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Transcriptomic response of *Escherichia coli* O157 isolates on meat: Comparison between a typical Australian isolate from cattle and a pathogenic clinical isolate

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ABSTRACT

The majority of foodborne illnesses associated with *E. coli* O157 are attributed to the consumption of foods of bovine origin. In this study, RNA-Seq experiments were undertaken with *E. coli* O157 to identify genes that may be associated with growth and survival on meat and the beef carcass at low temperature. In addition, the response of an *E. coli* O157 isolate representative of the general genetic 'type' found in Australia (*E. coli* O157:H$^-$ strain EC2422) was compared to that of a pathogenic clinical isolate (*E. coli* O157:H7 strain Sakai) not typically found in Australia. Both strains up-regulated genes involved in the acid stress response, cold shock response, quorum sensing, biofilm formation and Shiga toxin production. Differences were also observed, with *E. coli* O157:H7 Sakai up-regulating genes playing a critical role in the barrier function of the outer membrane, lipopolysaccharide biosynthesis, extracellular polysaccharide synthesis and curli production. In contrast, *E. coli* O157:H$^-$ EC2422 down-regulated genes involved in peptidoglycan biosynthesis and of the primary envelope stress response Cpx system. The unique gene expression profiles of the strains, indicate that these genotypes may differ in their ability to persist in the meat production environment and therefore also in their ability to cause disease.

1. Introduction

Enterohemorrhagic *Escherichia coli* (EHEC) is a subset of pathogenic *E. coli* that can cause non-bloody diarrhea, bloody diarrhea or hemorrhagic colitis in humans. Hemorrhagic colitis occasionally progresses to hemolytic uremic syndrome (HUS), an important cause of acute renal failure in children and morbidity and mortality in adults. *E. coli* O157 is the primary cause of HUS in many regions and countries (Elder et al., 2000; Masana et al., 2010; Mellor et al., 2013; Pollock et al., 2008; Pollock et al., 2010). However, the frequencies of HUS and incidences of infection differ substantially worldwide (Locking et al., 2011; Mellor et al., 2013; Prevention, 2011; Rivas et al., 2006; Tarr et al., 2005; Vally et al., 2012). The major transmission route for *E. coli* O157 is foodborne, with the majority of reported cases attributed to the consumption of foods of bovine origin (Rangel et al., 2005). Cattle are the main reservoir for *E. coli* O157 and harbor the pathogen in their gastrointestinal tract without developing clinical disease. During commercial slaughter of domestic animals, it is nearly impossible to guarantee that the carcass surface is not contaminated with faecal matter and while decontamination strategies can further reduce contamination, carcasses free from *E. coli* contamination cannot be guaranteed (Ross et al., 2003). Aside from devastating personal losses due to infection and illness, the economic costs can be substantial. For example, in the United States the costs of healthcare, social care and lost productivity are approximated to be around $600 million per year (Scharff, 2012), whereas costs to the food industry from product recalls and reduced trade can run to tens of millions of dollars (Matthews et al., 2013).

During food processing *E. coli* O157 encounter different stress conditions that might affect their fate along the food chain and therefore their transmission to humans. An understanding of how *E. coli* O157 survives and proliferates on beef would permit the development of effective interventions to inactivate or control this pathogen in the meat production environment. In Australia and New Zealand, carcasses are cooled by refrigerated air leading to exposure of contaminating microbes to both chilling and osmotic stresses, which have a significant impact on the growth of bacteria including *E. coli* (Gill and Bryant,

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1997; Lenahan et al., 2009). Several integrated transcriptomic and proteomic studies have been undertaken to assess how *E. coli* O157 responds to conditions of low water activity and low temperature stress (King et al., 2014; King et al., 2016; Kocharunchitt et al., 2012; Kocharunchitt et al., 2014). These *in vitro* studies investigated the transcriptional and proteomic responses of exponential phase *E. coli* O157:H7 Sakai grown under steady-state conditions (Kocharunchitt et al., 2012), during adaptation upon an abrupt downshift in water activity (a_w) (from 0.993 to a_w 0.967 (Kocharunchitt et al., 2014)) and temperature (from 35 °C to 14 °C (King et al., 2014)) and, during dynamic changes in temperature and water activity (from 35 °C a_w 0.993–14 °C a_w 0.967) (King et al., 2016). The results of these studies revealed that *E. coli* activates a number of different stress responses, which have the potential to impact its resistance or sensitivity to the conditions experienced upon subsequent food processing treatments and in the human gastrointestinal tract. As well as being able to adapt to diverse environmental stress conditions during food production, foodborne pathogens have to be able to withstand food associated stresses. Therefore, there is also a need to understand the physiological state of pathogens when present on food (Bergholz et al., 2014).

While a small number of transcriptomic and proteomic studies have been undertaken to characterise the response of various foodborne pathogens during adaptation and growth on specific food matrices (for example (Deng et al., 2012; Lippolis et al., 2008),), the potential for the analysis of gene and protein expression of pathogens in food environments has not yet been completely realized. Transcriptomic studies can lead to substantial technical challenges associated with accurately measuring bacterial gene expression in complex matrices, including the low yield and purity obtained during RNA extraction and the cross-contamination effect of the food matrix. To date, only one study has been undertaken to profile the whole genome transcriptomic response of *E. coli* in meat (Fratamico et al., 2011). Fratamico et al. (2011) compared the whole genome transcriptomic response of *E. coli* O157:H7 Sakai in raw ground beef extract after 2 h of incubation at 37 °C using microarrays (Fratamico et al., 2011). A few studies have also been undertaken using quantitative real-time reverse transcription-PCR (qRT-PCR) to monitor the expression of specific genes of interest in minced beef (Kjeldgaard et al., 2011; Mahmoudzadeh et al., 2016). Therefore, to date little is known of the whole genome expression response of *E. coli* O157 on beef (which is the major source of outbreaks associated with this pathogen) and how this might impact on its subsequent ability to cause human disease.

The differences in global disease burden may result from regional variations in *E. coli* O157 genotypes. Australia has a relatively low reported frequency of HUS cases (Vally et al., 2012) and the incidence of disease associated with *E. coli* O157 appears to be lower than the global average (Mellor et al., 2015). This is despite the fact that the prevalence of *E. coli* O157 in Australian cattle faeces is similar to that reported in countries where the incidence of human infection is high (Mellor et al., 2013). The similar rates of cattle prevalence but different rates of human infection between countries have been attributed to a number of factors, including differences in disease surveillance, food-handling practices, supply chains and host susceptibility (Mellor et al., 2013). Using a single nucleotide polymorphism (SNP) assay, Mellor et al. (2015) have also reported that *E. coli* O157 isolates clearly segregate into SNP lineages that are differentially associated with each isolate's country of origin (Mellor et al., 2015). Mellor et al. (2015) hypothesized that genotypic differences associated with isolates of differing geographical sources may contribute towards providing a basis for differences in the incidence and severity of *E. coli* O157-associated disease in each region (Mellor et al., 2015). *E. coli* O157 genotypes from geographically separated countries are also likely to differ in their physiological response on beef, impacting on their ability to survive and proliferate in the food production environment. In turn, these differences may contribute to the different disease burdens observed globally. Therefore, the aim of the current study was to analyse and compare the transcriptomic response of a pathogenic clinical isolate; *E. coli* O157:H7 Sakai and, a typical Australian isolate from cattle; *E. coli* O157:H$^-$ EC2422, on meat at low temperature.

