A Thesis for the Degree of Master of Science

Transcriptome analysis of Enterotoxigenic *Escherichia coli* (ETEC) isolates exposed to cabbage

양배추 접촉 장독소형 대장균의 전사체 분석

February, 2017

Seunghee Jeong

Department of Agricultural Biotechnology

College of Agriculture and Life Sciences

Seoul National University

석사학위논문

Transcriptome analysis of Enterotoxigenic *Escherichia coli* (ETEC) isolates exposed to cabbage

양배추 접촉 장독소형 대장균의 전사체 분석

지도교수 유 상 렬

이 논문을 석사학위논문으로 제출함
2017 년 2 월

서울대학교 대학원
농생명공학부
정승희

정승희의 석사학위논문을 인준함
2017 년 2 월

위 원 장 문 태 화 (인)

부위원장 유 상 렬 (인)

위 원 하 남 출 (인)

ABSTRACT

Enterotoxigenic *Escherichia coli* (ETEC) is a food-borne pathogen causing diarrhea among children in developing countries as well as travelers in ETEC endemic areas. Recent studies have revealed that many outbreaks of ETEC were mediated by fresh produce such as sprouts or lettuce. To understand how ETEC adapt to fresh produce, ETEC FORC31 isolated from stool of a patient suffered from foodborne disease in Korea was cultivated in the presence of cabbage (*Brassica oleracea* var. *capitata* L.). Planktonic cells exposed to cabbage were subjected to RNA sequencing to obtain bacterial gene expression profiles. As a result, among a total of 5,391 annotated coding DNA sequences, 1.13% and 0.74% of total genes were significantly up- or down-regulated when contacted with cabbage for 4 h. Differently expressed genes (p-value ≤ 0.05, fold-change ≥ 5) were grouped mainly into five categories according to COG designations: amino acid transport/metabolism, nucleotide transport/metabolism, cell motility, inorganic ion transport/metabolism, and signal transduction mechanisms. This indicates that ETEC FORC31 may utilize various nutritional factors released from cabbage to promote its growth and survival. Particularly, RNA-Seq revealed that molybdenum-related genes including molybdate transporter and molybdenum cofactor (Moco) biosynthesis operons are

differentially expressed when the bacteria were exposed to cabbage. Molybdate used for Moco biosynthesis, and Moco is found in a number of different molybdoenzymes, which involve carbon, sulfur, and nitrogen metabolisms. It was further demonstrated that ETEC FORC31 *ΔmoeAB* defective in Moco biosynthesis had reduced motility and changed curli production in the presence of excess nitrate. In addition, *ΔmoeAB* was more sensitive to organic acid than wild type. These results indicate that molybdenum metabolism significantly affects for survival of ETEC in cabbage. Therefore, to reduce ETEC contamination in cabbage, organic acid treatment is carefully conducted because up-regulated Moco biosynthesis genes may affect bacterial acid resistance.

Keywords: ETEC, Cabbage, Transcriptome, RNA-Seq, Molybdenum, Molybdoenzyme

Student Number: 2015-21793

CONTENTS

List of Figures

List of Tables

Ⅰ. INTRODUCTION

Enterotoxigenic *Escherichia coli* (ETEC) is a common cause of bacterial infection leading to acute watery diarrhea in infants and young children as well as travellers in ETEC endemic countries. The major virulence factors of ETEC include heat stable (ST) and/or heat labile (LT) toxin (Begum *et al.* 2016).

In recent studies, fresh produces (e.g. cilantro, parsley, sprout and lettuce) have been reported as the major cause of ETEC outbreaks (Ethelberg *et al.* 2010, Feng & Reddy 2014, Gomez-Aldapa *et al.* 2013). For examples, a massive ETEC outbreak reported in Korea in 2012 hospitalized children of seven schools. This outbreak was cause by ready-to-eat kimchi contaminated by ETEC O169 (Shin *et al.* 2016). Among sixty-six *E. coli* outbreak occurred from 2009 to 2010, the second most common subtype was ETEC (30.3%), following EPEC (39.4%). In Korea, outbreaks of foodborne or waterborne diseases by ETEC was also the second most common (20.7%) among *E.coli* outbreaks in 2014 (Oh *et al.* 2015).

Outbreaks related fresh produce has been increased because of growing consumption of fruits and vegetables (Pollack 2001). Due to

the rising demand for ready-to-eat foods, the amount of international trades and the storage period of fresh produce have risen, making it easier to contamination by food-borne pathogens (Deering *et al.* 2012). Fresh produces are contaminated by bacterial pathogens through primary contamination (during growing and harvest) or secondary contamination (while washing, slicing, soaking, packaging and preparation) (Gomez-Aldapa *et al.* 2013). It has been reported that more outbreaks were related to non–animal origin food such as leafy greens have increased (Nousiainen *et al.* 2016).

Many studies have reported that several food-borne pathogens can survive and grow to relatively high levels in vegetables. This suggests that food-borne pathogens in human can also make use of the plant-extracted nutrition source to grow itself (Cooley *et al.* 2003, Deering *et al.* 2012, Jablasone *et al.* 2005, Schikora *et al.* 2008). Up to now, a lot of researches about the persistence of food-borne pathogen in fresh vegetables and the bacteria-plant interactions have been reported (Bae *et al.* 2013, Crozier *et al.* 2016, Kyle *et al.* 2010). However, fresh vegetables used for study of interaction between plant and pathogen were biased toward lettuce, spinach and sprout which were major

sources of food poisoning outbreaks (Carey *et al.* 2009, Deering *et al.* 2012, Gorski *et al.* 2009). Therefore, it is required to study other fresh produces eaten raw because there are many fresh produces having potential to carry food-borne pathogens.

Recently, analysis of transcriptome has been applied to figure out the plant-bacterial interactions. This technique has made the researchers easily study genes which are differentially expressed during growth in fresh produce (Schenk *et al.* 2012). Searching essential genes to plant-microbe interaction will suggest effective method for bacterial reduction in fresh vegetable.

ETEC FORC31, a strain isolated from human stool (Centers for Disease Control & Prevention, Busan, Korea) has many virulence factors; heat labile toxin, type 1 secretion system, CFA/I fimbriae, pilus, type fimbriae, yersiniabactin, siderophore. Therefore, there is possibility of causing foodborne illness in human when they eat foods contaminated with ETEC FORC31. Many researches about plant-pathogen interaction have focused on food-borne pathogens with high mortality, such as *Salmonella*, *Listeria*, *E.coli* O157:H7 (Bae *et al.* 2013, Crozier *et al.* 2016, Deering *et al.* 2012, Gorski *et al.* 2009,

Goudeau *et al.* 2013, Kyle *et al.* 2010). On the other hand, there was no such study conducted on ETEC-plant interaction despite many ETEC outbreaks by plant contamination.

Cabbage (*Brassica oleracea* var. *capitata* L.) is one of the most common fresh produces and usually consumed with shredded forms. In 2014, the outbreak of *E. coli* O111-contaminated cabbage occurred in the Minnesota (Minnesota Department of Health 2015). Therefore, to reveal the mechanisms of bacterial contamination in nutritious cabbage, understanding interactions between cabbage and ETEC is important.

To know the interaction between cabbage and ETEC FORC31, Next Generation Sequencing (NGS) technologies, a powerful method for analyzing gene expression profiles, was used (Kimbrel *et al.* 2011). This approach may suggest how the ETEC outbreak is effectively controlled in the presence of the cabbage.

Ⅱ. MATERIALS AND METHODS

2.1. Preparation of cabbage

Cabbage (*Brassica oleracea* var. *capitata* L.), used in this study, was purchased from a commercial market located in Seoul, Korea. Before inoculation, two or three cabbage leaves were soaked in 2 L of sodium hypochlorite (100 ppm) for 5 minutes to remove residual bacteria. Soaked leaves were washed with 2 L of distilled water and dried for 10 minutes. After drying, cabbage leaves were sliced with sterile shredder.

2.2. Bacterial strains and growth condition

All bacterial strains and plasmid used in this study are listed in Table 1. Basically, all strains were incubated on Luria-Bertani (LB) media at 37°C. When needed, the following antibiotics were supplemented; kanamycin (25 or 50 μg/mL), ampicillin (50 μg/mL) or carbenicillin (100 μg/mL).

To set up the time point of bacterial RNA isolation, ETEC

FORC31 were grown in M9 glucose (0.04%) broth at 37°C, 220 rpm with or without cabbage. An overnight culture of ETEC FORC31 was subcultured into fresh M9 glucose media (20-fold dilution) and 10 g of sterilized shredded cabbage was added. For the control experiment, the cells were grown in M9 glucose media without cabbage. To check growth, the $O.D._{600}$ value was measured every 1 h. The experiment was biologically duplicated.

2.3. Construction and complementation of Δ*moeAB* mutant

Using the Lambda Red recombination technique with pKD13 as the template, the Δ*moeAB* mutant was constructed by replacing the coding region of the kanamycin resistance cassette (Murphy & Campellone 2003). PCR primers were designed on the basis of ETEC FORC31 genome (Table 2.).

ETEC FORC31 carrying plasmid pKD46 was grown in 30 mL of LB broth with ampicillin and 50 mM of L-arabinose at 30°C with shaking (220 rpm) until $O.D._{600}$ reached 0.6. The cells were harvested by centrifugation (10,000 x g, 5 min, 4°C), washed three times with distilled water (DW), and resuspended with 100µl of cold DW (4°C).

Washed cells were transformed by electroporation of PCR insert which was generated using primers with 40 base pair (bp) of homologous regions flanking start and stop condons of the target gene. Electroporation was performed to manufacturer's instructions using MicroPulser Electroporation System (Biorad, CA, USA). Then, cells were recovered with 1mL of super optimal catabolite (SOC) media at 30°C for 2 h in 220 rpm. To reduce stress, mutants were screened on LB agar containing low concentration of kanamycin (25 μg/mL) at 30°C (Jandu *et al.* 2009). To remove temperature-sensitive pKD46, mutants were grown at 42°C on LB agar containing kanamycin (50 μg/mL). Plasmid pCP20 was used to remove kanamycin resistance cassette (Datsenko & Wanner 2000). Primers listed in Table 2. were used for PCR to confirm homologous recombination and deletion site.

Complementation of Δ*moeAB* was performed using p*moeAB.* p*moeAB* plasmid was constructed by cloning *moeAB* operon into pUHE21-2*lacI*^{q}. Amplification of *moeAB* operon was performed by PCR using p*moeAB*_BamHI_F and p*moeAB*_HindIII_R primers containing enzyme site of BamHI and HindIII. All plasmids including p*moeAB* were inserted through electroporation into the cell.

2.4. RNA extraction

RNA was extracted from ETEC FORC31 grown in M9 glucose media with or without cabbage. RNA protect bacterial reagent (Qiagen, Hilden, Germany) was treated to 500 μL of each cells before RNA isolation. Total RNA was purified from samples by using RNeasy mini kit (Qiagen, Hilden, Germany) and residual DNA was degraded with Turbo DNA-*free*^{TM} (Ambion, Texas, USA). The quantity and quality of total RNA was visualized through Agilent 2100 Bioanalyzers (Agilent Technologies, CA, USA) and the RNA integrity number (RIN) were determined (Schroeder *et al.* 2006). Purified RNA was stored at -81°C until use.

2.5. RNA sequencing and analysis of transcriptomes

Chunlab, Inc. (Seoul, South Korea) performed RNA-sequencing. Five micrograms of total RNA was used for analysis. To remove rRNA, Ribo-Zero kit (Epicentre Biotechnomogies, Wisconsin, USA) was used. Messenger RNA sequencing libraries were prepared using Illumina TruSeq RNA Sample Preparation kit v.2 (Illumina, CA, USA) and sequenced on the Illumina Hiseq 2500 to obtain 100 bp of

paired-end reads (Inagaki *et al.* 2015). Reference for mapping was the genome of ETEC FORC31 and RNA reads were mapped by CLC Genomics Workbench 6.5.1 (CLC bio, Arahus, Denmark). The expression level of genes was normalized using normalized Trimmed Mean of *M*-value (TMM) conducted in the edgeR Bioconductor package (version 2.4.0.) (Maza 2016). The mapped data was visualized by CLRNAseq program (Chunlab, Seoul, South Korea).

