Escuela Agrícola Panamericana, Zamorano

Food Science and Technology Department

Food Science and Technology Major

Graduation Research Project

Survival of *E.coli* O157:H7 in Sprouts produced in home-scale hydroponic system

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Honduras, november 2023

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Resumen

El consumo de germinados, sobre todo de alfalfa, es el más consumido en los Estados Unidos debido a su valor nutricional y a los beneficios que se les atribuyen para la salud. Sin embargo, los germinados son susceptibles a una contaminación microbiana, lo que provoca enfermedades transmitidas por los alimentos. El objetivo de este estudio fue examinar la supervivencia de *E. coli* O157:H7 en germinados cultivados mediante sistemas hidropónicos caseros y comparar su crecimiento en la superficie de los tarros. Se eligió microorganismo *E. coli* O157:H7, una bacteria patógena. El experimento se llevó a cabo en el laboratorio International Center for Food Industry Excellence (ICFIE) de Texas Tech University, y la preparación del cultivo bacteriano implicó varios pasos para establecer una población microbiana controlada y estandarizada. Se inocularon las semillas con *E. coli* O157:H7 y se tomaron muestras del día 1 al 7 para su análisis. Los resultados indicaron que *E. coli* O157:H7 estaba presente tanto en las semillas como en la superficie del tarro y durante el crecimiento de los brotes. Se utilizó un diseño de Bloques Completos al Azar (BCA) para evaluar los tratamientos. La bacteria proliferó más en las semillas, alcanzando concentraciones de 7.64 Log UFC/g y en la superficie 5.25 Log UFC/g al cabo de 24 horas. La fase de germinación es fuente potencial de contaminación en los germinados, debido a las condiciones favorables que ofrece para el crecimiento bacteriano. Los resultados muestran la necesidad de aplicar prácticas higiénicas estrictas en todo el proceso de producción de germinados para mitigar los riesgos de enfermedades de transmisión alimentaria asociados a su consumo.

Palabras clave: Alfalfa, crecimiento, inoculo, patógeno.

Abstract

The consumption of sprouts, particularly alfalfa sprouts, is the most consumed in the United States due to their nutritional value and perceived health benefits. However, sprouts are susceptible to microbial contamination, leading to foodborne illnesses. This study aimed to examine the survival of *E. coli* O157:H7, a pathogenic bacterium, in sprouts grown using home-scale hydroponic systems and to compare its growth on the surface of the jars. *E. coli* O157:H7, was chosen as a model microorganism. The experiment was conducted at the laboratory of The International Center for Food Industry Excellence at Texas Tech University, and bacterial culture preparation involved various steps to establish a controlled and standardized microbial population. Seeds were inoculated with *E. coli* O157:H7 at 4 Log CFU/g and samples were taken at periods 0, 3, 6, 12, and 24 hours, 2, 3, 4, 5, 6, 7 days for analysis. Results indicated that *E. coli* O157:H7 was present in both seeds/sprouts and on the surface of the jar during the entire experiment period. A Randomized Complete Block was used to evaluate the treatments. The bacterium proliferated on the sprouts, reaching concentrations of 7.64 Log CFU/g and onto the surface 5.25 Log/CFU/ml after 24 hours. The study highlighted the importance of the germination phase as a potential source of contamination in sprouts due to the favorable conditions it provides for bacterial growth. Overall, the research demonstrated the capability of *E. coli* O157:H7 to proliferate during the sprouting process and indicated that the hydroponic environment of sprout production might enhance bacterial growth. The findings underscore the need for stringent hygiene practices throughout the sprout production process to mitigate foodborne illness risks associated with sprout consumption.

Keywords: Alfalfa, Growth, pathogen, inoculum

Introduction

Sprouts are germinated seeds of herbaceous plants (Mir et al., 2021) and are sold for human consumption in salads and sandwiches. Alfalfa sprouts are the most common crop/seed used for sprout production and consumption in the U.S. (Belabre et al., 2022). Eighty-five percent of this production comes from California, Idaho, Oregon, Washington and Nevada. Consumption of sprouts has been on the rise in recent decades owing to their nutritional value, consumer perceived health benefits and ease and rapid production (Ikram et al., 2021).

Raw sprouts have been identified to be an important risk food for the transmission of foodborne diseases (Barbosa et al., 2015). Microbial contamination of sprouts has been reported to occur due to the presence of pathogenic bacteria in seeds, contaminated water and the growth environment facilitating germination and the sprouting process provides optimal conditions for bacterial growth (Yang et al., 2013) . The contamination of sprouts has become a worldwide food safety concern. Between 2015 to 2020, 137 foodborne pathogen associated illnesses were reported resulting from contaminated sprouts in different states of the United States, 84 of which were caused by *E.coli* O157:H7 (Marler, 2022). Annually, almost 300 million illnesses and about 200,000 deaths are caused by diarrheagenic *Escherichia coli (E. coli)* worldwide and this varies by region. Approximately 265,000 illnesses and about 100 deaths are caused by the *E. coli* O157:H7 strain (North Carolina Public Health, 2019). *E. coli* species (including O157:H7) live in the intestine of mammals especially sheep, cattle and goats, but are not normally affected by this organism (Rovid Spickler, 2016). Many the foodborne reported outbreaks have been related to the ingestion of contaminated food with this pathogen (Centers for Disease Control and Prevention [CDC], 2014). Infectious with *E. coli* O157:H7 is associated with bloody diarrhea, nausea, vomiting, dehydration, asthenia and decreased diuresis (Ameer et al., 2023).

The Food Safety Modernization Produce Safety Rule (FSMA PSR) stablished specific rules for produce growers, this focuses on the prevention of contamination before, during, and after the

production of fresh fruits and vegetables, typically eaten raw (Food and Drug Administration [FDA], 2015). The requirements in 21 CFR Part 11, Subpart M (Sprouts) apply to the growing, harvesting, packing and holding of all sprouts except sprouts that are grown in soil or non-soil substrates (e.g., mats, perlite, or other growth media) and that are harvested above the soil or substrate line without their roots (§112.141) (FDA, 2019).

The FSMA PSR specifies that sprout growers must take measures reasonably necessary to prevent the introduction of known or reasonably foreseeable hazards into seeds or beans that will use for sprouting. They must visually examine seeds and beans, and the packaging used to ship seeds or beans, for signs or potential contamination with known or reasonably foreseeable hazards and use only seeds that have been treated with scientifically valid method to reduce microorganisms of public health significance and handle the seeds in a manner to minimize potential contamination. They must grow, harvest, pack, and hold the sprouts in a fully enclosed building, and food contact surfaces you use to grow, harvest, pack or hold sprouts must be cleaned and sanitized before contact with sprouts or seeds or bean used to grow sprouts. They must take samples of spent sprout irrigation water or sprouts and test for *E. coli* O157:H7, Salmonella species, and any pathogens and have a written corrective action plan if any of these pathogens are positive (CFR, 2016).

