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Characterization of ethylenediaminetetraacetic acid and acid tolerance of foodborne pathogenic bacteria

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Abstract

Foodborne pathogenic bacteria are subject to different stressful conditions due to process conditions, storage and composition of food. It is crucial to understand the survival characteristics of these bacteria to develop effective measures to limit or eliminate their survival in food. EDTA is a chelating agent and commonly used in food formulations for its function to prevent discoloration or flavor loss in food and to extend shelf life. Due to its common use in food industry, it is important to understand its antimicrobial function for possible interaction with other antimicrobials for elimination of foodborne pathogens. In this study, different foodborne pathogenic bacteria including two Gram-positive (*Listeria monocytogenes* and *Staphylococcus aureus*) and three Gram-negative (*Escherichia coli* O157:H7, *Pseudomonas aeruginosa* and *Salmonella typhimurium*) bacteria were characterized for their survival and growth in the presence of EDTA (0.01 and 0.05%) and under acidic condition (pH 5.0). The presence of EDTA in the growth media caused Gram-positive and Gram-negative bacteria to become more susceptible to subsequent stressful conditions compared to control ($p<0.05$). Gram-negative bacteria were more tolerant to acidic conditions as well as presence of EDTA compared to Gram-positive bacteria ($p<0.05$). This study provides insight on survival characteristics of foodborne pathogenic bacteria against selected stress conditions they are exposed in food and highlights the antimicrobial function of EDTA in food formulations.

Keywords: Stress response, Foodborne pathogens, *Listeria monocytogenes*, *Escherichia coli*, *Pseudomonas aeruginosa*, EDTA, Acid tolerance

Introduction

Food safety is a global public health concern and foodborne pathogenic organisms cause significant number of diseases and death worldwide. Food industry and government agencies are focusing on development of new methods or improvement of existing methods to minimize contamination of food with pathogenic microorganisms. The safety of food is ensured by application of different measures including processing, food composition and packaging. Foodborne pathogens are exposed to variety of different stressful conditions through their lifecycle. One key aspect in developing effective food safety measure is the understanding of ecology of foodborne pathogens

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and how they respond to stress conditions. These conditions may lead to selection of strains that develop resistance or tolerance to harsher stress conditions (Wesche et al. 2009).

Key intrinsic characteristics of food that allow control of growth or survival of pathogenic microorganisms include pH, salt content, water activity, etc. (Yeargin and Gibson 2019). Foodborne pathogens show different survival characteristics against these stressful conditions. In negative bacteria demonstrate increased tolerance to external stress factors, including lower pH, increased salt content and presence of detergents. This increased tolerance is mainly due to the differences in cell envelope assembly of Gram-negative and Gram-positive bacteria. Unlike Gram-positive bacteria, Gram-negative bacteria possess an outer membrane providing additional barrier (Jordan et al. 2008). The outer membrane of Gram-negative bacteria consists of lipopolysaccharide molecules making the exterior of bacterial cells hydrophobic and serves as a barrier for entrance of macromolecules and hydrophilic substances. This outer membrane improves the tolerance of Gram-negative bacteria against extracellular stresses. The outer membrane of Gram-negative bacteria could be modified by certain compounds through decomposition of the lipopolysaccharide layer increasing the permeability of the membrane (Vaara, 1992; Alakomi, et al. 2006; McBroom and Kuehn 2007). One such compound is a chelating molecule called Ethylenediaminetetraacetic acid, EDTA. EDTA is commonly used in food formulations for its stabilizer function. It delays or stops the chemical reactions in food that cause discoloration or texture and/or flavor loss. Besides its stabilizer function, it also has antimicrobial characteristics. As a chelator, EDTA, sequesters divalent cations of the outer membrane of Gram-negative bacteria. Divalent cations have a crucial role in enabling the electrostatic interconnections with proteins and lipopolysaccharides serving as the backbone of the stability of the outer membrane in Gram-negative bacteria. Supplementation of benzalkonium chloride with chelating compounds, such as EDTA and polyethylenimine, improved its activity in inhibition of Gram-negative bacteria *Pseudomonas* and *Stenotrophomonas* (Alakomi et al. 2006; Sim et al. 2019; Vale et al. 2019).