2.6. Quantitative real-time PCR (qRT-PCR) analysis

Before qRT-PCR, purified RNA was reverse-transcribed to complementary DNA by RNA to cDNA EcoDry™ Premix (Clontech, CA, USA) according to the manufacturer's instruction. All qRT-PCR was performed with cDNA, 2X iQ CYBR Green Supermix (Biorad, CA, USA), nuclease-free water and primers which are listed in Table 2. Amplification and detection were conducted on the CFX Connect™ Real-time PCR Detection System (Biorad, CA, USA). The house-keeping gene encoding glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used to normalize gene expression level. The data was analyzed using Bio-Rad CFX Manager™ 3.1. Software (Biorad, CA,

USA).

2.7. Swimming motility assay

Motility was assayed on soft agar swim plates inoculated with 1 μL of overnight culture of ETEC FORC31 WT, Δ*moeAB* and Δ*moeAB*::p*moeAB.* The plates contained 1% tryptone, 0.25% NaCl, and 0.3% Difco agar and was grown at 37°C for 11 h. For treatment with nitrate, the plates were supplemented with 15 mM KNO_3 (Showa, Tokyo, Japan). pUHE21-2 $lacI^q$ were inserted into all strains. All plates were supplemented by 10uM of IPTG for *moeAB* induction.

In addition, to check the growth effect in motility assay, all strains were grown at 37°C with agitation (220 rpm) in medium which contains 1% tryptone and 0.25% NaCl. If needed, potassium nitrate (KNO_3; 15 mM ; Showa, Japan) was added.

2.8 Assay for curli expression

Red color on Congo red plates (LB agar without NaCl containing 10 uM IPTG, 50 μg/mL ampicillin, 40 μg/mL Congo red

and 20 μg/mL Coomassie Blue) indicates production of curli fimbriae (Bak *et al.* 2015). Five microliters of overnight culture of ETEC FORC31 WT, Δ*moeAB* and Δ*moeAB*::p*moeAB* in LB broth without salt was spotted onto Congo red plates, and incubated for 24 h at 37°C.

2.9. Organic acid resistance test

Lactic acid (85% to 92% (v/v); Hayashi Pure Chemical Industries., Japan), acetic acid (100% (v/v); Merck, USA), propionic acid (99.5% (v/v); Sigma-Aldrich, USA), and citric acid monohydrate (50% (w/v); Sigma, USA) were used in organic acid resistance test.

To check growth effect, the ETEC FORC31 wild type (WT) and mutants (Δ*moeAB* and Δ*moeAB* and Δ*moeAB*::p*moeAB*) were grown into LB, M9 glucose or cabbage exposed media. To describe the growth curves, the $O.D._{600}$ value was measured every 1 h. The experiment was biologically duplicated.

For acid-resistance test in LB, overnight culture was subcultured into fresh LB (200-fold dilution). Cells were grown until $O.D._{600}$ reached 0.6. Two hundred microliters of culture were

transferred to 1.8 mL of LB containing organic acid for 1 h. The final concentrations of organic acid are shown in Fig 7.

For acid-resistance test in M9 glucose or cabbage exposed media, all strains were grown in the same condition of RNA extraction. Then, organic acid was treated to media and incubated for 1 h. The final concentrations of organic acids are shown in Fig 8. and Fig 9.

In all tests, ampicillin (50 μg/mL) was added before inoculation because pUHE21-2 *lacI* ^{q} was inserted into all strains. To express *moeAB* proteins, 10 uM of IPTG was added to media before inoculation. All strains were incubated at 37°C with agitation (220 rpm).

The CFU/mL of initial cell population was determined by plating serial dilutions in LB or M9 media at time zero. The CFU/mL of acid treated culture was determined as described above. Percentage of acid survival was calculated the remained number of CFU/mL after the acid treatment for 1 h divided by the initial cell population of CFU/mL at time zero. Each experiment was duplicated (Masuda & Church 2003).

Table 1. Strains used in this work, their genotype and source

Strain or plasmid	Relevant characteristics	Source or reference
Strains		
ETEC FORC31	Wild type, ETEC O6 isolate from human stool	Centers for Disease Control & Prevention (Busan, Korea)
Δ*moeAB*	ETEC FORC31 Δ*moeAB*	This study
Δ*moeAB* ::p*moeAB*	Δ*moeAB* with p*moeAB*	This study
Plasmids		
pKD13	FRT Km^R FRT PS1 PS4 *oriR6Kγ*;Ap^R	[25]
pKD46	P_{BAD}-*gam-beta-exo oriR101 repA101*^{ts};Ap^R	[25]
pCP20	*cI*857λP_R*flp oripSC101*^{ts};Ap^R Cm^R	[25]
pUHE21-2*lacI*^{q}	rep_{pMB1} Ap^R *lacI*^{q}	
p*moeAB*	pUHE21-2*lacI*^{q}-*moeAB*; Ap^R	This study

a, Ap^R, ampicillin resistant; Km^R, kanamycin resistant.

Table 2. Primers used in this study

Primer	Sequence (5'-3')
Construction of bacterial strain	
*moeAB*_Lambda_F	ATG GAA TTT ACC ACC GGA TTG ATG TCG CTC GAC ACC GCG CTG TAG GCT GGA GCT GCT TCG
*moeAB*_Lambda_R	TTA CTG CCC ACA CAC CTC ACA CCC CGG ATT ACG CAT CAG TAT TCC GGG GCT CCG TCG ACC
*moeAB*_Confirm_F	GTG GCA TAC AGT CTG TGG CGT
*moeAB*_Confirm_R	CCG GAC GTT CTG CAT CCT CAT
Lambda_Kan	CAG TCA TAG CCG AAT AGC CT
p*moeAB*_BamHI_F	AAA AGG ATC CAT GGA ATT TAC CAC CGG ATT GAT
p*moeAB*_HindIII_R	AAA AAG CTT TTA CTG CCC ACA CAC CTC AC
pUHE_F	AGA TTC AAT TGT GAG CGG ATA AC
pUHE_R	GGT CAT TAC TGG ATC TAT CAA CA
qRT-PCR	
*pmrK*_F	GTT TAT TGC CTG CTA TTA CCT G
*pmrK*_R	CAA TGC TGT TAA TCC AGT ATC C
*yadN*_F	CGG TGG TCA GTT AAA TAT CAG T
*yadN*_R	TGT TAC CAA AGA ATA CGG AAC C
*cblA*_F	ACT CTG TCG CAT TAA CCT ATT C
*cblA*_R	GAA GCA AAG TTC AGA TCA TTG G

*fliC*_F	ACC TGA ACA AAT CTC AGT CTT C
*fliC*_R	GAA ATA CCA TCA TTC GCG TTA C
*motA*_F	GAT ATT GAA AAT CCC CGT GAG A
*motA*_R	CTC AAT CTC TTC ATC CAT CAG G
*tar*_F	GTT TCC AAT CAA TTA CGG GAA C
*tar*_R	CTA TCG AGC AAT TCA ACT TTG G
*hybO*_F	GTA CAA AGG GCA GTA TGT GTT A
*hybO*_R	CCC GGA ATA TTG ATA ACG GTT T
*cysA*_F	GAA GAA CTA AAA TTC ACC AGC G
*cysA*_R	CCC ATA AAT TCG AGC ACA AAA C
*narG*_F	AGC TAC TCC TGG TAT CTT TAC A
*narG*_R	TCT TCA ATG ATA GAA GCC CAT G
*moeA*_F	CAC CAA AAC GAT TCT TGA AGA G
*moeA*_R	TGT ACC AGT TGA TAG AAG GTC A
*moaE*_F	CAT TAA CCC TCG AAC ACT ATC C
*moaE*_R	GCG GGT TTT GAG ATA ATC CAT A
*modA*_F	AAA AGA GAA AGA CGT GGA TGT G
*modA*_R	TAA TCC ATC CAT TTC TGA TCG G
GAPDH_F	ATG CTG AAA TAT GAC TCC ACT C
GAPDH_R	ACC AGT CAT AAC CAC TTT CTT C

III. RESULTS

3.1. Growth of ETEC FORC31 in cabbage.

Many researchers have revealed that food-borne pathogens can utilize fresh vegetable as their nutrient source (Cooley *et al.* 2003, Jablasone *et al.* 2005, Schikora *et al.* 2008). Therefore, to understand the response of pathogenic *E. coli* under exposure to plant-derived food, the transcriptome analysis of ETEC FORC31 was conducted to know which genes are differently expressed when exposed to cabbage. Growth of ETEC FORC31 was observed to determine proper time point for RNA extraction. Growth of ETEC FORC31 in M9 glucose media was higher when the cells were exposed to cabbage (Fig. 1.). In addition, cabbage media without bacteria was also observed to check effect of residual bacteria and it was not detected until 6 h post inoculation (Fig. 1.). As a result, ETEC FORC31 was harvested and their RNA was isolated at 4 h post inoculation which is mid-log phase.

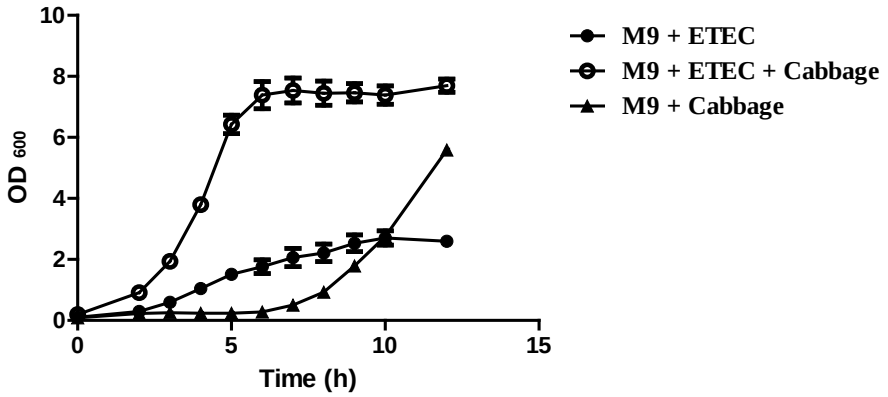

Figure 1. Growth of ETEC FORC31 with Cabbage. Growth of ETEC FORC31 with cabbage in M9 glucose media was observed for setting RNA isolation time point. Bacterial growth in cabbage contacted media was higher than compared to M9 glucose media. In addition, residual bacteria in cabbage were not detected until 6 h. The experiments were duplicated.

3.2. Gene expression profile of ETEC FORC31 exposed to cabbage.

Messenger RNA of ETEC FORC31 grown with or without cabbage was isolated. All RNA samples had RIN value over 8 and appropriate amount. Two different libraries were analyzed, and 136,321,058 sequences reads were obtained through RNA-sequencing. Among them, 129,304,194 (94.9%) sequence reads were mapped (Table 3.).

Because trimmed means of M-values (TMM) had lowest coefficients of variation value, TMM was applied for normalization (Table 4.) (Dillies *et al.* 2013). To get differentially expressed genes (DEGs), RNA-seq data was filtered above a fold change of 5 and below a p-value of 0.05 (Fig. 2.). After trimming the RNA-seq data, it was revealed that 1.13% (61/5,391) of the genes were up-regulated and 0.74% (40/5,391) of the genes were down-regulated in ETEC FORC31 exposed to cabbage for 4 h.

DEGs were grouped by Clusters of Orthologous Groups (COG) designations and its ratio was calculated (Fig. 3.) (Kyle *et al.* 2010). Among the COG categories, most of DEGs were classified into five categories; Amino acid transport/metabolism, Nucleotide transport/metabolism, Cell motility, Inorganic ion transport/metabolism,

and Signal transduction mechanisms.

Above five major categories were up-regulated in ETEC FORC31 exposed to cabbage for 4 h. It suggests that cabbage contacted cells utilize amino acid, nucleotide, inorganic ion from cabbage for growth because M9 glucose media has limited nutritious element. Different growth rate between cabbage and M9 glucose media might be caused by different nutrient conditions (Fig. 1.).

In addition, many genes involved in signal transduction mechanisms were up-regulated. It means that cabbage contains many bacterial signal molecules.

When exposed to cabbage, the most significantly up-regulated category was cell motility. Cell motility may be affected by nutrient and signal molecule from cabbage.