For *E. coli*, the focus is on the water use during growth (called agricultural water). The requirement for agricultural water used during cultivation activities is a microbial water quality profile (MWQP). There are two values that are included within the MWQP, Geometric Mean (GM) which specifies that there must be an average of less than 126 CFU of *E. coli* per 100ml of water Statistical Threshold Value (STV), which tells that the maximum is 416 CFU of *E. coli* per 100ml of water. The requirement for agricultural water used during and after harvest is not detectable, generic *E. coli* in 100 mL of water (CFR, 2016). Similar to other fresh vegetables, sprout production has embraced soilless production in controlled environment agricultural facilities such as hydroponics (Al-Kodmany, 2018). These facilities have been reported to have lower food safety risks in comparison to soil-based

production (Wang et al., 2020). Despite of this, recent reports have indicated food borne pathogen contamination in hydroponic produced vegetables (Sela Saldinger et al., 2023). The fact that hydroponic production of vegetables and sprouts is being adopted at home-scale level in urban centers where control is limited, poses a bigger food safety risk, which creates the need to examine the survival dynamics of the major foodborne pathogens in sprouts in similar homes units, especially where enteric pathogens such as *E. coli* can be introduced and persist in plants for longer periods compared to soil-grown crops (Xu y Warriner, 2005).

This study was aimed to:

First, to determine the differences between the two treatments growth in sprouts and the surface of the jar in terms of survival.

Second, to examine the survival of *E. coli* O157:H7 in sprouts, produced in home-scale hydroponic systems.

Third, to determine the survival of *E. coli* O157:H7 on the surface of the jar.

Materials and Methods

Location

The experiment was carried out at the International Center for Food Industry Excellence (ICFIE) in the Experimental Sciences Building at Texas Tech University in Lubbock, Texas, United States of America.

Bacterial Culture Preparation

For this study *E. coli* O157:H7 ATCC strain A4 was used, this strain was obtained from feces of a patient with hemolytic uremic syndrome. The first day, one loop of the isolated was taken and transferred to 10ml of Brain Heart Infusion (BHI), this was repeated in two more tubes. The culture was labeled and incubated for 24 hours at 37 °C. The next day, one tube was selected and conducted through serial dilutions, which were spread plated onto MacConkey selective agar and incubated for 24 hours at 37 °C, to determine the concentration of bacteria on the culture (Log CFU/ml). The third day, one colony was selected from the plates and spread plated to be put in 10ml BHI tubes with ampicillin antibiotic with different concentrations (10μg /ml, 25 μg /ml, and 50μg/ml). The fourth day, 1ml was transferred from the 10ml BHI tubes with different concentrations (10μg /ml, 25 μg /ml, and 50μg/m) to 9ml tubes of BHI without ampicillin antibiotic and were incubated for 24 hours at 37 °C. The fifth day, 1ml taken from all the 10 ml BHI tubes without ampicillin antibiotic was put into two new 9ml tubes of BHI with a concentration of 10 μg /ml ampicillin antibiotic to stabilize the strain and were incubated for 24 hours at 37 °C. On the day six, 1ml from the 10 ml BHI with a concentration of 10μg/ml tubes with the strain stabilized was put into new 9ml tubes of BHI with a concentration 25μg/ml ampicillin antibiotic to generate resistance, the tubes were incubated for 24 hours at 37 °C. There were made serial dilutions from the tube of concentration 10 μg/ml BHI plated onto MacConkey Agar with a concentration of 10 μg/ml ampicillin antibiotic to determine the log concentration of the bacteria and incubated for 24 hours at 37 °C. On the day 7, the CFU present in a concentration of 10 μg/ml ampicillin antibiotic was counted and 1ml was transferred from the 10ml BHI tubes with a

concentration of 25 μg/ml to a concentration of 50 μg/ml to be incubated for 24 hours at 37 °C to generate resistance. Also, were made serial dilutions from the tube concentration 25 μg/ml BHI and plated the inoculum onto MacConkey agar with 25 μg /ml of antibiotic to determine the log concentration of the bacteria. On the last day, there were made serial dilutions from the 10ml BHI tubes with a concentration of 25 μg/ml and plate it in MacConkey agar with 50 μg/ml of ampicillin (antibiotic) to establish resistant strains. There were resistant strains of *E. coli* because of the high concentration of background flora found on the seeds. Then, 1ml with a concentration of 25 μg/ml from the 10ml BHI tubes was transferred to a concentration of 50μg/ml BHI ampicillin and incubated 8 hours at 37 °C, until attaining 10^8 CFU/ml. 1ml of the concentrations that contains 50 μg/ml was put into 100ml BHI and incubated. After this inoculum was prepared, it was divided into five 20ml aliquots in centrifuge tubes. The samples were centrifuged for 10 minutes at 4,000 x g at room temperature, the supernatant was decanted carefully, to not lose the pellet. 10ml of BHI with 10% of glycerol were added and then the pellets were mixed well by shaking and suspended into one 50ml tube to bring the volume up to 50ml with BHI broth with 10% glycerol.

The biological safety hood was used to make the strains, all the materials were autoclaved before starting to pipette 1ml of the cocktail into sterile 2ml cryogenic tubes. Finally, the cryogenic tubes were labeled and put into a labeled freezer box at -80 °C.

Inoculation

One vial of the ampicillin resistant *E. coli* O157:H7 was taken from the -80 °C freezer, one loop into 10ml of BHI and was incubated at 37 °C overnight. After the incubation, the 10ml of ampicillin resistant *E. coli* were put into sterile 30ml high-strength centrifuge tubes. The sample was centrifugated for 10 minutes at 4,000 x g at room temperature, and it was added 10ml of sterile distilled water and mixed well by shaking and suspended into 200ml of sterile distilled water and then 40ml were divided into five beakers.

Alfalfa seeds were purchased from a commercial provider. The suppliers of the seeds provided a certification of analysis that the seeds were heat treated and negative for *E. coli* and *Salmonella*.

Each batch (20g) will be immersed in 40ml of *E. coli* O157:7 inoculum (original concentration 10^8 CFU/ml), gently swirling for 5 minutes at room temperature, to achieve the targeted 10^4 CFU/g level of inoculation on the seed. Excess bacterial suspension was drained, and the inoculated seed was spread over sterile absorbent sheets and air-dried overnight for 16 to 18 hours, under a biological safety hood. The inoculated seeds were soaked in sterile tap water and were left to soak at 25 °C for 8 hours, after which the excess will be drained. The seeds were 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 were inverted and kept at an angle of 45° in a tray to ensure proper drainage during the incubation. The sprouting jars were covered with a light blocking material at room temperature 25 °C with a relative humidity of 70 ± 5%. Germinating seeds were rinsed with 200 mL of sterile distilled water twice daily. The sprouts were exposed to light on day 6 and day 7 to the development of chlorophyll, and the leaves turn into a green color. The trial was done in 5 replicates.

