Membrane Lipid Homeostasis and Stress Resistance in *Escherichia coli* and *Lactobacillus plantarum*

by

Yuanyao Chen

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Department of Agricultural, Food and Nutritional Science
University of Alberta

Abstract

Food processing, preservation and storage impose stresses on food bacteria. The maintenance of membrane lipid homeostasis through modification of phospholipids is a key strategy for bacteria to adapt environmental changes and survival in food. Mechanisms of adaptation are generally comparable between probiotics and pathogens. This thesis research aimed to investigate the effect of the conversion of unsaturated fatty acids to hydroxy fatty acids or cyclopropane fatty acids (CFAs) on stress resistance in *Lactobacillus plantarum* and *Escherichia coli*. Firstly, *L. plantarum* TMW1.460 converts linoleic acids to 10-hydroxy-12-octadecenoic acid (10-HOE) and 13-hydroxy-9-octadecenoic acid (13-HOE) by linoleate 10-hydratase (LahA) and linoleate 13-hydratase (LahB). The role of 10-HOE in ethanol tolerance was studied through the disruption of *lahA* that encodes linoleate 10-hydratase. The results revealed that 10-HOE did not contribute to the ethanol tolerance, but LahA affected the cell surface hydrophobicity. Moreover, deletion of LahA in *L. plantarum* facilitated the purification of 13-HOE and the demonstration of its antifungal activity against *Penicillium roqueforti* and *Aspergillus niger.* Secondly, both heat and pressure resistant strain *E. coli* AW1.7 and sensitive strain *E. coli* MG1655 utilized all of the unsaturated fatty acids ($C_{16:1}$ and $C_{18:1}$) in the membrane and most of them were converted to CFAs after entry into the stationary growth phase. Through comparison of survival between wild-type and *cfa* deficient strains in *E. coli* AW1.7 and MG1655, this thesis demonstrated that the CFA synthesis in *E. coli* increased the heat, pressure and acid resistance. Thirdly, the cyclopropanation of membrane fatty acids also protected *E. coli* AW1.7 and its dried cells against the inactivation conducted by supercritical CO_2. The lethality of supercritical CO_2 was low on dry cells of *E. coli*. Treatments with gaseous CO_2 were more bactericidal for dry cells than supercritical CO_2 at 65°C. Remarkably, *E. coli* AW1.7 was more susceptible than AW1.7

deficient in *cfa* when subjected to the gaseous CO_2 treatment. This study suggested that CO_2-induced membrane fluidization and permeabilization are the major causes of microbial inactivation conducted by high pressure carbon dioxide. This thesis research improved our understanding of the contribution of bacterial membrane lipid homeostasis to stress resistance, which facilitates the optimization of the preparation method to protect probiotics and the design of the intervention method to control pathogens in food production.

Preface

This thesis is an original work by Yuanyao Chen, which is written according to FGSR thesis format requirement.

Chapter 1 is a literature review and is in preparation for submission. My contribution included literature search, collecting the information related to bacterial fatty acid biosynthesis, regulation, stress resistance in food bacteria and writing the manuscript. Dr. Gänzle provided his comments and further revised it.

Chapter 2 has been published as Chen YY, Liang NY, Curtis JM and Gänzle MG (2016), "Characterization of linoleate 10-hydratase of *Lactobacillus plantarum* and novel antifungal metabolites", *Frontiers in Microbiology*, 7: 1561. I completed the majority of laboratory work independently; with technical assistance from Nuanyi Liang for the extraction, purification and antifungal activity assay of hydroxy fatty acids. The manuscript was written by me and then revised by Dr. Gänzle, Ms. Liang and Dr. Curtis.

Chapter 3 has been published as Chen YY and Gänzle MG (2016), "Influence of cyclopropane fatty acids on heat, high pressure, acid and oxidative resistance in *Escherichia coli*", *International Journal of Food Microbiology*, 222: 16-22. The study was designed by Dr. Gänzle and me. I conducted the lab work, analyzed the data, and wrote the manuscript together with Dr. Gänzle.

Chapter 4 has been submitted as Chen YY, Temelli F and Gänzle MG, "Inactivation of dry *Escherichia coli* by high pressure carbon dioxide". The experiments related to high pressure carbon dioxide treatments were performed under the supervision of Dr. Gänzle, and collaborated with Dr. Temelli. I accomplished all the experiments, collected and analyzed the data. I wrote the manuscript, and revised based on the comments from Drs. Gänzle and Temelli.

Dedication

This Ph.D. thesis is dedicated to my beloved family.

Acknowledgements

To start with, I would like to express my sincere appreciation to my supervisor Dr. Michael Gänzle. I am grateful to you for accepting me as your Ph.D. student. During my Ph.D. study, none of my progress and improvements could be achieved without your substantial help and guidance. Your suggestions on experimental design, laboratory work, and scientific writing benefited me a lot and built up a solid foundation for my future academic work. For me, there is nothing more honorable than being your student. Your generous help will be kept in my mind forever.

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earnest friends in my life. You always passed on your knowledge to me, assisted me on troubleshooting, and answered my questions when I felt confused. Moreover, please allow me to convey my honest thanks to my previous and current lab friends: Drs. Petya Koleva, Fernanda Sanchez Maldonado, Brenna Black, Januana Texiera, Simmon Hofstetter, Yang Liu, and Jing Zhao; Xiaoyan Chen, Yalu Yan, Yan Yang, Hui Li, Yuan Fang, Ziyi Hu, Xin Zhao, Nuanyi Liang, Weilan Wang, and Qixing Ou. All of you make our lab 2-50 feel like a family.

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10,13-HOA	10,13-dihydroxy octadecanoic acid
10-HOE	10-hydroxy-12-octadecenoic acid
13-HOE	13-hydroxy-9-octadecenoic acid
a_W	Water activity
BCFAs	Branched-chain fatty acids
CFAs	Cyclopropane fatty acids
DHA	Ocosahexaenoic acid (22:6*n*-3)
EPA	Eicosapentaenoic acid (20:5*n*-3)
FAS II	Type II fatty acid synthase
GAD	Glutamate decarboxylase
GP	Generalized polarization
HPCD	High pressure carbon dioxide
IPTG	Isopropyl-β-D-thiogalactopyranoside
LAB	Lactic acid bacteria
LAH	Linoleate hydratase
LahA	Linoleate 10-hydratase
LahB	Linoleate 13-hydratase
LAURDAN	6-dodecanoyl-2-dimethylaminonaphthalene

LB	Luria–Bertani
LHR	Locus of heat resistance
MATS	Microbial adhesion to solvents
MES	4-morpholineethanesulfonic acid
MIC	Minimum inhibitory concentration
OD	Optical density
PBS	Phosphate-buffered saline
PUFAs	Polyunsaturated fatty acids
ROIs	Reactive oxygen intermediates
SFAs	Saturated fatty acids
STEC	Shiga-toxin producing *E. coli*
UFAs	Unsaturated fatty acids

Chapter 1. Bacterial lipid homeostasis and stress resistance of bacteria in food

1.1. Introduction

The bacterial cytoplasmic membrane, as the primary boundary between the cell and the external environment, is the frontline for sensing environmental changes. It is composed of a fluid lipid bilayer framework and embedded proteins. The main constituents of membrane lipids are glycerophospholipids that contain two acyl chains (Denich et al., 2003). The different types of acyl chains within the phospholipids determine lipid conformation and packing, which are reflected in membrane fluidity. The membrane fluidity is characterized by gel-to-liquid crystalline states. Under favorable growth conditions, bacterial membrane bilayer in the liquid crystalline state is considered to be biologically active, which is important for membrane-associated functions, including membrane proteins (Denich et al., 2003). Meanwhile, changes in the external environment may induce the alteration of membrane lipid composition, influence membrane fluidity, and further affect the activity of embedded proteins (Beney and Gervais, 2001). During food processing, preservation, and storage, bacteria in food are subjected to external environmental changes resulting from fermented products during microbial metabolism (e. g, lactic acid or ethanol), intervention treatments (e. g. heating, cooling, high pressure) and exposure to oxygen.

The capability of food bacteria to quickly respond to environmental stress is essential for

their survival and activity. Bacterial survival depends on their ability to adjust fatty acid composition to maintain membrane biophysical properties in different environments, which is referred to as membrane lipid homeostasis (Zhang and Rock, 2008). In response to perturbations, bacteria alter membrane lipid composition by employing a *de novo* fatty acid biosynthetic pathways and post-modification of pre-existing phospholipids, which allows them to protect themselves against adverse conditions and abrupt environmental changes. Membrane lipid homeostasis is achieved by the conversion of (1) saturated to *cis*-UFAs, (2) straight-chain to branched-chain fatty acids, (3) *cis*- to *trans*-UFAs, and (4) *cis*-unsaturated to CFAs; and by the formation of (5) polyunsaturated fatty acids.

In this review, I will discuss the biosynthetic mechanisms of various membrane lipids, and their respective transcriptional regulation mechanisms induced by environmental changes. I will also discuss whether food bacteria are able to enhance their survival by adjusting membrane lipid composition upon exposure to the stresses relating to food processing, preservation and storage, such as high temperature, low temperature, pressure, acid, ethanol, solvents, and oxidation.

1.2. The biosynthesis of bacterial membrane lipids

Type II fatty acid synthases (FAS II) are employed by most of the bacteria for *de novo* fatty acid biosynthesis, which is a fundamental process in construction of membrane architecture. The synthesis of membrane lipids is performed by a set of highly conserved

soluble enzymes that are encoded by discrete genes and each protein is responsible for an individual step in the type II pathway (White et al., 2005). The biosynthesis of a fatty acid chain is initialized by acetyl-CoA carboxylase (ACC), which catalyzes the conversion of the substrate acetyl-CoA to the intermediate malonyl-CoA. The malonyl group is then transferred to the terminal sulphydryl of acyl carrier protein (ACP) to form malonyl-ACP via the catalytic action of malonyl-CoA : ACP transacylase (FabD). In the elongation process, malonyl-CoA is utilized as a chain extender to catalyze the elongation of the fatty acyl-ACP with C_2 units until a C_{16} or C_{18} fatty acyl chain is formed. The biosynthetic process is shown in Figure 1-1. The antibiotic cerulenin is known to inhibit the activity of FabF and thus stall the cycle of the *de novo* fatty acid biosynthetic pathway (Vance et al., 1972; Buttke and Ingram, 1978; Garwin et al., 1980), and is commonly used in the investigation of post-modification pathways. Straight-chain SFAs synthesized by the type II pathway are linear and tightly packed together, which results in a rigid membrane (Table 1-1). To maintain membrane homeostasis under different environmental conditions, bacteria are capable of controlling the synthesis of new membrane lipids and modifying the pre-existing phospholipids (Zhang and Rock, 2008) (Figure 1-1).

1.2.1. *Cis*-unsaturated fatty acids

Bacteria not only synthesize straight-chain SFAs, but also incorporate double bonds into

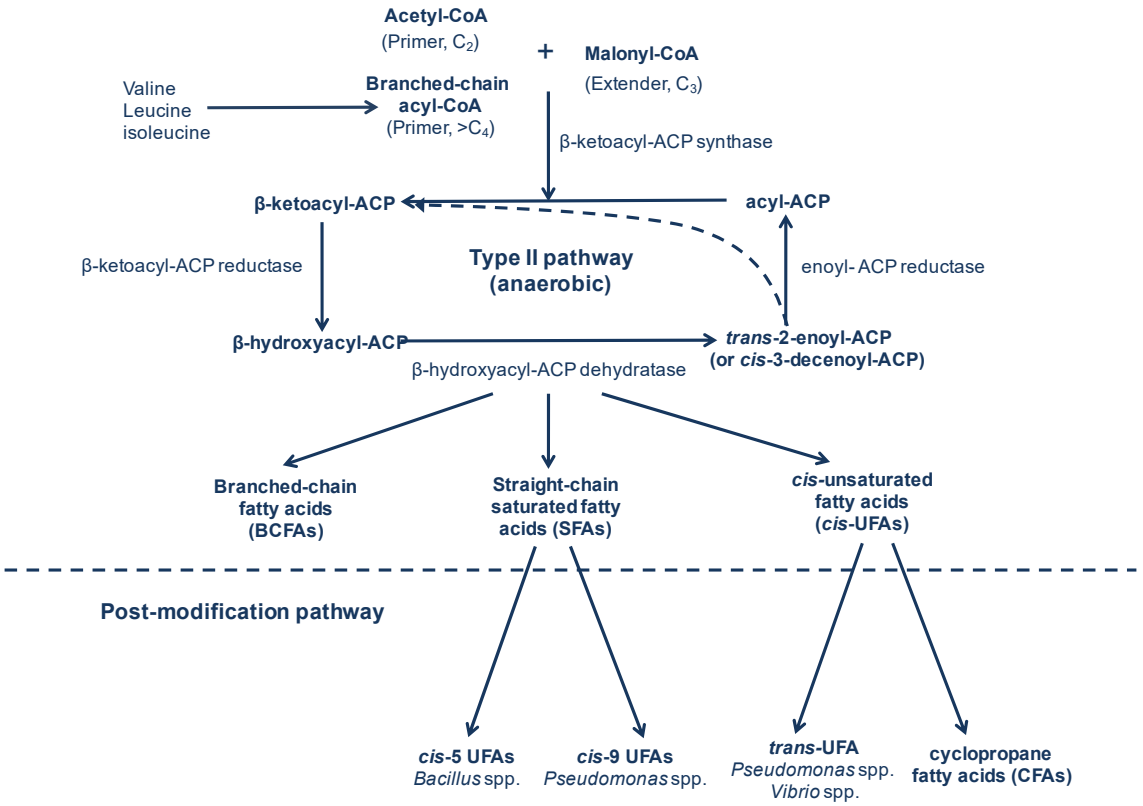
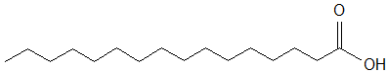
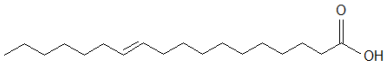
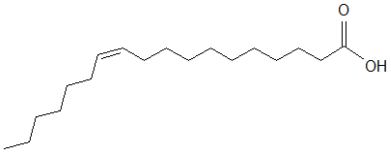
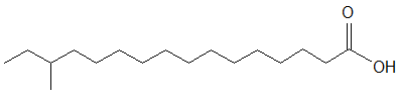
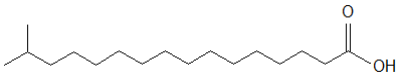
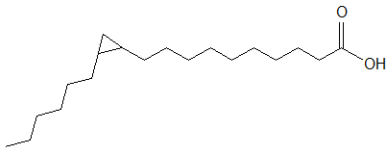


Figure 1-1. Summary of fatty acid biosynthetic pathways of bacterial membrane phospholipids. The biosynthesis of the straight-chain fatty acid chain is initialized by the condensation of acetyl-CoA and malonyl-ACP, which produces β-ketoacyl-ACP and CO_2. The first condensation is carried out by β-ketoacyl-ACP synthase III (FabH). NADPH-dependent β-ketoacyl-ACP reductase (FabG) produces β-hydroxyacyl-ACP, which is then dehydrated to *trans*-2-enoyl-ACP by FabA or FabZ (β-hydroxyacyl-ACP dehydratase). The first elongation is terminated by an NADPH-dependent enoyl-ACP reductase (FabI) or the other two isoforms (FabK and FabL) to form an acyl-ACP. Subsequently, 3-ketoacyl-ACP synthase (FabF or FabB) is used as a condensing enzyme, and the malonyl-CoA is used as a chain extender to elongate fatty acyl-ACP with a C_2 unit until a C_{16} or C_{18} fatty acid is formed. Bacteria, like *E. coli*, are capable of synthesizing *cis*-unsaturated fatty acids by introducing *cis*-double bonds into the growing acyl chain at the C_{10} position (*cis*-3-decenoyl-ACP) with enzyme FabA-FabB. When the growth medium is rich in valine, leucine, and isoleucine, branched-chain acyl-CoA is produced and used by FabH of some Gram-positive bacteria for biosynthesis of branched-chain fatty acids. Bacteria also modified pre-existing membrane fatty acids that are synthesized by the type II pathway. *Bacillus* and *Pseudomonas* spp. exclusively introduce a *cis* double bond to existing SFAs at the Δ5 and Δ9 position, respectively, by O_2-dependent desaturases. *Pseudomonas* and *Vibrio* spp. convert *cis*-UFAs to their *trans*-derivatives by *cis-trans* isomerase. The *cis*-UFAs are also converted to cyclopropane fatty acids by CFA synthases which are widely distributed in both Gram-positive and Gram-negative bacteria.

Table 1-1. The structure of membrane fatty acids and their effects on membrane fluidity

Fatty acid	Structure	Effect on membrane fluidity
C16:0		Decrease membrane fluidity
Trans-11-C18:1		Mimics saturated fatty acids
Cis-11-C18:1		Increase membrane fluidity
Anteiso-C17:0		Increase membrane fluidity compared with *iso*-chains
Iso-C17:0		Decrease membrane fluidity compared with *anteiso*-chains
Cyclopropane C19:0		Desrease membrane fluidity compared with unsaturated fatty acids

acyl chains to produce *cis*-UFAs. UFAs are formed by two major ways: most of the bacteria, including *Escherichia coli*, produce *cis*-UFAs ($C_{16:1\Delta9}$ and $C_{18:1\Delta11}$) anaerobically using soluble enzymes in the type II pathway; while *Bacillus* and *Pseudomonas* spp. insert the double bond into pre-existing SFAs via the catalytic action of O_2-dependent, membrane-bound desaturases (Mansilla et al., 2008). Compared with SFAs, the presence of UFAs with a *cis*-double bond causes the chain to become kinked and prevents them from packing closely together, resulting in a more fluid membrane (Hąc-Wydro and Wydro, 2007) (Table 1-1). In response to environmental changes, bacteria adjust the ratio

of SFAs / UFAs to modulate membrane fluidity.

E. coli introduce a *cis*-double bond into the growing acyl chain with the FabA-FabB catalytic system (Cronan Jr et al., 1969). The formation of a *cis*-double bond at the C_{10} position relies on FabA-mediated dehydration to produce *trans*-2-decenoyl-ACP followed by isomerization to generate *cis*-3-decenoyl-ACP (Kass and Bloch, 1967). FabB, a unique condensing enzyme, catalyzes the elongation of *cis*-3-decenoyl-ACP, while FabF is responsible for the elongation of *trans*-2-decenoyl-ACP (Rosenfeld et al., 1973; Feng and Cronan, 2009). Strains deficient in *fabB* are *E. coli* UFA auxotrophs, indicating that the FabB-mediated catalytic reaction is an essential step for *cis*-UFA biosynthesis (Feng and Cronan, 2009). Furthermore, the ratio of FabA and FabB in *E. coli* determines the metabolic flux between UFAs and SFAs (Xiao et al., 2013).

Gram-positive *Streptococcus pneumonia* do not possess the FabA homolog, but are still capable of synthesizing UFAs through FabM-mediated isomerization of *trans*-2-decenoyl-ACP to *cis*-3-decenoyl-ACP (Marrakchi et al., 2002). FabM is demonstrated to be the only enzyme that affects membrane fluidity in streptococci (Altabe et al., 2007). There are two FabZ isozymes in *Enterococcus faecalis*, one termed *Ef*FabN could catalyze dehydration/isomerization to replace the function of *E. coli* FabA, and the other termed *Ef*FabZ could only catalyze dehydration reaction like that of *E. coli* FabZ (Lu et al., 2005; Wang and Cronan, 2004). *Lactococcus lactis* contains two genes *fabZ1* and *fabZ2* which are identical to *EfFabN* and *EfFabZ* (75% and 69%, respectively)

in *E. faecalis* (Eckhardt et al., 2013).

Cis-UFAs are also synthesized by the catalytic action of O_2-dependent desaturases that are differentiated by their substrate specificity and preferred position of introducing double bonds. Δ5-Desaturase (Δ5-Des) in *Bacillus subtilis* exclusively catalyze the introduction of a *cis*-double bond to existing SFAs of membrane phospholipids at the Δ5 position (Altabe et al., 2003). *Pseudomonas aeruginosa* synthesize UFAs through FabAB-mediated *de novo* biosynthetic pathway (Hoang and Schweizer, 1997). Alternatively, the *P. aeruginosa* Δ*fabA* mutant is still capable of inserting the Δ9 double bond into the existing phospholipids at the *sn*-2 position or exogenous SFAs by the catalytic action of O_2-dependent desaturase DesA and DesB, respectively (Zhu et al., 2006).

1.2.2. Branched-chain fatty acids

Some Gram-positive bacteria also rely on the type II pathway to produce SFAs and branched-chain fatty acids (BCFAs) to adjust membrane fluidity. BCFAs include *iso*-, *anteiso*- and ω-alicyclic fatty acids and their phase transition temperatures are in the order: *anteiso*-BCFAs < *iso*-BCFAs < straight-chain fatty acids with same carbon numbers (Kaneda, 1991). The methyl branch of BCFAs disrupts acyl-chain packing, and *anteiso*-BCFAs, with a methyl group further from the end of the acyl chain, induce a more fluid membrane compared with *iso*-BCFAs (Zhang and Rock, 2008) (Table 1-1).

The only difference between the synthesis of straight-chain fatty acids and BCFAs is their respectively preferred initial substrate (primers). Acetyl-CoA is used as a primer for the synthesis of straight-chain fatty acids in *Escherichia coli*. The branched-chain α-keto acids, such as valine, leucine and isoleucine, are used as major primer sources in the biosynthesis of BCFAs. These amino acid precursors are first transaminated with branched-chain amino acid transaminase, and then oxidatively decarboxylated by branched-chain α-keto acid dehydrogenase (Bkd) to produce their corresponding short branched-chain acyl-CoA derivatives that serve as primers for BCFA synthesis (Zhu et al., 2005a). The resulting primers are used by FabH to initiate the first condensing reaction and malonyl-CoA acts as a chain extender for repeated condensation to achieve the acyl chain elongation. Growth of *Listeria monocytogenes* deficient in *bkd* was stimulated by supplementation with 2-methylbutyrate, isobutyrate or isovalerate, which are precursors of odd-numbered *anteiso*-fatty acids, even-numbered *iso*-fatty acids, or odd-numbered *iso*-fatty acids (Zhu et al., 2005a). This implies that these branched short-chain carboxylic acids also could be utilized as primer sources when they are exogenously supplied. In addition, short-chain fatty acids serve as growth factors and primer sources in the biosynthesis of BCFAs for *Bacillus subtilis bkd* mutant and *Staphylococcus carnosus* transaminase-deficient strain (Willecke and Pardee, 1971; Beck, 2005).

E. coli FabH uses acetyl-CoA instead of branched-chain substrates to produce straight-chain fatty acids. However, FabH homologs of *B. subtilis* heterogeneously

expressed in *E. coli* still synthesized BCFAs, suggesting that BCFA synthesis depends on the intrinsic property of FabH (Choi et al., 2000). Acetyl- and propionyl-CoA, which are used as preferred primers for *E. coli* FabH, result from the limited room of FabH primer binding pocket that cannot fit acyl chains > C_4 (Heath and Rock, 1996; Qiu et al., 2001). The crystal structure of *Staphylococcus aureus* FabH exhibits a significantly larger primer binding pocket than that of *E. coli*, in addition, FabH of *S. aureus* showed a much higher affinity for isobutyryl-, butyryl-, or isovaleryl-CoA than acetyl-CoA (Qiu et al., 2005).

1.2.3. Cyclopropane fatty acids

Cyclopropane fatty acids (CFAs) are widely distributed in both Gram-positive and Gram-negative bacteria, and are formed by post-biosynthetic modification of existing acyl chain of membrane phospholipids (Goldfine, 1982). CFA synthase, a cytosolic enzyme, catalyzes the generation of a *cis*-cyclopropane ring by transferring a methylene group from *S*-adenosyl-L-methionine to the *cis*-double bonds of UFAs ($C_{16:1}$ and $C_{18:1}$) in phospholipids (Cronan et al., 1979; Wang et al., 1992; Huang et al., 2002).

The formation of CFAs in membrane phospholipids protects bacteria grown in adverse environmental conditions, especially in acidic conditions (Chang and Cronan, 1999). The protective effect of CFAs is attributed to decreased H^+ permeability and increased H^+ extrusion capacity under acidic conditions (Shabala and Ross, 2008). Moreover, the

conversion of UFAs to CFAs induces a change in membrane fluidity that counteracts the effects of adverse environmental conditions. Substitution of saturated membrane lipids by CFAs increases the fluidity of lipid bilayers by disrupting lipid packing and increasing lipid diffusion; however, CFAs maintain a more rigid membrane when compared to their analogous UFAs (Poger and Mark, 2015) (Table 1-1).