2. Methods and materials

2.1. Bacterial strains

The strains used in this study were *E. coli* O157:H$^-$ EC2422 and *E. coli* O157:H7 Sakai, designated EC2873 and EC2898 in our culture collection, respectively. *E. coli* O157: H$^-$ EC2422 was isolated from grass-fed cattle faeces in Queensland (Australia). *E. coli* O157: H$^-$ EC2422 is one of the most common PFGE types from Australian cattle and is non-motile; as are ~60% of Australian isolates (Fegan et al., 2004; Pintara et al., 2018). *E. coli* O157: H$^-$ EC2422 is a Clade 7 strain (Mellor et al., 2012). Clade 7 strains dominate in both Australian cattle and human isolates (Mellor et al., 2012). *E. coli* O157:H7 Sakai was isolated from the Sakai outbreak in Japan involving radish sprouts, in which approximately 1000 patients were hospitalized with severe gastrointestinal symptoms and about 100 victims had complications of HUS, resulting in 3 deaths (Hayashi et al., 2001). *E. coli* O157:H7 Sakai is a Clade 1 strain (Riordan et al., 2008).

2.2. Preparation of meat and agarose plates

Post-rigor beef (*M. semitendinosus*) was obtained from a commercial butcher within 24 h of slaughter. All experiments were conducted on the same day as collection. In total, eight pieces of post-rigor beef were obtained. The outer surface of the beef muscle was flame sterilised, and the flamed surface was aseptically removed prior to cutting the muscle into pieces. From the eight sterilised post-rigor beef pieces, 30 circular pieces of meat were cut with a diameter of 8.2 cm (to fit in a standard petri dish) and an approximate thickness of 15 mm. The weight of each piece of meat was not determined. For the reference samples, 30 Brain-Heart Infusion agarose plates were made with Brain-Heart Infusion broth (BHI broth; Oxoid, CM1135) and 1% agarose. Agarose was used instead of agar, as anecdotal evidence indicates that the presence of agar may increase RNA degradation of the samples. The a_w of the post-rigor beef pieces and BHI agarose plates was determined with an AquaLab Series 3 (Decagon Devices, Inc., Pullman, USA). The a_w of the post-rigor beef pieces and BHI agarose plates was 0.98 ± 0.01 and 0.98 ± 0.00, respectively. The pH of the post-rigor beef pieces and BHI agarose plates was determined using a HI 2020-02 Edge Portable pH meter (Hanna Instruments, VIC, Australia). The pH of the meat was 5.61 ± 0.05 and the pH of the BHI agarose plates were 7.00 ± 0.02.

2.3. Comparison of the growth rate of the test strains in overnight culture and determination of their growth rate on meat

Triplicate growth curves were generated for each strain grown in BHI overnight at 37 °C using the automatic Bioscreen C system (Labsystems, Helsinki, Finland). No significant difference was observed in the growth curves of these strains under these conditions (results not shown).

Meat samples were inoculated with either of the two *E. coli* strains as described below. Growth was determined by viable count at 0, 1, 24 and 48 h. After the required time of incubation, meat samples were deposited into stomacher bags containing 50 ml of water and stomached for 1 min at setting 3 (BagMixer, Interscience). The resulting supernatant was serially diluted and plated onto the BHI agarose plates, before being incubated at 37 °C for 24 h.

2.4. Inoculation of meat and agarose plates

Thirty cultures in total, consisting of fifteen cultures each of *E. coli* O157:H$^-$ EC2422 and *E. coli* O157:H7 Sakai in 10 ml of BHI broth,

were incubated with shaking (100 rpm) at 37 °C for 20 h. Overnight cultures were centrifuged (5000 rpm for 5 min) and washed twice in 10 ml of sterile water. Pellets were resuspended in 1 ml of sterile water to yield a cell density of approximately 10^{10} CFU ml^{-1}, of which 250 µl (~2.5×10^9 CFU) was spread evenly with a sterile disposable microbiological spreader onto each meat sample and reference BHI agarose plate. In total there were 60 samples comprising of: 15 meat samples inoculated with *E. coli* O157:H$^-$ EC2422, 15 reference BHI agarose plates inoculated with *E. coli* O157:H$^-$ EC2422, 15 meat samples inoculated with *E. coli* O157:H7 Sakai and, 15 reference BHI agarose plates inoculated with *E. coli* O157:H7 Sakai. Samples were incubated for 1 h at 14 °C at an average humidity of 80%. A Tinytag View 2 logger (TV-4500, Gemini Data Loggers Ltd, Sussex, UK) was used to monitor the temperature and relative humidity during incubation of all samples.

2.5. Preparation of samples for transcriptomic analysis

After 1 h of incubation at 14 °C, meat samples were individually placed into filtered stomacher bags containing 15 ml of RNALater™ (Ambion) RNA stabilization solution. The bags were then heat sealed and hand massaged for 1 min, before the supernatant was decanted into individual 50 ml falcon tubes. After 1 h of incubation at 14 °C, 15 ml of RNALater™ was added to the BHI agarose plate samples and cells were dislodged with a sterile disposable microbiological spreader before the supernatant was poured into individual 50 ml falcon tubes. Bacteria were harvested from all tubes by centrifugation (6000 rpm for 10 min) and the supernatant was discarded. Pellets were placed on dry ice for a minimum of 20 min and were then stored at −70 °C.

2.6. RNA isolation and processing

Pellets were thawed on ice and washed twice with sterile RNase-free water prior to RNA extraction. The Qiagen RNAProtect® Bacteria Mini Kit RNeasy (Qiagen, Cat. No. 74,524) was used to extract RNA and remove genomic DNA from all samples. However, RNALater™ was substituted in place of RNAProtect® to improve the RNA quality of all samples. Additional changes were made to the manufacturer's protocol for enzymatic lysis and Proteinase K digestion, including the addition of 20 µl of Proteinase K and an increase in the amount of Lysozyme to 15 mg ml^{-1}. DNase treatment was performed on the column following the manufacturer's instructions.

Total RNA was quantified using a Nanodrop ND-1000 (ThermoFisher Scientific) and RNA quality was assessed using the Agilent RNA 6000 Nano Kit on the Agilent 2100 Bioanalyzer (Agilent, Santa Clara, CA). The rRNA was removed from the total RNA using an Epicentre Ribo-Zero kit (Epicenter, USA). rRNA depleted RNA was quantified using the Invitrogen Qubit® RNA HS Assay Kit with the Qubit® Fluorometer (Life Technologies).

The 60 rRNA depleted samples were sent to the Ramaciotti Centre for Genomics at The University of New South Wales (Sydney, Australia) for library preparation using the Illumina TruSeq Stranded kit and 100 bp length, paired-end Illumina sequencing on an Illumina HiSeq2500 sequencer.

2.7. Genome and virulence factor comparison between strains

Gene (cds) content and identity was compared between EC2422 and Sakai strains using a cluster-annotation approach (https://github.com/theo-allnutt-bioinformatics/pangenome_using_uclust) a similarity of 60% sequence identity between protein sequences was required to score presence in either genome. The presence of virulence factor genes was scored using the Centre for Genomic Epidemiology VirulenceFinder database (2017 version) https://cge.cbs.dtu.dk/services/VirulenceFinder/and a custom Python script (https://github.com/theo-allnutt-bioinformatics/scripts/blob/master/pathtype.py). Virulence factors were scored as present when DNA sequence identity was ≥ 90% and total length of match compared to the database was ≥ 50%.