Table 3. Information of RNA samples

No	Sample Name	Conc. (ng/µl)	Volume (µl)	Total amount (µg)	rRNA Ratio [23S/16S]	RIN Value	Results
1	Control_4 h	367.9	27	9.9	1.2	8.3	Pass
2	Cabbage_4 h	1135.8	13	14.8	2.4	9.9	Pass

Number of reads	Control (M9 only)_4 h	Cabbage_4 h
rRNA reads	294,142	335,008
mRNA reads	37,194,236	35,292,328
Intergenic reads	23,161,744	21,398,744
Unmapped reads	3,298,524	3,718,340
Total reads	69,359,608	66,961,450

Table 4. Comparing methods for normalization of RNA-seq data

	Normalization methods		
Coefficients of variation (CV)	**RPKM**	**RLE**	**TMM**
	0.281	0.1957	0.1937

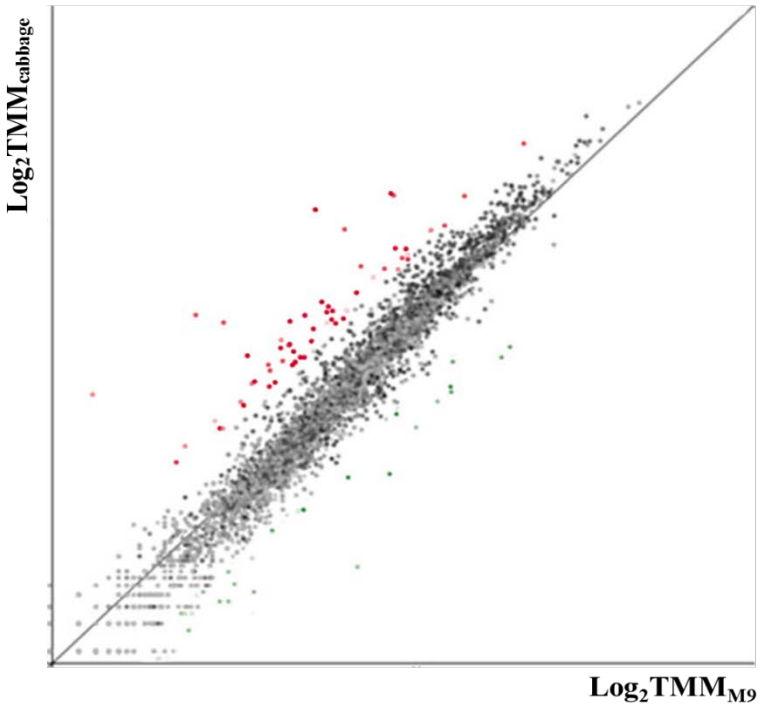

Figure 2. Differentially expressed genes (DEG) of ETEC FORC31 when exposed to cabbage. A graph shows differentially expressed genes of cells exposed to cabbage in exponential phase (4 h). The x-axis indicates log-scaled TMM values of control, and the y-axis indicates TMMs of cabbage-exposed cells. Red dots stand for up-regulated DEGs and green dots stand for down-regulated DEGS with cabbage contact (p-value ≤ 0.05, fold-change ≥ 5).

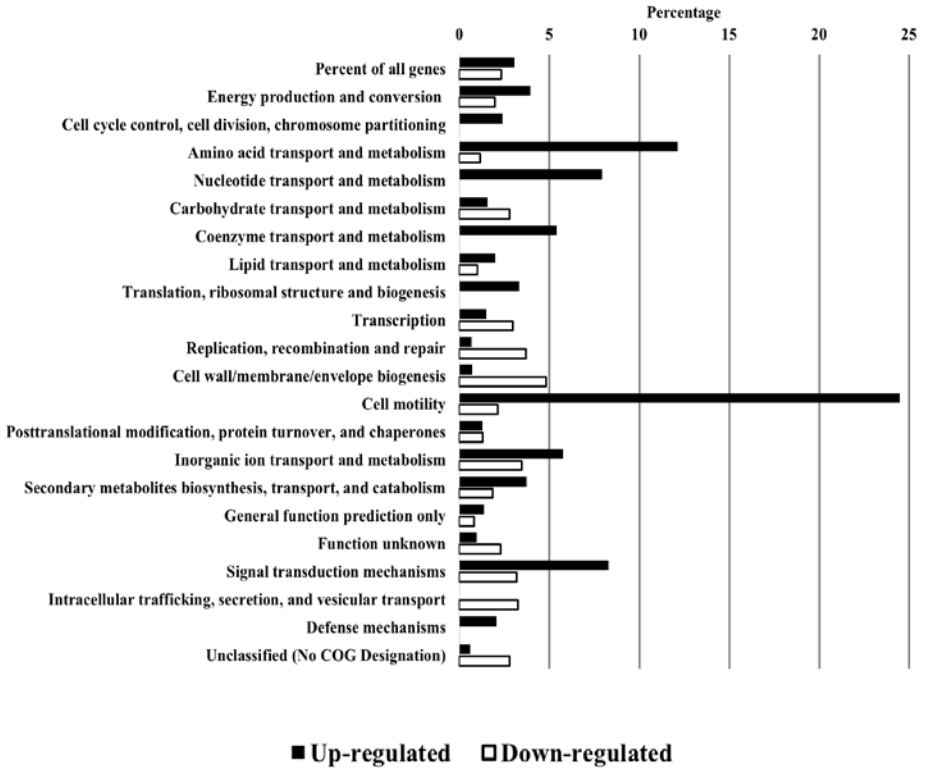

Figure 3. Grouping of DEGs in ETEC FORC31 under exposure to cabbage. Differentially expressed genes (DEG) were categorized by clusters of orthologous groups (COG) designations. Bars describe the percentage of induced or repressed DEGs compared to the total percentage of genes in a given categories after 4 h of cabbage exposure. (p-value ≤ 0.05, fold-change ≥ 5)

3.3. Gene expression patterns with known function.

Expression of many genes was up or down-regulated when exposed to cabbage. Operons or gene pathways which involved in DEGs and showed similar expression pattern were described in Table 5.; energy metabolism (molybdenum, hydrogen, sulfate, nitrogen), adhesion, motility, carbohydrate, amino acid, nucleic acid metabolism, and stress resistance.

It is known that most organisms require molybdenum, hydrogen, sulfur, and nitrogen as an essential element in various biological pathways (Crane *et al.* 1995, Dubini *et al.* 2002, Iobbi-Nivol & Leimkühler 2013, Lundberg *et al.* 2004).

In this study, molybdenum cofactor (Moco) biosynthesis (*moaABCDE*; FORC31_3188-92, *moeAB*; FORC31_3145-6, *mogA*; FORC31_4025, *mobA*; FORC31_4666) genes were up-regulated and molybdate ABC transporter (*modABC*; FORC31_3226-8) genes were down-regulated. Molybdenum is trace elements in molybdoenzymes. Molybdenum transport is performed in the form of the oxyanion molybdate (MoO_4^{2-}). Molybdate is required for Moco biosynthesis and Moco is used to form molybdoenzymes which involves nitrogen,

carbon, and sulfur metabolism (Iobbi-Nivol & Leimkühler 2013).

The *hybOABCDEFG* operon (FORC31_0819-26) encoding hydrogenase 2 which respiratory enzyme which couples hydrogen oxidation was down-regulated (Vignais & Colbeau 2004).

Genes involved in sulfate reduction (*cysJIH*; FORC31_1104-6, *cysDNC*; FORC31_1120-2) and sulfate uptake (*cysPUWAM*; FORC31_1460-3, *sbp*; FORC31_4598) were up-regulated. Sultfate adenylyltransferase (*cysND*), adenylylsulfate kinase (*cysC*), 3'-phospho-adenylylsulfate reductase (*cysH*), sulfite reductase (*cysJI*) are involved in assimilatory sulfate reduction to generate hydrogen sulfide (Crane *et al.* 1995, Liu *et al.* 1994, Segel *et al.* 1987). Sulfate ABC transporter takes up not only sulfate, but also other elements including thiosulfate, selenite, selenite and molybdate (Aguilar-Barajas *et al.* 2011).

Nitrate can be utilized as an alternative electron acceptor for energy generation (dissimilative nitrate reduction). *E.coli* has membrane-bound respiratory nitrate reductases (Lundberg *et al.* 2004). In transcriptomic data, the *narIJHG* operon (FORC31_2751-4), encoding nitrate reductase A was up-regulated. Similarly, genes related to

nitrate/nitrite uptake (*narK*; FORC31_2755) was up-regulated. Meanwhile, genes of nitrogen regulation (*GlnK*; FORC31_3540, *GlnALG*; FORC31_4649-51) were up-regulated. These genes control the nitrogen level inside the cell (Blauwkamp & Ninfa 2002, Magasanik 1989).

It is known that food-borne pathogens, especially *E.coli*, can adhere to plant tissues and form fimbriae, leading to persistence or resistance to disinfectants (Brankatschk *et al.* 2014, Fink *et al.* 2012, Yaron & Römling 2014). Fimbriae have a role in attachment of *E.coli* to plants like cabbage leaves (Yaron & Römling 2014). In this study, genes related to fimbrial protein (*ydeTSRQ*; FORC31_2511-4, *yadN*; FORC31_3885, *yadMLKC*; FORC31_3888-91) were significantly down-regulated. However, genes involved in fimbriae protein in plasmid (*cblDCAB*; FORC31_p356-9) were significantly up-regulated (Ghigo & Beloin 2011).

It has been revealed that, cell motility is closely related to bacterial colonization in plant (Crozier *et al.* 2016). The transcriptome data showed that many motility-related genes were significantly up-regulated. In detail, chemotaxis genes (*cheAW, tar, tap, cheRBYZ*;

FORC31_2106-13, *trg*; FORC31_2602, *yjcZ*; FORC31_4397, *tsr*; FORC31_4090) were significantly up-regulated (Porter *et al.* 2011). Almost operons involved in structure of flagella (*fliDST, fliC, fliAZ*; FORC31_2011-6, *motAB*; FORC31_2104-5, *flgKL*; FORC31_2884-5, *flgBCDEGHIJ*; FORC31_2886-94, *flgAMN*; FORC31_2896-8) were significantly up-regulated (Chevance & Hughes 2008). In addition, *pdeH* (FORC31_0284), encoding a c-di-GMP phosphodiesterase related to the flagellar motility and curli-mediated adhesion, and *ycgR* (FORC31_2790), encoding molecular brake that interacts with flagellar switch complex, are also up-regulated (Reinders *et al.* 2016).

There are four systems involving in carbohydrate transport in gram-negative; phosphotransferase (PTS) system, ATP-binding cassette (ABC) superfamily proteins, sugar-specific porins, and major facilitator superfamily (MFS) proteins (Kyle *et al.* 2010). In RNA-seq data, fructose PTS system including *fruBKA* (FORC31_1707-9) was up-regulated. In *E.coli*, fructose can offer the sole source of carbon (Kornberg 2001). *malM, lamB, malK* (FORC31_4480-2) and *malEFG* (FORC31_4484-6) were down-regulated. These genes involve maltose uptake through porin or ABC transporter systems (Gilson *et al.* 1986,

Panagiotidis *et al.* 1993, Wang *et al.* 1997).

Several genes involved in amino acid metabolism such as arginine, leucine, and asparagine showed significantly up-regulated expression level. Arginine succinyltransferase (AST) pathway is necessary for arginine catabolism in *E.coli* (Schneider *et al.* 1998). The *astCADBE* operon (FORC31_2259-63), gene cluster involved in the AST pathway was up-regulated. The *leuABCD* operon (FORC31_3953-6) which is related to L-leucine biosynethesis was up-regulated (Somers *et al.* 1973). *asnA* (FORC31_4764) or *asnB* (FORC31_3317) genes which function asparagine synthetase and synthesized L-asparagine from L-aspartate were up-regulated. (Felton *et al.* 1980, Humbert & Simoni 1980).

Bacteria can obtain pyrimidine via uracil degradation (Parales & Ingraham 2010). In nucleic acid metabolism, the *rutABCDEFG* operon (FORC31_2958-64) involved in uracil degradation was up-regulated. In addition, the *pyrBI* operon related to pyrimidine ribonucleotides biosynthesis was up-regulated (Donahue & Turnbough 1994).

By reducing net negative charge via lipid A modification, gram-negative bacteria show resistance to polymyxin which binds LPS and

kills bacteria through membrane lysis (Yu *et al.* 2015). In gene expression profile, genes encoding polymyxin resistance (*pmrD;* FORC31_1614*, pmrMLKJIFH*; FORC31_1615-21, *pmrG*; FORC31_1622) were down-regulated. In addition, *mdtJI* (FORC31_2412-3), transporter of a drug or spermidine coding gene, and *rspAB* (FORC31_2432-3), producing starvation sensing protein were down-regulated (Higashi *et al.* 2008, Huisman & Kolter 1994).