1.2.4. *Trans*-unsaturated fatty acids

UFAs synthesized in growing cells naturally exist in the *cis*-configuration. The isomerization of *cis*- to *trans*-UFAs is a post-biosynthetic modification, which is exclusive to the genera of *Pseudomonas* and *Vibrio* (Heipieper et al., 2003; Von Wallbrunn et al., 2003). The *cis*-double bond introduces a "kink" into the acyl chain that prevents tight packing of the chains; however, chains containing *trans* double bonds are straighter than *cis* chains (Roach et al., 2004). The *cis*- to *trans*-UFA isomerization increases the phase transition temperature and reduces membrane permeability, which mimics the properties of SFAs (Zhang and Rock, 2008) (Table 1-1). Compared with the synthesis of SFAs through the *de novo* pathway, the conversion of *cis*- to *trans*-UFAs is a much faster adaption mechanism which can respond to sudden environmental changes. *P. putida* in different growth phases utilizes different metabolic strategies for membrane lipid alteration: the synthesis of SFAs occurs in the exponential growth phase, while *trans*-UFA production in the non-growth phase (logarithmic growth phase) is used as an

emergency response for bacterial adaptation of environmental changes (Loffeld and Keweloh, 1996). The formation of *trans*-UFAs compensates for membrane perturbations caused by high temperature or the addition of heavy metals and organic solvents (Heipieper et al., 1996; Junker and Ramos, 1999).

The catalysis of *cis*-$C_{16:1}$ and *cis*-$C_{18:1}$ to their respective *trans*-derivatives is catalyzed by *cis-trans* isomerase (Cti) (Holtwick et al., 1997; Junker and Ramos, 1999). Cti is a cytochrome *c*-type protein, with a predicted heme-binding motif (Holtwick et al., 1999), which is located in the periplasmic space (Pedrotta and Witholt, 1999). The enzymatic reaction is initialized by the formation of a substrate-enzyme complex, and then the electrophilic iron (probably Fe^{3+}) at the heme domain removes an electron from the *cis* double bond resulting in the transformation of sp^2 linking to an sp^3, followed by a rotation to the *trans* configuration to complete the reconstitution of the double bond (Von Wallbrunn et al., 2003). There is no consumption of electron energy, thus this process does not require ATP or any other cofactors (Heipieper et al., 2003). The isomerization proceeds directly from *cis*- to *trans*-configuration without shifting the position of the double bond (Diefenbach and Keweloh, 1994).

1.2.5. Polyunsaturated fatty acids

Very long chain omega-3 polyunsaturated fatty acids (PUFAs), such as eicosapentaenoic acid (20:5*n*-3; EPA) and docosahexaenoic acid (22:6*n*-3; DHA) are prevalent in

psychrophilic and piezophilic marine bacteria that inhabit the deep sea environment, as well as the intestinal tract of deep-sea fish (Wirsen et al., 1986; Yano et al., 1994). Compared with mono-UFA (e.g. oleic acid), DHA renders a more flexible membrane with its helical conformation and broadly distributed angles formed by multiple double bonds (Fernandes et al., 2002). Deuterium (^{2}H) NMR analysis has revealed that the structural feature of DHA-containing lipids contributes to their specific biological function (Huber et al., 2002). Indeed, marine bacteria's adaptation to low temperatures and high pressure conditions was reported to be attributed to the presence of EPA and DHA, rather than mono-UFAs (Valentine and Valentine, 2004; Wang et al., 2009).

It was thought that PUFAs were synthesized by chain elongation and O_2-dependent desaturation of pre-existing fatty acids (Bajpai and Bajpai, 1993). However, PUFA biosynthesis in some prokaryotes and eukaryotes can be carried out through an anaerobic pathway without the involvement of fatty acid elongase and desaturase (Metz et al., 2001). *Moritella marina* or *Shewanella marinintestina* treated with cerulenin, an inhibitor of *de novo* fatty acid biosynthesis, increased PUFA production, indicating that PUFA synthesis was an independent process distinct from the type II pathway (Morita et al., 2005). The genes that encode PUFAs were explored. Recombinant *E. coli* with the introduced *pfa*A-D, a putative EPA synthase gene cluster of *Photobacterium profundum* strain SS9, failed to yield EPA (Allen and Bartlett, 2002). Similarly, the cloning of *pfa*A-D from DHA-producing *M. marina* strain MP-1 did not result in DHA production in recombinant

E. coli (Tanaka et al., 1999), whereas the incorporation of *pfaE* that encodes phosphopantetheinyl transferase (PPTase) renders its success (Orikasa et al., 2006a; Orikasa et al., 2006b).

Okuyama et al. (2007) revealed that the bacterial EPA and DHA biosynthesis are catalyzed by the expression of gene cluster *pfa*A-E, which encodes polyketide synthases (PKSs) including malonyl-coenzyme A:acyl carrier protein acyltransferase (AT), acyl carrier protein (ACP), 3-ketoacyl-ACP synthase (KS), 3-ketoacyl-ACP reductase (KR), 3-hydroxydecanoyl-ACP dehydratase (HD), enoyl-ACP reductase (ER) and phosphopantetheinyl transferase (PPTase) (Okuyama et al., 2007). It was also shown that bacteria commonly contain two or three HD domains and at least one of them is functional as FabA in *E. coli*. The PKS system catalyze elongation of the acyl chain with repeated condensations, and double bonds within PUFAs are formed by both *trans-cis* isomerization and enoyl reduction reactions that occur in selected cycles (Metz et al., 2001). Specifically, double bonds in the EPA and DHA products are introduced at every third carbon (Wallis et al., 2002).

1.3. The regulation of bacterial membrane lipid biosynthesis

1.3.1. The control of newly synthesized membrane lipids

The fatty acids synthesized through the *de novo* pathway are essential components of bacterial membrane phospholipids. For example, UFAs are some of the essential

membrane phospholipids, thus UFA auxotrophs of *E. coli* are difficult to store and require a *cis*-UFA supplementary medium for growth (Clark and Cronan, 1981). The cell membrane of *Listeria monocytogenes* contains more than 95% BCFAs when cultured in complex medium at 37°C (Annous et al., 1997). Only branched- and straight-chain membrane fatty acids were detected in *B. subtilis* grown at 37°C (Grau and De Mendoza, 1993). These fatty acids are synthesized when bacteria are grown in favorable conditions. Fatty acid synthesis is mainly triggered by sensing its deficiency in membrane phospholipids which is achieved by monitoring the alteration in the long-chain acyl-thioester pool. The regulation mechanisms of BCFA synthesis have not been well studied. However, some information is available on the regulation of UFAs at the transcriptional level.

The transcription of *fabA* and *fabB* ar precisely regulated to control the synthesis of membrane *cis*-UFAs in *E. coli* (Zhang and Rock, 2009). FadR binds at the -40 region of the major *fabA* promoter to activate gene transcription for UFA production, while the presence of another FadR-independent weaker promoter ensures the basal transcription of *fabA* to allow synthesis of UFAs for maintaining functional membrane (Henry and Cronan, 1991; 1992). FadR also positively regulates the transcription of *fabB*, the gene encoding for a second essential protein for the synthesis of UFAs in *E. coli* (Campbell and Cronan, 2001). Zhang et al. (2012) demonstrated that the overexpressed *fadR* of *E. coli* induces the up-regulation of *fabB*, *fabF* and *accA* which results in enhanced fatty

acid production, with an increase mainly in UFAs and slightly in SFAs.

The second transcriptional regulator FabR is functional as a repressor, which blocks the transcription of both *fabA* and *fabB* (Zhang et al., 2002; Feng and Cronan, 2009). *E. coli* FabR regulates gene transcription by monitoring the alteration in long-chain acyl-thioester pool. UFA-ACP or UFA-CoA are proposed triggers for a conformational change that enhances the binding of FabR to repress the transcription of *fabA* and *fabB*, and this conformational change is prevented by SFA-ACP or SFA-CoA (Zhu et al., 2009).

1.3.2. The modification of the pre-existing phospholipids

In response to abrupt environmental changes, bacteria tend to modify pre-existing membrane phospholipids to rapidly adapt to unfavorable conditions. Once the sudden environmental changes are sensed by bacteria, the regulation of enzyme synthesis or activation is immediately initialized.

Post-biosynthetic desaturation in *Bacillus* spp. is induced by low temperature (Aguilar et al., 1998; Aguilar et al., 1999). When growth medium contains isoleucine sources and α-keto acid derivative, the precursor of branched-chain fatty acid, the expression of *des* is repressed at 37°C (Cybulski et al., 2002). However, the transcription of *des* increased by 10- to 15- fold when the culture of *B. subtilis* was transferred from 37°C to 20°C (Aguilar et al., 1998). The signal transduction system composed by a thermo sensor DesK and a response regulator DesR regulates the transcription of *des* at low temperatures (Aguilar et

al., 2001). Actually, the alteration of membrane fluidity at low temperatures is reported to be the real signal that regulates the ratio of kinase to phosphatase activities of C-terminal domain of DesK (Albanesi et al., 2004). Decreased membrane fluidity sensed by transmembrane segments of DesK favors a kinase-dominant state of cytoplasmic C-terminal tail; the auto-phosphorylated DesK transfers its phosphoryl group to cytoplasmic regulator DesR (DesR-P) that increases its binding to upstream of *des* and initiates the synthesis of Δ5-Des; in contrast, the newly formed UFAs increase membrane fluidity which promotes the phosphatase activity of DesK and then causes the hydrolysis of DesR-P, release from DNA binding and termination of the transcription of *des* (Mansilla and De Mendoza, 2005).

E. coli AW1.7 is a heat and pressure resistant strain isolated from beef-packing plant (Aslam et al., 2004). In exponential-phase *E. coli* AW1.7 cells, a lower proportion of CFAs and a higher proportion of substrate UFAs were detected in the membrane (Ruan et al., 2011). When cells reached stationary phase, almost all of the UFAs were quantitatively converted to CFAs (Chen and Gänzle, 2016). The difference in CFA synthesis is caused by different transcription levels of *cfa* between growth phases. Meanwhile, the transcription of CFA synthase is regulated by two promoters: one is functional throughout the growth curve, while the other is activated by RpoS that is expressed from σ^S as cells enter the stationary phase of growth (Wang and Cronan, 1994). However, the increased level of CFA synthase is temporary, because it is quickly

degraded by RpoH-dependent proteolysis after modification of phospholipids (Chang et al., 2000). The transcription of *cfa* is not only activated during the growth phase transition but also induced by environmental changes. σ^s-mediated CFA expression renders stationary-phase *E. coli* cells more resistant to acid shock than exponential-phase cells; however, the survival of exponential-phase cells was fundamentally enhanced if they were exposed to pH 5 before a shift to pH 3, because moderate acid exposure can greatly stimulates *cfa* transcription (Chang and Cronan, 1999). Moreover, the transcription of *cfa* in *Lactococcus lactis* increased 9-fold when cells were stressed with 6% ethanol (Huong To et al., 2011).

The *cti* of *Pseudomonas putida* is expressed in exponential and stationary phase at fairly constant levels, whereas the presence of solvent (toluene) induces the up-regulated expression (Bernal et al., 2007). The regulation of the activity of constitutively expressed Cti is essential, and it is only active in resting cells subjected to the abrupt environmental changes. Cti is extremely sensitive to membrane fluidity; thus the state of the membrane is the determining factor for enzymatic activity (Heipieper et al., 2001). The active site of the periplasmic enzyme cannot easily reach the double bonds at a certain depth of the membrane (Heipieper et al., 2010). In the presence of solvent, an increase in membrane fluidity allows the double bonds to approach the membrane surface more frequently, which facilitates the interaction with Cti and results in an isomerization. Subsequently, the reaction would be inhibited along with the formation of *trans*-UFAs. Finally, the ratio

of *trans*- to *cis*-UFAs is reduced to normal level when cells re-enter the exponential growth phase and initiate *cis*-UFA formation.

1.4. Food bacterial membrane lipid alteration in response to environmental stresses

Food bacteria encounter adverse conditions during the food preservation. For example, thermal pasteurization is commonly used for the food decontamination. To retain the original properties of raw materials, dairy products and fruit juice are treated with the temperature between 73°C and 95°C for a short time (less than or equal to 1 min) (Lee and Coates, 2003; Champagne et al., 2005; Sheehan et al., 2007). Cold stress during the refrigerated storage is utilized to control the growth of food spoilage organisms. In addition, a non-thermal technique, high hydrostatic pressure (up to 600 MPa), is commercially used to decontaminate meat products (Hugas et al., 2002; Garriga et al., 2004). Chemical stresses such as, acid and ethanol produced during fermentation, pose a challenge to the survival of food bacteria. Some lactic acid bacteria (LAB) are selected as starter cultures and undergo acidic conditions caused by the organic acid production during food fermentation and storage. In yogurt production, the conversion of lactose to lactic acid leads to a pH decrease to 4.2-4.5, and even below 4.0 during subsequent storage (Leroy and De Vuyst, 2004). Ethanol generated by microorganisms is commonly found in fruit products, fermented foods and drinks. For instance, wine is a stressful environment because it contains high alcohol content, high SO_2 and low pH (Versari et

al., 1999). Bacteria grown in wine are exposed to ethanol at the concentration of 10-12% (Teixeira et al., 2002). Food bacteria also encounter oxidation stress throughout food storage. Spray-drying and freeze-drying techniques are used to preserve food ingredients with viable probiotics (Gardiner et al., 2000; Sleator and Hill, 2008); however, high and low processing temperatures are challenges for maintaining the viability of probiotics. Meanwhile, the oxidative stress triggered by spray-drying and freeze-drying processing is a hurdle for the survival of lactobacilli (Teixeira et al., 1996; Castro et al., 1997).

Bacterial survival is always challenged by environmental stresses throughout the growth. However, exposure to sub-lethal stress facilitates the development of bacterial adaptive responses, resulting in an increased survival when subjected to lethal conditions. The phospholipid homeostatic adaptation established in food bacteria in response to high temperature, low temperature, pressure, acid, ethanol, solvents and oxidation are discussed below (Table 1-2).

1.4.1. High Temperature

When the temperature is higher than the physiological range of limitation that bacteria normally experience, cell membrane lipids become more disordered and the membrane fluidity exceeds the optimum level, which results in a 'hyperfluid' state and may cause the loss of bilayer integrity (Hazel, 1995). Membrane lipid saturation was increased in thermally-adapted *Pseudomonas putida* P8 (Diefenbach et al., 1992). When the growth

temperature was increased from 15°C to 40°C, the SFA content of exponential-phase *P. aeruginosa* was increased from 25% to 39%, while UFA content was decreased from 66.5% to 51% (Dubois-Brissonnet et al., 2000). In the presence of cerulenin, which inhibits *de novo* fatty acid synthesis, a psychrophilic bacterium *Vibrio* ABE-1 converted *cis*-9 $C_{16:1}$ to *trans*-9 $C_{16:1}$ for adaptation in ambient temperature (20°C) (Okuyama et al., 1991). The formation of *trans*-UFAs was also essential for the growth of psychrophilic *P. syringae* at 28°C, while the mutant deficient in *cti* showed growth arrest (Kiran et al., 2004). The increased ratio of *trans*/*cis*-UFAs was only detected when *P. putida* S12 was exposed to high temperature (Heipieper et al., 1996). *E. coli* synthesized high-melting point membrane lipid, SFAs, and long-chain fatty acids in response to increased growth temperatures (Sinensky, 1974). As a major UFA in *E. coli*, *cis*-vaccenic acid was synthesized via the catalytic action of a temperature-sensitive enzyme and the formation progress of *cis*-vaccenic acid became slow at high temperature, as a strategy to maintain membrane fluidity (De Mendoza and Cronan Jr, 1983). In contrast, more $C_{16:0}$, $C_{18:1}$ and $C_{18:2}$ were detected in *Lactobacillus helveticus* when the temperature increased from 38°C to 54°C (Guerzoni et al., 2001).

It has been reported that a heat-resistant strain *E. coli* AW1.7 synthesized more CFAs than a heat-sensitive strain *E. coli* GGG10 during the exponential phase (Ruan et al., 2011), suggesting that CFAs protect stationary-phase *E. coli* cells against high temperatures. Similarly, the low UFA/SFA ratio with low membrane fluidity was

observed in heat resistant *Salmonella typhimurium*, and CFAs synthesized in acid-adapted strains played a critical role in heat tolerance (Álvarez-Ordóñez et al., 2008). Conversion of $C_{18:1}$ to $C_{19:0}$ CFA in stationary-phase cells of *Pediococcus* spp. was related to their thermal resistance (Annous et al., 1999). Heat-shocked *Lactococcus lactis* produced more $C_{19:0}$ CFA than non-treated cells (Broadbent and Lin, 1999).

1.4.2. Low Temperature

When bacteria survive at low temperature, their membrane turns into a gel-like state that influences the activity of membrane-bound proteins (Beales, 2004). The low-temperature adapted bacteria synthesize low-melting point phospholipids to induce a more fluid membrane, which is achieved by shortening the length of the lipid chain or by increasing the degree of fatty acid unsaturation and branching (Suutari and Laakso, 1994). The cell membrane of *L. monocytogenes* was composed of more than 95% branched-chain fatty acids, which mainly included *anteiso*-$C_{15:0}$, *anteiso*-$C_{17:0}$, and *iso*-$C_{15:0}$ when they were cultured in a complex medium at 37°C (Annous et al., 1997). In response to the low temperature, the alteration of the membrane lipid profile through fatty acid chain shortening and branching from *iso* to *anteiso*, which results in an increased amount of *anteiso*-$C_{15:0}$ (Annous et al., 1997; Zhu et al., 2005b). Indeed, *anteiso*-$C_{15:0}$ played in important role in maintaining the membrane fluidity of *L. monocytogenes* grown at refrigeration temperatures (Edgcomb et al., 2000). An increased amount of

anteiso-BCFAs as well as a decreased amount of *iso*-BCFAs were detected in *Bacillus subtilis* under the cold stress (Klein et al., 1999). When *B. subtilis* was shifted from 37°C to 20°C for 5h, $C_{16:1}$ was also detected and accounted for 20% of the total fatty acids (Grau and De Mendoza, 1993). Similarly, a decreased degree of membrane lipid saturation was observed in *Pseudomonas putida* S12 at low temperature (Heipieper et al., 1996). When the temperature decreased from 40°C to 20°C, the amount of *cis*-vaccenic acid increased in the membrane of *E. coli* (Aibara et al., 1972; Nishihara et al., 1976). Membrane phospholipids of cold-shocked *Lactococcus lactis* showed a decreased ratio of SFAs/UFAs (Broadbent and Lin, 1999). In addition to the reduction in the ratio of SFAs/UFAs, a decreased amount of $C_{19:0}$ CFA was observed in *Lactobacillus coryniformis* subjected to mild cold (26°C) (Schoug et al., 2008). Cold-stressed *Salmonella* spp. exhibited a higher UFA/SFA membrane ratio (Álvarez-Ordóñez et al., 2008; Álvarez-Ordóñez et al., 2009). Polyunsaturated fatty acids DHA and EPA are essential in membranes of deep sea bacteria that adapt to a cold-dependent lifestyle (Valentine and Valentine, 2004). EPA synthesis is necessary for the cold adaption of *S. livingstonensis* Ac10 (Sato et al., 2008), because EPA contributes to the membrane organization and cell division at low temperature (Kawamoto et al., 2009),

1.4.3. Pressure

Cell membranes are the main target of high hydrostatic pressure (HHP) treatment (Pagán

and Mackey, 2000; Ritz et al., 2000). HHP forces phospholipids in the membrane bilayer to pack more tightly and induces a transition from the liquid-crystalline state to the gel-phase, in a manner analogous to the effect of low temperature on membrane (Hazel and Williams, 1990). Lipid composition of marine barophilic bacteria shifts SFA to UFA as the membrane adaptats to increased pressure (Yano et al., 1998). Mono-UFAs were required for piezophilic bacterium CNPT3 and *Photobacterium profundum* SS9 growth at high pressure (DeLong and Yayanos, 1985; Allen et al., 1999). Disruption of *fabF* reduced the production of $C_{18:1}$ and increased the sensitivity of *P. profundum* SS9 to elevated hydrostatic pressure (Allen and Bartlett, 2000). PUFAs, mainly DHA and EPA, are found in barophilic bacteria inhabiting the intestinal tract of deep-sea fish, which contribute to bacterial adaptation in low temperature and high pressure environments (Yano et al., 1997). EPA was reported to be necessary for the growth of the high pressure adapted *Shewanella violacea* DSS12, and deficiency in EPA was not fully compensated by mono-UFAs or BCFAs (Kawamoto et al., 2011). PUFAs synthesized in *Vibrio marinus* MP-1 and *Alteromonas* spp. were proposed to maintain proper membrane fluidity against temperature and pressure challenges (Delong and Yayanos, 1986; Wirsen et al., 1986). EPA inhibited the formation of a hyperfluid membrane and stabilized the dynamic membrane structure in *S. violacea* DSS12 subjected to high pressure (Usui et al., 2012). In addition, EPA is very important for the late step of cell division of *S. violacea* DSS12 at high-hydrostatic pressure (Kawamoto et al., 2011). Compared with

Gram-negative bacteria, Gram-positive piezotolerant bacteria *Sporosarcina* sp. DSK25 used different mechanisms for high pressure adaptation; it not only produced mono-UFAs in rapid response, but also synthesized *anteiso*-BCFA and PUFAs as a long-term response for membrane adaption (Wang et al., 2014).

Increased pressure tolerance of another Gram-positive bacteria *L. plantarum* was also attributed to increased membrane lipid unsaturation (Ter Steeg et al., 1999). Moreover, CFA synthesis in *E. coli* increased high pressure resistance, demonstrated by the susceptibility of the *cfa*-deficient mutant to high pressure (Charoenwong et al., 2011).

1.4.4. Acid

At low pH, organic acids cross the microbial membrane in undissociated form. This acidifies the cytoplasm which causes the damage or modification to the functionality of enzymes, structural proteins and DNA, furthermore, malic and citric acids are capable of destabilizing the outer membrane through chelation and intercalation (Mani-López et al., 2012). Some bacteria are able to survive in acidic conditions by relying on their abilities to maintain the pH homeostasis. Acid habituation was explored in five *E. coli* strains: membrane monosaturated fatty acids ($C_{16:1}$ and $C_{18:1}$) were either converted to SFAs or their CFA derivatives ($C_{17:0cyclopropane}$ and $C_{19:0cyclopropane}$), which were related to the acid tolerance of individual strains (Brown et al., 1997). The protective effect of CFAs against acid stress is attributed to decreased proton permeability and increased proton extrusion

of the *E. coli* membrane (Shabala and Ross, 2008). In *Salmonella enterica*, full acid resistance required CFAs and UFAs (Kim et al., 2005). Significantly higher CFAs and SFAs were also detected in acid-adapted *Lactobacillus casei* ATCC 334 cells when they were challenged at pH 2.0 (Broadbent et al., 2010). Moreover, $C_{19:0}$ CFA synthesized in *Lactobacillus plantarum* was reported to be related to acid tolerance (Huang et al., 2016). The transcription of *cfa* in *Lactococcus lactis* MG1363 was highly induced by acid adapted conditions (pH 5), and higher levels of CFAs resulted in a more rigid membrane than control conditions (pH 7.2); however, the presence of CFAs in the wild-type strain did not confer a better survival compared with the *cfa* mutant when incubated at pH 3 for 3 h (Huong To et al., 2011). An acid-adaptive strain of *Streptococcus mutans* altered the membrane fatty acids profile from short-chain, SFAs at pH 7 to that of long-chain, monounsaturated fatty acids at pH 5, which provided protection from acidic conditions during the fermentation (Fozo and Quivey, 2004b). FabM-mediated monounsaturated fatty acid production explained acid tolerance of *S. mutans* (Fozo and Quivey, 2004a). In *L. monocytogenes*, an enhanced amount of *anteiso*-BCFAs and a decreased content of *iso*-BCFAs were observed when the pH reduced from 7.0 to 5.0 (Giotis et al., 2007).

1.4.5. Ethanol and organic solvents

Solvents such as ethanol are able to interact with phospholipid bilayers at the lipid-water interface, and this interfacial binding causes disorder in acyl chains and membrane

fluidization (Barry and Gawrisch, 1994). The conversion of *cis*-vaccenic acid to $C_{19:0}$ CFA compensated the increase in membrane fluidity that was induced by ethanol in *Oenococcus oeni* (Teixeira et al., 2002). Increased transcription level of *cfa* was detected when *O. oeni* was grown in the presence of 8% ethanol, and ethanol-grown cells had an increase in $C_{16:0}$ and $C_{19:0}$ CFA that corresponded to an elevated degree of saturation (Grandvalet et al., 2008). *O. oeni* cells, cultivated in a medium containing 8% ethanol, were able to adjust membrane permeability through decreasing fluidity at the lipid-water interface (Da Silveira et al., 2003). An increase in $C_{19:0}$ CFA and a reduction of oleic and vaccenic acids were demonstrated in *L. hilgardii* grown in an elevated concentration of ethanol (Couto et al., 1996).

