measured using a TD 20/20 illuminometer (Turner Designs, Sunnyvale, CA). To test reproducibility experiments were repeated; respiration and biofilm experiments were repeated 4 times, and the CFU/ml experiments were repeated 3 times.

An ANOVA was used to reduce the possibility of showing significance when no significance exists, a type one statistical error. Data was normalized using a natural log transformation than analyzed using an Analysis of Variance (ANOVA) with SAS 9.3 (SAS, Cary NC) to determine the statistical significance of the differences between wild-type and mutant, as well as significant differences in between individual chemicals within a plate (carbon or nitrogen). Significance was determined with a Dunnett's test with a p threshold of <0.05. Individual replicates were also tested to insure they did not vary in comparison to the others.

In a second analysis, results of all chemical filled wells were divided by the negative control well from the same plate to produce a ratio of chemical /control. Ratios were then averaged across replications and standard deviations were calculated. This was done separately for respiration/growth, bacterial cell count, and biofilm amounts. Chemicals were then ranked according to their effect on bacterial respiration, cell division rate, and biofilm amounts. Chemicals having the causing reductions and having low ratios in two of the three phenotypes examined in the wild-type were considered to be the top performing chemicals.

Determination of the IC_{50} values for the eight top performing chemicals

Top performing chemicals from the first screening experiment were suspended in ddH_2O to the concentrations listed in Table 1 and filter sterilized. Differences in concentrations used to account for different chemical solubility ddH_2O. 100 µl of these dilutions was added to each well of a 96 well plate. *E. coli* O157:H7 was then standardized in 2 x beef broth (6% beef extract, 10% peptone) to 0.2 OD_{600} to produce the inoculum. 100 µl of this solution was inoculated into each well and mixed with the chemical solution. Plates were incubated at 10°C for four days. At

the end of this incubation period, the same procedures were performed as for the initial screening experiment to determine chemical effects on OD_{600} and CFU/ml and to prepare the biofilm for the ATP measurement. Of the 200 µl mix of bacterial solution and BacTiter Glo, 50 µl was transferred from the test plate to a white 96 well plate and read with a gain of 100 on a Synergy H1 plate reader (Bio-Tek Instruments, Winooski, VT). OD_{600} and ATP measurements were repeated four times and the CFU/ml experiment was repeated three times.

Table 1. List of chemicals and the concentrations used for the determination of IC_{50} experiment

Chemical	Concentration 1	Concentration 2	Concentration 3	Concentration 4	Concentration 5
Acetoacetic acid	150 mg/mL	70 mg/mL	15mg/mL	7mg/mL	1.5 mg/mL
L-lyxose	250 mg/mL	50 mg/mL	10 mg/mL	2 mg/mL	0.4 mg/mL
D,L-α-glycerol-P	250 mg/mL	50 mg/mL	10 mg/mL	2 mg/mL	0.4 mg/mL
D,L-α-Amino-Caprylic acid	10 mg/mL	2 mg/mL	0.4mg/mL	0.08 mg/mL	0.016 mg/mL
D-Mannosamine	330 mg/mL	65 mg/mL	13 mg/mL	2.5 mg/mL	0.5 mg/mL
β-Phenylethylamine	150 mg/mL	70 mg/mL	15 mg/mL	7 mg/mL	1.5 mg/mL
Thymine	10 mg/mL	2 mg/mL	0.4mg/mL	0.08 mg/mL	0.016 mg/mL
D-Asparagine	10 mg/mL	2 mg/mL	0.4mg/mL	0.08 mg/mL	0.016 mg/mL

Chemicals considered top performing from Phenotype MicroArray experiments and the concentrations used for the determination of IC_{50}

Data were analyzed by dividing the values for each chemical and concentration by those of the negative control that contained unsupplemented liquid beef broth alone. Averages and standard deviations were calculated across the replicate experiments and plotted against the concentration of the respective chemical. Each resulting curve was then analyzed using MasterPlex ReaderFit 2010 (Hitachi Solutions LTd. 2012) to determine trends and calculate the concentration of the chemical needed to inhibit OD_{600}, ATP, and CFU/ml by 50% (IC_{50}). ReaderFit 2010 was used to compare multiple regression models and determine best fit trend. Trend lines with positive slopes were not calculated. Those chemicals with trends having an R^2 value greater than 0.9 and with IC_{50} concentrations of less than 10 mg/ml in both CFU/ml and

ATP were considered top performing chemicals. These chemicals were selected for further testing on beef.

Effect of β-phenylethylamine and acetoacetic acid on bacterial cell counts of E. coli O157H:7 on beef

Beef round roast purchased from a local retailor was submerged in boiling water for 45 seconds. Cooked meat was aseptically removed from the roast producing clean beef muscle. Clean muscle was cubed to approximately 25g and 13 pieces were placed on separate clean trays. Table 2 summarizes how the beef pieces were treated with the chemicals and inoculated with the bacteria.