Microbial Enumeration

Before inoculation, samples of the jar surface and sprout seeds were taken, to establish that no foodborne pathogens were present prior to the experiment. After inoculating the ampicillin resistant *E. coli* O157:H7, samples of the seed or sprouts were collected at the time 0 h, 3 h, 6 h, 12 h, 24 h, 48 h, 72 h, 4 days, 5 days 6 days and 7 days from the inner surface of the lid (5 cm^2) (S), and sprouts (SP). For each sample time, 2 g of sprouts were taken for microbial analysis. The sample sprouts were aseptically placed within a pre-weighed stomacher bag, and the mass was determined. The bags were transferred immediately to the lab where 18ml of Buffer Peptone Water (BPW) was added and the mixture was pummeled for 2 minutes with a stomacher (Lab Blender 400, Seward Laboratory, Worthington, UK). Plant tissues were further mashed manually by pressing with a thumb through the bag to facilitate the release of potentially internalized bacteria. The lid surface was

swabbed on the inside (5 square cm) and then placed in a pre-weighed stomacher bag with BPW. Samples were then directly plated on MacConkey Agar amended with 50 µg/ml ampicillin.

Experimental Design

Two treatments were evaluated, five replicates were established in the experiment for a total of ten experimental units. A Randomize Complete Block Design was used to compare the growth of E. coli O157:H7 over time (0 hours to 7 days)

An ANOVA and a DUNCAN mean separation were performed to estimate significant differences between treatments ($P < 0.05$). The Statistical Analysis System® (SAS version 9.4) was used.

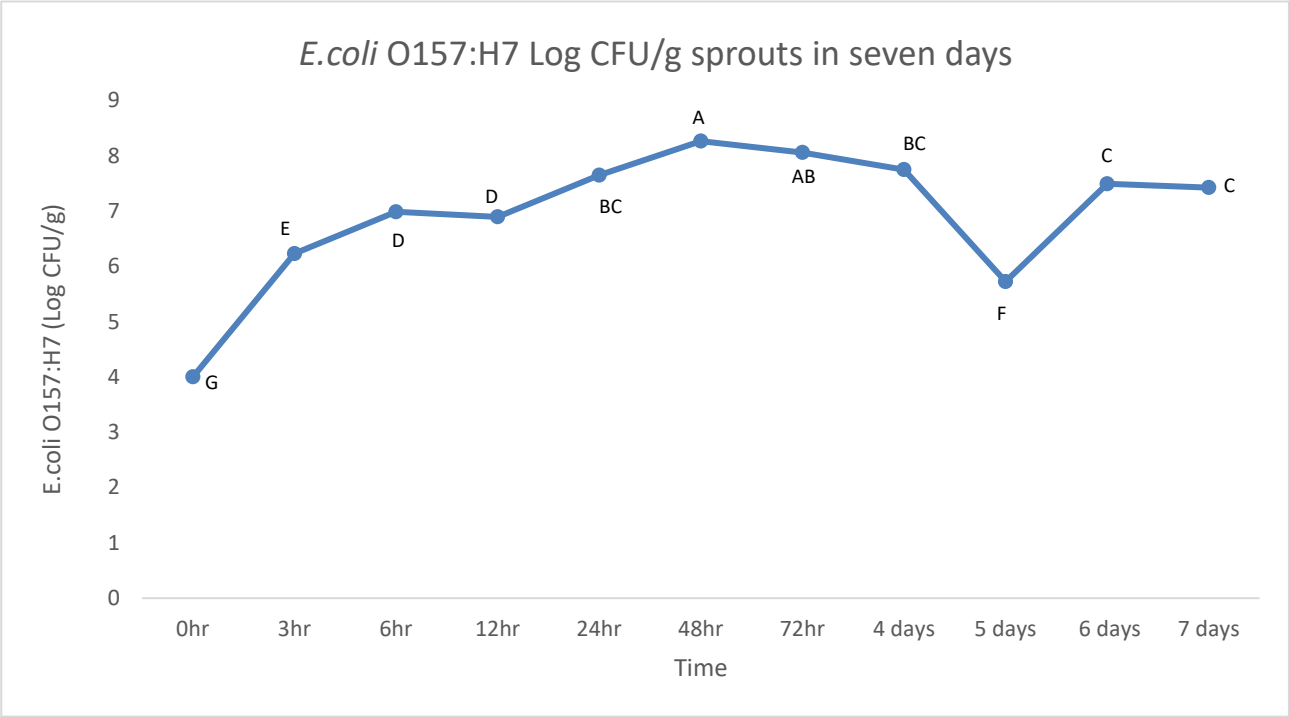
Results and Discussion

According to Hamilton y Vanderstoep (1979) the germination step is the main source of contamination in sprouts as bacteria present in the seeds may become internalized during sprouting. As Robertson et al. (2002) mentioned, seeds can harbor high levels of bacteria ranging from 3.00 to 6.00 Log CFU/g. For this reason, this step focuses not only on the sources of contamination in the sprouts but also on the seeds (Figure 1).

The seeds of alfalfa were germinated at (70 ± 5%) of moisture and warmth temperatures around 21 °C to 26 °C (Benincasa et al., 2019), these factors are ideal for pathogen growth onto the sprouts. According to Peñas et al. (2009) during the germination of the seeds, macromolecules such as lipids and proteins are broken down to form nutrients that are more easily digested and absorbed for *E. coli* O157:H7. For this reason, the pathogen grows during the sprouting process (Figure 1).

Figure 1

Growth of E. coli O157:H7 on seeds/sprouts over seven days



Data also shows differences between treatment and periods of time. *E.coli* O157:H7 can reach concentrations of 6.1 Log CFU/g in seeds around 24hr, this can occur at 3 Log CFU/g initial concentration (Shaw et al., 2016). Similarly, in this study *E. Coli* O157:H7 could proliferate from 4.00 Log CFU/g to 7.6482 Log CFU/g in 24 hours (Table 1).

Table 1 provides data on the growth of *E. coli* O157:H7 with measured given in hours (seed) and days (sprouts). There are significant differences between growth of *E. coli* O157:H7 and time periods. At the beginning 0 hours (group G), sprouts had a relatively low bacterial contamination of 4.00 log CFU/g. That indicates the initial level of contamination. Between 3 hours and 12 hours (groups D), there is a significant increase in the bacterial population in the sprouts. Suggests that *E. coli* O157:H7 adapted and multiplied rapidly during this period. Comparing the groups with different treatments (A, AB, BC, and C). It can observe the duration of exposure to *E. coli* O157:H7 significantly influences the bacterial populations in sprouts. Longer exposure times generally result in higher bacterial loads.