E. coli utilize a different strategy in membrane lipid modulation against the toxic effect of ethanol. The content of *trans*-9-octadecenoate (*trans*-9, $C_{18:1}$) was ten-fold higher in 5% ethanol-stressed *E. coli* O157:H7 cells compared with that of non-ethanol treated cells (Chiou et al., 2004). However, the presence of *trans*-octadecenoate was questioned due to the lack of *cis-trans* isomerase in *E. coli*, which needed to be further investigated (Heipieper, 2005). It was possible to differentiate the octadecenoate isomers through employing a polar RTx-2330 capillary column, where a higher amount of *trans*-9-octadecenoate and a lower amount of $C_{19:0cyclo}$ were confirmed in *E. coli* O157:H7 cells grown in a medium with 5% ethanol (Ku et al., 2007). Compared with *cis* isomers, *E. coli* cells containing membrane *trans*-$C_{18:1}$ were more resistant to ethanol

stress, where *trans*-UFAs were obtained from the medium through the fatty acid transport systems (Keweloh and Heipieper, 1996). Membrane bilayer packing of phospholipids containing *trans*-UFAs mimics the physiology of those containing SFAs, which is considered as a strategy to reduce membrane fluidity and permeability (Zhang and Rock, 2008). In addition, the introduction of *fabA* in *E. coli* resulted in an increased amount of SFAs that protected the cells against ethanol, while the recombinant *E. coli* harboring *des* were ethanol sensitive because of the increased amount of UFAs in the cell (Luo et al., 2009). Phenol-tolerant *E. coli* also possessed increased membrane saturation (Keweloh et al., 1991).

Pseudomonads are known to live in soil and water contaminated with organic solvents (Cronan, 2002). The *cis*-*trans* isomerization system in *Pseudomonas* spp. is activated by organic solvents (Junker and Ramos, 1999; Pedrotta and Witholt, 1999). *Pseudomonas putida* was able to grow in the presence of 50% toluene (v/v), and a higher *trans*/*cis* ratio was detected in adapted cells (Weber et al., 1994). The conversion from *cis*- to *trans*-UFAs compensates for the enhanced membrane fluidity caused by solvents. *Pseudomonas putida* DOT-T1E mutant deficient in *cfaB* was a solvent sensitive strain, suggesting that CFAs increase the tolerance of *P. putida* to organic solvent (Pini et al., 2009).

1.4.6. Oxidation

Reactive oxygen intermediates (ROIs) are reduced forms of atmospheric oxygen (O_2) that freely oxidize cellular components, such as membrane lipids, protein and DNA, ultimately leading to the cell death (Mittler, 2002). The cellular water loss causes several changes that promote the accumulation of ROIs in the cell (França et al., 2007). When a cell is dehydrated, the membrane is perturbed and becomes more fluidized and susceptible to the attack of ROIs (Crowe et al., 1989; França et al., 2007). Membrane lipid oxidation accompanied with a decreased ratio of UFAs/SFAs was observed in *Lactobacillus bulgaricus* after spray-drying or freeze-drying (Teixeira et al., 1996; Castro et al., 1997). Fatty acids rich in double bonds are the main targets for the oxidative attack (Singh et al., 2002). Cell membranes composed of large amounts of PUFAs are more susceptible to the oxidation; the resulting lipid peroxidation causes changes in membrane fluidity and permeability, which ultimately influences cellular metabolism (Bandyopadhyay et al., 1999). Compared with UFAs, CFAs are less susceptible to ROIs attack (Grogan and Cronan Jr, 1997). *E. coli* UFA-auxotroph strain supplemented with CFAs showed higher resistance to hyperbaric oxygen than those supplemented with mono-UFAs or PUFAs (Harley et al., 1978). The presence of BCFAs, particularly *anteiso*-$C_{15:0}$, in the cytoplasmic membrane increased resistance to oxidation, as demonstrated by lower growth rate of *Staphylococcus aureus bkd* mutant as compared with the wild-type strain grown in 8.8 mM H_2O_2 (Singh et al., 2008). Inconsistent with previous studies, marine microorganisms with DHA and EPA (in vivo) were reported to

be stable under the oxidative stress (Okuyama et al., 2008). Meantime, UFAs synthesized under heat stress are capable of consuming O_2 and thus preventing further damage to cellular DNA, which was considered to be the cross-resistance to oxidation in *L. helveticus* (Guerzoni et al., 2001).

1.5. Conclusions

Generally, food bacteria evolved membrane lipid homeostasis to resist harsh conditions and sudden environmental changes. However, to survive in various growth conditions, different food bacteria developed their own specific lipid homeostatic adaption. A better understanding on mechanisms of bacterial lipid adjustment and regulations of fatty acid biosynthesis is the prerequisite to improve current intervention methods for pathogen control, and to optimize preparation methods for probiotics protection in food industry. Subsequent chapters will explore the role of hydroxylation and cyclopropanation of membrane fatty acids on food bacterial stress resistance.

Table 1-2. Food bacterial membrane lipid alteration in response to environmental stresses

Stress	Modulation of membrane fatty acids	Organisms (genus)	Reference
High temperature	SFAs (+), UFAs (-), *trans*-UFAs (+)	*Pseudomonas*	(Diefenbach et al., 1992; Dubois-Brissonnet et al., 2000; Kiran et al., 2004; Heipieper et al., 1996)
	trans-UFAs (+)	*Vibrio*	(Okuyama et al., 1991)
	SFAs (+), long-chain fatty acids (+), *cis*-UFA (-), CFAs (+)	*Escherichia*	(Sinensky, 1974; De Mendoza and Cronan Jr, 1983; Ruan et al., 2011)
	SFAs(+), UFAs (+)	*Lactobacillus*	(Guerzoni et al., 2001)
	UFA/SFA (-), CFAs (+)	*Salmonella*	(Álvarez-Ordóñez et al., 2008)
	CFA (+)	*Pediococcus*	(Annous et al., 1999)
	CFA (+)	*Lactococcus*	(Broadbent and Lin, 1999)
Low temperature	*anteiso*-BCFAs (+)	*Listeria*	(Annous et al., 1997; Zhu et al., 2005b)
	anteiso-BCFAs (+), *iso*-BCFAs (-), *cis*-UFAs (+)	*Bacillus*	(Klein et al., 1999; Grau and De Mendoza, 1993)
	SFAs (-)	*Pseudomonas*	(Heipieper et al., 1996)
	UFAs/SFAs (+)	*Salmonella*	(Álvarez-Ordóñez et al., 2008; Álvarez-Ordóñez et al., 2009)
	cis-UFAs (+)	*Escherichia*	(Aibara et al., 1972; Nishihara et al., 1976)
	SFAs/UFAs (-)	*Lactococcus*	(Broadbent and Lin, 1999)
	SFAs/UFAs (-), CFAs (-)	*Lactobacillus*	(Schoug et al., 2008)
	EPA (+)	*Shewanella*	(Sato et al., 2008)
Pressure	*cis*-UFAs (+)	*Photobacterium*	(DeLong and Yayanos, 1985; Allen et al., 1999; Allen and Bartlett, 2000)
	EPA (+)	*Shewanella*	(Kawamoto et al., 2011; Usui et al., 2012)
	PUFAs (+)	*Vibrio*	(Delong and Yayanos, 1986)
	PUFAs (+)	*Alteromonas*	(Wirsen et al., 1986)
	anteiso-BCFA (+); PUFAs (+); *cis*-UFAs (+)	*Sporosarcina*	(Wang et al., 2014)
	cis-UFAs (+)	*Lactobacillus*	Ter Steeg et al., 1999
Acid	SFAs (+), CFAs (+)	*Escherichia*	Brown et al., 1997
	CFAs (+), *cis*-UFAs (+)	*Salmonella*	Kim et al., 2005
	CFAs (+), SFAs (+)	*Lactobacillus*	Broadbent et al., 2010; Huang et al., 2016
	CFAs (+)	*Lactococcus*	Huong To et al., 2011
	long-chain fatty acids (+), *cis*-UFAs (+)	*Streptococcus*	Fozo and Quivey, 2004b
	anteiso-BCFAs (+), *iso*-BCFAs (-)	*Listeria*	Giotis et al., 2007
Ethanol	CFAs (+), SFAs (+)	*Oenococcus*	Teixeira et al., 2002; Grandvalet et al., 2008
	CFAs (+), *cis*-UFAs (-)	*Lactobacillus*	Couto et al., 1996
	trans-UFAs (+); SFAs (+)	*Escherichia*	Chiou et al., 2004; Ku et al., 2007; Keweloh and Heipieper, 1996; Luo et al., 2009
Solvent	SFAs (+)	*Escherichia*	Keweloh et al., 1991
	trans/*cis* UFAs (+)	*Pseudomonas*	Weber et al., 1994
Oxidation	UFAs/SFAs (-)	*Lactobacillus*	Teixeira et al., 1996; Castro et al., 1997
	anteiso-BCFAs (+)	*Staphylococcus*	Singh et al., 2008

1.6. Research hypothesis and objectives

The hypothesis of this thesis research is that the conversion of UFAs to CFAs or hydroxy fatty acids is related to the survival of food bacteria under a wide range of environmental conditions. To test the hypothesis, my objectives include:

1. To review current knowledge on membrane fatty acid synthesis, regulation and the role in stress resistance for food applications.
2. To investigate whether the synthesis of 10-HOE is a strategy to maintain membrane lipid homeostasis when *Lactobacillus plantarum* were challenged by ethanol stress.
3. To evaluate whether cyclopropanation is a general protection for *E. coli* against environmental stresses, especially to intervention treatments, such as high temperature, high pressure, high pressure carbon dioxide, acid and oxidation.

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Chapter 2. Influence of 10-hydroxy-12-octadecenoic acid on ethanol tolerance in *Lactobacillus plantarum*

2.1. Introduction

Antifungal metabolites of lactic acid bacteria have potential for applications as antifungal preservatives in cereal products, and in silage (Magnusson et al., 2003; Oliveira et al., 2014). Several hydroxy fatty acids have antifungal activity (Hou and Forman Iii, 2000; Hou, 2008) and antifungal 3-hydroxy fatty acids of C_{10} to C_{14} chain lengths are formed by *Lactobacillus plantarum* MiLAB 14 (Sjögren et al., 2003). *L. hammesii* accumulates 10-hydroxy-12-octadecenoic acid (10-HOE), an antifungal compound that increased the mold-free shelf life of bread (Black et al., 2013a and 2013b). The biosynthesis of antifungal hydroxy fatty acids and the application of hydroxy fatty acids in food are dependent on knowledge of enzymes involved in microbial fatty acid hydroxylation (Kim and Oh, 2013).

Linoleate hydratase activity was first characterized in *Streptococcus pyogenes*; the hydratase was previously described as myosin cross reactive antigen (Kil et al., 1994). The FAD containing linoleate hydratase in *S. pyogenes* hydrates the *cis*-9 and *cis*-12 double bonds of C_{16} and C_{18} fatty acids to produce 10-hydroxy and 10,13-dihydroxy fatty acids (Volkov et al., 2010). Remarkably, the linoleate hydratases of bifidobacteria, lactobacilli, and *Nocardia spp.* exclusively hydrate the *cis*-9 double bond (Koritala and Bagby; Rosberg-Cody et al., 2011; Yang et al., 2013). The crystal structure of the linoleate hydratase from *Lactobacillus acidophilus* provided the structural basis for the

selective substrate recognition of linoleate 10-hydratase (Volkov et al., 2013). A second linoleate hydratase in *L. acidophilus* hydrates the *cis*-12 double bond to produce 13-hydroxy fatty acid (Kim et al., 2015; Park et al., 2015).

The physiological and ecological function of linoleate hydratases have been investigated. The linoleate hydratase / myosin cross reactive antigen of *S. pyogenes* mediated adherence to human keratinocytes; linoleate hydratase was also suggested to detoxify linoleic acid by conversion to a hydroxyl-product with lower antibacterial activity (Volkov et al., 2010). Similarly, a *L. acidophilus* mutant with truncated linoleate hydratase exhibited a decreased adherence to intestinal epithelial cells, and was more sensitive to stresses (O'Flaherty and Klaenhammer, 2010). Heterologous expression of a linoleate hydratase of *Bifidobacterium breve* in *Lactococcus lactis* increased resistance to heat and solvent stresses (Rosberg-Cody et al., 2011). Linoleate 10-hydratase is predicted to be a membrane-associated protein with one trans-membrane helix (Kil et al., 1994; Rosberg-Cody et al., 2011), and hydration of linoleic acid occurs in the cell periphery (Kishino et al., 2011). However, the effect of linoleic acid hydratase on properties of cell membranes and the cell surface have not been demonstrated. Moreover, 10-HOE has antifungal activity (Black et al., 2013b) but a corresponding activity of alternative hydration products of fatty acids remains unknown.

This study aimed to characterize linoleic acid hydratases in *L. plantarum*, *L. reuteri*, *L. hammesii*, and *L. spicheri*. Strain selection included strains which produce only 10-HOE

and strains that produce 10-HOE and 13-hydroxy-9-octadecenoic acid (13-HOE) (Black et al., 2013b). Four enzymes were characterized by heterologous expression in *E. coli*. The physiological function of the linoleate 10-hydratase (LahA) and its product 10-HOE of *L. plantarum* were studied in more detail by comparison of the ethanol resistance and cell surface properties of *L. plantarum* TMW1.460 and its linoleate 10-hydratase deficient mutant Δ*lahA*. The antifungal activity of 13-HOE was compared to the activities of other hydroxy fatty acids.

2.2. Materials and methods

2.2.1. Bacterial strains and fermentation

L. reuteri LTH2584, *L. plantarum* TMW1.460, *L. hammesii* DSM16381, *L. spicheri* Lp38 and *L. sanfranciscensis* ATCC 27651 were anaerobically cultivated at 37°C (*L. reuteri*) or 30°C (all other strains) in modified De Man Rogosa Sharpe (mMRS) medium containing 0.1% Tween 80 (mMRS-Tween 80) (Zhang et al., 2010). mMRS-Tween 20 was prepared by replacing Tween 80 with an equal weight of Tween 20. *Escherichia coli* DH5α (New England Biolabs, Massachusetts, USA) served as a host for plasmids in the cloning procedures, and *E. coli* BL21 Star (DE3) (ThermoFisher Scientific, Massachusetts, USA) was used for protein overexpression. *E. coli* strains were cultivated in Luria–Bertani (LB) medium (BD, Mississauga, CA) with agitation at 200 rpm and 37°C. Antibiotic-resistant *E. coli* carrying plasmid pET-28a(+), pUC19 or pJRS233 were

cultured in media containing 50 mg/L kanamycin, 50 mg/L ampicillin or 500 mg/L erythromycin, respectively. Erythromycin-resistant *L. plantarum* was grown in presence of 5 mg/L erythromycin. *Aspergillus niger* FUA5001 and *Penicillium roqueforti* FUA5005 were grown at 25°C for 72 h on malt extract agar.

Linoleic acid metabolism of lactobacilli was analyzed after incubation in mMRS-Tween80 broth supplied with 5% inoculum and 4 g/L linoleic acid anaerobically for 4 days. Lipids were isolated by addition of one volume of 85:15 (vol/vol) chloroform-methanol to cultures prior to incubation at 4°C overnight; cultures were then extracted twice with additional two volumes of chloroform–methanol (85:15, vol/vol). The organic solvent was evaporated under reduced pressure and the dry residue stored at −20°C under nitrogen.

2.2.2. DNA manipulations

Genomic DNA was isolated using the Blood & Tissue Kit (Qiagen, Hilden, Germany). Plasmid DNA from *E. coli* was extracted with a QIAprep Spin Miniprep kit (Qiagen). PCR primers used in this study were synthesized by Integrated DNA Technologies (SanDiego, USA). The Taq DNA polymerase was purchased from TaKaRa Bio (Shiga, Japan). T4 DNA ligase and restriction enzymes were obtained from Thermo Scientific (Mississauga, CA). PCR products were purified by using the DNA gel extraction kit (Qiagen). DNA sequencing was performed by Macrogen (Maryland, USA).

2.2.3. Sequence and phylogenetic analysis of linoleate hydratases in lactobacilli

Genes of putative linoleate hydratases in *L. spicheri* Lp38, *L. reuteri* LTH2584 and *L. plantarum* TMW1.460 were amplified with primers that are specific to linoleate hydratases in genome-sequenced strains of these species (Table 2-1). To identify the linoleate hydratase in *L. hammesii*, *lah* sequences from the closely related *L. spicheri* and *L. brevis* were aligned and specific primers targeting conserved sequences upstream and downstream of *lah* coding sequences were designed (Table 2-1). The four *lah* genes were sequenced by service of Macrogen (Rockville, MD, U.S.A.).

Table 2-1. Primers used in this study

Primers (forward, F; reverse, R)	Sequence (5'-3')	Restriction site
DSM16381 sequencing, F1	5'-TACGGAGGTGTTTTTTGATGGT-3'	—
DSM16381 sequencing, R1	5'-CGTAAATTCATAAATCATTTGGTGCATGTA-3'	—
DSM16381 sequencing, F2	5'-TACATGCACCAAATGATTTATGAATTTACG-3'	—
DSM16381 sequencing, R2	5'-TACTTCGTCTTAGGTGACCA-3'	—
LTH2584 cloning, F	5'-CGCCATATGTACTATTCAAACGGAAATTATG-3'	NdeI
LTH2584 cloning, R	5'-ATTTGCGGCCGCTTAAAGTAAATGTTGTTCTTCCATT-3'	NotI
TMW1.460 cloning, F	5'-CCGGAATTCATGGTTAAAAGTAAAGCAATTATGA-3'	EcoRI
TMW1.460 cloning, R	5'-ATTTGCGGCCGCTTAATCAAACATCTTCTTAGTTGC-3'	NotI
DSM16381 cloning, F	5'-CGCGGATCCATGGTTAAAACAAAAGCAGTAATG-3'	BamHI
DSM16381 cloning, R	5'-CCCAAGCTTTTAGCTAAACATCCGCTTCGTTGC-3'	HindIII
Lp38 cloning, F	5'-CCGGAATTCATGGTTAAGACAAAAGCTGTAATG-3'	EcoRI
Lp38 cloning, R	5'-ATAGTTTAGCGGCCGCGTTAACTAAACATTTTCTTCGTTGCC-3'	NotI
lah-upstream A, F	5'-AACTGCAGGCCTAAAACGAGCTAAACGAC-3'	PstI
lah-upstream A, R	5'-ACATGCATGCCCCGGCACCAATCATAATTGCTTTAC-3'	SphI
lah-downstream B, F	5'-ACATGCATGCAAGAAGATGTTTGATTAATTAAA-3'	SphI
lah-downstream B, R	5'-CCCAAGCTTATGAAAAAATTAACATCAGTCG-3'	HindIII

The phylogenetic analysis of linoleate hydratases included the type strains of the 24 groups of the genus *Lactobacillus sensu lato* (Zheng et al., 2015) and the genera *Weissella, Leuconostoc,* and *Oenococcus*. Sequences of biochemically characterized linoleate 10-hydratase and linoleate 13-hydratase from *Lactobacillus* spp., *Bifidobacterium* spp. and *Streptococcus* spp. were included in the phylogenetic analysis (Volkov et al., 2010; Rosberg-Cody et al., 2011; Yang et al., 2013; Kim et al., 2015). Protein sequences of LAHs were retrieved from GenBank (http://www.ncbi.nlm.nih.gov), using NCBI BLAST analysis with organism specific search. Protein phylogenetic tree was built in MEGA7.

2.2.4. Cloning and heterologous expression of linoleate hydratases of lactobacilli

Coding regions of the four *lah* were amplified from genomic DNA of the respective strains with primers listed in Table 2-1. Amplicons were purified and cloned into pGEM-T Easy vector (Promega, Madison, USA). The *lah* fragments in recombinant pGEM-T vectors were digested with restriction endonucleases (Table 2-1), purified and ligated into expression vector pET-28a(+) (Novagen, Ontario, Canada), yielding the respective constructs pET28a/LAH for each strain. Recombinant plasmids were introduced into chemically competent *E. coli* BL21 Star (DE3) (Thermo Fisher Scientific) and transformants were plated on LB agar containing 50 mg/L kanamycin. The gene cloning was verified by PCR amplification and sequencing.

Four recombinant *E. coli* BL21 (DE3) strains were grown to an optical density (OD) at

600 nm of 0.6. Protein expression was induced by the addition of isopropyl-β-D-thiogalactopyranoside (IPTG) to a final concentration of 1.0 mM, followed by incubation for 4 h and harvesting of cells by centrifugation at 4°C.

2.2.5. Purification of linoleate hydratases.

Overexpressed linoleate hydratases were present mainly as inclusion bodies. Solubilization and refolding of inclusion bodies were carried out with the protein refolding kit (Novagen) according to the manufacturer's instructions. The refolded proteins were concentrated by using a 10 KDa Amicon Ultra-15 centrifugal filter Unit (Millipore, Germany).

After concentration, the His-tagged linoleate hydratases were purified by Ni-NTA spin columns (Qiagen). The purified LAHs were finally dialyzed against 50 mM 4-morpholineethanesulfonic acid (MES) buffer (pH 6.1) (Sigma-Aldrich) overnight at 4°C. The purified enzymes were assessed by SDS–PAGE and visualized by staining with Coomassie blue. FAD was added to the purified enzymes at a final concentration of 0.2 mM and incubated at 4°C for 24 h (Joo et al., 2012).

2.2.6. Enzymatic activity assay and fatty acid analysis

To determine the enzymatic activity, 4.5 mg linoleic acid and 25 μg purified LAHs were incubated in 1 ml of 50 mM MES buffer (pH 6.1) containing 50 mM NaCl, 2% ethanol, and 10% glycerol at 25°C for 3 h. Fatty acids were extracted following the procedure

described above. The organic phase of the extracts was collected and analyzed with LC-APPI-MS as described (Black et al., 2013a; Black et al., 2013b).

2.2.7. Construction of *L. plantarum* TMW1.460Δ*lah* by double-crossover mutagenesis

Gene disruption of *lah* in *L. plantarum* TMW1.460 was achieved by an in-frame, unmarked deletion (Su et al., 2011) (Figure 2-1). The approximately 900 bp 5'-flanking regions (fragment A) and 1000 bp 3'-flanking regions (fragment B) of *lah* were amplified by PCR with primers listed in Table 2-1. The fragment A was digested with PstI, SphI, and fragment B was digested with SphI, HindIII. The resulting fragments were purified and sequentially ligated into vector pUC19 to generate pUC19/AB. The AB fragment in pUC19/AB was confirmed by sequencing and subcloned into the PstI and HindIII restriction sites of pJRS233 to create pJRS233/Δ*lah*. Recombinant plasmids were transformed into electrocompetent *L. plantarum* TMW1.460 at 12.5 kV/cm, 25 μF and 200 Ω. The cells were grown in mMRS-Tween80 broth containing 5 mg / L erythromycin at 42°C for 80 generations to select for single-crossover mutants. Several colonies were isolated, cultured in mMRS-Tween80 broth for approximately 100 generations, and plated on mMRS-Tween80 agar at 30°C. The colonies were replica plated on mMRS-Tween80-erythromycin agar to identify erythromycin-sensitive double-crossover mutants. Gene replacement was confirmed by PCR amplification and sequence analysis. The phenotype was determined by LC-APPI-MS analysis of culture supernatant of

L. plantarum TMW1.460Δ*lah* grown in mMRS-Tween80 supplemented with 4 g/L linoleic acid.

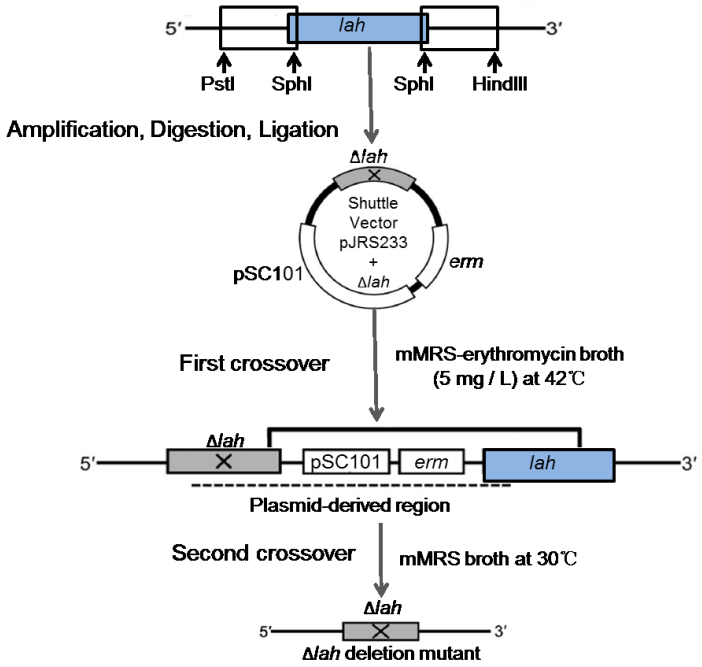


Figure 2-1. A schematic diagram of construction of *L. plantarum* TMW1.460Δ*lah*

2.2.8. Determination of ethanol resistance

Ethanol tolerance of *L. plantarum* TMW1.460 and TMW1.460Δ*lah* was carried out with strains grown in mMRS-Tween 80, or mMRS-Tween 20. Ethanol was added as indicated and media were sterilized by filtration after addition of ethanol. Stationary phase cells were harvested by centrifugation and resuspended in the same volume of mMRS-Tween 80 or -Tween 20 containing 20% ethanol. A 100 μl aliquot of cell suspension was analyzed as an untreated control. The samples were incubated in 30°C and aliquots were

removed in 1.5 h intervals and serially diluted in 0.85% NaCl. The appropriate dilutions were surface plated in duplicate on respective mMRS-Tween80 or -Tween20 agar and incubated at 30°C for 24 h. Ethanol resistance was determined in three independent experiments.

