Escuela Agrícola Panamericana, Zamorano

Food Science and Technology Department

Food Science and Technology Major

Graduation Research Project

Survival of nalidixic acid strains of *Salmonella* Enteritidis on mung bean sprouts and surfaces produced in a home-scale hydroponic system.

Presented by

Elva Alcira Hernández Guatemala

Advisers

Angela Shaw, Ph.D.

Ligia Elizabeth Luna Jarrin, Msc.

Honduras, noviembre 2023

Contents

List of Tables

List of Figures

List of Annexes

Abstract

The production of sprouts in hydroponic systems is increasing worldwide, including alfalfa, mung bean, clover, etc., which are generally consumed raw or minimally processed. Mung bean sprouts are often contaminated with pathogens during production, one of the main pathogens is *Salmonella* Enteritidis. The presence of *Salmonella* in sprouts carries a high risk during their production as disinfection methods are often ineffective in inhibiting them. This study was based on examining the survival of *Salmonella Enteritidis* resistant to nalidixic acid in mung bean sprouts produced in hydroponic systems for 7 days under domestic conditions to evaluate its behavior. To evaluate this, mung bean seeds were grown in jars and exposed to distilled water contaminated with *Salmonella* at room temperature (25 °C) and 70% relative humidity. Samples were taken from the sprouts and surface of the jars and sown on xlt4 agar with nalidixic acid to detect only *Salmonella*. *Salmonella* Enteritidis was quantified by the plate count method and was detected at levels from 4 log CFU/g to 7 log CFU/g. *Salmonella* Enteritidis *growth* was higher in the sprouts than on the surface, reaching a level of 7.35 log CFU/g, however, on the surface there was growth, but it was not significant over time, reaching levels of 6.19 log CFU/mL. It was detected that *Salmonella* tends to move to surfaces through the water since seed rinsing is a risk of contamination of sprouts and surfaces by *Salmonella* Enteritidis under domestic conditions, so it is necessary that producers implement the requirements by the food safety rule focused on the production of sprouts.

Keywords: Survival, antibiotic, *Salmonella* Enteritidis, mung bean, sprouts, behavior, temperature, home, rinsing, hydroponic.

Resumen

La producción de germinados en sistemas hidropónicos está aumentando en todo el mundo, incluyendo alfalfa, judía mungo, trébol, etc., que generalmente se consumen crudos o mínimamente procesados. Los brotes de judía mungo suelen contaminarse con patógenos durante su producción, siendo uno de los principales *Salmonella* Enteritidis. La presencia de *Salmonella* en los germinados conlleva un alto riesgo durante su producción, ya que los métodos de desinfección suelen ser ineficaces para inhibirla. Este estudio se basó en examinar la supervivencia de *Salmonella* Enteritidis resistente al ácido nalidíxico en brotes de judía mungo producidos en sistemas hidropónicos durante 7 días en condiciones domésticas para evaluar su comportamiento. Para evaluarlo, se cultivaron semillas de frijol mungo en frascos y se expusieron a agua destilada contaminada con *Salmonella* a temperatura ambiente (25 °C) y 70% de humedad relativa. Se tomaron muestras de los brotes y de la superficie de los frascos y se sembraron en agar xlt4 con ácido nalidíxico para detectar únicamente *Salmonella*. *Salmonella* Enteritidis se cuantificó por el método de recuento en placa y se detectó en niveles de 4 log UFC/g a 7 log UFC/g. El crecimiento de *Salmonella* Enteritidis fue mayor en los brotes que en la superficie, alcanzando un nivel de 7,35 log UFC/g, en la superficie hubo crecimiento, pero no fue significativo a lo largo del tiempo, alcanzando niveles de 6,19 log UFC/mL. Se detectó que *Salmonella* tiende a moverse a las superficies a través del agua ya que el enjuague de las semillas es un riesgo de contaminación de los germinados y superficies en condiciones domésticas, por lo que es necesario que los productores apliquen los requerimientos por la norma de seguridad alimentaria enfocada a la producción de germinados.

Palabras clave: Supervivencia, antibiótico, *Salmonella* Enteritidis, frijol mungo, germinados, comportamiento, temperatura, doméstico, enjuague, hidropónico.

Introduction

Sprouts are germinated seeds that develop into young plants. They are generally consumed raw and are used to add flavor to salads, soups, and other dishes. The varieties of sprouts that are most consumed are alfalfa, mung bean, red clover, radish, broccoli, and wheatgrass (Clemson University, 2023).

The consumption of sprouts has become more popular in North America and other parts of the world, due to the increased consumption of fresh fruits and vegetables. The U.S. market is valued at approximately $ 25 million with over 400 growers producing 300,000 tons of sprouts annually (Thompson, 2000). Alfalfa sprouts are the most widely consumed variety of sprouts in the United States. Eighty-five percent of its production comes from Oregon, Washington, California, Idaho, and Nevada (International Specialty Supply, 2018). Mung bean sprouts are the most consumed around the world, in the United States between 15-20 million pounds of mung bean sprouts are consumed annually (Thompson, 2000). Alfalfa sprouts are the most widely consumed variety of sprouts in the United States. Eighty-five percent of its production comes from Oregon, Washington, California, Idaho, and Nevada (International Specialty Supply, 2018). Mung bean sprouts are the most consumed around the world, in the United States between 15-20 million pounds of mung bean sprouts are consumed annually (Grant, 2016), 75% of the mung beans consumed in the United States are imported from China and Japan, and those produced domestically are grown mostly in Oklahoma (Sesauer, 2019). Also, mung bean is one of the most important edible legumes, grown on more than 6 million hectares worldwide (about 8.5% of the world's legume area) (Dianzhi Hou et al., 2019), and 15 million pounds of sprouts are grown annually in the United States (Ge et al., 2014a).

