Identification of the response pathways of *Escherichia coli* and *Enterococcus faecalis* to glyphosate and it's major breakdown product Aminomethyl phosphonic acid (AMPA)

KS Stenger

orcid.org 0000-0002-9217-1063

Dissertation submitted in fulfilment of the requirements for the degree *Master of Science in Environmental Sciences* at the North-West University

Supervisor:	Prof CC Bezuidenhout
Co-supervisor:	Dr AS Okoli

Graduation May 2019

23591382

ACKNOWLEDGEMENTS

“My wish is not that you should dance in the clouds with tomorrow's dawn - merely that you scale heights enough to gather the pure fruits of respect from the trees, to salute the sun and rise above vipers' lies and the acidic smiles that precede handshakes gloved in cyanide and morphine - drenched barbed-wire words.” Avarshinah.

My supervisors, Prof. Carlos Bezuidenhout and Prof. Arinze Okoli, thank you for your time, guidance and opportunity provided to embark on this journey of growth. Without you all this wouldn’t have been possible, I will forever be grateful.

Mr. Earl Forbes, Miss. Kathrina Pedersen, and Mr. Magnus Bjørsvik thank you for your contribution towards this research project.

To the “Halala” team, thank you for making this journey so much fun.

Everyone from the microbiology department, thank you for such a stimulating environment.

To my “friendship” Moitshepi Plaatjie, thank you for your support and encouragement.

Portcia Lungile, thank you for helping me stay cool in the heat of the furnace.

My family, may God bless you with many more fruitful years.

“Forsake not [Wisdom], and she will keep, defend, and protect you; love her, and she will guard you.” Proverbs 4:6.

ABSTRACT

Glyphosate is the active ingredient in non-selective herbicides, and disrupts the shikimate pathway of plants and bacteria by inhibiting the synthesis of aromatic amino acids. Application of glyphosate has increased exponentially worldwide due to the increasing adoption of genetically modified crops. Studies have shown that residues of glyphosate and its major break-down product aminomethyl phosphonic acid (AMPA) accumulate in some genetically modified (GM) food crops including soybeans, cowpea, coffee, as well as meat and dairy products from cattle and pig. Glyphosate can also disperse into the ecosystem and reach water systems. Therefore, resident bacteria of the gut will be exposed, in their respective niches, to glyphosate and AMPA at varying sub-lethal concentrations. Limited studies have been conducted on the response of gut bacteria to glyphosate and AMPA at sub-lethal concentrations. This study is especially important because of the crucial role played by gut bacteria in human and animal health. The aim of this research was to employ proteomic approaches to analyse expression profiles of *Escherichia coli* and *Enterococcus faecalis* exposed to sub-lethal concentrations of glyphosate and AMPA. Extraction of proteins was done using a commercially available kit, followed by quantification with the bicinchoninic acid assay. The TheromFisher Scientific TMT Mass Tagging Kits and Reagents were employed for labelling peptides. Data analysis was done using mass spectreophotometer and Proteome Discovere software. Pathways were analysed and mapped using KEGG PATHWAY and STRING online protein databases. Glyphosate seem to greatly affect nitrate metabolism and iron uptake in *E. coli* through inhibition of respiratory nitrate reductase (NarGH) and up-regulation of enterobactin biosynthesis (EntA, B, E, F, H) and iron transport proteins (TonB, FepA, ExbD), respectively. AMPA exerts a similar response on iron uptake in *E. coli*. In *E. faecalis* glyphosate and AMPA interferes with translation through up-regulated of proteins involved in aminoacyl-tRNA biosynthesis. Interestingly, glyphosate and AMPA may induce oxidative stress at sub-lethal concentrations in *E. coli* and *E. faecalis*. At sub-lethal concentrations glyphosate and AMPA negatively affect energy and growth of *E. coli* through nitrate metabolism pathway and stress response pathway. Additionally, glyphosate and AMPA may stimulate pathogenesis in *E. coli* through increasing the bacteria iron scavenging potential. Glyphosate and AMPA interrupt cell proliferation in *E. faecalis* through antimicrobial activity on aminoacyl-tRNA biosynthesis pathway and ribosomes. Glyphosate and AMPA also caused changes in expression of hypothetical proteins in *E. coli* and *E. faecalis,* indicating that some physiological responses remain uncharacterized. Majority of deferentially expressed proteins are involved in energy metabolism, iron uptake and transport, carbohydrate metabolism, transport and stress response. An indication of a complex set of interactions indicating glyphosate affects pathways other than the shikimate pathway. These interactions serve an important role in bacterial fitness, survival, and adaptive resistance to antibacterial

agents. Thus, glyphosate and AMPA may serve as environmental cue for antibiotic resistance, virulence expression and habitat adaptation of *E. coli* and *E. faecalis*.

Key terms: Glyphosate, AMPA, *E. coli*, *E. faecalis*, Proteomic, Shikimate pathway, Sub-lethal concentrations.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABBRIVIATIONS

Amp- Ampicillin

AMPA- (Aminomethyl)phosphonic acid

BHI- Brain Heart Infusion media

Bt- *Bacillus thuringiensis*

C. botulinum- *Clostridium botulinum*

CO_2- Carbon dioxide

Cip- ciprofloxacin

COGs- Clusters of Orthologous Groups

D. magna- *Daphnia magna*

DNA- Deoxyribonucleic acid

E. coli- *Escherichia coli*

E. faecalis- *Enterococcus faecalis*

EFSA- European Food Safety Authority

EPA- Environmental Protection Agency

EPSP- 5-enolpyruvylshikimate 3-phosphate

EPSPS- 5-enolpyruvylshikimate 3-phosphate synthase

FASTA- Fast Alignment

GDP- Gross domestic product

Glyphosate- *N* - (phosphonomethyl)glycine

GR- Glyphosate resistant

IARC- International Agency for Research on Cancer

IPA- Isopropylamine

iTRAQ- Isobaric Tags for Absolute and relative quantification

LB- Luria Bertani media

LC- Liquid gas Chromatography

MIC- Mininum inhibitory concnetration

MRL- Maximum residue level

MS- Mass spectrometry

NOAEL- no-observed-adverse-effect-level

PEP- Phosphoenol pyruvate

RNA- Ribonucleic acid

S3P- shikimate-3-phosphate

Spp- species

TMT- Tandem Mass Tags

USA- United States of America

WHO- World health organisation

CHAPTER 1

INTRODUCTION

1.1. GENERAL INTRODUCTION AND PROBLEM STATEMENT

Glyphosate is the active ingredient in the majority of the most successful (Giesy *et al*., 2000) and widely applied herbicide such as Roundup, Touchdown and PowerMax (Duke and Powles, 2008; Saunders and Pezeshki, 2015). Apart from its use in the agricultural sector where it is used to eradicate weeds, glyphosate is also used in residential weed management, right of way management and forestry practices (Giesy *et al*., 2000). Commercially available glyphosate formulations consist of an IPA salt, surfactant and water (Saunders & Pezeshki, 2015). Glyphosate undergoes degradation to form AMPA and sarcosine, with AMPA being the dominant break down product (Borggaard & Gimsing, 2008). AMPA undergoes further degradation to form methylamine and inorganic phosphate (Duke *et al*., 2012). Glyphosate half-life varies significantly in soil (Bai & Ogbourne, 2016), with a 90-day half-life in water (Schuette, 1998).

Glyphosate functions as an amino acid inhibitor because it blocks the synthesis of phenylalanine, tyrosine and tryptophan by inhibiting 5-enolpyruvylshikimate-3-phosphate (EPSP) synthase in the shikimate pathway. The pathway is needed for chorismate production, which serves as an intermediate precursor molecule for aromatic amino acids and a number of secondary metabolites (Giesy *et al*., 2000; Saunders & Pezeshki, 2015). Disruption of the enzyme leads to unregulated carbon flow into the pathway (Carvalho *et al*., 2016), which causes an imbalance in the metabolism resulting in growth inhibition, starvation for aromatic amino acids, and energy drainage (Fischer *et al*., 1986). Glyphosate target site is the shikimate pathway, but its effects extend to non-shikimate pathways such as cell motility, energy production, and carbohydrate metabolism (Lu *et al*., 2013).

Transcriptional changes in *Escherichia coli* after treatment with glyphosate showed that glyphosate induces differential expression of genes involved in amino acid metabolism and transport, cell motility, energy production and conversion, carbohydrate metabolism and transport, and function unknown COGs functional categories. The changes in expression of hypothetical genes indicate that some physiological responses following glyphosate exposure remain uncharacterized (Lu *et al*., 2013).

Escherichia coli is a diverse species and forms an important component of the biosphere. It is capable of inhabiting a range of habitats such as animal intestines as harmless commensals (Lu *et al*., 2013). *Escherichia coli* is extensively utilized as a model organism in molecular genetics, because it is well understood and intensively studied (Taj *et al*., 2014). *Enterococcus faecalis* is predominantly present in the digestive tract, but can also be found in the environment, and food. *Enterococcus faecalis* is harmless to healthy people, but can pose health risks to individuals with weakened immune system especially in hospitals. *Enterococcus faecalis* is highly sensitive towards glyphosate exposure. Exposure of *E. faecalis* to glyphosate may directly or indirectly disturb the normal gut bacterial community (Shehata *et al*., 2013).

Several crops including soybean, canola, cotton, corn, and maize have been genetically modified to tolerate glyphosate-derived herbicides. Application of these herbicides is on the increase because genetically modified herbicide-tolerant crops are increasingly being cultivated. Residues of glyphosate and AMPA have been detected in food, feed, and drinking water (Anadón et al., 2009; Cuhra *et al*., 2016) in varying amounts. Therefore, bacteria in the environment, including resident bacteria of the gut will be exposed to varying concentrations of glyphosate and AMPA in their respective niches.

Very little information exists on the response of bacteria to sub-lethal concentrations of glyphosate and AMPA, hence, it is of interest to explore this niche, owing to the ever increasing usage of glyphosate and its occurrence in water and food sources. For example, it is important to determine whether oral exposure to glyphosate residues has the potential to modulate the human gut microbiota at a level of concern for human health (Nielsen *et al*., 2018). Therefore, this study was undertaken to elucidate the response pathways of *E. coli* and *E. faecalis* to sub-lethal glyphosate and AMPA concentrations using changes in the proteome of the bacteria.

1.2. AIM AND OBJECTIVES

The aim of the study is to identify the global response pathways of *Escherichia coli* and *Enterococcus faecalis* to sub-lethal concentrations of glyphosate and AMPA. To achieve the aim, the following objectives will be carried out:

- Determination of sub-lethal concentrations of glyphosate and AMPA
- Determination of protein expression profiles of the bacteria exposed to sub-lethal glyphosate and AMPA concentrations

- Metabolic pathway analyses of *Escherichia coli* and *Enterococcus faecalis*

CHAPTER 2

LITERATURE REVIEW

2.1. History and properties of glyphosate

N - (phosphonomethyl)glycine (glyphosate) was invented in 1950 by Henri Martin of a small pharmaceutical company Cilag, but had no pharmaceutical applications (Dill *et al*., 2010). Glyphosate was only synthesized and tested as a herbicide in 1970 by John E Franz of Monsanto Co. The herbicide became commercially available in 1974 as a post-emergence, non-selective herbicide branded Roundup® (Duke & Powles, 2008). Glyphosate is a broad-spectrum herbicide initially used to control perennial weeds in ´right of ways´ and other areas. Application of glyphosate in the agriculture was limited because it also killed crops, but its use increased with the evolution of conservative tillage practices and introduction of glyphosate resistant (GR) GM crops (Johnson *et al*., 2009).

Glyphosate is a white and odorless crystalline solid comprised of one basic amino function and three ionizable acidic sites. As an acid, glyphosate is moderately soluble in water (1.16 g/L at 25°C), but its solubility increases dramatically when converted to monobasic salts like isopropylamine, sodium, potassium, trimethylsulfonium, or ammonium. As such most commercial herbicidal products are formulated as concentrated water solutions in the form of monobasic salts (Dill *et al*., 2010). The herbicidal products also contain other inert ingredients like solvents and antifoam compounds. These inert ingredients increase the efficacy of the herbicide against target plants and also the toxicity to non-target organisms (Pérez *et al*., 2011). Glyphosate has an advantageous environmental profile in that it has a low volatility (2.59 $x10^{-5}$ Pa at 25°C) and a high density (1.75 g/cm^3), which indicate that chances of glyphosate evaporating from treated areas and landing on non-target sources are very low. Glyphosate does not undergo chemical decomposition in the environment because it is stable to hydrolytic degradation and photo-degradation (Rueppel *et al*., 1977). However, glyphosate undergoes degradation by microorganisms in soil (under aerobic and anaerobic conditions) and water sources (Franz *et al*., 1997 cited by Dill *et al*., 2010). Upon microbial degradation, glyphosate forms AMPA as a major breakdown product and other metabolites.