Foodborne bacteria are exposed to acid stress in food materials and pH adjustment of food is commonly used as a food protection measure. Some stress response mechanisms that bacteria uses to minimize the lethal impact of acid stress includes membrane composition change, increase in protein efflux, increase in amino acid catabolism, and induction of DNA repair enzymes (Siegumfeldt et al., 2000). These response mechanisms could be initiated by other stress factors or they could be complemented by other response mechanisms to allow bacteria become more tolerant to acidic stress. The impact of stress adaptation of bacteria on increased tolerance to subsequent stressful conditions has been reported in number of were reported in a number of previous studies, Cheng et al. (2003) indicated that acid adaptation of cells increased acid tolerance and this increase was dependent on strain, acid adaptation time, and pH of the acid challenge.

EDTA is commonly used in food industry for its food stabilizer function. Due to its antimicrobial property EDTA could provide additional protection along with other antimicrobial compounds in food or could activate stress response mechanisms of pathogenic bacteria that could increase survival of these bacteria and cause risk in terms of foods safety. Therefore, the impact of EDTA on their survival in acidic conditions was characterized within the scope of this study. Furthermore, the effect of prior adaptation of pathogenic bacteria within different concentrations of EDTA on their growth in specific acidic conditions was studied. These findings would serve as a reference in food product formulation to ensure the safety of the food supply.

Materials and Methods

Bacterial Strains and Growth Conditions

For evaluation of EDTA and acid stress tolerance behavior of bacteria, variety of foodborne pathogens that are most commonly associated with foodborne outbreaks were included in this study. Stress response of two Gram-positive and three Gram-negative foodborne pathogenic bacteria were characterized. Tested Gram-positive bacteria included: a clinical isolate of each of *Listeria monocytogenes* and *Staphylococcus aureus*. Tested Gram-negative bacteria included: a clinical isolate of each of *Escherichia coli* O157:H7, *Pseudomonas aeruginosa* and *Salmonella typhimurium*. Each bacterial isolate were grown on Brain-Hearth Infusion (BHI) agar at 37°C for 24 hours, transferred into BHI broth (BHI-B) (Sigma-Aldrich, St. Louis, MS) and incubated at 37°C for 18 hours before the subsequent stress characterization test.

Adaptation of Bacteria to Presence of EDTA

Prior to evaluation of stress tolerance of each bacteria, the cells were adapted to presence of EDTA in growth media. For this purpose, following overnight growth at 37°C in BHI-B, each culture of bacteria was transferred to BHI-B containing 0.01% or 0.05% EDTA (Sigma-Aldrich, St. Louis, MS). Following inoculation in BHI-EDTA broth, the cultures were incubated at 37°C for 18 hours statically. These cultures were

transferred to following stress conditions including acid or EDTA stress.

Screening of Acid Tolerance of EDTA Adapted Bacteria

The growth of selected bacteria was screened in BHI-B adjusted to pH of 5.0. The pH of the growth media is adjusted to desired acidity using HCl. Following the pH adjustment, the media were sterilized before testing. Bacteria grown in 0.01% EDTA containing BHI-B, 0.05% EDTA containing BHI-B or BHI-B were inoculated in BHI-B at pH 5.0. Regular BHI-B without pH adjustment was tested as control. Starting microbial load of each culture was ~10^5 cfu/ml. Growth of each culture was assessed by measurement of optical density at 600nm (OD600). The OD600 were measured every 30 minutes for 24 hours using a plate reader (Tecan, Switzerland). Each condition for each of the tested foodborne bacteria was screened in four independent replicates.

Impact of Presence of EDTA in Acidified Media

Possible effect of presence of EDTA in stress tolerance of bacteria in acidified media was also evaluated. The pH of BHI-B was adjusted to 5.0, and 0.01% of EDTA was supplemented to the media. The growth of each bacteria at 37°C under these conditions were evaluated as described above: measuring OD600 in 30 minutes intervals for 24 hours using a plate reader (Tecan, Switzerland).

