Evaluation of the pathogenic potential of Escherichia coli in ready-to-eat spinach and alfalfa sprouts

By Sean West and Vanessa Segovia **7/11/2014**

Abstract

The purpose of this investigation was to detect the presence of *Escherichia coli* (*E. coli*) on ready to eat alfalfa sprouts and spinach leaves purchased locally. This involved the use of both, phenotypic and genotypic analysis. The methodology of this study was based on the isolation of bacteria present in the vegetables sampled, phenotypic tests (Gram-stain technique, selective media, and biochemical tests), DNA extraction and amplification (PCR for 16S ribosomal RNA, ERIC sequences and stx_1 genes), DNA-amplified purification, and sequencing. According to the phenotypic tests, the presence of *E. coli* in the samples was unlikely; these results were confirmed and supported by the results based on molecular methods obtained. Sequencing results indicated the presence of *Aeromonas media* (an opportunistic pathogen), and *Pseudomonas koreensis* (usually found in agricultural soil). These results were consistent with phenotypic results obtained. In conclusion, phenotypic methods represent an important contribution for bacteria identification; however, molecular methods are highly precise and accurate in comparison to them. This sample may be considered satisfactory and acceptable for human consumption.

Introduction

E.coli is a gram-negative, facultative anaerobe, bacilli bacterium that is commonly found in the lower intestine of warm-blooded organisms. (Yao et al. 2014) It is an essential part of the natural flora of the human gut. Along with a whole community of other bacteria they play a role in metabolism by fermentation of non-digestible dietary residue, the salvage of energy as short-chain fatty acids, production of vitamin K and absorption of ions. In addition to digestive functions they serve to control proliferation of epithelial cell. And finally they contribute to homoeostasis of the immune system along with protection against pathogens. (Guarner & Malagelada 2003) (Eckburg et al. 2005) However because *E.coli* is one of the most studied organisms on the planet it has been found that when these bacteria have the ability to share DNA, which can be done between E.coli themselves and other cases different species of bacteria. One of the mechanisms by which the DNA is shared is the infection by prophages (when a phage inadvertently transfers some of its hosts DNA to its next hosts during reassembly between infections). *E.coli* has been shown to have acquired many abilities like new metabolic capabilities or virulence factors by comparative bioinformatics over the course of their evolution. (Perna et al. 2001)

One such disease is known as haemolytic uremic syndrome (HUS) caused by shigatoxigenic *E.coli* (STEC). In 2011, STEC O104:H4 resulted in a large outbreak of HUS in Germany and 15 other countries in Europe and North America. At the time this outbreak brought about a public health crisis and caused more than two billion US dollars in economic losses; partly, due to the disruption of imports from suspected countries. As early as 2001 there was a patient that presented that present with HUS like symptoms who had their stool sample investigated and had found to contain the O104:H4 serotype however the case was not followed up. The Robert Koch institute (RKI), the University Medical Center Hamburg-Eppendorf, and The Health Department of the Hamburg Northern District reported that the epidemic profile of this outbreak was caused by Shiga toxin-producing *E.coli* (Cui, Li & Yang 2013). The present study followed some the genotypic

methods used in this investigation to detect and identify genes for virulence factors (Bielaszewska et al. 2011)

The present investigation used the method for separating microorganisms from ready to eat vegetables as outlined in Australia standard AS5013 for testing for microbes in ready to eat food. This involved the use of peptone water for the recovery of bacteria from samples. It is commonly used in testing of *E.coli* because it allows for the recovery of injured cells (McFeters, Cameron & LeChevallier 1982). The use of selective media is essential for the microbial isolation. For instance, on ChromoCult® agar the *E.coli* appears blue colour due to the media being hydrolyzed by β-glucuronidase enzyme produced by *E.coli*. While other coliforms produce a salmon colour (Ogihara *et al.* 2004). However the control *E.coli* 0157:H7 lacks the ability to produce beta-glucuronidase; thus, also produces pink colonies. (Frampton & Restaino 1993). Usually, *E.coli* can easily differentiated from other bacteria on EMB agar due to the characteristic reaction (green metallic sheen) produced by the fermentation of lactose and the movements of the flagella (Acumedia, 2011; Edens *et al.* 1997). The phenotypic bacterial identification is complicated and non-accurate; however, it have been useful for general bacterial identification (genus). Some basic biochemical tests used are Gram staining, catalase, oxidase, indole, methyl red and Voges–Proskauer tests. Differences in season or any physical characteristics of the sample may affect their outcome. While additionally infection by virus may also produce unstable phenotypes. So this has led to the development of other typing methods based on the microbial genotype to minimize problems with reproducibility (Madigan, 2012; Olive & Bean 1999).