Table 5. Gene expression profile of ETEC FORC31 when exposed to cabbage

Category	Locus_tag	Log_2FC	Gene annotation
Molybdenum metabolism			
Moco synthesis	FORC31_3188	2.25	Molybdopterin synthase large subunit moaE
	FORC31_3189	2.30	Molybdopterin synthase small subunit moaD
	FORC31_3190	**2.37**	Cyclic pyranopterin monophosphate synthase moaC
	FORC31_3191	**2.40**	Molybdenum cofactor biosynthesis protein moaB
	FORC31_3192	1.55	Molybdopterin biosynthesis protein moaA
	FORC31_3145	1.38	Molybdopterin biosynthesis protein moeA
	FORC31_3146	1.35	Molybdopterin biosynthesis protein moeB
	FORC31_4025	0.94	Molybdopterin adenylyltransferase mogA
	FORC31_4666	0.35	Molybdopterin-guanine dinucleotide biosynthesis mobA
Molybdate transport	FORC31_3226	**-2.47**	Molybdenum transporter, ATP-binding protein modC
	FORC31_3227	**-3.43**	Molybdenum transporter, membrane subunit modB
	FORC31_3228	**-3.76**	Molybdenum transporter, periplasmic modA
Nitrogen metabolism			
Nitrate Reductase	FORC31_2751	**2.47**	Respiratory nitrate reductase gamma chain narI
	FORC31_2752	1.78	Respiratory nitrate reductase delta chain narJ
	FORC31_2753	1.44	Respiratory nitrate reductase beta chain narH

	FORC31_2754	2.07	Respiratory nitrate reductase alpha chain narG
Transport	FORC31_2755	1.67	Nitrate nitrite transporter narK
	FORC31_3539	**5.38**	Ammonium transporter amtB
Regulatory protein	FORC31_3540	**6.05**	Nitrogen regulatory protein P-II, glnK
	FORC31_4649	**2.65**	Glutamine synthetase type I glnA
	FORC31_4650	**2.89**	Nitrogen regulation protein NtrB glnL
	FORC31_4651	**2.81**	Nitrogen regulation protein NR(I) glnG
Hydrogen metabolism			
Hydrogenase	FORC31_0819	**-4.25**	Uptake hydrogenase small subunit precursor hybO
	FORC31_0820	**-3.07**	Fe-S-cluster-containing hydrogenase component1 hybA
	FORC31_0821	-2.26	Ni Fe-hydrogenase2B-type cytochrome subunit hybB
	FORC31_0822	-1.10	Uptake hydrogenase large subunit hybC
	FORC31_0823	-0.44	Hydrogenase maturation protease hybD
	FORC31_0824	-0.43	Hydrogenase2 operon protein hybE
	FORC31_0825	-0.08	[NiFe] hydrogenase nickel incorporation protein hybF
	FORC31_0826	-0.12	[NiFe] hydrogenase metallocenter assembly hybG
Sulfur metabolism			
Sulfate Reduction	FORC31_1104	2.09	Sulfite reductase [NADPH] flavoprotein cysJ
	FORC31_1105	**3.16**	Sulfite reductase [NADPH] hemoprotein cysI
	FORC31_1106	1.50	Phosphoadenylyl-sulfate reductase cysH
	FORC31_1120	2.11	Sulfate adenylyltransferase subunit 2 cysD
	FORC31_1121	2.19	Sulfate adenylyltransferase subunit 1 cysN

	FORC31_1122	2.23	Adenylylsulfate kinase cysC
Sulfate Transport	FORC31_1460	1.63	Sulfate and thiosulfate binding protein cysP
	FORC31_1461	1.13	Sulfate transport system permease protein cysT
	FORC31_1462	1.75	Sulfate transport system permease protein cysW
	FORC31_1463	**2.82**	Sulfate and thiosulfate import ATP-binding cysA
	FORC31_4598	1.24	Sulfate-binding protein Sbp
Adhesion			
Fimbriae	FORC31_2511	-1.15	Type 1 fimbriae anchoring protein fimD
	FORC31_2512	-1.31	Type 1 fimbrae adaptor subunit fimF
	FORC31_2513	**-2.39**	Type 1 fimbrae adaptor subunit fimG
	FORC31_2514	-0.83	Mannose-specific adhesin fimH
	FORC31_3885	**-2.50**	Fimbrial protein yadN
	FORC31_3888	-0.94	Fimbrial protein yadM
	FORC31_3889	-0.64	Fimbrial protein yadL
	FORC31_3890	-0.53	Fimbrial protein yadK
	FORC31_3891	-1.24	Fimbrial protein yadC
	FORC31_p356	0.81	Alpha-fimbriae tip adhesin cblD
	FORC31_p357	0.47	CFA/I fimbrial subunit C usher protein cblC
	FORC31_p358	**2.65**	Alpha-fimbriae major subunit cblA
	FORC31_p359	2.32	Alpha-fimbriae chaperone protein cblB
Cell motility			
Chemotaxis	FORC31_2106	**3.66**	Signal transduction histidine kinase cheA

	FORC31_2107	**4.01**	Positive regulator of cheA protein activity, cheW
	FORC31_2108	**3.30**	Methyl-accepting chemotaxis protein II, tar
	FORC31_2109	**3.17**	Methyl-accepting chemotaxis protein IV, tap
	FORC31_2110	**2.48**	Chemotaxis protein methyltransferase cheR
	FORC31_2111	**2.89**	Chemotaxis response regulator protein-glutamate cheB
	FORC31_2112	**3.25**	Chemotaxis protein cheY
	FORC31_2113	**3.75**	Protein phosphatase cheZ
	FORC31_4090	**3.03**	Methyl-accepting chemotaxis protein I, tsr
	FORC31_4397	1.10	Methyl-accepting chemotaxis protein, yjcZ
	FORC31_2602	1.14	Methyl-accepting chemotaxis protein III
Flagellar	FORC31_2011	0.94	Flagellar biosynthesis protein fliT
	FORC31_2012	1.33	Flagellar biosynthesis protein fliS
	FORC31_2013	**2.43**	Flagellar hook-associated protein fliD
	FORC31_2014	**5.14**	Flagellar biosynthesis protein fliC
	FORC31_2015	**3.12**	Flagellar biosynthesis sigma factor fliA
	FORC31_2016	0.90	Flagellar biosynthesis protein fliZ
	FORC31_2104	**2.47**	Flagellar motor rotation protein motA
	FORC31_2105	**2.47**	Flagellar motor rotation protein motB
	FORC31_2790	**3.66**	Inner membrane protein, flagellar brake protein ycgR
	FORC31_2884	**2.52**	Flagellar hook-associated protein flgL
	FORC31_2885	**3.02**	Flagellar hook-associated protein flgK
	FORC31_2886	0.59	Flagellar protein flgJ

	FORC31_2887	1.04	Flagellar P-ring protein flgI
	FORC31_2888	0.88	Flagellar L-ring protein flgH
	FORC31_2889	1.90	Flagellar basal-body rod protein flgG
	FORC31_2890	1.90	Flagellar basal-body rod protein flgF
	FORC31_2891	2.08	Flagellar hook protein flgE
	FORC31_2892	**2.44**	Flagellar basal-body rod modification protein flgD
	FORC31_2893	**2.62**	Flagellar basal-body rod protein flgC
	FORC31_2894	**2.65**	Flagellar basal-body rod protein flgB
	FORC31_2896	1.69	Flagellar basal body P-ring biosynthesis protein flgA
	FORC31_2897	**2.85**	Negative regulator of flagellin synthesis flgM
	FORC31_2898	1.89	Flagellar biosynthesis protein flgN
	FORC31_0284	**4.19**	c-di-GMP phosphodiesterase pdeH
Carbohydrate Metabolism			
Fructose Transport	FORC31_1707	**2.77**	Phosphocarrier, HPr family protein fruB
	FORC31_1708	1.98	1-phosphofructokinase fruK
	FORC31_1709	1.48	PTS system, fructose-specific IIBC component fruA
Maltose Transport	FORC31_4480	-1.11	Maltose operon periplasmic protein malM
	FORC31_4481	-2.11	Maltoporin lamB
	FORC31_4482	-1.69	Maltose maltodextrin transport ATP-binding malK
	FORC31_4484	**-2.45**	Maltose maltodextrin ABC transporter, substrate malE
	FORC31_4485	-1.31	Maltose maltodextrin ABC transporter, permease malF
	FORC31_4486	-1.62	Maltose maltodextrin ABC transporter, permease malG

Amino acid Metabolism			
Arginine	FORC31_2259	**2.91**	Succinylornithine transaminase astC
	FORC31_2260	**3.23**	Arginine N-succinyltransferase astA
	FORC31_2261	**3.41**	Succinylglutamic semialdehyde dehydrogenase astD
	FORC31_2262	**3.24**	Succinylarginine dihydrolase astB
	FORC31_2263	**2.99**	Succinylglutamate desuccinylase astE
Leucine	FORC31_3953	1.79	2-isopropylmalate synthase leuA
	FORC31_3954	1.59	3-isopropylmalate dehydrogenase leuB
	FORC31_3955	1.84	3-isopropylmalate dehydratase large subunit leuC
	FORC31_3956	**2.38**	3-isopropylmalate dehydratase small subunit leuD
Asparagine	FORC31_4764	**3.64**	Aspartate-ammonia ligase asnA
	FORC31_3317	1.88	Asparagine synthetase asnB
Nucleic Acid Metabolism			
Uracil degradation	FORC31_2958	**3.10**	Pyrimidine monooxygenase rutA
	FORC31_2959	**3.03**	Predicted amidohydrolase rutB
	FORC31_2960	**2.85**	Possible ring-opening amidohydrolase rutC
	FORC31_2961	2.09	Possible hydrolase or acyltransferase rutD
	FORC31_2962	1.89	Predicted reductase rutE
	FORC31_2963	**2.54**	Predicted flavin reductase rutF
	FORC31_2964	1.59	Uracil permease rutG
Pyrimidine synthesis	FORC31_4247	**2.67**	Aspartate carbamoyltransferase pyrB
	FORC31_4248	**2.36**	Aspartate carbamoyltransferase regulatory chain pyrI

Stress resistance			
Polymyxin resistance	FORC31_1614	-0.99	Polymyxin resistance protein pmrD
	FORC31_1615	-0.99	Polymyxin resistance protein pmrM
	FORC31_1616	-0.68	Polymyxin resistance protein pmrL
	FORC31_1617	-0.75	Polymyxin resistance protein pmrK
	FORC31_1618	-0.85	Polymyxin resistance protein pmrJ
	FORC31_1619	-1.17	Bifunctional polymyxin resistance protein pmrI
	FORC31_1620	-1.38	Polymyxin resistance protein pmrF
	FORC31_1621	-1.56	UDP-4-amino-4-deoxy-L-arabinose-oxoglutarate pmrH
	FORC31_1622	**-2.76**	Polymyxin resistance protein pmrG
Multidrug	FORC31_2412	**-2.47**	Spermidine/multidrug export protein mdtJ
	FORC31_2413	-1.97	Spermidine/multidrug export protein mdtI
Starvation	FORC31_2432	**-3.32**	Starvation sensing protein rspA
	FORC31_2433	**-3.24**	Starvation sensing protein rspB

*The black color indicates up-regulated genes and the gray color indicates down-regulated genes.

* Log_2FC; $Log_2(TMM_{Cabbage}/TMM_{M9})$, Significantly DEGs are in bold (p-value ≤ 0.05, fold-change ≥ 5),

3.4. qRT-PCR for validation of RNA-sequencing data.

To validate reliability of RNA-sequencing data, qRT-PCR of twelve genes was performed. These genes were selected as candidate of virulence factor on behalf of eight major groups specifically regulated by exposure to cabbage; polymyxin resistance (*pmrK*), fimbriae (*yadN*, *cblA*), flagellar (*fliC*, *motA*), chemotaxis (*tar*), hydrogenase (*hybO*), sulfate transport (*cysA*), nitrogen reductase (*narG*), molybdenum metabolism (*moeA*, *moaE*, *modA*). Although fold change values of cabbage were quite different between qRT-PCR and RNA-Seq, the tendency of up- or down-regulation in qRT-PCR corresponded with those of RNA-Seq. This result supports that the transcriptomic data is biologically significant (Fig 4.).