Statistical Analyses

Each experiment in this study was replicated four times, and the impact of each condition was statistically assessed by comparison of the growth rates and OD600 value at stationary growth phase using one-way ANOVA. The growth rate in logarithmic growth phase for each test was calculated by following formula: $\ln (OD600_{t2}/OD600_{t1})/(t_2-t_1)$ in which t_1 is the time the bacteria started its logarithmic growth phase and t_2 is a selected later time in its logarithmic growth phase. Statistical significance is defined at $p<0.05$.

Results and Discussion

The composition of food has significant impact on survival and growth of microorganisms in food. It is important to understand how pathogenic bacteria react to the stressful conditions of food due to food's intrinsic properties. EDTA is commonly used as a food stabilizer as it interacts with the chemical reactions within the food to inhibit or stop discoloration or texture and/or flavor losses. Besides its quality improvement function, EDTA has antimicrobial properties to inhibit growth or survival of microorganisms present in food. Although the antimicrobial function of EDTA is known, there are limited studies that focused on its impact against foodborne pathogens and its interaction with other stress conditions within the food. Therefore, in this study the impact of EDTA on acid stress tolerance of five different foodborne pathogenic bacteria associated with majority of foodborne outbreaks was studied.

Impact of EDTA adaptation on growth of Gram-positive and Gram-negative bacteria

Adaptation of *L. monocytogenes* and *S. aureus* in BHI-B with 0.1% or 0.5% EDTA had significant impact on subsequent growth in regular BHI-B ($p<0.05$; Figure 1). Both cells of *L. monocytogenes* and *S. aureus* showed impaired growth in BHI-B following adaptation in the presence of EDTA. The cells adapted to EDTA showed longer lag-phase compared to control cells (Figure 1). This indicates that presence of EDTA caused injury of cells of Gram-positive bacteria and leads to longer lag-phase for cells to recover. On the contrary, the tested Gram-negative bacteria didn't show similar impairment in their growth following adaptation to EDTA. The growth behavior of EDTA adapted and control cells were similar to each other (data not shown). This could be explained due to the chelating function of EDTA against the cell membrane of Gram-positive bacteria. The outer membrane of the Gram-negative bacteria serves as a protectant against the damaging effect of EDTA of cells and prevents injury of the cells (Gill and Holley 2003). Similar enhanced sensitivity of Gram-positive bacteria to other stress conditions was reported in other studies (Guardabassi et al. 2010; Khazandi, et al. 2019; Vale et al. 2019).

Effect of EDTA in acid tolerance of Gram-positive and Gram-negative bacteria

The impact of prior EDTA adaptation in acid tolerance showed similarity between *L. monocytogenes* (Figure 2a) and *S. aureus* ($p>0.05$; data not shown). On the other hand, the impact of EDTA adaptation on their acid tolerance showed significant difference from Gram-negative bacteria tested in this study, *E. coli* O157:H7 (Figure 2b), *S. typhimurium* and *P. aeruginosa* (Figure 2c) ($p<0.05$). This finding highlights the difference between Gram-positive and Gram-negative bacteria in their response to the presence of EDTA and their survival in acidic conditions. Gram-negative bacteria showed more tolerance to the stressful conditions of EDTA compared to the Gram-positive bacteria. This difference in the EDTA and acid stress response could be explained by the differences in the cell wall structure between Gram-positive and Gram-negative bacteria and the presence of outer membrane in Gram-negative bacteria that provides additional protection against the stressful conditions (Alakomi et al. 2006; Khazandi, et al. 2019).