One widely used genetic technique for typing is the polymerase chain reaction (PCR), which is a process used to replicate DNA by imitating the processes that replicate DNA naturally. It uses an *in vitro* process that has tight controls on temperature and chemical conditions that can be tuned based on the replication needed. The difference is that DNA is replicated in shorter runs rather than the whole genome. To check for the presence of toxin genes PCR is run with specific templates called primers that match the gene in question and allow for a run of DNA to be copied, in a cell the template would be the complementary stand of the DNA (Marchesi *et al*. 1998). Universal primers (such as 27 forward and 1492 reverse) are highly useful for the 16S rRNA PCR assay for identification purposes. This is possible since it is a very highly conserved gene among bacteria as it is essential to the function of the organism. Mutations only remain in the gene if they are not detrimental to the organism, but these happen at random over millions of years (Stackebrandt & Goebel 1994). In view of this, the sequence of two closely related bacteria will be more similar than that of more distantly related bacteria. Furthermore the exact nucleotide sequences of samples can be compared to a database of already established sequences. Primers used for the Enterobacterial Repetitive Intergenic Consensus (ERIC) sequences matches up with a common sequence of repetitive nucleotides that exist between genes in enterobacterial species (Versalovic et al, 1991). When PCR is run with this primer these repetitive sequences are amplified up to such large numbers that they can be visualised in a characteristic certain pattern on an electrophoresis gel. ERIC sequences are useful for fingerprinting bacteria similarly to how junk DNA is used in humans (Wilson & Sharp 2006). The stx_1 primer set encodes for the shiga-like toxin type 1, if the gene is present in the genome of bacteria it will be amplified and able to be visualised products with an specific size; for instance ~ 366 bp long. (Herrera-Luna *et al.* 2009)

Another important molecular technique is electrophoresis, which uses electricity to separate molecules from each other when they pass through a non-reactive matrix. Most commonly used electrophoresis is gel electrophoresis where the matrix is composed of either agarose gel or polyacrylamide gel. Agarose is used to separate large molecules like DNA from each other while polyacrylamide gel is used on smaller molecules like proteins. The rate of migration through the gel in the case of DNA is proportional to how many bases make up the molecule, so a heavy molecule will travel slower than a small one producing a pattern that can be compared to ladders that have markers of a known amount of bases. The molecules themselves move because of the resistance of the molecules own electric charge against the charge generated by the electrodes placed at either end of the container that the gel is sitting in. Fragments that are imbedded in the gel can be stained and visualised under ultraviolet light (Jorgenson 1986).

The purpose of this experiment was to isolate, identify, and detect the presence of potential pathogenicity of *E.coli* from ready-to-eat spinach and alfalfa sprouts by phenotypic and genotypic techniques.

Materials and methods

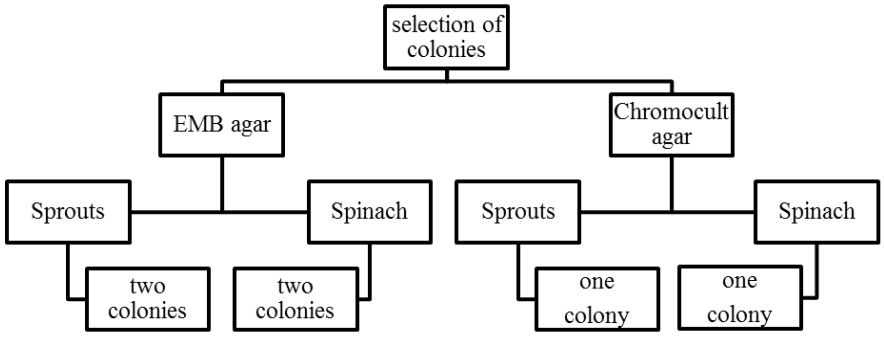
Sample collection and preparation

One sample of spinach and alfalfa sprouts were obtained from a randomly selected supermarket. The preparation of the samples was based on the FSANZ guidelines, 10 g of each sample were weighed using sterile stomacher bags; and then, diluted in 90 mL of peptone water. Following that, each bag was placed into a stomacher during 2 minutes to improve the sample homogenisation and organism recovery.

Isolation of the organisms

100 µL of each sample were immediately placed and spreader by duplicate onto selective media agar plates (EMB agar and Chromocult agar). As positive control, one colony of *E. coli* 0157:H7 was streaked by duplicated onto the same type media. All six plates were incubated aerobically at 37 ºC during 48 hours. After that, the agar plates of the sample were compared with the positive control agar plates to evidence the presence or absence of similarities between the morphology of the colonies forming units (CFU). Next, coliform-like colonies observed on the EMB agar (dark blue-black colour colonies) and chromocult agar (salmon colour colonies) inoculated with the samples were selected as is shown in the figure 1. Furthermore, one colony of the positive control was selected from the EMB agar. All colonies selected were streaked onto nutrient agar plates and incubated aerobically at 37 ºC during 48 hours. The rest of the plates were kept at ~ 4 ºC. Following that, the colonies isolated were transferred to pre-labelled tubes with nutrient broth (1 to 3 for colonies from sprouts; 4 to 6 for colonies from spinach; 7 for positive control), and incubated under identical conditions previously mentioned. Results were recorded, tabulated and analysed.

Figure 1. Protocol followed for the selection of colonies to be isolated.



Phenotypic characterization

Morphology and chemical cell wall constitution of the bacteria isolated were examined by the Christian Gram stain method (1884) using light microscopy. After that, biochemical tests available and considered relevant for the identification of *E. coli* were performed (catalase, oxidase, lactose and glucose fermentation, indole, Vogues Prokauer (VP), methyl red (MR). To perform the catalase test one colony of interest was placed on a glass slide; then, three drops of hydrogen peroxide were added onto the colony. The oxidase test was executed by using oxidase detection strips (Oxoid, 2014). Indole, VP, MR, lactose and glucose fermentation test tubes were inoculated with 100 µL of the nutrient broth cultures, and incubated at 37 ºC during 24 hours. Later, results obtained and expected were, recorded, tabulated, compared and analysed.