Table 1

Growth of E. coli O157:H7 on seeds/sprouts over seven days after inoculum and the differences between time periods

Group	*E. coli* O157:H7 seed/sprout Log CFU/g	Time
G	4.0000	0hr
E	6.2308	3hr
D	6.9860	6hr
D	6.8944	12hr
BC	7.6482	1 day
A	8.2634	2 days
AB	8.0572	3 days
BC	7.7484	4 days
F	5.7244	5 days
C	7.4924	6 days
C	7.4244	7 days
CV (%)	4.46	

Note. A-G: Different letters represent differences between treatment and time. C-C: Same letters represent that there are not differences.

AB: Letters in pairs do not represent differences with any of the periods that contains one of the letter of the group. Hr: Hours.

The way *E. coli* O157:H7 interacts in the soil and hydroponic systems is different. The hydroponic system provides a more favorable condition for bacterial growth because it has more moisture and water. *E. coli* O157:H7 is more movable in hydroponic environment according to Erickson et al. (2010) for this reason the pathogen could proliferate in the surface of the jar and attached to wet surfaces once the jar drained. According to Redondo et al. (2019) *E. coli* O157:H7 can survive for months in waters containers .

E. coli O157:H7 exhibited significant growth, on the surface, from 5.00 Log CFU/ml to 8.00 Log CFU/ml (Figure 2). Therefore, on day five of germination, alfalfa sprouts have a higher nutrient content compared to the beginning of the germination process, minerals and nutrients increase in a 70% more and the minerals could be dispersed around the jar according to López Hernández et al. (1990). The sprouts were already in direct contact with the surface of the jar and the microorganism was able to again more strength on day five too.

Figure 2

Growth of E. coli O157:H7 onto the surface of the jar over seven days

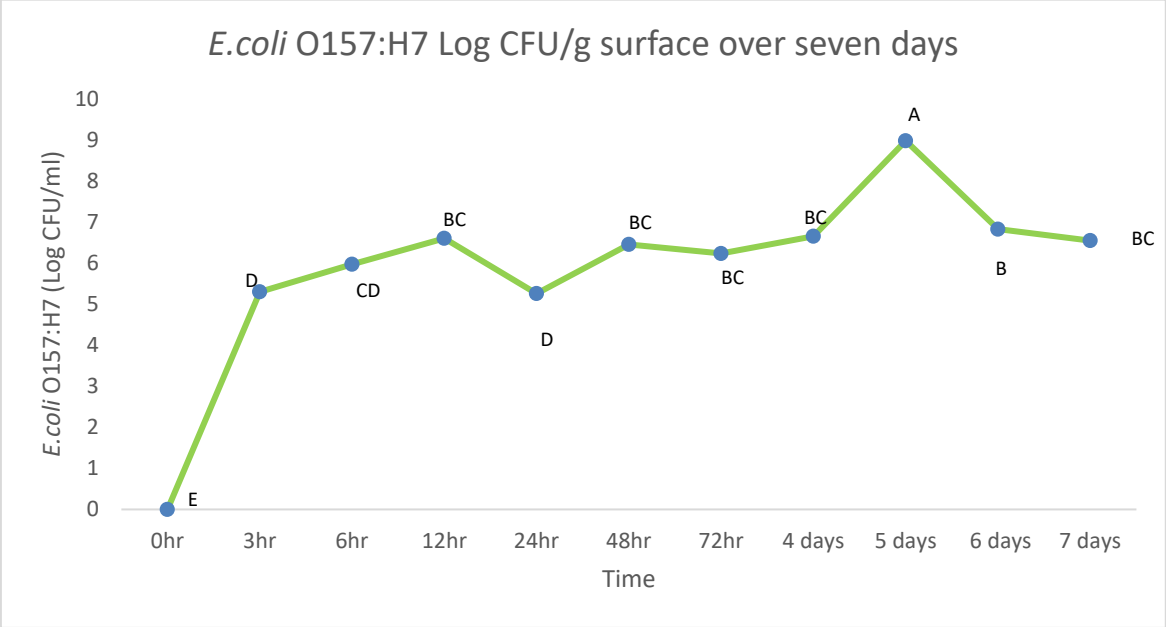


Table 2 presents the growth of *E. coli* O157:H7 on the surface of the jar, with measurements given by hours and days. At the beginning 0 hours (group E), there is no detectable bacterial contamination on the surface of the jar. Indicates that the surface is initially clean and uncontaminated. Between 3 hours and 12 hours (groups D, BC), there is a significant increase in the bacterial population on the surface of the jar. *E. coli* O147:H7 adapted rapidly during this period, leading to a substantial increase bacterial load. Comparing the groups (A, BC, CD and D). It can be observed the duration of exposure to *E. coli* O157:H7 significantly influences the bacterial population on the jar.

Table 2

Growth of E. coli O157:H7 onto the surface of the jar after the first rinse over seven days

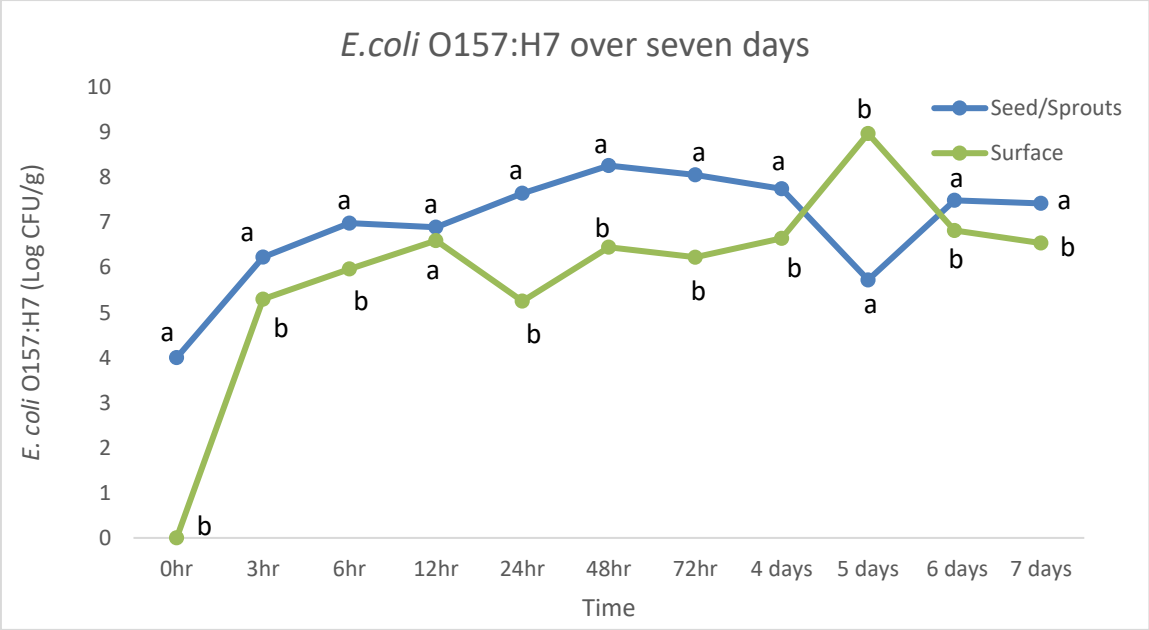
Group	*E. coli* O157:H7 surface Log CFU/ml	Time
E	0.00	0hr
D	5.2982	3hr
CD	5.9682	6hr
BC	6.5992	12hr
D	5.2544	1 day
BC	6.4508	2 days
BC	6.2298	3 days
BC	6.6492	4 days
A	8.973	5 days
B	6.8218	6 days
BC	6.5434	7 days
CV (%)	9.7	

Note. A-B: Different letters represent differences between treatment and time. D-D: Same letters represent that there is no differences. BC: Letters in pairs do not represent differences with any of the periods that contains one of the letter of the group. Hr: Hours.