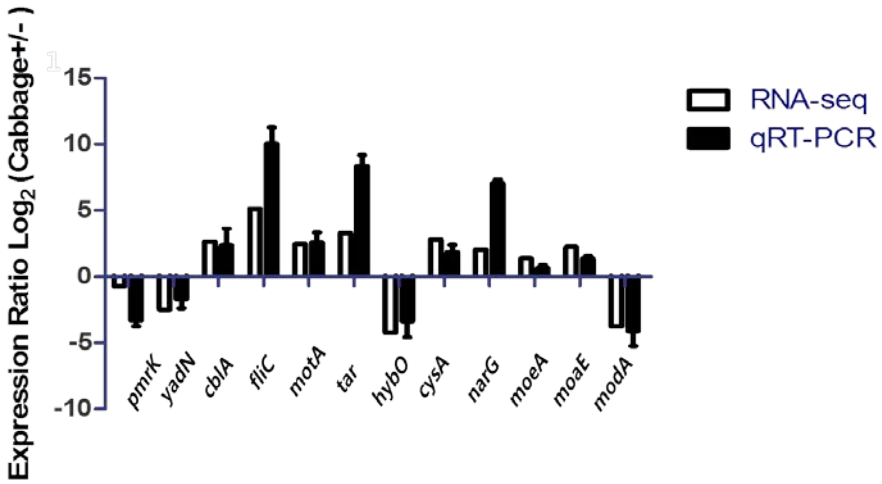

Figure 4. Relative expression ratio of significantly changed genes by exposure to cabbage. Twelve genes of eight major groups which were specifically regulated when exposed to cabbage were validated by qRT-PCR under the same condition of RNA-sequencing.

3.5. Transcriptional response of genes related to molybdenum metabolism.

Molybdenum is trace elements required in many biological processes. Molybdenum is bioavailable and transported in the form of the oxyanion molybdate (MoO_4^{2-}). Molybdate is used in biosynthesis of molybdenum cofactor (Moco) which is coordinated in the active site of molybdoenzymes which catalyze oxo-transfer reactions in the metabolisms of nitrogen, carbon, and sulfur compounds (Iobbi-Nivol & Leimkühler 2013).

Genes involved in biosynthesis of Moco are in five gene loci in *E.coli* (*moa, mob, moe, mog, moc*) and its pathway is divided into four steps; (1) Formation of cyclic pyranopterin monophosphate (cPMP) from GTP, (2) Conversion of cPMP to Molybdopterin (MPT), (3) Insertion of molybdate to form Moco (Mo-MPT), (4) Modification of Mo-MPT (Fig. 5.). There are three kinds of modified Moco structure; di-oxo Moco, *bis*-MGD, sulfurated MCD (Iobbi-Nivol & Leimkühler 2013). Sulfite oxidase family contains di-oxo Moco which is modified from Mo-MPT without the aid of proteins. *mobA* is involved in formation of MGD which is cofactor of DMSO reductase family and

mocA is involved in synthesis of MCD which is cofactor of xanthine oxidase family (Iobbi-Nivol & Leimkühler 2013) .

In transcriptomic data, *mobA* is up-regulated but *mocA* was down-regulated. It means that when exposed to cabbage, formation of MCD is lower than bis-MGD and xanthine oxidase (XO) family is less synthesized compared to sulfite oxidase (SO) and DMSO reductase family (Fig 5.).

In cultivation of plant, Mo is often added to irrigation water to enhance the growth and nutrient value such as soluble sugar and chlorophyll concentrations because it enables the plant to get the nitrate from the soil (Nie *et al.* 2007, Weir 2004). The Cruciferae, including cabbage, is high molybdenum demanded crops. Thus, molybdenum is mixed with fertilizer in form of ammonium molybdate or sodium molybdate to cultivate cabbage (Weir 2004). It means that cabbage has abundant molybdate, thus it makes change of transcriptomic profile of ETEC FORC31. In RNA-seq data, the expression level of all operons related to molybdenum metabolism was significantly changed (Table 5., Fig. 5.). Genes related to Moco biosynthesis operons were slightly up-regulated at 4 h post inoculation and molybdate transporter operon was

down-regulated (Fig. 5.).

There are three molybdate transport systems in *E.coli*; molybdate ABC transporter, sulfate transporter, and selenite-sensitive anion transporter (Kaiser *et al.* 2005). In the RNA-seq data, high-affinity molybdate transporter is down-regulated and sulfate transporter, secondary molybdate transport system, is up-regulated (Fig. 5.).

Recent some papers have reported that molybdoenzymes are linked to virulence in several bacteria, but there was no study related to virulence of molybdoenzymes in pathogenic *E.coli* (Andreae *et al.* 2014, Dhouib *et al.* 2015, Périnet *et al.* 2016). Thus, *moeAB* (Moco biosynthesis gene) deletion mutant of ETEC FORC31 was made to know relationship between virulence and molybdo-enzymes in ETEC. A removal of *moeAB* should lead to a loss of all molybdoenzyme activities.

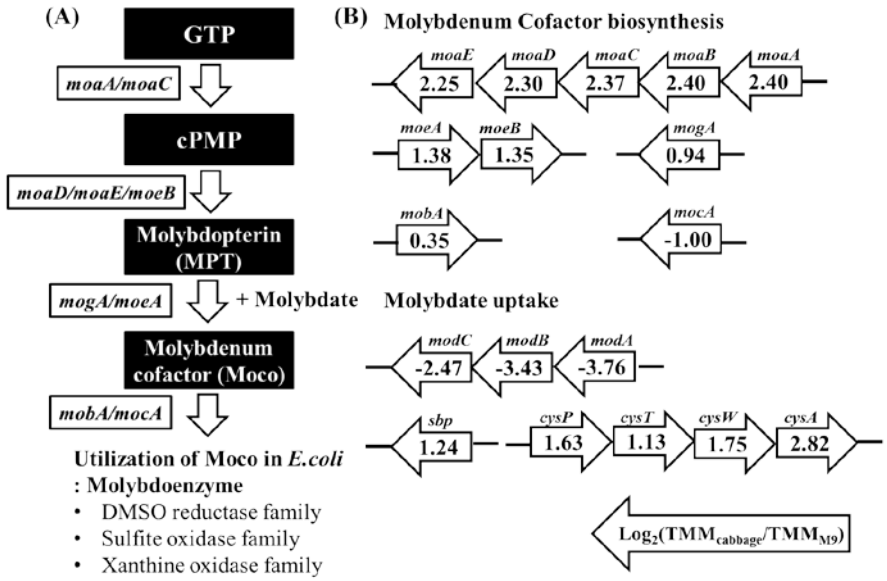

Figure 5. Moco biosynthesis pathway and expression level of ETEC FORC31 operons related to molybdenum metabolism under exposure to cabbage. (A) A scheme shows the Moco biosynthetic pathway in *E.coli* and the proteins involved in this pathway. Three family molybdoenzymes which contain Moco are described at the end of scheme. (B) ETEC FORC31 operons related to molybdate uptake and Moco biosynthesis. Genes are represented in order of location and direction of transcription in the operons. Each number in arrow shows the relative fold change of TMM (log2) at 4 h exposure to cabbage compared to M9 glucose media. Gene names according to function is represented above the arrow.

3.6. Molybdenum cofactor affects motility and curli production of ETEC FORC31 in nitrate supplementation.

Nitrate reductase, belonging to molybdoenzyme, requires an active Moco in the Mo-bis guanine dinucleotide (*bis*-MGD) form, for the reduction of nitrate to nitrite (Andreae *et al.* 2014). In nutritional aspects, fresh produce including cabbage has a large amount of nitrate that microorganisms can utilize as nitrogen source [53, 54]. In transcriptomic data, genes related to cell motility were up-regulated and also genes involved in nitrate reduction were differently expressed when exposed to cabbage (Table 5.). It can be a sign that ETEC FORC31 can utilize nitrate of cabbage for their survival.

Because it is known that nitrate affect chemotaxis as chemoattractant, the motility of ETEC FORC31 *ΔmoeAB* that is unable to synthesize Moco was tested (Rivera-Chávez *et al.* 2016). Nitrate supplementation increase motility of WT, but motility of Δ*moeAB* was not changed compared to control (without nitrate addition). Motility of Δ*moeAB*::p*moeAB* strain restored WT level (Fig. 6.).

In addition, to check the growth effect in motility assay, all strains were grown in medium which contain same component without agar.

Growth of *ΔmoeAB* was not significantly different compared to WT (data not shown).

When the bacteria are inoculated on low-osmomolarity nutrient agar plates with the diazo dye Congo red, the production of the extracellular matrix components such as cellulose and curli fimbriae is characterized by a red, dry and rough (rdar) morphotype (Anwar *et al.* 2014). The rdar morphology of ETEC FORC31 WT and mutant was checked on Congo red agar after 24 h incubation. When nitrate was added in Congo red agar, Red zone of WT disappeared, but Δ*moeAB* showed similar red morphology regardless of nitrate. Red zone of Δ*moeAB*::p*moeAB* strain was similar to WT (Fig. 6.).

In previous study, genes involved in curli production repress motility (Anwar *et al.* 2014). This result indicates that nitrate supplementation may increase the motility of ETEC FORC31.

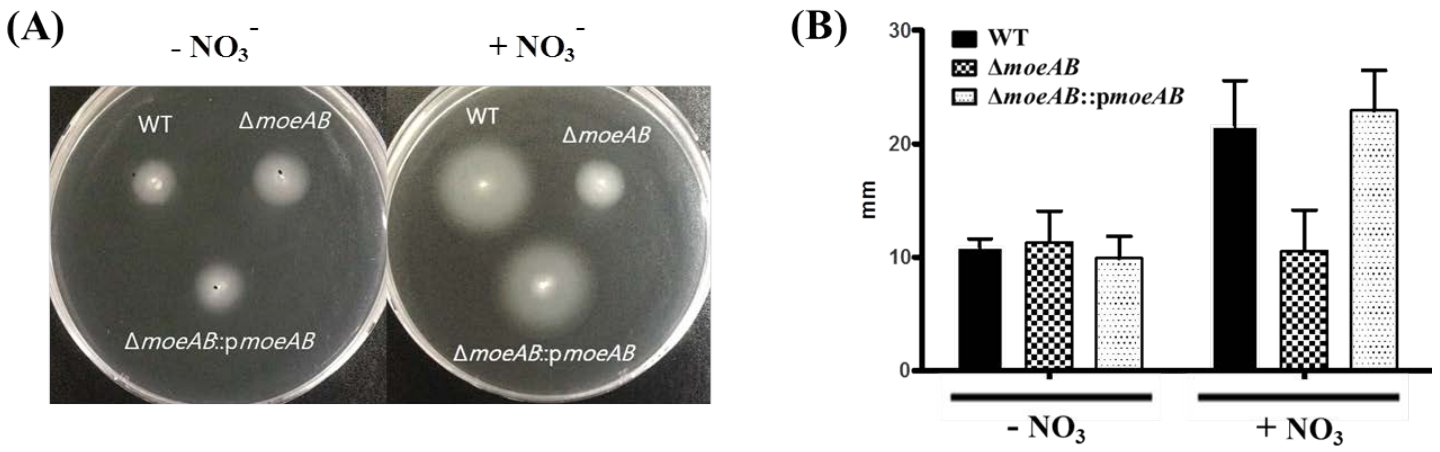
(A)
- NO3-
+ NO3-
WT
ΔmoeAB
ΔmoeAB::pmoeAB
WT
ΔmoeAB
ΔmoeAB::pmoeAB
(B)
WT
ΔmoeAB
ΔmoeAB::pmoeAB
mm
30
20
10
0
- NO3
+ NO3
(C)
- NO3-
+ NO3-
W
Δ
C
W
Δ
C
W WT
Δ ΔmoeAB
C ΔmoeAB::pmoeAB

Figure 6. Nitrate addition changes motility and the rdar morphology in ETEC FORC31 WT, but not in *ΔmoeAB*. (A) Representative examples of motility assay after 11 h. Δ*moeAB* showed motility defect by nitrate addition. (B) The graph shows motility zone after 11 h incubation. Nitrate supplementation increased motility of WT, but not Δ*moeAB*. Motility of Δ*moeAB*::p*moeAB* strain restored WT level. Error bars indicate standard deviation (SD) from three independent experiments. (C) Assay for curli production on Congo red agar after 24 h incubation. Red zone of WT disappeared by adding nitrate. Δ*moeAB* showed red morphology regardless of nitrate. The morphology of Δ*moeAB*::p*moeAB* strain is similar to WT.

3.7. Molybdenum cofactor is related to organic acid resistance of ETEC FORC31.

Organic acids are generally known as safe (GRAS). Some organic acids have been made as sanitizers on fresh produces. In many study, organic acids have shown antimicrobial activity (Park *et al.* 2011). In this study, the effects of four organic acids (lactic, citric, acetic, and propionic acid) were tested as candidate of sanitizer for ETEC contaminated cabbage.