Table 2. List of samples used for beef experiment

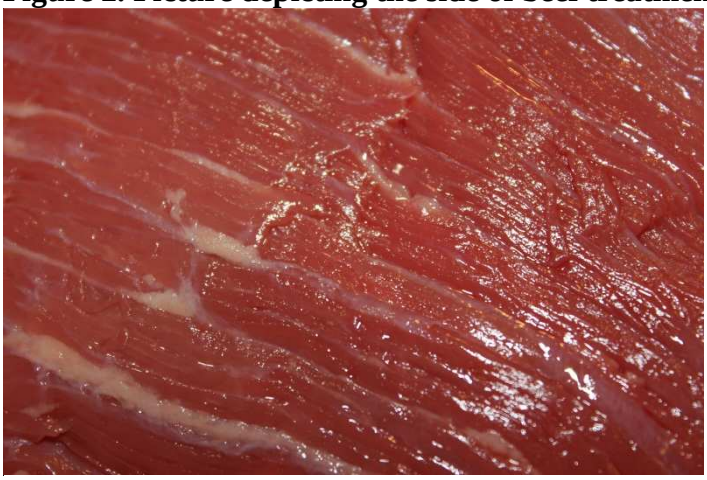
Sample	Chemical	Concentration	Inoculate
1	None	None	None
2	None	None	2.0×10^5 CFU
3	ddH_2O	None	2.0×10^5 CFU
4	β-Phenylethylamine	150 mg/mL	2.0×10^5 CFU
5	β-Phenylethylamine	70 mg/mL	2.0×10^5 CFU
6	β-Phenylethylamine	15mg/mL	2.0×10^5 CFU
7	β-Phenylethylamine	7mg/mL	2.0×10^5 CFU
8	β-Phenylethylamine	1.5 mg/mL	2.0×10^5 CFU
9	Acetoacetic acid	150 mg/mL	2.0×10^5 CFU
10	Acetoacetic acid	70 mg/mL	2.0×10^5 CFU
11	Acetoacetic acid	15mg/mL	2.0×10^5 CFU
12	Acetoacetic acid	7mg/mL	2.0×10^5 CFU
13	Acetoacetic acid	1.5 mg/mL	2.0×10^5 CFU

Sample layout for treatments used for the beef experiment including the concentrations and the inoculum used.

Two pieces were left untreated with chemicals, one as a beef control (sample 1) which was not inoculated to test whether beef was contaminated prior to treatmentand one as a nontreated/negative control (sample 2) which was inoculated with the tested strain. One piece

was treated with ddH_2O to act as a solvent control (sample 3) to test whether the ddH_2O had an effect on CFU/ML. Five dilutions of β-phenylethylamine and acetoacetic acid were prepared in ddH_2O to use as treatments (sample 4-13). 200 µl of each chemical treatment was pipetted over the grained side of the beef Figure 2.

Figure 2. Picture depicting the side of beef treatment was applied to



Beef pieces were treated by pipetting 200µl of treatment onto the grained side of the beef. The side of beef muscle displayed above. This side was used to simulate the outside of beef carcass.

The *E. coli* O157:H7 strain was standardized in ddH_2O to 0.1 OD_{600} to produce the inoculum. This inoculum was serially diluted and plated onto LB agar plates to determine the CFU/ML of the inoculum and estimate the number of bacteria the beef was inoculated with. Beef was then inoculated with 20 µl of the inoculum (˜2.0 x 10^5 CFU), which was pipetted onto the treated side of the beef. Beef was incubated for 1 week at 10°C. After incubation, each piece of beef was placed in a sterile stomacher bag with 100 ml of Tryptic Soy Broth (TSB) (17g tryptone, 3g soytone, 2.5g dextrose, 5g NaCl, 2.5g dipotassium phosphate, and 1L ddH_2O) and homogenized at 200 RPM for one minute in a Stomacher 400 Circulator (Seward Ltd, West Sussex United Kingdom). This liquid was serially diluted and plated on LB and streptomycin Sorbitol MacConkey agar plates (SMAC) (peptones 20g, D-sorbitol 10g, bile salts 1.5g, sodium chloride 5.0g, neutral red 0.03g, crystal violet 0.001g, agar 15g, ddH_2O 1 l), supplemented with

1000 µg/ml streptomycin to calculate recoverable CFU/ML. This experiment was repeated 4 times to test reproducibility. Data was then analyzed using a *t*-test to test for the statistical significance of the reduction in CFU/ML that the treatments caused.

RESULTS

Phenotypic MicroArray screening of the effect of carbon and nitrogen sources on E. coli O157:H7 growth/respiration, biofilm amounts and bacterial cell counts

The ANOVA output (Table 3) for the PM1 plates (carbon sources) showed that there were statistical differences between the two strains in regard to OD_{600}. The *flhC* mutants produced higher OD_{600} (indicative of growth/respiration), but no significant differences in regards to ATP and CFU/ml. The ANOVA results showed significance in the reduction of CFU/ml with the addition of acetoacetic acid to the well in comparison to the negative control. Figures 3-8 show chemicals with the greatest effect on the wild-type for given parameter and compares those effects to the mutant.

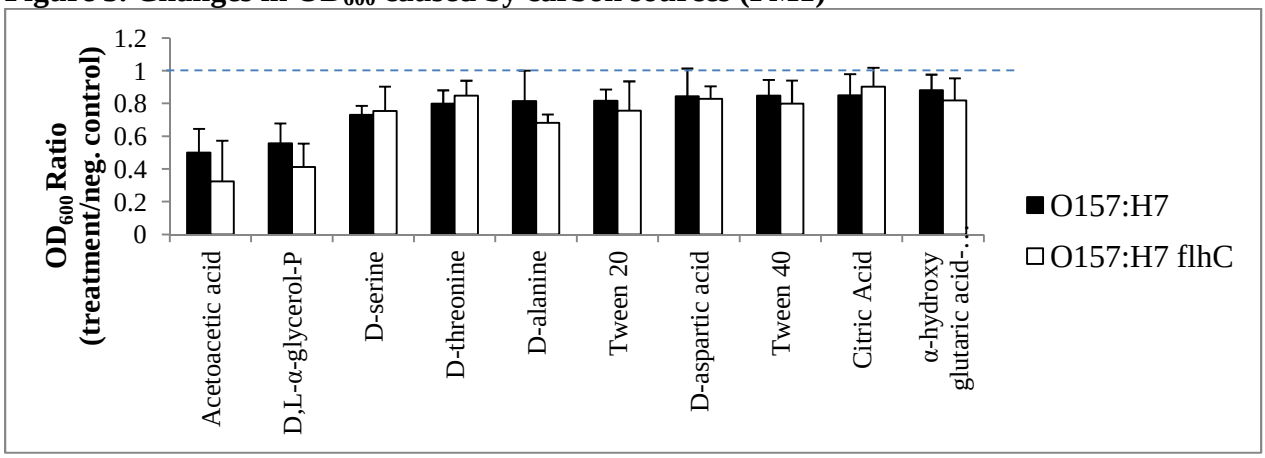
Table 3. ANOVA outputs for Phenotypic MicroArray Experiments