Genomic DNA extraction

The isolation of genomic DNA from Gram-negative bacteria was performed by following the guidelines displayed in the Wizard® genomic DNA purification kit used (Promega, 2010). First, an aliquot of 1.8 mL of each overnight bacterial suspension (samples) was placed into pre-labelled (1 to 7) 2 mL collection tubes. Next, the tubes were centrifuged at high speed (14000 rpm) for 1 minute, and the supernatant was discarded. The pellets were resuspended by adding 500 µL of extraction buffer. Afterwards, 20 µL of Lysozyme (10 mg/mL) were added to each tube to cause the bacterial cell lysis. The tubes were sealed and vortex thoroughly. Later, the tubes were incubated on ice during 15 minutes, followed by 5 minutes at 80 ºC, and 5 minutes more on ice to cool them down. Following that, 250 µL of 6 M ammonium acetate (stored on ice) were added to each tube, which were then sealed and vortex vigorously, and leaved to stand during 10 minutes on ice. After that, the tubes were centrifuged at 14000 rpm for 5 minutes to collect the precipitated proteins and cellular biomass. Subsequently, 600 µL of the supernatant were recovered into new pre-labelled microcentrifuge tubes containing 360 µL of iso-propanol. The tubes were inverted around 5 times; then leaved during 5 minutes to allow the DNA to precipitate; and centrifuged (14000 rpm) for 5 minutes to pellet the DNA. The supernatant was tipped off, and the remaining fluid was drained off by inversion of the tubes onto a piece of paper towel. Successively, 500 µL of 70% ethanol were added to the tubes to wash the pellets. The tubes were then centrifuged (14000 rpm) for 5 minutes, and the supernatant discarded. The pellets (containing the DNA extracted) were resuspended in 100 µL of sterile water and 2 µL of RNase (10 mg/mL). Finally, the quantity of DNA was measured by spectrophotometry using Biowave II and Hellma cell (Biochrom, 2006); and the quality was measured by electrophoresis. Results obtained and expected were, recorded, tabulated, compared and analysed.

Quality examination

The quality of the genomic DNA extracted was determined by 1% agarose gel electrophoresis technique. To perform this technique, the first step was the preparation of the 1% agarose gel. 0.25 g of UltraPure™ agarose (Invitrogen, 2010) were weighed and placed into a flask; then, 30 mL of Tris-Acetate-EDTA (TAE) Buffer (previously diluted 30 mL in 270 mL of distilled water) were added to the flask, which was later placed into a microwave during ~ 45 seconds or till evidence total dissolution of the solute. After 2-3 minutes, 2.5 µL of red gel were added to the flask and carefully shaken during 10 seconds. The content of the flask was poured into a casting tray with a comb. After solidification of the agarose gel, 5 µL of a molecular weight marker (Bioline 2014) was loaded into the first well (lane 1) of the gel, followed by 5 µL of each sample into the remaining wells. Later, the casting tray is placed into a gel box, which is then filled with diluted TAE buffer. The electrophoresis was run at 100 volts during 45 minutes. The gel was visualised under UV transilluminator. Results expected and obtained were recorded, compared and analysed.

Polymerase chain reaction (PCR) for 16S rRNA, PCR, Stx_1 and ERIC sequences

In order to detect and identify *E. coli* from the samples (genomic DNA extracted), a selected fractions of the gen that encodes for the 16S rRNA was amplified by PCR technique using the 27-forward (27F) and 1492-reverse (1492R) universal primers (product ~1500 bp) (Casas et al. 2004). The stx_1F and stx_1R primers were used to amplify DNA fractions of the gene that encodes for the Stx_1 (product ~366 bp) (Brian et. al, 1992). To compare the fingerprinting pattern of the ERIC sequences of *E. coli* with the patters of the samples, the ERIC1R and ERIC2 primers were used (product ~126 bp) (Versalovic et al, 1991). To perform the PRC assays, a total volume of 50 µL of each sample were prepared in eppendorf for PCR tubes as is displayed in the table 1. Details about the primers and the conditions used for each PCR assay performed can be observed in the table 2.

Table 1. PCR sample preparation protocol

	16S rRNA	**Stx_1**	**ERIC**
Mango Mix*	25 µl	25 µl	25 µl
Forward primer	1 µl	1 µl	1 µl
Reverse primer	1 µl	1 µl	1 µl
Sample	15 µl	15 µl	15 µl
Water	8 µl	8 µl	8 µl
Total volume preparer per sample	50 µl	50 µl	50 µl
Number of samples	7	7	7

Table 2. Primers and the conditions used for 16S rRNA, Stx_1, and ERIC PCR assays performed

Primer	**Sequence 5' → 3'**	**Product expected**	**PCR conditions**		
27F	AGA GTT TGA TCC TGG CTC AG	~1500 bp	Initial Denaturalization: 94 ºC	1c	5 min
			Denaturalization: 94 ºC		1 min

			Annealing: 65 ºC	34 c	1 min
1492R	GGT TAC CTT GTT ACG ACT T		Elongation: 72 ºC		1 min
			Final elongation: 72 ºC	1c	10 min
			Final hold:15 ºC	1c	∞
stx_1F	AAA TCG CCA TTC GTT GAC TAC TTC T	~366 bp	Initial Denaturalization: 94 ºC	1c	5 min
			Denaturalization: 94 ºC	34 c	1 min
			Annealing: 65 ºC		1 min
stx_1R	TGC CAT TCT GGC AAC TCG CGA TGC A		Elongation: 72 ºC		1 min
			Final elongation: 72 ºC	1c	10 min
			Final hold:15 ºC	1c	∞
ERIC1R	ATG TAA GCT CCT GGG GAT TCA C	~126 bp	Initial Denaturalization: 95 ºC	1c	7 min
			Denaturalization: 90 ºC	30 c	30 sec
			Annealing: 52 ºC	1c	1 min
			Elongation: 65 ºC	1c	8 min
ERIC2	AAG TAA GTG ACT GGG GTG AGC G		Final elongation: 65 ºC	1c	16 min
			Final hold:15 ºC	1c	∞

After the sample preparation, three agarose gels (1%) were prepared in an identical routine as was explained previously ("Quality examination"). Moreover, the molecular weight marker (Bioline 2014) and the samples were loaded following the same pattern mentioned.