2.2. Glyphosate mode of action

Glyphosate has a unique mode of action; it is the only molecule that is extremely effective at inhibiting the 5-enolpyruvyl-shikimate-3-phosphate synthase. Glyphosate is a strong transition state analog of phosphoenylpyruvate (PEP) (Duke & Powles, 2008). By binding more tightly to EPSP synthase than PEP, glyphosate prevents PEP from binding to the enzyme (Salman *et al*., 2016). 5-enolpyruvylshikimate 3-phosphate (EPSP) synthase (EC 2.5.1.19) is an enzyme involved in the shikimate pathway. The shikimate pathway is found in bacteria, fungi, algae and higher plants. The pathway is responsible for the synthesis of aromatic amino acids and other aromatic compounds. EPSP synthase is located on the sixth position (the penultimate step) in the shikimate pathway (Figure 1). It catalyzes the transfer of the enolpyruvyl moiety from PEP to shikimate-3-phosphate (S3P), resulting in the formation of 5-enolpyruvylshikimate 3-phosphate and inorganic phosphate as products (Dill, 2005). This step is crucial because EPSP is used as an intermediate in the biosynthesis of chorismate, which is used in the synthesis of aromatic compounds and other necessary metabolites (Duncan *et al*., 1984, Dill 2005). Inhibition of EPSPS causes a disruption in the shikimate pathway, which leads to uncontrolled carbon flow. Distortion in carbon flow then leads to subsequent disruption of the organism's metabolism (Duke *et al*., 2003).

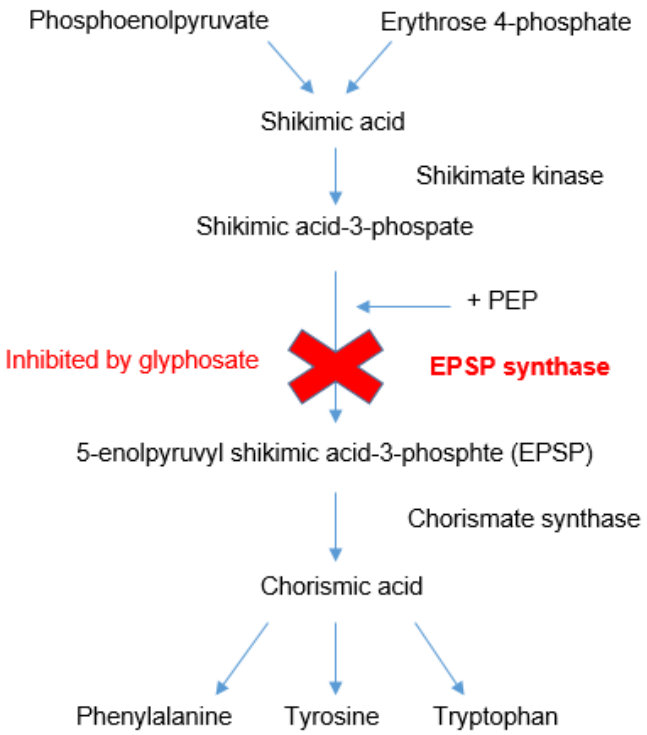


Figure 1: Glyphosate inhibition of the shikimate pathway

2.3. Glyphosate uptake and translocation

Plants take up glyphosate mainly through leaves and roots, after application by spraying. Surfactants assist glyphosate to penetrate the plants by reducing the surface tension between the plant surface and the applied glyphosate (Kirkwood *et al*., 2000). Once it is inside the plant, glyphosate undergoes systemic translocation via the phloem to the metabolic sink tissue (CaJacob *et al*. 2004 cited by Huang *et al*., 2015). Subsequently glyphosate is delivered throughout the plant and reaches actively growing tissue. The differences in glyphosate susceptibility between species can be attributed to difference in leaf uptake rates, among other factors (Duke & Powles, 2008). Glyphosate has a slow mode of action and as such it allows glyphosate to access most parts of the plant before tissue damage reaches levels that inhibit translocation (Huang *et al*., 2015). Rapid uptake, absence or *limited in-planta* degradation, systemic translocation to growing points, and slow mode of action are the primary attributes for the excellent herbicidal efficacy and popularity of glyphosate (Duke & Powles, 2008; Huang *et al*., 2015).

2.4. Environmental profile of glyphosate

2.4.1. Soil

Once glyphosate reaches the soil it binds tightly to soil constituents (Duke & Powles, 2008). Glyphosate has a high affinity for soil particles (soil organic matter, clay minerals, oxides and hydroxides) which limit its movement in the environment, thus making it environmentally friendly to some degree (Giesy *et al*., 2000; Saunders & Pezeshki, 2015). Glyphosate degradation in soil is attributed to microorganisms. Microbial degradation is considered the primary degradation mechanism of glyphosate, which forms AMPA (major metabolite), methylphosphonic acid, glycine and sarcosine as products (Borggaard & Gimsing, 2008; Kwiatkowska *et al*. 2014; Saunders & Pezeshki, 2015; Bai & Ogbourne, 2016). Glyphosate degradation can occur via two pathways. One leads to the formation of sarcosine and the other forms AMPA (Borggaard & Gimsing, 2008). The rate at which degradation of glyphosate take place is highly dependent on soil microbial activity among other factors (Saunders & Pezeshki, 2015). Factors that increase the rate of degradation are soil texture (clay>silt), acidic or basic pH, high organic matter, high temperature and aerobic conditions in well-drained soil. While factors that decrease the rate of degradation include soil texture (sand), neutral pH, low organic matter, low temperatures and anaerobic conditions in waterlogged soil (Saunders & Pezeshki, 2015).

Glyphosate half-life is based on soil types and environmental conditions (Bai & Ogbourne, 2016). Glyphosate has been reported to have a half-life ranging from 0.8 to 151 days in loam and clay, respectively (Mamy *et al*., 2005; Bergström *et al*., 2011). As part of their conclusion Borggaard and Gimsing (2008) wrote that the similarity in bond sharing between glyphosate and phosphate is an indication that the two chemicals may compete on sorption sites which may affect retention and degradation of glyphosate. The extent to which phosphate influences sorption and degradation of glyphosate varies between soils, i.e. in some soils pre-sorption of phosphate can almost eliminate glyphosate sorption, while in others it has little influence. Phosphate can also stimulate glyphosate degradation in some soils, while it has little to no effect in others.

2.4.2. Leaching of glyphosate

Glyphosate and AMPA transportation can occur as solutes or can be co-transported bonded to soil colloids from terrestrial environments to aquatic environments. Transportation can happen through either subsurface runoff (ends up in drainage and groundwater) or surface runoff (ends up in streams, lakes etc.). Transport of glyphosate (and AMPA) in uniform, non-structured soils (sandy soil) can be referred to as piston flow, while in structured soils (clay soils) it can be described as preferential flow (Borggaard & Gimsing, 2008). Glyphosate leaching in many sandy soils is limited, thus, risk of contamination of water sources by glyphosate and AMPA is considered to be low, but with long-term glyphosate use on coarse-textured soil materials to control weeds, contamination of groundwater may increase (Borggaard & Gimsing, 2008). Leaching of glyphosate is highly variable in fields, as it depends on a number of factors such as soil properties, environmental conditions (temperature etc.), and method of application (Borggaard & Gimsing, 2008, Duke *et al*., 2012).

Glyphosate leaching is a growing concern as there is an increased frequency of glyphosate and APMA residues reported in water systems (Bai & Ogbourne, 2016). A study done by Battaglin *et al*. (2014), between 2001 and 2010 reported that glyphosate was detected in 40% of 3700 soil, water and sediment samples collected from 38 sites in the USA, while AMPA was detected in 55% of the samples. None of the reported samples concentrations exceeded 700 µg/L, which is the accepted maximum contamination level in the US. Nevertheless, concentrations ranging up to 1237 µg/L have been reported in literature from surface waters contaminated with glyphosate (Monsanto, 1990 as cited by Villeneuve, 2011). Findings like these promote an increased awareness that excessive glyphosate runoff on fields do in fact exist, contrary to conventional wisdom (Saunders & Pezeshki, 2015).

2.4.3. Glyphosate in water

Despite glyphosate being reported to be biodegraded in soil and having a high affinity for soil particles, glyphosate is frequently detected in freshwater ecosystems (Giesy *et al*., 2000; Villeneuve *et al* 2011; Saunders & Pezeshki, 2015). Glyphosate in water ecosystems undergoes a similar microbial biodegradation as in the soil. It also ends up being adsorbed onto sediments with further degradation by microbes under anaerobic condition; degradation usually occurs slower as compared to that in soil (Ghassemi *et al*., 1981). Glyphosate (Round up) has a reported half-life of up to 90 days in water with low microbial activity (Schuette, 1998). Glyphosate presence in ground water is very low, for example, according to a study done by EPA over six years only seven groundwater samples from a total of 27,877 tested samples contained glyphosate. The maximum detected concentration was 1.1 µg/L, a very low concentration compared to the maximum contaminant limit (700 µg/L) for glyphosate (Saunders & Pezeshki, 2015).

Glyphosate exposure would originate mostly from runoff or accidental glyphosate spills (Bai & Ogbourne, 2016). Thus, proper management practises, reduced application frequencies and using vegetation buffers may contribute largely to reducing glyphosate contamination of aquatic environments (Saunders & Pezeshki, 2015; Bai & Ogbourne, 2016), since contamination of surface waters by herbicides in agricultural landscapes heavily depends on the methods and levels of application and general agricultural practices (Huber *et al*., 2000 as cited by Villeneuve *et al*., 2011). For example, Shipitalo & Owens (2006), concluded that glyphosate concentrations tend to be higher in runoff from no-till watersheds than from other tillage practices, regardless of similar amounts of total runoff. As such it should be considered when evaluating the impact of glyphosate-tolerant crops on surface water quality. According to Bai & Ogbourne (2016), the acute toxicity risks posed to humans are minimal since most of the reported residue concentrations of glyphosate in water sources are below the maximum contamination levels. Treatment of water to remove glyphosate is crucial to reduce the risk of human exposure to glyphosate residues through drinking water.

Information on herbicide contamination of surface freshwater ecosystems varies greatly from country to country (Villeneuve *et al*., 2011), as seen in the following studies.

A two year study of surface waters in Southern Ontario, Canada, showed that only 2-5% of 502 samples collected from sites considered typical of agricultural and urban drainages exceeded the analytical limit quantitation of 17 µg/L, and none of the samples exceeded the

Canadian Water Quality Guideline for glyphosate of 65 µg/L (Struger *et al*., 2008). According to data reported by Peruzzo *et al*. (2008), after monitoring streams in Argentina near transgenic soybean cultivation area between 2003 and 2004, glyphosate concentrations in the water ranged from 100 µg/L to 700 µg/L. Scribner *et al*. (2007), detected glyphosate concentrations up to 427 µg/L and maximum AMPA concentrations of 41 µg/L in their report after sampling streams, rivers, lakes, wetlands and vernal pools in the United States from 2001 through 2006.

According to Villeneuve *et al*. (2011), an annual report done by the French Institute for environment published data in 2007 based on monitoring 453 pesticides at 2023 groundwater and river sampling sites. Their results showed detection of pesticides at almost 91% of the sampling sites, at mean annual concentrations of <0.5 µg/L. AMPA was most often detected in French streams followed by diuron and glyphosate. Pesce and co-workers (2008) reported glyphosate concentrations between 0.23 µg/L and 0.74 µg/L and AMPA concentrations between 0.17 µg/L and 3.76 µg/L in a French river over a 1-year sampling period in 2003. AMPA was largely predominant among the total substances detected, which is an indication of an increasing use of glyphosate. Another study done on French streams and rivers by Horth and Blackmore in 2006 (cited by Villeneuve *et al*., 2011), detected glyphosate concentrations ranging from 2.0 µg/L to 34.0 µg/L and AMPA concentrations were between 2.2 µg/L and 27.5 µg/L.

A study done by Battaglin *et al*. (2005) on 51 streams in 9 Midwestern states in 2002, reported that glyphosate and AMPA were detected in 35% and 53% of pre-emergence, 40% and 83% of post-emergence, and 31% and 73% of harvest season samples respectively. Glyphosate concentration ranged between 0.1 µg/L and a maximum of 8.7 µg/L while AMPA had a minimum detected concentration of 0.1 µg/L and a maximum of 3.6 µg/L. Sampling was done after application of pre-emergence herbicides, after application of post-emergence herbicides, and during harvest season. None of the samples exceeded the U.S. Environmental Protection Agency's maximum contamination level. A separate study conducted by Battaglin *et al*. (2014), in various sources in the US from 2001 through 2010, indicated that glyphosate and AMPA are quite mobile as they occur widely in the environment and can originate from agricultural and urban sources. Results indicated that glyphosate and AMPA are more abundant in surface waters than in groundwater and soil-water, as they were detected in 59% of 470 surface water sites and only in 8.4% of 820 groundwater and soil-water sites (Battaglin *et al*., 2014).

As part of their results after sampling effluent and streams in the US during 2002 Kolpin *et al*. (2006), found that glyphosate and AMPA were present in 67.5% of 40 streams and WWTP effluent samples collected (present at generally low concentration). AMPA was more frequent (67.5%) than glyphosate (17.5%). The results also suggested that wastewater treatment plants effluents contribute to both glyphosate and AMPA concentrations in streams, as both AMPA and glyphosate had a two-fold increase in frequency between stream samples located upstream and those located downstream of the wastewater treatment plants. Glyphosate detection between sampling points (up- and downstream) decreased by 45%, while AMPA only decreased by about 3%. This could be an indication that AMPA may be more mobile and more persistent than glyphosate. They also found that the overall results suggest that glyphosate and AMPA are more mobile and persistent in aquatic environments, than previously thought.