	Experiment	Wild Type vs Mutant	Chemicals Causing a Significant Decrease
Carbon PM1	OD_{600}	Mutant Greater	No significant Decrease
	ATP	No Significant Differences	No significant Decrease
	CFU	No Significant Differences	Acetoacetic acid
Nitrogen PM3	OD_{600}	No Significant Differences	Hydroxylamine D,L-α-Amino-Caprylic Acid
	ATP	Mutant Greater	Hydroxylamine D,L-α-Amino-Caprylic Acid L-Glutamic Acid Nitrate D-Aspartic Acid D-Mannosamine
	CFU	Mutant Greater	No significant Decrease

ANOVA outputs showing significant differences between strains for all 3 experiment parameters when grown in the presence of carbon sources in regards to $OD_{600,}$ and ATP and CFU/ml for nitrogen sources, as well as showing chemicals that significantly decreased any of the evaluated phenotypes.

Acetoacetic acid had the greatest effect on OD_{600}, reducing it by 50%. D,L-α-glycerolphosphate, D-Serine and D-threonine also reduced OD_{600}, by 44% and 26% and 20% respectfully (Figure 3).

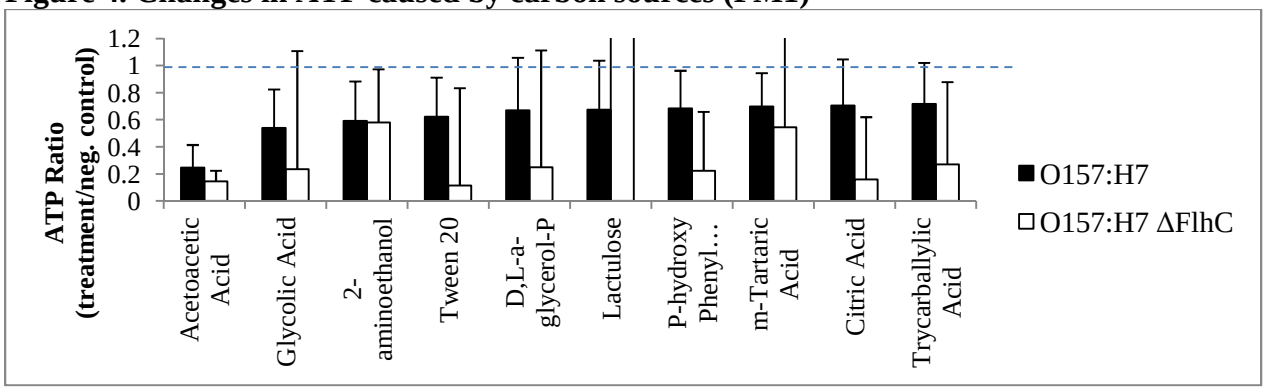
Figure 3. Changes in OD_{600} caused by carbon sources (PM1)



Change in OD_{600} produced by individual carbon sources on both wild-type and mutant in the Phenotypic MicroArray experiments. Chemicals are ranked by greatest effect on the wild-type, with dashed line representing normal growth of strains in beef broth.

Acetoacetic acid showed the greatest reduction in ATP as well, reducing it by 75%, followed by glycolic acid with a reduction by 46%, 2-aminoethanol by 40%, tween 20 by 37% and D,L-a-glycerol-P by 33% (Figure 4).

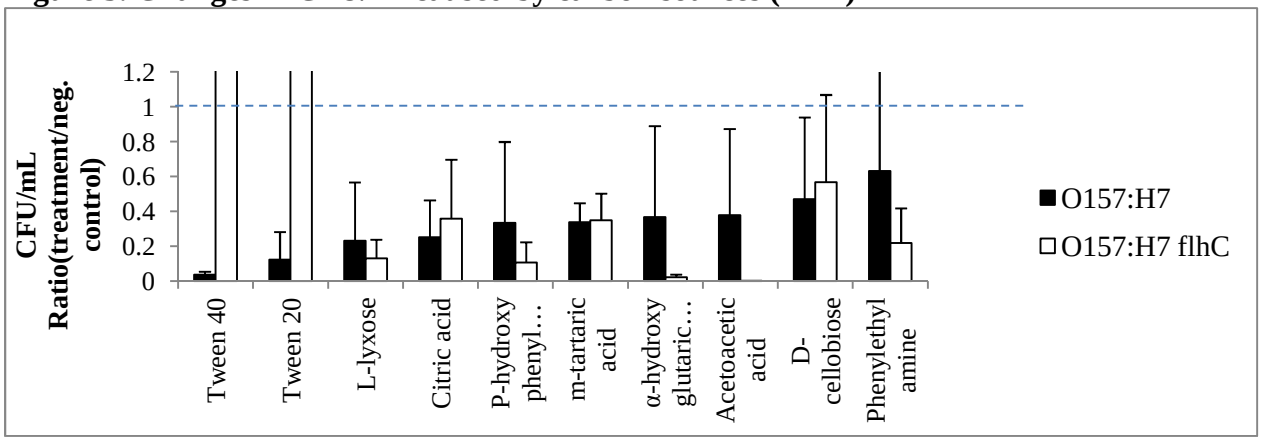
Figure 4. Changes in ATP caused by carbon sources (PM1)



Change in ATP produced by individual carbon sources on both wild-type and mutant in the Phenotypic MicroArray experiments. Chemicals are ranked by greatest effect on the wild-type, with dashed line representing normal growth of strains in beef broth.