Interestingly, inclusion of EDTA to the acidified media fur-

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ther inhibited the growth of both Gram-positive and Gram-negative bacteria. The cells of *L. monocytogenes* were incapable of survival and growth under the stressful conditions of combination of EDTA and high acidity. Similar impact of presence of EDTA was observed in *S. aureus* (data not shown). This indicates that the cells of tested Gram-positive bacteria were injured in the presence of EDTA and their growth in the presence of EDTA under acidic conditions were completely inhibited (Figure 3a). Although, the cells of *E. coli* O157:H7 showed growth under EDTA and high acid stress, it was significantly less than the cells grown only under acid stress ($p<0.05$; Figure 3b). The presence of EDTA (0.01%) in the growth media that is pH adjusted to 5.0 cause cells to be more susceptible and the growth rate and the cell density at stationary growth phase were significantly lower than the cells grown in BHI-B at pH 5.0 ($p<0.05$; Fig. 3b). *Salmonella typhimurium* showed similar behavior as *E. coli* O157:H7 strain tested in this study (data not shown).

Other tested Gram-negative bacteria, *Pseudomonas aeruginosa,* showed significantly different growth behavior compared to the foodborne pathogenic bacteria tested in this study ($p<0.05$). The EDTA adaptation of *P. aeruginosa* at 0.5% significantly increased the lag-time for the growth of the cells at pH 5.0 compared to the cells adapted to 0.1% EDTA or control cells ($p<0.05$; Figure 2c). On the other hand, interestingly the growth rate and the density of the cells at stationary growth phase were similar to each other regardless of the adaptation or the pH adjustment of the growth media (Fig. 2c & 3c).

This study highlights the differences of EDTA and acid tolerance of different foodborne pathogens and shows the antimicrobial activity of EDTA. The chelating properties of EDTA possibly cause formation of pores in cell membrane of the bacteria and cause leakage of the cell or increase the uptake of the other antimicrobial compounds inside the cell (Alakomi et al. 2006; Sim et al. 2019; Vale et al. 2019). It is worthwhile to note that sublethal levels of EDTA in food could select for more tolerant strains of pathogenic bacteria and could pose threat to food safety. Further phenotypic and genotypic characterization of additional strains of these foodborne pathogens is needed to better understand the stress response mechanisms of these pathogenic microorganisms and develop better control measures to eliminate them from food sources.

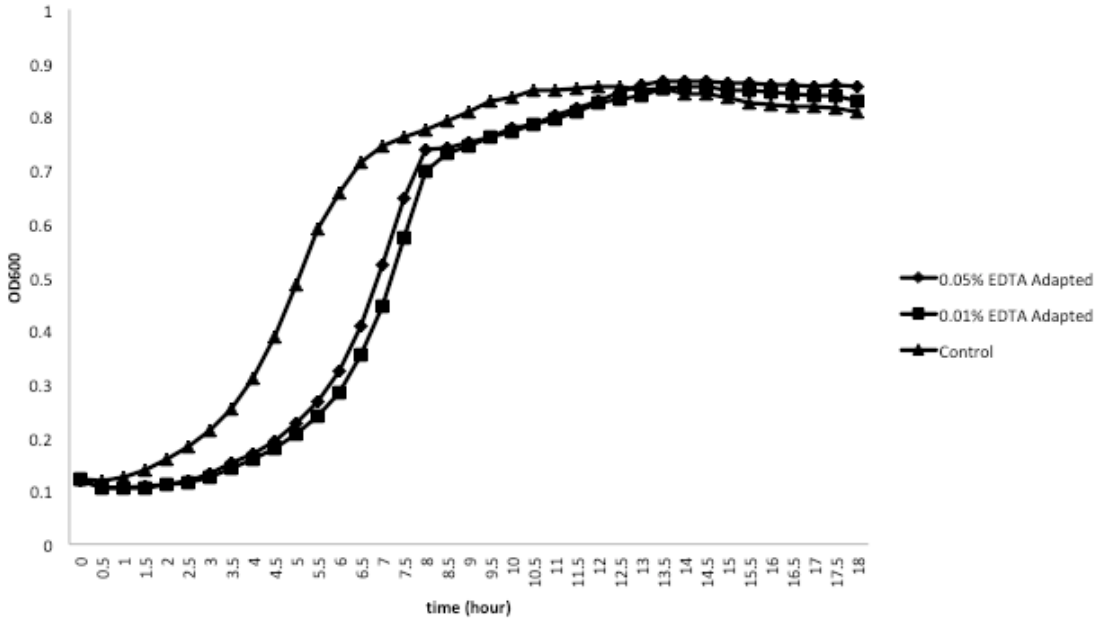

Figure 1- Growth of *L. monocytogenes* in BHI-B following overnight adaptation in BHI-B supplemented with 0.01% EDTA (■) or 0.05% EDTA (◆). Control *L. monocytogenes* only grown in BHI-B is represented by filled triangles (▲).