2.5. Aminomethylphosphonic acid (AMPA)

AMPA is a product of glyphosate degradation primarily by microbial processes. Glyphosate is cleaved by glyphosate oxidoreductase to glyoxylate and AMPA (Duke *et al*., 2012). AMPA can also be formed following the degradation of phosphonic acids found in some households and industrial detergents and cleaning products (Skark *et al*., 1998; Nowack, 2003 cited by Battaglin *et al*., 2014). Majority of AMPA frequently detected in the environment is due to glyphosate degradation, since phosphonic acids are recalcitrant to biological or non-biological degradation and are strongly adsorbed to sediments and suspended particles (HERA, 2004 cited by Battaglin *et al*., 2014). For example, Battaglin *et al*. (2014) indicated that AMPA was detected without glyphosate in 17.9% of all samples collected, while it was uncommon for glyphosate to be detected without AMPA. Another source of AMPA is artificial sweetener. AMPA can also originate from artificial sweetener (acesulfame), which can serve as a possible source of AMPA in water. But there is a strong correlation between glyphosate and AMPA concentrations in collected samples, suggesting that AMPA is more likely to be a product of glyphosate rather than acesulfame (Van Stempvoort *et al*., 2014).

AMPA is very water soluble and undergoes degradation but at a slower rate than glyphosate. Upon degradation AMPA forms inorganic phosphate, methylamine, ammonium and carbondioxide (Borggaard & Gimsing, 2008; Duke *et al*., 2012). AMPA has been documented to have a soil half-life of between 60 and 240 days and a similar aquatic half-life as glyphosate (Giesy *et al*., 2000; Bergström *et al*., 2011 cited by Battaglin *et al*., 2014). AMPA has a longer half-life than glyphosate, which means soil contamination could accumulate with

the extensive use of glyphosate (Mamy *et al*., 2005). AMPA has a high detection frequency in streams and rivers than glyphosate and this can be due to differences in source proximity, water travel time, water residence time, degradation processes, and other natural processes (Battaglin *et al*., 2014). AMPA is also detected at higher concentrations than glyphosate in aquatic environments (Pesce *et al*., 2008; Struger *et al*., 2008; Battaglin et al., 2014). For example, the maximum glyphosate and AMPA concentrations in samples from rivers in the USA was 3.08 µg/L and 4.43 µg/L, respectively (Battaglin *et al*., 2014).

2.6. Glyphosate effect on bacteria

Herbicides can alter the composition of freshwater microbial communities by providing competitive advantage to tolerant microbes. A similar response can also be observed in the gut microorganisms. Herbicides can affect microbial communities directly since they share similar physiological metabolisms with plants. The observed change in microbial communities is achieved by modifying the equilibrium between species and their interactions (Dorigo *et al*., 2007). Gut bacteria play a crucial role in human physiology and well-being, through an integrated bio-semiotic relationship with the human host (Samsel & Seneff, 2013). Gut bacteria assist the human host with a range of beneficial metabolic and physiological contributions such as aiding digestion, synthesizing vitamins, detoxifying xenobiotics, and also part-taking in immune system homeostasis and gastrointestinal tract permeability (Littman & Pamer, 2011). The increase in incidences of inflammatory bowel diseases through Western Europe and the US, led to a suspected correlation between the impact of glyphosate on gut bacteria and these disease conditions (Samsel & Seneff, 2013),. Similarly glyphosate may pose as a culprit in increased cases of *Clostridium botulinum* mediated diseases in animals, due to the loss of antagonist effect of beneficial bacteria on *C. botulinum* (Shehata *et al*., 2013, Krüger *et al*., 2013).

Kurenbach *et al*. (2015), conducted a study on *Escherichia coli* and *Salmonella enterica serovar Typhimurium*. The aim of this was to determine the impact of different commercial herbicides (such as dicamba (3,6-dichloro-2-methoxybenzoic acid; Kamba), 2,4 dichlorophenoxyacetic acid (2,4-D), and glyphosate [N- (phosphonomethyl)glycine; Roundup]) on the susceptibility of select bacteria to antibiotics including ampicillin (Amp, beta-lactams), ciprofloxacin (Cip; fluoro-quinolones), chloramphenicol (Cam), kanamycin (Kan; amino- glycosides), and tetracycline (Tet). The maximum concentrations of herbicides used in the study were all well below the MIC for each of the herbicides.

The results showed that the susceptibility of bacteria to antibiotics can change when exposed to the antibiotic and herbicide at the same time. They observed that this variation in susceptibility differed according to species. Glyphosate increased the tolerance of *E. coli* to Kan and Cip but did not affect or reduce susceptibility to Amp, Cam, and Tet. While the exposure of *S. typhimurium* to glyphosate induced tolerance to Kan and Cip but did not affect or reduce susceptibility to Amp, Cam, and Tet. *E. coli* and *S. typhimurium* had similar response patterns to sub-lethal concentrations of kamba and 2,4-D, as they both increased tolerance to Amp, Cam, Cip, and Tet and increased susceptibility to Kan.

Lu and colleagues (2013) conducted a study to evaluate the genome-wide transcriptional responses of *E. coli* to glyphosate in 2013. What they found was that glyphosate induced metabolic starvation and energy drain, as most genes involved were down-regulated. Non-shikimate related effects were also observed, such as cell motility, energy production, and carbohydrate metabolism. Glyphosate caused an up-regulation of genes involved in regulation of cell motility and chemotaxis. In this case glyphosate functions as a repellent rather than an attractant, which results in negative chemotaxis. What they also observed was that genes encoding proteins involved in the shikimate and aromatic amino acid pathway were down-regulated. Genes modulated include aromatic amino acid biosynthesis genes such as *asd* gene, AcroK, AcroA and AcroC; central carbon metabolism genes such as *zwf*, *ybhE*, *dld*, *mdh*, and *sdhCDAB* operon; and energy production and conversion genes such as *atpB*, *atpE*, *atp*F, *tpH*, and nuo genes. In total, 1040 genes were differentially expressed following exposure to glyphosate representing 23.3% of the entire genome, from which 34% were hypothetical genes representing uncharacterized physiological responses.

Fei *et al*. (2013) conducted a study to identify regulated genes that confer resistance to high concentrations of glyphosate in a new strain of *Enterobacter*. The results of the study indicated that gene expression occurred directly or indirectly in response to glyphosate. The genes expressed are involved in many pathways which are vital to bacterial fitness. The results also indicated that a combination of glyphosate-resistant *epsps* and the DEGs that are induced by osmotic, acidic and oxidative stresses may confer resistance to glyphosate. This is a reflection of the complex nature of genes needed to lead to glyphosate resistance.

Newman *et al*. (2016) identified 67 differentially expressed bacterial transcripts from the rhizosphere after long term glyphosate (PowerMAX) exposure at recommended field rate. Fourty five of the differentially expressed genes were down-regulated and 22 were up-regulated. Majority of down-regulated genes were involved in carbohydrate metabolism and amino acid metabolism, and extent to other functions including fatty acid and lipid

metabolism, iron acquisition and metabolism, nitrogen metabolism, protein metabolism, membrane transport, cell division, cell wall and capsule formation. A large portion of up-regulated genes were involved in protein metabolism and respiration, and extend to other functions including carbohydrate metabolism, stress response, RNA metabolism, amino acid metabolism, motility and chemotaxis, nucleoside and nucleotide metabolism, and membrane transport.

Pathways down regulated in the study include synthesis of amino acids (alanine, methionine, glutamine, etc.), the Entner-Doudoroff pathway, the pentose phosphate pathway, ferric iron ABC transport, and ammonia assimilation. The results of the study suggest that glyphosate leads to modifications in rhizosphere bacterial community, due to increase in respiration and expression of transcripts involved in protein degradation while lowering amino acid synthesis and expression of transcripts involved in the Entner-Doudoroff pathway. Similar findings were reported by Zabaloy *et al.* (2012) whereby respiration was increased due to stress response following glyphosate exposure, which is accounted for by a greater proportion of glyphosate-sensitive species in soil with no glyphosate application history.

Glyphosate toxicity varies between microorganisms. Beneficial commensal bacteria appear to be highly sensitive to glyphosate as compared to pathogenic bacteria which are greatly resistant. *Escherichia coli* showed the highest resistance with MIC value of 5 mg/ml, *S. aureus* and *S. lentus* showed moderate resistant with MIC value of 0.60 and 0.30 mg/ml, respectively. High sensitivity was shown among beneficial bacteria such as *E. faecalis*, *E. faecium, B. cereus*, and *B. adolescentis* with MIC value of 1.5 $x10^{-4}$, 3 $x10^{-4}$, and 7.5 $x10^{-5}$ mg/ml, respectively (Shehata *et al*., 2013). *Enterococcus faecalis, E. faecium and B. badius* has a toxic effect on *C. botulinum* (Shehata *et al*., 2013, Krüger *et al*., 2013), which helps to minimize its growth. Loss of microbiota such as *Enterococcus* spp. and *B. badius* due to sensitivity to glyphosate may lead to over-growth of *C. botulinum* in animals promoting disease, an indication that glyphosate cause disturbance in the gut microbial community (Shehata *et al*., 2013; Krüger *et al*., 2013; Nielsen *et al*., 2018).

Additionally, bifidobacteria exhibited high sensitivity towards glyphosate (Shehata *et al*., 2013). Bifidobacteria generate unfavourable growth conditions for pathogens that are more tolerant to glyphosate such as *Salmonella* (Isolauri *et al*., 2001). Therefore, glyphosate may modulate gut bacteria indirectly by inhibiting bifidobacteria and allowing growth of *Salmonella* (Shehata *et al*., 2013). A study on green turtles (*Chelonia mydas*) indicated that glyphosate causes reduced density of the gut bacterial community, after examining exposure of mixed bacterial communities from the gastrointestinal tract to varying glyphosate concentrations

over a period of 24 h. Changes may also extend to species composition and lowered species diversity in green turtles exposed to glyphosate (Kittle *et al*. 2018). This is an indication that the effects of glyphosate on gut bacteria community is not only limited to animals receiving feed contaminated with glyphosate.

A recent study by Aitbali *et al*. (2018) describes increased anxiety and depression-like behaviours paralleled with decreased total bacterial count and altered gut microbial composition in terms of *Firmicutes*, *Bacteroidetes* and *Lactobacillus* in mice, following sub-chronic and chronic treatment with 250 or 500 mg/kg/day of glyphosate-based herbicide. This study highlight a crucial connection between toxicity of glyphosate-based herbicides on gut microbial community and the implications thereof on the host. Similar to the aforementioned study Lozano *et al*. (2018) reported gut microbial community imbalance following long term-term exposure to Roundup. Alteration of the *Firmicutes* to *Bacteroidetes* ratio brought about by Roundup exposure in the study may have a role in the epidemic of intestinal disorders. In contrast, study done by Nielsen *et al*. (2018) showed very limited effects of pure glyphosate and glyphosate-based herbicide on gut microbial community composition in rats over a 2-week oral exposure at a concentration of 50x allowed daily intake for humans. The lack of variation to gut bacterial community may be caused by alleviation of antimicrobial effect of glyphosate due to supplementation of aromatic amino acid in the gut environment. However, one cannot rule out negative effect of glyphosate in malnutrition or individuals on special diets that may lead to lower levels of available amino acids.

2.7. Genetically Modified crops

Unlike traditional selective breeding, genetic engineering provides a platform for breeders to obtain DNA from basically anywhere in the biosphere, insert it in crops and confer desired traits such as increased yield, growth with limited irrigation or irrigation with salty water, produce fruits and vegetables resistant to mold and rot, insect resistance or resistance to herbicides (Landrigan & Benbrook, 2015). Glyphosate tolerant crops can be engineered through expression of a microbial CP4-EPSPS or mutated EPSPS that is insensitive to glyphosate (Pollegioni *et al* 2011). Crops can be grown with a single trait or containing multiple traits also referred to as stacked traits (Huang *et al*., 2015).

Herbicide resistance is the leading trait introduced to crops, mainly in corn and soybean. Naturally all crops were vastly susceptible to glyphosate, until the introduction of genetically

modified soybean and canola in 1996, cotton in 1997, corn, and maize in 1998 (Huang *et al*., 2015; Duke, 2014; Dill 2005). Introduction of these plants revolutionised the agricultural landscape and also caused a steady increase in glyphosate usage worldwide (Saunders & Pezeshki, 2015). Genetically modified crops have become abundant worldwide. Greatly because of the ease of weed management under glyphosate application, while leaving crops unharmed (Duke, 2014; Landrigan & Benbrook, 2015). Unintentional glyphosate tolerance can also occur mainly in weeds. There are 225 reported cases of 29 resistant weed species worldwide (Shaner *et al*., 2012).