AAA also reduced the CFU/ml by 62%, but was out preformed in this test by tween 40 which reduced the growth by 94%. The following chemicals reduced CFU/ml as well: tween 20 by 87%, L-lyxose by 77%, citric acid by 75%, P-hydroxy phenyl acetic acid by 66%, m-tartaric acid by 66%, and α-hydroxy glutaric acid-lactone by 63% (Figure 5). As a note of interest, CFU/ml values of the mutant were unaffected by both tweens.

Figure 5. Changes in CFU/ml caused by carbon sources (PM1)

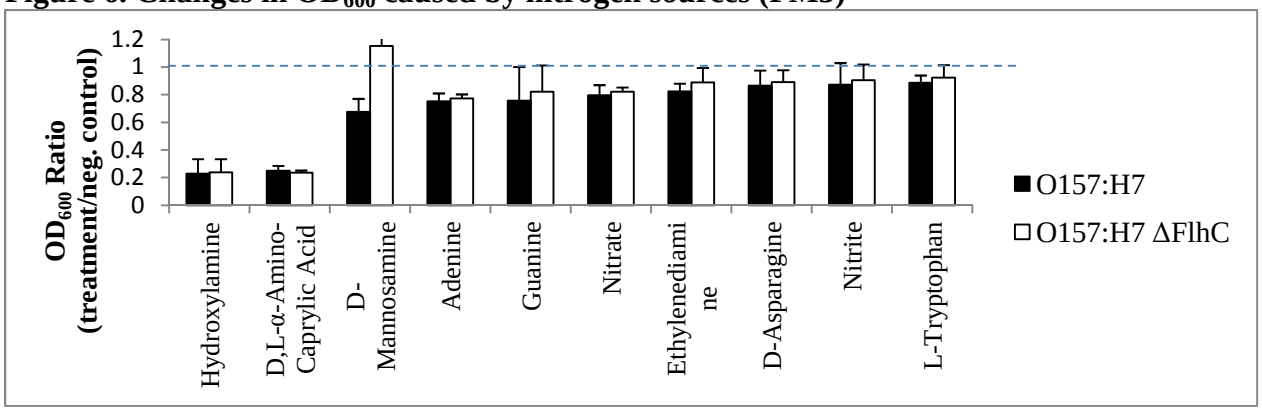


Change in CFU/ml produced by individual carbon sources on both wild-type and mutant in the Phenotypic MicroArray experiments. Chemicals are ranked by greatest effect on the wild-type, with dashed line representing normal growth of strains in beef broth.

The ANOVAs for the PM3 (nitrogen sources) plate determined that the two strains did significantly differ with the mutant having a higher amount of biofilm production and higher cell division rates, and individual nitrogen sources did impact both biofilm formation and cell division (Table 3). Hydroxylamine and D,L-α-amino-caprylic acid significantly reduced Respiration and biofilm amounts. L-glutamic acid, nitrate, D-aspartic acid, and D-mannosamine also significantly reduced biofilm formation.

The greatest reduction in OD_{600} for both strains was in hydroxylamine by 77% and D,L-α-amino-caprylic acid by 75%. D-mannosamine, adenine, guanine, and nitrate also all reduced OD_{600} by 32%, 24%, 23% and 19% respectively (Figure 6).

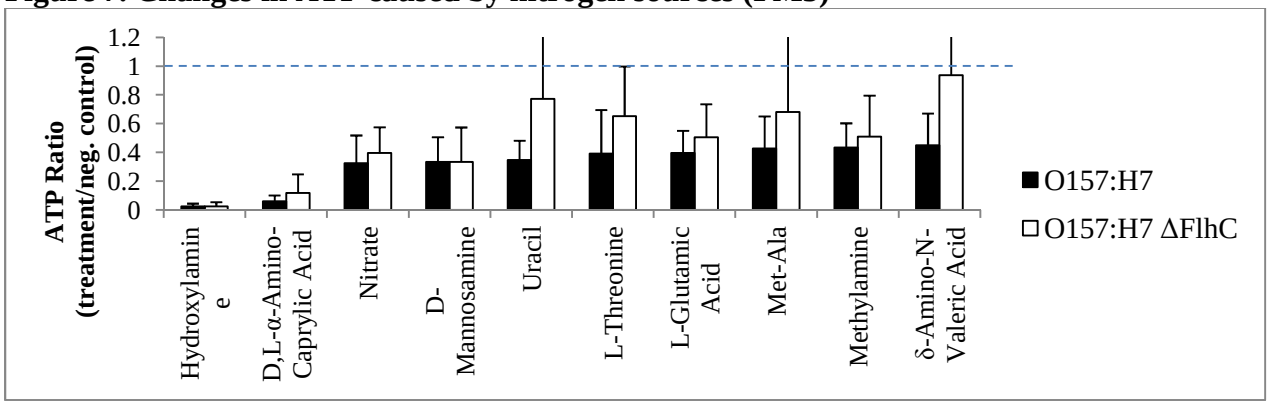
Figure 6. Changes in OD_{600} caused by nitrogen sources (PM3)



Change in OD_{600} produced by individual nitrogen sources on both wild-type and mutant in the Phenotypic MicroArray experiments. Chemicals are ranked by greatest effect on the wild-type, with dashed line representing normal growth of strains in beef broth.