2.2.9. Determination of the membrane fluidity under ethanol stress

To investigate the membrane fluidity, LAURDAN (6-dodecanoyl-2-dimethylaminonaphthalene) (Thermo Fisher Scientific) was employed to measure generalized polarization (GP) value. *L. plantarum* TMW1.460 and TMW1.460Δ*lah* were cultivated at 30°C for 20 h in mMRS-Tween80 or mMRS-Tween 20. The membrane fluidity of the cells influenced by ethanol at the concentration ranging from 0% to 16% was assessed as described (Molina-Höppner et al., 2004). The effect was determined in three independent experiments.

2.2.10. Physicochemical properties of cells surface

Cell surface hydrophobicity was assessed by quantification of microbial adhesion to solvents (MATS) (Kankaanpää et al., 2004). Solvents used were: chloroform (polar and electron acceptor) and tetradecane (nonpolar), ethyl acetate (polar and electron donor) and octane (nonpolar). The MATS is based on the comparison between microbial cell affinity to a polar and nonpolar solvent within a couple that pose similar van der Waals surface tension components.

L. plantarum TMW1.460 and TMW1.460Δ*lah* were grown in mMRS-Tween 20, or mMRS without Tween but supplemented with 1 g/L oleic acid or linoleic acid. Cells were harvested by centrifugation, washed twice and resuspended in 150 mM NaCl to a final cell concentration of 10^8 CFU/ml. A 1 ml aliquot was removed as untreated control (A_0). A 2.4 ml aliquot of cell suspension was mixed with 0.4 ml of solvent by vortexing for 60s, respectively. The emulsified mixture was allowed to stand for 20 min to ensure the complete separation of the two phases. A sample (1 ml) was carefully taken from the aqueous phase (A). The optical cell density of sample A_0 and A was measured at 600 nm. The microbial adhesion percentage to each solvent was calculated with the equation: percent affinity=$100\times[1-(A/A_0)]$. Each measurement was determined in three independent experiments.

2.2.11. Extraction and purification of 13-HOE and 10-HOE

Extraction and purification of 13-HOE and 10-HOE were based on a protocol developed for 10-HOE (Black et al., 2013b). Fermentation of *L. hammesii* or *L. plantarum* TMW1.460Δ*lah* in mMRS-Tween 80 with 4 g/L linoleic acid was conducted for the production of 10-HOE or 13-HOE, respectively. The crude extraction of cellular lipids was performed as described above. Subsequently, the purification of 10-HOE or 13-HOE was based on a protocol described previously (Black et al., 2013b). The fermentation of *L. hammesii* or *L. plantarum* TMW1.460Δ*lah* in mMRS-Tween 80 with 4 g/L linoleic acid and the crude extraction of fatty acid mixtures were performed as described above. For

further purification, 25 mg of sample dissolved in chloroform was applied to a 500-mg Sep-Pak silica cartridge (Waters Ltd., Mississauga, ON, Canada) previously equilibrated with 6 ml chloroform. The cartridge was successively washed with the following gradient of isopropanol in chloroform: 35 ml of chloroform, followed by 18 ml of 1%, 5%, 10%, and 50% (vol/vol) isopropanol in chloroform. Eluates were collected and concentrated to dryness under nitrogen. The dry residues were dissolved in chloroform for analysis by LC-APPI-MS to identify the product, and the removal of contaminating lipids.

2.2.12. Determination of the antimicrobial activity of fatty acids

The minimum inhibitory concentration (MIC) was determined to assess the toxicity of linoleic acid to lactobacilli, and to determine the antifungal activity of 10-HOE, 13-HOE, coriolic acid, ricinoleic acid and linoleic acid. Lipids dissolved in ethanol were serially diluted two-fold in mMRS-Tween80 using 96-well microtiter plates. Ethanol in the samples was evaporated under a sterile laminar flow hood before inoculation with indicator strains. Stationary phase cells of lactobacilli were harvested by centrifugation, washed twice in mMRS-Tween80 and diluted to approximately 10^7 CFU/ml in the same medium. The plates were incubated at 30°C.

For evaluation of the antifungal activity, lipids diluted with serial two fold dilutions. Conidiospores of *Aspergillus niger* and *Penicillium roqueforti* were prepared as reported (Black et al., 2013b). The plate was inoculated with *A. niger* as an indicator organism and incubated at 25°C for 2 days, while the plate inoculated with *P. roqueforti* was incubated

for 3 days. Fungal growth without addition of lipids served as the positive control and media alone as the negative control. The MIC was defined as the lowest concentration of lipids that inhibited the growth of fungi when growth was visible in the positive control. MIC values were determined by six independent experiments.

2.2.13. Statistical analysis

Data analysis was conducted with R 3.1.2 (R Core Team, 2014). Significant differences in the assessment of cell survival and membrane fluidity under ethanol stress were determined by one-way analysis (ANOVA); post-hoc pairwise comparisons were conducted using the Tukey Honest Significant Differences (TukeyHSD) method. Significance was assessed at a 5% probability of error ($P<0.05$).

2.2.14. Accession numbers

The sequences of linoleate 10-hydratase in *L. reuteri*, *L. plantarum*, *L. hammesii* and *L. spicheri* were deposited in GenBank with accession numbers KX827285, KX827286, KX827287, KX827288 respectively.

2.3. Results

2.3.1. Identification of the products of linoleate conversion by lactobacilli

The products of linoleic acid conversion by five lactobacilli were analyzed by negative ion LC/APPI-MS/MS (Table 2-2). MS/MS spectra of the products confirmed the position

of hydroxyl groups (Figure 2-2) (Black et al., 2013a). The strains of lactobacilli differed in with respect to their conversion of linoleic acid to hydroxy fatty acids (Table 2-2). *L. reuteri*, *L. hammesii* and *L. spicheri* produced 10-HOE only, while *L. plantarum* produced 10-HOE, 13-HOE and 10,13-dihydroxy octadecanoic acid. *L. plantarum* TMW1.460Δ*lahA* produced 13HOE but not 10HOE or 10,13-dihydroxy octadecanoic acid, demonstrating that their formation by *L. plantarum* TMW1.460 is attributable to a dedicated linoleate 13-hydratase acting on linoleic acid and 10-HOE, respectively. *L. sanfranciscensis* did not convert linoleic acid.

2.3.2. Phylogenetic analysis of linoleate hydratase

Phylogenetic relationships of putative linoleate hydratases from lactobacilli were compared to the corresponding enzymes of the 24 type strains in the genus *Lactobacillus,* and type strains from the genera *Pediococcus*, *Weissella, Leuconostoc,* and *Oenococcus* (Figure 2-3). All four hydratases from lactobacilli that were investigated in this study belonged to myosin cross reactive antigen family. *L. sanfranciscensis* harboured no linoleate hydratase. The topology of the protein tree did not conform to the evolutionary relationship of the organisms (Zheng et al., 2015). The tree displayed two clusters but the two clusters do not differentiate between linoleate 10-hydratases and linoleate 13-hydratases (Figure 2-3).

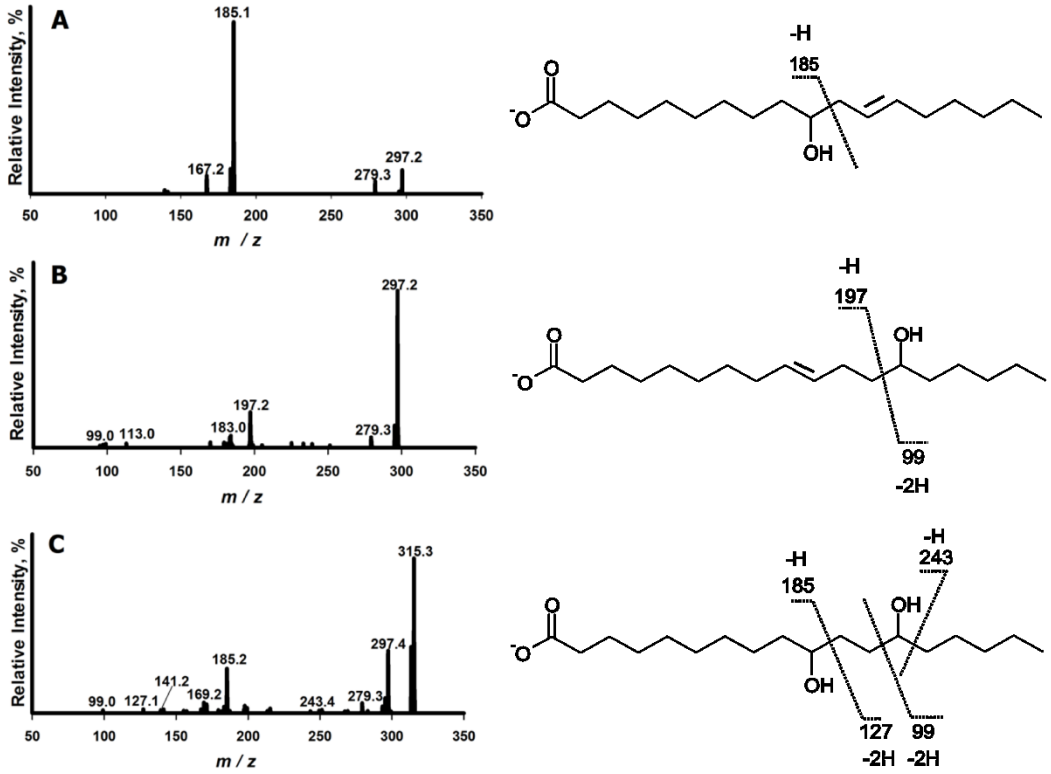


Figure 2-2. Fragmentation pattern and APPI-MS/MS spectra of hydroxy fatty acids produced when linoleic acid was used as the substrate. A, mass spectrum of 10-hydroxy-12- octadecenoic acid; B, mass spectrum of 13-hydroxy-9-octadecenoic acid; C, mass spectrum of 10,13-dihydroxy octadecanoic acid.

2.3.3. Characterization of linoleate 10-hydratase

Protein sequence analysis did not distinguish 10-hydratases from 13-hydratases, therefore (Figure 2-3), LAHs from four strains of lactobacilli were characterized biochemically after heterologous expression in *E. coli* and purification by affinity chromatography. A single band was observed by SDS-PAGE analysis after purification of the four enzymes (Figure 2-4); this band was absent in crude cellular extracts of *E. coli* strains prior to induction (data not shown). The genes from *L. reuteri*, *L. plantarum*, *L. hammesii* and *L. spicheri* encoded a protein of 590, 564, 564, 564 amino acids, respectively, matching the

Table 2-2. Comparison of products obtained from strain fermentation and enzymatic reaction with linoleic acid as substrate

Strain	Products of fermentation	Products of apoenzyme+FAD	Products of apoenzyme	Fragmentation ions (*m/z*)
L. reuteri LTH2584	10-HOE[a)]	10-HOE	No products	297[M-H]$^-$, 185
L. hammesii DSM16381	10-HOE	10-HOE	No products	297[M-H]$^-$, 185
L. spicheri LS38	10-HOE	10-HOE	No products	297 [M-H]$^-$, 185
L. plantarum TMW1.460	10-HOE, 13-HOE[a)] 10,13-HOA[a)]	10-HOE	No products	297 [M-H]$^-$, 185 297 [M-H]$^-$, 99, 197 315 [M-H]$^-$, 99, 127, 185, 243
L. plantarum TMW1.460 Δ*lah*	13-HOE	---	---	297 [M-H]$^-$, 99, 197
L. sanfranciscensis ATCC 27651	No products	---	---	---

[a)] 10-HOE, 10-hydroxy-12-octadecenoic acid; 13-HOE, 13-hydroxy-9-octadecenoic acid; 10,13-HOA, 10,13-dihydroxy octadecanoic acid

size of the major band observed by SDS-PAGE analysis (Figure 2-4). The addition of cofactor FAD to apoenzyme (refolded and purified LAH from inclusion body) was essential for activity (Table 2-2). LC-APPI-MS analysis revealed that all four recombinant proteins produced 10-HOE from linoleic acid, as demonstrated by the fragment ion at *m*/*z* 185.1 in the MS/MS spectra (Table 2-2). Hence, all four recombinant proteins were shown to be 10-hydratases. The substrate specificity of the linoleate 10-hydratase of *L. plantarum* TMW1.460 confirmed that formation of 13-HOE and 10,13-dihydroxy octadecanoic acid by this strain is likely attributable to a second and

dedicated linoleate 13-hydratase.

2.3.4. Most lactobacilli require oleic acid or linoleic acid for growth

Tween 80 is a derivative of oleate and a component of mMRS (Johnsson et al., 1995; Gänzle et al., 2000). mMRS-Tween20 did not support the growth of *L. reuteri*, *L. hammesii* and *L. sanfranciscensis* but did support the growth of *L. plantarum* and its Δ*lahA* mutant (data not shown). However, unsaturated fatty acids (UFAs) may also exhibit antibacterial activity (Desbois and Smith, 2010). Therefore, the MICs of oleic and linoleic acid to lactobacilli including *L. plantarum* TMW1.460Δ*lahA* were assessed. The MICs of oleic and linoleic acids were > 8 g/L for all strains, which indicates a high tolerance towards oleic and linoleic acid. *L. sanfranciscensis* showed less tolerance and its growth was inhibited by 1 g/L oleic acid and 0.5 g/L linoleic acid.

2.3.5. The effect of *lah* on stress tolerance in *L. plantarum*

To investigate the effect of hydroxy fatty acids or LahA itself on ethanol resistance, the survival of *L. plantarum* TMW1.460 and TMW1.460Δ*lahA* was assessed in mMRS-Tween80 and mMRS-Tween20 containing 20% ethanol. The ethanol tolerance of *L. plantarum* TMW1.460 and TMW1.460Δ*lahA* did not differ (Figure 2-5). However, the presence of Tween 80 in the growth medium enhanced bacterial survival (Figure 2-5).

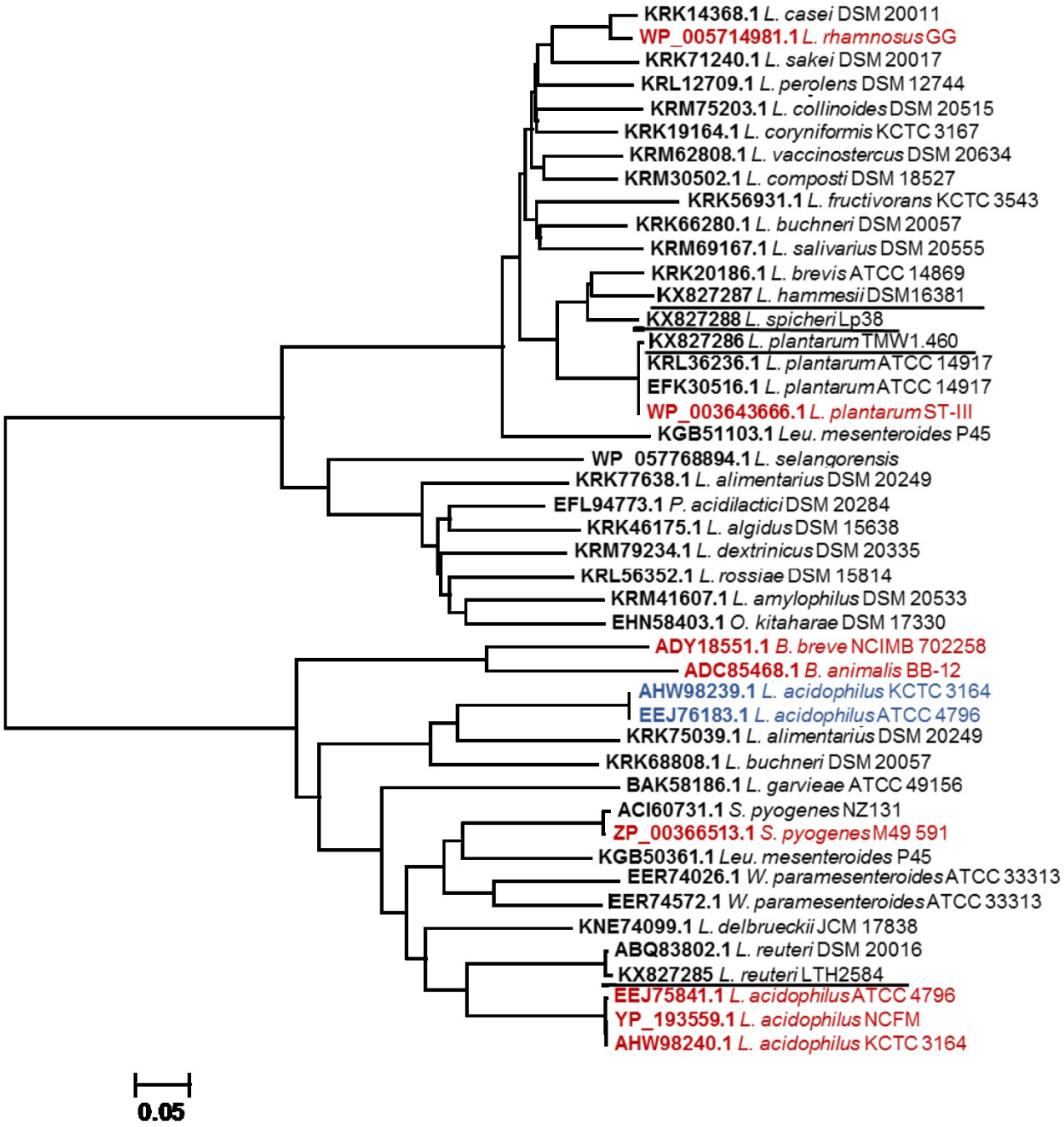


Figure 2-3. Phylogenetic tree of linoleate hydratases. The evolutionary relationships are shown with scale bar line which represents an evolutionary distance of 0.05. Linoleate 10-hydratases reported in the literature are highlighted in red, linoleate 13-hydratases are highlighted in blue, linoleate 10-hydratases that were characterized in this study are underlined.

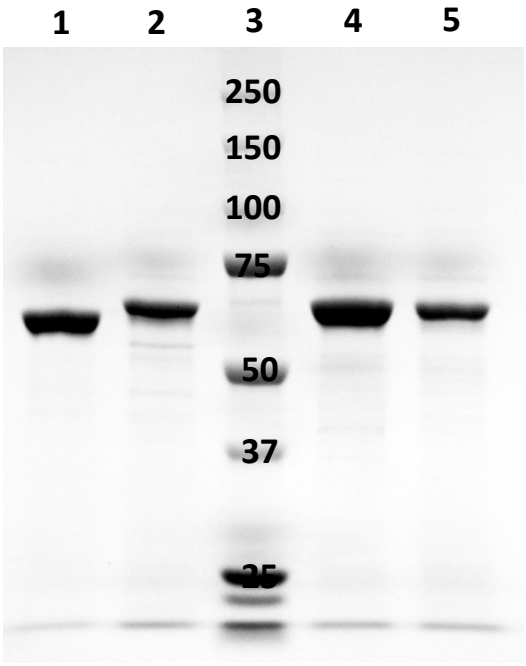


Figure 2-4. SDS-PAGE analysis of purified linoleate hydratases expressed from respective recombinant *E. coli* BL21 (DE3) cells. Lane 1, LAH of *L. reuteri*; lane 2, LAH of *L. plantarum*; lane 3, molecular mass marker proteins (250, 150, 100, 75, 50, 37 and 25 kDa); lane 4, LAH of *L. hammesii*; lane 5, LAH of *L. spicheri*.

2.3.6. The effect of *lahA* on ethanol-dependent membrane fluidity in *L. plantarum*

The ethanol-dependent membrane phase behavior of strains grown in mMRS-Tween80 or Tween 20 was analyzed (Figure 2-6). The GP values decreased with increasing ethanol concentration, indicating that ethanol increased the fluidity of the membrane. The response of the membrane fluidity of *L. plantarum* TMW1.460 and TMW1.460Δ*lahA* to ethanol was similar. Tween 80 supplemented medium induced a more fluid membrane in both strains.

2.3.7. Influence of *lahA* on cell surface properties in *L. plantarum*

The MATS of *L. plantarum* TMW1.460 and TMW1.460Δ*lahA* cultivated in mMRS

-Tween20, or mMRS-Tween20 supplemented with oleic or linoleic acid are shown in Figure 2-7. The deletion of *lahA* modified the properties of the cell surface. The adhesion of *L. plantarum* TMW1.460Δ*lahA* was more solvent-dependent when compared to the wild-type strain and the affinity of cells to the four solvents was generally higher for Δ*lahA* mutant than for wild-type strain. Similar trends were noted with strains grown in mMRS with different supplements, suggesting that the differences between wild-type and mutant strains are attributable to LahA, not the product of this enzyme. Similar behavior was observed when strains cultivated in Tween 80 (Data not shown).

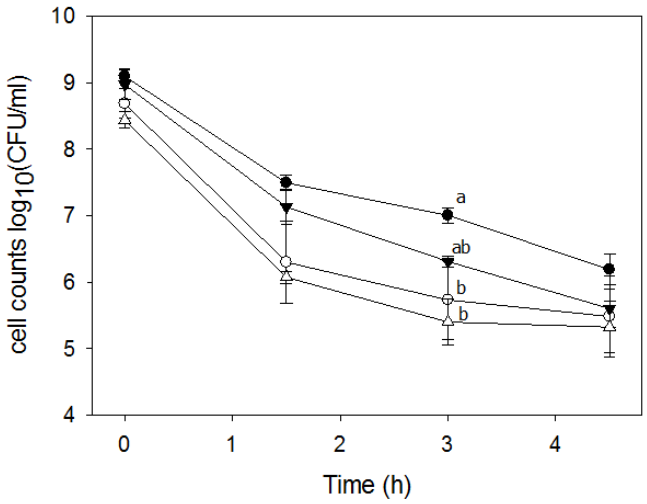


Figure 2-5. Survival of *L. plantarum* and its *lah* deficient derivative under 20% ethanol treatment. *L. plantarum* TMW1.460 was incubated in mMRS-Tween80 (closed circles) or mMRS-Tween20 (open circles); *L. plantarum* TMW1.460Δ*lah* was grown in mMRS-Tween80 (closed triangle) or mMRS-Tween 20 (open triangle) during treatment. Values obtained at the same treatment time that does not share common superscripts are significantly different ($P<0.05$). Data represent means ± standard deviations of three independent experiments with duplicate determinations of cell counts.

In this study, both wild-type and mutant strains exhibited low affinity for tetradecane and octane (nonpolar solvents), indicating that the cell surface of both strains was hydrophilic rather than hydrophobic. To determine the effect of deletion of *lahA* on the Lewis electron donor / electron acceptor property, the bacterial affinity to chloroform and ethyl acetate between different strains were also compared (Figure 2-7). *L. plantarum* wild-type strain showed similar adhesion ability to chloroform and ethyl acetate. However, regardless of the different supplement in medium, the adhesion of the mutant strain was always higher to chloroform (electron acceptor and acidic solvent) than to ethyl acetate (electron donor and basic solvent).

2.3.8. Antifungal properties of purified hydroxy fatty acids

13-HOE and 10-HOE were purified from organic extracts of cultures of *L. plantarum*TMW1.460Δ*lah* and *L. hammesii,* respectively when 4 g/L linoleic acid was used as the substrate. The major impurity detected in the organic phase was linoleic acid; other oxidized forms of hydroxy C_{18} fatty acids were also present. The compounds were purified by silica solid phase microextraction and analyzed by LC-APPI-MS/MS.