2.8. Glyphosate and AMPA residues in crops

A number of studies have reported residual glyphosate in crops and feed. Bøhn and colleagues (2014), reported mean residues of glyphosate and AMPA of up to 3.3 mg/kg and 5.7 mg/kg respectively in glyphosate tolerant soybean, while other studies reported concentrations ranging from 1.1 mg/kg to 15.1 mg/kg of glyphosate residues in tolerant soybean cultivated in USA (Cuhra *et al*., 2015). These reported residues are below regulatory limits. Maximum residue level (MRL) for soybean are 10 mg/kg in Brazil, 20 mg/kg in Europe (Bøhn *et al*., 2014), and 40 mg/kg in the US (Cuhra *et al*., 2015). But glyphosate residue levels surpassing regulatory guideline limits have been reported (Then, 2014). Glyphosate and AMPA residues have also been reported in crops such as maize, cannabis, cowpea and corn. But glyphosate residue detected is not restricted to these crops, residues are also detected in dairy products, coffee, and even in honey (Reddy *et al*., 2008; Rubio *et al*., 2014; EFSA, 2015). Despite these reports, published data on glyphosate in glyphosate tolerant crops is scant (Cuhra *et al*., 2016, Cerdeira & Duke, 2006).

2.9. South African context

South Africa is highly depended on the agricultural sector for economic growth and food security. This is mostly observable in majority of poor communities and in rural areas that heavily depend on agriculture as their main source of income. The agricultural sector is vital in South Africa as in 2009, it contributed approximately 12% to the GDP and employs about 30% of the formal workforce when non-registered farm workers and subsistence farmers in rural areas are included (Quinn *et al*., 2011). Glyphosate was registered in South Africa in the 1970s. Since glyphosate was introduced it has been used to control weeds in field crops, timber, horticulture, sugar and viticulture industries. Since the introduction of glyphosate in South Africa it is marketed under more than 20 trade-names, however it is not clear as to

how much of this herbicide has been used prior to 2012 nor its value in the economy or environmental impact.

Globally glyphosate usage skyrocketed from a total of 67 million kg applied in/outside agriculture in 1995 to 826 million kg in 2014, while glyphosate usage in the agricultural sector grew from 51 million kg to 747 million kg (a 14.6-fold). Glyphosate used for non-agricultural purposes globally rose from 16 million kg to 76 million kg from 1995 to 2014. Only between 2005 and 2014 61 billion kg of glyphosate have been used, which accounts for 71.6 % of the global glyphosate used since it reached the markets (Benbrook, 2016). In South Africa more than 23 million litres of glyphosate was sold at an estimate value of R 641 million in 2012. Sixty-five percent of the total sold glyphosate was utilized on maize, wheat and soybean farms. Moreover, majority of glyphosate was applied on maize farms (46%) followed by wheat (13%), industrial use (8%) and soybean (6%). Glyphosate is immensely valuable in South African agricultural sector. Depending on potential yield loss assumptions, glyphosate is valued at an estimate of between R525 million and R2.203 billion with GM famers benefiting the most from the technology. For wheat farmers glyphosate was at an estimate of between R123 million and R485 million, the value of glyphosate for soybean farmer was estimated to be between R148 million and R693 million and the most probable value lying at R412 million (Gouse, 2014).

South Africa is the only developing country in which GM varieties of the basic staple food crop are grown. The basic staple food is white-grained maize and yellow-grained maize which is grown extensively but used primarily as feed (Gouse *et al*., 2005). Maize can be prepared in a variety of maize-based foods such as whole-maize foods, wet-ground maize foods, snacks and bread, maize sourdough and dumplings, porridges and beverages (Ekpa *et al*., 2018). Low income groups in South Africa are equipped to acquire mainly low cost staple foods such as maize meal porridge, with limited added variety of fruits and vegetables (Schönfeldt *et al*., 2018). As such they heavily depend on maize and other grains such as sorghum and soybean. These grains are associated with heavy glyphosate usage. In addition consuming a monotonous diet consisting of starch with low essential nutrients such as vitamins, minerals, and essential amino acids (Ranum *et al*., 2014; Schönfeldt *et al*., 2018), may lead to malnutrition which may pose health risks with combination of accumulating residual glyphosate and AMPA in staple foods.

2.10. Different methodologies and media used

2.10.1. Luria Bertani medium

The LB medium is extensively used to culture members of the *Enterobacteriaceae* (Bertani, 2004). LB medium has become a commonly used bacterial culture medium, as it allows fast growth and decent growth yields for many bacteria (Sezonov *et al*., 2007). LB is widely applied in recombinant DNA work. Other applications include usage as a general-purpose bacterial culture medium for a variety of facultative organisms (MacWilliams & Liao, 2006).

2.10.2. Brain Heart Infusion (BHI) medium

Brain Heart Infusion Agar is a solid medium suitable for the culture of a wide range of microorganisms, including bacteria, yeasts and moulds (Murray *et al*., 2007). It is especially useful for the isolation and culture of fastidious bacteria. The medium is recommended for the cultivation of fastidious organisms. BHI medium derives its nutrients from the brain heart infusion, peptone and glucose components. The peptones and infusion provide ideal sources of organic nitrogen, carbon, sulfur, vitamins and trace substances (Sigma-Aldrich, 2013).

2.10.3. Protein extraction approaches

Purification of protein forms a crucial part in protein research, as it serves as the first step in any proteomic experiment. Understanding protein function is important because proteins function partly or completely in DNA synthesis activity (Cilia *et al*., 2009; Tan & Yiap, 2009), also a reliable and comprehensive protein extraction is the closest proteomic equivalent to a fully sequenced and annotated genome (Cilia *et al*., 2009). The initial step of any purification procedure starts with extraction of protein from the source (De Mey *et al*., 2008; Ganesh & Lin, 2011). To liberate the cytoplasmic content of the host requires the cells to be disintegrated by physical, chemical, or enzymatic processes (De Mey *et al*., 2008). Mechanical procedures include ultrasonic disruption and mechanical agitation. Enzymatic procedures include nonionic detergents. There are many different extraction and analysis methods that exist (De Mey *et al*., 2008). These protein extraction procedures can differ widely in reproducibility and representation of the total proteome (Cilia *et al*., 2009). Identifying the right method is crucial for accuracy and precision, as it is the starting point for downstream processes (Tan & Yiap, 2009).

In this study the Bugbuster extraction kit was utilised for experimentation, as it provide a milder and simpler chemical cell disruption alternative compared to mechanical methods, and

it is simple, rapid, cost effective. Additionally, this kit is frequently utilized for the small-scale extraction of soluble target proteins (Novagen, 2008; Ganesh & Lin, 2011). Bugbuster extraction was used in combination with the bicinchoninic acid (BCA) assay for estimation of the concentration of protein, as it gives the best results (De Mey *et al*., 2008). BCA was chosen because it is stable under alkali conditions, it can be carried out in a one-step process, and it is not affected by a range of detergents and denaturing agents such as urea and guanidinium chloride (Walker, 2002).

2.10.4. Mass Spectrophotometry

Prior to the genomic revolution, the structure of proteins was widely investigated using chemical or enzymatic methods like the Edman degradation. Overtime mass spectrometry was introduced (Domon & Aebersold, 2006). Mass spectrometry is widely used due to its diverse application across different fields, unparalleled sensitivity, low detection limits, and high speed (de Hoffmann & Stroobant, 2007). Mass spectrometry (MS)-based protein quantification is a powerful technique for proteome wide quantification of differentially regulated protein. In combination with isotopic labelling of biological samples before MS analysis, it has proven to be a prolific method for protein quantification (Rauniyar *et al*., 2013).

The basic proteomic workflow consists of sample preparation, protein and peptide separation, MS, and data analysis (Bantscheff *et al*., 2012). Investigation of proteins using MS-based procedures follows three stages (1) sample preparation, (2) sample ionization, and (3) mass analysis. Sample preparation is mainly achieved by using 1- or 2-D polyacrylamide gel containing protease followed by peptide purification. Sample ionization allows the sample to be analysed by MS, through converting the sample into desolvated ions. This is accomplished by using electrospray ionization (ESI) and matrix-assisted laser desorption/ionization (MALDI). These two techniques dramatically changed proteomic analysis and made polypeptides accessible to mass spectrometric analysis (Domon & Aebersold, 2006).

The mass analyser is the heart of the mass spectrometer, as it is responsible for sensitivity, resolution, mass accuracy and generation of information-rich ion mass spectra from peptide fragments (Aebersold & Mann, 2003; Domon & Aebersold, 2006). In this study a Thermo Scientific Q-Exactive mass spectrometer was utilized to analyse separated peptides.

2.10.4. Isobaric tags

Isobaric reagents contain a reporter ion group, a mass normalization group, and an amine reactive group. The amine reactive group help attach the isobaric tags to the peptides by reacting with the N-terminal amine groups and ε-amine groups of lysine residues. The specificity of amine groups makes it possible to use almost all peptides for quantification. The mass normalization groups balance the mass differences between reporter ion groups so that the different isotopic variants of the tag have the same mass. The reporter ion group appear in low mass range, which is different from peptide fragment peaks. The remainder of the sequence informative *b*- and *y*-ions remain as additive isobaric signals, which aids in sensitivity (Bantscheff et al., 2012; Rauniyar *et al*., 2013). Most commonly used chemical labelling methods are tandem mass tags and relative quantification (iTRAQ).

In the study we used Tandem Mass Tags (TMT) because they (i) allow multiplexed analysis of different biological samples, (ii) the complexity of LC separations is not increased because the labelled peptides are precisely co-eluted, and the complexity of peptide mass spectra is not increased since the differentially labelled peptides are isobaric (Bantscheff *et al*., 2012), and (iii) provides an increased signal to noise ratio of reporter-ions used for quantification, because chemical noise is removed in the second stage of mass spectrometry (Dayon *et al*., 2010).

2.10.5. Proteome Discoverer

The Proteome Discoverer software package is a client-server application that uses workflows to process and report mass spectrometry data. Proteome Discoverer does a comparison of the raw data obtained from the mass spectrometry or spectral libraries with information from a selected FASTA database, to identify proteins from mass spectra of digested fragments (Thermo Scientific, 2014). In the current study, Proteome Discoverer™ 2.1 software 1 (Thermo Scientific, USA) which is commercially available was used to process qualitative and quantitative protein data that was collected using a mass spectrometer. The search output obtained post processing by Proteome Discoverer was exported to Microsoft Excel outputs for further processing and analysis. High-throughput proteomics is largely restricted by the availability of comprehensive sequence databases, since protein identifications rely on matches with sequence databases (Aebersold & Mann, 2003).

Chapter summary

N - (phosphonomethyl) glycine (glyphosate) is a broad-spectrum herbicide function by inhibiting the 5-enolpyruvyl-shikimate-3-phosphate synthase of the shikimate pathway found in bacteria, fungi, algae and higher plants. The pathway synthesizes aromatic amino acids and other aromatic compounds, inhibition by glyphosate leads to uncontrolled carbon flow and consequently disrupt the organism's metabolism. Glyphosate undergoes degradation by microbial activity forming AMPA (major breakdown product), methylphosphonic acid, glycine and sarcosine. Glyphosate has a half-life ranging from 0.8 to 151 days in soil, and up to 90 days in water. AMPA has a half-life of 60 to 240 days in soil and a 90 day half-life in water.

Glyphosate contamination varies a lot in water, due to management practises, application frequencies and method of application in agricultural landscapes. In general glyphosate and AMPA residues reported have been low and not exceeding the Water Quality Guideline for glyphosate but the residues are widespread epcecieally in areas with GM crops. For example, glyphosate and AMPA contamination varies between 0.1 µg/L up to 700 µg/L across the US, Canada, Argentina, and France in water. These concentrations pose minimal risks to humans but treatment of water to remove glyphosate is crucial to reduce the risk of human exposure to glyphosate residues through drinking water. Residues have also been reported in food crops including soybean (1.1 mg/kg to 15.1 mg/kg), maize, cowpea, and dairy products.

Bacteria has varying susceptibilities to glyphosate and beneficial commensal bacteria show high susceptibility while pathogenic bacteria exhibits high tolerance. For instance, *E. coli* has MIC value of 5 mg/ml, while beneficial bacteria such as *E. faecalis*, *E. faecium, B. cereus*, and *B. adolescentis* have MIC values of 1.5 $x10^{-4}$, 3 $x10^{-4}$, and 7.5 $x10^{-5}$ mg/ml, respectively. This imbalance in susceptibility may lead to a shift in gut bacterial community leading to overgrowth of pathogenic bacteria, promoting disease in human and animals. Glyphosate has the potential to increase or decrease the antibiotic susceptibility of bacteria well below the MIC, which may pose as a gate way to adaptive resistance to antibiotics. Glyphosate also causes differential expression of carbohydrate metabolism, amino acid metabolism, fatty acid and lipid metabolism, iron acquisition and metabolism, nitrogen metabolism, protein metabolism, membrane transport, cell division, cell wall and capsule formation, and stress response.

South Africa heavily depend on the agricultural sector, and with its stable food crop being GM glyphosate usage is high. In 2012 South Africa used 23 million litres of glyphosate, of which 46% was used on maize farms, 13% on wheat, 6% on soybean, and 8% for industrial

use. Considering residues found in other parts of the world in soybean (1.1 mg/kg to 15.1 mg/kg) and corn (308 ng/g), the South African communities may be exposed to these concentrations or even higher since low income households heavily depend on grains especially maize. As such continuous exposure may pose negative health effects especially in individuals suffering from malnutrition.