Sample preparation for sequencing

Due to the high phenotypical similarities evidence between bacteria in samples 1-2 and 4-5, only four of the six samples were selected to be sequenced (2, 3, 4 and 6). This samples were re-labelled as VS1, VS2, VS3 and VS4, respectively. After that, the DNA from the 16S rRNA PCR amplification was purified by centrifugation following the protocol of the Wizard® SV Gel and PCR Clean-Up System kit (Promega, 2002). To perform this, four minicolumns (one per sample) from the kit were placed in a collection tube. Then, the PCR products were transferred to the minicolumns and incubated at room temperature (RT) for 1 minute. Later, the minicolumns were centrifuged at 13000 rpm during 1 minute. Following that, the minicolumns were removed from the Spin Column assembly; the liquid contained in the collection tubes was discarded; and the minicolumns were returned to the collection tubes. Subsequently, the columns were washed by adding 700 µL of Membrane Wash Solution diluted with 95% ethanol to the minicolumn. The tubes were centrifuged at 13000 rpm during 1 minute. Next, the liquid contained in the collection tubes was discarded and the minicolumns placed back in the Collection Tubes. After that, the columns

were washed a second time with 500 µL of Membrane Wash Solution, and centrifuged during 5 minutes at 13000 rpm. The liquid into the collection tubes was discarded, and the microcolumns centrifuged again at 13000 rpm for 1 minute with the microcentrifuge lid off to allow the evaporation of any residual of ethanol. Then, the microcolumns were carefully transferred to clean and pre-labelled 1.5 mL microcentrifuge tubes. Successively, 50µl of nuclease-free water were applied directly to the centre of the microcolumns (without touching the membrane). The tubes were incubate at RT for 1 minute, and later centrifuged during 1 minute (13000 rpm). The minicolumns were discarded. After that, the concentration of DNA in the samples was measured by spectrophotometry with a NanoDrop (Thermo_scientific 2013) and considered to prepare the sample to be sent to sequencing; one 1.5 mL microcentrifuge tube with a unique primer-27 forward (1 µL), sample (7 µL) and pure water (4 µL) with a final volume of 12 µL and a concentration between 30 and 75 ng/12 µL (Australian Genome Research Facility 2014).

Bioinformatics

The suitable samples were compared to the GenBank database. This is the method used to decide upon which samples could be sequenced and how the samples were compared to the database using the basic local alignment search tool (BLAST) (Found at *www.ncbi.nlm.nih.gov/**blast**/).*

Of the data received from the Australian Genome Research Facility Ltd there were four files. Firstly, there was the Applied Biosystems file (.ab1) which gave a chromatogram of the results; secondly, there were three raw sequence files that were not of interest in this experiment. The sequence file that contained data as recorded by the instrumentation without selection for quality (.seq); the version of the previous file that had been analysed by a computer for accuracy and edited to produce what it determined to be the best quality parts of the sequence (.fa); and a version of the file but converted into a format that would easily allow it to be uploaded to the BLAST tool (.bn).

The Applied Biosystems chromatogram files obtained from auto sequencer instruments were opened by a free program called Bioedit. When this file is opened by in Bioedit it will display a graph along with a sequence. Firstly, the sequence was trimmed to obtain a final sequence that resembled figure 2 with non-overlapped peaks; by cutting out the portions that resembled figure 3 at the extreme right and figure 4 at the extreme left as detailed by Hall (2004).

Figure 2. Chromatogram in the most accurate region of its run where peaks are easily distinguishable.

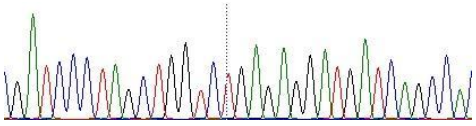


Figure 3. **C**hromatogram which has reached the end of its accurate run of bases.

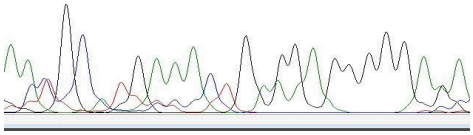
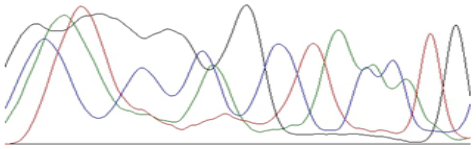


Figure 4. Contaminated sample that cannot be accurately read (typical of what would be seen at the start of a chromatogram).