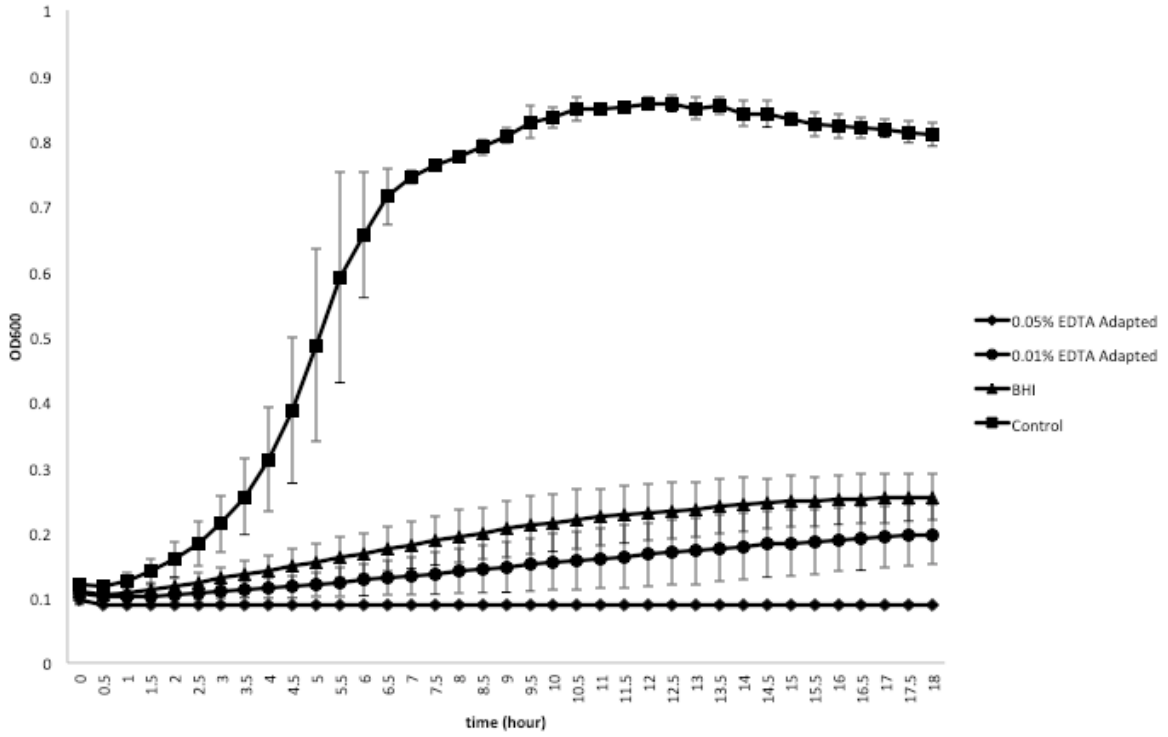

Figure 2a- Growth of *L. monocytogenes* in BHI at pH 5.0 supplemented with different concentrations of EDTA (0.01% and 0.05%) following growth in BHI-B. Control is the cells grown in BHI-B with no EDTA supplementation.