Many chemicals reduced the amount of biofilm. Hydroxylamine and D,L-α-amino-caprylic acid both reduced the ATP by 98% and 94%. They were followed by nitrate, D-mannosamine, uracil, L-threonine, L-glutamic acid, Met-Ala, methylamine, and δ-amino-N-valeric acid, all of which reduced biofilm by more than 50% (Figure 7).

Figure 7. Changes in ATP caused by nitrogen sources (PM3)

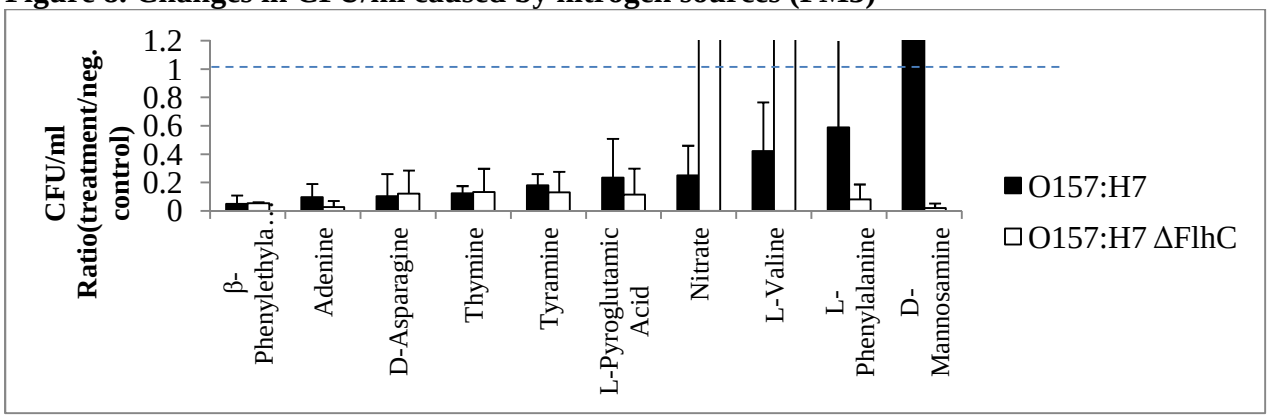


Change in ATP produced by individual nitrogen sources on both wild-type and mutant in the Phenotypic MicroArray experiments. Chemicals are ranked by greatest effect on the wild-type, with dashed line representing normal growth of strains in beef broth.

Many chemicals reduced the CFU/ml. The greatest reduction in CFU/ml was by β-phenylethylamine which reduced CFU/ml by 95% followed by adenine which reduced CFU/ml

by 90%, D-asparagine by 89%, thymine by 87%. Tyramine, L-pyroglutamic acid, nitrate, and L-valine all reduced CFU/ml by more than 50% (Figure 8).

Figure 8. Changes in CFU/ml caused by nitrogen sources (PM3)



Change in CFU/ml produced by individual nitrogen sources on both wild-type and mutant in the Phenotypic MicroArray experiments. Chemicals are ranked by greatest effect on the wild-type, with dashed line representing normal growth of strains in beef broth.

Over all, the top performing chemicals from PM1 plates were selected as follows: Acetoacetic acid is a byproduct of fatty acid metabolism (ketone body) so it is already present in the human body making it a possible candidate for treating meat. D,L-α-glycerol-phosphate, a food additive used as a stabilizer, is already used in food processing making it a good candidate for treatment on meat. L-lyxose is a rare 5 carbon sugar produced by some bacteria. *E. coli* cannot use this sugar for growth unless it is specially adapted to do so (Badia *et al.* 1991). This makes it a good candidate as a food additive to reduce growth of *E.coli* O157:H7. Many other chemicals performed well in this experiment, but were eliminated from the next experiment for various reasons. These include tween 20/40, which are emulsifiers and wetting agents that have a very low allowable safe dose of 0.25 mg/kg, making it possibly dangerous as a food additive. In addition, tweens may affect meat flavor and texture. For this reason, we eliminated it from further screenings. Citric acid is a natural food preservative already common in the food industry, but has been shown to have little effect on *E. coli* O157:H7 which wears quickly losses

its effectiveness (Brackett *et al.*1994). Citric acid effects on *E coli* O157:H7 has been shown to have inconsistent effects (Laury *et al.* 2009; Yang *et al.* 2009). Because these two reasons, we eliminated citric acid from further experiments. M-tartaric acid, a common wine additive and by product, has a very low maximum usable limit of 10 g/100 l, making it possibly dangerous as a food additive.

The treatments with the best performance on PM3 Plates were as follows: D-Asparagine is a non-essential amino acid so it would be safe for food processing uses. Thymine is a deoxyribonucleotide of DNA. β-phenylethylamine is a naturally occurring trace amine found in chocolate and has GRAS status making it ideal for use as a treatment for beef (Granvogl *et al.* 2006). D-mannosamine is a monosaccharide used for multiple cellular processes that is already found in the body, making it a possible candidate for beef treatment. D,L-α-amino-caprylic acid is an unnatural amino acid, or more simply non encoded amino acid (Ma 2003). Many such chemicals have shown to have antimicrobial properties making it a possible treatment (Hicks et al. 2013). As seen in the PM1 results, many chemicals that performed well were eliminated from further experiments. Nitrate is already used as a curing agent in food processing, but has been linked to cancer as previously described (Bryan *et al.* 2013). Hydroxylamine is an intermediate in nitrogen metabolism that can cause health problems (Desesso & Goeringer 1990). Table 4 summarizes the chemicals and their properties. Included are properties that disqualified the respective chemical form our next experiment (underlined), as well as chemicals that we did use for the determination of IC_{50} (bolded).