2.8. Analysis of RNA-seq data

A total of 60 samples were analysed, consisting of fifteen replicates each of the four strain/media combinations. RNA extractions were carried out on two separate days. The effect of RNA extraction day was examined using glmQL tests and was shown to have no significant effect on any genes ranked in the top 20 fold-change for matrix effect (results not shown). Read files were clipped and quality filtered using ea-utils (v1.04.676) fastq-mcf command (Aronesty, 2013) to remove residual Illumina adaptors with a minimum size of 70 bp, minimum quality of 20 (Phred + 33) averaged over a 5 bp window, less than 50% homopolymer in read and less than 70% low complexity sequence. No samples were outliers in terms of number of filtered reads remaining.

Read mapping was performed using the program, BBmap v33, (https://sourceforge.net/projects/bbmap/). Observed read coverage and number of replicates was expected to be sufficient for approximately 95% confidence in detecting a 1.8 fold change in expression between growth matrices (Hart et al., 2013). Reference genomes used were strains *E. coli* O157:H7 Sakai (Genbank: BA000007) and *E. coli* O157:H$^-$ EC2422 (using PacBio draft assembly). Both strains were re-annotated using the program, Prokka v1.11 (Seemann, 2014) and a custom *E. coli* database to ensure that genes were equivalently annotated in both strains prior to differential expression analysis. The coding sequence (cds) locus identifiers used in the present analysis refer to the re-annotated genomes. *E. coli* O157:H7. Sakai cds were given locus identiers prefixed 'sakai_' and *E. coli* O157:H$^-$ EC2422 cds were given locus identifiers prefixed 'EC2422_'. The abbreviation, 'EC' used here for the EC2422 strain should not be confused with 'Enzyme Commission' numbers. Both re-annotated genomes are available here: https://github.com/theo-allnutt-bioinformatics/King_etal_2018. Read counts per cds were obtained using the program, featureCounts v1.5.0 (Liao et al., 2014).

Statistical tests for differential gene expression were performed using the R package, edgeR v3.6.8. The Generalised Linear Model Quasi-Likelihood (glmQL) test was used with default settings. The Supplementary Material contains an annotated list of all genes differentially expressed by *E. coli* O157:H$^-$ EC2422 (Table S1) and *E. coli* O157:H7 Sakai (Table S2) on meat and agarose.

2.9. Functional profile analysis

An analysis was performed using SUPER-FOCUS (Silva et al., 2016). SUPER-FOCUS is a homology-based approach that maps reads to functional gene subsystems of the SEED database (http://edwards.sdsu.edu/superfocus/), to report the subsystems present in the datasets and profile their abundances. Kruskal-Wallis non-parametric H- tests (K-W) were conducted for differential expression of each functional group for the categories: substrate (regardless of strain); *E. coli* O157:H$^-$ EC2422 only substrate (meat *vs* agarose); *E. coli* O157:H7 Sakai only substrate (meat *vs* agarose); and strain to strain. SUPER-FOCUS was originally designed for functional analysis of metagenomic data, however, we have found it performs very well on our RNA-Seq data. The SUPER-FOCUS functional analysis enabled us to reveal hierachical groups of genes' functions that showed differential expression and, then to examine individual genes' expression within those groups.

2.10. Validation of RNA-seq data by qRT-PCR

The RNA-Seq data was validated using quantitative real-time reverse transcription-PCR (qRT-PCR). The siroheme synthase gene (*cysG/ECs4219*) was included for normalization within samples (Zhou et al., 2011). Forward and reverse PCR primers for five genes were designed using Primer3 software (Rozen and Skaletsky, 2000) and are listed in

Table 1
Primer pairs used for validation of RNA-Seq data by qRT-PCR.

Gene/Annotation[a]	Forward primer (5' - 3′)	Reverse primer (5' - 3′)
cysG	TAC CGC AGG AGG AGA TTA AC	CAC AGT GTT TCC AGC TCT TC
hdeB	CAC TGG TGA ACG CAC AAT CT	AAC CGG GGT CAT TGC TTT
Acid shock protein precursor	CGC TGC TAT GGG TCT GTC TT	CAG GTG CTG CTT TAT GCT GT
Trehalose-6-phosphate phosphatase	GTC GAG ATC AAA CCG AGA GG	GAC TGC GAA GCC AGA TTC AT
puuD	TGA GGC GGT TAG TGT CAT CA	GGT GAT AAA GCC CTC GAA CA
chaA	GCC TTT ATG ACG GGT AAC GA	CGC CAT TGA GCA CGT TAG TA

[a] See Table 4 for the corresponding Locus tag for each strain.

Table 2
A comparison of the genomes of *E. coli* O157:H7 Sakai and *E. coli* O157:H$^-$ EC2422.

Genome		Size (bp)	% GC	CDS	% unique CDS	% unique sequence
Sakai	Chromosome	5,498,450	50.53	5344	10.90 (553 unique CDS)	3.42
	AB011549 (pO157)	92,721	47.60	84	16.87	5.50
	AB011548 (pOSAK1)	3306	43.41	3	N/A	N/A
EC2422	Chromosome	5,404,896	48.57	4997	7.64 (377 unique CDS)	4.56
	pO157	93,342	54.14	81	17.50	6.48

Table 1. All qRT-PCR reactions were carried out using the SensiFAST^{TM}SYBR$^{®}$ No-ROX Kit (Bioline Pty. Ltd., Australia), according to the manufacturer's instructions. All real-time PCR reactions were performed on the LightCycler 480 (Roche Applied Science, West Grove, PA) under the cycling conditions of: 45 °C for 10 min, 95 °C for 2 min, 45 consecutive cycles consisting of 95 °C for 5 s and 60 °C for 10 s and, 72 °C for 5 s. Melting curve analysis (55–95 °C, increment of 0.11 °C/s) was performed to ensure PCR specificity. The method described by Pfaffl (2001) was employed to determine the expression fold changes of the target gene in cultures on meat, compared with the agarose reference culture.

2.11. RNA-seq accession number

The RNA-seq data was deposited with links to BioProject accession number PRJNA524020 in the NCBI BioProject database (https://www.ncbi.nlm.nih.gov/bioproject/).

3. Results and discussion

RNA-Seq technology was used to identify genes that were differentially expressed in *E. coli* O157:H7 Sakai and *E. coli* O157:H$^-$ EC2422 on beef compared with BHI agarose, to identify similarities and differences between the strains in the expression of genes that may be associated with growth and survival on beef.

3.1. Growth rate of E. coli O157:H7 sakai and E. coli O157:H$^-$ EC2422 on meat

As growth rate affects gene expression (Klumpp and Hwa, 2014), it was important to establish whether the strains were capable of growth under the test conditions of this study. As described above, viable counts of both strains were determined on meat samples at 14 °C at 0 and 1 h. There was no growth for either strain after 1 h of incubation on meat, as indicated by comparison to its inoculated reference sample at 0 h (P value $>$ 0.05) (results not shown). Our results are supported by those of Huang (2010) who reported a lag phase for *E. coli* O157:H7 grown on mechanically-tenderized beef at 15 °C (Huang, 2010). The SUPER-FOCUS analysis of the transriptomic response of both strains on meat also revealed the significant (K-W false detection rate probability (FDR_P) $<$ 0.05) down-regulation of genes involved in cell division.