Despite their large consumption in the world and the United States, they have been linked as an important source of foodborne illness because they need temperature and humidity for their growth, which are the same ideal conditions for pathogens such as *Salmonella*, *Listeria* y *E.coli* O157: H7 (Western Kentucky University, 2022). Also, the microbial contamination of sprouts has been reported to occur due to the presence of pathogenic bacteria in seeds, and facilitating germination

and sprouting process that provides optimal conditions for bacterial growth (Ding et al., 2013). Many of these outbreaks associated with sprout consumption have occurred mainly in the United States, United Kingdom, Canada, and Europe (Yang et al., 2013). Since 1996, FDA has been reported in 48 outbreaks associated with sprouts, resulting in 2499 cases, 179 hospitalizations, and 3 deaths (Food and Drug Administration [FDA], Thu, 2020). In 2016, 230 *Salmonella* cases associated with mung bean sprouts were reported in South Australia, between 2000 and 2016, at least 17 salmonellosis outbreaks linked to the consumption of raw sprouts were documented internationally, nine of them in the United States and between 2010 and 2017, 12 multistate outbreaks linked to sprouts occurred because of S. enterica contamination that affected a range of three to 26 states (Reed et al., 2018). Most outbreaks were attributed to alfalfa sprouts, followed by clover, mung bean, and sprouted chia powder. *Salmonella* Enteritidis was the most common pathogen identified, followed by *E. coli, and Listeria* (Gensheimer & Gubernot, 2016).

Due to the large number of cases associated with the consumption of sprouts and fresh produce that continued to be reported in the United States, the U.S. government released new legislation in 2011 called the Food Safety Modernization Act, which was developed to protect public health by improving the food safety system from the farm to the consumer (United States Department of Agriculture [USDA], 2019).

The FSMA is comprised of seven fundamental rules, including the Produce Safety Rule (21 CFR 112). The Produce Safety Rule addresses the standards for the Growing, Harvesting, Packing, and Holding of fruits and vegetables grown for human consumption. This rule is made up of seventeen subparts, including subpart M, which focuses on sprouts. Subpart M includes requirements for growing, harvesting, packaging and preserving sprouts, testing, and sampling for spent irrigation water and sprouts, and actions to be taken if tests are positive for pathogens (Code of Federal Regulation, 2023a).

The measures and requirements addressed by this subpart mention that producers must treat seeds to be used for production with a scientifically valid method to reduce microorganisms, and clean

and disinfect surfaces used for growing, harvesting, packing, or storing sprouts before coming into contact with them, etc. (Code of Federal Regulations [CFR], 2015).

Salmonella is one of the main pathogens associated with the consumption of sprouts, mainly mung bean and alfalfa sprouts that have occurred in countries such as the United States, Germany, Holland, Sweden, among others. Mung bean has been cultivated especially in Asia, Australia, United States and Africa, this is best known for producing bean sprouts, whose production has been very convenient at home or industrial scale (Ge et al., 2014b). Therefore, due to the large number of outbreaks that have been associated with the different varieties of sprouts caused mainly by *Salmonella*, this research will focus on to examine the survival of nalidixic acid resistant *Salmonella* Enteritidis in mung bean sprouts, produced in home-scale hydroponic systems over seven days and Evaluate the survival of nalidixic acid resistant *Salmonella* Enteritidis in the surface associated to mung beans sprouts produced in home-scale hydroponic systems over seven days.

Materials And Methods

Culture Resistant Preparation

Salmonella Enteritidis ATCC 13076(46), BAA-708(10), I3 and IV were obtained from the ICFIE lab, Department of Animal and Food Sciences, Texas Tech University. The four strains were prepared separately with overnight incubation at 37 °C in three 10 mL test tubes with brain heart infusion (BHI) broth. From the three tubes with each culture, one was selected, streaked, and spread on a selective agar plate (XLT4), and incubated overnight. One healthy colony was selected from streaked plates and introduced in 10 mL Brain Heart Infusion (BHI) broth without antibiotic and with antibiotic (nalidixic acid) a different concentration 10, 25, and 50 µg/mL and incubated overnight at 37 °C. The nalidixic acid was used to ensure that background flora did not interfere with the results. The *Salmonella* Enteritidis strains that were resistant were plated from the incubated amended broth onto XLT4 agar and in two tubes 10 mL Brain Heart Infusion (BHI) to generate a resistance and incubated at 37 °C for 24 h. Finally, the strain was plated in XLT4 with 50 µg/mL nalidixic acid and incubated at 35 °C for 24 h. The procedure was repeated two additional times until the cell count reached 10^8 CFU/mL. Cells were harvested by centrifugation at 4,000 × g for 10 min at 24 °C and washed with sterile water, and the centrifugation and rinsing process was repeated two more times. The culture was then resuspended in BHI amended with 10% Glycerol and further diluted in BHI amended with glycerol before transfer to 1 mL cryotubes and storage at -80 C. The cells of one of the cryotubes were plated on XLT4 agar amended with 50 µg/mL nalidixic acid overnight at 35 °C to establish the cell count in each of the tubes. Prior to inoculation, the culture in each tube was diluted in sterile distilled water to obtain the desired cell concentrations for seed inoculation.