Moco is cofactor of molybdoenzymes. In previous study, *ydeP* belonging to molybdoenzyme is related to acid resistance in log phase of acidic LB broth (pH2.5) (Masuda & Church 2003). As *ΔmoeAB* cannot synthesize Moco, *ΔmoeAB* may produce defective molybdoenzymes, leading to reduced resistance to acids. To know whether *ΔmoeAB* shows defective acid survival compared to WT, organic acids were treated to the cells in mid-log phase for 1 h.

Before acid resistance test, *ΔmoeAB* was grown in LB, M9, and M9 exposed to cabbage media to check the growth effects. Growth of *ΔmoeAB* was not significantly different compared to WT (data not shown).

In the experiment in LB, *ΔmoeAB* is more sensitive to all of four organic acids compared to WT (Masuda & Church 2003). Acid survival of complementation strain was restored WT level (Fig. 7.).

To test the organic acid survival of *ΔmoeAB* after 4 h of subculture in M9 with cabbage which is RNA-seq condition, organic acids were treated for 1 h. As a result, *ΔmoeAB* was more sensitive to lactic acid compared to WT. Other organic acid survivals were not significantly different between WT and *ΔmoeAB* at two different concentration (Fig. 8.).

To test the effect of cabbage to organic acid resistance, organic acid survival test of WT was performed in 1% organic acid for 1 h. As a result, only lactic acid showed acid resistance compared to M9 in ETEC FORC31 WT although lactic acid showed the most effective antimicrobial action among four organic acids. Other organic acid survival of FORC31 WT was not significantly different between M9 and cabbage media (Fig. 9.).

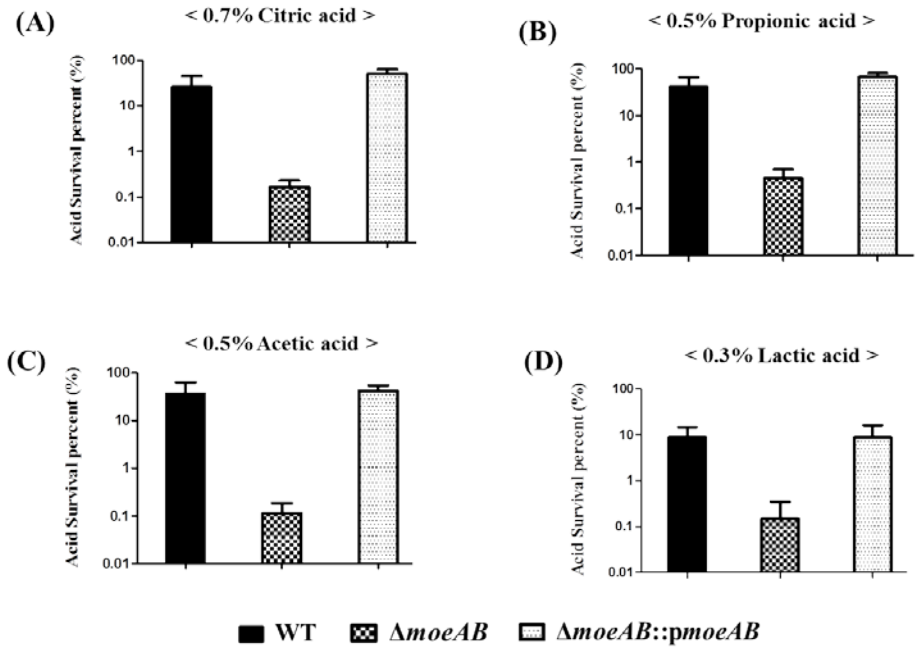

Figure 7. Organic acid survival of *ΔmoeAB* in LB. *ΔmoeAB* was more sensitive to four organic acids compared to WT in LB. Acid survival of complementation strain was restored WT level. Error bars indicate SD from three times experiments.

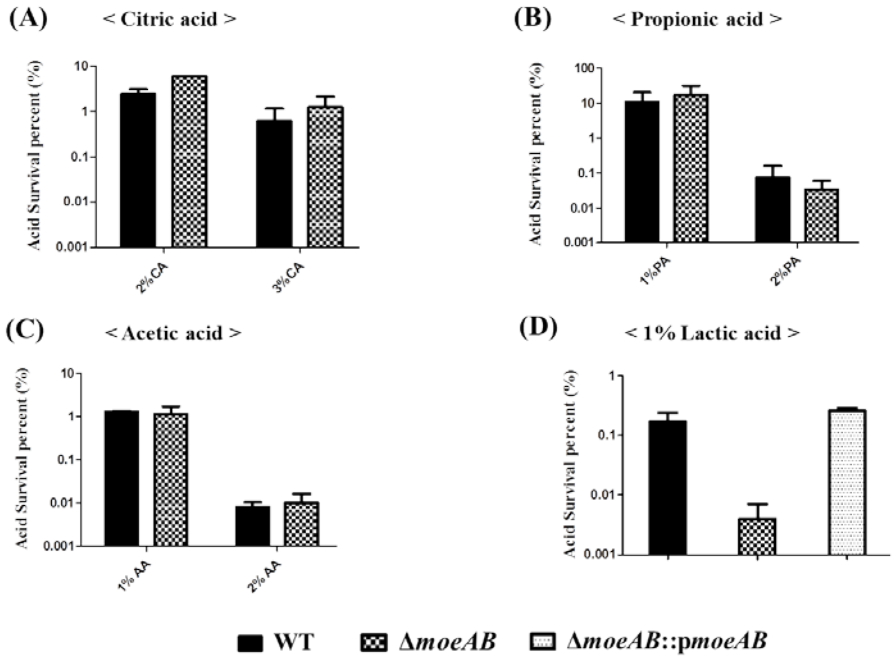

Figure 8. Organic acid survival of *ΔmoeAB* in M9 exposed to Cabbage. *ΔmoeAB* was more sensitive to lactic acid than WT. Error bars indicate SD from duplicate experiments. M9; M9 glucose media.

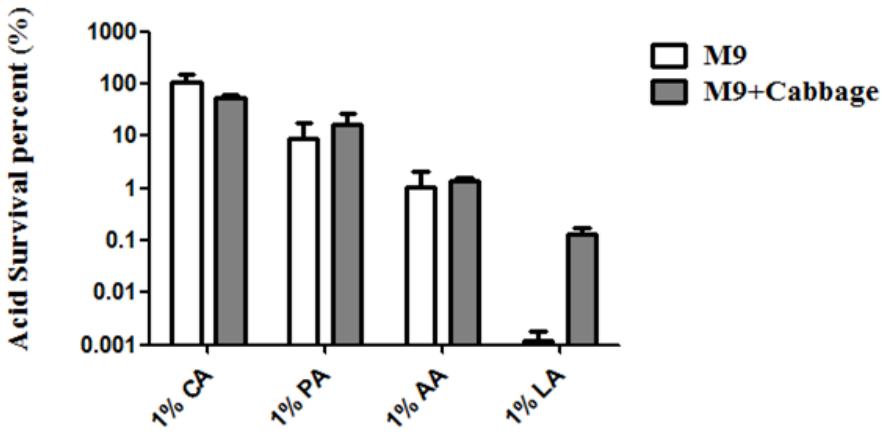

Figure 9. Effect of 1% organic acid survival on ETEC FORC31 WT between M9 and M9 exposed to Cabbage. Among four organic acids, ETEC FORC31 exposed to cabbage showed only lactic acid resistance compared to M9. Error bars indicate SD from duplicate experiment. M9; M9 glucose media, CA; Citric acid, PA; Propionic acid, AA; Acetic acid, LA; Lactic acid.

Ⅳ. DISCUSSION

There were many studies about the interactions between food borne pathogens and fresh produce such as lettuce or sprout (Brankatschk *et al.* 2014, Crozier *et al.* 2016, Fink *et al.* 2012, Gorski *et al.* 2009, Kyle *et al.* 2010). Using RNA sequencing technology, this study shows the whole transcriptome of ETEC FORC31 grown with cabbage. Before this study, there was no study about ETEC response to vegetables or gene expression of other foodborne pathogens exposed to cabbage. Therefore, it continuously needs to study bacterial transcriptional profile when exposed to plant to understand bacteria-plant interactions and survival strategy of bacteria in fresh produce.

The higher growth level of cabbage co-cultured cells indicates that ETEC FORC31 can utilize nutritional contents eluted from cabbage (Fig. 1.). Gene expression profile of ETEC FORC31 also supports this result. Many genes grown with cabbage were changed. Especially, the expression level of genes involved in transport or metabolism of extracellular nutrients (ex. Amino acid, carbohydrate, nucleic acid, inorganic ion) were significantly different when the cells were cultured with cabbage (Fig. 3., Table 5.). These genes are

ultimately needed to grow bacteria. It suggests that FORC31 may proliferate by using nutrient of fresh produce. Furthermore, the result indicates that FORC31 might grow well with cabbage during the process of distribution in environment.

In this study, ETEC FORC31 exposed to cabbage showed a more transcribed set of genes for arginine, leucine, and asparagine metabolism and fructose transporter. According to the database of United States Department of Agriculture (ndb.nal.usda.gov/ndb/search/list), the cabbage contains 1.3% of protein and 5.8% of carbohydrate and bacteria can utilize it as a carbon and amino acid source. In nucleic acid metabolism, uracil degradation and UMP biosynthesis genes were up-regulated. It supports that nucleic acids from cabbage can be also utilized as nutrient. Especially, UMP biosynthesis modulates curli/cellulose production which expressed by *csgDEFG* (Garavaglia *et al.* 2012). It suggests that up-regulation of the *pyrBI* operon play a role for curli/cellulose production in cabbage.

Genes involved in curli, fimbriae, flagella machinery, and chemotaxis are related to colonization in plant (Brankatschk *et al.* 2014, Crozier *et al.* 2016, Deering *et al.* 2012, Kyle *et al.* 2010). In this study,

most of genes encoding fimbrial proteins were down-regulated patterns while flagella machinery and chemotaxis genes showed up-regulated patterns after 4 h incubation with cabbage (Table. 5.). Because the bacterial RNA was isolated from planktonic cells, the nutrient from cabbage is leached in media via the agitation so that bacteria might not need to adhere to cabbage to get abundant nutrient. Activation of motility means getting out of biofilm, which leads to find new habitats for colonization (Anwar *et al.* 2014). Thus, these up-regulated motility genes contribute to colonization of cabbage.

Considering genes related to sulfate/nitrate transporter and reduction is up-regulated, ETEC FORC31 might get electrons from the cabbage by assimilatory sulfate and nitrate reduction (Crane *et al.* 1995, Liu *et al.* 1994, Lundberg *et al.* 2004). Genes involved in starvation sensing were found to be less transcribed in the presence of cabbage. Because M9 medium contains limited nutrients, starvation sensing might be active in M9 than M9 plus cabbage media (Huisman & Kolter 1994). Meanwhile, bacterial signal molecule from cabbage affected gene expression pattern. It is known that *narL*, which is nitrate and nitrite sensing regulator, affects expression of other genes. According to

BioCyc (biocyc.org), *narL* repress hydrogenase genes, but induce Moco biosynthesis (*moeAB*), nitrate/nitrite transporter, nitrate reductase. In addition, it is known that expression of the *rutABCDEFG* operon is positively regulated by nitrogen regulatory protein C (*ntrC*) which is up-regulated when cells were exposed to cabbage (Zimmer *et al.* 2000). Furthermore, inorganic ion might be affect gene expression. It is known that low Mg^{2+} stimulates expression of genes involved in polymyxin resistance (Yu *et al.* 2015). Since cabbage contains high magnesium, it may be affects down-regulated polymyxin resistance genes.

Molybdenum is an essential trace element for various biological function but it is toxic at high concentrations. Therefore, genes related to molybdate transporter must be tightly regulated (Iobbi-Nivol & Leimkühler 2013). Molybdate ABC transporter can be eliminate transport activity at higher concentrations (> 1 mM) (Kaiser *et al.* 2005). It is known that molybdate transport is regulated by *modE* (Molybdate-responsive transcription factor) protein in *E. coli*. *modE* negatively regulates tansporter and activate Moco synthesis genes by binding molybdate. In RNA-Seq analysis in this study, Moco synthetic genes were up-regulated and high affinity molybdate transporter was

down-regulated (Fig. 5.).

In *E. coli modABC* mutants, sulfate transporters can carry out molybdate at a lower affinity (K_M 100 uM). In double mutants lacking both the *modABC* and sulfate transport, low affinity selenite-sensitive anion transporters can transport molybdate but the K_M for this transporter is unknown (Kaiser *et al.* 2005). Genes related to second molydate transport system (*sbp*/*cysTWA*) was up-regulated (Table 5.). These gene expressions imply that molybdate eluted cabbage is rich and transported a lot into the cells, so bacteria do not have to get molybdate by energy-consuming high-affinity transporter.