Rather than relying on a computer algorithm to trim the results as was available in the ".fa" and ".bn" files, the above method was used to select the sequence data that would be used for analysis. After the selection of the sequence, it was then copied to the clipboard. From the BLAST webpage a Standard Nucleotide BLAST was selected with the following parameters for database "16S ribosomal RNA sequence (Bacteria and Archea)" was selected and the search program selection was set to "Optimize for Highly similar sequences (megablast)". Then in the text box below "Enter accession number(s), gi(s), or FASTA sequence(s) "the following was inputted a "> " character and then a note or name for the sample. On the next line the trimmed sequence obtained previously was pasted in and the sequence run by clicking the "BLAST" button. Then after some time, a list was generated of the mostly likely matches to the nucleotide sequence. Then with some deduction and weighing up the coverage of the sample, the percentage match of results it was concluded which sample was most likely to be the organism present. The organisms with the highest percentage match was selected and used for the results.

Results

Phenotypic characterization

Results obtained from the examination of the morphology and chemical composition of cell walls based on the Gram-stain method were identical for all of the samples and the control. All bacteria observed were Gram-negative (pink) bacilli (rod shaped).

The physical appearance description of the bacteria presents in the samples on the selective media can be observed and compared with the results expected in the following table.

Table 3. Bacterial cell colonies from sprouts and spinach samples on EMB and Chromocult agar. Positive control: *E coli.*

	Results expected		**Results obtained**	
EMB agar	Control (E. coli)	Dark-blue or black colonies with green metallic sheen in reflected light.	control	Dark-blue or black colonies with green metallic sheen in reflected light.
	Coliforms	Dark-blue or black colonies.	Sprouts	-Dark-blue or black colonies. -Colourless colonies.
	Non-coliforms	Colourless colonies.	Spinach	-Dark-blue or black colonies. -Colourless colonies.

Chromocult agar	control	Blue-violet colour colonies.	control	Salmon-red colour colonies.
	Coliforms	Salmon-red colour colonies.	Sprouts	Salmon-red colour colonies. Yellow colour colonies.
	Non-coliforms	Yellow colour colonies.	Spinach	Salmon-red colour colonies. Yellow colour colonies.

Biochemical test results were summarised in the following table. Non-identical results between the positive control *(E. coli*) and the samples were observed.

Table 4. Biochemical tests results expected and obtained.

Test	**Expected**	**Sprouts**			**Spinach**		
	Control +	1	2	3	4	5	6
Catalase	+	+	+	+	+	+	+
Oxidase	-	-	-	-	+	+	+
Voges Proskauer	-	+	+	+	-	-	D/+
Methyl red	+	-	-	-	+	+	-
Indole	+	-	-	+	+	+	-
Lactose	+	+	+	+	+	+	-
Glucose	+	+	+	+	+	+	+

Quantification of DNA

The following table shows the concentration of genomic DNA (Biowave II and Hellma cell), and the concentration of the amplified products obtained from the PCR 16S rRNA in the samples destined to be sequenced (Nano Drop).

Table 5. Concentration of DNA in the samples measured by spectrophotometry.

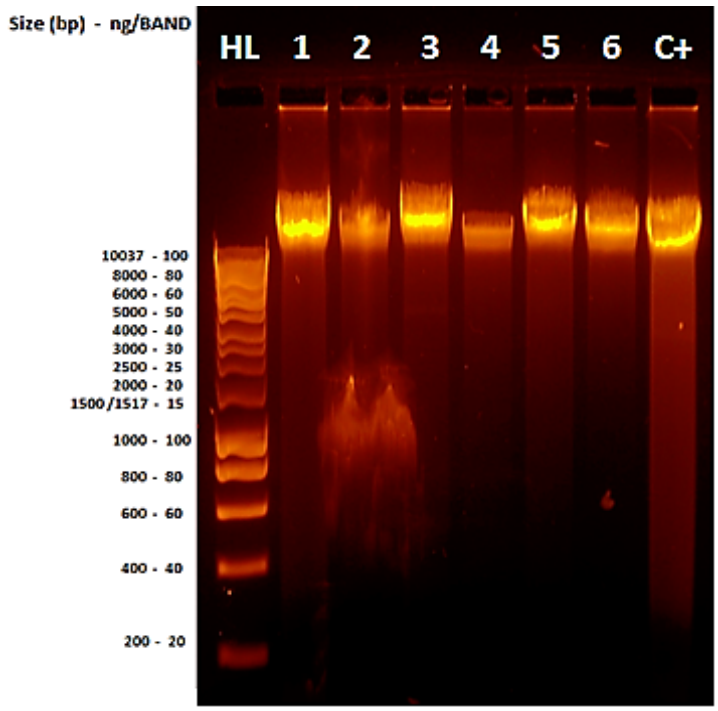
Sample	**Concentration of genomic DNA (ng/µL)**	**Concentration of 16S rRNA amplified DNA (ng/µL)**
1	120	
2	74	15.9
3	131	13.4
4	27.5	13.8
5	61	
6	52.5	13.4

7 (positive control)	134	
8 (negative control)	0	0

DNA extraction

The following figure shows the result obtained from the DNA extraction assay performed. The presence of thick bands with a size > 10000 bp, and concentrations over 100 ng/band were evidenced.

