GLUTATHIONE UTILIZATION IN *LACTOBACILLUS FERMENTUM* CECT 5716

BY

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THESIS

Submitted in partial fulfillment of the requirements
for the degree of Master of Science in Food Science and Human Nutrition
in the Graduate College of the
University of Illinois at Urbana-Champaign, 2018

Urbana, Illinois

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ABSTRACT

Glutathione, a tripeptide antioxidant, has recently been shown to be either utilized or synthesized by gram-positive bacteria, such as Lactic Acid Bacteria. Glutathione plays an important role in countering environmental stress, such as oxidative stress. In this study, cellular activity regarding glutathione in *Lactobacillus fermentum* CECT 5716 is characterized. We demonstrate that *L. fermentum* CECT 5716 has a better survival rate in the presence of glutathione under both oxidative and metal stress. As *L. fermentum* CECT 5716 does not possess the ability to synthesize glutathione, it shows the ability to uptake both reduced and oxidized glutathione from the environment, regenerate reduced glutathione from oxidized glutathione, and perform secretion of glutathione to the environment.

ACKNOWLEDGEMENTS

I would like to thank my adviser, Dr. Michael Miller for his constant guidance and tremendous support during completion of my research and this thesis. I am grateful for his helpfulness and his patience with my stubbornness and overthinking habit. I would also like to thank Drs. Elizabeth Jeffery and Matthew Stasiewicz for taking the time to serve on my committee.

I would like to thank my current and former labmates: Luis Ibarra, Dr. Lili Zhang, Shannon Rezac, Suneet Takhar, Eric Fritz, Dr. Jennifer Hoeflinger; and especially to Dr. Christine Xiaoji Liu whom I bother relentlessly for both research/science-related matter and unimportant things. I am also thankful for the collaborator from Jeffery lab, Yanling Wang.

I am thankful for my family: my father Tjandra Heru who constantly calls me to give me support; my mother Dr. Meilinah Hidayat, who keeps me in her prayer and shares my interest in research and health-related issue; my brother Gradi Desendra who keeps supporting me for my thesis completion as we were in the same boat. I would like to thank Dr. Rachmat Mauludin, my supportive undergraduate thesis adviser, who kept motivating me to pursue graduate degree.

I also would like to express my utmost gratitude to the late Senator J. William Fulbright for this invaluable opportunity to study in the USA under Fulbright Scholarship administered by Institute of International Education. Your legacy to promote mutual understanding truly impacted millions of people to yearn and struggle for mutual understanding and peace.

Lastly, I would thank God my Father, whose great faithfulness keeps me strengthened. All of these would have been futile without Your Divine Providence.

For my late grandmothers,

Oma as my first inspiration to study abroad

Emak as a great example of perseverance

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CHAPTER 1: INTRODUCTION

1.1 Background

Glutathione is an antioxidant found in almost all species but was believed not to be present in most gram-positive bacteria[1]. In more recent discoveries, cytoplasmic glutathione was discovered in several gram positive bacteria and reported to contribute to increase of survival rate under several stress conditions[2]

Lactobacilli, as a member of gram-positive bacteria; was initially considered to be devoid of glutathione. As their metabolism is strictly anaerobically fermentative[3], molecular oxygen and reactive oxygen species may pose substantial harm for them. Lactobacilli have developed means to counter oxidative stress, from directly reducing molecular oxygen to regenerating antioxidant that directly attacks the oxidant. Putative glutathione system, albeit not necessarily present in complete form like in higher organisms, have been found in various *Lactobacillus*[4].

As environmental stress inevitably surrounds lactobacilli, antioxidants play indispensable role to ensure their survival. Glutathione, possibly utilized by various species of lactobacilli, may provide additional protection from oxidative and other stresses. Since lactobacilli are generally known for their probiotic property, increasing their survivability may be beneficial for industrial-scale culture. Glutathione utilization by lactobacilli may contribute to their survival of harsh competition in gut microbial community as well.

1.2 Objective and results

The objectives of this research were to 1) evaluate protective effect of glutathione on *Lactobacillus fermentum* CECT 5716 under oxidative and metal stress; 2) to evaluate utilization of glutathione in non-glutathione-synthesizing strain (*L. fermentum* CECT 5716); and 3) to propose a model of the glutathione system in this strain.

In this study, *Lactobacillus fermentum* CECT 5716 showed better survivability under oxidative stress caused by hydrogen peroxide with glutathione supplementation in their growth medium. Metal stress with copper was also alleviated with glutathione supplementation. As they showed capability to uptake both reduced and oxidized glutathione, they also showed the ability to reduce glutathione; despite lacking the capability to synthesize glutathione. Glutathione was also found to be actively transported outside the cytoplasm, which suggests it as a method to counter external environmental stress. Based on these activities related to glutathione, a transmembrane glutathione recycling model was proposed, that provide explanation regarding antioxidant effect of *Lactobacillus fermentum* in vitro and in animal model[5,6].

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CHAPTER 2: LITERATURE REVIEW

2.1 Lactic Acid Bacteria

Lactic acid bacteria (LAB) are defined as a group of bacteria which produce lactic acid, have low percentage of G and C, and are non-spore-forming. Generally, most LAB are facultative anaerobic, fermentative, catalase-negative, and acid tolerant. LAB often require rich and complex medium for their growth; while some of them possess the ability to grow in unlikely environment, such as high acidity or bile. Historically LAB had been known to be lacking cytochrome and heme-linked electron transport proteins, while in more recent discovery it was proven that respiratory pathways were found in some of LAB[1].

LAB perform biotransformation of fermented foods, such as dairy, meats and vegetables. Silage, cocoa, sourdough, and numerous indigenous food fermentation are also attributed to LAB[2]. Some LABs are also important pathogens, such as *Streptococcus pyogenes*. LAB belong to gram-positive bacterial phylum Firmicutes. LAB are classified into six families, which are *Aerococcaceae, Carnobacteriaceae, Enterococcaceae, Lactobacillaceae, Leuconostoccaceae, Streptococcaceae*. Other LAB-associated group, which is Bifidobacteria, belongs to phylum Actinobacteria; which share great similarity albeit containing high percentage of G+C[3]. Thus LAB are not phylogenetic classification, but rather representing metabolic capabilities of this group[4].

Many species of Lactobacillus are probiotics[5,6]. International definition of probiotics is live microorganisms that, when administered in adequate amounts, confer a health benefit on the host[7]. LAB have several health effects, which including food nutritional quality improvement,

colon cancer risk reduction, gut microbiota stabilization, stimulating vitamin and enzyme production and serum cholesterol reduction[8].

LAB are found in various habitats and environmental conditions. LAB are used as starters for fermented food production. Most fermented foods are produced using LAB. LAB are responsible for food preservation by oxidizing carbohydrates and producing acids, alcohol and carbon dioxide. Fermentation by LAB prevents growth of other microbes, results in more digestible products compared to their respective ingredients, and creates specific flavor, aroma, and texture in food[9].

Lactobacillus is the most diverse among LAB. Lactobacilli can be classified based on source of isolation, which are plant, cereal, meat products, dairy products, wine products, animal gastrointestinal tract, non-gastrointestinal parts of animal, and environment in general[10].

By their metabolic pathways for glucose fermentation and their ability to metabolize pentose, LAB are classified as homofermentative, facultative heterofermentative and obligate heterofermentative. Homofermentative LAB ferment glucose through glycolysis solely into lactic acid and do not metabolize pentoses. Facultative heterofermentative LAB do glucose fermentation through glycolysis pathway while fermenting pentose sugars via the phosphoketolase pathway. Obligate heterofermentative LAB ferment glucose and pentose through phosphoketolase pathway. Glycolysis resulting in lactate while phosphoketolase pathway producing acetate, carbon dioxide, and ethanol[11]. LAB require complicated growth media formulation and some species require nutrient rich media to grow[1].

2.2 Genus Lactobacillus

Lactobacillus belongs to Lactobacilliaceae, first classified by Winslow et al[12], that also consists of *Pediococcus, Paralactobacillus* genera. Members of Lactobacillaceae require nutrient-rich environment to grow, and Lactobacillaceae are part of microbial community in human gastrointestinal tract, mouth, and genitalia[13].

Members of the genus *Lactobacillus* are generally aerotolerant, mostly rod-shaped, non-motile, non-sporeforming. They can grow in between pH of 3 to 8, and between 2-53 °C, while the optimal temperature for their growth is typically between 30 to 40 °C. *Lactobacillus* mainly utilize Embden-Meyerhof-Parnas pathway or glycolysis to metabolize glucose. This genus was classified by Beijerinck in 1901[14].