On the meat carcass surface several factors strongly influence the potential for, and rate of, growth of bacteria; including temperature, water activity, pH and lactic acid concentration (Ross et al., 2003). In contrast to many other nations, Australian meat chilling is based on air cooling, resulting in water activity decreases at the carcass surface (Lovett, 1978; Salter, 1998) that are sufficient to severely inhibit *E. coli* growth rate (Ross et al., 2003). Circulation of cold air can dry the surface of the carcass and reduce the water activity to 0.95 and it can remain at levels below 0.97 for 20–30 h during and after chilling (Lovett, 1978; Salter, 1998). It is important to note that the water activity of the inoculated post-rigor beef pieces in this study was relatively high (0.98 ± 0.01) and therefore, the transcriptomic responses observed in this study may not reflect the osmotic stress response likely to be mounted by *E. coli* on the carcass.

3.2. Genomic features of E. coli O157:H7 sakai and E. coli O157:H$^-$ EC2422

The *E. coli* O157:H7 Sakai chromosome is 5.5 Mbp and encodes 5361 protein-coding sequences (Hayashi et al., 2001). In addition, *E. coli* O157:H7 Sakai possesses a large virulence plasmid of 92,721 bp (pO157) and a cryptic plasmid of 3306 bp (pOSAK1) (Makino et al., 1998). Table 2 displays a comparison summary of the genomes of *E. coli* O157:H7 Sakai and *E. coli* O157:H$^-$ EC2422 as annotated in the current study.

Table 3 contains a comparison of the virulence factors of *E. coli* O157:H7 Sakai and *E. coli* O157:H$^-$ EC2422. Both strains possess Shiga toxin producing genes. *E. coli* O157:H7 Sakai possesses *stx1* and *stx2a*, while *E. coli* O157: H$^-$ EC2422 possesses *stx1* and *stx2c*. The production of Shiga toxin is an essential virulence mechanism of pathogenic STEC (pSTEC), but this alone does not appear to indicate an isolate's propensity to cause human disease (Boerlin et al., 1999). The variety and complexity of factors that contribute to the pathogenic potential of an organism make it difficult to categorically define pSTEC (Mellor et al., 2016). However, definitions to categorically define pSTEC have been proposed based on the presence of key virulence factors strongly associated with human clinical isolates (Boerlin et al., 1999; Brooks et al., 2005; Wickham et al., 2006). Intimin (*eae*) is one such factor that, together with translocated intimin receptor (*tir*), facilitates the intimate adhesion of pSTEC to human epithelial cells. As can be seen in Table 3, *E. coli* O157:H7 Sakai and *E. coli* O157:H$^-$ EC2422 both possess a chromosomal copy of intimin (*eae*) and the intimin receptor (*tir*).

3.3. Transcriptomic responses of E. coli O157:H7 sakai and E. coli O157:H$^-$ EC2422 on meat at 14°C

Transcriptomic profiling experiments were undertaken using RNA-

Table 3
Virulence factors of *E. coli* O157:H7 Sakai and *E. coli* O157:H$^-$ EC2422.

Gene	Locus tag		Annotation	Sakai Chromosome	EC2422 Chromosome	Sakai pO157	EC2422 pO157
	E. coli strain Sakai	*E. coli* strain EC2422					
eae	*sakai_04586*	*ec2422_03167*	Intimin	+ [a]	+		
ehxA	*sakai_05444*	*ec2422_05091*	Enterohaemolysin			+	+
espA	*sakai_04583*	*ec2422_04784*	Type III secretion system	+	+		
espB	*sakai_04581*	*ec2422_03162*	Secreted protein B	+	+		
espJ	*sakai_02699*	*ec2422_01311*	Prophage-encoded Type III secretion system effector	+	+		
espP	*sakai_05503*	*ec2422_05066*	Putative exoprotein precursor			+	+
etpD	*sakai_05429*	*ec2422_05076*	Type II secretion protein			+	+
gad	*sakai_04422*	*ec2422_00719*	Glutamate decarboxylase	+	+		
	sakai_00849	*ec2422_04784*					
iha	*sakai_01353*	*ec2422_00138*	Adherence protein	+	+		
katP	*sakai_05500*	*ec2422_05064*	Plasmid-encoded catalase peroxidase			+	+
nleA	*sakai_01788*	*ec2422_00433*	Non-LEE encoded effector A	+	+		
nleB	*sakai_00849*	*ec2422_04784*	Non-LEE encoded effector B	+	+		
	sakai_00848	*ec2422_02463*					
nleC	*sakai_00849*	*ec2422_04784*	Non-LEE encoded effector C	+	+		
		ec2422_00827					
stx1	*Not annotated* [b]	*ec2422_01819*	Shiga toxin 1	+	+		
stx2a	*Not annotated* [b]	-	Shiga toxin 2a	+			
stx2c	-	*ec2422_04784*	Shiga toxin 2c		+		
tccP	*Not annotated* [b]	-	Tir-cytoskeleton coupling protein	+			
	Not annotated [b]						
tir	*Not annotated* [b]	*ec2422_03169*	Translocated intimin receptor protein	+	+		
toxB	*sakai_05483*	*ec2422_04784*	Toxin B			+	+

[a] "+" denotes the presence of the gene.
[b] The locus tags for the original *E. coli* O157:H7 Sakai strain were generated using GenBank, whereas the locus tags for *E. coli* O157:H7 Sakai strain EC2898 were generated using Prokka. Therefore, in some instances matching annotation was not found.

Table 4
Gene expression fold changes[a] resulting from RNA-Seq analysis and qRT-PCR for five selected genes.

Gene/Annotation	Locus tag		*E. coli* strain Sakai		*E. coli* strain EC2422	
	E. coli strain Sakai	*E. coli* strain EC2422	RNA-Seq	qRT-PCR	RNA-Seq	qRT-PCR
hdeB	*sakai_04412*	*ec2422_02999*	40.090[b]	24.850 ± 6.803	13.093	25.188 ± 0.324
Acid shock protein precursor	*sakai_02284*	*ec2422_00898*	64.765	45.708 ± 17.104	14.295	47.441 ± 9.963
Trehalose-6-phosphate phosphatase	*sakai_02594*	*ec2422_01206*	36.523	5.457 ± 2.943	8.246	3.944 ± 4.151
puuD	*sakai_01848*	*ec2422_00495*	0.194	0.283 ± 0.322	0.230	0.083 ± 0.031
chaA	*sakai_01693*	*ec2422_00362*	0.091	0.422 ± 0.194	0.089	0.134 ± 0.040

[a] RNA-Seq results are shown as fold change values (as opposed to logFC) to ease comparison with the qRT-PCR results.
[b] RNA-Seq and qRT-PCR results are shown to a maximum of three decimal places.

Seq technology. As a first step in the data analyses, any genes that had zero mapped reads for both strains were removed, resulting in mapping of 4418 out of the 4,420 genes comprising the *E. coli* reference genome (O157:H7 Sakai), indicating > 99% coverage of the whole transcriptome of *E. coli*. The results of the RNA-Seq experiments were also validated using qRT-PCR (Table 4). While the standard deviation for some of the qRT-PCR results was high for some genes, in all cases the results displayed the same trend in expression as the RNA-Seq results.