Inoculation

Inoculation of seeds with *Salmonella* Enteritidis will follow the methodology of Kocharunchitt, et al. (2009) and Xiao et al. (2014) with modifications. Mung bean seeds will be purchased from a commercial provider. The seeds were confirmed to be free of *Salmonella* Enteritidis *prior* to the experiment. Each batch (20 g) will be immersed in 40 mL of the *Salmonella* Enteritidis *inoculum*

(original concentration of 10^8 cfu/mL), gently swirling for 5 min at room temperature, to achieve targeted ~4 log cfu/g level of inoculation on the seed. The excess bacterial suspension will be removed/drained, and the inoculated seeds will be spread over sterile absorbent sheets and air-dried overnight (16–18 h) under a biological safety hood. The inoculated seeds (20 g) will be soaked in sterile tap water and left to soak at 25 °C for 8 h, after which the excess fluid will be drained. The seeds will be sprouted by aseptically transferring them to a new sterile glass jar containing a small amount of sterile tap water in the base to maintain uniform moisture. Sprouting jars will be inverted and kept at an angle in a tray to ensure proper drainage during the incubation. The sprouting jars will be covered with a light blocking material and incubated at 25 °C with a relative humidity of 70 ± 5% in the dark for five days. Germinating seeds will be rinsed with 200 mL sterile distilled water twice daily to remove by-product residues of growth (e.g., ethylene gas and carbon dioxide) from the sprouted seeds and to prevent them from overheating. The sprouts will be exposed to light on day 6 and day 7, this helps them to turn green and ready for harvest, it is a practice done by most home growers. The trial will be done in five replicates.

Microbial Enumeration

Before inoculation, the sampling will be on the water to be used, jar surface, and sprout seeds, to establish base pathogen count. After inoculation, the sampling time will be 0 h, 3h, 6 h, 12 h, 24h, 48 h, 72 h, 4 days, 5 days, 6 days, and 7 days. Samples will be taken from the inner surface of the lid (5 square cm) (S), and sprouts (SP). For each sampling time, ~2g of sprouts will be taken for microbial analysis. The sampled sprouts will be aseptically placed within a pre-weighed stomacher bag, and the mass determined. The bags will be transferred immediately to the lab where ~18 mL of BPW will be added, and the mixture pummeled for 2 min with a stomacher (Lab Blender 400, Seward Laboratory, Worthington, UK). Plant tissues will further be mashed manually by pressing with a thumb through the bag to facilitate the release of potentially internalized bacteria. The lid surface will be swabbed on the inside (5 square cm) and then placed in a pre-weighed stomacher bag with BPW. Samples will then be directly plated on XLT4 agar amended with 50 µg/mL nalidixic acid.

Results and Discussion

Germination is one of the main factors for sprout production, however, pathogen growth has been shown to occur during germination. Table 1 shows the growth of *Salmonella* Enteritidis in the sprouts and the surface. In the sprouts during the 7 days there was a significant growth, at hours 0, 3, 6, and 12 the population was similar, on day 1, 5, 6, and 7 the growth remained similar but different at 0, 3, 6, 12 hours and days 2, 3 and 4, however during days 2, 3 and 4 the growth was higher than the rest of the days, reaching up to 7.38 log CFU/g of the 4 log CFU/g initially inoculated in the seeds.

Table 1

Survival of Salmonella in sprout and surface over 7 days.

Time (Hours)	TREATMENT	
	Sprouts (Log CFU/g ± SD)	Surface (Log CFU/ mL ± SD)
0	4.00 ± 0.47^{c}	0 ± 0^{b}
3	4.10 ± 1.2^{c}	0 ± 0^{b}
6	4.19 ± 0.33^{c}	3.56 ± 1.06^{a}
12	3.77 ± 0.34^{c}	4.14 ± 0.19^{a}
24 (Day 1)	6.02 ± 0.23^{b}	5.75 ± 0.20^{a}
48 (Day 2)	7.35 ± 0.18^{a}	4.89 ± 0.56^{a}
72 (Day 3)	7.08 ± 0.17^{a}	6.05 ± 0.27^{a}
96 (Day 4)	7.23 ± 0.39^{a}	6.19 ± 0.24^{a}
120 (Day 5)	6.31 ± 0.39^{b}	5.37 ± 0.12^{a}
144 (Day 6)	6.30 ± 0.29^{b}	5.51 ± 0.40^{a}
168 (Day 7)	6.19 ± 0.23^{b}	5.34 ± 0.23^{a}
CV (%)	8.61	19.51