Kandler and Weiss[15] classified Lactobacilli into 3 groups, which are obligate hormofermentative (group A), facultative homofermentative (group B), and obligate fermentative (group C), while more recent studies suggested the contradiction with their phylogenetic tree within the group[16]. Collins et al.[17] grouped Lactobacilli into three phylogenetic group, which are *Lb. delbrueckii* group, *Lb. casei-Pediococcus* group, and *Leuconostoc* group. The latest classification by Salvetti et al.[18] based on the 16s rRNA divides Lactobacilliaceae into several groups, which are *L. delbrueckii group, L. salivarius group, L. reuteri group, L. buchneri group, L. alimentarius group, L. brevis group, L. collinoides group, L. fructivorans group, L.plantarum group, L. sakei group, L. casei group, L. coryniformis group, L. manihotivorans group, L.perolens group, L. vaccinostercus group, Pediococcus group*, and non-associated single species.

Lactobacilli obtain most of their energy through substrate-level phosphorylation. Most common pathway utilized by lactobacilli is Embden-Mayerhof-Parnas (EMP) pathway, while

some also utilize pentose phosphate pathway. Homolactic fermentation mostly occurs via EMP Pathway, while heterolactic fermentation occurs via the phosphoketolase[14]. Homo-fermentative lactobacilli have the ability to utilize pentose phosphate pathway to metabolize certain substrate. Free saccharides are uptaken into their cytoplasm using phosphotransferase system (PTS) or permease system[19].

Lactobacilli acquire amino acids from external source through proteolytic systems. Proteolytic system consists of proteinase, transport system, and peptidase. High molecular weight proteins are digested by secretion of proteinases to form oligopeptides, uptaken into cytoplasm, and hydrolyzed into amino acids by peptidases[20,21]. Each species has various and diverse requirement of amino acids. Proteolysis results in changes in chemical composition and organoleptic properties of fermented product[22]. Proteolysis contributes to suppression of antigenic response to Beta-lactoglobulin content in milk[23].

2.3 *Lactobacillus fermentum*

Lactobacillus fermentum is facultative anaerobic, strictly obligate heterofermentative, member of *L. reuteri* group. It is found in dairy products, sourdough, oral cavity and feces. The cells are non-motile, rod-shaped, have 0.5-0.9 μm thickness. The cells can be either single or in pairs, and have high variability of length. *Lactobacillus fermentum* requires vitamin B5, B3, and B1, while not necessarily require vitamin B2, B6, and folic acid. They bear great resemblance to *L. reuteri*, and are only distinguishable by genomic difference, as they share similar physiological property which makes phenotypic classification challenging[14].

Lactobacillus fermentum CECT 5716 was isolated from breast milk. The genome of *Lactobacillus fermentum* CECT 5716 was sequenced and found to be highly similar with

Lactobacillus fermentum IFO 3956, which its circular chromosome contains 2,100,449 base pairs, GC content of 51.49% and possesses no plasmid[24]. This strain is a good probiotic candidate as it shows high degree of survivability of gastrointestinal tract-like environment, has strong adherence to intestinal cell, stimulation of mucin-encoding genes, and possess in-vitro and in-vivo immunomodulatory and antibacterial property against pathogenic bacteria[25,26]. This strain was reported to prevent and revert intestinal damage on Trinitrobenzenesulfonic acid-induced colitis in mice[27]. Effects on influenza vaccination was reported to be enhanced with oral intake of this strain as well[26].

2.4 Oxidative Stress

By-products of molecular oxygen breakdown, which are superoxide anion radical (O_2-), hydrogen peroxide, and highly reactive hydroxyl radicals are produced in cells that are grown with the presence of oxygen. Biological targets for the reactive species of oxygen are DNA, RNA, proteins and lipids. Most impactful cellular damage is caused by hydroxyl radicals (·OH). The hydroxyl radicals are generated from hydrogen peroxide through Fenton reaction, which requires divalent metal ions, such as iron and copper, and metal reducers. Polyunsaturated fatty acids in membrane can be attacked by free oxygen radicals and lipid peroxidation will occur. Lipid peroxidation will result in the reduction of membrane fluidity, which modify the properties of membrane and may significantly compromise membrane-bound protein[28].

At cellular level, exposure of proteins to reactive oxygen species will result in modifications of amino acid side chains, and thus altering the protein structure. Such modification results in change in functional property and metabolism disturbances. Irreversible amino acid residue oxidation in protein can undergo through two mechanisms, which are

ionizing radiation, and metal ion-catalyzed oxidation reactions. Ionizing radiation can radiolyze water molecule, creating hydroxyl radicals. The radical species can react with alpha hydrogen atom of amino acid residue to form carbon centered radical. This alkoxyl radical may yield cleavage of the peptide bond under presence of oxygen[28].

As cysteine residue in cytosolic protein are maintained in reduced form due to the effect of the disulfide bond to protein stability, changes in reducing environment may alter protein folding and activity. Under oxidizing environment, unwanted formation of disulfide bond can occur in native-form cysteine residue in protein[28].

Metal-catalyzed oxidation of protein occurs by the reaction of radical oxygen species that oxidizes amino acid residue at or near cation-binding site. Metal-catalyzed oxidation can result in alteration of cytosolic proteins, which are carbonyl group formation, loss of catalytic activity, amino acid modification, increase in acidity, decrease in thermal stability, change in viscosity, change in fluorescence, fragmentation, formation of protein-protein cross-links, formation of disulfide bridges, and increased susceptibility to proteolysis[28].

2.5 Antioxidant system of Lactobacilli

Within *Lactobacillus*, several enzymes have been attributed to quench molecular oxygen and its toxic derivatives (**Table 2.1**). These reactions aim to reduce either molecular oxygen or its reactive species into less or non-reactive species. Decarboxylation of pyruvate catalyzed by pyruvate oxidase turns molecular oxygen into hydrogen peroxide[29]. NADH:H_2O_2 oxidase, found in *Lactobacillus plantarum*[30], *Lactobacillus delbrueckii subsp. bulgaricus*[31] and *Lactobacillus sanfranciscensis*[29]; possesses the ability to reduce molecular oxygen into hydrogen peroxide. *Lactobacillus sanfranciscensis* CB1[32] and *Lactobacillus sakei*[33] can convert radical molecular

oxygen, O_2-, into hydrogen peroxide with superoxide dismutase. However the accumulation of hydrogen peroxide also contributes to oxidative stress[31]. Hence, peroxide can be detoxified with NADH:H_2O oxidase, that uses a molecule of NADH to reduce peroxide into 2 molecules of water. NADH:H_2O oxidase is reported to be present in *Lactobacillus plantarum* and *Lactobacillus casei*[34]. Despite LAB was initially considered to be catalase-negative, *Lactobacillus sakei* possesses a heme-dependent catalase; which converts two molecules of peroxide into two molecules of water and molecular oxygen[35]. Two molecules of NADH can also be used to reduce molecular oxygen directly into water and oxygen, catalyzed by NADH peroxidase that is found in *Lactobacillus plantarum*[30] and *Lactobacillus sanfranciscensis*[29].

Other enzymes do not directly detoxify molecular oxygen or radical oxygen species, but rather reduce back the oxidized enzymes or cofactors. Glutathione reductase was characterized in *Lactobacillus sanfranciscensis*, which converts glutathione disulfide into reduced glutathione with NADPH as reducing cofactor[36]. Thioredoxin reductase, which was characterized in *Lactobacillus casei*[37], can reduce thioredoxin disulfide into thioredoxin. Thioredoxins are generally 12 kDa reductases, possessing active -CGPC- conserved site motif, which is used to catalyze protein disulfide change. Thioredoxin also has a role as antioxidant against oxidative stress. Thioredoxin system comprises of thioredoxin, thioredoxin reductase, and NADPH as reducing cofactor[38].

2.6 Glutathione

Glutathione, or gamma-L-glutamyl-L-cysteinyl-glycine (**Figure 2.1**) , is a tripeptide comprised of cysteine, glutamate, and glycine residue. Presence of gamma-glutamyl bond enables glutathione to resist intracellular peptidase, and thiol functional group serves as electron donor[39]. Glutathione is considered to be the most important low molecular weight peptide in

cells in most species, which thiol functional group is responsible over reduction and conjugation reactions[40]. Glutathione is present in virtually all eukaryotic cells, while traditionally in prokaryotes glutathione presence restricted to proteobacteria and cyanobacteria, with some exceptions[41]. Glutathione in E. coli, a gram-negative bacterium and member of proteobacteria, serves as detoxifying agent, maintaining thiol status, anti-oxidant, and source of cysteine. Glutathione detoxifies electrophilic xenobiotics, as the conjugates are formed either spontaneously or catalyzed by glutathione-s-transferase. Conjugates are generally excreted from the cells[39].