10-HOE or 13-HOE were eluted in the 1% isopropanol fraction and a single peak was observed in the LC-APPI-MS/MS chromatogram after purification. The antifungal activities of purified 13-HOE and 10-HOE against *A. niger* and *P. roqueforti* are shown in Table 2-3, and compared to reference lipids differing in the number and position of

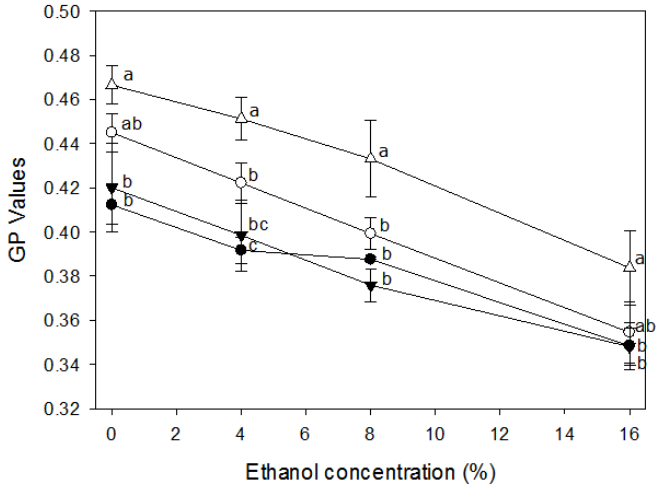


Figure 2-6. General polarization (GP) values of *L. plantarum* and its Δ*lah* derivative stained with LAURDAN under ethanol stress. *L. plantarum* TMW1.460 was cultivated in mMRS-Tween80 (closed circles) or mMRS-Tween 20 (open circles) prior to staining; *L. plantarum* TMW1.460Δ*lah* strain was cultivated in mMRS-Tween80 (closed triangle) or mMRS-Tween 20 (open triangle) prior to staining. Values obtained at the same treatment time that do not share common superscripts are significantly different ($P<0.05$). Data represent means ± standard deviations of three independent experiments with duplicate determinations of cell counts.

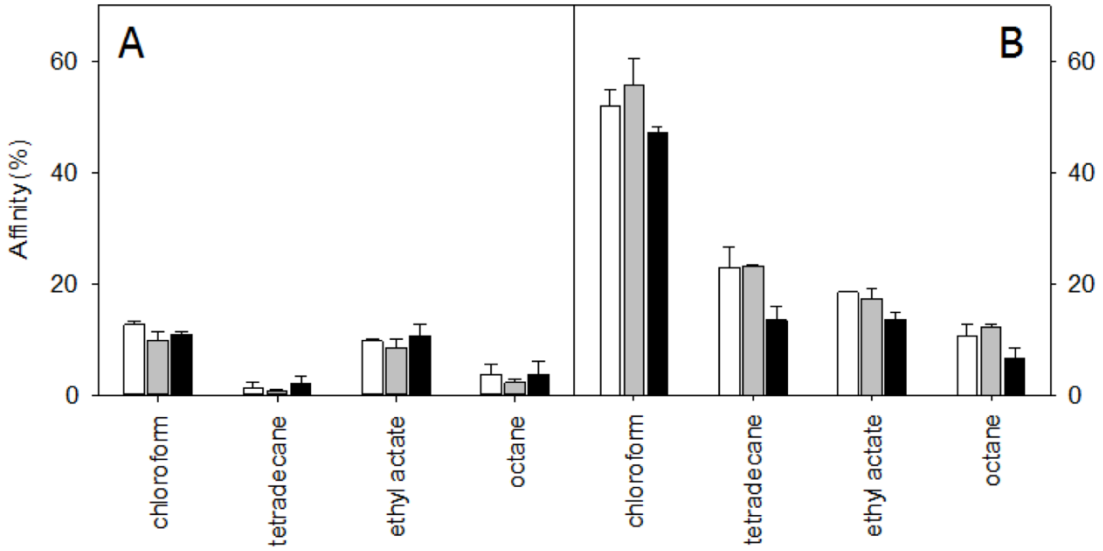
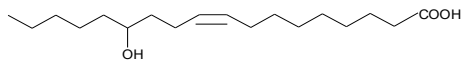
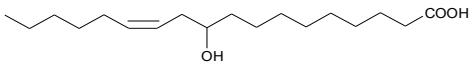
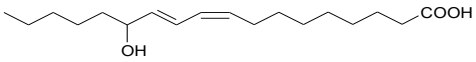
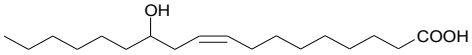
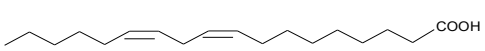


Figure 2-7. Effect of *lahA* on cell surface properties of *L. plantarum* grown in different media. Cell surface hydrophobicity was measured using the MATS method. Panel A: *L. plantarum* TMW1.460; Panel B: *L. plantarum* TMW1.460Δ*lah*. White bar indicates % affinity to solvents when cells were grown in mMRS -Tween 20; gray bar indicated in mMRS (Tween 20) supplemented with 1 g/L oleic acid; black bar indicated in mMRS (Tween 20) supplemented with 1 g/L linoleic acid. Data represent means ± standard deviations of three independent experiments.

hydroxyl groups or double bonds. Both coriolic acid and ricinoleic acid were active against all fungi indicators with MICs between 0.26 and 0.29. Linoleic acid showed the lowest antifungal activity.

Table 2-3. MICs of hydroxy fatty acids extracted from cultures of *L. hammesii* and *L. plantarum* Δ*lahA* and reference fatty acids (n=3)

structure	compound	MIC (g/L)	
		A. niger	*P. roqueforti*
	13-HOE	0.25±0.00	0.38±0.14
	10-HOE	0.42±0.13	0.38±0.14
	Coriolic acid	0.26±0.09	0.26±0.09
	Ricinoleic acid	0.29±0.10	0.29±0.10
	Linoleic acid	4.00±0.00	5.33±2.07

2.4. Discussion

Linoleate hydratases are highly conserved in both Gram-positive and Gram-negative bacteria (Volkov et al., 2010). This study revealed that linoleate 10-hydratase and linoleate 13-hydratase or enzymes that produce both 10-HOE and 13-HOE are not distinguished by phylogenetic and sequence analysis. All hydratases from lactobacilli examined in this study were determined as LahA, however, they are distributed in two different clusters which also contain linoleate 13-hydratase. Moreover, the topology of the protein tree disagreed with the evolutionary relationship of the organisms (Zheng et

al., 2015), indicating that linoleate hydratases are accessory proteins (Wyllie et al., 2000). Linoleate 10-hydratase is a FAD-containing enzyme and exhibits flavin-like UV absorbance (Rosberg-Cody et al., 2011). FAD cofactor is bound to the conserved FAD binding motif of LahA and stabilizes the active conformation of the enzyme but it is not directly involved in catalysis (Volkov et al., 2010; Yang et al., 2013). The non-covalently bound FAD is easily lost in the purification process (Volkov et al., 2013). This study confirmed that LahA is a FAD-dependent enzyme (Joo et al., 2012) and demonstrated that purification of active LahA from inclusion bodies required the addition of FAD. Different from the linoleate hydratase of *S. pyogenes*, which catalyses the formation of 10-HOE and 13-HOE (Volkov et al., 2010), the LahA characterized in this study formed exclusively 10-HOE from linoleate. The comparison of products formed by *L. plantarum* TMS1.460, the LahA deficient mutant of this strain, and the LahA of this strain strongly suggest that 13-HOE and 10,13 dihydroxy octadecanoic acid formation by this strain is attributable to a linoleate 13-hydratase that was recently characterized in *L. acidophilus* (Park et al., 2015).

UFAs are essential for growth of many lactic acid bacteria (LAB) (Johnsson et al., 1995; Gänzle et al., 2000) but high concentrations may inhibit the growth of LAB (Guerini et al., 2002). In contrast, in the present work oleic and linoleic acids stimulated the growth of *L. reuteri*, *L. hammesii* and *L. sanfranciscensis*. The bacteriostatic and bactericidal activities exerted by UFAs against lactobacilli is strain-dependent (Jenkins and Courtney, 2003); the observation that the LAH-negative *L. sanfranciscensis* was the only strain that

was inhibited by oleic and linoleic acids suggests that LAH contributes to these strain-specific differences.

Oleic acid in Tween 80 modulated membrane fluidity and influenced the ethanol tolerance of both *L. plantarum* TMW1.460 and TMW1.460Δ*lahA*. Consistent with my results, the addition of Tween 80 to the growth medium increased the viability of *Oenococcus oeni* in wine (Guerrini et al., 2002) and supplementation of UFAs to *Saccharomyces cerevisiae* protected against ethanol stress (Mishra and Prasad, 1989). However, there is no difference in ethanol resistance between *L. plantarum* TMW1.460 and TMW1.460Δ*lahA.* The protective effect of LahA that was previously observed in exponential phase bifidobacteria may relate to the presence of the enzyme rather than its products (Rosberg-Cody et al., 2011; O'Connell et al., 2013).

The physiochemical properties of the cell surface plays a critical role in the adhesion of pathogens and probiotics to intestinal surfaces. An evaluation of surface properties is achieved by determination of MATS (Bellon-Fontaine et al., 1996; Rosenberg, 2006). Both *L. plantarum* wild-type and *lahA* mutant strains displayed a hydrophilic surface character with weak adhesion to nonpolar solvents. Similar results were obtained in other *Lactobacillus* spp. and *Lactococcus* spp. (Pelletier et al., 1997; Boonaert and Rouxhet, 2000; Ly et al., 2006). The hydrophobicity of the cell surface changed when the bacteria were cultivated in medium with the addition of UFAs (Kankaanpää et al., 2004; Muller et al., 2011), which was not observed in my study. Bacteria grown in mMRS medium with or without different supplements exhibited similar affinity to solvents. However, *lahA*

deficiency resulted in a fundamental change in the profile of solvent affinity. Compared with wild type strain, *L. plantarum* TMW1.460Δ*lahA* presented more basic and electron-donating properties. The bacteria with basic character are considered to possess COO^- and HSO_3^- chemical groups on their cell surface (Pelletier et al., 1997). *L. casei* BL83, BL208 and BL229 also displayed low cell adhesion that was associated with their basic surface property (Munoz-Provencio et al., 2009). Indeed, the deletion of LahA was involved in the reduced adherence to human keratinocytes by *S. pyogenes* and to human intestinal epithelial cells by *L. acidophilus* (O'Flaherty and Klaenhammer, 2010; Volkov et al., 2010). In my study, LahA mediated differences in cell surface properties may explain the changed cell adhesion to human cells in the previous reports.

Linoleic acid possesses antifungal activity against the plant pathogenic fungi, especially for *Crinipellis perniciosa* at the concentration of 100 μM (Walters et al., 2004). In my study, linoleic acid was less active against *A. niger* and *P. roqueforti*, fungi that are commonly found in cereals and cereal products (Legan, 1993; Sjögren et al., 2003). Remarkably, the MIC of 13-HOE was approximately 15 times lower than that of linoleic acid. Therefore, 13-HOE may be as suitable as 10-HOE as an antifungal agent in foods (Black et al., 2013b). The antifungal activity of hydroxy fatty acids is likely related to their partitioning into lipid bilayers, thus increasing membrane permeability (Sjögren et al., 2003). The MICs of 13-HOE, 10-HOE along with coriolic acid and ricinoleic acid were all comparable, suggesting that all unsaturated monohydroxy fatty acids of C_{18} varying in their hydroxyl group position or degree of unsaturation exert antifungal

properties. Similar inhibition activities of *Aspergillus* and *Penicillium spp.* were detected among 9- and 13-hydroxy of C_{18} unsaturated fatty acid analogues of plant oxylipins (Prost et al., 2005). In contrast, dihydroxy saturated C18 fatty acids do not display antifungal activity (Black et al., 2013b).

In conclusion, my study revealed that the differentiation of accessory proteins between linoleate 10-hydratase and linoleate 13-hydratase cannot be achieved by phylogenetic analysis. Thus the linoleate hydratases from lactobacilli were characterized by heterologous expression and identified as FAD-dependent linoleate 10-hydratases. Generation of a LahA-deficient mutant of *L. plantarum* demonstrated that 13-HOE generated by a different and dedicated hydratase, and is a novel antifungal hydroxy fatty acid. The most prominent physiological difference of the LahA deficient mutant and the wild type strain was the altered surface hydrophobicity of the bacterial cells. *L. plantarum* is part of the phyllosphere of many plants (Minervini et al., 2015) and oxylipids are an important component of the plant defense against pathogens (Prost et al., 2005). It is possible that the lipid-converting properties of linoleate hydratases and their influence on cell surface properties are components of host-microbe interactions.

2.5. References

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Chapter 3. Influence of cyclopropane fatty acids on heat, high pressure, acid and oxidative resistance in *Escherichia coli*

3.1. Introduction

Pathogenic strains of *Escherichia coli* are a major public health concern. Pathovars in the species *E. coli* include enterotoxigenic and enteropathogenic *Eschericha coli*, which are significant causes of childhood diarrhea, Shiga-toxin producing *E. coli* (STEC), which cause the hemolytic-uremic syndrome in humans, and *Shigella* spp., a human adapted pathovar which is a major cause of childhood diarrhea in the developing world (Croxen et al., 2013; Lanata et al., 2013; Niyogi 2005; Rasko et al., 2011). *Shigella* spp. and STEC have a very low infectious dose, which necessitates elimination of even low levels of contamination. Control of *E. coli* in food, however, is challenged by its resistance to heat, pressure, acid, and oxidative stress. *E. coli* is relatively resistant to short-term exposure to oxidative stress or low pH (Foster, 2004; Storz and Imlay, 1999). *E. coli* also exhibits strain-specific resistance to heat (Juneja et al., 1997; Liu et al., 2015; Mercer et al., 2015; Smith et al., 2001). Strains of *E. coli* are among the most pressure resistant vegetative bacterial cells (Vanlint et al., 2012); some STEC strains resist application of 600 MPa (Liu et al., 2015, Gänzle and Liu, 2015).

Maintaining or establishing food safety by pathogen intervention technologies requires an improved understanding of the mechanism in stress resistance and cross-resistance to different environmental stressors. The cytoplasmic membrane is the main barrier of

defense against environmental stresses. Bacterial survival depends on the ability to adjust lipid composition to acclimatize cells to different environments (Zhang and Rock, 2008). The synthesis of cyclopropane fatty acids (CFA) is a modification of the membrane phospholipids that occurs in early stationary phase (Wang and Cronan Jr, 1994; Zhang and Rock, 2008). CFA are formed by a soluble enzyme, CFA synthase, which transfers a methylene group from *S*-adenosyl-L-methionine to *cis* double bonds of unsaturated fatty acids in membrane phospholipids (Cronan Jr et al., 1979; Huang et al., 2002). CFA in the cytoplasmic membrane protect bacterial cells against several environmental stressors including ethanol (Grandvalet et al., 2008; Teixeira et al., 2002), high osmotic pressure (Asakura et al., 2012; Guillot et al., 2000), low pH (Brown et al., 1997; Chang and Cronan Jr, 1999), and repeated freeze-thaw cycles (Grogan and Cronan Jr, 1986; Zavaglia et al., 2000). A high CFA content in membrane lipids also improves survival after freeze-drying (Muñoz-Rojas et al., 2006). The mutational disruption of *cfa* also increased the sensitivity of *E. coli* to pressure (Charoenwong et al., 2011). The heat resistant strain *E. coli* AW1.7 exhibits high levels of CFAs in the cytoplasmic membrane, which suggests a contribution to the exceptional heat resistance of this strain (Ruan et al., 2011). However, the role of CFAs in heat resistance of *E. coli* is poorly documented and its relevance to the survival of *E. coli* during food processing remains unknown.

This study aimed to investigate the role of CFA on stress resistance in the heat- and pressure resistant beef isolate *E. coli* AW1.7 (Dlusskaya et al., 2011; Liu et al., 2012) and the reference strain *E. coli* MG1655. The role of CFA in stress resistance was evaluated

by comparison of CFA levels in the cytoplasmic membrane, by disruption of *cfa* in both strains and characterization of the stress resistance of mutant strains, and by assessing their resistance to heat treatment in food.

3.2. Materials and methods

3.2.1. Bacterial strains and culture conditions

Bacterial strains and plasmids used in this study are described in Table 3-1. Strains *E. coli* AW1.7 and MG1655 are genome-sequenced and non-pathogenic strains of *E. coli* (Mercer et al., 2015). *In silico* analysis of their serotypes (https://cge.cbs.dtu.dk/services/SerotypeFinder/, Joensen et al., 2015) predicted their serotypes as *E. coli* O128:H12 AW1.7 and *E. coli* O16:H48 K12 MG1655. *E. coli* DH5α served as a host for plasmids in the cloning procedures. *E. coli* strains were cultivated in Luria-Bertani (LB) medium (BD, Mississauga, CA) with agitation at 200 rpm and 37°C. Antibiotic-resistant *E. coli* carrying plasmid pUC19 or pKOV were selected by addition of 50 mg/l ampicillin or 34 mg/l chloramphenicol, respectively. Working cultures were activated by streaking strains from a -80°C stock culture onto LB agar, followed by two successive 18 h subcultures to obtain stationary phase cultures.

3.2.2. DNA manipulation

Genomic DNA was isolated with the Blood & Tissue Kit (Qiagen, Hilden, Germany). Primers were synthesized by Integrated DNA Technologies (SanDiego, USA). The Taq

DNA polymerase was purchased from TaKaRa Bio (Shiga, Japan) and T4 DNA ligase and restriction enzymes from Thermo Scientific (Mississauga, CA). The Miniprep and DNA gel extraction kits were obtained from Qiagen. The DNA sequencing was conducted by Macrogen (Maryland, USA).

Table 3-1. Bacterial strains and plasmids used in this study

Strains	Description	Reference or Source
E. coli AW1.7	heat- and pressure- resistant strain, isolated from beef-packing plant	(Aslam et al., 2004)
E. coli K-12 MG1655	heat- and pressure- sensitive reference strain	(Guyer et al., 1981)
E. coli DH5α	Cloning host for plasmids	New England Biolabs
E. coli AW1.7 Δ*cfa*	*E. coli* AW1.7 with truncated *cfa*	This study
E. coli MG1655 Δ*cfa*	*E. coli* MG1655 with truncated *cfa*	This study
Plasmids	**Description**	**Reference or Source**
pUC19	lacZα promoter; cloning vector used in *E. coli*; Amp^r	New England Biolabs
pUC19/A-AW	pUC19 plasmid with 1 kb fragment of *cfa* upstream region in *E. coli* AW1.7; Amp^r	This study
pUC19/B-AW	pUC19 plasmid with 1 kb fragment of *cfa* downstream region in *E. coli* AW1.7; Amp^r	This study
pUC19/AB-AW	pUC19 plasmid with 2 kb of *cfa* upstream and downstream fragment of *E. coli* AW1.7; Amp^r	This study
pUC19/A-MG	pUC19 plasmid with 1 kb fragment of *cfa* upstream region in *E. coli* MG1655; Amp^r	This study
pUC19/B-MG	pUC19 plasmid with 1 kb fragment of *cfa* downstream region in *E. coli* MG1655; Amp^r	This study
pUC19/AB-MG	pUC19 plasmid with 2 kb of *cfa* upstream and downstream fragment of *E. coli* MG1655; Amp^r	This study
pKOV	Temperature sensitive pSC101; Sac B; Cm^r	Link et al., 1997
pKOV/Δ*cfa*-AW	pKOV plasmid with 2 kb of *cfa* flanking fragment of *E. coli* AW1.7; resulting *cfa* deletion; Cm^r	This study
pKOV/Δ*cfa*-MG	pKOV plasmid with 2 kb of *cfa* flanking fragment of *E. coli* MG1655; resulting *cfa* deletion; Cm^r	This study

Amp^r: ampicillin-resistance gene; Cm^r: chloramphenicol -resistance gene.

3.2.3. Construction of *E. coli* mutant strains

To disrupt the gene *cfa* from the genome of *E. coli* AW1.7 and MG1655 (Accession number LDYJ00000000 and NC_000913.3, respectively), an in-frame, unmarked deletion was performed by using the method of Link et al. (1997). The approximately 1000 bp 5'-flanking regions (fragment A) and 1000 bp 3'-flanking regions (fragment B) of the target genes *cfa* in *E. coli* AW1.7 and MG1655 were amplified by PCR with primers listed in Table 3-2. The fragment A was digested with XbaI, SphI, and fragment B was digested with SphI, HindIII. The resulting fragments were purified and sequentially ligated into vector pUC19 to generate pUC19/AB. The AB fragments in pUC19/AB were confirmed by sequencing, amplified with primers carrying an additional BamHI restriction site, and subcloned into the XbaI and BamHI restriction sites of the pKOV plasmid to create pKOV/Δ*cfa*-AW and pKOV/Δ*cfa*-MG. The resulting recombinant plasmids were transformed into electrocompetent *E. coli* at 12.5 kV/cm, 25 μF and 200 Ω. The cells were plated on chloramphenicol-LB plates and incubated at 43°C to select for single-crossover mutants. Several colonies were isolated, serially diluted in LB broth, and plated on 5% (wt/vol) sucrose-LB plates and incubated at 30°C. Sucrose-resistant colonies were subsequently replica plated on chloramphenicol plates at 30°C to identify the double-crossover mutants. Gene replacement was confirmed by PCR amplification and sequencing.

3.2.4. Membrane fatty acid composition

For total fatty acid extraction, cells grown to stationary phase were collected by centrifugation, washed twice with 10 mM phosphate-buffered saline (PBS) (pH 7.2), and lipids were extracted using a modified Bligh and Dyer (1959) procedure. Briefly, lipids in the fresh cell pellet were extracted twice with chloroform–methanol–water (1:2:0.8), followed by addition of chloroform-water (1:1) to allow partitioning water and organic phase. The lower chloroform phase was removed and evaporated to dryness under nitrogen. Lipids were converted to fatty acid methyl esters (FAME) by base-catalyzed transesterification. Fatty acid samples (less than 50 mg) were saponified with 1 ml dry toluene and methylated with 0.5 M sodium methoxide in 2 ml anhydrous methanol for 30 min at 50°C. The reaction was terminated by addition of 0.1 ml glacial acetic acid and 5 ml water. FAME were extracted twice with 5 ml hexane, and the solvent was removed under a stream of nitrogen. FAME were redissolved in 1 ml of hexane and analyzed with gas chromatography–mass spectrometry (GC-MS) (Model 7890A/5975C; Agilent Technologies, Santa Clara, CA, USA) fitted with a silica capillary column HP-5 ms (30 m length; 0.25 mm inner diameter; 0.25 μm film thickness). The splitless injection volume was 1 μl, and the temperature of the injector was 250°C. Helium was used as the carrier gas with a flow rate of 4.4 ml/min. The oven temperature program was 50°C held for 2 min, increased at a rate of 5°C/min to 325°C, and 325°C held for 5 min. The mass spectrometer was performed with a full scan model, from 50 to 400 m/z. The temperature of mass source and mass quadrupole was 230°C and 150°C, respectively. FAME were

identified by comparing their retention times with those of a standard mixture (463 NuChek Prep, Elysian, USA), and by comparing the mass spectra with reference spectra (NIST 05). Furthermore, compounds were quantified by comparing individual peak area with those in a standard mixture of known concentration. The fatty acid composition was expressed as relative percentages (weight/weight).