CHAPTER 3

MATERIAL AND METHODS

3.1. Growth response of *Escherichia coli* and *Enterococcus faecalis* to sub-lethal concentrations of glyphosate and AMPA

Bacterial strains used for this study were *E. coli* JM109, *E. coli* EC100, *E. coli* DH5-alpha and *E. faecalis*. Bacteria were revived on agar plates, followed by overnight incubation in 10 ml of Luria Bertani media (Sigma-Aldrich, Germany for *E. coli* strains) and Brain heart infusion media (Sigma-Aldrich, for *E. faecalis*) at 37°C. Glyphosate, *N*-(Phosphonomethyl)glycine monoisopropylamine salt (Sigma-Aldrich, Germany) and AMPA, (Aminomethyl)phosphonic acid (Sigma-Aldrich, Germany) were used in the study. Reported MICs in literature for *E. coli* include 1.2 mg/ml, 2.4 mg/ml and 80 mg/ml (Shehata *et al*., 2013; Shehata *et al*., 2014; Nielsen *et al*., 2018), respectively, while for *E. faecalis* include 0.15 mg/ml, 0.3 mg/ml and 80 mg/ml (Shehata *et al*., 2013; Shehata *et al*., 2014; Nielsen *et al*., 2018). These varying MICs are due to different formulation of glyphosate used in the different studies. In the current study MICs where not determined as this was not the aim of the study. The study set out to determine concentration sub-lethal to the bacteria, which are concentrations that showed the least resistance to growth of the bacteria.

To determine concentrations of glyphosate and AMPA that are sub-lethal to *E. coli* and *E. faecalis*, bacterial response curves where used in the study. From which exposure times and concentrations were determined and chosen to represent early log phase, mid-log phase and late log-phase. For *E. coli* 10 mM (91.3 mg/ml) glyphosate and 50 mM (138.8 mg/ml) AMPA where chosen as sub-lethal concentrations. Experiment times were determined to be 2, 3, and 8 h. Concentrations sub-lethal to *E. faecalis* were 0.1 mM (0.91 mg/ml) glyphosate and 50 mM (138.8 mg/ml) AMPA. Experiment times chosen were 3 and 5 h for *E. faecalis* which are different times to *E. coli*, due to the difference in physiological growth of the bacterial cells.

3.2. Protein extraction and quantification

Triplicates of fresh 25 ml broth containing pre-determined sub-lethal concentrations of glyphosate or AMPA were inoculated with culture (OD between 0.1 and 0.13), and incubated at 37°C, 200 rpm over 2, 6, and 8 h for *E. coli*; 3 and 5 h for *E. faecalis*. The CFU/ml was

determined at 0 h and at the end of incubation. Protein synthesis was terminated at the end of incubation using 20 µl of 0.02 µg/ml Rifampicin. This was to ensure that no further protein synthesis occurred as a result of factors other than the effects of glyphosate and AMPA. Samples were centrifuged at 4700 rpm for 25 minutes at 4°C, supernatant was discarded and pellets were suspended in 1 ml 0.2 M ice-cold sucrose followed by centrifugation at 14000 rpm for 15 minutes at 4°C. Pelleted cells were stored at -80°C until protein extraction.

Protein extraction from bacteria pellets was conducted using a protein extraction kit (BugBuster®, Sigma-Aldrich, Germany) according to the manufacturer´s guidelines. The protein content of the cell-free extracts was estimated by the bicinchoninic acid assay employing a microtiter protocol (ThermoFisher Scientific, US). Optical densities were measured at 595 nm using a Beckman Du-7500 spectrophotometer to measure the absorbances of the copper complexes in both samples and standards. The protein concentration of each sample was calculated based on a calibration curve (Okoli, 2010).

3.3. Protein labelling

Tandem Mass Tag (TMT) was done using the TheromFisher Scientific TMT Mass Tagging Kits and Reagents (ThermoFisher Scientific, US). This approach allows multiplex relative quantitation by mass spectrometry. For each sample, a unique reporter in the low mass regions of the MS/MS spectrum (126-127 Da for TMT^2 and 126-131 Da for TMT^6) is used to measure relative protein expression levels during peptide fragmentation (ThermoFisher Scientific, US). Protein pellets (100 µg) for all samples was suspended in 100 µl of 100 mM TEAB followed by addition of 2.5 µl of trypsin. Sample was digested overnight at 37°C. After overnight incubation, 41 µl of TMT reagent was added to each sample and incubated for 1 hour at room temperature. Thereafter, peptide digestion was quenched using 8 µL of 5% hydroxylamine and incubated for 15 minutes.

3.4. Mass spectrometry

Equal aliquots of labelled peptides from the samples under investigation were pooled together and were submitted to the Proteomic Facility for mass spectrometry. OMIX C18 tips (Varian, Inc., Palo Alto, CA) was used for sample cleanup and concentration. Peptide mixtures containing 0.1% formic acid were loaded onto a Thermo Fisher Scientific EASY-nLC1000 system and EASY-Spray column (C18, 2 µm, 100 Å, 50 µm, 50 cm). Peptides were fractionated using a 2-100% acetonitrile gradient in 0.1% formic acid over 180 min at a flow rate of 250 nl/min. The separated peptides were analyzed using a Thermo Scientific Q-Exactive mass spectrometer.

3.5. Bioinformatic analysis

Data was collected in data dependent mode using a Top10 method. Protein identification and TMT ratio calculation was done by Thermo Scientific Proteome Discoverer™ 2.1 software (ThermoFisher Scientific). The fragmentation spectra were searched against *E. coli* and *E. faecalis* tandem mass spectra of peptides on FASTA Databases using the Sequest search engine in Proteome discoverer. The protein relative abundance data were converted to log2, and 0.5log2, which is equivalent to 1.5-fold change at P-value < 0.01 was set as cut-off for differentially expressed (up-regulated or down-regulated) proteins of glyphosate/AMPA-exposed bacteria (experimental) relative to unexposed (control) bacterial cells. Thus, a minimal fold change of ≥± 1.5, where >+ 0.5log2 is equivalent to minimal 1.5-fold increase in protein relative abundance, for example, up-regulation; and < -0.5log2 is equivalent to minimal 1.5-fold decrease in protein relative abundance, i.e., down-regulation, indicated differentially expressed proteins (Paulo *et al*., 2015).

3.6. Categorization of proteins according to functional groups

Additional functional analyses were conducted using UniProt (https://www.uniprot.org/), InterPro (https://www.ebi.ac.uk/interpro/) and NCBI-Protein (https://www.ncbi.nlm.nih.gov/protein). Protein accession numbers were utilized to search the databases. KEGG PATHWAY (https://www.genome.jp/kegg/pathway.html) was utilized for functional group categorization of differentially expressed proteins. Microsoft Excel was then utilized for further data analysis.

3.7. Pathway analysis

Pathway analysis was performed with KEGG PATHWAY and STRING (version 10.5) databases (https://string-db.org/). KEGG PATHWAY was utilised as the primary database to identify protein pathway interactions. Protein gene codes were used to locate pathways in which the differentially expressed proteins belong. The search was done against pre-existing pathway maps within the database. After positive identification of protein/s in the pathway map, images (png) of the pathway maps were downloaded from the site. The images were further edited with Photoscape (version 3.7) to elucidate proteins belonging to the same pathway and their interactions. STRING database was used as a secondary source to identify protein pathways which were not readily identifiable in KEGG PATHWAY.

3.8. Statistical analysis

Discriptive statistical calculations of averages, logarithmic values, standard deviations, and p-values were done using Microsoft Excel 2013. Spectral data from the mass spectrometer was analysed through the Thermo Scientific Proteome Discoverer™ 2.1 software (ThermoFisher Scientific). All experiments where done in triplicate.

CHAPTER 4

RESULTS

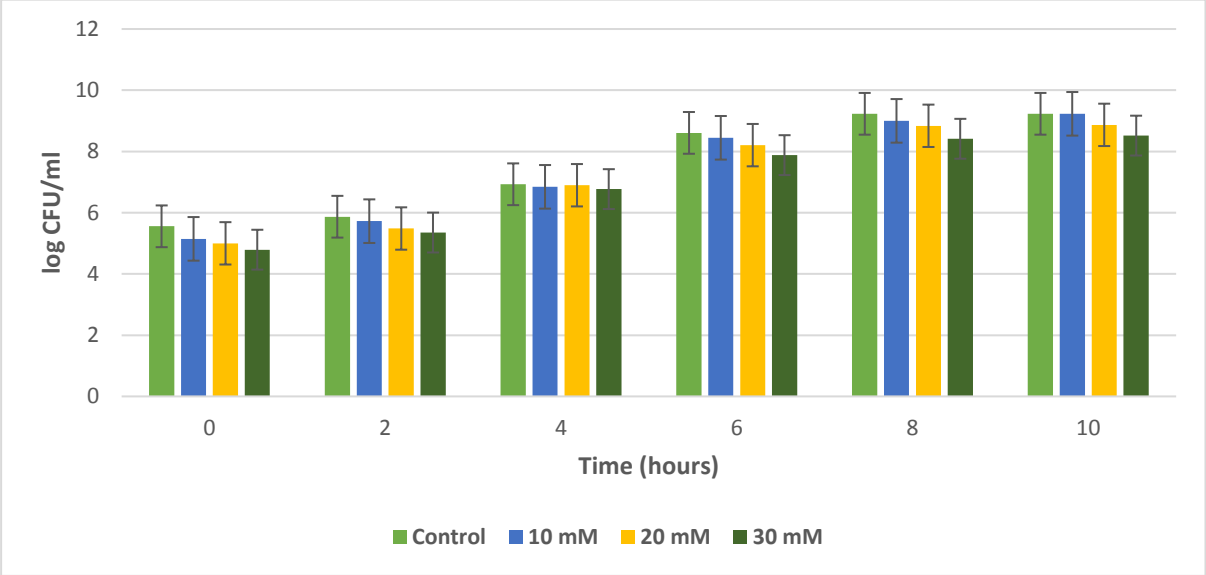
4.1. Growth response of *Escherichia coli* (RM 109) and *Enterococcus faecalis* exposed to sub-lethal concentrations of glyphosate and AMPA

The aim of the study was to determine the molecular response of *E. faecalis* and *E. coli* to sub-lethal concentrations of glyphosate and AMPA. To identify the appropriate sub-lethal concentrations for proteomic studies, the bacteria were exposed to different sub-lethal concentrations of glyphosate and AMPA. *Escherichia coil* RM109 was exposed to non-lethal glyphosate concentrations of 0 mM, 10 mM, 20 mM and 30 mM glyphosate (Figure 1A). AMPA concentrations tested were 50 mM and 100 mM, and 50 mM was determined as sub-lethal to *E. coli* RM109 (Figure 1B).

For *E. faecalis*, sub-lethal concentration of 0.1 mM glyphosate, and 50 mM AMPA were determined (Figure 2A, 2B). The range of concentrations of glyphosate and AMPA that have been identified in the environment is 3.3 mg kg^{-1} (0.003 mg/ml) in soybean (Bøhn *et al*., 2014), 308 ng g^{-1} ($3.08x10^{-4}$ mg/ml) in corn (Reddy *et al*., 2008), and range from 0.23 µg/L to 700 µg/L ($2.3x10^{-7}$ mg/ml to $7x10^{-4}$ mg/ml) in water (Pesce *et al*., 2008, Peruzzo *et al*., 2008). These concentrations may increase over time with increased application and accumulation of glyphosate. Therefore, bacteria will potentially encounter very low concentrations of glyphosate and AMPA in their respective environmental niches. Thus, sub-lethal concentrations that showed the least effect on bacteria were preferred for proteomic investigations as it reflected the bacterial response to low glyphosate and AMPA concentrations in the environment.

Figure 2 and 3 represent bacterial growth curves of *E. coli* and *E. faecalis* in response to sub-lethal concentrations of glyphosate and AMPA.