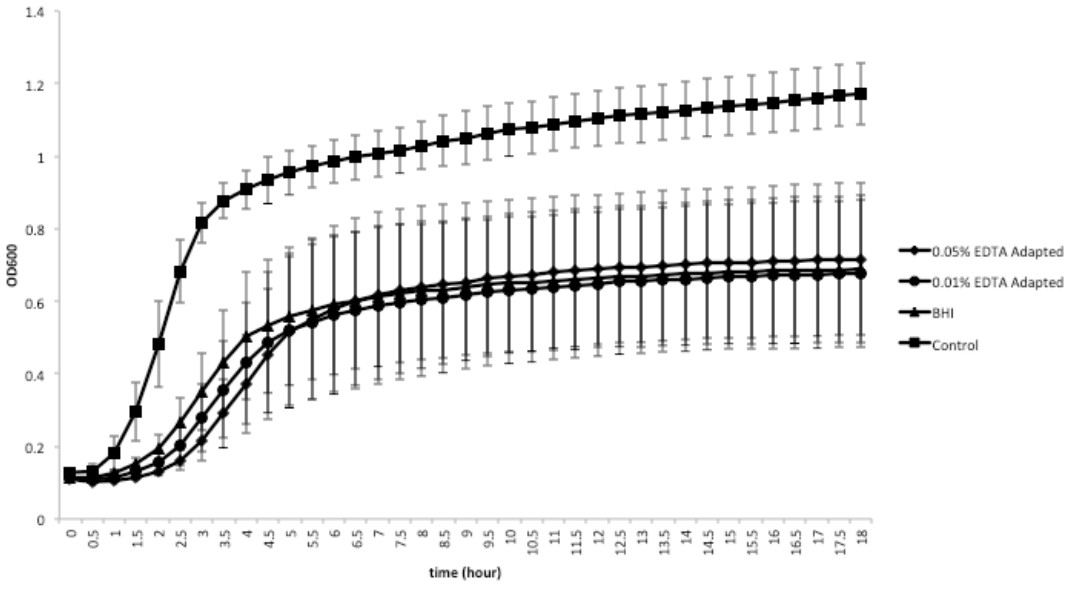

Figure 2b- Growth of *E. coli* O157:H7 in BHI at pH 5.0 supplemented with different concentrations of EDTA (0.01% and 0.05%) following growth in BHI-B. Control is the cells grown in BHI-B with no EDTA supplementation.

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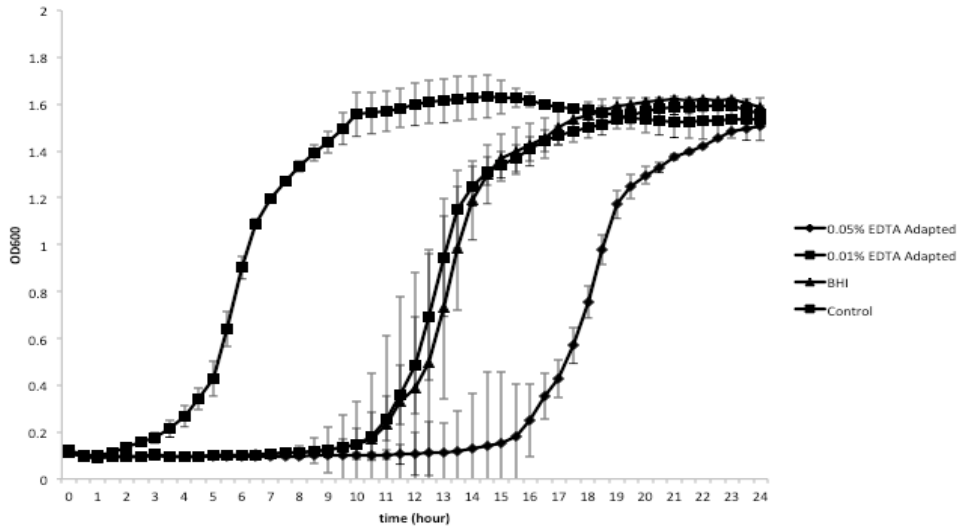

Figure 2c- Growth of *Pseudomonas aeruginosa* in BHI at pH 5.0 supplemented with different concentrations of EDTA (0.01% and 0.05%) following growth in BHI-B. Control is the cells grown in BHI-B with no EDTA supplementation.