The counts of *E. coli* O157:H7 in the two treatments increased from the initial inoculum level by about 2 Log CFU/ml in 3 hours into the seed and 5 Log CFU/ml onto the surface after the inoculum (Figure 3).

Figure 3

Growth of E. coli O157: H7 on seeds/sprouts and the jar surface over a seven days periods



There was a higher growth of *E. coli* O157:H7 in the sprouts compared to the surface of the jars. Over time, the growth of *E. coli* O157:H7 on the seeds increased in both treatments. Even though, on day five there was a lower count in the sprouts due to the germination process, which is completed between day 3-8 according to Jiménez Pérez (2004), that is when they are ready to be harvested and are directly touching the surface of the jar which makes the pathogens more likely to stay on the surface on the jar instead of the sprouts. According to Macarisin et al. (2013) the pathogens are able to attach and even proliferate on plants and abiotic surfaces.

As was expected, due to the sprouts provides favorable nutritional conditions not only for its growth but also for other organisms that associate with it, seeking food and survival opportunities like pathogens (Arriagada Ríos, 2000).

The growth of *E. coli* O157:H7 in the sprouts shows a higher concentration compared to the surface.

In this study, we identified that *E. coli* O157:H7 was capable to proliferate by at least 4.00 Log CFU/g to 8.2634 Log CFU/g during sprouting and 0.00 Log CFU/ml to 8.9739 onto the surface in a short time of period generating a risk for the consumer from the beginning of germination process and from the begging of the first rinsed (Table 3). The results showed significant differences in the treatments ($P<0.05$) except at 12 hours ($P>0.05$), the bacteria could stabilize in both treatments (Table 3).

Table 3

Differences between growth of E. coli O157:H7 on seeds/sprouts and onto the surface of the jar over seven days

Time	*E. coli* O157:H7 seed/sprout Log CFU/g ± SD	*E. coli* O157:H7 surface Log CFU/ml ± SD	CV (%)
0hr	4.0000 ±0.00[a]	0.00[b]	0.0
3hr	6.2308± 0.32[a]	5.2982 ± 0.70[b]	7.09
6hr	6.9860 ± 0.31[a]	5.9682 ± 0.56[b]	3.25
12hr	6.8944 ± 0.39[a]	6.5992 ± 0.11[a]	4.11
1 day	7.6482 ± 0.29[a]	5.2544 ± 1.61[b]	17.49
2 days	8.2634 ± 0.54[a]	6.4508 ± 0.20[b]	4.47
3 days	8.0572 ± 0.33[a]	6.2298 ± 0.22[b]	1.78
4 days	7.7484 ± 0.39[a]	6.6492 ± 0.21[b]	3.66
5 days	5.7244 ± 0.15[a]	8.9730 ± 0.24[b]	2.64
6 days	7.4924 ± 0.24[a]	6.8218 ± 0.16[b]	3.27
7 days	7.4244 ± 0.08[a]	6.5434 ± 0.37[b]	3.97

Note. CV (%): Coefficient of variation. CFU: Colony Forming Units. Log: Logarithms. g: Gram. ml: Milliliter SD: Standard Deviation. [ab]Different letters in each column denote significant differences between treatments ($P<0.05$) . [aa]Same letters do not denote differences between treatments. Hr: Hours.

Conclusions

E. coli O157:H7 is able to increase by 4-7 log Colony Forming Units (CFU) from 0 hours to 7 days onto the seeds/sprouts.

E. coli O157:H7 can migrate from sprouts to the surface of the jar once the jar is rinsed and maintain viability for a period of seven days.

E. coli O157:H7 can persist and proliferate in both environments sprouts and surfaces, this characteristic represents a risk, since it could lead an easy spread of the bacteria in hydroponic systems.

Recommendations

Take samples of the water that is used for the germination and sprouting process. Ensure that samples are taken at different time intervals to obtain a comprehensive understanding of *E. coli* O157:H7 can growth on water.

Evaluate the cotyledons of sprouts or the short stem to pinpoint the regions with the greatest pathogen presence. This will help identify if one part of the sprout is more susceptible to contamination than others.

Evaluate the reduction of bacterial load using a commercial disinfectant available at home. Perform efficacy test with the disinfectant in a series of controlled experiments to assess its ability to reduce bacterial contamination in sprouts.

Conduct a comparative analysis of the bacterial contamination levels between *E. coli* O157:H7 and *L. Monocytogens* in sprout samples. This assessment will provide insights into the relative prevalence and potential risks associated with these two pathogens, aiding in the development of targeted mitigation strategies for sprout safety.

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Annex

Annex A

Statistical analysis seed

R-Square	Coeff Var	Root MSE	Surv Mean
0.985847	4.707581	0.310142	6.588145

Annex B

Statistical analysis of surface

R-Square	Coeff Var	Root MSE	Surv Mean
0.948496	9.711664	0.571999	5.889818

Annex C

Significance of treatment

Significance of treatment		
	Hour 3	
TRT	Pr>F	0.0227
	Hour 6	
TRT	Pr>F	0.0016
	Hour 12	
TRT	Pr>F	0.1683
	Hour 24	
TRT	Pr>F	0.0285
	Hour 48	
TRT	Pr>F	0.001
	Hour 72	
TRT	Pr>F	<.0001
	Day 4	
TRT	Pr>F	0.0027
	Day 5	
TRT	Pr>F	<.0001
	Day 6	
TRT	Pr>F	0.0107
	Day 7	
TRT	Pr>F	0.0074

Note. TRT: Treatment.