A



B

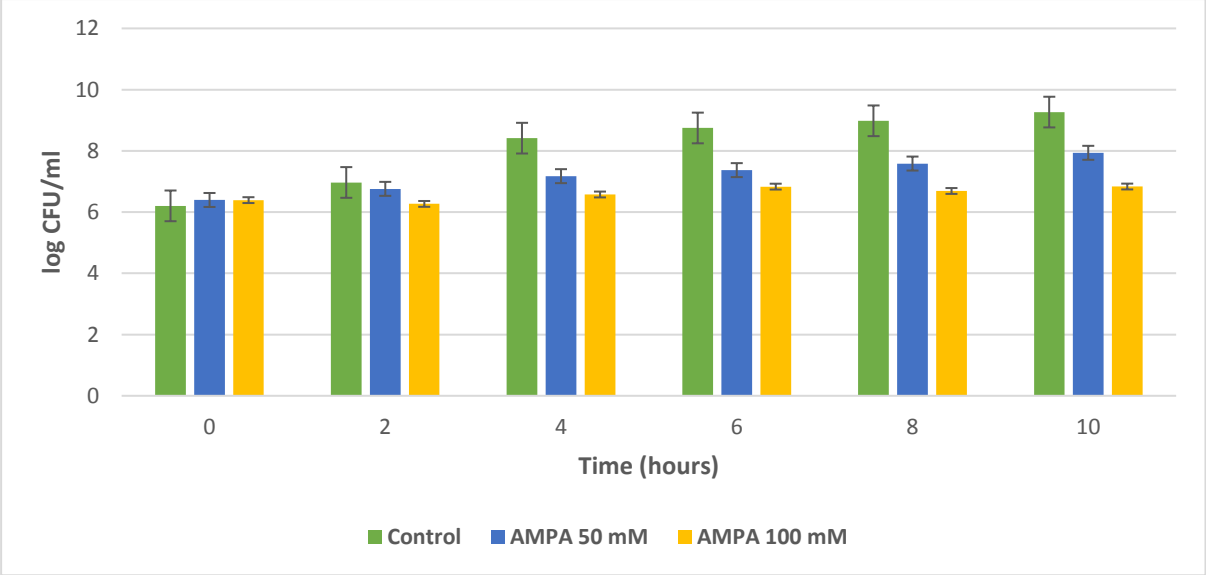
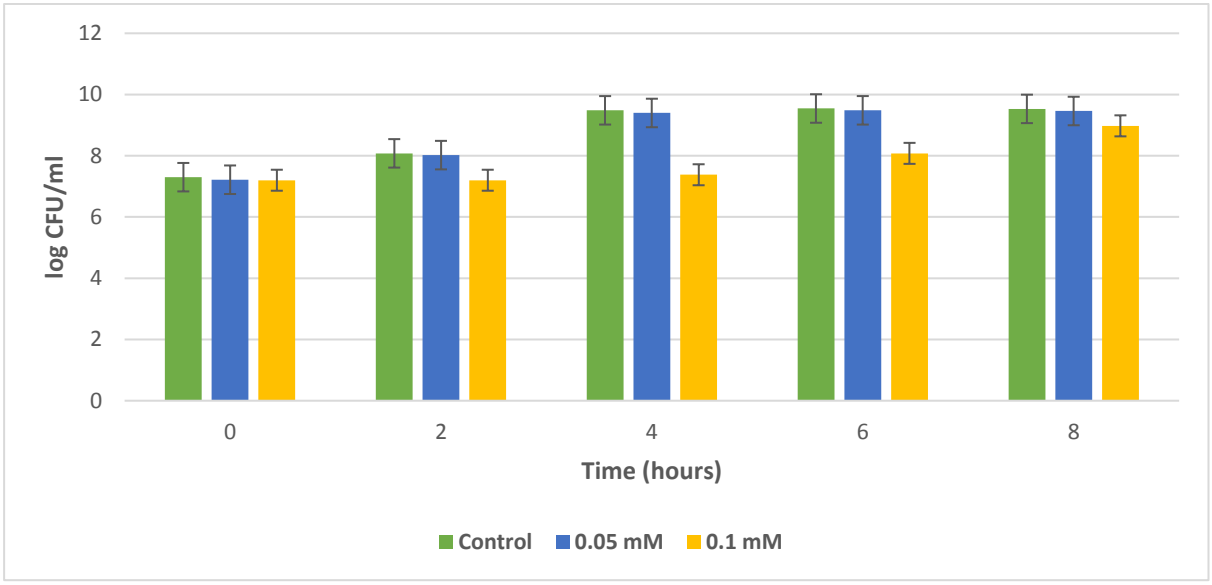


Figure 2: Bacterial growth curve in response to sub-lethal glyphosate and AMPA concentrations. [A]: *E. coli* RM109 in response to different glyphosate concentrations, ranging from 10 mM to 30 mM over time. CFU/ml: Colony Forming Units per milliliter; [B]: *E. coli* RM109 in response to different AMPA concentrations, of 50 mM and 100 mM over time.

CFU/ml: Colony Forming Units per milliliter, AMPA: Aminomethyl phosphonic acid.

A



B

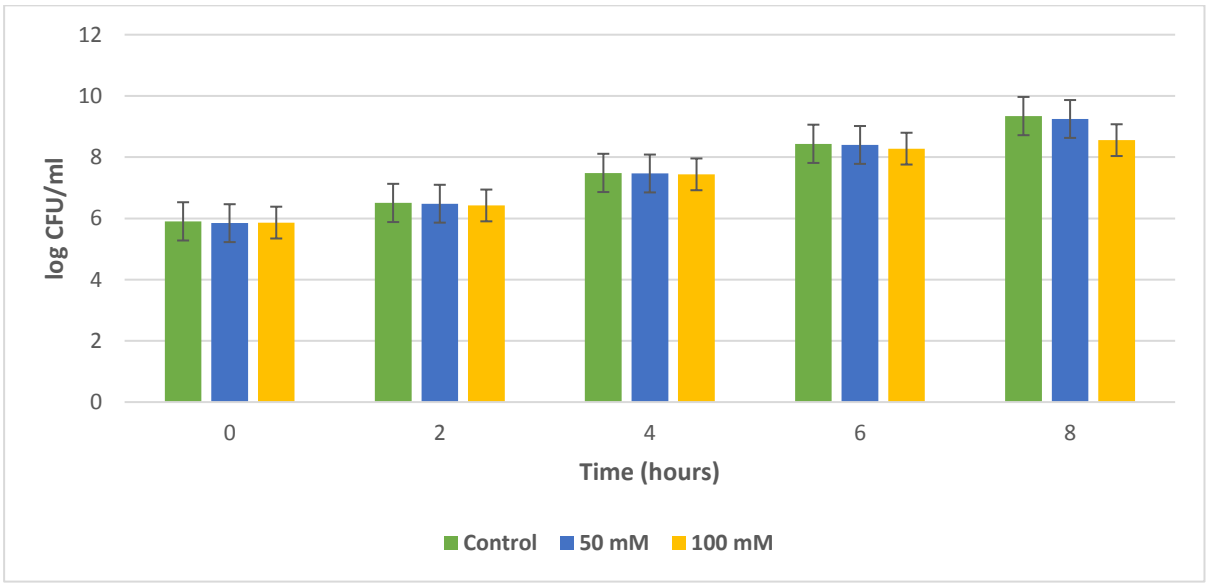


Figure 3: Bacterial growth curve in response to sub-lethal glyphosate and AMPA concentrations. [A]: *E. faecalis* in response to 0.1 mM glyphosate over a period of 8 hours CFU/ml: Colony Forming Units per milliliter; [B]: *E. faecalis* in response to 50 mM and 100 mM AMPA over a period of 10

hours. CFU/ml: Colony Forming Units per milliliter, AMPA: Aminomethyl phosphonic acid.

The tested concentrations of glyphosate in the current study were set at 10 Mm, 20 mM as well as 30 mM. No major differences in the growth of the bacterium were observed in relation to the control. The least resistance was observed at 10 mM glyphosate toward *E. coli* growth, while 10 mM and 30 mM showed the highest resistance as compared to the control. The log phase comenced from 2 h to 8 h and the bacteria entered stationary phase thereafter. In response to low doses of AMPA *E. coli* showed slight difference in growth between the control and tested concentrations, with 100 mM showing the highest resistance to growth and 50 mM showing the least resistance to growth. The log phase started from 4 h and extended to 10 h. In response to sub-lethal doses of AMPA *E. coli* showed some differences in growth between the control and tested concentrations. Highest resistance towards growth was at 100 mM, whereas 50 mM showed the least resistance to growth.

Enterococcus faecalis growth response curve to glyphosate indicated that the bacterium goes through log-phase between 2 h and 6 h, and enters stationary phase shortly thereafter. When exposed to 0.05 mM glyphosate the growth of the bacterium is almost the same as in the absence of glyphosate. When the concentration increases to 0.1 mM the bacterium experiences a longer lag-phase as compared to the control, which is an indication of glyphosate shock factor. When exposed to AMPA the growth of the bacterium is not drastically hindered by 50 mM and 100 mM AMPA for the first 6 hours, but as the bacteria enters stationary phase 100 mM AMPA resistance towards growth increase while growth under 50 mM AMPA remained unchanged.

Concentrations that were chosen for proteomic analysis were 10 mM and 0.1 mM glyphosate for *E. coli* and *E. faecalis*, respectively, and 50 mM AMPA for both *E. coli* and *E. faecalis*. The concentrations show the least resistance towards growth and were ideal to show the effects towards bacteria when encountering these concentrations in their respective environments.

4.2. Number of modulated proteins as a measure of bacteria protein dynamic in response to sub-lethal concentrations of glyphosate and AMPA

E. coli RM109 differentially expressed a greater number of proteins in response to glyphosate than AMPA (Table 1). A greater number of proteins (162) were differentially expressed by RM109 after 2 hours exposure compared to the expressed 32 and 42 proteins after 6 and 8 hours exposures respectively. Furthermore, both *E. coli* strains and *E. faecalis*

employed up-regulation of protein levels/activities as a general means to mitigate the effects of glyphosate and AMPA. Differences in the number of expressed proteins between *E. coli* strains (RM109, EC100 and DH5-alpha) indicated strain differences in response to glyphosate and AMPA. For example, in response to glyphosate there was a difference in the total number of regulated proteins for RM109 (32), EC100 (164) and DH5-alpha (66). *Enterococcus faecalis* differentially expressed more proteins in response to AMPA (258 proteins at 3 hours and 276 proteins at 5 hours) than glyphosate (123 proteins at 3 hours and 83 proteins at 5 hours), even though glyphosate is more toxic than AMPA (Table 1).

Table 1: An overview of the number of proteins that were differentially expressed by different strains of *E. coli* and *E. faecalis* under the influence of glyphosate and AMPA sub-lethal concentrations.

Bacterial culture/ Chemical treatment	Time (hours)	Genome Size (kbp)	Total identified proteins	Total regulated proteins	Up regulated	Down regulated
*E. coli** RM109 Glyphosate	2	4377	1900	162	68	94
	6			32	18	14
	8			41	27	14
*E. coli** RM109 AMPA***	2	4377	1895	32	25	7
	6			40	32	8
	8			39	33	6
*E. coli** DH5α Glyphosate	6	4359	1229	66	38	28
*E. coli** DH5α AMPA***	6	4359	1482	88	78	10
*E. coli** EC100 Glyphosate	6	4377	1156	164	134	30
*E. coli** EC100 AMPA***	6	4377	1163	51	49	2
E. faecalis Glyphosate	3	3344	733	123	110	13
	5			83	67	16
*E. faecalis*** AMPA***	3	3344	674	258	162	96
	5			276	172	104

Different exposure times were used, due to different physiologies between *E. coli* and *E. faecalis*. **Escherichia coli*, ***Enterococcus faecalis*, ***Aminomethyl phosphonic acid

4.3. Functional categories of differentially expressed proteins of *E. coli* strains in response to glyphosate

4.3.1. RM109 response to glyphosate and AMPA

Several proteins that participate in both primary and secondary metabolism were differentially expressed by *E. coli* RM109 as a response mechanism to the effects of the sub-lethal concentrations of glyphosate and AMPA (Figures 4 & 5). In response to glyphosate, the bacterium differentially expressed proteins that function in iron transport & uptake, nitrate and nucleotide metabolism, transcription and translation processes, and stress response, in addition to proteins whose functions are yet to be characterized (Figure 4). Other differentially expressed proteins of RM109 in response to glyphosate included, dipeptide and tripeptide permease A (DtpA), which is involved in peptide transport; phospholipid ABC transporter-binding protein (MlaB); and selenium-binding protein (YdfZ), which functions in the uptake of selenium (Figure 4). Given that glyphosate may impact nitrogen metabolism by uncoupling synthesis of aromatic amino acids (Vivancos *et al*., 2011), it was interesting to observe that the bacterium down-regulated all the identified differentially regulated protein that participate in nitrate metabolism. In contrast, all the four identified proteins involved in transcription and translation processes were up-regulated. The stress response protein, arsenic pump-driving ATPase (ArsA), was up-regulated, while autonomous glycyl radical cofactor (GrcA) and HTH-type transcriptional regulator (PerR), which are also stress response proteins, where down-regulated (Figure 4).

The response of RM109 to AMPA involved regulation of the protein functional category of iron transport & uptake, carbohydrate metabolism, transcription & translation processes, and stress response, but not nucleotide and nitrate metabolism (Figure 5), which differs with the bacterium response to glyphosate where several nucleotide and nitrate metabolic proteins were differentially expressed (Figure 4). In the functional category of iron transport & uptake, the regulation of a number of specific proteins employed in RM109 response to AMPA was similar to the bacterium response to glyphosate. For example, FepA, EntB, EntE, TonB, ExbD and Flu which were up-regulated in response to AMPA (Figure 5) were also up-regulated in response to glyphosate (Figure 4). Similarly, the regulation pattern (up-regulation) of RpmF and GrcA, which are respectively involved in transcription & translation process, and stress response were the same in both AMPA and glyphosate (Figures 4 & 5). The bacterium also down-regulated selenoprotein (YdfZ) in response to both AMPA and glyphosate. However, the expression of stress response proteins by RM109 in response to

AMPA was different from its response to glyphosate both in number and type of stress response proteins and pattern of regulation (Figure 4 & 5), suggesting that the two chemicals induced different types of stress reponse in the bacterium.