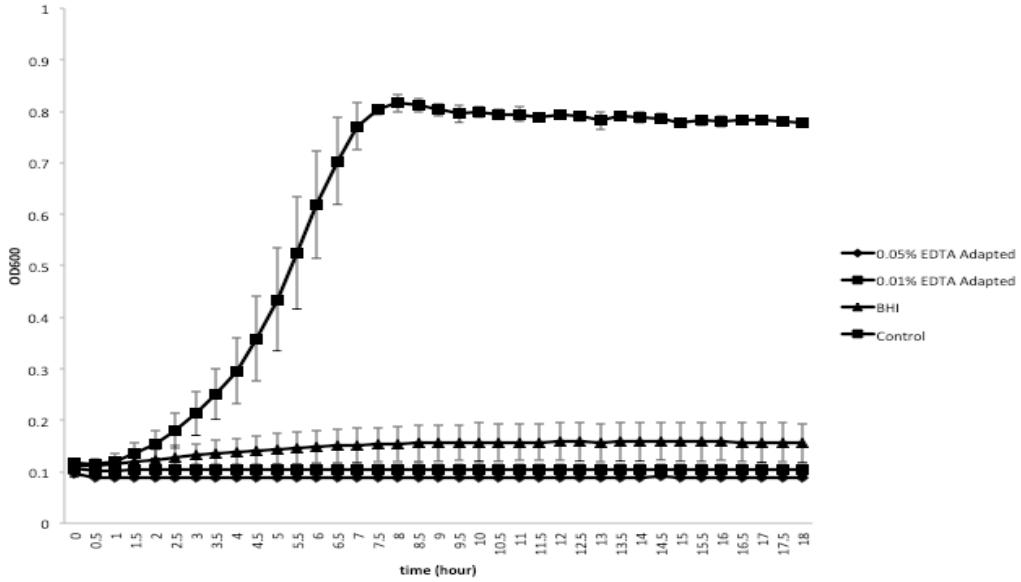

Figure 3a- Growth of *L. monocytogenes* in BHI at pH 5.0 supplemented with different concentrations of EDTA (0.01% and 0.05%) following growth in BHI-B supplemented with 0.01% EDTA. Control is the cells grown in BHI-B with no EDTA supplementation.

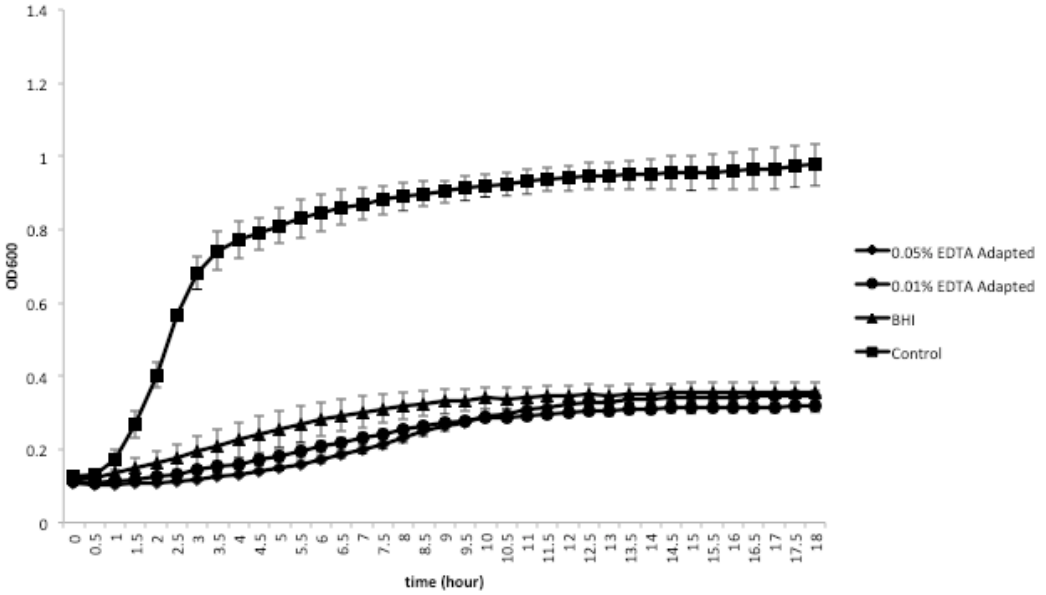

Figure 3b- Growth of *E. coli* O157:H7 in BHI at pH 5.0 supplemented with different concentrations of EDTA (0.01% and 0.05%) following growth in BHI-B supplemented with 0.01% EDTA. Control is the cells grown in BHI-B with no EDTA supplementation.