3.3.1. Stress responses

Transcriptomic profiling revealed a high degree of similarity in the response of *E. coli* O157:H7 Sakai and *E. coli* O157:H$^-$ EC2422, with the most highly up-regulated genes for both strains involved in the acid stress response (Table 5). Interestingly, the magnitude of the fold-change of up-regulated genes was generally higher for *E. coli* O157:H7 Sakai than *E. coli* O157:H- EC2422 (see Table 5 and Supplemental Tables S1 and S2). A number of factors are known to account for strain variation in gene expression, including differences in DNA sequence and transcriptional regulation. While beyond the scope of the current study, further work is warranted to investigate the factor/s accounting for this observed trend and, whether this phenomenon is also observed in the protein expression profiles of these strains.

The most highly expressed gene for both strains was the acid shock protein precursor (*sakai_02284* and *ec2422_00898*). In addition, also within the top 20 most highly up-regulated genes of both strains on meat, were the acid stress chaperone protein precursors (*hdeA* and *hdeB*), the glutamate decarboxylase enzyme (*gadA*) and the transcriptional regulator *gadE* (see Supplemental Tables S1 and S2). For both strains on meat, the SUPER-FOCUS analysis also revealed significant (FDR_P < 0.05) up-regulation of genes involved in the periplasmic acid stress response (*hdeD*, *hdeB*, *gadE*, *hdeA* and *yhiD*). The inner membrane protein encoding gene *yohK*, previously shown to be universally up-regulated by *E. coli* O157:H7 Sakai and *E. coli* K-12 strain MG1655 during exposure to inorganic and inorganic acids (King et al., 2010), was also amongst the most highly up-regulated genes in both *E. coli* O157:H7 Sakai and *E. coli* O157:H$^-$ EC2422 on meat (see Supplemental Tables S1 and S2). Also amongst those functions defined as part of the universal acid response (King et al., 2010), *E. coli* O157:H7 Sakai and *E. coli* O157: H$^-$ EC2422 up-regulated genes involved in iron uptake (*efeUOB*) (see Supplemental Tables S1 and S2). In addition, *otsB* encoding trehalose-6-phosphate phosphatase was amongst the most highly up-regulated genes by both *E. coli* O157:H7 Sakai (*sakai_02594*) and *E. coli* O157: H$^-$ EC2422 (*ec2422_01206*) on meat (see Supplemental Tables S1 and S2). Trehalose is as an important

Table 5
Gene expression log fold change of the ten most highly expressed genes for *E. coli* O157:H$^-$ EC2422 and *E. coli* O157:H7 Sakai on meat.

Strain	Locus tag	Annotation	Log fold change
EC2422	*ec2422_00898*	Acid shock protein precursor	3.84
	ec2422_00454	Anthranilate synthase component 1	3.76
	ec2422_02999	Acid stress chaperone HdeB precursor	3.71
	ec2422_00050	Ferrous iron permease EfeU	3.64
	ec2422_00051	Iron uptake system component EfeO precursor	3.60
	ec2422_01587	Inner membrane protein YohK	3.58
	ec2422_00453	Bifunctional protein TrpGD	3.53
	ec2422_00052	Deferrochelatase/peroxidase EfeB precursor	3.51
	ec2422_03000	Acid stress chaperone HdeA precursor	3.46
	ec2422_03015	Inner membrane metabolite transport protein YhjE	3.32
Sakai	*sakai_02284*	Acid shock protein precursor	6.02
	sakai_03019	Inner membrane protein YohK	6.00
	sakai_04413	Acid stress chaperone HdeA precursor	5.41
	sakai_04412	Acid stress chaperone HdeB precursor	5.33
	sakai_02594	Trehalose-6-phosphate phosphatase	5.19
	sakai_03020	Cytidine deaminase	4.82
	sakai_02075	Glutamate decarboxylase beta	4.81
	sakai_01808	Anthranilate synthase component 1	4.78
	sakai_04422	Glutamate decarboxylase alpha	4.71
	sakai_02074	Putative glutamate/gamma-aminobutyrate antiporter	4.70

osmoprotectant and stress protectant in *E. coli* and up-regulation of the trehalose biosynthetic operon (*otsBA*) during exposure to acidic pH has previously been described (Kannan et al., 2008; Weber et al., 2005). The up-regulation of genes encoding acid stress response proteins is not surprising, with the average pH of ten pieces of meat from five different animals recorded to be pH ~5.6, in comparison to the BHI agarose at a pH ~7 (results not shown). This mimics the conditions after slaughter, where glycogen in muscles breaks down to form lactic acid over a period of 48 h and the pH falls from 7.3 to about 5.4–5.6 (McNeil et al., 1991). The pH on carcasses during and after chilling is usually in the pH range 5.4–7.0 (Ross et al., 2003). The up-regulation of acid response genes also aligns with the findings of Fratamico et al. (2011), indicating that *E. coli* O157:H7 found on beef may become more acid tolerant and potentially increase survival in the host (Fratamico et al., 2011).

The SUPER-FOCUS analysis also revealed that both strains significantly (FDR_P < 0.05) up-regulated genes on meat associated with the cold shock CspA family of proteins. *E. coli* O157:H7 Sakai significantly (FDR < 0.05) up-regulated *cspE* (*sakai_00656*), while *E. coli* O157:H$^-$ EC2422 significantly up-regulated *cspD* (*ec2422_04906*) and *cspG* (*ec2422_00020*) (see Supplemental Tables S1 and S2). This was unexpected, as both the meat and BHI agarose reference samples were incubated at 14 °C. This supports the work reported by Duffitt et al. (2011), in which numerous cold shock genes were significantly expressed in *E. coli* O157:H7 incubated in soil at 15 °C compared to cells grown in LB at 15 °C (Duffitt et al., 2011). It has been reported that *csp* genes are not exclusively induced by cold shock (for a review see (Wouters et al., 2001)). Indeed previously (King et al., 2010), *E. coli* K-12 MG1655 and O157:H7 Sakai were reported to display a universal gene expression response during exposure to a variety of organic and mineral acids that involved the up-regulation of cold shock-associated genes. As discussed above, in the current study genes of the acid stress response were the most highly up-regulated by both strains. Taken together, the results of the current and previous study (King et al., 2010) suggest an association between the acid and cold shock responses.

The SUPER-FOCUS analysis also revealed that only *E. coli* O157:H7 Sakai significantly (FDR_P < 0.05) up-regulated genes involved in the osmotic stress response on meat. Amongst these genes, *E. coli* O157:H7 Sakai significantly up-regulated a high affinity transporter (*proV/sakai_03545)* involved in the import of the potent osmoprotectant glycine betaine (see Supplemental Table S2). The SUPER-FOCUS analysis also revealed that both strains significantly (FDR_P < 0.05) up-regulated genes involved in the biosynthesis of the osmoprotectant trehalose. In the current study the water activity recorded for the BHI agarose reference samples and meat samples was 0.98–0.99 and 0.98, respectively (results not shown). It was therefore not expected that either strain would mount a response to osmotic stress. However, there is overlap in the expression of genes induced under conditions of osmotic stress and acid stress (Weber et al., 2005). The up-regulation of genes of the osmotic stress response aligns with the fact that genes of the acid stress response were amongst the most highly up-regulated by both strains on meat. As previously discussed, circulation of cold air can dry the surface of the carcass and reduce the water activity where it can remain at levels below 0.97 for 20–30 h during and after chilling (Lovett, 1978; Salter, 1998). Therefore, it will be important to determine the response of the strains when also subjected to a water activity more closely mimicking that of meat in an abattoir/retail or home setting.