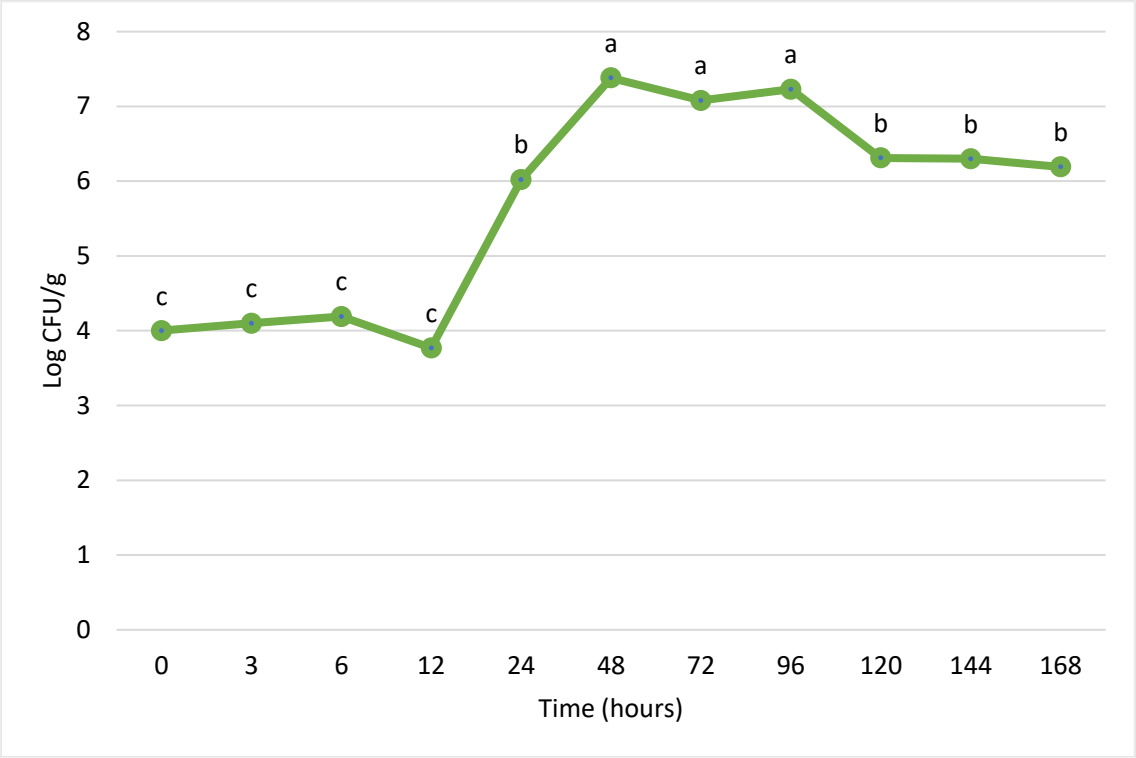
Note. abc: Different letters in the same columns present a significant statistical difference ($P < 0.05$) in each treatment; CV%: coefficient of variation. At 0 and 3 hours were sampled mung bean seeds.

According Andrews et al. (1982) the exudates of germinating seeds are what attract and promote microbial growth in plants during the early sprouting phase. This can be related to Figure 1 where at 6 hours the seed begins to sprout and *Salmonella* Enteritidis growth increases.

Similarly according to Ge et al. (2014b) *Salmonella* can be internalized in mung bean sprouts at levels of 3 and 5 CFU/g for 7 days and between 2 and 5 CFU/g for 1 day during growth, which is related to the study in which *Salmonella* growth was between 4 CFU/g and 7. 39 CFU/g in the sprouts.

Figure 1

Survival of Salmonella Enteritidis in mung bean sprouts for 7 days.



Additionality, in a study evaluating the internalization of *Escherichia coli* and *Salmonella* Montevideo bioluminescents in growing bean sprouts, it was shown that *Salmonella* can internalize in growing sprouts once the seeds are inoculated (Warriner et al., 2003), which was verified in this study, since the presence of *Salmonella* could be evidenced in sprouts that came from seeds contaminated in the laboratory. This means that growers must use seeds from a certified source with good agricultural practices for hydroponic cultivation. Seed contamination can occur in the field and during harvest. In the field it can come from animal manure, wild animals, irrigation water, unsanitary practices, and improper handling of seeds by workers; during harvest, seeds can be contaminated by transport vehicles, workers, equipment, pests, and rodents (Food and Drug Administration, 2022).

Sprout growers should follow guidelines for testing spent irrigation water, disinfecting seed, and discontinuing the use of seed lots when contaminated. As for seed disinfection, it is necessary