Table 4. List of chemicals with greatest reduction from PM experiment

	Chemical	Chemical Properties
Carbon	**acetoacetic acid**	Produced as a byproduct of fatty acid metabolism
	tween 20	Allowable safe daily amount is 0.25mg/Kg body weight
	citric acid	Little effect on *E.coli* O157:H7
	m-tartaric acid	Maximum limit of 10g/100L
	D,L-α-glycerol-phosphate	Food additive used as a stabilizer
	L-lyxose	Rare 5 carbon sugar produced by some bacteria.
Nitrogen	**D-asparagine**	Non-essential amino acid
	thymine	Nucleic base of DNA
	nitrate	Possible carcinogen, currently used as a curing agent
	hydroxylamine	Toxic to humans
	β-phenylethylamine	Natural occurring trace amine found in chocolate
	D-mannosamine	Monosaccharide used for multiple cellular processes
	D,L-α-amino-caprylic acid	Unnatural amino acid

List of top preforming chemicals from the PM experiments and their properties; underlined properties caused us to eliminate these chemicals from future experiments. Bold chemicals are the chemicals that were used in the determination of IC_{50} experiments.

Determination of IC_{50} for the eight top performing chemicals from the initial screening experiment

The effect of the eight chemicals was tested as supplements to liquid beef broth at a range of concentrations. The highest concentration of acetoacetic acid reduced OD_{600} by 83%, ATP by 99%, but only reduced the CFU/ml by 71%. The highest concentration of β-phenylethylamine reduced OD_{600} by 75%, ATP by 98% and CFU/ml to undetectable. The highest concentration of L-lyxose reduced OD_{600} by 87%, APT by 75% and the CFU/ml by 80%. The highest concentration of D-mannosamine reduced OD_{600} by 83% and CFU/ml by 75%, but increased ATP, producing a positively sloped correlation. D,L-α-glycerol-phosphate, thymine, D,L-α-amino-caprylic acid and, D-asparagine all produced varying effects on all three experimental phenotypes including increasing the overall growth, having large errors or producing positive slopes. The inconsistent results are probably related to these chemicals having a low solubility in water. D,L-α-glycerol-phosphate reduced OD_{600} by 23%, APT by 5%, but doubled CFU/ml.

Thymine decreased OD_{600} by 69%, APT by 98% and the CFU/ml by 59%. D,L-α-amino-caprylic acid decreased OD_{600} by 80%, APT by 90% and the CFU/ml by 63%. D-asparagine reduced the ATP by 99% and the CFU/ml by 40% but also doubled the OD_{600}.

All negative slopes were analyzed using Masterplex ReaderFit 2010 (Hitachi Solutions Ltd 2011) to determine trends and match them to best fit models before regression could be done. For our data, the best fit model was run on each chemical and cellular phenotype to determine which model fit best for this experiment. The five parameter logistic regression which allows for asymmetry (Gottschalk & Dunn 2005) was the best model for our data. This curve fitting to determine IC_{50} has been used previously in testing antimicrobials (Carpenter *et al.* 2012; Tenorio-Borroto *et al.* 2012). Table 5 lists the calculated IC_{50} and the R^2 values for each chemical.

Table 5. Calculated IC_{50}

	IC_{50}					
	OD_{600}		ATP		CFU	
Chemical	IC_{50} mg/ml	R^2	IC_{50} mg/ml	R^2	IC_{50} mg/ml	R^2
Acetoacetic acid	18.33	0.999	9.17	0.999	1.1	0.984
β-Phenylethylamine	19.08	0.995	0.34	0.999	0.32	0.999
L-lyxose	111.810	0.980	75.61	0.97	72.16	0.95
D-Mannosamine	162.68	0.73	Positive Slope		8.34	0.614
D,L-α-glycerol-P	23.54	0.785	Positive Slope		Positive Slope	
Thymine	0.08	0.892	1.71	0.944	5.05	0.205
D,L-α-Amino-Caprylic acid	0.12	0.973	1.84	0.999	1.96	-0.25
D-Asparagine	Positive Slope		0.2	0.955	0.62	0.13

Masterplex ReaderFit 2010 calculated IC_{50} and R^2 values for all chemicals with that negatively impacted phenotypic expression tested for. Positive sloped trends were not calculated.

R^2 values above 0.9 were considered valid models for regression and calculation of IC_{50}. The IC_{50} for acetoacetic acid was 18 mg/ml for OD_{600}, 9.2 mg/ml for ATP and 1.1 mg/ml for CFU. All R^2 values for these regressions were greater than 0.98. Acetoacetic acid had the

greatest reduction in ATP and provided good R^2 values and IC_{50} concentrations of less than 10 mg/ml for ATP and CFU. We used this as criteria to select it as one of two top preforming chemicals in this experiment.

β-Phenylethylamine produced the greatest reduction in CFU, and produced high R^2 values (>0.99). It also produced low IC_{50} concentrations of 19 mg/ml for OD_{600}, 0.34 mg/ml for ATP, and 0.32mg/ml for CFU. These properties make it the second one of our top preforming chemicals.