Annex D

Counts of E. coli O157:H7

Time (hr)	Type	Jar	Plate 1	Plate 2	Average	Dilution	CFU/mL	Log CFU/mL
3	Seed	J1	94	75	84.5	10000	845000	5.927
	Seed		17	12	14.5	100000	1450000	6.161
	Seed	J2	TMTC	TMTC	TMTC	100	TMTC	TMTC
	Seed		77	84	80.5	10000	805000	5.906
	Seed		13	9	11	100000	1100000	6.041
	Seed	J3	TMTC	TMTC	TMTC	100	TMTC	TMTC
	Seed		TMTC	TMTC	TMTC	10000	TMTC	TMTC
	Seed		114	11	62.5	100000	6250000	6.796
	Seed	J4	TMTC	TMTC	TMTC	100	TMTC	TMTC
	Seed		98	65	81.5	10000	815000	5.911
	Seed		5	45	25	100000	2500000	6.398
	Seed	J5	TMTC	TMTC	TMTC	100	TMTC	TMTC
	Seed		97	157	127	10000	1270000	6.104
	Seed		28	9	18.5	100000	1850000	6.267

Note. TMTC: Too many to count Numbers To Count. CFU: Colony Forming Units. Log: Logarithms. mL: Milliliters.

Time (hr)	Type	Jar	Plate 1	Plate 2	Average	Dilution	CFU/mL	Log CFU/mL
6h	Seed	J1	TMTC	TMTC	TMTC	100	TMTC	TMTC
	Seed		TMTC	TMTC	TMTC	10000	TMTC	TMTC
	Seed		61	70	65.5	100000	6550000	6.816
	Seed	J2	TMTC	TMTC	TMTC	100	TMTC	TMTC
	Seed		TMTC	TMTC	TMTC	10000	TMTC	TMTC
	Seed		39	44	41.5	100000	4150000	6.618
	Seed	J3	TMTC	TMTC	TMTC	100	TMTC	TMTC
	Seed		TMTC	TMTC	TMTC	10000	TMTC	TMTC
	Seed		249	234	241.5	100000	24150000	7.383
	Seed	J4	TMTC	TMTC	TMTC	100	TMTC	TMTC
	Seed		TMTC	TMTC	TMTC	10000	TMTC	TMTC
	Seed		250	89	169.5	100000	16950000	7.229
	Seed	J5	TMTC	TMTC	TMTC	100	TMTC	TMTC
	Seed		TMTC	TMTC	TMTC	10000	TMTC	TMTC
	Seed		79	74	76.5	100000	7650000	6.884

Time (hr)	Type	Jar	Plate 1	Plate 2	Average	Dilution	CFU/mL	Log CFU/mL
12h	Seed	J1	TMTC	TMTC	TMTC	100	TMTC	TMTC
	Seed		TMTC	TMTC	TMTC	10000	TMTC	TMTC
	Seed		187	212	199.5	100000	19950000	7.300
	Seed	J2	TMTC	TMTC	TMTC	100	TMTC	TMTC
	Seed		TMTC	TMTC	TMTC	10000	TMTC	TMTC
	Seed		151	201	176	100000	17600000	7.246
	Seed	J3	TMTC	TMTC	TMTC	100	TMTC	TMTC
	Seed		TMTC	TMTC	TMTC	10000	TMTC	TMTC
	Seed		19	26	22.5	100000	2250000	6.352
	Seed	J4	TMTC	TMTC	TMTC	100	TMTC	TMTC
	Seed		TMTC	TMTC	TMTC	10000	TMTC	TMTC
	Seed		6	9	7.5	1000000	7500000	6.875
	Seed	J5	TMTC	TMTC	TMTC	100	TMTC	TMTC
	Seed		TMTC	TMTC	TMTC	10000	TMTC	TMTC
	Seed		7	3	5	1000000	5000000	6.699

Time (hr)	Type	Jar	Plate 1	Plate 2	Average	Dilution	CFU/mL	Log CFU/mL
24h	Seed	J1	TMTC	TMTC	TMTC	100	TMTC	TMTC
	Seed		TMTC	TMTC	TMTC	10000	TMTC	TMTC
	Seed		64	61	62.5	1000000	62500000	7.796
	Seed	J2	TMTC	TMTC	TMTC	100	TMTC	TMTC
	Seed		TMTC	TMTC	TMTC	10000	TMTC	TMTC
	Seed		49	29	39	1000000	39000000	7.591
	Seed	J3	TMTC	TMTC	TMTC	100	TMTC	TMTC
	Seed		TMTC	TMTC	TMTC	10000	TMTC	TMTC
	Seed		110	128	119	1000000	119000000	8.076
	Seed	J4	TMTC	TMTC	TMTC	100	TMTC	TMTC
	Seed		TMTC	TMTC	TMTC	10000	TMTC	TMTC
	Seed		12	48	30	1000000	30000000	7.477
	Seed	J5	TMTC	TMTC	TMTC	100	TMTC	TMTC
	Seed		TMTC	TMTC	TMTC	10000	TMTC	TMTC
	Seed		19	21	20	1000000	20000000	7.301

Time (hr)	Type	Jar	Plate 1	Plate 2	Average	Dilution	CFU/mL	Log CFU/mL
48	Seed	J1	226	200	213	1000000	213000000	8.328
	Seed		62	35	48.5	10000000	485000000	8.686
	Seed	J2	206	126	166	1000000	166000000	8.220
	Seed		18	53	35.5	10000000	355000000	8.550
	Seed	J3	31	19	25	1000000	25000000	7.398
	Seed		4	8	6	10000000	60000000	7.778
	Seed	J4	TMTC	TMTC	TMTC	1000000	TMTC	TMTC
	Seed		101	89	95	10000000	950000000	8.978
	Seed	J5	48	75	61.5	1000000	61500000	7.789
	Seed		11	6	8.5	10000000	85000000	7.929

Time (hr)	Type	Jar	Plate 1	Plate 2	Average	Dilution	CFU/mL	Log CFU/mL
72	Seed	J1	239	140	189.5	1000000	189500000	8.278
	Seed		33	13	23	10000000	230000000	8.362
	Seed	J2	129	80	104.5	1000000	104500000	8.019
	Seed		20	15	17.5	10000000	175000000	8.243
	Seed	J3	32	67	49.5	1000000	49500000	7.695
	Seed		8	4	6	10000000	60000000	7.778
	Seed	J4	243	246	244.5	1000000	244500000	8.388
	Seed		28	30	29	10000000	290000000	8.462
	Seed	J5	43	56	49.5	1000000	49500000	7.695
	Seed		4	5	4.5	10000000	45000000	7.653

Day	Type	Jar	Plate 1	Plate 2	Average	Dilution	CFU/mL	Log CFU/mL
4	Seed	J1	82	108	95	1000000	95000000	7.978
	Seed		4	17	10.5	10000000	105000000	8.021
	Seed	J2	107	100	103.5	1000000	103500000	8.015
	Seed		12	16	14	10000000	140000000	8.146
	Seed	J3	53	70	61.5	1000000	61500000	7.789
	Seed		6	6	6	10000000	60000000	7.778
	Seed	J4	65	56	60.5	1000000	60500000	7.782
	Seed		9	4	6.5	10000000	65000000	7.813
	Seed	J5	12	17	14.5	1000000	14500000	7.161
	Seed		2	0	1	10000000	10000000	7.000