Table 3-2. Primers used in this study

Primers (forward, F; reverse, R)	Sequence (5'-3')	Restriction site
AW-upstream A, F	5'-CTAGTCTAGACTGAATGGGCAGCAAAAAGAAGAAGCAGGG-3'	XbaI
AW-upstream A, R	5'-ACATGCATGCTGTGCAGGTGCTTTCCGCGCCCGTG-3'	SphI
AW-downstream B, F	5'-ACATGCATGCGGCTATACCGGCACGGCTAAGTAATTCG-3'	SphI
AW-downstream B, R1	5'-CCCAAGCTTTATCGCGGCAACGTGCTGATATACGCG-3'	HindIII
AW-downstream B, R2	5'-CGCGGATCCCCCAAGCTTTATCGCGGCAACGTGCTGATATACG-3'	BamHI
MG-upstream A, F	5'-CTAGTCTAGATATCGCGGCAACGTGCTGATATACGCAGCC-3'	XbaI
MG-upstream A, R	5'-ACATGCATGCGGCTATACCGGCACGGCTAAGTAATTC-3'	SphI
MG-downstream B, F	5'-ACATGCATGCTGTGCAGGTGCTTTCCGCGCCCGTG-3'	SphI
MG-downstream B, R1	5'-CCCAAGCTTCTGAATGGGCAGCAAAAAGAAGAAGCAGGG-3'	HindIII
MG-downstream B, R2	5'-CGCGGATCCCCCAAGCTTCTGAATGGGCAGCAAAAAGAAGAAGCAG-3'	BamHI

3.2.5. Determination of stress resistance

3.2.5.1. Determination of heat resistance

Cells from stationary-phase cultures (18 h) were harvested by centrifugation and resuspended in an equal volume of LB broth, yielding a final cell concentration of about 10^8 CFU/ml. Three 200 µl aliquots of cell suspensions were placed in 1.5 ml Eppendorf tubes (Fisher Scientific, Toronto, CA) and heated in a water bath. The heat resistant strain *E. coli* AW1.7 was treated at 60°C, while the heat sensitive strain *E. coli* MG1655 was

treated at 57°C to achieve a comparable reduction of cell counts. Samples were submerged in ice water after 15 and 30 min treatments. To determine viable cell counts, appropriate dilutions were surface plated in duplicate on LB agar by using spiral plater (Don Whitley Scientific Limited, West Yorkshire, UK). Colonies were counted after the plates were incubated at 37°C for 24 h. Survival was expressed as $\log(N_0/N)$ where N_0 designates cell counts of untreated cultures and N the cell counts of treated cultures. Heat resistance was determined in three independent experiments analyzed in duplicate.

3.2.5.2. Determination of pressure resistance

The pressure resistance of strains of *E. coli* was assessed with stationary-phase cells that were prepared as described above. Cell suspensions were packed into sterile 3 cm E3603 tubing (Fisher Scientific, Akron, OH, USA), heat sealed from both sides, and kept on ice before treatment. The samples were placed in a 2.2 ml pressure vessel (Micro-system, Unipress, Warsaw, Poland) filled with bis (2-ethylhexyl) sebacate (Sigma-Aldrich, Germany) as pressure-transmitting fluid. The pressure vessel was submerged in a water bath maintained at 20°C. *E. coli* AW1.7 cells were treated at 500 MPa for 10 and 30 min, while MG1655 cells were treated for 5 and 10 min. The rates of compression and decompression were 277.8 MPa/min. The temperature in the pressure vessel was monitored by an internal thermocouple and the temperature changes during compression and decompression did not exceed 4.5°C. The viable cell counts in untreated and pressure-treated samples were determined as described above. The pressure resistance

was determined in three independent experiments analyzed in duplicate.

3.2.5.3. Determination of acid resistance

The acid tolerance of *E. coli* was carried out in LB broth adjusted to pH 2.5 with HCl and sterilized by filtration. Stationary phase cells from 1 ml of culture were harvested by centrifugation and resuspended in the same volume of acidified LB broth to a cell density of approximately 10^8 CFU/ml. A 100 µl aliquot of cell suspension was analyzed as an untreated control. The samples were incubated at 37°C and aliquots were removed in 1 h intervals transferred into saline. Subsequent serial dilutions were plated on LB agar. The acid resistance was determined in three independent experiments analyzed in duplicate.

3.2.5.4. Determination of resistance to oxidative stress

Oxidative stress resistance of *E. coli* was assessed by exposure to 50 mM hydrogen peroxide (Elkins et al., 1999). Stationary phase cells were harvested by centrifugation, washed in 10 mM phosphate-buffered saline (PBS) (pH 7.2), and diluted to a cell count of approximately 10^7 CFU/ml. H_2O_2 (Sigma-Aldrich, Germany) was added to the cell suspension at a final concentration of 50 mM and cell suspensions were incubated at 37°C. Aliquots were withdrawn after 10 and 20 min treatments, serially diluted in PBS containing 0.2 % sodium thiosulfate (w/v) (Sigma-Aldrich, Germany) to neutralize the H_2O_2 before plating. The resistance to oxidative stress was determined in three independent experiments analyzed in duplicate.

3.2.6. Heat treatment in ground beef

The heat treatment of ground beef patties inoculated with *E. coli* AW1.7, MG1655 or their respective Δ*cfa* mutants was performed as described (Liu et al., 2015). In brief, lean ground beef with 4% or 10 % fat was obtained from a local processing plant on the day of processing and stored at -20°C until use. Coliform counts of the ground beef were determined by plating on violet red bile agar. Coliform counts in ground beef were less than 250 CFU/g and coliforms were eliminated to counts below the detection limit after grilling. Cells from 10 ml of 10^8 CFU/ml stationary-phase culture were mixed with 200 g ground beef in a sterile plastic bag and homogenized for 2 min with a Stomacher Lab-Blender. To determine cell counts before cooking, 20 g of inoculated meat were removed and transferred into a sterile plastic bag containing 20 ml of ice-chilled 0.1 % peptone water (BD, Mississauga, CA). The remaining meat was molded into a patty with a Single Hamburger Press (Weston Brand Pragotrade, Strongsville, OH, USA). The beef patties were grilled in a clam shell grill (Cuisinart 5-in-1 Griddler, Woodbridge, CA) to an internal temperature of 71±0.3°C. The internal temperature was monitored by a digital thermometer (Barnant Company, Barrington, USA) inserted into the geometric center of the patty. The cooked beef patty was immediately placed into 200 ml ice-chilled 0.1% peptone water. Both uncooked control and cooked samples were stomached for 2 min separately, serially diluted in 0.1% peptone water, and surface plated on LB agar for colony enumeration. The heat resistance in ground beef was determined in three

independent experiments analyzed in duplicate.

3.2.7. Statistical analysis

Data analysis was conducted with R 3.1.2 (www.r-project.org/). Significant differences in the membrane fatty acid composition were determined by one-way analysis (ANOVA); post-hoc pairwise comparisons were conducted using the Tukey Honest Significant Differences (TukeyHSD) method. Significant differences in the resistance of strains of *E. coli* to stress were determined by using T-test. Significance was assessed at a 5% probability of error ($P<0.05$); an error probability of 5 to 10% ($0.05 <P<0.1$) was reported as a trend.

3.2.8. Nucleotide sequence accession numbers

The sequences of the truncated *cfa* gene in *E. coli* AW1.7 and MG1655 were deposited in GenBank with accession numbers KT591116 and KT591115 respectively.

3.3. Results

3.3.1. Construction of *E. coli* mutant strains

To investigate the potential role of CFAs on stress tolerance, the mutants deficient in CFA synthesis were generated with the suicide vector pKOV. The *cfa* of *E. coli* AW1.7 is 99% identical to *cfa* in *E. coli* K-12 MG1655. To compare the influence of *cfa* on stress tolerance between heat- and pressure-resistant strain *E. coli* AW1.7 and susceptible strain

E. coli MG1655, *cfa* was inactivated in both strains with an in-frame, unmarked deletion method, which does not introduce polar effects on the downstream genes (Link et al., 1997). The mutations of *cfa* were confirmed by PCR amplification and sequencing.

3.3.2. Membrane fatty acid composition of wild-type and mutant strains

In *E. coli* wild-type cells, three main fatty acids were detected, $C_{16:0,}$ $C_{17:0}$cyclopropane and $C_{19:0}$cyclopropane fatty acids (Table 3-3), which represented about 85% of membrane fatty acids in *E. coli* AW1.7 and MG1655. Membranes from these cells also contained $C_{14:0}$, $C_{15:0}$ and $C_{17:0}$ fatty acids. The phenotype of *cfa* deletion mutants were confirmed by analysis of membrane fatty acids (Table 3-3). Disruption of *cfa* dramatically altered fatty acid profiles when compared to the corresponding wild-type strains. The major effect of *cfa* disruption was the absence of CFA and the accumulation of the corresponding substrates, $C_{16:1}$ and $C_{18:1}$ unsaturated fatty acids. Moreover, the abundance of the saturated $C_{16:0}$ was decreased in both Δ*cfa* mutant strains; this difference was significant (P<0.05) for the comparison of *E. coli* MG1655; a trend (P<0.1) was observed when comparing *E. coli* AW1.7 and the corresponding Δ*cfa* mutant.

The proportion of $C_{18:1}$ fatty acid in both *cfa*-deficient mutants was approximately equivalent to the proportion of $C_{19:0}$ cyclopropane fatty acid- in the respective wild-type strains. Interestingly, the proportion of $C_{16:1}$ in mutant cells was approximately equal to the sum of its homologous $C_{17:0}$ cyclopropane fatty acid plus the increased portion of

$C_{16:0}$ in respective wild-type cells. The saturation index of lipids was 1.00 and 0.48 in stationary phase *E. coli* AW1.7 and AW1.7Δ*cfa*, and 1.00 and 0.53 in stationary phase *E. coli* MG1655 and MG1655Δ*cfa*, respectively. The increased level of unsaturated fatty acids in the two Δ*cfa* mutant strain reflects the accumulation of $C_{16:1}$ and $C_{18:1}$ unsaturated fatty acids.

The proportion of $C_{19:0}$ cyclopropane fatty acid in *E. coli* AW1.7 was significantly higher when compared to *E. coli* MG1655 (P<0.05). Correspondingly, *E. coli* AW1.7Δ*cfa* had a higher proportion of $C_{18:1}$, the substrate of $C_{19:0}$ cyclopropane fatty acid, and a lower proportion of $C_{16:0}$ when compared to *E. coli* MG1655Δ*cfa* (P<0.05). These differences also resulted in a lower saturation index for AW1.7Δ*cfa* than MG1655Δ*cfa.*

3.3.3. Stress resistance of *E. coli* cells

To examine whether differences in the composition of the cytoplasmic membrane between *E. coli* strains relates to the difference in stress tolerance, survival under different stress conditions was quantified. Stress conditions were selected to reduce cell counts of the wild type strains by 1 – 6 $\log_{10}$ (CFU/mL). During exposure to each stress condition, survival of wild-type strains and the corresponding Δ*cfa* mutant strains was monitored after several time points. To illustrate the difference in stress tolerance in a more concise overview, only two treatment times are displayed in Figure 3-1.

The wild strains were more heat resistant when compared to the corresponding Δ*cfa* mutants. Disruption of *cfa* increased the reduction of cell counts of *E. coli* AW1.7 after

Table 3-3. Fatty acid composition of lipids extracted from stationary-phase *E. coli* AW1.7 and MG1655 and the cognate Δ*cfa* mutant strains.

Fatty acid	% of total membrane fatty acids [1)]			
	AW1.7	AW1.7Δ*cfa*	MG1655	MG1655Δ*cfa*
$C_{14:0}$	7.30±2.08[a2)]	6.07±0.29[a]	7.74±1.40[a]	6.12±0.78[a]
$C_{15:0}$	4.29±0.69[a]	3.50±0.14[a]	4.30±0.33[a,]	3.59±0.27[a]
$C_{16:1}$	ND[3)]	32.27+0.98[a]	ND	33.11±1.00[a]
$C_{16:0}$	38.84±0.94[bc]	34.63±0.90[c]	45.37±3.32[a]	39.57±0.74[b]
$C_{17:0}$cyclopropane	27.47±2.57[a]	ND	26.43±1.82[a]	ND
$C_{17:0}$	3.61±0.37[a]	4.12±0.29[a]	3.98±1.20[a]	4.12±0.83[a]
$C_{18:1}$	ND	19.41±0.23[a]	ND	13.50±0.7[b]
$C_{19:0}$cyclopropane	18.49±0.84[a]	ND	12.18±2.62[b]	ND
SI[4)]	1.00±0.00[a]	0.48±0.01[c]	1.00±0.00[a]	0.53±0.01[b]

[1)] % of total membrane fatty acids; values are expressed as mean ± standard deviation of three independent experiments.
[2)] Within a row, values with different superscripts are different at $P<0.05$.
[3)] ND, not detected.
[4)] SI, saturation index, sum of saturated fatty acids including CFAs /100%

treatment at 60°C for 30 min by 1.1 $\log_{10}$ (CFU/ml). Cell counts of *E. coli* MG1655 were additionally reduced by 0.9 $\log_{10}$ (CFU/ml) after treatment of the corresponding Δ*cfa* mutant at 57°C for 15 min (Figure 3-1). Likewise, *cfa* disruption increased the lethality of pressure treatment at 500 MPa by equal to or greater than one $\log_{10}$ (CFU/ml) for various time points. Collectively, these data confirm the heat and pressure resistance of *E. coli* AW1.7 (Dlusskaya et al., 2011; Garcia-Hernandez et al., 2015) and demonstrate that CFA synthase contributes to the resistance to both stress conditions. In keeping with prior data on the role of CFAs in acid tolerance of *E. coli*, the survival of both Δ*cfa* mutant strains in acidified LB broth (pH 2.5) was significantly reduced ($P<0.05$) when compared

to the survival of the respective wild-type strains (Figure 3-1). *E. coli* AW1.7 was less acid resistant than *E. coli* MG1655. Survival of the two wild-type strains and the respective Δ*cfa* mutant strains in presence of 50 mM H_2O_2 was similar (Figure 3-1).

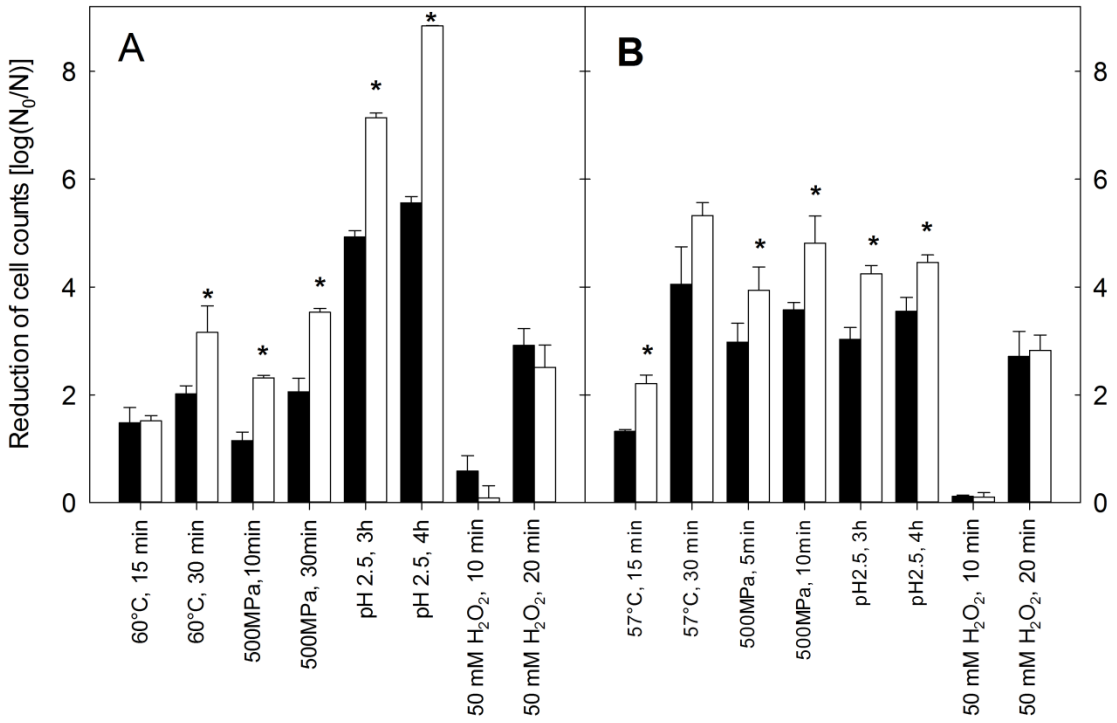

Figure 3-1. Resistance of strains of *E. coli* to heat, pressure, acid, and oxidative stress. Panel A: *E. coli* AW1.7; Panel B: *E. coli* MG1655. Black bars indicate wild-type strains; white bars indicate Δ*cfa* mutant strains. Asterisks indicate significant differences between wild-type and respective Δ*cfa* mutant strains (* $P < 0.05$). Data represent means ± standard deviations of three independent experiments with duplicate determinations of cell counts.

3.3.4. Thermal inactivation of *E. coli* cells in ground beef

To validate the contribution of CFAs to heat resistance in food, beef patties with 4% or 10% fat were individually inoculated with *E. coli* AW1.7, MG1655 or their Δ*cfa* mutants were grilled to an internal temperature of 71°C. The cell counts in uncooked control were

approximately 10^7 CFU·g^{-1}. *E. coli* MG1655 was more resistant to treatment than the Δ*cfa* mutant strain (P<0.05). *E. coli* AW1.7 was more resistant to treatment in beef with 4% fat; a similar trend was noted in beef with 10% fat (Figure 3-2).

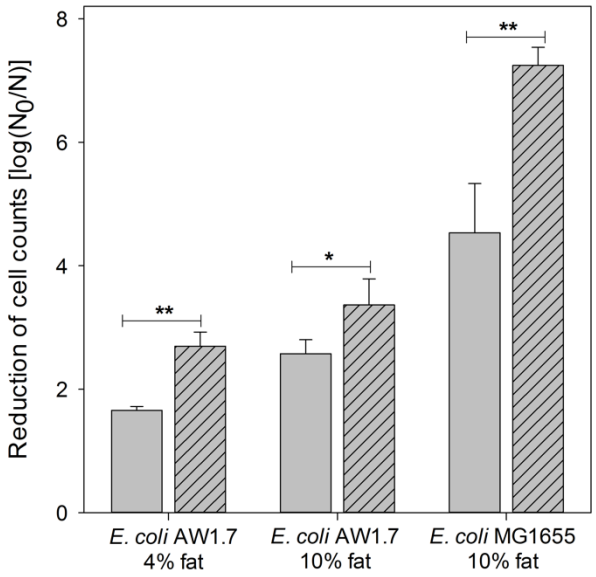

Figure 3-2. Survival of *E. coli* AW1.7 and *E. coli* MG1655 and their respective Δ*cfa* mutant strains (hatched bars) in beef patties cooked to an internal temperature of 71±0.3°C. Ground beef contained 4% or 10% fat as indicated. Asterisks indicate a trend (*, 0.05<P < 0.1) or a significant difference between wild-type and the respective Δ*cfa* mutant strains (**, P < 0.05). Data represent means ± standard deviations of three independent experiments with duplicate determinations of viable cell counts.

3.4. Discussion

CFAs are major components of membrane phospholipids in many bacteria including *E. coli* (Grogan and Cronan Jr, 1997). The conversion of unsaturated fatty acids by CFA synthase renders the membrane more stable to environmental insults (Zhang and Rock, 2008). The contribution of CFAs to bacterial heat resistance, however, remains poorly documented and its contribution to survival during food processing has not been

documented. In this study, two *cfa*-deficient mutant strains were generated. To explore the role of CFAs in stress tolerance, survival of wild-type and the respective Δ*cfa* mutant strains was compared after exposure to heat, high pressure, acid, and oxidation, and after cooking of beef to a core temperature of 71°C.

Deletion of *cfa* substantially altered the profile of membrane phospholipids. Stationary phase cells of wild type strains contained high levels of CFAs but no unsaturated fatty acids. In contrast, membranes of *cfa* mutant strains contained high levels of unsaturated fatty acids. Membranes of exponentially growing cells of *E. coli* AW1.7 had a lower proportion of CFAs but a higher proportion of UFAs (Ruan et al., 2011) when compared to data obtained in this study for the same strain. The different in CFA levels in exponentially growing and stationary phase cells are attributable to the σ^S-mediated increased expression of CFA synthase during the stationary phase of growth (Pagán and Mackey, 2000; Wang and Cronan Jr, 1994).

Alteration of the composition of the cytoplasmic membrane is a primary mechanism of bacterial adaptation to environmental stress (Denich et al., 2003; Yoon et al., 2015). The current study demonstrated that the presence of CFAs increased resistance to heat, pressure, and acid in the heat- and pressure-resistant strain *E. coli* AW1.7 and susceptible strain *E. coli* MG1655. Substitution of saturated membrane lipids by CFAs increases the fluidity of lipid bilayers through disruption of lipid packing and increasing the lipid diffusion; however, substitution of unsaturated fatty acids by their analogous CFAs decreases the fluidity of the membrane (Poger and Mark, 2015).

The effect of CFAs on acid resistance of *E. coli* is well documented (Brown et al., 1997; Chang and Cronan Jr., 1999). The protective effect of CFAs relates to reduced proton permeability as well as an increased ability to extrude protons (Shabala and Ross, 2008). CFA synthesis in *E. coli* was proposed to increase resistance to oxidative stress (Grogan and Cronan Jr, 1997). The ability of *Mycobacterium tuberculosis* to convert mycolic acids to cyclopropane derivatives increased resistance to hydrogen peroxide (Yuan et al., 1995). However, an effect of CFA- formation on the oxidative stress resistance in *E. coli* has not been determined experimentally and was not observed in this study. *E. coli* harbours multiple glutathione- and thioredoxin-dependent systems for reduction of peroxides (Carmel-Harel and Storz, 2000), which may mitigate H_2O_2-induced oxidation of unsaturated fatty acids under conditions used in this study.

Differences in pressure resistance between stationary-phase and exponentially growing cells of *E. coli* were attributed to σ^S-mediated changes in the cytoplasmic membrane, including CFA formation (Pagán and Mackey, 2000). A comparison of the pressure resistance of *E. coli* BW2952 with mutant strains from the Keio collection demonstrated that the *cfa*, *rpoS*, and *trxB* deficient strains were most sensitive to pressure (Charoenwong et al., 2011). My study extended knowledge on the role of CFAs in pressure resistance to the heat- and pressure resistant food isolate *E. coli* AW1.7. The resistance of *E. coli* to pressure is linked to altered membrane properties as well as its resistance to oxidative stress (Malone et al., 2006; Gänzle and Liu, 2015). The contribution of CFAs to pressure resistance may thus relate to the reduced membrane

fluidity but may also involve increased resistance of membrane lipids to oxidative attack.

A role of membrane properties on heat resistance of *E. coli* and *Salmonella* spp. was inferred from the increased resistance of heat-adapted cells (Katsui et al., 1981; Alvarez-Ordóñezet al., 2008). Ruan et al. (2011) noted an increased proportion of CFAs in the membrane of *E. coli* AW1.7 when compared to heat-sensitive *E. coli*. The current study provided direct evidence for a role of *cfa* in heat resistance of *E. coli*. Moreover, this protective effect was confirmed in food. The effect of *cfa* deletion on heat resistance in LB broth and ground beef was comparable to the effect on pressure resistance. Although heat and pressure resistance in different strains of *E. coli* are not correlated (Liu et al., 2015), CFA synthesis is thus an important factor for the cross-resistance of *E. coli* to multiple stressors including heat and pressure. The accumulation of compatible solutes also accounts for cross-resistance of *E. coli* to heat and pressure (Pleitner et al., 2012; Vanlint et al., 2013).

The two strains of *E. coli* showed substantial differences with respect to their stress tolerance. *E. coli* AW1.7 was more resistant to heat and pressure but less resistant to acid stress than *E. coli* MG1655. CFA accumulation increased heat resistance in both strains; however, the difference in heat resistance between the two wild type strains is mainly attributable to a genomic island termed locus of heat resistance (LHR) in *E. coli* AW1.7 (Mercer et al., 2015). This 14 kbp genomic island encodes 16 putative proteins that are predicted to contribute to turnover of misfolded proteins, solute accumulation, and thiol homeostasis (Mercer et al., 2015).

In conclusion, my study demonstrates that the *cfa* gene in *E. coli* contributes to heat resistance in this species. Remarkably, cell counts of *E. coli* AW1.7 are reduced by less than 3 $\log_{10}$(CFU/g) after cooking of meat according to Health Canada's recommendations for ground beef and poultry (Anonymous, 2014). This level of heat resistance is exceptional but not unprecedented (Dlusskaya et al., 2011; Luchansky et al., 2013; Liu et al., 2015). Improved knowledge on mechanisms of stress resistance will facilitate the design of intervention methods for improved pathogen control in food production. This study, therefore, enables further investigations to control *E. coli*, including pathogenic strains of the species, by application of suitable pathogen intervention technologies in food processing.

3.5. References

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Chapter 4. Inactivation of dry *Escherichia coli* by high pressure carbon dioxide

4.1. Introduction

Microbial inactivation by high pressure carbon dioxide (HPCD) was first proposed in 1951 (Fraser, 1951). HPCD has gained increasing interest as a non-thermal preservation technology to meet the consumers' requirements for minimally processed food products (Garcia-Gonzalez et al., 2007). The effect of HPCD on vegetative bacterial cells was mainly evaluated in liquid foods (Damar and Balaban, 2006; Werner and Hotchkiss, 2006; Liao et al., 2007; Spilimbergo et al., 2007; Spilimbergo and Ciola, 2010) and solid foods with high water activity (Ferrentino and Spilimbergo, 2011); however, foodborne pathogens in low water activity (a_W) foods are more resistant to the pathogen intervention treatments that are lethal to the cells at high a_W (Beuchat et al., 2013). Only a few studies investigated the bactericidal effect of HPCD on dry bacterial cells.