Figure 4 represent functional categories of differentially expresses proteins of *E. coli* (RM109) in response to glyphosate

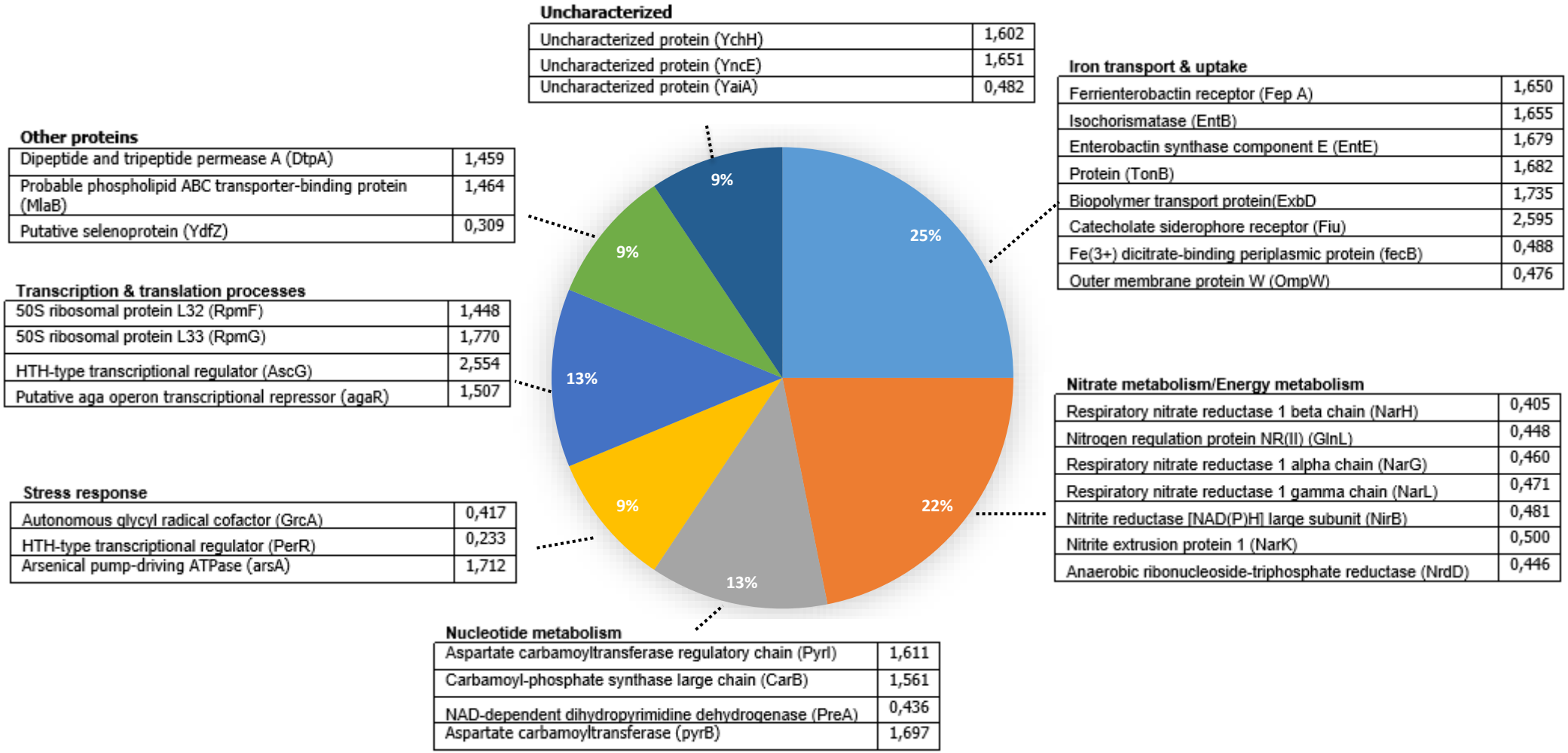


Figure 4: Functional categories of differentially expresses proteins of *E. coli* (RM109) to glyphosate. Statistical significance level was set at $p<0.01$ with a minimal fold change of $\pm$ 1.5. $\geq$ + 1.5 fold change indicates up-regulation; $\leq$ - 1.5 fold change indicates down-regulation.

The figure below represent functional categories of differentially expresses proteins of *E. coli* (RM109) in response AMPA

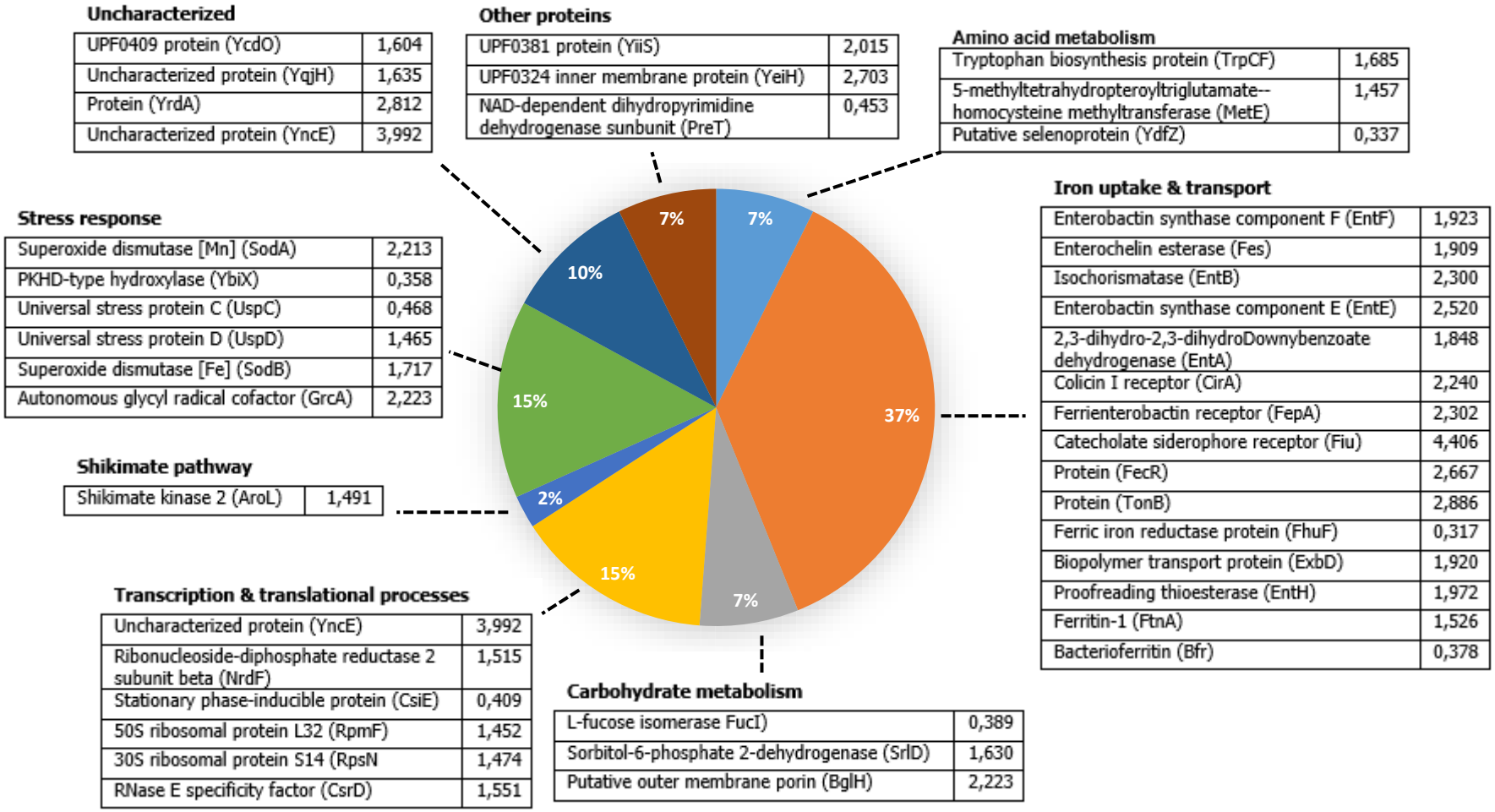


Figure 5: Functional categories of differentially expresses proteins of *E. coli* (RM109) to AMPA. Statistical significance level was set at $p<0.01$ with a minimal fold change of $\pm$ 1.5. $\geq$ + 1.5 fold change indicates up-regulation; $\leq$ - 1.5 fold change indicates down-regulation.

4.3.2. EC100 response to glyphosate and AMPA

In response to glyphosate, EC100 regulated a wide array of proteins (66 proteins) belonging to the functional groups of amino acid metabolism, carbohydrate metabolism, iron transport & uptake, lipid/cell wall, nitrate assimilation, stress response, transcription & translation processes, transport, and proteins with yet-to-be characterized functions (Figure 6). Remarkably, only 6 proteins were differentially expressed by the bacterium in response to AMPA, belonging to the functional categories of carbohydrate metabolism, transcription & translational process, iron transport & uptake and stress response (Figure 7). Majority of the glyphosate-induced (88%) differentially expressed proteins were up-regulated (Figure 6) and the entire 6 differentially expressed proteins in response to AMPA were also up-regulated (Figure 7). Of the proteins that were down-regulated in response to glyphosate, majority belong to the carbohydrate metabolism (7 out of 12 proteins), amino acid metabolism (3 out of 5 proteins) and stress response (3 out of 8 proteins). In addition to the fewer number of proteins that were expressed in response to AMPA compared to glyphosate, functional groups of amino acid metabolism, lipid/Cell wall, nitrate assimilation which were regulated in response to glyphosate were completely absent in EC100 response to AMPA (Figures 6 & 7).

4.3.3. DH5-alpha response to glyphosate and AMPA

Similar functional groups were involved in DH5-alpha response to glyphosate and AMPA, namely iron transport and uptake, transcription & translation, and stress response (Figures 8 & 9), although, specific regulated proteins were different within each functional group. For example, in the transcription & translation process, the 50S ribosomal protein L35 (RpmI) and HTH-type transcriptional repressor (FabR) were differentially expressed in response to glyphosate (Figure 8), but in response to AMPA the differentially expressed protein of transcription & translation process was UPF0135 protein (YbgI) (Figure 9). Similarly, the stress response proteins that were differentially expressed in response to glyphosate were autonomous glycyl radical factor (GrcA), Probable pyridine nucleotide-disulphide oxidoreductase (RdA) and Lead, cadmium and mercury-transporting ATPase (ZntA), but in response to AMPA, protein (HdeB) and universal stress protein D (UspD) were the stress response proteins that were differentially expressed (Figures 8 & 9). Remarkably, all the 5 differentially expressed proteins, which were up-regulated, in response to glyphosate were also up-regulated in response to AMPA, however, the bacterium up-regulated additional 9 proteins of the functional categories of iron transport & uptake in response to AMPA, which

indicates a strong requirement (greater in response to AMPA than to glyphosate) for uptake of Iron by DH5-alpha in response to the effect of the two chemicals.

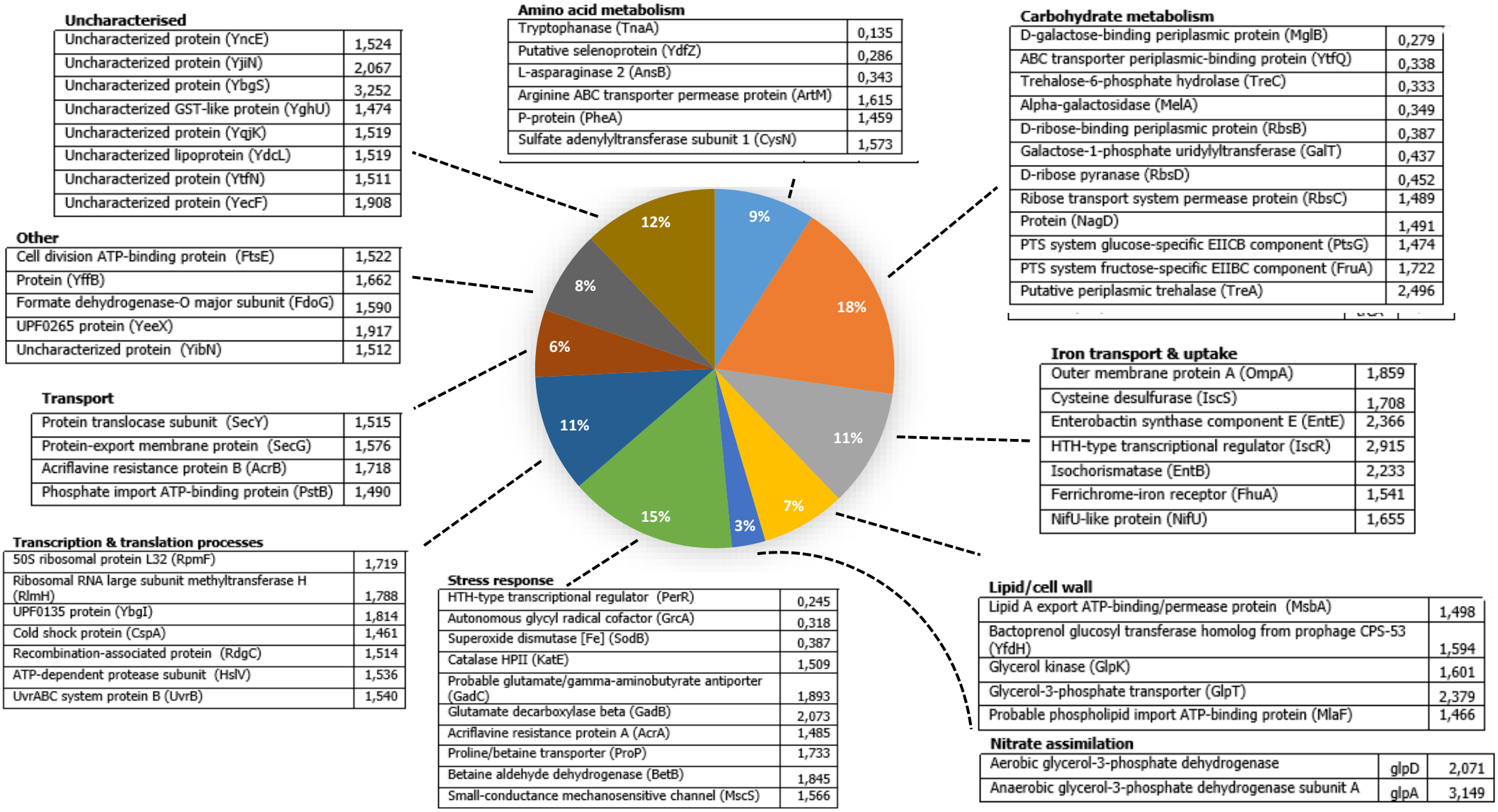


Figure 6: Functional categories of differentially expresses proteins of *E. coli* (EC100) to glyphosate. Statistical significance level was set at $p<0.01$ with a minimal fold change of $\pm$ 1.5. $\geq$ + 1.5 fold change indicates up-regulation; $\leq$ - 1.5 fold change indicates down-regulation.