Many researchers reported that molybdoenzymes which contains Moco are related to virulence phenotype such as motility and biofilm formation, acid resistance (Andreae *et al.* 2014, Masuda & Church 2003, Périnet *et al.* 2016). In this work, ETEC FORC31 *ΔmoeAB*, disrupted in Moco synthesis, had reduced motility compared to WT when nitrate was supplemented (Fig. 6.). In previous researches, NO supplementation increased motility in *E.coli* (Partridge *et al.* 2009). In *p. aeruginosa*, when nitrate was added, swarming motility of *narGH* mutant reduced compared to WT because of the reduced formation of

NO, a signaling molecule for rhamnolipid production (Van Alst *et al.* 2007). NO is intermediate during nitrate reducing process. Because *ΔmoeAB* produces defective molybdoenzymes including nitrate reductase, *ΔmoeAB* could not reduce nitrate to nitrite which is the first step of nitrate reduction and not ultimately produce NO in nitrate supplementation. As a result, WT showed increased motility with nitrate condition, while the motility of *ΔmoeAB* was not increased (Fig. 6.). In addition, red zone of WT on Congo red agar disappeared by adding nitrate but *ΔmoeAB* showed increased curli production compared to WT with or without nitrate (Fig. 6.). Previous study revealed that curli production represses motility (Anwar *et al.* 2014). It suggests that increased motility phenotype is related to disappeared curli production. However, because other molybdoenzymes are also related to motility, reduced motility of *ΔmoeAB* may not be due to the nitrate reductase activity alone, and further study is required (Andreae *et al.* 2014).

A number of plants contain high level of nitrate derived from fertilizer (Sušin *et al.* 2006, Zhong *et al.* 2002). Genes involved in nitrate sensing and nitrate reductases were up-regulated in cabbage

exposure (Table 5.). It suggests that the bacteria expresses nitrate reductases to utilize nitrate of cabbage. The result that motility of ETEC FORC31 was increased by adding nitrate implies that when exposed to cabbage, motility genes are up-regulated by nitrate in it (Fig. 6., Table 5.).

In LB condition, *ΔmoeAB*, which produce defective molybdoenzymes, showed sensitive to all of four organic acids (Fig. 7.). It means that Moco is important for bacterial organic acid survival in nutrient-rich condition. In previous study, *ydeP*, belonging to molybdoenzyme, showed acid resistance and is a putative oxidoreductase which is homologous to the alpha subunit of *E.coli* formate dehydrogenase H (Masuda & Church 2003). Formate dehydrogenase catalyzes formate to carbon dioxide and hydrogen which are less acidic than formate and three formate dehydrogenases belong to molybdoenzyme in *E.coli* (Iobbi-Nivol & Leimkühler 2013). Therefore, it is possible that decreased acid survival of *ΔmoeAB* may not be caused only by *ydeP* activity.

In M9 media supplemented with cabbage, *ΔmoeAB* is sensitive to lactic acid and resistant to other three organic acids. Acid survial of

complemented strain (*ΔmoeAB*::p*moeAB)* was restored to WT levels. It can be explained that Moco is important for bacterial lactic acid survival in M9 supplemented with cabbage (Fig. 8.). In addition, at the same concentration, only lactic acid showed acid resistance compared to M9 in ETEC FORC31 WT although lactic acid showed the most effective antimicrobial action among four organic acids (Fig. 9.). Considering genes involved in Moco biosynthesis were up-regulated in cabbage-contacted condition (Fig. 5.), this up-regulated Moco biosynthesis genes might affect lactic acid resistance in cells exposed to cabbage. Considering the importance of molybdenum for acid susceptibility of bacteria in cabbage, disrupting Moco synthesis might prevent fresh produce from bacterial contamination. However, until now, there is no non-toxic chelator of molybdenum (Neumann & Leimkuhler 2008). Therefore, to reduce ETEC contamination in cabbage, organic acid treatment should be carefully conducted because up-regulated Moco biosynthesis genes may lower the acid effect on biocontrol.

V. CONCLUSION

In conclusion, the complete transcriptome of ETEC FORC31 exposed to cabbage gave insight into the overall interaction between cabbage and ETEC. In RNA-Seq results, genes related to nutrient transport and metabolism, which are needed for survival of food-borne pathogens, especially on fresh produce, showed significantly different expression level when exposed to cabbage. In particular, molybdenum metabolism which is an important for motility, curli production, and acid survival was significantly changed during the cabbage exposure. These changes may significantly affect the survival of ETEC in cabbage, especially acid susceptibility. Therefore, organic acid treatment should be carefully conducted to reduce ETEC in cabbage because the bacteria may show high acid resistance activated by Moco biosynthesis genes.

VI. REFERENCES

Aguilar-Barajas E, Díaz-Pérez C, Ramírez-Díaz MI, Riveros-Rosas H, Cervantes C. 2011. Bacterial transport of sulfate, molybdate, and related oxyanions. *Biometals* 24: 687-707

Andreae CA, Titball RW, Butler CS. 2014. Influence of the molybdenum cofactor biosynthesis on anaerobic respiration, biofilm formation and motility in *Burkholderia thailandensis. Research in microbiology* 165: 41-49

Anwar N, Rouf SF, Römling U, Rhen M. 2014. Modulation of biofilm-formation in *Salmonella enterica* serovar Typhimurium by the periplasmic DsbA/DsbB oxidoreductase system requires the GGDEF-EAL domain protein STM3615. *PLoS One* 9: e106095

Bae D, Seo KS, Zhang T, Wang CL. 2013. Characterization of a Potential *Listeria monocytogenes* Virulence Factor Associated with Attachment to Fresh Produce. *Applied and Environmental Microbiology* 79: 6855-61

Bak G, Lee J, Suk S, Kim D, Lee JY, *et al*. 2015. Identification of novel sRNAs involved in biofilm formation, motility, and fimbriae formation in *Escherichia coli*. *Scientific Reports* 5

Begum YA, Talukder KA, Azmi IJ, Shahnaij M, Sheikh A, *et al*. 2016. Resistance Pattern and Molecular Characterization of Enterotoxigenic *Escherichia coli* (ETEC) Strains Isolated in Bangladesh. *PLoS One* 11: 11

Blauwkamp TA, Ninfa AJ. 2002. Physiological role of the GlnK signal transduction protein of *Escherichia coli*: survival of nitrogen starvation. *Molecular Microbiology* 46: 203-14

Brankatschk K, Kamber T, Pothier JF, Duffy B, Smits TH. 2014. Transcriptional profile of *Salmonella enterica* subsp. *enterica* serovar Weltevreden during alfalfa sprout colonization. *Microbial biotechnology* 7: 528-44

Carey CM, Kostrzynska M, Thompson S. 2009. *Escherichia* coli O157: H7 stress and virulence gene expression on Romaine lettuce using comparative real-time PCR. *Journal of microbiological methods* 77: 235-42

Chevance FF, Hughes KT. 2008. Coordinating assembly of a bacterial macromolecular machine. *Nature Reviews Microbiology* 6: 455-65

Cooley MB, Miller WG, Mandrell RE. 2003. Colonization of Arabidopsis thaliana with *Salmonella enterica* and Enterohemorrhagic *Escherichia coli* O157 : H7 and competition by *Enterobacter asburiae*. *Applied and Environmental Microbiology* 69: 4915-26

Crane BR, Siegel LM, Getzoff ED. 1995. Sulfite reductase structure at 1.6 Angstrom: evolution and catalysis for reduction of inorganic anions. *Science* 270: 59

Crozier L, Hedley PE, Morris J, Wagstaff C, Andrews SC, *et al*. 2016. Whole-Transcriptome Analysis of Verocytotoxigenic *Escherichia coli* O157: H7 (Sakai) Suggests Plant-Species-Specific Metabolic Responses on Exposure to Spinach and Lettuce Extracts. *Frontiers in Microbiology* 7

Datsenko KA, Wanner BL. 2000. One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR

products. *Proceedings of the National Academy of Sciences* 97: 6640-45

Deering AJ, Mauer LJ, Pruitt RE. 2012. Internalization of *E. coli* O157: H7 and *Salmonella* spp. in plants: a review. *Food Research International* 45: 567-75

Dhouib R, Othman DSP, Essilfie A-T, Hansbro PM, Hanson JO, *et al*. 2015. Maturation of molybdoenzymes and its influence on the pathogenesis of non-typeable *Haemophilus influenzae. Frontiers in microbiology* 6

Dillies MA, Rau A, Aubert J, Hennequet-Antier C, Jeanmougin M, *et al*. 2013. A comprehensive evaluation of normalization methods for Illumina high-throughput RNA sequencing data analysis. *Briefings in Bioinformatics* 14: 671-83

Donahue JP, Turnbough CL. 1994. Nucleotide-specific transcriptional pausing in the *pyrBI* leader region of *Escherichia coli* K-12. *Journal of Biological Chemistry* 269: 18185-91

Dubini A, Pye RL, Jack RL, Palmer T, Sargent F. 2002. How bacteria get energy from hydrogen: a genetic analysis of periplasmic hydrogen oxidation in *Escherichia coli*. *International Journal of Hydrogen Energy* 27: 1413-20

Ethelberg S, Lisby M, Bottiger B, Schultz AC, Villif A, *et al*. 2010. Outbreaks of gastroenteritis linked to lettuce, Denmark, January 2010. *Euro Surveill* 15

Felton J, Michaelis S, Wright A. 1980. Mutations in two unlinked genes are required to produce asparagine auxotrophy in *Escherichia coli*. *Journal of bacteriology* 142: 221-28

Feng PCH, Reddy SP. 2014. Prevalence and Diversity of Enterotoxigenic *Escherichia coli* Strains in Fresh Produce. *J. Food Prot.* 77: 820-23

Fink RC, Black EP, Hou Z, Sugawara M, Sadowsky MJ, Diez-Gonzalez F. 2012. Transcriptional responses of *Escherichia coli* K-12 and O157: H7 associated with lettuce leaves. *Applied and environmental microbiology* 78: 1752-64

Garavaglia M, Rossi E, Landini P. 2012. The pyrimidine nucleotide biosynthetic pathway modulates production of biofilm determinants in *Escherichia coli*. *PLoS One* 7: e31252

Ghigo JM, Beloin C. 2011. The sweet connection: Solving the riddle of multiple sugar-binding fimbrial adhesins in *Escherichia coli*. *Bioessays* 33: 300-11

Gilson E, Rousset J-P, Charbit A, Perrin D, Hofnung M. 1986. *malM*, a new gene of the maltose regulon in *Escherichia coli* K12: I. *malM* is the last gene of the *malK-lamB* operon and encodes a periplasmic protein. *Journal of molecular biology* 191: 303-11

Gomez-Aldapa CA, Rangel-Vargas E, Bautista-De Leon H, Vazquez-Barrios ME, Gordillo-Martinez AJ, Castro-Rosas J. 2013. Behavior of enteroaggregative *Escherichia coli*, non-O157-shiga toxin-producing *E. coli*, enteroinvasive *E. coli*, enteropathogenic *E. coli* and enterotoxigenic *E. coli* strains on mung bean seeds and sprout. *International Journal of Food Microbiology* 166: 364-68

Gorski L, Duhe JM, Flaherty D. 2009. The Use of Flagella and Motility for Plant Colonization and Fitness by Different Strains of the

Foodborne Pathogen *Listeria monocytogenes*. *PLoS One* 4: e5142

Goudeau DM, Parker CT, Zhou YG, Sela S, Kroupitski Y, Brandl MT. 2013. The *Salmonella* Transcriptome in Lettuce and Cilantro Soft Rot Reveals a Niche Overlap with the Animal Host Intestine. *Applied and Environmental Microbiology* 79: 250-62

Higashi K, Ishigure H, Demizu R, Uemura T, Nishino K, *et al*. 2008. Identification of a spermidine excretion protein complex (MdtJI) in *Escherichia coli*. *Journal of bacteriology* 190: 872-78

Huisman GW, Kolter R. 1994. Sensing starvation: a homoserine lactone-dependent signaling pathway in *Escherichia coli*. *Science* 265: 537-39

Humbert R, Simoni R. 1980. Genetic and biomedical studies demonstrating a second gene coding for asparagine synthetase in *Escherichia coli*. *Journal of bacteriology* 142: 212-20

Inagaki S, Henry IM, Lieberman MC, Comai L. 2015. High-throughput analysis of T-DNA location and structure using sequence capture. *PLoS One* 10: e0139672