Glutathione also works as antioxidant, by reducing endogenous hydrogen peroxide catalyzed by selenium-dependent glutathione peroxidase. Oxidized glutathione can be reduced back catalyzed by glutathione reductase with NADPH as reducing cofactor[42]. Glutathione reductase from different sources are known to be highly homologous and conserved[39].

Mechanism of glutathione synthesis in most organisms involves two ATP-dependent enzymes-catalyzed reactions. The first step is to form gamma-glutamylcysteine from glutamate and cysteine. This first step is the rate limiting step catalyzed by glutamate-cysteine ligase (GCL). The second step is catalyzed by glutathione synthetase (GS), which yields glutathione from gamma-glutamylcysteine and glycine[43].

Glutamate-cysteine ligase in yeast and bacteria has a single polypeptide, contrary to GCL in animals which has a catalytic and modifier subunit. The second step in glutathione synthesis is to form complete glutathione, from gamma-glutamylcysteine with glycine catalyzed by glutathione synthetase[43]. Overexpression of glutathione synthetase was found not to increase the glutathione synthesis rate[44].

Gamma-Glutamyl transpeptidase (GGT) is a key enzyme for glutathione breakdown. GGT in *E. coli* has the active catalytic side in the outer side of inner membrane. Glutathione in *E. coli* is degraded by exporting glutathione from cytoplasm, and GGT cleaves glutathione into L-cysteinyl-glycine and a glutamyl residue bound with enzyme. L-cysteinyl-glycine can be broken down with dipeptidase and readily absorbed into the cell. The glutamyl residue can be transferred onto an amino acid or returned into the cytoplasm[39].

Several glutathione-related components have been found in LAB. Glutathione-S-transferase, an enzyme required to conjugate glutathione with xenobiotics, was putatively found in *Lactobacillus casei* through genome sequencing and bioinformatics approach[45]. Glutathione reductase, which catalyzes reduction of oxidized glutathione into reduced form with NADPH as cofactor, was described in *Lactobacillus sanfranciscensis*[36]. Putative gene that was proposed to be glutathione peroxidase, an enzyme utilized for hydrogen peroxide neutralization, was found in *Lactobacillus hokkaidonensis* through genomic sequence[46]. Both activities related to glutathione peroxidase and glutathione reductase were found in *L. fermentum* ME-3 [47]. Putative glutathione reductase and glutathione peroxidase activity were also found through bioinformatics approach in several strains of *Streptococcus thermophilus, Lactobacillus casei, Lactobacillus rhamnosus, Lactobacillus plantarum, and Lactobacillus fermentum*; however, these putative proteins have not been characterized experimentally[48].

2.7 Glutathione Synthesis in Bacteria

Several gram negative bacteria, like *Pseudomonas aeruginosa*[49], *Escherichia coli*[50] and *Sinorhizobium meliloti*[51] are known to utilize glutathione, and synthesize it in "conventional"

two steps reactions, catalyzed by Glutamyl Cysteine Ligase (GCL) and Glutathione synthase (GS).

However an unusual mechanism of glutathione synthesis was discovered in *Listeria monocytogenes*[52] and *Streptococcus agalactiae*[53]. That unique enzyme, encoded by gene called GshF, has both functions of GCL and GS. Such enzyme shows similarity with *E.coli* GCL, but the putative GS domain does not have sequence similarity with GS from *E. coli*[53]. GshF is reported to be present in several Actinobacteria, which belong to gram-positive bacteria[54]. Several LAB are reported to possess putative enzyme through bioinformatics approach[48]. Overexpression of GshF from *Lactobacillus plantarum* and *Lactobacillus casei* in *E. coli* showed increased cytoplasmic glutathione yield[55]. However, GshF is not exclusively found in gram-positive bacteria, as it was discovered in gram-negative *Pasteurella multocida*[56].

2.8 Glutathione Transport in Bacteria

Glutathione is known to be imported by several species of bacteria. *E. coli* can perform glutathione import with an ATP-binding protein encoded by yliABCD (also known as gsiABCD), which consist of ATP-binding subunit, periplasmic binding subunit, and inner membrane component[57]. *Haemophilus influenzae* was reported to uptake glutathione through DppBCDF, an ABC transporter, primed with periplasmic solute-binding protein GbpA[58]. *Lactococcus lactis*, which does not synthesize glutathione, was reported to accumulate glutathione when grown in milk via an unknown mechanism[59]. *Streptococcus mutans* was reported to uptake glutathione[60] through a mechanism explained below.

Glutathione is also known to be exported by several bacteria. GSH was transported and the concentration increased during exponential phase and peaked in early-stationary phase

culture in *Salmonella typhimurinum* LT-2 and *E. coli* K-12 [61]. Export of glutathione in *E. coli* is mediated by the CydDC complex, a heterodimeric ATP-binding cassete-type transporter[62], and such activity was reported to combat stress caused by reactive nitrogen species[63]. Hence glutathione was proposed to be cycled transmembranely in *E. coli*, based on activity of glutathione importer (yliABCD), glutathione exporter (cydDC), and gamma-glutamyl transpeptidase that cleaves glutathione[50].

Glutathione transporters in many species are known to have certain degree of affinity to cysteine or cysteine. In *Saccharomyces cerevisiae,* GSH-P1, which is a glutathione transporter, was mildly inhibited by cysteine[64]. Reduced glutathione, glutathione disulfide, and cysteine were reported to compete for uptake by a common transporter in *Streptococcus mutans*[65,66]. Glutathione uptake in *Streptococcus mutans* is performed through priming by GshT, a solute binding protein, with TcyABC, an L-cystine ABC transporter[67]. CydDC, a membrane-bound respiratory oxidases, was reported in *E. coli* to be capable of transporting cysteine[68] and also reported to transport glutathione as well[62]. Pophaly et al[69] proposed that CydDC is the responsible transporter over glutathione import in LAB, based on bioinformatics analysis. However, this hypothesis was not supported by any experimental evidence. There is a possibility that this protein perform glutathione transport bi-directionally; however, it requires experimental evidence for confirmation.

2.9 Glutathione Status in Lactic Acid Bacteria

Glutathione has been known for a long period to be present in gram negative bacteria, while reported to be absent in most gram positive bacteria[70]. However, glutathione presence in growth medium was reported to alleviate cold-related stresses in *L. sanfranciscensis*[71].

Glutathione was involved in acid resistance in *Lactobacillus salivarius*[72] and *Lactococcus lactis*[73]. Lactococcus lactis were reported to survive better with glutathione supplementation under oxidative stress[74]. Glucose metabolism and protein synthesis of *Lactobacillus reuteri* was reported to be enhanced with glutathione supplementation[75]. Intracellular glutathione was also found in various species among LAB[69].

Glutathione in LAB can be either transported, synthesized, or both transported and synthesized. First LAB which was found to contain glutathione is *Lactococcus lactis*[70]. The fact that *Lactococcus lactis*, which lack the ability to synthesize glutathione, can accumulate glutathione which increases resistance towards oxidative stress suggested the capability of glutathione import[74]. *Streptococcus mutans* was also reported to import glutathione under glutathione supplementation in the glutathione-depleted medium[60]. Several strains of *Lactobacillus casei, Lactobacilus rhamnosus, and Lactobacillus fermentum* do not contain glutathione when grown in glutathione-free medium. However, glutathione can be found in those strains grown with glutathione supplementation[48].

Some bacteria that were grown without glutathione presence in the medium were found to contain glutathione inside their cytoplasm, such as *Streptococcus agalactiae*. That strain synthesizes glutathione with bifunctional GshF[53]. Presence of putative GshF gene in several strains of *Streptococcus thermophilus, Lactobacillus casei, Lactobacillus rhamnosus, and Lactobacillus plantarum* were reported; and glutathione content was found in several strains of *Streptococcus thermophilus* that were grown in glutathione-free medium[48] (Pophaly et al, 2017).