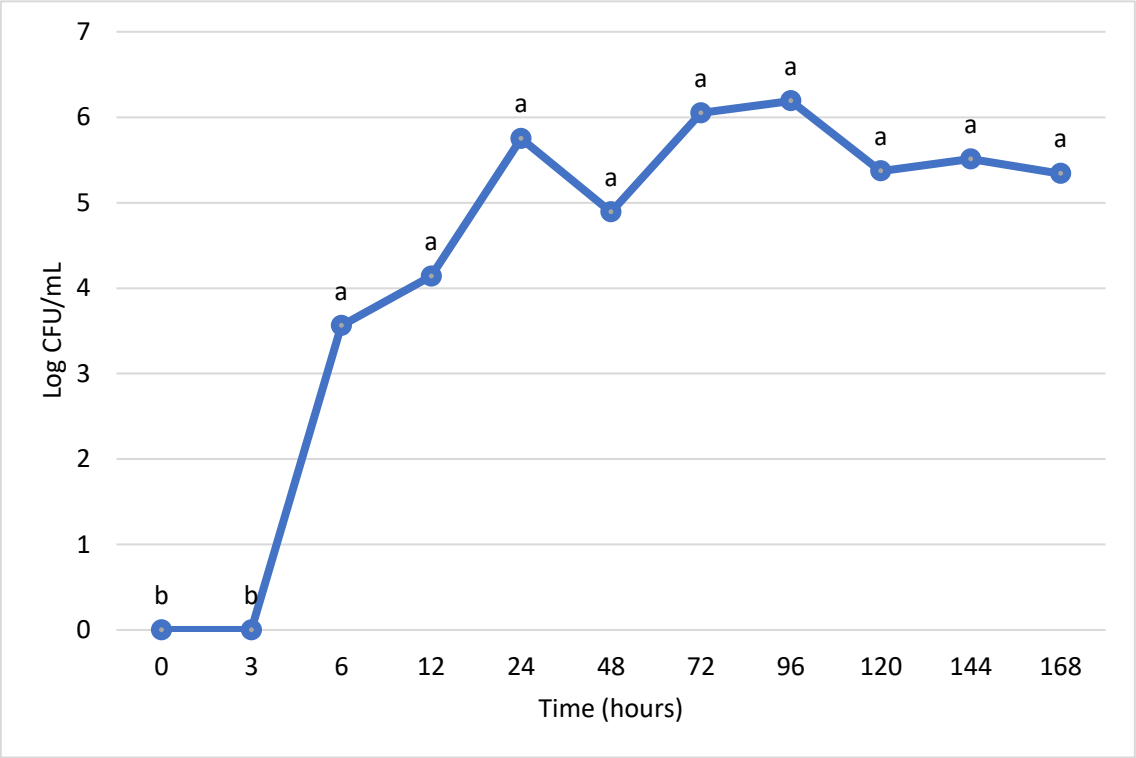
that growers comply with FDA disinfection guidelines, which state that seeds should be disinfected with 20,000 ppm of calcium hypochlorite solution.

On the other hand, in the surface of the jars the presence and growth of *Salmonella* was not observed until 6 hours, however its growth was not significantly different over time. The *Salmonella* population remained similar during the 7 days of germination with values between 3.56 log CFU/mL and 6.19 log CFU/mL (Table 2).

In Figure 2, it can be observed that the behavior of *Salmonella* Enteritidis on the surface is not constant because *Salmonella* use water of the rinse as transport to travel from the sprouts to the surface and different parts of the sprouts could show a different concentration of *Salmonella* present in sprouts. *Salmonella* can survive and adhere to abiotic surfaces (Lim et al., 2020), this is consistent with the results of the study, where the increase in *Salmonella* was observed up to day 3, from 3.56 log cfu/mL to 6.05 log cfu/mL.

Figure 1

Survival of Salmonella on the surface of jars for 7 days.



Although its growth was not significant, there was presence and survival on the surfaces. The irrigation water has been recognized as an important pathway for the contamination of fresh produce with pathogens, therefore, the rinsing of mung bean seeds with distilled water is an important factor in the drift of *Salmonella* to the surface (Ilic et al., 2022). Even so, there was presence and survival on these surfaces which means that cross-contamination is likely. Irrigation water has been recognized as an important pathway for the contamination of fresh produce with pathogens. (Ilic et al., 2022). Therefore, rinsing mung bean seeds with distilled water is an important factor in the *Salmonella* drift to the surface. According to a study in which the fate and mitigation of contaminated *Salmonella* in lettuce (*Lactuca sativa*) grown in a hydroponic system was evaluated, once *Salmonella* is introduced into hydroponic systems, it spreads rapidly to the rest of the system (Li et al., 2022).

According to the results, after inoculating mung bean seeds at a level of 4 log CFU/g in 20 g, *Salmonella* growth was higher on the seeds than on the surface of the jars. For this purpose, the mean

log CFU/g and log CFU/mL were compared. Table 2 shows the growth of *Salmonella* between the seeds and the surface over time. A significant difference in *Salmonella* growth was found at hours 0, 3, days 2, 3, 4, 5, 6, and 7 between seeds and surface. However, no significant differences were observed at 6 and 12 hours, and day 1 ($P < 0.05$).

Table 2

Survival of Salmonella between sprouts and surface over 7 days.

Time (Hours)	Treatment		
	Sprouts (Log CFU/g ± SD)	Surface (Log CFU/ mL ± SD)	C. V (%)
0	4.00 ± 0.47^{a}	0^{b}	23.57
3	4.10 ± 1.2^{a}	0 ± 0^{b}	41.85
6	4.19 ± 0.33^{a}	3.56 ± 1.06^{a}	53.07
12	3.77 ± 0.34^{a}	4.14 ± 0.19^{a}	9.31
24 (Day 1)	6.02 ± 0.23^{a}	5.75 ± 0.20^{a}	2.94
48 (Day 2)	7.38 ± 0.18^{a}	4.89 ± 0.56^{b}	6.65
72 (Day 3)	7.08 ± 0.17^{a}	6.05 ± 0.27^{b}	1.96
96 (Day 4)	7.23 ± 0.39^{a}	6.19 ± 0.24^{b}	4.42
120 (Day 5)	6.31 ± 0.39^{a}	5.37 ± 0.12^{b}	5.31
144 (Day 6)	6.30 ± 0.29^{a}	5.51 ± 0.40^{b}	4.89
168 (Day 7)	6.19 ± 0.23^{a}	5.34 ± 0.23^{b}	3.04