Day	Type	Jar	Plate 1	Plate 2	Average	Dilution	CFU/mL	Log CFU/mL
5	Seed	J1	52	77	64.5	10000	645000	5.810
	Seed		3	4	3.5	100000	350000	5.544
	Seed	J2	79	82	80.5	10000	805000	5.906
	Seed		7	4	5.5	100000	550000	5.740
	Seed	J3	81	41	61	10000	610000	5.785
	Seed		12	5	8.5	100000	850000	5.929
	Seed	J4	48	40	44	10000	440000	5.643
	Seed		1	3	2	100000	200000	5.301
	Seed	J5	36	45	40.5	10000	405000	5.607
	Seed		12	7	9.5	100000	950000	5.978

Day	Type	Jar	Plate 1	Plate 2	Average	Dilution	CFU/mL	Log CFU/mL
6	Seed	J1	TMTC	TMTC	TMTC	100000	TMTC	TMTC
	Seed		52	60	56	1000000	56000000	7.748
	Seed	J2	TMTC	TMTC	TMTC	100000	TMTC	TMTC
	Seed		29	32	30.5	1000000	30500000	7.484
	Seed	J3	36	25	30.5	1000000	30500000	7.484
	Seed		1	0	0.5	10000000	5000000	6.699
	Seed	J4	31	28	29.5	1000000	29500000	7.470
	Seed		5	4	4.5	10000000	45000000	7.653
	Seed	J5	37	34	35.5	1000000	35500000	7.550
	Seed		6	2	4	10000000	40000000	7.602

Day	Type	Jar	Plate 1	Plate 2	Average	Dilution	CFU/mL	Log CFU/mL
7	Seed	J1	198	214	206	100000	20600000	7.314
	Seed		69	55	62	1000000	62000000	7.792
	Seed	J2	222	185	203.5	100000	20350000	7.309
	Seed		44	38	41	1000000	41000000	7.613
	Seed	J3	121	155	138	100000	13800000	7.140
	Seed		44	35	39.5	1000000	39500000	7.597
	Seed	J4	156	151	153.5	100000	15350000	7.186
	Seed		32	26	29	1000000	29000000	7.462
	Seed	J5	157	182	169.5	100000	16950000	7.229
	Seed		36	44	40	1000000	40000000	7.602

Time (hr)	Type	Jar	Plate 1	Plate 2	Average	Dilution	CFU/mL	Log CFU/mL
3	Surface	J1	TMTC	TMTC	TMTC	100	TMTC	TMTC
	Surface		8	1	4.5	10000	45000	4.653
	Surface		0	1	0.5	100000	50000	4.699
	Surface	J2	152	73	112.5	100	11250	4.051
	Surface		11	3	7	10000	70000	4.845
	Surface		0	0	0	100000	0	TMTC
	Surface	J3	TMTC	TMTC	TMTC	100	TMTC	TMTC
	Surface		82	38	60	10000	600000	5.778
	Surface		6	7	6.5	100000	650000	5.813
	Surface	J4	TMTC	TMTC	TMTC	100	TMTC	TMTC
	Surface		89	81	85	10000	850000	5.929
	Surface		18	16	17	100000	1700000	6.230
	Surface	J5	TMTC	TMTC	TMTC	100	TMTC	TMTC
	Surface		32	32	32	10000	320000	5.505
	Surface		3	3	3	100000	300000	5.477

Time (hr)	Type	Jar	Plate 1	Plate 2	Average	Dilution	CFU/mL	Log CFU/mL
6h	Surface	J1	TMTC	TMTC	TMTC	100	TMTC	TMTC
	Surface		33	76	54.5	10000	545000	5.736
	Surface		8	2	5	100000	500000	5.699
	Surface	J2	TMTC	TMTC	TMTC	100	TMTC	TMTC
	Surface		13	4	8.5	10000	85000	4.929
	Surface		4	0	2	100000	200000	5.301
	Surface	J3	TMTC	TMTC	TMTC	100	TMTC	TMTC
	Surface		TMTC	TMTC	TMTC	10000	TMTC	TMTC
	Surface		54	14	34	100000	3400000	6.531
	Surface	J4	TMTC	TMTC	TMTC	100	TMTC	TMTC
	Surface		162	146	154	10000	1540000	6.188
	Surface		13	53	33	100000	3300000	6.519
	Surface	J5	TMTC	TMTC	TMTC	100	TMTC	TMTC
	Surface		80	117	98.5	10000	985000	5.993
	Surface		21	15	18	100000	1800000	6.255

Time (hr)	Type	Jar	Plate 1	Plate 2	Average	Dilution	CFU/mL	Log CFU/mL
12h	Surface	J1	TMTC	TMTC	TMTC	100	TMTC	TMTC
	Surface		TMTC	TMTC	TMTC	10000	TMTC	TMTC
	Surface		34	47	40.5	100000	4050000	6.607
	Surface	J2	TMTC	TMTC	TMTC	100	TMTC	TMTC
	Surface		TMTC	TMTC	TMTC	10000	TMTC	TMTC
	Surface		52	22	37	100000	3700000	6.568
	Surface	J3	TMTC	TMTC	TMTC	100	TMTC	TMTC
	Surface		TMTC	TMTC	TMTC	10000	TMTC	TMTC
	Surface		48	27	37.5	100000	3750000	6.574
	Surface	J4	TMTC	TMTC	TMTC	100	TMTC	TMTC
	Surface		TMTC	TMTC	TMTC	10000	TMTC	TMTC
	Surface		58	64	61	100000	6100000	6.785
	Surface	J5	TMTC	TMTC	TMTC	100	TMTC	TMTC
	Surface		TMTC	TMTC	TMTC	10000	TMTC	TMTC
	Surface		39	19	29	100000	2900000	6.462

Time (hr)	Type	Jar	Plate 1	Plate 2	Average	Dilution	CFU/mL	Log CFU/mL
24h	Surface	J1	TMTC	TMTC	TMTC	100	TMTC	TMTC
	Surface		43	45	44	10000	440000	5.643
	Surface		0	0	0	1000000	0	1.000
	Surface	J2	TMTC	TTC	TMTC	100	TMTC	TMTC
	Surface		135	130	132.5	10000	1325000	6.122
	Surface		2	2	2	1000000	2000000	6.301
	Surface	J3	TMTC	TMTC	TMTC	100	TMTC	TMTC
	Surface		158	171	164.5	10000	1645000	6.216
	Surface		1	2	1.5	1000000	1500000	6.176
	Surface	J4	TMTC	TMTC	TMTC	100	TMTC	TMTC
	Surface		TMTC	TMTC	TMTC	10000	TMTC	TMTC
	Surface		3	11	7	1000000	7000000	6.845
	Surface	J5	TMTC	TMTC	TMTC	100	TMTC	TMTC
	Surface		249	247	248	10000	2480000	6.394
	Surface		0	0	0	1000000	0	1.000