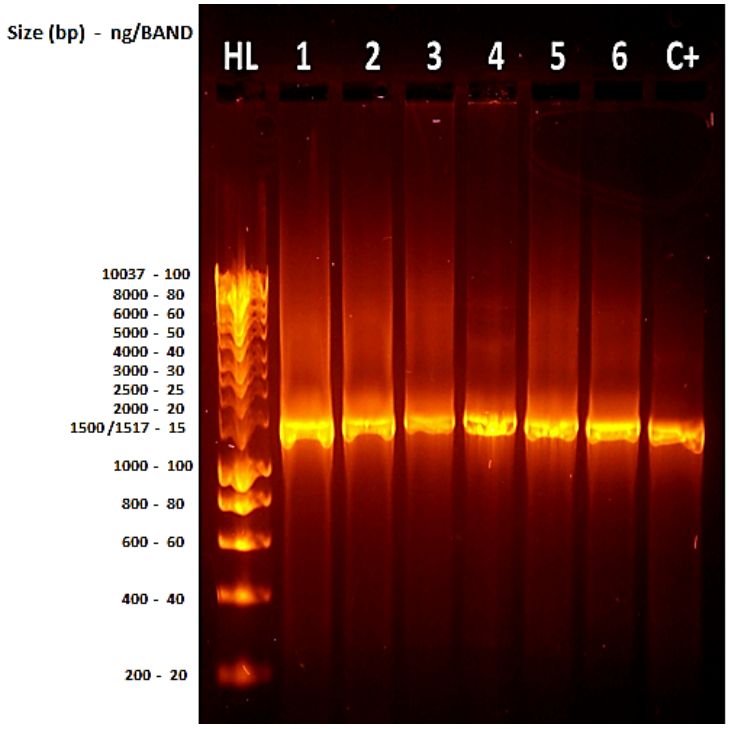
Figure 5. Genomic DNA on garose gel (1%). Lane 1: molecular weight marker; lanes 2 to 7: samples 1 to 6; lane 8: positive control (*E. coli*).



PCR for 16S rRNA

Figure 6 displays the results obtained from the PCR for 16S rRNA performed. It could be observed the presence of a band (product) of approximately 1500 bp size and a minimum concentration of 30 ng/band in all of the lanes. Product expected ~1500 bp.

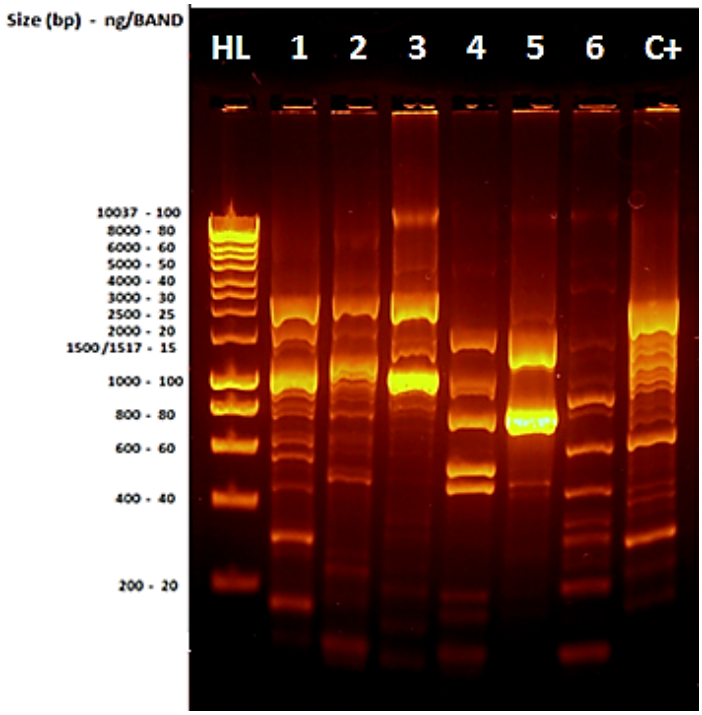
Figure 6. Products obtained from PCR rRNA on agarose gel (1%). Lane 1: molecular weight marker; lanes 2 to 7: samples 1 to 6; lane 8: positive control (*E. coli*).



PCR for ERIC sequences

The following figure illustrates the results obtained from the PCR for ERIC sequences performed. The presence of numerous products was evidenced in all of the bands. Non-identical pattern of products were observed between *E. coli* (C+) and the samples (1 to 6); nor between the samples.

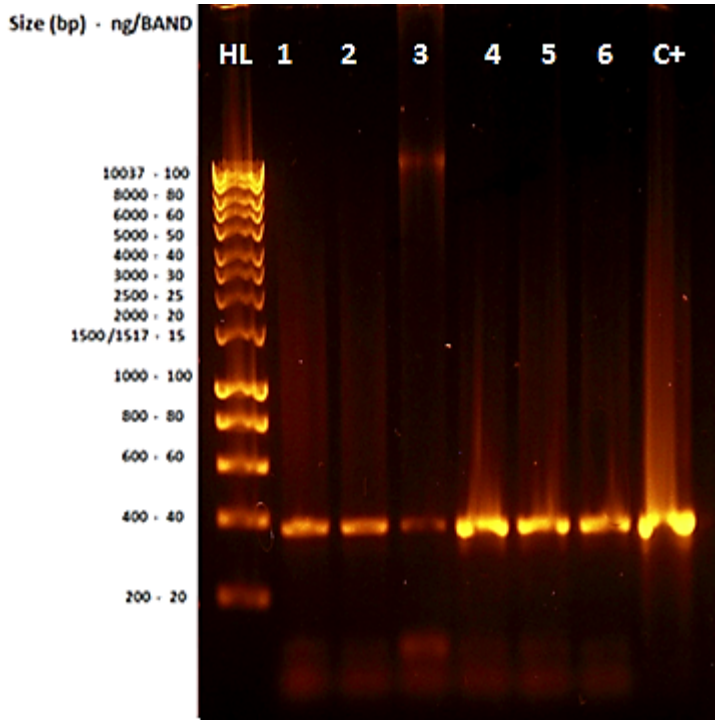
Figure 7. Products obtained from PCR of ERIC sequences on a agarose gel (1%). Lane 1: molecular weight marker; lanes 2 to 7: samples 1 to 6; lane 8: positive control (*E. coli*).