RpoS controls the general stress response in *E. coli* (for a review, see (Weber et al., 2005)). Previously (King et al., 2016), under *in vitro* conditions of combined cold and osmotic stress in a time-series experiment, *E. coli* O157:H7 Sakai was observed to significantly up-regulate the RpoS regulon. In the current study, of 304 RpoS-dependent genes, on meat *E. coli* O157:H7 Sakai and *E. coli* O157:H$^-$ EC2422 significantly up-regulated (FDR < 0.05) 114 and 89 genes, respectively (Supplemental Table S3). Of these RpoS-dependent genes, 87 (75%) were commonly up-regulated by both *E. coli* O157:H7 Sakai and *E. coli* O157:H$^-$ EC2422. These genes confer resistance to a diverse variety of environmental stresses, including those involved in resistance to acid, osmotic and oxidative stress. Both strains also up-regulated expression of the RpoS-dependent gene *bolA* (*ec2422_04416* and *sakai_00481*). The chromosomal copy of *bolA* of *E. coli* O157:H$^-$ EC2422 shares a 99% identity alignment to the *E. coli* O157:H7 Sakai *bolA* gene. BolA is a transcription factor that regulates the expression of genes involved in the morphology of the cell to confer protection to the cell, specifically under stress conditions and during stationary phase (Freire et al., 2009; Santos et al., 2002). BolA induced changes in the outer membrane make the cell less permeable to harmful agents through modulation of the OmpF/OmpC porin balance (Freire et al., 2006). *bolA* induces higher levels of OmpC to the detriment of OmpF levels, thus reducing the permeability of the outer membrane. The stress response regulator OmpR is also a key element in the regulation of porin expression levels. A stress-related increase of phospho-OmpR in the cell leads to an increase in outer membrane OmpC levels and a concomitant reduction of OmpF (Egger et al., 1997). Both strains contain two copies of *ompR*. *ec2422_02856* and *sakai_04270* are identical and share 86% similarity to the identical *ec2422_03655* and *sakai_05125*. *E. coli* O157:H7 Sakai significantly (FDR < 0.05) up-regulated *ompC/ECs3104* and up-regulated one *ompR* variant (*sakai_05125*) and down-regulated the other variant (*sakai_04270*) (see Supplemental Table S2). Whereas, *E. coli* O157:H$^-$ EC2422 significantly (FDR < 0.05) down-regulated *ompC/ec2422_01661* and *ompR/ec2422_02856* (no significant differential expression was observed for *ompR/ec2422_03655*) (see Supplemental Table S1). BolA also regulates flagellar biosynthesis pathways, induces biofilm formation through the production of fimbria-like adhesins and curli (Dressaire et al., 2015) and, impairs cell growth rate (Guinote et al., 2011). Distinct differences were observed between the strains in relation to the expression pattern of genes associated with curli fimbriae production and are discussed in the sections below.

The strains also differed in their expression of RpoS-dependent genes. *E. coli* O157:H$^-$ EC2422 uniquely up-regulated genes encoding a dihydroxy-acid dehydratase (*ec2422_03314*) and a formate hydrogenlyase complex iron-sulfur subunit (*ec2422_02176*). *E. coli* O157:H7 Sakai uniquely up-regulated 27 RpoS-dependent genes, of which over a third encode hypothetical proteins. Other RpoS-dependent genes

uniquely up-regulated by *E. coli* O157:H7 Sakai included an alkyl hydroperoxide reductase (*sakai_00639*); involved in scavenging of endogenous hydrogen peroxide, chaperones involved in the heat shock response (*dnaJ/sakai_00013*, *grpE* and heat shock protein 90) and a gene encoding the lipid A biosynthesis palmitoleoyl acyltransferase; which is involved in membrane integrity. Of those RpoS-dependent genes significantly down-regulated (FDR < 0.05) by *E. coli* O157:H7 Sakai (92) and *E. coli* O157:H$^-$ EC2422 (68), over 60% (61) were commonly down-regulated by both strains. Those genes commonly down-regulated included genes required for cell division (*ftsZ*), cell shape determination (*mreBC*) and encoding the primary sigma factor controlling the heat shock response (*rpoH*). Interestingly, *E. coli* O157:H7 Sakai also uniquely down-regulated the housekeeping sigma factor encoded by *rpoD* (*sakai_03957*).

3.3.2. Virulence gene expression

The SUPER-FOCUS analysis revealed that on meat, both strains significantly down-regulated (FDR_P < 0.05) genes involved in virulence. However, the results of the SUPER-FOCUS analysis also showed that both strains significantly (FDR_P < 0.05) up-regulated genes of the Shiga toxin cluster. On meat, *E. coli* O157:H7 Sakai up-regulated *stx1* (*ECs2974*), Shiga toxin subunit B precursor (*sakai_02958*) and Shiga toxin subunit A precursor (*sakai_02959*) (see Supplemental Table S2). While *E. coli* O157:H$^-$ EC2422 up-regulated genes encoding the Shiga toxin subunit A precursor (*ec2422_01819*) and Shiga toxin subunit B precursor (*ec2422_01818*) (see Supplemental Table S1). DNA damage can induce *stx* expression (Kimmitt et al., 2000) and the SUPER-FOCUS analysis revealed that both strains significantly (FDR_P < 0.05) up-regulated genes involved in DNA repair. Both strains also up-regulated *toxB* (*sakai_03874* and *ec2422_05127*), encoding the ToxB protein that contributes to adherence of EHEC to epithelial cells (Tatsuno et al., 2001) (see Supplemental Tables S1 and S2).

3.3.3. Cell envelope

The envelope of Gram-negative bacteria plays a crucial defensive role against various environmental assaults. The envelope consists of two concentric membranes, the outer membrane and inner membrane, which are separated by an aqueous periplasmic space that houses the peptidoglycan cell wall. Lipopolysaccharide plays a critical role in the barrier function of the outer membrane of Gram-negative bacteria (Silhavy et al., 2010). The SUPER-FOCUS analysis revealed that on meat *E. coli* O157:H7 Sakai significantly (FDR_P < 0.05) up-regulated polysaccharide associated genes. A number of genes involved in lipopolysaccharide biosynthesis were significantly (FDR < 0.05) up-regulated by *E. coli* O157:H7 Sakai (*ECs0202*, *ECs0249*, *ECs1738*, *ECs4497*, *ECs4498*, *ECs4500*, *ECs4501*, *ECs4502*, *ECs4503*, *ECs4504*, *ECs4505*, *ECs4506*, *ECs4507*, *ECs4508*, *ECs4721*) (see Supplemental Table S2). Direct comparison of the response of *E. coli* O157:H7 Sakai on meat under chill temperature in the current study to the response of this strain in previous *in vitro* experiments (King et al., 2016) under conditions of combined cold and osmotic stress, is complicated by the difference in the exact environmental parameters under study. However, the cell envelope appears to be a key line of defence for *E. coli* O157:H7 Sakai under the environmental conditions experienced in both studies. In comparison, *E. coli* O157:H$^-$ EC2422 significantly (FDR < 0.05) down-regulated a number of genes involved in lipopolysaccharide biosynthesis (*ec2422_01438*, *ec2422_03123*, *ec2422_03276*, *ec2422_03381*, *ec2422_04136*, *ec2422_04955*) (see Supplemental Tables S1).