2.10 Tables and Figures

Figure 2. 1 Glutathione Structure

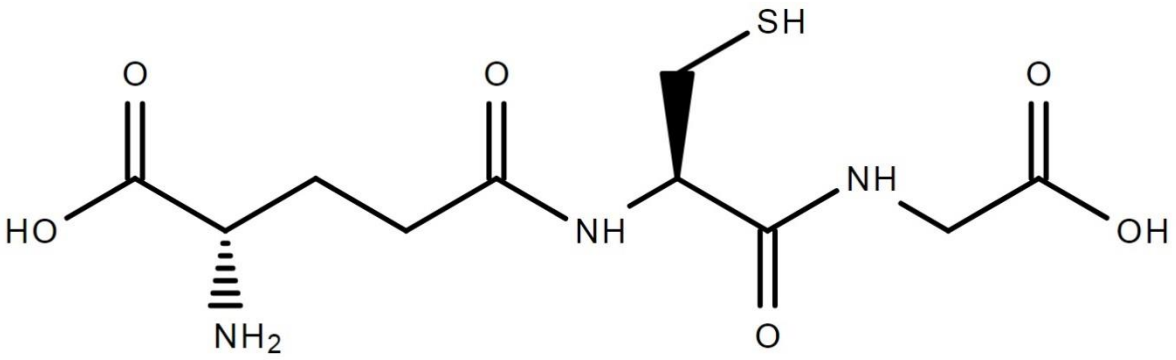

Table 2. 1 Antioxidant System in Lactobacilli

Enzyme	Reaction	Host	Reference
NADH:H_2O_2 Oxidase	NADH + H^+ + O_2 -> NAD^+ + H_2O_2	*L. plantarum*, L. sanfranciscensis*, L delbrueckii subsp. bulgaricus**	Stoltz et al (1995) Götz et al (1980) Marty-Teysset et al. (2000)
NADH:H_2O Oxidase	2 NADH + 2 H^+ + O_2 -> 2 NAD^+ + H_2O	*L. plantarum* L. casei**	Condon (1987)
NADH: peroxidase	NADH + 2 H^+ + O_2 -> NAD^+ + 2 H_2O	*L. plantarum* L. sanfranciscensis**	Stolz et al. (1995) Götz et al. (1980)
Superoxide dismutase	2 H^+ + $O_2^{\cdot-}$-> H_2O_2	*L. sanfranciscensis CB1*, L. sakei**	De Angelis and Gobbetti (1999), Amanatidou et al. (2001)
Heme-dependent catalase	H_2O_2 -> 2 H_2O+ O_2	*L. sakei**	Knauf et al. (1992)
Thioredoxin reductase	Trx disulfide + NADPH -> $Trx_{(SH2)}$ + NADP+	*L. casei*[#]	Serata et al. (2012)
Pyruvate oxidase	Pyruvate + Phosphate + O2 -> Acetyl phosphate + CO_2 + H_2O_2	*L. delbrueckii subsp. bulgaricus*, L. casei*, L. plantarum**	Götz et al. (1980)

Table 2. 2 (cont.)

Cysteine Uptake	Cysteine -> Cystine	*L. fermentum**	Turner et al. (1999)
Glutathione reductase	NADPH + GSSG -> $NADP^+$ + GSH	*L. sanfranciscensis***	Jansch et al. (2007)

#Bioinformatics prediction

*Experimentally confirmed

Adapted from De Angelis and Gobbetti[73] with necessary changes

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CHAPTER 3: GLUTATHIONE UTILIZATION AS ANTIOXIDANT IN *LACTOBACILLUS FERMENTUM CECT 5716*

3.1 Abstract

Glutathione, a tripeptide antioxidant, has recently been shown to be either utilized or synthesized by gram-positive bacteria, such as Lactic Acid Bacteria. Glutathione plays an important role in countering environmental stress, such as oxidative stress. In this study, cellular activity regarding glutathione in *Lactobacillus fermentum* CECT 5716 is characterized. We demonstrate that *L. fermentum* CECT 5716 has a better survival rate in the presence of glutathione under both oxidative and metal stress. As *L. fermentum* CECT 5716 does not possess the ability to synthesize glutathione, it shows the ability to uptake both reduced and oxidized glutathione from the environment, regenerate reduced glutathione from oxidized glutathione, and perform secretion of glutathione to the environment.

3.2 Introduction

Glutathione or γ-L-glutamyl-L-cysteinyl-glycine, the most abundant non-protein thiol found in many organisms[1], is an important low molecular weight antioxidant. Glutathione is known to be able to neutralize reactive oxygen species, such as hydrogen peroxide in combination with glutathione peroxidase[2]. The resultant oxidized glutathione can be reduced by glutathione reductase with NADPH as the electron donor[3]. Glutathione has also been shown to neutralize xenobiotics with glutathione-s-transferase[4]. In addition, glutathione acts a protein reductant in the cytoplasm; either directly or indirectly through the glutaredoxin system[5].

Glutathione is synthesized in two steps by proteins GshA and GshB or by the more recently discovered bifunctional GshF which enables glutathione to be synthesized in one step[6].

Glutathione was historically considered to be neither synthesized nor imported by most gram positive bacteria[7], including Lactic Acid Bacteria (LAB). However, glutathione supplementation was subsequently found to alleviate the impact of various environmental stresses in LAB, such as alleviating the effect of cold-related stresses in *Lactobacillus sanfranciscensis*[8], and being involved in acid stress response[9] and increasing survival rate under oxidative stress in *Lactobacillus salivarius*[10]. This suggests that at least some LAB possess the ability to synthesize and/or utilize glutathione which has been confirmed in *Streptococcus pneumoniae*[11] and *Streptococcus mutans* [12,13]. In addition, glutathione utilization components have been characterized in several LAB including; *Lactobacillus sanfranciscensis* with glutathione reductase[14], and *Lactobacillus fermentum* ME-3 has glutathione reductase and glutathione peroxidase[15].

This study aims to characterize glutathione utilization in glutathione-importing *Lactobacillus fermentum.* Previous screening performed by Pophaly et al.[16] has demonstrated the lack of the complete glutathione synthesis system in several strains of *L. fermentum*. We have investigated the protective effect of glutathione in *L. fermentum* CECT 5716, a commercially available probiotic[17,18] under several environmental stressors. Glutathione provided *L. fermentum* CECT 5716 protection from oxidative stress. We confirmed that reduced and oxidized glutathione can be imported by this strain. Furthermore, we determined that glutathione can be exported from the cells. Promoting survivability of a probiotic strain over stress conditions may promote their survival in gastrointestinal tract and during industrial culture production.

3.3 Materials and Methods

Bacterial Strain and Chemical Reagents

Pure culture of *Lactobacillus fermentum* CECT 5716 was provided by Biosearch Life (Granada, Spain). Fructose, magnesium sulfate heptahydrate, reduced (GSH) and oxidized glutathione (GSSG) were purchased from Sigma-Aldrich (St. Louis, MO, USA). MRS broth and Bacto Peptone were purchased from BD Biosciences (Franklin Lakes, NJ, USA); Tween 80, agar powder, yeast extract, and potassium phosphate dibasic anhydrous, copper (II) sulfate pentahydrate and ammonium citrate tribasic were purchased from Thermo Fisher Scientific (Waltham, MA, USA); PBS tablets and manganese sulfate were purchased from MP Biomedicals (Solon, OH, USA); and peroxide solution (3%) was purchased from local retail stores.

GSH-treated Cell Preparations.

Overnight culture of *L. fermentum* CECT 5716 from -80°C grown in semisynthetic medium[19] was sub-cultured three times, incubated in 37°C incubator, each for 24 hours in the semisynthetic growth medium containing 1% (w/v) fructose or the same media which was supplemented by glutathione. Glutathione supplementation was performed by adding 76.83 mg of reduced glutathione into 50 mL of 1%(w/v) fructose semisynthetic media to result in 50μM of supplemented reduced glutathione, then filtering the medium aseptically with sterile 0.22 μM membrane (Thermo Fisher Scientific, Waltham, MA, USA) under sterile condition. Glutathione content in the base medium was measured with LC-MS/MS, which was 2 μM, exclusively present in oxidized form, while GSH supplementation of this medium increased the amount of total glutathione to 52 μM. These cell suspensions were used for the peroxide stress-response

assay, cytoplasmic glutathione content, and extracellular and intracellular glutathione under sub-lethal peroxide treatment.

Peroxide Stress-Response Assay.