Establishing food safety for solid foods by employing HPCD technology needs an improved understanding of the mechanism of inactivation of bacterial cells (Damar and Balaban, 2006; Garcia-Gonzalez et al., 2007; Perrut, 2012). Supercritical CO_2 at temperature and pressure conditions above its critical point (31 ^oC, 7.4 MPa) is characterized by its gas-like diffusivity and liquid-like density, exhibiting physico-chemical properties in between those of a liquid and a gas (Garcia-Gonzalez et al., 2007). Supercritical CO_2 inactivates wet cells more efficiently than either gaseous or

liquid CO_2 (Lin et al., 1992; Lin et al., 1993). Several factors are thought to contribute to bacterial inactivation by HPCD. First, HPCD diffuses into the cell membrane, accumulates in the hydrophobic interior, and disturbs the phospholipid bilayer. The resultant decrease of the order of the lipid chains increases membrane fluidity and permeability, which is also referred to as the anesthesia effect (Isenschmid et al., 1995). The modification of the cell membrane may lead to leakage of cytoplasmic solutes (Hong and Pyun, 1999). Second, supercritical CO_2 may disturb membrane function through the removal of some membrane phospholipids (Kamihira et al., 1987). The content of phosphatidylglycerol in membrane lipids of *Escherichia coli* was substantially reduced after treatment with supercritical CO_2 (Tamburini et al., 2014). Third, bactericidal effects of pressurized CO_2 may relate to cytoplasmic acidification. Pressurized CO_2 dissolves in water to form H_2CO_3 (Meyssami, 1992). CO_2 and H_2CO_3 are membrane permeable and intracellular dissociation of carbonic acid acidifies the cytoplasm (Garcia-Gonzalez et al., 2007). Accumulation of CO_2 in the cytoplasm decreases the intracellular pH, which may contribute to cell death after HPCD treatment (Spilimbergo et al., 2005; Spilimbergo et al., 2010). The lower cytoplasmic pH may also inhibit enzymes that are involved in the essential metabolic pathways (Ballestra et al., 1996; Hong and Pyun, 2001; Damar and Balaban, 2006). However, low a_W conditions influence the inactivation mechanisms to different extents but not enough is known about the effect of HPCD in a low a_W environment.

This study investigated the bactericidal effect of HPCD on *E. coli* at low a_W. *E. coli* AW1.7, a heat and pressure resistant strain, was used as a model organism (Dlusskaya et al., 2011; Mercer et al., 2015). To gain insight into the mechanisms of microbial inactivation, three derivatives of *E. coli* AW1.7 were also investigated: *E. coli* AW1.7Δ*cfa* is deficient in cyclopropane fatty acid synthase and was used to probe the role of the cell membrane (Chen and Gänzle, 2016); *E. coli* AW1.7ΔpHR1 lacks the locus of heat resistance and was used to probe the role of heat resistance (Mercer et al., 2015); *E. coli* AW1.7Δ*gadAB* is deficient in glutamate decarboxylase activity and was generated to probe the role of acid resistance (Castanie-Cornet et al., 1999; Garcia-Gonzalez et al., 2007). Treatments were performed with liquid, gaseous, and supercritical CO_2 to determine processing conditions permitting efficient inactivation.

4.2. Materials and methods

4.2.1. Bacterial strains and culture conditions

E. coli AW1.7 and derivative strains (Table 4-1) and *E. coli* DH5α were cultivated aerobically at 37°C in Luria-Bertani (LB) medium (Becton Dickinson (BD), Mississauga, ON, Canada) containing tryptone (10 g/L), yeast extract (5 g/L) and sodium chloride (10 g/L). Antibiotic-resistant *E. coli* harboring plasmid pUC19 or pKOV were cultured with 50 mg/L ampicillin or 34 mg/L chloramphenicol, respectively.

Table 4-1. Bacterial strains and plasmids used in this study

Strains	Description	Reference or Source
E. coli AW1.7	Heat- and pressure- resistant strain, isolated from a beef-packing plant	(Aslam et al., 2004)
E. coli AW1.7Δ*cfa*	*E. coli* AW1.7 with truncated *cfa*	(Chen and Gänzle, 2016)
E. coli AW1.7ΔpHR1	Heat sensitive derivative of *E. coli* AW1.7 deficient in the genomic island termed locus of heat resistance	(Mercer et al., 2015)
E. coli AW1.7Δ*gadAB*	*E. coli* AW1.7 with truncated *gadA and gadB*	This study
E. coli DH5α	Cloning host for plasmids	New England Biolabs
Plasmids	**Description**	**Reference or Source**
pUC19	lacZα promoter; cloning vector used in *E. coli*; Amp^r	New England Biolabs
pUC19/*gadA*-X	pUC19 plasmid with 1.2 kb fragment of *gadA* upstream region in *E. coli* AW1.7; Amp^r	This study
pUC19/*gadA*-Y	pUC19 plasmid with 1 kb fragment of *gadA* downstream region in *E. coli* AW1.7; Amp^r	This study
pUC19/*gadA*-XY	pUC19 plasmid with 2.2 kb of *gadA* upstream and downstream fragment of *E. coli* AW1.7; Amp^r	This study
pUC19/*gadB*-X	pUC19 plasmid with 1 kb fragment of *gadB* upstream region in *E. coli* AW1.7; Amp^r	This study
pUC19/*gadB*-Y	pUC19 plasmid with 1 kb fragment of *gadB* downstream region in *E. coli* AW1.7; Amp^r	This study
pUC19/*gadB*-XY	pUC19 plasmid with 2 kb of *gadB* upstream and downstream fragment of *E. coli* AW1.7; Amp^r	This study
pKOV	Temperature sensitive pSC101; Sac B; Cm^r	(Link et al., 1997)
pKOV/Δ*gadA*	pKOV plasmid with 2.2 kb of *gadA* flanking fragment of *E. coli* AW1.7; resulting *gadA* deletion; Cm^r	This study
pKOV/Δ*gadB*	pKOV plasmid with 2 kb of *gadB* flanking fragment of *E. coli* AW1.7; resulting *gadB* deletion; Cm^r	This study

Amp^r: ampicillin-resistance gene; Cm^r: chloramphenicol -resistance gene.

4.2.2. DNA manipulation

Genomic DNA in *E. coli* AW1.7 was extracted using the Blood & Tissue Kit (Qiagen, Hilden, Germany). Plasmid DNA was isolated with a QIAprep Spin Miniprep kit (Qiagen). Primers (Table 4-2) were synthesized by Integrated DNA Technologies (San

Diego, CA, USA). The Taq DNA polymerase was obtained from TaKaRa Bio (Shiga, Japan). The PCR products were purified with DNA gel extraction kits (Qiagen). T4 DNA ligase and restriction enzymes were purchased from Thermo Scientific (Mississauga, ON, Canada). The DNA sequencing was completed by Macrogen (Rockville, MD, USA).

Table 4-2. Primers used in this study

Primers (forward, F; reverse, R)	Sequence (5'-3')	Restriction site
gadA-upstream X, F	5'-CGCGGATCCCTATAATCTTATTCCTTCCGCAGAACGGTCAG-3'	BamHI
gadA-upstream X, R	5'-CTAGTCTAGACTCAGCGATCACCCGAAACTGCAGGGTATTG-3'	XbaI
gadA-downstream Y, F	5'-CTAGTCTAGATGCGCCAAAACGTGAATCGAGTAGTTCTGAGCGG-3'	XbaI
gadA-downstream Y, R	5'-CGACGTCGACACATCGATTGGTGTTGGTGGCGCAG-3'	SalI
gadB-upstream X, F	5'-CGCGGATCCAATGACCATCGCAATAGACAAACCACC-3'	BamHI
gadB-upstream X, R	5'-CTAGTCTAGACTCAGCGATCACCCGAAACTGCAGGG-3'	XbaI
gadB-downstream Y, F	5'-CTAGTCTAGACGCACCAAAACGTGAATCGAGTAGTTCCG-3'	XbaI
gadB-downstream Y, R1	5'-CCCAAGCTTGCGACGGACAACGCATCGGTTACTGTAAAAG-3'	HindIII
gadB-downstream Y, R2	5'-CGCGGATCCCCCAAGCTTGCGACGGACAACGCATCGGTTACTG-3'	BamHI/ HindIII

4.2.3. Construction of *E. coli* AW1.7Δ*gadAB*

Gene disruption of *gadA* and *gadB* encoding glutamate decarboxylases in *E. coli* AW1.7 was carried out with an in-frame, unmarked deletion method (Link et al., 1997; Chen and Gänzle, 2016). The approximately 1000 bp 5'-flanking regions (fragment X) and 1000 bp 3'-flanking regions (fragment Y) of gene *gadA* and *gadB* were amplified by PCR with primers listed in Table 4-2. The fragments X and Y were digested with the respective restriction enzymes and purified. Fragments were sequentially cloned into the BamHI and SalI restriction sites of pUC19 to generate pUC19/*gadA*-XY, or cloned into BamHI &

HindIII for the generation of pUC19/*gadB*-XY. The authenticity was confirmed by sequencing. The XY fragments in pUC19/*gadA*-XY and pUC19/*gadB*-XY were amplified by PCR; the primer for amplification from pUC19/*gadB*-XY included an additional BamHI restriction site (Table 4-2). The subcloning into plasmid pKOV was carried out to create pKOV/Δ*gadA* and pKOV/Δ*gadB*. Recombinant plasmid pKOV/Δ*gadA* was transformed into electrocompetent *E. coli* AW1.7. The cells were plated on chloramphenicol-LB plates and incubated at 43°C to select for single-crossover mutants. Several colonies were isolated, serially diluted in LB broth, and plated on 5% (w/v) sucrose-LB plates at 30°C. Sucrose-resistant colonies were subsequently replica plated on chloramphenicol plates at 30°C to identify the double-crossover mutant *E. coli* AW1.7Δ*gadA*. The second gene deletion was performed by introducing pKOV/Δ*gadB* into the resulting mutant and following a similar screening procedure as above to create *E. coli* AW1.7Δ*gadAB.* The double knockout mutant was confirmed by PCR amplification and sequencing.

4.2.4. Sample preparation

Cultures for use in HPCD treatments were prepared from frozen stock (-80°C) cultures by streaking on LB agar, followed by subculture in LB broth to stationary phase. An aliquot (100 µl) of stationary phase culture was spread over the agar plate and incubated

at 37°C for 24 h. The plate was then washed with 1 ml of saline containing 1 g peptone / L and 8.5 g NaCl / L, yielding a final cell concentration of approximately 10^9 CFU/mL. The cell suspension (20 µL) was placed in a sterilized glass vial and dried under a laminar flow of sterile air for 5-6 h. The dried cells were equilibrated in desiccators containing deionized water or saturated NaCl solution at 37°C for 16 h to achieve water activity of 1.0 or 0.75, respectively (Mathlouthi, 2001). The saturated NaCl solution was prepared by dissolving salt in boiling deionized water (100°C), which was then cooled down and incubated at 37°C overnight for the formation of crystals.

HPCD treatments using gaseous, liquid or supercritical CO_2 were carried out after overnight equilibration of the cells to a_W = 1.0 or 0.75. Cells were equilibrated in 1.5 ml glass vials (12 mm × 30 mm × 4.6 mm; Sigma-Aldrich), which were then assembled with septum-fitted screw caps. Septa (8 mm diameter) were cut from 0.20 µm nylon membrane (Merck Millipore Ltd., County Cork, Munster, Ireland). The vial caps were loosely closed to ensure equilibration with CO_2 after pressurization.

4.2.5. Experimental apparatus

The schematic diagram of the apparatus for treatment with HPCD (phase equilibria apparatus, SITEC-Sieber Engineering AG, Maur/Zurich, Switzerland) was described previously (Zhao and Temelli, 2015). In brief, the apparatus consisted of a high pressure cell with an internal volume of 10 mL connected to an ISCO 260D syringe pump

(Teledyne Isco, Lincoln, NE, USA). The pressure inside the vessel was measured by a pressure transducer with an accuracy of 0.01 MPa. A heating jacket installed around the vessel was filled with circulating water at the desired temperature supplied by a water bath (Model 260, Thermo Fisher Scientific Inc., Waltham, MA, USA). The temperature in the pressure vessel was monitored by an internal thermocouple. An on/off valve was located at the upper level exit of the vessel, while a micrometering valve was installed at the lower level exit to control the depressurization rate.

4.2.6. HPCD treatment

Prior to each treatment, the inside surface of the high pressure vessel was disinfected with 70% aqueous ethanol solution, and the remaining ethanol in the vessel was removed by flushing the vessel with CO_2 for 5 min. The loosely-capped vial containing the cell suspension at a certain water activity was loaded into the preheated vessel. The vessel was immediately sealed and gently flushed with CO_2 at tank pressure for 1 min to remove the air trapped in the vessel. After the exit valves were closed, CO_2 (99.9% pure, < 3 ppm H_2O; Praxair Canada Inc., Mississauga, ON, Canada) was pumped into the vessel until the desired pressure was reached. *E. coli* cells were treated at various pressure levels in the range of 5.7 MPa to 40 MPa for 15 min. The temperature of the vessel was maintained at a constant level within the range of 25°C to 65°C. At the end of the treatment time, the micrometering valve was slowly opened to achieve a decompression

rate of 5-6 MPa/min. The position of the micrometering valve was kept constant at each run to achieve a similar depressurization rate. The sample was removed from the vessel immediately after decompression for microbiological analysis. The treatment time was defined as the period from the point of the vessel reaching the experimental pressure to the start of the CO_2 release.

4.2.7. Determination of viable cell counts

The survival of *E. coli* cells after HPCD treatment was determined by viable cell counts. The cell suspension was serially diluted, and appropriate dilutions were surface plated on LB agar. Colonies were counted after 24 h of incubation at 37°C. The equilibrated cells without being subjected to HPCD treatment served as untreated control. Bacterial survival was expressed as $\log_{10}$ (N_0/N) with N_0 representing the cell counts of the untreated control and N representing the cell counts of treated samples. The effect of HPCD treatment on bacterial survival was determined based on triplicate independent experiments.

4.2.8. Statistical analysis

Data analysis was carried out using the software R 3.1.2 (R Core Team, 2014). Significant differences in the assessment of cell survival after supercritical, gaseous and liquid CO_2 treatment were determined by using one-way analysis of variance (ANOVA);

post-hoc pairwise comparisons were conducted using the Tukey Honest Significant Differences (TukeyHSD) method. Significance was assessed at a 5% probability of error ($P < 0.05$); an error probability of 5 to 10% ($0.05 < P < 0.1$) was reported as trend.

4.3. Results

4.3.1. Generation of *E. coli* AW1.7Δ*gadAB* strain

The glutamate decarboxylase (GAD) system is the major acid resistant mechanism in *E. coli* (Castanie-Cornet et al., 1999) and was suggested to contribute to resistance to high pressure CO_2 (Garcia-Gonzalez et al., 2007). The two GAD structural genes in *E. coli*, *gadA* and *gadB*, share 98% identity at the nucleotide level (Smith et al., 1992). These two GAD structural genes in *E. coli* AW1.7 were disrupted to explore the effect of acid-resistance on the response of *E. coli* to HPCD. The double mutant was generated with an in-frame, unmarked deletion method without introducing polar effects on the downstream genes (Link et al., 1997). The contribution of GadA and GadB in *E. coli* AW1.7 to acid resistance was verified by comparing the survival of *E. coli* AW1.7 and AW1.7Δ*gadAB* in phosphate buffer at pH 2.5, or phosphate buffer (pH 2.5) that was supplemented with 20 mM glutamine or glutamate. The survival of wild-type strain was higher than that of the Δ*gadAB* mutant strain (data not shown).

4.3.2. Influence of water activity on cell survival after supercritical CO_2 treatment

The effect of supercritical CO_2 treatment on the inactivation of *E. coli* cells was investigated at a_W 1.0 and 0.75, and at 10, 20, and 40 MPa (Table 4-3) (Figure 4-1). Cell counts of *E. coli* AW1.7 and mutants were reduced by more than 3 $\log_{10}$ (CFU/ml) after supercritical CO_2 treatment at 35°C and an a_W of 1.0 (Table 4-3). The inactivation of *E. coli* AW1.7, AW1.7Δ*cfa*, and AW1.7ΔpHR1 was significantly enhanced by increasing treatment pressure. *E. coli* AW1.7Δ*cfa* was the only derivative of *E. coli* AW1.7 that was more susceptible to supercritical CO_2 than the wild type strain (P<0.05). Treatments at an a_W of 0.75 but otherwise identical HPCD conditions reduced cell counts by less than 0.5 $\log_{10}$ (CFU/ml) (Figure 4-1). All *E. coli* strains exhibited a similar resistance. The lethality of supercritical CO_2 thus depends on the pressure, the water activity, and the membrane properties of the treated cells.

4.3.3. Influence of temperature on the lethality of supercritical CO_2 treatment

Dry *E. coli* were resistant to supercritical CO_2 treatment at 35°C; further experiments were thus carried out with increasing treatment temperature to explore the influence of temperature on microbial inactivation (Figure 4-2). Because *E. coli* AW1.7Δ*gadAB* behaved similarly as the wild type strain *E. coli* AW1.7 in the previous set of experiments (Figure 4-1), it was not included in this experiment. When dry *E. coli* cells (a_W = 0.75) were treated with supercritical CO_2 at 55°C, the cell counts were reduced by 0.4 to 1.0

$\log_{10}$ (CFU/ml) (Figure 4-2A); corresponding to only a slight increase in process lethality when compared to treatments at 35°C (Figure 4-1). The pressure level did not affect the tolerance of *E. coli* strains to supercritical CO_2 at 55°C (Figure 4-2A). The survival of *E. coli* AW1.7 and AW1.7ΔpHR1 at a treatment temperature of 65°C was comparable to treatments at 55°C. However, the survival of AW1.7Δ*cfa* was significantly reduced (P<0.05) by treatments at 65°C (Figure 4-2B) when compared to treatments at lower temperatures.

Table 4-3. Inactivation of *E. coli* AW1.7 and derivative strains (a_W 1.0) treated with supercritical CO_2 at 10, 20 or 40 MPa for 15 min at 35°C

strain	Pressure[1)]		
	10 MPa	20 MPa	40 MPa
E.coli AW1.7	2.98 ± 0.23 [b2), y3)]	4.81 ± 0.61 [x]	5.45 ± 0.30 [b, x]
E.coli AW1.7Δ*cfa*	4.78 ± 0.20 [a, z]	5.99 ± 0.16 [y]	7.54 ± 0.25 [a, x]
E.coli AW1.7ΔpHR1	3.75 ± 0.44 [ab, y]	5.02 ± 0.50 [xy]	5.54 ± 0.18 [ab, x]
E.coli AW1.7Δ*gadAB*	3.99 ± 0.30 [ab]	4.31 ± 0.36	4.76± 0.79 [b]

[1)] Data represent means ± standard error of the mean for three independent experiments.
[2)] Within a column, values with different superscripts (a or b) are different at $P < 0.05$.
[3)] Within a row, values with different superscripts (x, y or z) are different at $P < 0.05$.

4.3.4. Inactivation of dry *E. coli* cells treated by liquid and gaseous CO_2

To determine whether bacterial inactivation by high pressure CO_2 occurs only at supercritical conditions, the effects of liquid and gaseous CO_2 treatments on the survival of dry *E. coli* AW1.7 and AW1.7Δ*cfa* were also evaluated. Treatments were performed with gaseous CO_2 at 5.7 MPa and 30, 35 or 65°C, and with liquid CO_2 at 10 MPa and 25

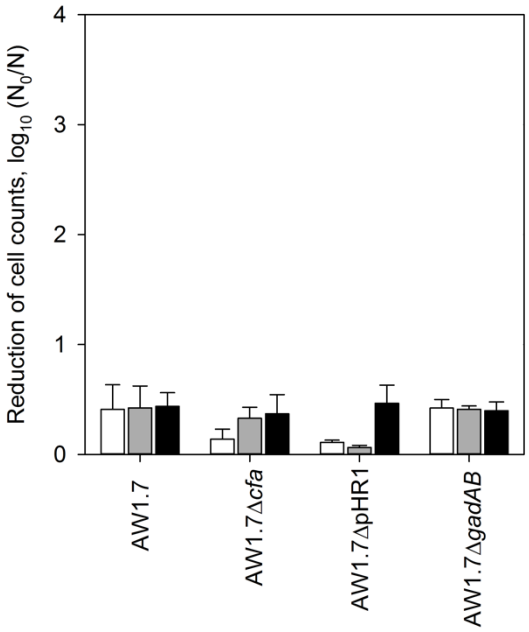

Figure 4-1. Inactivation of *E. coli* AW1.7 and derivative strains treated with supercritical CO_2. Strains were air dried and equilibrated to a_W 0.75. CO_2 treatment was done at 10 MPa (white bar), 20 MPa (gray bar) or 40 MPa (black bar) for 15 min at 35°C. Data represent means ± standard error of the mean for three independent experiments.

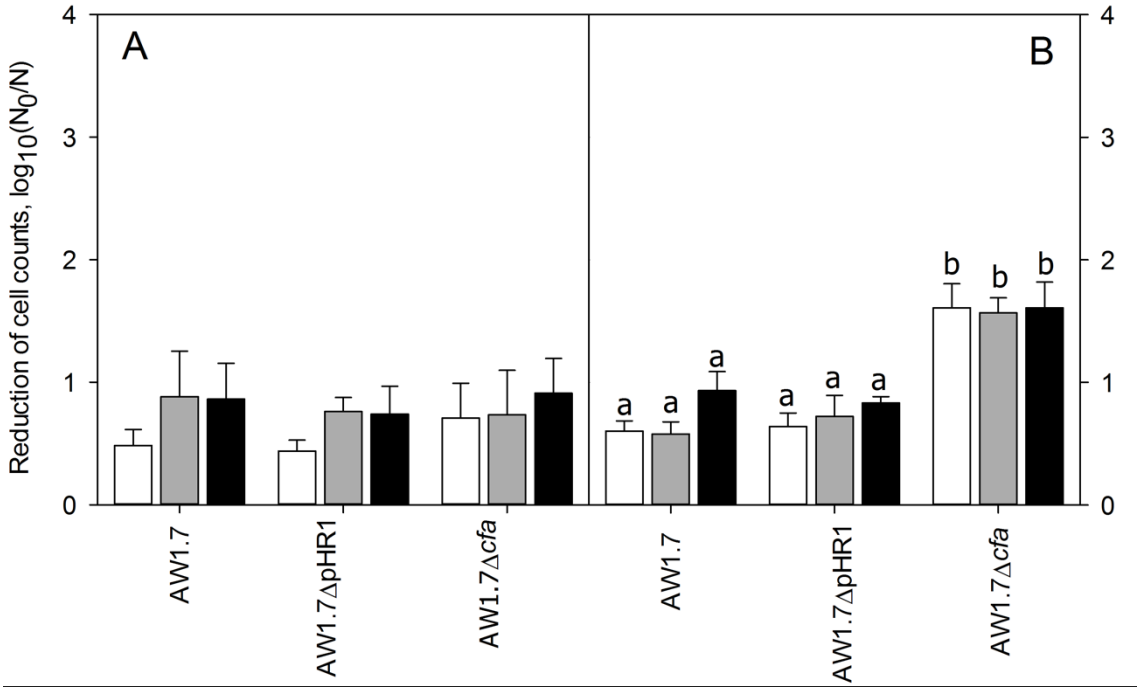

Figure 4-2. Inactivation of *E. coli* AW1.7 and derivative strains by supercritical CO_2. Strains were air-dried, equilibrated to a_W 0.75, and treated with CO_2 at 10 MPa (white bar), 20 MPa (gray bar) and 40 MPa (black bar) for 15 min at 55°C (Panel A) or 65°C (Panel B). Values for different strains at the same treatment pressure that do not share a common superscript are significantly different (P<0.05). Data represent means ± standard error of the mean for three independent experiments.

or 30°C (Figure 4-3). An increase in temperature enhanced the antimicrobial effect of both gaseous and liquid CO_2. *E. coli* AW1.7 and AW1.7Δ*cfa* showed similar resistance to liquid CO_2 treatment and cell counts were reduced by less than one $\log_{10}$(CFU/ml). Remarkably, *E. coli* AW1.7 was more susceptible to gaseous CO_2 than *E. coli* AW1.7Δ*cfa* at a_W of 0.75 (P<0.05 at 30°C and 65°C; P<0.1 at 35°C) (Figure 4-4). Cell counts of *E. coli* AW1.7 and AW1.7Δ*cfa* were reduced by 3.4 and 2.6 $\log_{10}$(CFU/ml) respectively, after treatment at 5.7 MPa and 65°C (Figure 4-3). The effect of membrane composition on the resistance to gaseous CO_2 treatment is thus opposite to its effect on resistance to supercritical CO_2 treatment.