E. coli possesses five known sets of genes promoting EPS production, including the *wca* operon; responsible for colanic acid synthesis (Ionescu and Belkin, 2009). Previously, the Rcs phosphorelay system involved in colanic acid biosynthesis was reported to be significantly up-regulated by *E. coli* O157:H7 Sakai during planktonic growth under combined conditions of cold and osmotic stress (King et al., 2016). In the current study, the SUPER-FOCUS analysis revealed significant (FDR_P < 0.05) up-regulation of the four genes of the *yjbEFGH* operon in *E. coli* O157:H7 Sakai. This operon is induced during growth on solid surfaces and is involved in the production of an unknown extracellular polysaccharide (EPS) (Ionescu and Belkin, 2009). Ionescu and Belkin (2009) report that even when RpoS activity is diminished, expression of the EPS encoded by the *yjbEFGH* operon may allow cells to survive and proliferate without general stress protection. In contrast to *E. coli* O157:H7 Sakai, the SUPER-FOCUS analysis revealed that *E. coli* O157:H− EC2422 displayed significant (FDR_P < 0.05) down-regulation of the four genes of the *yjbEFGH* operon.

Peptidoglycan maintains cell shape and provides mechanical strength to resist osmotic challenges (for a review, see (Typas et al., 2011)). *E. coli* O157:H$^-$ EC2422 significantly (FDR < 0.05) down-regulated genes involved in the biosynthesis of peptidoglycan (*ec2422_00331*, *ec2422_02666*, *ec2422_03523*, *ec2422_03947*, *ec2422_04046*, *ec2422_04048*, *ec2422_04049*, *ec2422_04050*, *ec2422_04859*) (see Supplemental Table S1).

Also in comparison to *E. coli* O157:H7 Sakai, on meat *E. coli* O157:H$^-$ EC2422 down-regulated elements involved in monitoring and maintaining the integrity of the cell envelope. *E. coli* O157:H$^-$ EC2422 significantly (FDR < 0.05) down-regulated expression of *cpxR* (*ec2422_03457*) and *cpxA* (*ec2422_03456*), as well as a number of genes positively controlled by CpxRA (Price and Raivio, 2009) (*ec2422_00261*, *ec2422_00673*, *ec2422_00825*, *ec2422_01049*, *ec2422_01137*, *ec2422_01210*, *ec2422_02058*, *ec2422_02573*, *ec2422_02822*, *ec2422_02852*, *ec2422_03397*, *ec2422_03733*, *ec2422_04117*, *ec2422_05011*) (see Supplemental Table S1). In *E. coli* the Cpx two-component system is one of the primary envelope stress response systems, sensing and responding to protein misfolding in the periplasm and inner membrane (for a review, see (Grabowicz and Silhavy, 2017)).

3.3.4. Bacterial surface structures

The ability of an organism to attach to food surfaces may influence its persistence during manufacturing and at retail and consequently its ability to cause infection (Kumar and Anand, 1998). Bacterial surface structures, including flagella, have been reported to be an important factor in the attachment process (BOUTTIER et al., 1997; Butler et al., 1979; Chung et al., 1989; Pratt and Kolter, 1998). The SUPER-FOCUS analysis revealed a significant (FDR_P < 0.05) increase in the expression of genes involved in flagellar motility for both *E. coli* strains on meat. *E. coli* O157:H7 Sakai significantly (FDR < 0.05) up-regulated genes encoding the flagellar motor switch protein FliM (*sakai_02671*), flagellar biosynthesis sigma factor FliA (*sakai_02650*), flagellar basal body P-ring biosynthesis protein FlgA (*sakai_01434*), flagellar basal-body rod protein FlgB (*sakai_01435*), flagellar biosynthesis protein FlhA (*sakai_00255*) and a flagellar protein (*sakai_02577*) (see Supplemental Table S2). *E. coli* O157:H$^-$ EC2422 significantly (FDR < 0.05) up-regulated genes encoding flagellin (*ec2422_01262*), flagellar motor switch protein FliG (*ec2422_01276*), flagellar M-ring protein FliF (*ec2422_01275*), flagellar basal-body rod protein FlgF (*ec2422_00226*), flagellar biosynthesis protein FlhA (*ec2422_01190*), flagellar biosynthetic protein FlhB (*ec2422_01191*), flagellar transcriptional regulator FlhC (*ec2422_01202*), flagellar hook-basal body complex protein FliE (*ec2422_01274*) and flagellar assembly protein FliH (*ec2422_01277*) (see Supplemental Table S1). *E. coli* O157:H$^-$ EC2422 is a non-motile strain and the up-regulation of genes involved in motility either indicates a futile expense of energy, or that these genes play a role in adherence or another function yet to be determined.

Curli fimbriae are also involved in adhesion to surfaces and act as virulence factors by promoting attachment to eukaryotic cells (Kikuchi et al., 2005; Uhlich et al., 2002). Curli fimbriae, encoded on two divergently transcribed operons *csgDEFG* and *csgBAC*, are expressed in response to environmental stress factors such as low temperature and low osmolarity (Barnhart and Chapman, 2006; Blanco et al., 2012). The

SUPER-FOCUS analysis revealed a difference in the pattern of expression of genes involved in curli production, with a significant (FDR_P < 0.05) increase observed for *E. coli* O157:H7 Sakai and a significant (FDR_P < 0.05) decrease observed for *E. coli* O157:H$^-$ EC2422. Curli also play an important role in biofilm development; along with flagella, pili and exopolysaccharide and, act as a scaffolding agent in biofilms (Evans and Chapman, 2014).

Surface fimbriae of pathogenic *E. coli* facilitate sensing, adhesion and even invasion of host epithelial cells. *E. coli* O157:H$^-$ EC2422 down-regulated a number of fimbrial genes (*ec2422_00705*, *ec2422_00706*, *ec2422_00708*, *ec2422_00709*, *ec2422_00710*, *ec2422_01780*, *ec2422_03863*, *ec2422_03864*, *ec2422_03866*) (see Supplemental Table S1).

3.3.5. Quorum sensing and biofilm formation

The SUPER-FOCUS analysis revealed a significant (FDR_P < 0.05) increase in the expression of genes involved in quorum sensing and biofilm formation by both *E. coli* strains on meat. Low temperature has previously been reported to increase expression of biofilm associated genes in *E. coli* (White-Ziegler et al., 2008). Different quorum sensing systems have been identified in *E. coli*: that mediated by the LuxR homolog SdiA; the LuxS/autoinducer 2 (AI-2) system, an AI-3 system and, a signalling system mediated by indole (Li et al., 2007). Of these systems, both strains up-regulated genes of the AI-2 system. AI-2 has been reported to control biofilm formation in *E. coli* (González Barrios et al., 2006). Genes of the AI-2 system are organized in two divergent operons, *lsrACDBFG*, which comprises genes encoding an AI-2-specific uptake system and two enzymes (LsrF and LsrG) involved in AI-2 degradation and recycling and, the regulatory *lsrRK* operon (Rossi et al., 2018). *E. coli* O157:H7 Sakai and *E. coli* O157:H$^-$ EC2422 up-regulated all genes within the *lsrACDBFG* and *lsrRK* operons (see Supplemental Tables S1 and S2).

AI-3 was first described as a crucial player in the pathogenesis of EHEC O157:H7, where it controls the expression of different virulence loci (Sperandio et al., 2003). This molecule is sensed by the QseC membrane histidine kinase, also potentially involved in AI-2 sensing, that undergoes autophosphorylation upon binding to AI-3 (Rossi et al., 2018). *E. coli* O157:H$^-$ EC2422 up-regulated genes of the AI-3 system, with up-regulation of the genes encoding the sensor protein QseC (*ec2422_02504*) and the transcriptional regulatory protein QseB (*ec2422_02505*) (see Supplemental Table S1).