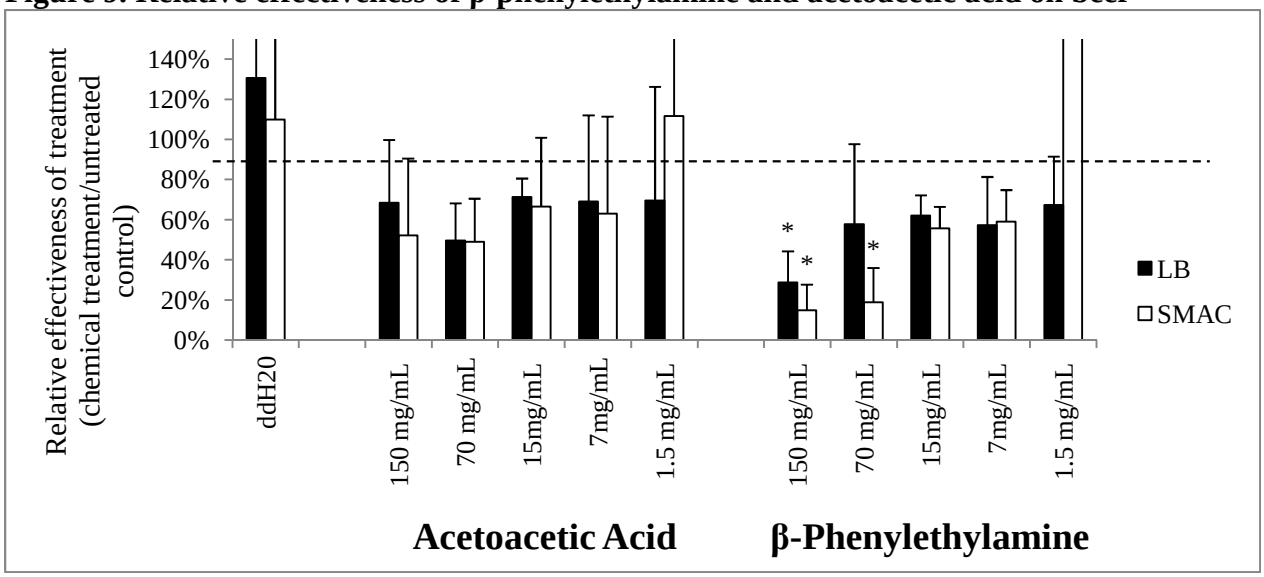
L-lyxose also produced R^2 values above 0.95, showing that calculated IC_{50} values of 110 mg/ml for OD_{600}, 76 mg/ml for ATP, and 72 mg/ml for CFU/ml were valid. Because of the high concentrations necessary to achieve 50% inhibition for all cellular phenotypes, this chemical was not considered top preforming.

Thymine and D,L-α-amino-caprylic acid produced low R^2 values for ATP, indicating that IC_{50} may not be valid. D-Mannosamine, D,L-α-glycerol-phosphate, and D-Asparagine all produced a positive correlation in at least one of the experimental phenotypes. Altogether, the determination of IC_{50} values led to two top performing chemicals, β-phenylethylamine and acetoacetic acid.

Effect of β-phenylethylamine and acetoacetic acid on bacterial cell counts of E. coli O157H:7 on beef

The two top performing chemicals from the previous experiment were tested for their effect on bacterial cell counts on beef meat pieces. CFU/ml obtained from treated samples 3-13 was divided by the CFU/ml of untreated control sample (sample 2) to produce a ratio. Ratios were averaged across replicates and standard deviations calculated. Figure 9 summarizes these results.

Figure 9. Relative effectiveness of β-phenylethylamine and acetoacetic acid on beef



Averages and standard deviations of relative effectiveness ratios recovered after 7 days. Ratios represent CFU/ml on treated pieces in comparison to untreated control 100%. DdH_20 acted as a solvent control. * indicates significant difference from control, as determined by *t*-test. Dashed line representing normal recover CFU/ml on untreated meat.

All uninoculated untreated beef meat pieces produced bacterial cell counts of zero, indicating that our beef was free of *E .coli* O157:H7 at purchase. DdH_20 treatment (sample 3) did not cause a reduction in the CFU, relative to the untreated, inoculated control (sample 2). Recovered CFU/ml for the highest concentrations of β-phenylethylamine and acetoacetic acid were 9.8×10^4 and 2.8×10^5 in comparison to the untreated control (sample 2) with 4.7×10^5. The 150 mg/ml concentration of β-phenylethylamine reduced the recovered CFU/ml on both LB and SMAC plates by was 71% (*p*-value = 0.0014) and 85% (*p*-value = 0.0076), respectively when compared to the untreated control. When reduced to 70 mg/ml concentration, β-phenylethylamine caused a reduction in CFU/ml that SMAC plates estimated at 81% (*p*-value = 0.0082). LB plates did not detect this difference. None of the tested concentrations of acetoacetic acid produced a significant difference in CFU/ml.

DISCUSSION

This study identified multiple chemicals for use by the food industry to help reduce the chances of outbreaks of *E. coli* O157:H7. All the chemicals tested in this study are nutrients for the bacteria and are known to enter into metabolic pathways. The metabolic pathways can in turn affect the bacteria's phenotype. Many bacteria use these same central pathways, suggesting treatments while tested only on *E. coli* O157:H7 may also affect other microorganisms in a similar way. This may include not only other pathogenic serotypes of *E. coli,* but possibly other enteric bacteria. Because all of the chemicals tested were nutrients for the bacteria, they should exert reduced selective pressure in comparison to antimicrobial treatments focused on killing the bacteria. Reduced selective pressure would allow treatments to be effective over a longer timespan because there is either less resistance or it may take longer to develop.

As discussed previously, both QS and 2CSTS can be used to control bacterial phenotype characteristics including biofilm formation. Which one (or combination) of the 37 2CSTSs (Zhou *et al.* 2003) of *E. coli* may be involved in signaling β-phenylethylamine and acetoacetic acid was not tested in this study. Repeating either the liquid beef broth or the meat experiment with a collection of 37 mutants would be one extensive future experiment.