Time (hr)	Type	Jar	Plate 1	Plate 2	Average	Dilution	CFU/mL	Log CFU/mL
48	Surface	J1	199	202	200.5	10000	2005000	6.302
	Surface		12	4	8	1000000	8000000	6.903
	Surface	J2	169	151	160	10000	1600000	6.204
	Surface		6	0	3	1000000	3000000	6.477
	Surface	J3	159	132	145.5	10000	1455000	6.163
	Surface		11	3	7	1000000	7000000	6.845
	Surface	J4	247	227	237	10000	2370000	6.375
	Surface		15	2	8.5	1000000	8500000	6.929
	Surface	J5	140	131	135.5	10000	1355000	6.132
	Surface		1	2	1.5	1000000	1500000	6.176

Time (hr)	Type	Jar	Plate 1	Plate 2	Average	Dilution	CFU/mL	Log CFU/mL
72	Surface	J1	27	25	26	100000	2600000	6.415
	Surface		5	2	3.5	1000000	3500000	6.544
	Surface	J2	17	13	15	100000	1500000	6.176
	Surface		3	3	3	1000000	3000000	6.477
	Surface	J3	15	13	14	100000	1400000	6.146
	Surface		1	0	0.5	1000000	500000	5.699
	Surface	J4	22	24	23	100000	2300000	6.362
	Surface		3	1	2	1000000	2000000	6.301
	Surface	J5	11	19	15	100000	1500000	6.176
	Surface		1	1	1	1000000	1000000	6.000

Day	Type	Jar	Plate 1	Plate 2	Average	Dilution	CFU/mL	Log CFU/mL
4	Surface	J1	172	278	225	10000	2250000	6.352
	Surface		33	26	29.5	100000	2950000	6.470
	Surface	J2	TMTC	TMTC	TMTC	10000	TMTC	TMTC
	Surface		69	68	68.5	100000	6850000	6.836
	Surface	J3	TMTC	TMTC	TMTC	10000	TMTC	TMTC
	Surface		82	81	81.5	100000	8150000	6.911
	Surface	J4	TMTC	TMTC	TMTC	10000	TMTC	TMTC
	Surface		52	31	41.5	100000	4150000	6.618
	Surface	J5	TMTC	TMTC	TMTC	10000	TMTC	TMTC
	Surface		25	34	29.5	100000	2950000	6.470

Day	Type	Jar	Plate 1	Plate 2	Average	Dilution	CFU/mL	Log CFU/mL
5	Surface	J1	TMTC	TMTC	TMTC	1000000	TMTC	TMTC
	Surface		148	159	153.5	10000000	1.54E+09	9.186
	Surface	J2	TMTC	TMTC	TMTC	1000000	TMTC	TMTC
	Surface		65	57	61	10000000	6.1E+08	8.785
	Surface	J3	TMTC	TMTC	TMTC	1000000	TMTC	TMTC
	Surface		213	162	187.5	10000000	1.88E+09	9.273
	Surface	J4	TMTC	TMTC	TMTC	1000000	TMTC	TMTC
	Surface		71	84	77.5	10000000	7.75E+08	8.889
	Surface	J5	TMTC	TMTC	TMTC	1000000	TMTC	TMTC
	Surface		58	50	54	10000000	5.4E+08	8.732

Day	Type	Jar	Plate 1	Plate 2	Average	Dilution	CFU/mL	Log CFU/mL
6	Surface	J1	60	50	55	100000	5500000	6.740
	Surface		3	3	3	1000000	3000000	6.477
	Surface	J2	64	70	70	100000	7000000	6.845
	Surface		12	5	5	1000000	5000000	6.699
	Surface	J3	29	50	39.5	100000	3950000	6.597
	Surface		15	11	13	1000000	13000000	7.114
	Surface	J4	55	73	64	100000	6400000	6.806
	Surface		4	9	6.5	1000000	6500000	6.813
	Surface	J5	126	141	133.5	100000	13350000	7.125
	Surface		5	15	10	1000000	10000000	7.000

Day	Type	Jar	Plate 1	Plate 2	Average	Dilution	CFU/mL	Log CFU/mL
7	Surface	J1	52	63	57.5	100000	5750000	6.760
	Surface		1	0	0.5	1000000	500000	5.699
	Surface	J2	61	62	61.5	100000	6150000	6.789
	Surface		8	2	5	1000000	5000000	6.699
	Surface	J3	123	114	118.5	10000	1185000	6.074
	Surface		21	18	19.5	100000	1950000	6.290
	Surface	J4	144	132	138	10000	1380000	6.140
	Surface		68	65	66.5	100000	6650000	6.823
	Surface	J5	TMTC	TMTC	TMTC	10000	TMTC	TMTC
	Surface		112	129	120.5	100000	12050000	7.081

Annex E

Test on the seed to verify that not have E. coli O157:H7

Seed at hour 0					
Dilutions	Plate 1	Plate 2	Average	Average*Dil	
10^1	0	0	0	0	1
	0	0			
10^2	0	0	0	0	1

Annex F

Ampicillin requirement

Ampicillin requirement

Stock Concentration ampicillin 50µg/ml

4mg/ml using sterile distilled water and stored at 4 °C.

Was added 12.5ml of stock to 1 L sterile distilled water to achieve final concentration of 50µg/ml.

50µg/ml of ampicillin in 1 L of sterile distilled water

$$\frac{4mg}{ml} \times 12.5ml = 50\mu g/ml$$

25 µg /ml of ampicillin in 1 L of sterile distilled water

$$12.5ml \times \frac{25\mu g}{ml} \times \frac{ml}{50\mu g} = 6.5ml$$

10 µg /ml of ampicillin in 1 L of sterile distilled water

$$12.5ml\ x\ \frac{10\ \mu g}{ml} \times \frac{ml}{50\mu g} = 2.5ml$$

Annex G

Results of bacterial culture preparetion

Day three of culture			
Strain A4	10^7	10^8	10^9
Plate 1	23	9	1
Plate 2	46	14	2
Counts	8.537819095	9.060698	9.176091

Day six of culture (10µg/ml)			
Strain A4	10^7	10^8	10^9
Plate 1	46	3	1
Plate 2	43	7	0
	8.648360011	8.69897	8.69897

Day seven of culture (25 µg/ml)	
Strain A4	10^7
Plate 1	14
Plate 2	19
Counts	8.217484

Day eight of culture (50 µg/ml)		
Strain A4	10^6	10^8
Plate 1	50	1
Plate 2	52	0
Counts	7.707570176	7.69897