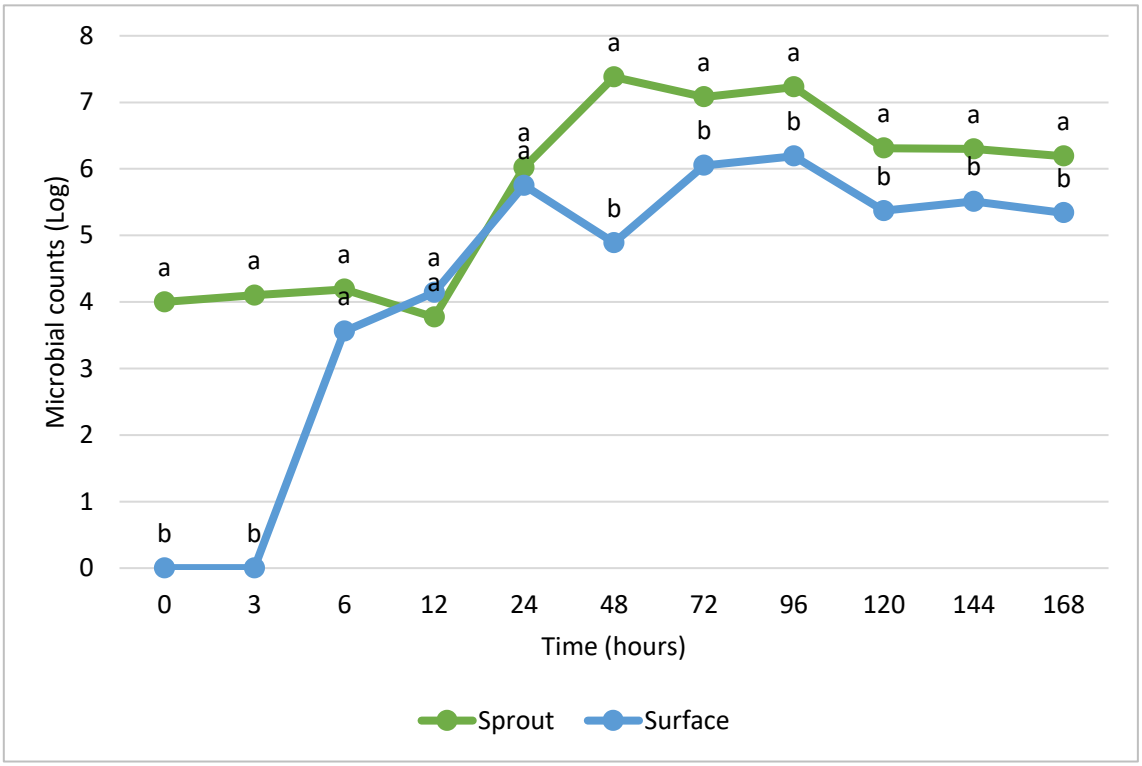
Note. Ab: Different letters present a significant statistical difference ($P < 0.05$) between treatments; CV%: coefficient of variation.

At 0 and 3 hours were sampled mung bean seeds.

At hour 0 and 3, there was no growth of *Salmonella* Enteritidis on the surface, but from hour 6 growth was observed, so it could be assumed that *Salmonella* displaced to the surface through the water used and that during hours 6, 12 and day 1 and day 2 *Salmonella* was adapting to the surface, reaching a peak on the fourth day with a value of 6.19 log UFC/mL and showing a decrease on days 5, 6 and 7. The same behavior can be observed in the sprouts, in which at the beginning *Salmonella* Enteritidis is adapts to the medium (sprouts) until reaching a maximum growth on days 2, 3 and 4 and a decrease on days 5, 6 and 7. The behavior of *Salmonella* Enteritidis in the sprouts and on the surface can be associated to the growth curve of the bacteria, which shows the phase of adaptation, exponential and stationary.

Figure 2.

Survival of Salmonella Enteritidis between sprouts and surface for 7 days.



Mung bean sprouts were produced in jars under conditions commonly used at home on a laboratory scale. For this purpose, ambient conditions (25 °C and 70% RH) were maintained and with twice-daily rinsing of the seeds. The production of sprouts in commercial scale germination equipment has frequent irrigation and drainage, which allows controlling the proliferation of pathogens, while when produced in a closed environment such as jars, in which only the seeds and sprouts are rinsed, *Salmonella* tends to grow (Fu et al., 2008), this is in accordance with was observed in this investigation, where *Salmonella* was detected on the mung bean sprouts and the surface of the jars. Figure 3 shows that from day 2 to 7 *Salmonella* had a similar behavior both in sprouts and on the surface, because when the seeds were rinsed, *Salmonella* is dragged to the surface with a different concentration than that of the sprouts.

A key factor when producing sprouts is to verify that the seeds are free of pathogens, since, if they are contaminated, the pathogen population tends to increase during germination and sprouting,

which are characterized by high humidity and temperatures between 21 °C and 25 °C, that is, they offer an ideal environment for their distribution (Peter J. Taormina et al., 1999).

Similarly, *Salmonella* is a pathogen that tends to survive in humid and sun-shaded environments, the same as the environmental conditions of the system in which the mung bean sprouts were grown, demonstrating its survival and multiplication for 7 days until reaching a growth of 7.39 log CFU/g in the sprouts and 6.19 log CFU/mL on the surface of the jars. Also is a microorganism that survives hostile environmental and is therefore associated with its survival on the surfaces of the system. Additionality, lack of cleaning practices or insufficient and inefficient cleaning practices favor the fixation and permanence of bacteria on food contact (Abban et al., 2012).