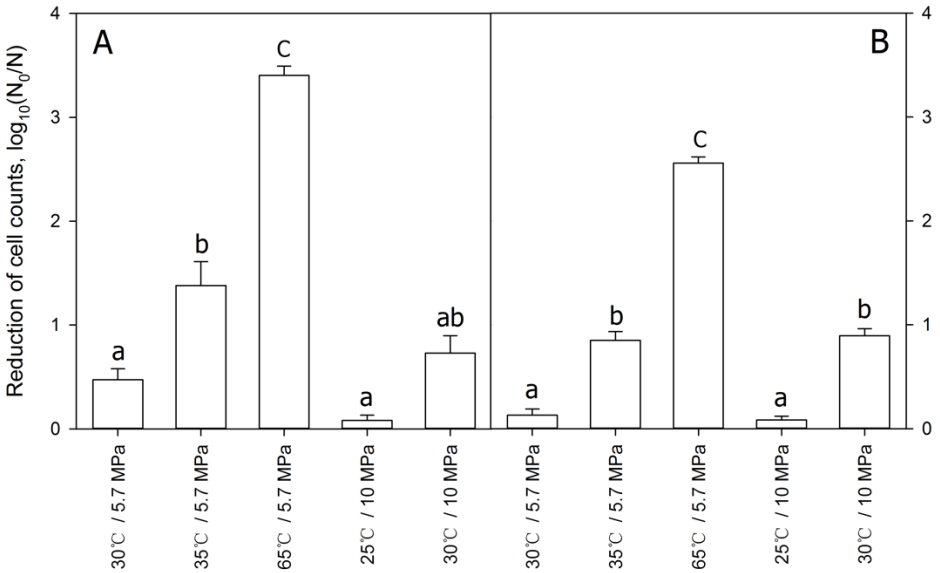

Figure 4-3. Inactivation of *E. coli* AW1.7 (panel A) and AW1.7Δ*cfa* (panel B) by high pressure CO_2. Strains were air-dried, equilibrated to a_W 0.75, and treated with CO_2 at the T/p conditions as indicated. Values for same strain treated at different T/p conditions that do not share a common superscript are significantly different (P<0.05). Data represent means ± standard error of the mean for three independent experiments.

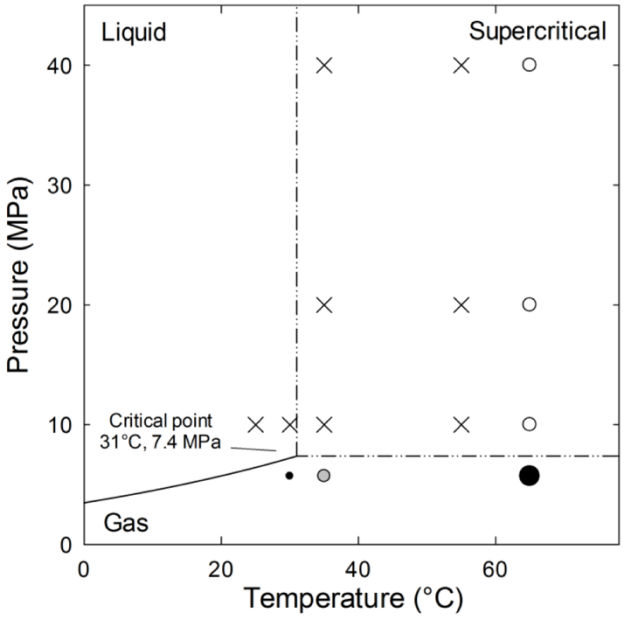

Figure 4-4. Lethal effect of CO_2 towards dry (a_W = 0.75) *E. coli* AW1.7 and AW1.7Δ*cfa* at different temperature and pressure conditions. Cross marks indicate no difference in sensitivity of *E. coli* AW1.7 and AW1.7Δ*cfa*, and a reduction of cell counts of $\log_{10}(N_0/N)$ < 1.0; white circles indicate that *E. coli* AW1.7Δ*cfa* was more sensitive than *E. coli* AW1.7 (P<0.05), black and gray circles indicate that *E. coli* AW1.7 was more sensitive than *E. coli* AW1.7Δ*cfa* at P<0.05 and P<0.1, respectively. The circle area is proportional to process lethality towards the more sensitive strain.

4.4. Discussion

Desiccated microorganisms generally tolerate environmental insults that are lethal to the same organism at high water activity (Beuchat et al., 2013; Syamaladevi et al., 2016). In this study, the effect of HPCD on the inactivation of *E. coli* AW1.7 and several isogenic mutant strains was evaluated at high and low water activities. Treatment with supercritical CO_2 was lethal to *E. coli* at high a_W; however, treatments of *E. coli* at a_W of 0.75 were lethal only when combined with elevated temperature. Remarkably, treatment with gaseous CO_2 at 65°C was the most effective in reducing cell counts of dry *E. coli*,

and the effect of membrane composition on resistance to gaseous CO_2 was opposite to its effect on resistance to supercritical CO_2.

4.4.1. Effect of water activity, temperature and pressure during supercritical CO_2 treatment

A higher water content of cells was proposed to increase the cellular absorption of CO_2 (Kumagai et al., 1997), resulting in an enhanced diffusion of CO_2 into the cells (Dillow et al., 1999). A high water content also favors the formation of extracellular HCO_3^-, which interacts with the charged phospholipid head groups and membrane proteins and thus influences the membrane stability (Jones and Greenfield, 1982). Moreover, higher pressure enhances the solubilization of CO_2 in water and thus favors cellular penetration (Lin et al., 1994). The increased inactivation of wet cells with increasing pressure (Hong et al., 1999; Erkmen, 2000), and the resistance of dry *E. coli* to supercritical CO_2 conforms to previous observations (Kamihira et al., 1987; Dillow et al., 1999).

Generally, the treatment temperature increases the lethality of HPCD on bacterial cells (Liao et al., 2010; Yuk et al., 2010). In the present study, dry cells of *E. coli* were resistant to supercritical CO_2 below 55°C; the lethality of supercritical CO_2 against *E. coli* at 65°C did not increase with pressure. The resistance of *E. coli* AW1.7 and AW1.7ΔpHR1 to supercritical CO_2 treatment at 65°C was comparable although these two strains differ substantially in their heat resistance (Mercer et al., 2015). Heat resistance of *E. coli*

AW1.7 is conferred by the locus of heat resistance (LHR), which encodes heat shock proteins, and proteins involved in thiol and ion homeostasis (Mercer et al., 2015). Moreover, the tolerance of *E. coli* AW1.7 and AW1.7Δ*gadAB* to supercritical CO_2 was comparable; indicating that intracellular acidification by CO_2 was not a major contributor to treatment lethality.

4.4.2. Effect of the physical state of CO_2

An overview of the process lethality against dry cells of *E. coli* AW1.7 and *E. coli* AW1.7Δ*cfa* in relation to the phase diagram of CO_2 is shown in Figure 4-4. Treatments with liquid CO_2 were not bactericidal. The lethality of treatments with gaseous CO_2 was higher than the lethality of treatments with supercritical CO_2 (Figure 4-4). *E. coli* AW1.7Δ*cfa* was the only derivative strain showing differential inactivation; remarkably, the resistance of *E. coli* AW1.7Δ*cfa* to gaseous CO_2 was higher than that of the wild type strain but its resistance to supercritical CO_2 was lower (Figure 4-4).

Liquid CO_2 was lethal to *E. coli* at high a_W, and process lethality was related to membrane permeabilization (Oulé et al., 2006). In contrast, the present study demonstrated that dry cells of *E. coli* resisted treatments with liquid CO_2 (Figure 4-4). Dry cells of *E. coli* were tolerant to gaseous CO_2 when the treatment was performed at room temperature (Debs-Louka et al., 1999); however, gaseous CO_2 was lethal for *E. coli* AW1.7 at 35 and 65°C (Figure 4-4). Density, diffusivity, and solubility of CO_2 in

aqueous solution, as well as the solvent power of CO_2 are different in the gaseous, liquid and supercritical states (Zhang et al., 2006).

4.4.3. Effect of membrane properties

E. coli AW1.7Δ*cfa* was the only derivative of *E. coli* AW1.7 that showed a differential response to HPCD. Remarkably, the relative sensitivity of dried *E. coli* AW1.7 and AW1.7Δ*cfa* depended on the state of CO_2 (Figure 4-4). *E. coli* AW1.7 was more resistant to treatment with supercritical CO_2 but *E. coli* AW1.7Δ*cfa* was more resistant to treatment with gaseous CO_2. Disruption of *cfa* in *E. coli* abolishes the formation of cyclopropane fatty acids (CFAs). The lack of CFAs is compensated by incorporation of $C_{16:1}$ and $C_{18:1}$ unsaturated fatty acids in the membrane (Chen and Gänzle, 2016); this substitution increases the fluidity of the membrane (Poger and Mark, 2015). The membrane fluidity is further increased by supercritical CO_2 (Bothun et al., 2005) and high temperatures. Higher temperatures also enhance the diffusivity of CO_2 and thus accelerate the penetration of CO_2 into the cells (Hong et al., 1997; Damar and Balaban, 2006).

Supercritical CO_2 possesses a high affinity to the hydrophobic core of the membrane (Spilimbergo et al., 2002). CO_2 disorders the hydrocarbon fatty acyl chains and disrupts the lipid-protein interactions, referred to as 'anaesthesia effect' (Isenschmid et al., 1995; Oulé et al., 2006). Incorporation of CO_2 into the membrane was also proposed to expand the volume of the phospholipid bilayer (Bothun et al., 2005). Taken together, the

interaction of supercritical CO_2 with the membrane results in increased membrane fluidity and permeability, and alters its composition (Tamburini et al., 2014). Supercritical CO_2-induced membrane permeabilization leading to a loss of cytoplasmic content was suggested as a mechanism of microbial inactivation at high a_W (Garcia-Gonzalez et al., 2010). My study supports this mechanism by investigation of the response of *E. coli* AW1.7Δ*cfa*, which has a more fluid membrane than the wild type strain. In addition, my study demonstrates that the increased membrane fluidity and permeability induced by supercritical CO_2 and high temperatures are also the main contributors to the inactivation of dry cells of *E. coli*.

Gaseous CO_2 inactivated dry *E. coli* via different mechanisms than supercritical CO_2, as demonstrated by the inverse relative sensitivities of *E. coli* AW1.7 and AW1.7Δ*cfa*. Gaseous CO_2 possesses lower solubility in the membrane bilayer compared to supercritical CO_2 (Spilimbergo et al., 2002). However, gaseous CO_2 has higher mass transport properties and a lower density than supercritical and liquid CO_2, and thus diffuses more rapidly into the cell. Diffusion of CO_2 is further enhanced by increased temperatures, which is consistent with the increased lethality of gaseous CO_2 at high temperatures (Figure 4-4). Intracellular CO_2 may induce the precipitation of Ca^{+2}, Mg^{+2} as bicarbonates (Jones and Greenfield, 1982; Lin et al., 1993). Thus, inactivation of dry *E. coli* by gaseous CO_2 appears to be governed by the diffusion of CO_2 into the cell; the replacement of unsaturated fatty acids with cyclopropane fatty acids in membrane

phospholipids may increase this diffusion. At a high a_W, CO_2 needs to be solubilized in the extracellular water first before interacting with the cells, which may reduce the lethality of gaseous CO_2 at high a_W. Since the solubility of CO_2 in water and the phospholipid bilayer increases with pressure, the lethality of supercritical CO_2 was high at high a_W conditions.

In conclusion, gaseous CO_2 treatment at high temperature (65°C) inactivates dry *E. coli*. This result provides a novel and alternative method for the food industry to enhance the safety of low a_W products. My study further identified the CO_2-induced membrane fluidization and changes in membrane barrier properties as a major cause of cell death by HPCD; however, mechanisms of CO_2 mediated inactivation of dry *E. coli* depended on the physical state of CO_2. Liquid CO_2 was ineffective, the effectiveness of supercritical CO_2 related to its interaction with the phospholipid bilayer, and the effectiveness of gaseous CO_2 was related to the diffusivity of CO_2. The different mechanisms of bacterial inactivation by gaseous and supercritical CO_2 may allow tailored engineering of novel preservation processes to match the requirements of specific dry food products.

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Chapter 5. General discussion and future directions

Food bacteria developed stress response mechanisms during the evolvement to ensure their survival and robustness under environmental insults. To adapt the environmental changes, bacteria need to maintain their membrane biophysical properties through the alteration of their fatty acid compositions (Zhang and Rock, 2008). The ability of modifying the pre-existing phospholipids allows bacteria to adjust themselves to abrupt environmental changes rapidly. The research of this Ph.D. project aimed to explore the effect of the conversion of unsaturated fatty acids (UFAs) to hydroxy fatty acids or cyclopropane fatty acids (CFAs) on the stress resistance in food bacteria. Through this work, it enhanced our understanding of the relationship between the bacterial membrane homeostasis and stress resistance.

5.1. Linoleate 10-hydratase and its products are related to the ecological roles of *L. plantarum*.

Unsaturated fatty acids (e.g. linoleic acid), are necessary supplements of the medium for the growth of certain lactobacilli (Gänzle et al., 2000). The conversion of linoleic acid to 10-hydroxy-12-octadecenoic acid (10-HOE) was conducted by linoleate 10-hydratase (LahA), and the synthesis of 10-HOE was detected in most of the lactobacilli used in the current study. I proposed that the formed 10-HOE could be incorporated into the membrane, which might contribute to the bacterial membrane lipid homeostasis under stresses. *L. plantarum* TMW1.460, as a beer spoilage isolate, is tolerant to ethanol (Ulmer

et al., 2000). To investigate the role of 10-HOE in ethanol tolerance, disruption of *lahA* was conducted in *L. plantarum* TMW1.460. However, the synthesis of 10-HOE in wild-type strain was not involved in ethanol resistance compared to Δ*lahA* mutant strain. Moreover, 10-HOE was not found in membrane phospholipids of *L. plantarum* TMW1.460 (data not shown). And thus the 10-HOE production in *L. plantarum* TMW1.460 is not related to the ethanol stress. However, the physiological and ecological function of LahA and its hydroxy product 10-HOE in *L. plantarum* remains unknown. To fill this gap, more experiments were conducted. *L. plantarum* are commonly regarded as probiotics and used in the food industry (De Vries et al., 2006; Son et al., 2009; Zago et al., 2011). The ability of their adhesion to human intestinal surfaces is considered to be an important factor for probiotics to achieve the health benefit. In this study, LahA affected the cell surface properties of *L. plantarum*, which promoted the bacterial adhesion to intestinal surfaces. In addition, *L. plantarum* is part of the phyllosphere of many plants (Minervini et al., 2015). The production of 10-HOE and 13-HOE with antifungal properties are involved in plant defense against pathogens. Therefore, LahA and its products play important roles in establishing and maintaining the ecological fitness of *L. plantarum*.

5.2. Cyclopropanation is a general mechanism of the bacterial stress response.

The rapid modification of membrane lipids occurred in pathogens against intervention treatments is the causation of food-borne infection. For example, heat and high pressure

resistant strains of pathogenic *Escherichia coli* are a challenge to food safety (Liu et al., 2015). To enhance pathogen inactivation in food, it is necessary to improve my understanding on bacterial membrane lipid modification in response to stresses. CFAs are major components of membrane phospholipids in *E. coli* (Grogan and Cronan, 1997). This thesis research hypothesized that CFAs generally protect *E. coli* cells against various intervention stresses, and thus allow *E. coli* to survive in a wide range of environmental conditions. To test this hypothesis, the effect of CFAs on bacterial survival was assessed under multiple stress conditions in heat and pressure resistant *E. coli* AW1.7 and sensitive strain *E. coli* MG1655. Both stationary-phase *E. coli* AW1.7 and MG1655 completely utilized UFAs ($C_{16:1}$ and $C_{18:1}$) in the membrane and most of them were converted to CFAs after entry into the stationary growth phase. Membrane CFAs protected *E. coli* cells under acid, heat, high pressure and high pressure carbon dioxide (HPCD) treatments, suggesting that CFAs are a general protector for *E. coli* against environmental stresses. In addition, this study is the first to report that CFAs conferred heat resistance in food. My study demonstrated that CFA synthesis is related to the food bacterial tolerance to multiple intervention treatments, suggesting that it is possible to design effective intervention method for the pathogen control in food production through targeting their membrane.

Although the CFA synthases widely exist in both Gram-positive and Gram-negative bacteria (Figure 5-1), whether the CFA synthesis is a common strategy of stress resistance in food bacteria is still an open question. This research explored the role of CFAs in the

stress resistance of *E. coli* that is the model organism representing Gram-negative bacteria. However, some Gram-positive pathogens, such as *Listeria monocytogenes*, *Staphylococcus aureus*, *Bacillus cereus* and *Clostridium* spp., also lead to foodborne diseases (Farber and Peterkin, 1991; Rupnik, 2007; Bennett et al., 2013) and thus need to be eliminated from food as well. Do CFAs also protect Gram-positive bacteria when they survive under adverse environmental conditions? Till now, for Gram-positive bacteria, only limited studies focused on the relationship between CFAs and stress resistance, and they only suggested that the CFA formation was mainly related to ethanol, thermal and acid stresses. For example, the gene expression level of *cfa* was up-regulated and the amount of the corresponding $C_{19:0cyclopropane}$ fatty acids were increased in both *O. oeni* and *L. hilgardii*, when they were exposed to ethanol (Couto et al., 1996; Grandvalet et al., 2008). The conversion of $C_{18:1}$ to $C_{19:0cyclopropane}$ fatty acid was involved in the thermal resistance of stationary-phase *Pediococcus* spp. (Annous et al., 1999). Moreover, $C_{19:0cyclopropane}$ fatty acid synthesized in *L. plantarum* was reported to be related to acid tolerance (Huang et al., 2016). The current study built up a series of approaches and strategies to study the effect of CFAs on bacterial stress resistance, which can be applied in Gram-positive bacteria in the future to help us have a comprehensive understanding of the effect of CFAs in all food pathogens.

5.3. The mechanisms of bacterial inactivation of HPCD

This study investigated the bacterial membrane lipid homeostasis in food bacteria and

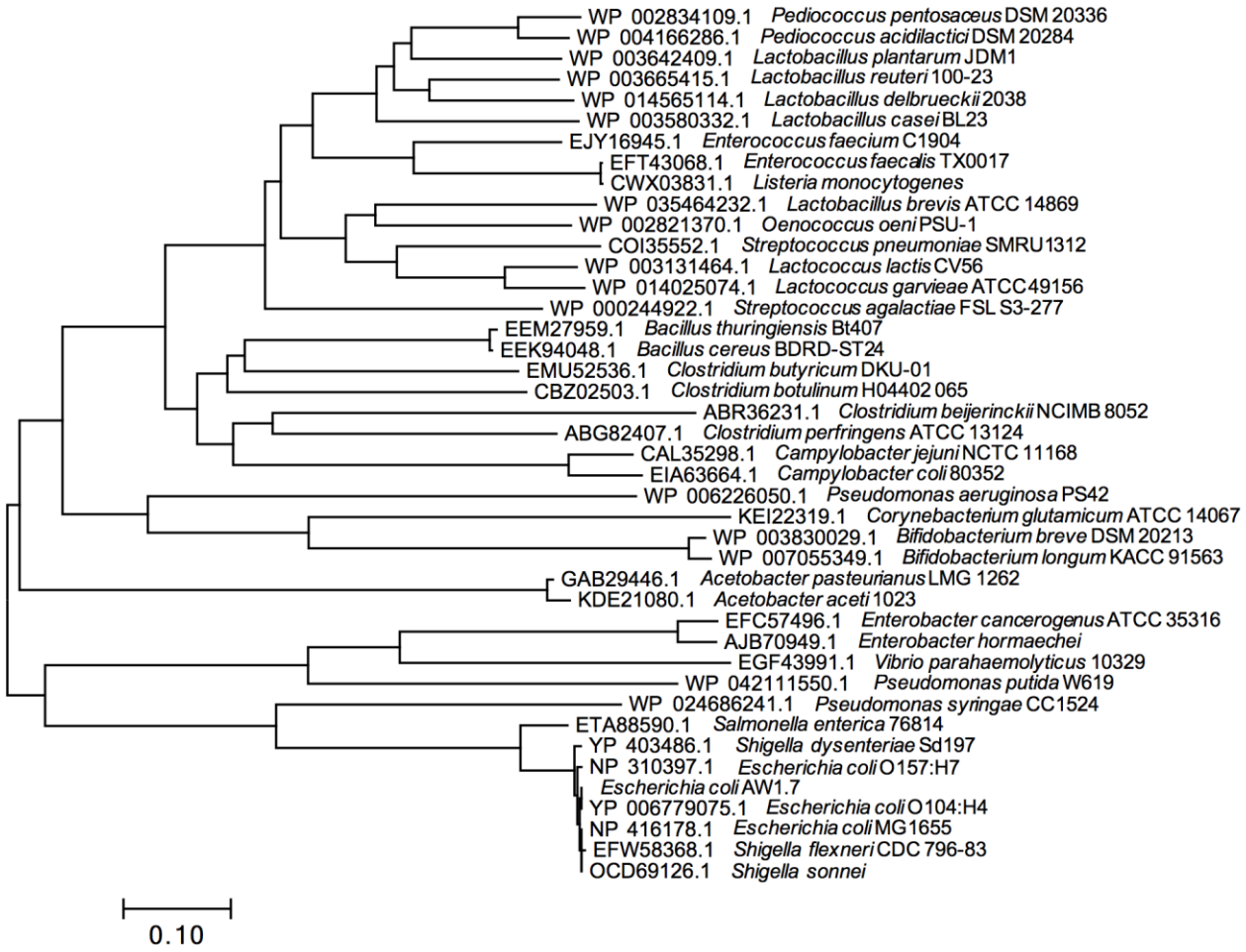

Figure 5-1. Phylogenetic tree of cyclopropane fatty acid synthases in Gram-positive and Gram-negative bacteria. The evolutionary relationships are shown with scale bar line which represents an evolutionary distance of 0.10.

aimed to provide the theoretical foundation for optimizing intervention methods. Improving the food safety requires a comprehensive understanding of mechanisms of the bacterial stress response, as well as the bacterial inactivation principles of pathogen intervention techniques. As a recent and promising food preservation technology, HPCD has not been widely applied in the food industry (Garcia-Gonzalez et al., 2007). One of the reasons is that the mechanism of bactericidal effect of HPCD has not been well

understood, and thus it is hard to optimize the treatment conditions of HPCD. To gain deeper insight into the microbial inactivation mechanism of HPCD, several mutants of *E. coli* AW1.7 were generated to test the proposed hypothetical mechanisms, including cell membrane perturbation and intracellular acidification (Garcia-Gonzalez et al., 2007). Firstly, it is known that CFAs in phospholipids render a more rigid membrane compared with UFAs (Poger and Mark, 2015). The different survival between *E. coli* AW1.7 wild type and *cfa* mutant strains was observed in this study, suggesting that CO_2-induced membrane fluidization and changes in membrane barrier properties are major causes of cell death by HPCD. Secondly, this study indicated that moisture content is an important factor for bacterial inactivation of supercritical CO_2, as demonstrated that the treatment with supercritical CO_2 was lethal to *E. coli* at high water activity (a_W), but not to the cells at low a_W. Thirdly, the inactivation efficiency of gaseous CO_2 is higher than supercritical CO_2 on dry *E. coli* cells, providing a novel and alternative method for the food industry to enhance the safety of products with low water content. This study revealed that many factors, including pressure, temperature, the physical state of CO_2 and the water content in food, all influence the bacterial inactivation efficiency of HPCD, which could provide clues for further optimizing conditions of HPCD to effectively treat food products.

5.4. Future directions

The study in the thesis suggests that CFA synthesis is a general protection strategy of food bacteria against environmental stresses. However, the protective role of CFAs

against stresses was only assessed in Gram-negative bacteria (e. g. *E. coli*) cells. As we discussed above, the CFA synthase also exists in Gram-positive bacteria (Figure 5-1) but I have not studied it yet. Whether CFAs protect Gram-positive bacteria against stresses, especially against high pressure and HPCD treatments, requires further studies to verify my speculation.

The study of the thesis indicates that gaseous and supercritical CO_2 could be utilized to inactivate bacteria in food with different moisture contents. Remarkably, *E. coli* AW1.7 was more susceptible to gaseous CO_2 than its Δ*cfa* strain, and it is opposite under supercritical CO_2 treatment. In the future, the effects of the different physical states of CO_2 on membrane property need to be further characterized, which will help us to better explain the survival of *E. coli* under supercritical and gaseous CO_2 treatments.

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