PCR stx_1

The visualization of bands of ~366 bp in all of the lanes (2 to 8) indicated the presence of Stx_1 products in the control and in all of the samples.

Figure 8. Products obtained from PCR of Stx_1 on a agarose gel (1%). Lane 1: molecular weight marker; lanes 2 to 7: samples 1 to 6; lane 8: positive control (*E. coli*).



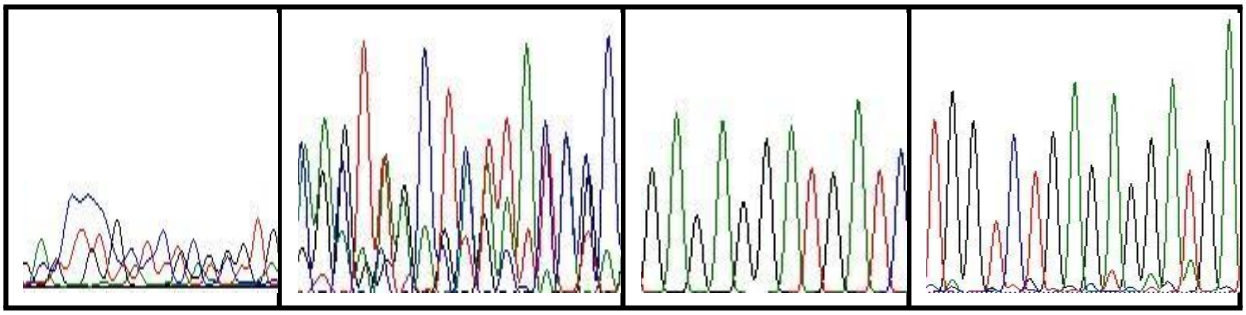
Bioinformatics

The following table (table 6) shows the results obtained from the sequencing of the 16S rRNA gene for the samples selected, re-labelled, and sent to AGRF. The figure 9 displays the resulting quality and intensity of the data corresponding to each sample based on their chromatograms.

Table 6. Summary table about the success of the four samples that were sent for sequencing.

Sample Name	**Q20 Bases**	**Intensity**
VS1_C05	463	281
VS2_C06	189	254
VS3_C07	1100	225
VS4_C08	1125	233

Figure 9. From left to right, quality and the intensity of each sample (1, 2, 3 and 4).



Blast output

Table 7 reflects the results obtained from the BLAST analysis performed.

Table 7. BLAST results.

Sample	**Percentage match (%)**	**Identification**
1	Not applicable	Not applicable
2	Not applicable	Not applicable
3	98% (1096/1121 bp)	*Aeromonas media* strain RM
4	99% (994/997 bp)	*Pseudomonas koreensis* strain Ps 9-14

Discussion

The gram stain technique performed results showed that all of the bacteria present in the samples exhibit the same rod-shaped morphology (bacilli) and cell wall chemical composition (gram negative/thin peptidoglycan layer) than *E. coli* (Madigan, 2012).

Results obtained from the EMB medium were not consistent with the result expected. Typical colonies of *E. coli* were not observed in either of the EMB agar inoculated with the samples (spinach and sprouts). However, not all strains of E. coli produce a green metallic sheen in reflected light on the EMB medium, this is considered a limitation of the this medium (Acumedia, 2011). The result expected in the Chromocult agar for *E. coli* (blue colonies) was not observed even in the control plate. This is related with the fact that the strain of *E. coli* used as positive control (0157:H7) does not produce the enzyme glucuronidase (which is the responsible of the blue colour in this media). In view of this, this bacteria cannot be differentiated from others coliforms. (Merck, 2012; Yang et al. 2004).

Regarding the biochemical tests, the oxidase test permitted to clearly distinguish between the two different groups of bacteria isolated. In the sprouts sample, all of the bacteria isolated were oxidase-negative; by contrast, only catalase-positive bacteria were isolated from the spinach sample. *E coli* and others enterobacteria are described as non-cytochrome oxidase enzyme producers (oxidase-negative microorganisms). Some morphologically similar bacteria (non-enteric Gram-negative bacteria or miscellaneous Gram-negative bacilli/MGNB) oxidase-positive are the *Pseudomonas, Aeromonas, Vibrio,* and the like) (Madigan 2012; O'Hara, 2005). The summary of the results obtained indicated that samples 1-2 and 4-5 exhibit identical results; hence, it is possible the presence of the same oxidase positive bacteria in the samples 1 and 2; and, the same oxidase-positive bacterial genus in samples 4 and 5. Moreover, none of the bacteria present in the samples possess the same biochemical requirements and behaviour than the positive control, which

results corresponded entirely with *E coli*. According to Brenner (2005), results obtained are roughly consistent with *Klebsiella, Enterobacter, Pseudomonas,* and *Aeromonas* bacteria. Despite the group biochemical tests used to distinguish *E. coli* cells from others bacteria, they were not sufficient to detect *E. coli* 0157:H7. One of the tests that were not used in this study due to it unavailability, but that could be valuable to detect the presence of this strain of *E. coli* is the sorbitol (Brenner, 2005 p.684)