GSH-treated cells prepared as above were harvested by centrifugation at 16,000 x g for 2 minutes. Then the cell pellets were washed twice by resuspending the pellet in PBS buffer, centrifugation at 16,000 x g for 2 minutes, and supernatant removal. After washing, cells were resuspended in semisynthetic growth medium without glutathione supplementation. The cell suspensions were divided with half used for the control and half for the treatment group. In the treatment group, hydrogen peroxide was added to the resuspended cells to yield 3mM in suspension, before incubating for 60 minutes at 37°C. For the control group, cells were incubated in the same condition without addition of hydrogen peroxide.

Viable cell number was quantified by serial dilutions in PBS and plating on de Man-Rogosa-Sharpe agar according to the Miles-Misra[20] method. The cell counts were determined following incubation of the plates for 24 hours at 37°C anaerobically (90% N_2, 5% CO_2, 5% H_2). The entire experiment was repeated 5 times independently.

Copper Stress-Response Assay.

An overnight culture of *L. fermentum* CECT 5716 from a glycerol stock stored at -80°C, was sub-cultured three times, each for 24 hours in 1% (m/v) fructose-containing semisynthetic growth media. For the treatment group the same medium was used but containing 125 or 250 μM of copper sulfate. The media were filtered aseptically with sterile 0.22 μm membrane (Thermo Fisher Scientific, Waltham, MA, USA).

Cell suspensions were homogenized by vortexing, subcultured by 1% (v/v) in 200µL glutathione-supplemented (containing 50 µM of supplemented reduced glutathione) or non-supplemented 1% fructose-containing semisynthetic media. Cellular growth profile was determined with Bioscreen C (Growth Curves USA, NJ, USA) for 24 hours by measuring OD_{600nm} every 10 minutes with 5 seconds of shaking before each measurement in an anaerobic chamber maintained at 37°C. The entire procedures were performed in 4 independent biological replicates and measured in 3 technical replicates. The growth curve was analyzed using the algorithm developed by Hoeflinger et al.[21,22] in MATLAB (MathWorks, Natick, MA, USA) to determine the lag phase time, maximum cell density, and maximum growth rate.

Cytoplasmic Glutathione Content.

The intracellular content of glutathione in *L. fermentum* CECT 5716 was determined. GSH-treated cells were prepared according to GSH-treated cell preparation above, except the glutathione was supplemented in either reduced form (50 µM) or oxidized form (25 µM) to yield equal sulfur content. Cells were harvested by centrifugation at 16,000 x g for 2 minutes. Then the cell pellets were washed three times by resuspending the pellet in PBS buffer, centrifugation at 16,000 x g for 2 minutes, supernatant removal. After the third cell-washing, cells were resuspended in PBS buffer. Viable cell counts were determined by plating on MRS agar as described above.

The resuspended cell pellets in PBS were homogenized by bead-beating for 10 minutes in an anaerobic environment (90% N_2, 5% CO_2, 5% H_2). Cell lysates were stored at -20°C until analysis. The entire experiment was repeated in 3 independent biological replicates. The samples were measured with LC/MS/MS as described below.

Extracellular and Intracellular Glutathione under Sub-lethal Peroxide treatment.

The cells of *L. fermentum* CECT 5716 were prepared according to GSH-treated cell preparation above. The cells were harvested by centrifugation at 3,220 x g for 5 minutes. Cell washing was performed 3 times, by resuspending in PBS buffer, centrifugation at 3,220 x g for 5 minutes, and supernatant removal.

Recovered cell pellets were resuspended in PBS buffer to 1/5 of the original volume. The treatment group was added hydrogen peroxide to yield 0.25 mM, incubated for 30 minutes at 37 °C. The peroxide treatment was repeated once for a total treatment time of 1 hour. The control group was incubated for 1 hour in 37 °C without peroxide treatment.

The cell suspension were centrifuged in 3220 x g for 5 minutes, the supernatant was decanted and recovered for analysis. The supernatants were placed in o-ring (Thermo Fisher, Waltham, MA, USA) tubes sealed with Parafilm (Bernis NA, Neenah, WI, USA), and kept at -20°C until measurement.

Cells were resuspended back into its original volume with PBS buffer. Viable cell counts were determined by plating on MRS agar as described above. Resuspended cell pellets were homogenized with bead-beater for 10 minutes under anaerobic condition (90% N_2, 5% CO_2, 5% H_2) and cell lysate were placed in o-ring tubes sealed with Parafilm. Cell lysates were kept at -20°C until measurement. The samples were measured with LC/MS/MS as described below.

LC-MS/MS Measurements.

GSH, GSSG, cysteine, and cystine were analyzed with the 5500 QTRAP LC/MS/MS system (Sciex, Framingham, MA) in the Metabolomics Lab of Roy J. Carver Biotechnology

Center, University of Illinois at Urbana-Champaign. Software Analyst 1.6.2 was used for data acquisition and analysis. The 1200 series HPLC system (Agilent Technologies, Santa Clara, CA). The LC separation was performed on an Agilent Eclipse Plus XDB-C18 column (4.6 x 150mm, 5μm) using mobile phase A (0.1% formic acid in water) and mobile phase B (0.1% formic acid in acetonitrile). The flow rate was 0.35 mL/min. The linear gradient was as follows: 0-3min, 100%A; 10-11 min, 0%A; 12-18 min, 100%A. The autosampler was set at 10°C. The injection volume was 10 μL. Mass spectra were acquired under positive electrospray ionization (ESI) with the ion spray voltage of +5000 V. The source temperature was 450 °C. The curtain gas, ion source gas 1, and ion source gas 2 were 32, 50, and 65, respectively. Multiple reaction monitoring (MRM) was used for quantitation: Cystine m/z 122.0 --> m/z 59.0; Cysteine m/z 122.0 --> m/z 59.0; GSSG m/z 613.3 --> m/z 355.2; GSH m/z 308.1 --> m/z 179.2.

Statistical Analysis. Experiment data with multiple factors were statistically analysed for the interference between each factors within the statistical model using JMP (SAS Institute, NC, USA). Each of the factors were further analysed with ANOVA to determine the significant difference. When multiple factors affect the parameter, Tukey Test was used to determine significance; when only a single factor affected the parameter, Student's t-test was used. In all cases, significance was defined as $p < 0.05$.

3.4 Result and Discussion

Peroxide stress Response Assay.

In this assay, stress-response of peroxide was performed to determine the protective effect of reduced glutathione supplementation on *L. fermentum* CECT 5716 viability. Initial dose

testing determined that 3 mM hydrogen peroxide was sufficient to generate an approximate 5-log reduction. To prevent additional environmental stress to the cells, plated cells were grown in anaerobic condition. We hypothesized that resistance to peroxide can only occur if glutathione is used by the cells.

Under peroxide treatment, surviving cells of the *L. fermentum* CECT 5716 grown in glutathione-minimum growth medium (2 µM of oxidized glutathione) were reduced by 5-log compared to the control group, as shown in **Figure 3.1**. Under the same peroxide treatment, cells that were grown with abundant glutathione in their growth medium (52 µM total glutathione) had a better survival rate, only suffering 3-log reduction.

This result confirmed that glutathione supplementation increases the survival rate of *L. fermentum* CECT 5716 under peroxide stress. A similar protective effect by glutathione against oxidative stress has been reported in *L. plantarum*[10]. Since the stress in this assay was provided from an outside source, there were two possibilities regarding the intracellular glutathione with oxidative stress. Either the oxidant enters the cytoplasm and detoxification of the external oxidant occurs exclusively inside the cell, or the cell may export glutathione to the environment before the external oxidant penetrates into the cytoplasm.

As cell washing was performed prior to the stress treatment, resulting in a similar extracellular environment for both glutathione supplemented and non-supplemented groups; in that the external GSH/GSSG concentrations were identical for the two treatments. Thus, the difference in survival rate under the same peroxide treatment can only be caused by the intracellular content. Therefore, it can be suggested that *L. fermentum* CECT 5716 actively uptakes glutathione into its cytoplasm and the intracellular glutathione resulted in an increased survival rate.

Copper stress Response Assay.

To determine glutathione's role in metal-induced stress, cells pretreated with copper (II) sulfate were evaluated for growth in the presence and absence of glutathione supplementation. The copper stress-response assay was designed to induce internal metal stress; while glutathione supplementation was performed latter to provide sufficient glutathione to growing pre-stressed cells. Cellular growth was measured in an anaerobic environment to prevent additional environmental stress.

Cells that were grown in the presence of glutathione showed shorter lag phase compared to cells that lack glutathione in their growth medium (**Figure 3.2**). Additionally, copper-pretreated cells showed a longer lag phase compared to the untreated group. Glutathione presence resulted in similar lag phases between copper pretreated group and the control group. In all cases, the glutathione-supplemented growth medium does not alter the *L. fermentum* CECT 5716 doubling rate.