For those reasons, it is important that growers follow FDA requirements regarding food contact and non-contact surfaces used to grow, harvest, package, or hold sprouts. They should clean and disinfect food contact surfaces used to grow, harvest, pack, or hold sprouts before they contact seed for growing, sample food contact and non-food contact surfaces and other surfaces in the growing, harvesting, packing, and storage environment, conduct additional testing of areas and surfaces surrounding areas where pathogens were detected, and clean and disinfect surrounding areas and affected surfaces (Code of Federal Regulation, 2023b).

Conclusions

Salmonella Enteritidis can survive and grow during the production of mung bean sprouts over a period of 7 days in small-scale hydroponic systems, i.e., grown on a domestic scale under uncontrolled conditions (ambient temperature 25 °C and 70% relative humidity) and the germination provides an ideal condition for growth *Salmonella* due to the high humidity and ideal temperatures (25 °C) and the seed rinsed allows the reduction of *Salmonella* proliferation control.

Salmonella Enteritidis can survive on surfaces because it can form biofilms allowing it to attach and persist on these surfaces, and it also reaches the surfaces through the water used for rinsing the sprouts; however, its growth is not significant over time during the production of mung bean sprouts.

Recommendations

Evaluate the growth and survival of *Salmonella* on a commercial scale under controlled conditions of hydroponic systems (pH, nutrient solution, temperature, light, air, heat, etc.).

Evaluate the growth and internalization of *Salmonella* separately in leaves, stems, and roots in mung bean sprouts and the nutrient solution of hydroponic systems.

References

Abban, S., Jakobsen, M., & Jespersen, L. (2012). Attachment behaviour of Escherichia coli K12 and Salmonella Typhimurium P6 on food contact surfaces for food transportation. *Food Microbiology*, *31*(2), 139–147. https://doi.org/10.1016/j.fm.2012.04.003

Andrews, W. H., Mislivec, P. B., Wilson, C. R., Bruce, V. R., Poelma, P. L., Gibson, R., Trucksess, M. W., & Young, K. (1982). Microbial Hazards Associated with Bean Sprouting. *Journal of AOAC INTERNATIONAL*, *65*(2), 241–248. https://doi.org/10.1093/jaoac/65.2.241

Clemson University. (2023). *The Food Safety of Sprouts Factsheet*. https://www.clemson.edu/extension/food/food2market/factsheets/7-the-food-safety-of-sprouts-factsheets.html

Code of Federal Regulation. (2023a). *Standards for the growing, harvesting, packing, and holding of produce for human consumption.: Sprouts*. https://www.accessdata.fda.gov/scripts/cdrh/cfdocs/cfcfr/CFRSearch.cfm?CFRPart=112&showFR=1&subpartNode=21:2.0.1.1.12.12

Code of Federal Regulation. (2023b). *Standars for the growing, harvesting, packing, and holding of produce for human consumption: Subpart M-Sprouts*. https://www.accessdata.fda.gov/scripts/cdrh/cfdocs/cfcfr/CFRSearch.cfm?CFRPart=112&showFR=1&subpartNode=21:2.0.1.1.12.12

Code of Federal Regulations. (2015). *21 CFR Part 112 -- Standards for the Growing, Harvesting, Packing, and Holding of Produce for Human Consumption*. https://www.ecfr.gov/current/title-21/chapter-I/subchapter-B/part-112

Dianzhi Hou, Laraib Yousaf, Yong Xue, Jinrong Hu, Jihong Wu, Xiaosong Hu, Naihong Feng, & Qun Shen (2019). Mung Bean (Vigna radiata L.): Bioactive Polyphenols, Polysaccharides, Peptides, and Health Benefits. *Nutrients*, *11*(6). https://doi.org/10.3390/nu11061238

Ding, H., Fu, T.-J., & Smith, M. A. (2013). Microbial contamination in sprouts: How effective is seed disinfection treatment? *Journal of Food Science*, *78*(4), R495-501. https://doi.org/10.1111/1750-3841.12064

Food and Drug Administration (Thu, 2020, February 7). FDA Concludes Major Sampling Study of Sprouts. *FDA*. https://www.fda.gov/food/cfsan-constituent-updates/fda-concludes-major-sampling-study-sprouts

Food and Drug Administration (2022). *Reducing Microbial Food Safety Hazards in the Production of Seed for Sprouting: Guidance for Industry*. https://www.fda.gov/regulatory-information/search-fda-guidance-documents/guidance-industry-reducing-microbial-food-safety-hazards-production-seed-sprouting

Fu, T.-J., Reineke, K. F., Chirtel, S., & VanPelt, O. M. (2008). Factors influencing the growth of Salmonella during sprouting of naturally contaminated alfalfa seeds. *Journal of Food Protection*, *71*(5), 888–896. https://doi.org/10.4315/0362-028X-71.5.888

Ge, C., Rymut, S., Lee, C., & Lee, J. (2014a). Salmonella Internalization in Mung Bean Sprouts and Pre- and Postharvest Intervention Methods in a Hydroponic System. *Journal of Food Protection*, *77*(5), 752–757. https://doi.org/10.4315/0362-028X.JFP-13-370