Concerning the genotypic analysis, the gel electrophoresis image (figure 2) based on the DNA extraction showed typical bands that correspond with genomic DNA (Hartl and Ruvolo, 2011) in all of the samples (including the positive control). This reflects that the extraction of DNA was correctly performed and successful. Therefore, the samples were suitable to be used for the PCR assays. In the gel electrophoresis that correspond with the PCR for 16S rRNA (Figure 3) the size of the products obtained (approximately 1500 bp) (Casa *et al.* 2004) for all of the samples was consistent with the result expected using the 27F and 1492R primers. Hence, the samples were totally suitable to be purified and sequenced. In the gel electrophoresis image (figure 4) that illustrates the results obtained from the PCR for ERIC sequences performed, the pattern of the products obtained were not consistent with the pattern of the product expected (*E. coli)*. Due to the fact that the ERIC sequences are highly conserved (~126 bp) but the location within genus is different it is possible to fingerprint and compare two or more bacteria (Hulton *et al*. 1991). It could be observed that the bacteria isolated 1-2 and 4-5, which showed identical biochemical tests, were not the same bacteria; thus, to obtain an accurate bacterial identification, the molecular technology is an effective method. By contrast, the image that corresponds with the PCR assay for the stx_1 gene (figure 5) shown that the gene stx_1, which encodes for the stx1, was present in all of the bacteria isolated. Hence, the bacteria present in the samples bacteria possess the potential to be pathogenic (to cause HUS). However this is attached to the infectious dose, which vary depending on the bacteria; for instance, the infectious dose of *E coli* 0157:H7 is less than 2000 cells) (Marriott & Gravani, 2006) and to the expression of the gene and the factors that can induce it, which are directly related with a stress response of the bacteria; for instance some type of antibiotics (such as ciprofloxacin and trimetroprim sulfamethoxazole) (McGannon et al. 2010).

The data received from the Australian Genome Research Facility Ltd showed that the results obtained for samples 1 and 2 were not suitable for sequencing due to their poor quality (less than 50 bp). This could be observed in the table 6 and figure 9. These samples gave poor quality readings as shown by their Q scores of 189, 463 (samples 1 and 2 respectively). Because poor Q scores are based on the probability of the bases that are called at that position compared to the other three possible bases; thus, the presence of overlapping peaks it is likely to be related with some contamination in the samples due to improper purification handling techniques (such as failure to spin at 14000 rpm) (Promega, 2002; Horsey 2008; Huse *et al*. 2007). Conversely, the Q scores from the two samples that were able to be sequenced (samples 3 and 4) were both above 1000; therefore their quality could be considered acceptable. The intensity denotes how strong the signal is for each peak of the chromatogram. The included documentation states that an intensity of less than 700 is ideal; otherwise, the adjacent signals may be overtaken (Livak *et al*. 1995). In light of this, the combination of both, quality and intensity is important to obtain accurate sequences. The actual sequencing data analysis gives results that must be understood in terms of how they are generated.

They are the output of a computer program that can only give answers of the same quality as the data that is inputted into it. This means that any error submitted to the BLAST will be taken into account as any actual base so it is very important to trim the sequence data based on its quality (Huse et al. 2007; Livak et al. 1995). With a 98% of similarity, *Aeromonas media* was identified in the sample 3. This result was consistent with biochemical tests results obtained in this study, and with the description of this bacterium defined by Brenner (2005). *A. media* have been isolated from river and drinking water (Allen *et al*., 1983; Hanninen & Siitonen, 1995) and stool specimens from patients with diarrhoea (Pablos et al., 2010. This bacterium is capable of growth at temperatures between 4-37 ºC; however, as others *Aeromonas* species, *A. media* cannot growth at 42 ºC (Brenner, 2005). It is considered an opportunistic pathogen, predominantly for children under five years old (Pereira et. al, 2008). Results obtained from the PCR for the stx_1 gene indicated that this bacterium possess the gene for the shiga-like toxin type 1. According to Alperi & Figueras (2010), this gene is present in *Aeromonas* isolated from stool specimens of humans with HUS, specifically, in *A. caviae, A. hydrophila, A. veronii,* but no in A. *media.* In view of this, results obtained in this study that reflect the potential pathogenicity of *A. media* may contribute to the scientific community. Similarly, the stx_1 gene was found in *Pseudomonas koreensis,* a mesophilic bacteria usually isolated in agricultural soil (Kwon *et al.* 2003) water, and in infected eyes of golden fishes (Shahi & Mallik, 2014). No information to support the presence of stx_1 gene in *P. koreensis* or any isolates of this bacterium in humans was found. However, the stx_1 gene has been detected in *Pseudomonas aeruginosa* (Narayanan *et al.* 2013). In light of this, the presence of this gene in the *A. media* isolated could be caused by genetic transference, which is highly possible since both, *P. aeroginosa* and *P. media* have been found inhabiting the same environments (Mena & Gerba, 2009; Marques et al., 1979).

Conclusion:

In this experiment there were no *E.coli* found on the ready-to-eat spinach or alfalfa sprouts. This was evidenced by comparing samples collected with control *E.coli* in a variety of tests. However in the bacteria that were present there was the gene for the production of shiga-like toxin type 1 which can cause Shigellosis in humans. Though of the bacteria that could be positively identified none were known to be a problem for healthy people. They were also both known to be normal inhabitants of soil and their presence would be expected on a food sample. Along with this the bacterial load of the salad would also be a deciding factor of whether someone could become ill after consuming this particular salad. This wasn't studied because this was a qualitative rather than quantitative study.

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