The significantly shorter lag phase for the cells grown in glutathione-supplemented medium, compared to their respective control groups, suggests that glutathione can be utilized by *L. fermentum* CECT 5716. Since the lag phase represents adaptation to the new medium and environment, a shortened lag phase suggests that glutathione utilization by *L. fermentum* CECT 5716 facilitated a better adaptation before transitioning to the exponential phase. Such activity may be attributed antioxidant or conjugating property of glutathione[23,24].

Pre-treatment of copper sulfate was designed so that the cells would accumulate copper. Internalized copper mediated stress on the cells, as demonstrated by the longer lag phase for copper-pretreatment group. Since copper-pretreated cells were passed into fresh media, we

assume copper was exclusively available inside the cytoplasm. Despite copper increasing lag phase in all tested conditions, we were unable to provide statistically significant evidence that suggested a specific interaction between glutathione supplementation and the copper-induced stress. Hence, glutathione's activity of reducing lag time was independent of the presence of copper.

Cytoplasmic Glutathione content.

The previous results provided evidence suggesting that glutathione was utilized by *L. fermentum* CECT 5716 and glutathione supplementation has a protective effect on this strain under oxidative stress. However, the presence of cytoplasmic glutathione was not determined and how glutathione is utilized was not characterized. To characterize the cytoplasmic GSH and GSSG, cell lysates, grown under supplementation with either GSH of GSSG, were analyzed by LC/MS/MS.

Under supplementation of 50 µM of GSH, both oxidized and reduced glutathione were found in lysed cells, as shown in **Table 3.1**. Cells grown with 25 µM of supplemented GSSG also contained both GSH and GSSG inside their cytoplasm. Meanwhile, the groups that were not supplemented with glutathione (containing 2 µM of GSSG in growth medium) did not contain detectable glutathione in the cell lysate with LC/MS/MS.

Since GSH supplementation results in intracellular reduced and oxidized glutathione, we can imply that *L. fermentum* CECT 5716 possesses the ability to import GSH. With GSSG supplementation and without GSH present, cytoplasmic GSH can be detected. The presence of cytoplasmic GSH under GSSG supplementation implies *L. fermentum* CECT 5716 possesses the capability of glutathione reduction activity. Such activity is supported by a screening performed

by Pophaly et al's which showed a putative protein with great resemblance to glutathione reductase gene *L. fermentum*[16] genome.

GSSG supplementation with 25 μM caused *L. fermentum* CECT 5716 to uptake GSSG, as GSSG was not uptaken at lower GSSG concentrations in the non-supplemented medium (2 μM). This suggests that there may be a minimum threshold of GSSG before influx can occur, and/or low affinity of the transporter for GSSG.

While the non-supplemented growth medium contained 2 μM of GSSG intrinsically, there was no GSH or GSSG detected from any of the cells growing in such medium. Another study conducted by Pophaly et al[16] has reported the inability of glutathione synthesis in several strains of *L. fermentum*. Although we were unable to locate glutathione synthesis genes, it may be possible that the experimental conditions were insufficient for *L. fermentum* CECT 5716 to synthesize glutathione.

Intracellular and Extracellular Glutathione Under Stress.

The previous data suggests the presence of cytoplasmic glutathione under supplementation and the ability to regenerate GSH from GSSG in *L. fermentum* CECT 5716. However, whether peroxide inactivation was performed either in intra- or extracellularly was unclear. As peroxide treatment can provide both external and internal oxidative stress, both intracellular and extracellular glutathione contents of *L. fermentum* CECT 5716 were measured to determine compartmentalization of glutathione in this strain. We used a sub-lethal peroxide treatment, validated by comparing the viability between peroxide-treated and the control group that was not statistically significant, to prevent the presence of unwanted extracellular glutathione that may be caused by cell lysis.

We found that the incubation of cells resuspended in glutathione-free PBS to contain extracellular oxidized glutathione, as shown in **Table 2.**. It also showed that under sublethal treatment of peroxide, the glutathione concentration in the PBS supernatant was not substantially altered. However, cystine was found in PBS supernatant, albeit in smaller amount compared to glutathione. Under sub-lethal stress treatment of peroxide present in the growth medium, we found no significant difference in both intracellular and extracellular glutathione.

Glutathione was present in PBS supernatant after 1 hour of incubation, despite washing the cells, in order to remove glutathione from the original supernatant, 3 times. This implies that *L. fermentum* CECT 5716 possesses the ability to export glutathione into the environment. Glutathione that is present in the supernatant is exclusively found in oxidized form; which was caused by either export of reduced glutathione that got oxidized during incubation period or export of oxidized glutathione. This finding is consistent with those of Peran et al.[25]

Hypothetically, efflux of oxidized glutathione will not deactivate extracellular oxidant, unlike efflux of reduced glutathione. Efflux of oxidized glutathione will only diminish their glutathione reserve without providing protection. Exporting the readily-oxidized GSH will enable *L. fermentum* CECT 5716 to recycle glutathione by uptaking extracellular non-enzymatically oxidized GSH. However, we cannot provide evidence that only reduced glutathione was transported into the extracellular environment, based on the assumption that excreting oxidized glutathione does not add value for their survivability. We proposed that the cells export glutathione in GSH form, without disregarding the possibility of GSSG export. Cystine presence in the supernatant may suggest that cysteine was also transported outside the cells. Cystine presence was likely caused by efflux of cysteine that was non-enzymatically oxidized in the presence of oxygen.

Limiting the amount of environmental stress during this experiment was challenging. Presence of oxidized glutathione in the group untreated with peroxide may imply that oxidant was inherently present during the cell incubation. The incubation was not performed in anaerobic conditions, as added peroxide may be spontaneously reduced by the reducing environment of an anaerobic chamber.

Exploratory bioinformatics analysis of Glutathione-related protein

The previous experiments showed evidence that *L. fermentum* CECT 5716 possesses glutathione-related activity, but may not synthesize glutathione by itself. Glutathione synthesis typically requires the two enzymes, GshA and GshB, and only the former is predicted through bioinformatics to be present in *L. fermentum* CECT 5716. Additionally, the genome lacks any putative gene that resembles the relatively recently discovered GshF, a bifacial enzyme which performs the activity of both GshA and GshB[16]. The fact that *L. fermentum* possesses an incomplete glutathione synthesis may imply that it has lost its capability to synthesize glutathione. The presence of the putative protein that resembles GshA may suggest that this strain exports a glutathione precursor and relies on other organisms to complete the glutathione synthesis.

Therefore, we proposed an antioxidative glutathione utilization model (**Figure 3)**, based on the activity towards glutathione of *L. fermentum* CECT 5716. We showed *L. fermentum* CECT 5716 is able to uptake both oxidized and reduced glutathione, perform reduction of GSSG to GSH intracellularly, export glutathione into their environment, and uses glutathione to counter intracellular oxidative stress. Glutathione that has been exported can be oxidized non-

enzymatically. This proposed mechanism can explain the observed, *in vitro*, antioxidant property across several strains of *Lactobacillus fermentum*[26–28].

Glutathione reductase is an enzyme that reduces oxidized glutathione using NADPH as its cofactor, and reported to be present in *Lactobacillus sanfranciscensis* DSM 20451[14]. We found a putative protein (LC40_1022) in *L. fermentum* CECT 5716 genome from our exploratory analysis, using BLASTp[29], that was highly similar (E-value: $5e^{-69}$) to the glutathione reductase of *L. sanfranciscensis* DSM 20451. Based on our findings of reduced glutathione in the cell's cytoplasm under oxidized glutathione supplementation, we propose that step 6 in the model (**Figure 3**) was performed by this putative glutathione reductase.

Glutathione uptake has been reported in many species of LAB. However, the protein responsible for such activity has not been clearly studied. Our exploratory analysis could not locate putative protein from *L. fermentum* CECT 5716 that bears resemblance to the γ-glutamyl-transpeptidase of *E. coli*, a transmembrane protein that facilitates glutathione uptake by hydrolyzing it into its constituent amino acids[30]. Pophaly et al.[31] have suggested that the putative cydDC complex in many species of *Lactobacillus* is responsible for glutathione import. cydDC complex, an ABC transporter, known to have affinity with glutathione and cysteine; has actually been shown to perform export of cysteine[32] and glutathione[33] in *E. coli* instead of import. Meanwhile, another well-characterized glutathione importer utilized by *E. coli*; is yliABCD complex, that consists of 4 subunits[34]. Our exploratory analysis with BLASTp showed that the yliA subunit has some mild similarity with a putative methionine ABC transporter found in the *L. fermentum* CECT 5716 genome.