Iobbi-Nivol C, Leimkühler S. 2013. Molybdenum enzymes, their maturation and molybdenum cofactor biosynthesis in *Escherichia coli*. *Biochimica et Biophysica Acta (BBA)-Bioenergetics* 1827: 1086-101

Jablasone J, Warriner K, Griffiths M. 2005. Interactions of *Escherichia coli* O157 : H7, *Salmonella typhimurium* and *Listeria monocytogenes* plants cultivated in a gnotobiotic system. *International Journal of Food Microbiology* 99: 7-18

Jandu N, Ho NK, Donato KA, Karmali MA, Mascarenhas M, *et al*. 2009. Enterohemorrhagic *Escherichia coli* O157: H7 Gene Expression Profiling in Response to Growth in the Presence of Host Epithelia. *PLoS One* 4: e4889

Kaiser BN, Gridley KL, Brady JN, Phillips T, Tyerman SD. 2005. The role of molybdenum in agricultural plant production. *Annals of Botany* 96: 745-54

Kimbrel JA, Di Y, Cumbie JS, Chang JH. 2011. RNA-Seq for Plant Pathogenic Bacteria. *Genes* 2: 689-705

Kornberg HL. 2001. Routes for fructose utilization by *Escherichia coli*. *Journal of Molecular Microbiology and Biotechnology* 3: 355-59

Kyle JL, Parker CT, Goudeau D, Brandl MT. 2010. Transcriptome Analysis of *Escherichia coli* O157:H7 Exposed to Lysates of Lettuce Leaves. *Applied and Environmental Microbiology* 76: 1375-87

Liu C, Martin E, Leyh TS. 1994. GTPase activation of ATP sulfurylase: the mechanism. *Biochemistry* 33: 2042-47

Lundberg JO, Weitzberg E, Cole JA, Benjamin N. 2004. Nitrate, bacteria and human health. *Nature Reviews Microbiology* 2: 593-602

Magasanik B. 1989. Regulation of transcription of the *glnALG* operon of *Escherichia coli* by protein phosphorylation. *Biochimie* 71: 1005-12

Masuda N, Church GM. 2003. Regulatory network of acid resistance genes in *Escherichia coli*. *Molecular microbiology* 48: 699-712

Maza E. 2016. In Papyro Comparison of TMM (edgeR), RLE (DESeq2), and MRN Normalization Methods for a Simple Two-Conditions-Without-Replicates RNA-Seq Experimental Design. *Frontiers in Genetics* 7

Minnesota Department of Health. 2015. Annual Summary of Communicable Diseases Reported to the Minnesota Department of Health, 2014. *Disease Control Newsletter* 42: 1-32

Murphy KC, Campellone KG. 2003. Lambda Red-mediated recombinogenic engineering of enterohemorrhagic and enteropathogenic *E. coli*. *BMC molecular biology* 4: 1

Neumann M, Leimkuhler S. 2008. Heavy metal ions inhibit molybdoenzyme activity by binding to the dithiolene moiety of molybdopterin in *Escherichia coli*. *Febs Journal* 275: 5678-89

Nie ZJ, Hu CX, Sun XC, Tan QL, Liu HE. 2007. Effects of molybdenum on ascorbate-glutathione cycle metabolism in Chinese cabbage (*Brassica campestris* L. ssp *pekinensis*). *Plant and Soil* 295: 13-21

Nousiainen L-L, Joutsen S, Lunden J, Hänninen M-L, Fredriksson-Ahomaa M. 2016. Bacterial quality and safety of packaged fresh leafy vegetables at the retail level in Finland. *International journal of food microbiology* 232: 73-79

Oh K-H, Chung GT, Yoo C-K. 2015. Waterborne and Foodborne Disease by Pathogenic *Escherichia coli* in Korea, 2014. *Public Health Weekly Report, KCDC* 8: 1250-54

Périnet S, Jeukens J, Kukavica-Ibrulj I, Ouellet MM, Charette SJ, Levesque RC. 2016. Molybdate transporter *ModABC* is

important for *Pseudomonas aeruginosa* chronic lung infection. *BMC research notes* 9: 1

Panagiotidis C, Reyes M, Sievertsen A, Boos W, Shuman H. 1993. Characterization of the structural requirements for assembly and nucleotide binding of an ATP-binding cassette transporter. The maltose transport system of *Escherichia coli*. *Journal of Biological Chemistry* 268: 23685-96

Parales RE, Ingraham JL. 2010. The Surprising Rut Pathway: an Unexpected Way To Derive Nitrogen from Pyrimidines. *Journal of Bacteriology* 192: 4086-88

Park SH, Choi MR, Park JW, Park KH, Chung MS, *et al*. 2011. Use of organic acids to inactivate *Escherichia coli* O157: H7, *Salmonella Typhimurium,* and *Listeria monocytogenes* on organic fresh apples and lettuce. *Journal of Food Science* 76: M293-M98

Partridge JD, Bodenmiller DM, Humphrys MS, Spiro S. 2009. NsrR targets in the *Escherichia coli* genome: new insights into DNA sequence requirements for binding and a role for NsrR in the regulation of motility. *Molecular Microbiology* 73: 680-94

Pollack SL. 2001. Consumer demand for fruit and vegetables: the US example. *Changing structure of global food consumption and trade* 6: 49-54

Porter SL, Wadhams GH, Armitage JP. 2011. Signal processing in complex chemotaxis pathways. *Nature Reviews Microbiology* 9: 153-65

Reinders A, Hee C-S, Ozaki S, Mazur A, Boehm A, *et al*. 2016.

Expression and Genetic Activation of Cyclic di-GMP-Specific Phosphodiesterases in *Escherichia coli*. *Journal of bacteriology* 198: 448-62

Rivera-Chávez F, Lopez CA, Zhang LF, García-Pastor L, Chávez-Arroyo A, *et al*. 2016. Energy Taxis toward Host-Derived Nitrate Supports a *Salmonella* Pathogenicity Island 1-Independent Mechanism of Invasion. *mBio* 7: e00960-16

Schenk PM, Carvalhais LC, Kazan K. 2012. Unraveling plant–microbe interactions: can multi-species transcriptomics help? *Trends in biotechnology* 30: 177-84

Schikora A, Carreri A, Charpentier E, Hirt H. 2008. The Dark Side of the Salad: *Salmonella typhimurium* Overcomes the Innate Immune Response of *Arabidopsis thaliana* and Shows an Endopathogenic Lifestyle. *PLoS One* 3

Schneider BL, Kiupakis AK, Reitzer LJ. 1998. Arginine catabolism and the arginine succinyltransferase pathway in *Escherichia coli*. *Journal of bacteriology* 180: 4278-86

Schroeder A, Mueller O, Stocker S, Salowsky R, Leiber M, *et al*. 2006. The RIN: an RNA integrity number for assigning integrity values to RNA measurements. *BMC molecular biology* 7: 1

Segel IH, Renosto F, Seubert PA. 1987. Sulfate-activating enzymes. *Methods in enzymology* 143: 334-49

Shin J, Yoon K-B, Jeon D-Y, Oh S-S, Oh K-H, *et al*. 2016. Consecutive Outbreaks of Enterotoxigenic *Escherichia coli* O6 in Schools in South Korea Caused by Contamination of Fermented Vegetable Kimchi. *Foodborne Pathogens and Disease* 13: 535-43

Somers J, Amzallag A, Middleton R. 1973. Genetic fine structure of the leucine operon of *Escherichia coli* K-12. *Journal of bacteriology* 113: 1268-72

Sušin J, Kmecl V, Gregorčič A. 2006. A survey of nitrate and nitrite content of fruit and vegetables grown in Slovenia during 1996–2002. *Food additives and contaminants* 23: 385-90

Van Alst NE, Picardo KF, Iglewski BH, Haidaris CG. 2007. Nitrate sensing and metabolism modulate motility, biofilm formation, and virulence in *Pseudomonas aeruginosa*. *Infection and immunity* 75: 3780-90

Vignais PM, Colbeau A. 2004. Molecular biology of microbial hydrogenases. *Current Issues in Molecular Biology* 6: 159-88

Wang Y-F, Dutzler R, Rizkallah PJ, Rosenbusch JP, Schirmer T. 1997. Channel specificity: structural basis for sugar discrimination and differential flux rates in maltoporin. *Journal of molecular biology* 272: 56-63

Weir RG. 2004. Molybdenum deficiency in plants. *NSW department of primary industries* 2: 1-4

Yaron S, Römling U. 2014. Biofilm formation by enteric pathogens and its role in plant colonization and persistence. *Microbial biotechnology* 7: 496-516

Yu Z, Qin W, Lin J, Fang S, Qiu J. 2015. Antibacterial mechanisms of polymyxin and bacterial resistance. *BioMed research international* 2015

Zhong W, Hu C, Wang M. 2002. Nitrate and nitrite in vegetables from north China: content and intake. *Food Additives &*

Contaminants 19: 1125-29

Zimmer DP, Soupene E, Lee HL, Wendisch VF, Khodursky AB, *et al*. 2000. Nitrogen regulatory protein C-controlled genes of *Escherichia coli*: Scavenging as a defense against nitrogen limitation. *Proceedings of the National Academy of Sciences of the United States of America* 97: 14674-79

국문 초록

장독소형대장균 (Enterotoxigenic *Escherichia coli*, ETEC)은 주요 식중독 유발균으로 주된 감염 경로는 ETEC 에 의해 오염된 음식과 물이며, 장관독소 (Enterotoxin) 를 생산하는데 열민감성 독소 (LT) 와 열 저항성 독소 (ST) 중 하나 또는 두 가지 모두를 인간과 동물의 소장에서 생산하여 복통을 수반한 장염을 일으킨다. 하지만 식물성 식품 내 ETEC 감염 기작이 구체적으로 밝혀지지 않아 이에 관한 연구가 필요하다. 따라서 본 연구는 인체 분변에서 분리된 ETEC FORC31 이 날것으로 자주 섭취되는 양배추에 접촉 시 전사체 분석을 통하여 식물성 식품 내 ETEC 의 적응 기작을 규명하여 식물성 식품 속 ETEC 저감화 방안을 모색하였다. 양배추로부터 나오는 잔여 균의 영향이 나타나지 않으며, 양배추에 접촉한 균주와 접촉하지 않은 균주의 생장곡선에서 모두 대수기에 있는 4시간을 RNA 추출시간으로 선정하여 박테리아의 RNA-Sequencing 을 하였다. 양배추에 접촉한 균주와 접촉하지 않은 균주의 전사체를 비교한 결과, 식품 접촉시 운동성,

에너지 생성, 아미노산 수송과 대사와 연관된 유전자들의 발현양이 유의적으로 차이를 나타냈다. 특히, 양배추를 비롯한 십자화과에 많이 존재한다고 알려진 몰리브덴 대사에 연관된 유전자들이 특이적으로 발현양의 차이를 보였다. 양배추 접촉 시 몰리브덴 특이적인 수송 유전자는 발현양이 감소하였고, 몰리브덴을 이용해 여러 산화환원 반응과 연관된 몰리브도 효소의 합성에 관여하는 몰리브덴 보조인자 (Moco) 합성 유전자는 양배추 접촉 시 발현양이 증가하였다. 전사체 결과를 바탕으로 몰리브덴 기작과 연관된 유해인자 가능성을 탐색하기 위해 Moco 합성에 관여하는 오페론인 moeAB 유전자가 결여된 균주와 보완 균주를 구축하여 병원성 표현형 실험을 진행하였다. moeAB 유전자가 결여된 돌연변이 균주는 질산염을 첨가한 조건에서 야생형에 비해 운동성의 감소와 Curli의 증가를 확인하였다. 반면, 보완균주는 야생형과 유사한 표현형을 보였다. 또한 유기산 처리 후 균의 생존율이 돌연변이 균주에서 야생형에 비해 유의적으로 감소함을 확인하여, 양배추에 있는 몰리브덴을 이용하여 산에서

생존능력을 향상 시킬 수 있음을 확인하였다. 이는 나아가 양배추에 접촉한 야생형 ETEC FORC31 에서 유기산 처리할 경우 증가된 Moco 합성에 의해 산 저항성을 나타낼 수 있음을 시사한다. 종합하면, 양배추 내 ETEC 전사체 발현 변화 연구를 통해 식물성 식품 내 ETEC 의 적응 기작에 대한 이해를 높였으며, 이 중 몰리브덴 대사의 유해인자 가능성을 확인할 수 있었다.

주요어 : 장독소형 대장균, 양배추, 전사체, 몰리브덴, 몰리브덴 보조인자, 몰리브도 효소.

학번 : 2015-21793