This study included an *E. coli* O157:H7 strain with a mutation in the *flh*C gene resulting in a nonfunctional FlhDC. Previous research showed this mutant was characterized by an increased cell division rate and biofilm amounts (Sule *et al.* 2011). There is some possibility that chemicals with no reduction in CFU/ml and ATP in the mutant and reduction in the wild-type might have this effect due to up-regulation of FlhDC. However, none of the top performing chemicals conclusively showed this trend, though both Tween 20 and 40 showed large reduction in biofilm amounts in the wild-type and increased amounts in the *flh*C mutant. This means that

regulation of the phenotype brought on by the top performing chemicals most likely did not require FlhDC. For this reason, we did not include the *flhC* mutant in the second and third experiment with the *flhC* mutant

The Phenotype MicroArray experiments identified eight chemicals that reduced CFU, OD_{600}, and/or ATP. The three carbon sources are acetoacetic acid, D,L-α-glycerol-phosphate, and L-lyxose. The five nitrogen sources are D-asparagine, thymine, β-phenylethylamine, D-mannosamine, D,L-α-amino-caprylic acid. While this information provided some insight, we were unable to retrieve any information as to the concentration of nutrients on the PM plates (patented technology). The top performing chemicals from the PM experiments were then used at various concentrations in the liquid beef broth experiment with the goal to identify the concentrations that are required to inhibit OD_{600}, ATP, and CFU/ml by 50%.

The IC_{50} experiments, as a generalized trend, showed that decreased concentrations of the respective nutrients caused an increase in the standard deviation. Many of the chemicals tested did show large standard errors, causing difficulties in correlating trends. D,L-α-glycerol-phosphate, thymine, D,L-α-amino-caprylic acid and, D-asparagine all produced very large errors in the experiments to determine IC_{50}. This error may be related to their low solubility in water. Because the PM plates from Biolog are standardized to carbon or nitrogen atoms in the wells, the amounts of each chemical dried to the wells would vary. If the chemical had a low solubility in the solvent used (ddH_2O in these experiments), it would stay coated to the sides of the well and possibly prevent the bacteria from forming biofilm. These chemicals may provide an excellent preventative to whole carcass treatments if applied as a dried aerosol or for developing *E. coli* O157:H7 resistant surfaces for food processing equipment or packaging.

For this study chunks of beef roast were used to simulate a hot carcass, freshly slaughtered whole carcass treatment. Though the fat content may vary greatly between carcasses, we used a

lean bottom roast because excessive fat can interfere with BAX testing, a quantitative PCR test that is an industry standard. If we consider a 100% bacteria recovery, meaning no bacteria was retained on the beef, two conclusions can be drawn. The first conclusion is that acetoacetic acid did slow growth of *E. coli* O157:H7 on beef, because the ~2.0 x 10^5 inoculated cells increased to 2.9 x 10^5. This increase in CFU /ml was lower than the increase in seen on the untreated beef, which increased from ~2.0 x 10^5 to 4.7 x 10^5. The second conclusion is β-phenylethylamine not only reduced bacterial growth, from 4.7 x 10^5 to 9.8 x 10^4, but may also reduce the bacterial ability to survive at 10°C, because 9.8 x 10^4 is lower than the amount inoculated ~2.0 x 10^5. These conclusions are based on a 100% recovery rate which may not be possible considering the ability of *E. coli* O157:H7 to make biofilm. The reduction in recovered bacterial numbers caused by β-phenylethylamine on the meat may be a reduction in the ability to recover the colonies from the meat. This reduced ability to recover bacteria from the meat may be due to increased biofilm strength. In our experiments to determine the IC_{50} we did see a reduction in biofilm amounts caused by β-phenylethylamine in beef broth. Meaning β-phenylethylamine treatment did reduce the amount of biofilm produced by *E. coli* O157:H7 in beef broth. But it is still possible that β-phenylethylamine could cause an increase in the ability of E. *coli* O157:H7 biofilms to withstand shearing forces. Current work in our lab is looking at developing a method to measure biofilm strength, which could then be used to test and see if these chemicals cause an increase in biofilm strength.

Treatment of meat varies as to the amount of processing the meat goes through. This research looked at whole carcass treatment but the chemicals identified may also be considered for use after testing and approval in further processing such as a curing additive or possible as a packaging material.

None of these chemicals, if FDA approved, are suggested as a cure all treatment and are better viewed as additional hurdles for food processers to use. Even those with little effect may be used as an additional hurdle considering current treatments, such as hot citric acid reduce *E. coli* O157:H7 by 0.3 log (Birk *et al.* 2010) and vaccines can be used to reduce its ~65% (Hurd & Malladi 2012; Moxley *et al.* 2009; Varela *et al*. 2012). If both these are used along with our treatment of β-phenylethylamine the chance of *E. coli* O157:H7 outbreaks can be greatly reduced.

Conclusions and future perspective

β-phenylethylamine, the best performing chemical at reducing *E. coli* O157:H7 in beef broth, also showed to be the most effective at reducing *E. coli* O157:H7 on beef, reducing the recovered bacteria by 75%. While not FDA approved for treatment on meat, β-phenylethylamine has GRAS status (Duke 2001) granted by Flavoring and Extract Manufacturing Association (FEMA #3220) and is found in many different foods (Burdock 1994). β-phenylethylamine have shown to be able to increase dopamine levels in the brain (Murata *et al.* 2009). Because of β-phenylethylamine's effect on neurobiology it is sold as a health supplement to support both mood and weight loss (Yen *et al.* 1984).

Current research in our lab is focused on developing a technique to accurately measure biofilm strength. Current plans will look at testing β-phenylethylamine effects on biofilm. Future experiments will look at testing whole untreated beef muscle as well as undecided vegetable/s. These experiments will also look at food qualities including, looks, taste, texture, and smell. These experiments will also test the ability of β-phenylethylamine to reduce E. *coli* O157:H7 on non-treated foods, since my research used store-bought beef. Experiments that test β-phenylethylamine should also be done using multiple strains, and serotypes to insure its effects are not isolated to our strain.

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