From our exploratory analysis, GshT of *Streptococcus mutans*, which was characterized by Vergauwen et al.[13], has only a mild resemblance to a putative ABC transporter protein found

in *L. fermentum* CECT 5716 genome. GshT is able to bind with different forms of glutathione, which facilitates glutathione uptake by TcyABC, a cysteine transporter. Similarly, there is a possibility *L. fermentum* CECT 5716 utilizes a cysteine transporter to import glutathione. *L. fermentum* CECT 5716 is known to possess a solute-binding cysteine transporter, BspA/CyuC[35]. However, besides the putative cydDC, we failed to locate any other putative protein in *L. fermentum* CECT 5716 that may have affinity with glutathione. Some studies[36–38] have reported reported that ABC transporters may work bidirectionally, which may explain the efflux and influx activity of cydDC. However, no study has shown that cydDC possesses bidirectional activity.

The intracellular glutathione utilization mechanism of *L. fermentum* CECT 5716 is a black box as well. Despite showing the capability of utilizing glutathione intracellularly to counter oxidative stress, our exploratory analysis failed to locate any candidate proteins that may be responsible for such activity. We could not find any putative protein in the *L. fermentum* CECT 5716 genome that resembles glutathione peroxidase, the enzyme that catalyzes hydrogen peroxide deactivation[39]. We also could not find any putative glutaredoxin in *L. fermentum* CECT 5716, which acts as an antioxidant system utilizing GSH as a reducing cofactor[40]. *L. fermentum* CECT 5716 does possess a putative protein similar to NrdH, a glutaredoxin-like protein from *E. coli*. However, NrdH has been reported to lack the GSH-binding residue unlike glutaredoxin[41]. Our exploratory analysis found that *L. fermentum* CECT 5716 putatively possesses a complete set of thioredoxin system, comprised of thioredoxin and thioredoxin reductase proteins which may be employed as their endogenous antioxidant system. However, the relationship between the thioredoxin system and glutathione in *L. fermentum* CECT 5716 is currently unknown.

Conclusion

In this study, glutathione utilization in *L. fermentum* CECT 5716 was characterized. *L. fermentum* CECT 5716 was shown to be capable of importing glutathione both in the reduced and oxidized form. They also possess the ability to regenerate GSH by reduction of GSSG; and are able to export glutathione. These findings guided activities were distilled into the proposed glutathione utilization model for this strain. Although our findings support this model, the exact mechanisms for some of the steps need to be further studied.

3.5 Tables and Figures

Figure 3. 1 Peroxide Stress Response Assay

L. fermentum CECT 5716 was passed three times with or without glutathione (GSH) supplementation, respectively; and treated/untreated with hydrogen peroxide (H_2O_2) for 1 hour. Cells were then enumerated. Data are reported as the mean ± the standard error of the mean. Values with no letters in common are significantly different ($p<0.05$), as determined by Tukey's mean separation test. Error bars represent standard Error.

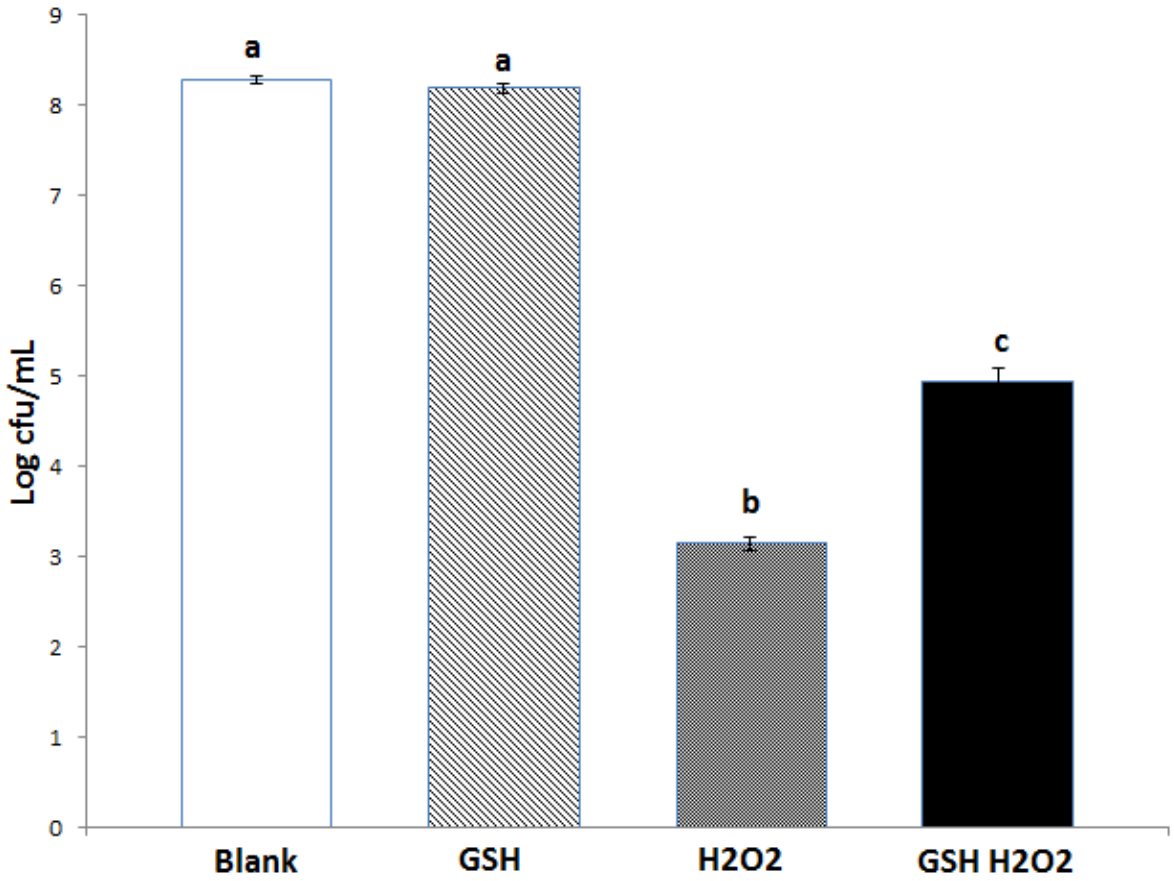

Figure 3. 2 Copper Stress Response Assay

L. fermentum CECT 5716 was grown in the presence of 125 µM or 250 µM copper (II) sulfate., and both copper-treatment and control groups were passed into media with/without GSH supplementation. Glutathione supplementation reduced the lag time across treatments while the pre-treatment of copper increased the lag time. Statistical modeling, using JMP software, determined that the glutathione's lag time reduction was independent of the presence of copper. Values with no letters in common are significantly different ($p<0.05$), as determined by Tukey's mean separation test. Error bars represent Standard Error.

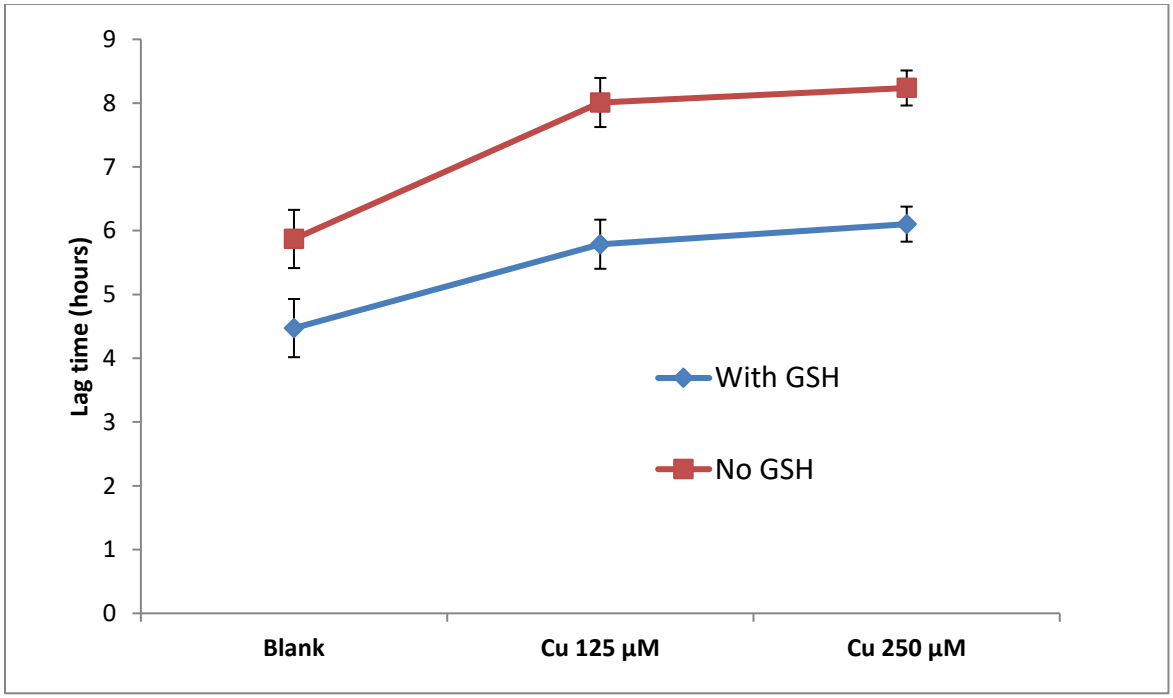

Figure 3. 3 Proposed Model for Anti-oxidative Glutathione Utilization in *L. fermentum* CECT 5716.