3.3.6. Metabolism

Beef contains protein, has a high sulfur content (Masters and McCance, 1939) and includes all of the essential amino acids (lysine, threonine, methionine, phenylalanine, tryptophan, leucine, isoleucine, valine), vitamin B12 and, other B vitamins, selenium, zinc, iron and, fat (Fratamico et al., 2011; Williams, 2007). BHI is a nutritionally rich media, with components (g/L) including brain infusion solids (12.5), beef heart infusion solids (5), proteose peptone (10), glucose (2), sodium chloride (5) and disodium phosphate (2.5). Only BHI contains glucose (2 g/L). The SUPER-FOCUS analysis revealed that on meat, both strains significantly (FDR_P < 0.05) up-regulated genes of the Entner-Doudoroff pathway, involved in glycolysis and gluconeogenesis, the TCA cycle, pyruvate metabolism, fermentation, electron transport and photophosphorylation and, nitrogen metabolism. The SUPER-FOCUS analysis also revealed that on meat, both strains significantly (FDR_P < 0.05) up-regulated genes involved in sulfur metabolism and in the biosynthesis of two sulfur-containing amino acids; cysteine and methionine. Activation of specific amino acid biosynthesis pathways or amino acid supplementation can lead to tolerance to certain environmental stresses (Horinouchi et al., 2010). The SUPER-FOCUS analysis revealed that on meat, both strains significantly (FDR_P < 0.05) up-regulated genes involved in biosynthesis of tryptophan, glutamine, glutamate, aspartate, asparagine and aromatic amino acids and their derivatives. The SUPER-FOCUS analysis revealed that on meat *E. coli* O157:H7 Sakai significantly (FDR_P < 0.05) up-regulated genes involved in histidine biosynthesis. This finding is in line with our previous studies (King et al., 2014; King et al., 2016; Kocharunchitt et al., 2012), in which *E. coli* O157:H7 Sakai up-regulated histidine biosynthesis at chill temperature. An increase in the cellular content of histidine may reflect the importance of this amino acid in mediating growth and/or survival of *E. coli* O157:H7 Sakai under the test conditions. However, if this is the case, the specific function/s histidine plays in mediating growth and/or survival under these conditions is yet to be determined.

The SUPER-FOCUS analysis revealed that on meat both strains significantly (FDR_P < 0.05) up-regulated genes involved in the transport of zinc and iron acquisition and metabolism. The SUPER-FOCUS analysis also revealed that on meat both strains significantly (FDR_P < 0.05) up-regulated genes encoding the low-pH-inducible ferrous iron transporter EfeUOB (Roy and Griffith, 2016). *E. coli* O157:H7 Sakai was previously shown to up-regulate genes and proteins involved in iron transport during exposure to chill temperature (King et al., 2014; King et al., 2016). In addition (King et al., 2010), *E. coli* O157:H7 Sakai and *E. coli* K-12 MG1655 were shown to display a universal gene expression response during exposure to organic and mineral acids that involved the up-regulation of genes involved in iron uptake.

On meat, in comparison to on BHI agarose, both strains displayed significant up-regulation of a number of metabolic pathways. This indicates that BHI agarose should not be used as a surrogate for meat in experiments designed to determine the physiological response of *E. coli* on meat.

3.4. Limitations of this study

One of the major limitations of this study was the inability to conduct gene expression profiling experiments under conditions exactly mimicking that likely to be encountered in the production environment. Specifically, the experiments conducted in the current study did not mimic the likely cell density of *E. coli* present on meat or account for the presence of background flora. While there is variation in the published literature on the methods employed to sample and determine the prevalence and level of *E. coli* O157 on the beef carcass in the processing plant, the density of culture on the agarose and meat in our study is undoubtedly much higher. Fegan et al. (2005) reported that the range of *E. coli* O157 counts from positive samples collected from individual animals at an Australian abattoir were < 0.1 to 0.12 MPN/cm^2 for prechill carcasses (Fegan et al., 2005). Brichta-Harhay et al. (2007) reported that the median count for *E. coli* O157:H7 from beef carcasses from several processing plants throughout the United States is 1.6×10^2 CFU $(100\ cm^2)^{-1}$ (Brichta-Harhay et al., 2007). Carney et al. (2006) conducted a study in a beef slaughter plant in Ireland and detected *E. coli* O157:H7 on beef carcasses at concentrations of up to 1.41 $\log_{10}$ CFU g^1 (Carney et al., 2006). In addition, the current study investigated the transcriptomic response of pure cultures of *E. coli* O157. As noted by Fratamico et al. (2011), there may be additional differences in gene expression on beef in the presence of the natural microflora. However, these types of studies in actual food products are difficult to perform using currently available technologies (Fratamico et al., 2011).

In addition, on the carcass surface spaces can be formed by shrinkage of muscle fibers during rigor, which results in the formation of channels into which bacteria may enter (Warriner et al., 2001). These entrapped bacteria may be protected from outside influences, such as bactericides and other decontamination procedures (Lillard, 1988). It is increasingly recognized that by using averaged molecular or phenotypic measurements of a whole population to describe cell behaviors, conclusions could be biased by the expression profiles of outliers; meanwhile, these unique patterns could be distinctive functional behaviors at a given place and time and therefore important to analyse and describe (Wang et al., 2015). While to date most transcriptome studies are conducted on a 'population level' (Saliba et al., 2014),

advances in single-cell transcriptomics could offer insight into the transcriptomic response of entrapped bacteria and the identification of effective treatments to remove or kill these cells.

4. Conclusions

The results of this study have shown that *E. coli* O157 elicits a gene expression response that is specific to the meat matrix. In comparison to BHI, on meat both *E. coli* O157 strains up-regulated genes involved in carbohydrate metabolism, amino acid biosynthesis, iron acquisition and metabolism, sulfur metabolism and nitrogen metabolism. On meat, both strains also up-regulated genes involved in the acid stress response, cold shock response, quorum sensing, motility, biofilm formation and Shiga toxin production. The *E. coli* O157 strains also exhibited unique gene expression profiles on meat, indicating that they may differ in their ability to persist in the meat production environment and therefore also in their ability to cause disease. *E. coli* O157:H7 Sakai invested in a long-term sustainable strategy of enhancing the barrier function of its outer membrane, through the up-regulation of genes involved in lipopolysaccharide biosynthesis, EPS synthesis and curli production. In contrast, *E. coli* O157:H$^-$ EC2422 significantly down-regulated genes involved in the biosynthesis of peptidoglycan and of the Cpx two-component system. The relatively low rate of infection in Australia from *E. coli* O157, may therefore be in part due to Australian strains not being as resilient to the types of stresses experienced in the meat production environment. It is also possible that Australian carcass cooling regimes involving refrigerated air, are more effective than those processes in place in other countries. Further work is required to determine if the transcriptomic responses observed are part of an initial or sustained response and, how this affects the survival and growth of *E. coli* O157. It will also be important to determine whether the response elicited is a strain- or a clade-specific response. Further insight would also be gained in comparing these responses to that of Clade 8 strains; which are associated with the highest rates of severe disease (Manning et al., 2008). Finally, it will be important to determine if the response observed is mainly due to the influence of a single or combination of factors, and what happens to the observed response of each strain when also subjected to a water activity more closely mimicking that of meat in an abattoir/retail or home setting.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fm.2019.03.008.

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