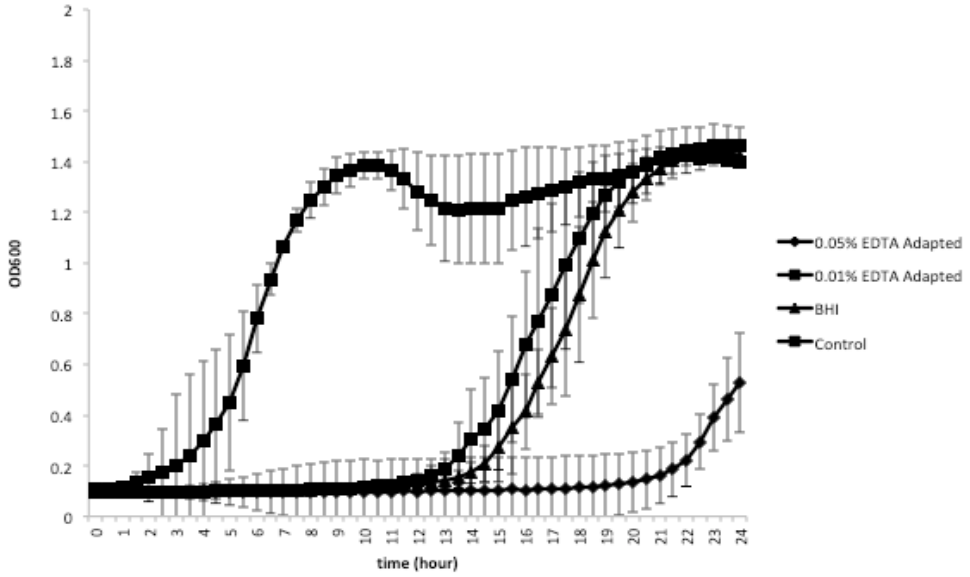

Figure 3c- Growth of *P. aeruginosa* in BHI at pH 5.0 supplemented with different concentrations of EDTA (0.01% and 0.05%) following growth in BHI-B supplemented with 0.01% EDTA. Control is the cells grown in BHI-B with no EDTA supplementation.

Conclusion

Foodborne diseases impact large population around the world causing severe illnesses, therefore it poses a significant public health problem. Foodborne bacteria are usually capable to survive or grow in food and under food storage conditions. In order to develop effective measures to eliminate or limit the occurrence of food-associated illnesses, it is important to understand the ecology of pathogenic microorganisms, and the mechanisms that provide protection for them to survive and grow in food. Therefore, it is crucial to understand how the pathogenic bacteria survive under the stressful conditions of food. In this study, the survival and growth of selected Gram-positive and Gram-negative foodborne bacteria were characterized under the presence of EDTA and acidic conditions. This study showed that the presence of EDTA caused all the tested bacteria become more susceptible to the acidic conditions and possibly to other stress conditions. The differences in EDTA and acid stress response of various foodborne pathogens were also presented. This study highlights the fundamental response of foodborne pathogens against EDTA and acidic conditions. Further phenotypic and genotypic characterization of commonly isolated foodborne pathogenic bacteria would improve our understanding on the stress response mechanisms and allow development of effective methods to eliminate foodborne pathogens from foods.

Compliance with Ethical Standards

Conflict of interest

The author declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Author contribution

The author read and approved the final manuscript. The author verifies that the Text, Figures, and Tables are original and that they have not been published before.

Ethical approval

Not applicable.

Funding

No financial support was received for this study.

Data availability

Not applicable.

Consent for publication

Not applicable.

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