Activity and mechanism related to the models are:
1. Non-enzymatic GSH oxidation. Based on absence of GSH in supernatant but not GSSG
2. GSH uptake. Observed activity, currently unclear mechanism. Likely performed by the same protein with no.4
3. GSH export. Implied activity from observation, currently unclear mechanism. Likely performed by the same protein with no.5
4. GSSG uptake. Observed activity, currently unclear mechanism. Likely performed by the same protein with no.2
5. GSSG export. Implied activity from observation, currently unclear mechanism. Likely performed by the same protein with no.3
6. GSSG reduction. Observed activity, likely performed by putative Glutathione Reductase (LC40_1022.
7. GSH oxidation. Observed activity, with no known candidate protein

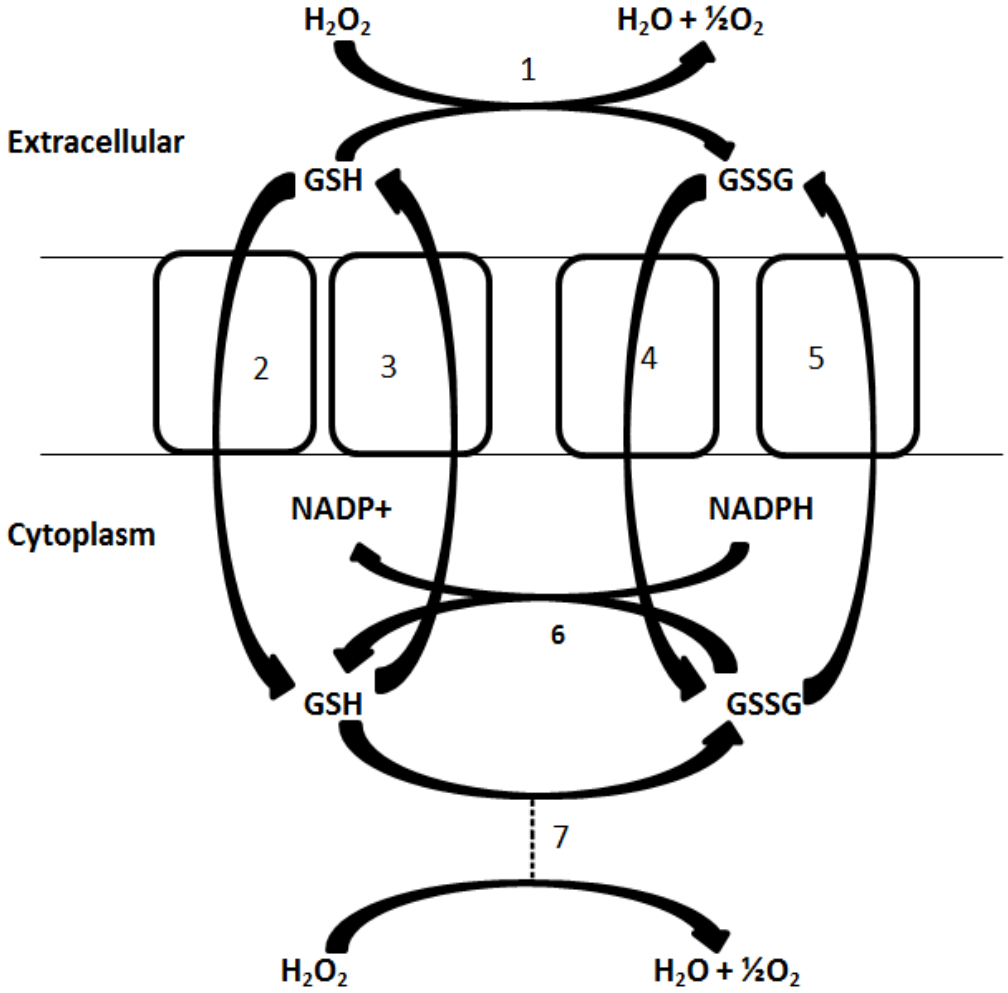

Table 3. 1 Cytoplasmic Glutathione species under glutathione supplementation.

	Cytoplasmic Content	
	GSH (ng/10^7CFU)	GSSG (ng/10^7CFU)
GSH Supplementation	1.32 ± 0.69	91.30 ± 6.71
GSSG Supplementation	3.17 ± 2.31	1594.62 ± 770.18
Control	BDL	BDL

BDL: Below Detection Limit (0.2ng/10^7cfu)

Results are mean ± SEM

Table 3. 2 Extracellular and Intracellular glutathione under sublethal stress.

		Sublethal H_2O_2 treatment	Untreated
Extracellular content	GSH (ng/10^7 CFU)	BDL	BDL
	GSSG (ng/10^7CFU)	55.35 ± 48.78	63.48 ± 57.10
	Cysteine (ng/10^7CFU)	BDL	BDL
	Cystine (ng/10^7 CFU)	0.16 ± 0.03	0.19 ± 0.01
Intracellular content	GSH (ng/10^7 CFU)	1.62 ± 0.17	1.32 ± 0.69
	GSSG (ng/10^7 CFU)	119.18 ± 8.97	91.30 ± 6.71
	Cysteine (ng/10^7CFU)	BDL	BDL
	Cystine (ng/10^7 CFU)	BDL	BDL
Plate Count (10^7 cfu)		1.78 ± 0.22	2.21 ± 0.19

BDL: Below Detection limit (0.2 ng/ 10^7 cfu)
Results are mean ± SEM.

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CHAPTER 4: SUMMARY AND FUTURE DIRECTION

4.1 Summary and future directions

In this study, glutathione is found to be used by *Lactobacillus fermentum* CECT 5716, and contributes to alleviate oxidative and metal stress. This strain shows the ability to uptake both reduced glutathione and glutathione disulfide, reduce glutathione disulfide into reduced form, and excrete glutathione to extracellular compartment. Despite those activities regarding glutathione were observed, exact mechanism and the responsible protein over those activities are not well established.

Pophaly et al.[1] proposed the putative cydDC, that were found in *Lactobacillus* through bioinformatics exploration, to be the responsible protein over glutathione uptake. However in other species, similar protein was found to perform export of both glutathione and cysteine; instead of import[2,3]. The presence of putative cydDC in *Lactobacillus fermentum* CECT genome may indicate glutathione transport through membrane, although whether it performs export or import is currently unclear. Further research on glutathione import and export should revolve around this putative cydDC. While at the same time, cysteine importer possessed by *Lactobacillus fermentum* can be a candidate for glutathione transporter, as glutathione and cysteine are found to pass through the same protein channel in other species[4]. Both cysteine and glutathione free medium would be the key to elaborate this question.

As glutathione supplementation allow *Lactobacillus fermentum* CECT 5716 to survive better under hydrogen peroxide stress, its mechanism is currently unclear. No putative gene found in this strain that bears resemblance to glutathione peroxidase. This may imply that glutathione does not directly detoxify oxidant, but rather primed with other antioxidant system.

This strain possesses putative complete thioredoxin system and a putative glutaredoxin-like protein, NrdH. Glutathione may act as reducing agent to these antioxidant; however, direct relationship between thioredoxin system or NrdH with glutathione is currently unclear. Biochemical approach to study the mechanism will be more favorable, as microbiological approach will require multiple knockouts to prove their relationship.

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