Ge, C., Rymut, S., Lee, C., & Lee, J. (2014b). Salmonella internalization in mung bean sprouts and pre- and postharvest intervention methods in a hydroponic system. *Journal of Food Protection*, *77*(5), 752–757. https://doi.org/10.4315/0362-028X.JFP-13-370

Gensheimer, K., & Gubernot, D. (2016). 20 Years of Sprout-Related Outbreaks: FDA's Investigative Efforts. *Open Forum Infectious Diseases*, *3*(suppl_1), Article 1438. https://doi.org/10.1093/ofid/ofw172.1140

Grant, A. (2016). Mung Beans Information – Learn How To Grow Mung Beans. *Gardening Know How*. https://www.gardeningknowhow.com/edible/vegetables/beans/how-to-grow-mung-beans.htm

Ilic, S., Moodispaw, M. R., Madden, L. V., & Lewis Ivey, M. L. (2022). Lettuce Contamination and Survival of Salmonella Typhimurium and Listeria monocytogenes in Hydroponic Nutrient Film Technique Systems. *Foods (Basel, Switzerland)*, *11*(21). https://doi.org/10.3390/foods11213508

International Specialty Supply. (2018). *Qualitative Risk Assessment of Sprouted Seeds - International Specialty Supply*. https://sproutnet.com/qualitative-risk-assessment-of-sprouted-seeds/

Li, Y., Zwe, Y. H., Tham, C. A. T., Zou, Y., Li, W., & Li, D. (2022). Fate and mitigation of Salmonella contaminated in lettuce (Lactuca sativa) seeds grown in a hydroponic system. *Journal of Applied Microbiology*, *132*(2), 1449–1456. https://doi.org/10.1111/jam.15295

Lim, S.-M., Lim, E. S., Kim, J.-S., Paik, H.-D., & Koo, O. K. (2020). Survival of foodborne pathogens on stainless steel soiled with different food residues. *Food Science and Biotechnology*, *29*(5), 729–737. https://doi.org/10.1007/s10068-019-00705-6

Peter J. Taormina, Larry R. Beuchat, & Laurence Slutsker (1999). Infections Associated with Eating Seed Sprouts: An International Concern - Volume 5, Number 5—October 1999 - Emerging Infectious Diseases journal - CDC. *Centers for Disease Control and Prevention*, *5*(5). https://doi.org/10.3201/eid0505.990503

Reed, E., Ferreira, C. M., Bell, R., Brown, E. W., & Zheng, J. (2018). Plant-Microbe and Abiotic Factors Influencing Salmonella Survival and Growth on Alfalfa Sprouts and Swiss Chard Microgreens. *Applied and Environmental Microbiology*, *84*(9). https://doi.org/10.1128/AEM.02814-17

Sesauer. (2019). *Sprouts - General*. https://www.texasrealfood.com/promptuary/sprouts/sprouts-general/

Thompson, S. (2000). *Risks Associated With The Consumption of Fresh Sprouts*. https://sproutnet.com/risks-associated-with-the-consumption-of-fresh-sprouts/

United States Department of Agriculture. (2019). *What is the Food Safety Modernization Act and How Does it Apply to Produce? | Food and Nutrition Service*. https://www.fns.usda.gov/f2s/what-is-the-food-safety-modernization-act

Warriner, K., Spaniolas, S., Dickinson, M., Wright, C., & Waites, W. M. (2003). Internalization of bioluminescent Escherichia coli and Salmonella Montevideo in growing bean sprouts. *Journal of Applied Microbiology*, *95*(4), 719–727. https://doi.org/10.1046/j.1365-2672.2003.02037.x

Western Kentucky University. (2022). *Good Agriculture Practices for Sprouts* [Thesis]. Western Kentucky University, United States. chrome-extension://efaidnbmnnnibpcajpcglclefindmkaj/https://www.nifa.usda.gov/sites/default/files/resource/Sprouts%20-%20corrected%20508.pdf

Yang, Y., Meier, F., Ann Lo, J., Yuan, W., Lee Pei Sze, V., Chung, H.-J., & Yuk, H.-G. (2013). Overview of Recent Events in the Microbiological Safety of Sprouts and New Intervention Technologies. *Comprehensive Reviews in Food Science and Food Safety*, *12*(3), 265–280. https://doi.org/10.1111/1541-4337.12010

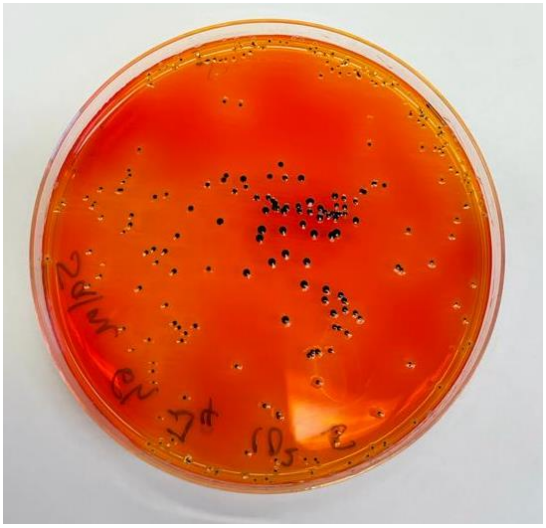
Appendices

Appendix A

Germination jars at room temperature 25 °C and 70% relative humidity.

Annex B

Salmonella Enteritidis images by spread plate method.



Annex C

Preparation of nalidixic acid antibiotic