Figure 7 represent functional categories of differentially expresses proteins of *E. coli* (EC100) in response AMPA

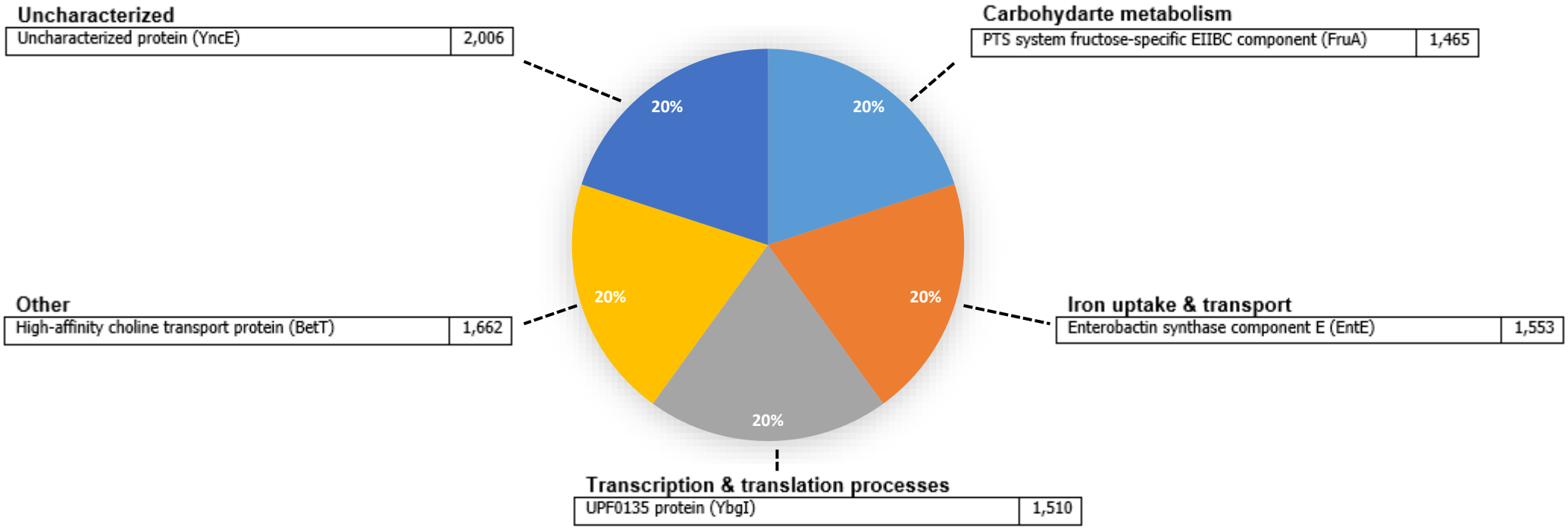


Figure 7: Functional categories of differentially expresses proteins of *E. coli* (EC100) to AMPA. Statistical significance level was set at $p<0.01$ with a minimal fold change of $\pm$ 1.5. $\geq$ + 1.5 fold change indicates up-regulation; $\leq$ - 1.5 fold change indicates down-regulation.

Figure 8 represent functional categories of differentially expresses proteins of *E. coli* (DH5-alpha) in response AMPA

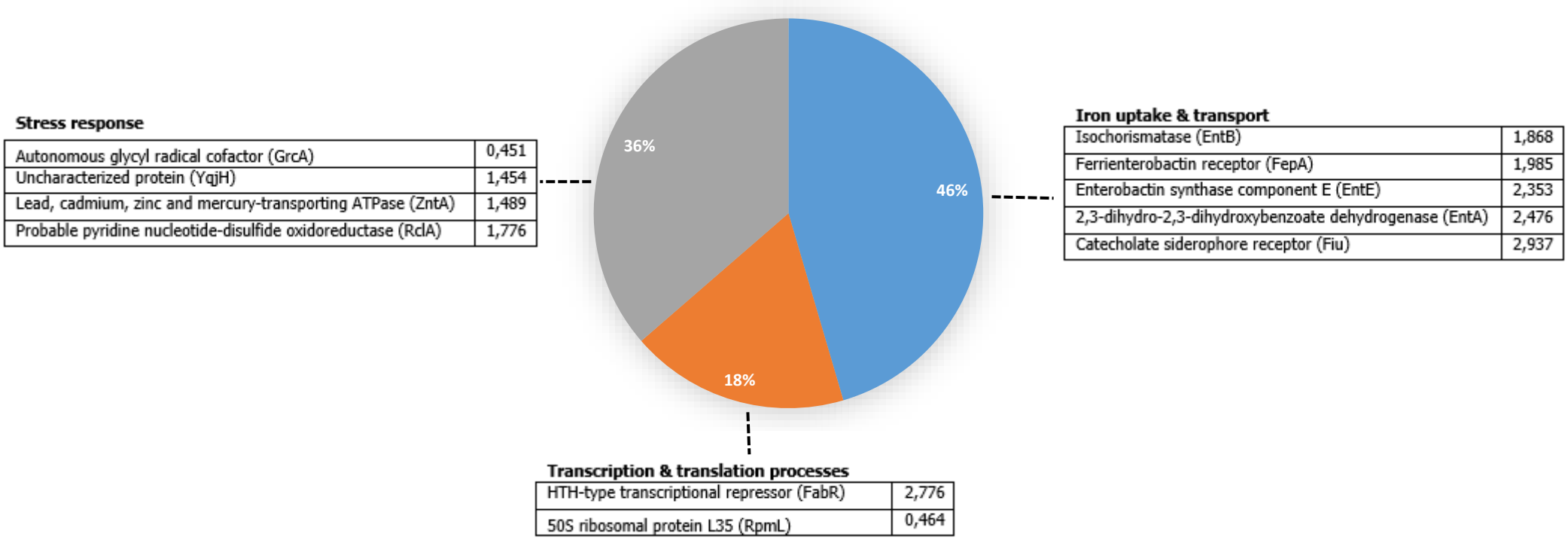


Figure 8: Functional categories of differentially expresses proteins of *E. coli* (DH5-alpha) to glyphosate. Statistical significance level was set at $p<0.01$ with a minimal fold change of $\pm$ 1.5. $\geq$ + 1.5 fold change indicates up-regulation; $\leq$ - 1.5 fold change indicates down-regulation.

Figure 9 represent functional categories of differentially expresses proteins of *E. coli* (DH5-alpha) in response AMPA

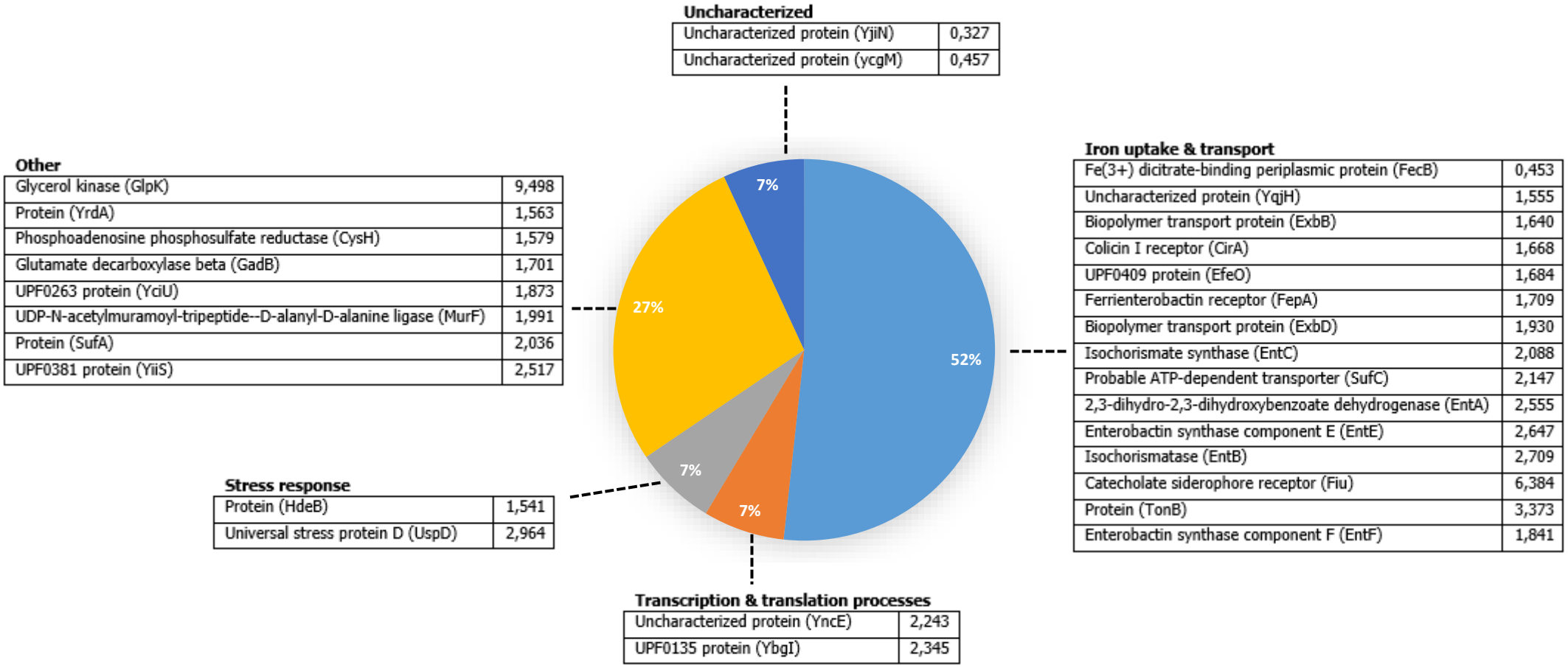


Figure 9: Functional categories of differentially expresses proteins of *E. coli* (DH5-alpha) to AMPA. Statistical significance level was set at $p<0.01$ with a minimal fold change of $\pm$ 1.5. $\geq$ + 1.5 fold change indicates up-regulation; $\leq$ - 1.5 fold change indicates down-regulation.

4.3.4 Comparison between RM109, EC100 and DH5-alpha response to glyphosate and AMPA

Large differences were identified in the proteins deployed by the 3 *E. coli* strains in response to glyphosate and AMPA; however, a number of similar proteins were also employed (Table 2). The 3 *E. coli* strains up-regulated EntB and EntE that were employed in iron transport & uptake, while RM109 and DH5-alpha additionally up-regulated FepA and Flu. Compared to RM109 and EC100, DH5-alpha exhibited the highest level of protein up-regulation (DH5-alpha: 2.742-4.715 fold; RM109: 1.650-2.595 fold; EC100: 1.798-2.366 fold) (Table 2). In Stress response, the 3 bacterial strains commonly down-regulated YdfZ, while RM109 and DH5-alpha additionally down-regulated GrcA. Among the 3 bacteria strains, RM109 and DH5-alpha regulated more number of proteins in a similar manner (Table 2). Greater similarity was also observed between RM109 and DH5-alpha in response to AMPA. RM109 and DH5-alpha up-regulated EntF, EntB, EntE, EntA, CirA, FepA, Flu, TonB and ExbD (Table 2). However, the 3 strains up-regulated EntB and EntE, similar to their regulation of the 2 proteins in response to glyphosate. Furthermore, RM109 and DH5-alpha similarly up-regulated proteins involved in stress response and other proteins whose functions are yet to be characterized (Table 2).

Table 2 shows differentially expressed proteins that are similar between RM109, EC100 and DH5-Alpha after exposure to glyphosate and AMPA.

Table 2: Similar differentially expressed proteins that are present between *E. coli* strains after glyphosate and AMPA exposure

Chemical	Description	Code	Categories	RM109	EC100	DH5alpha
Glyphosate	Ferrienterobactin receptor	FepA	Iron transport & uptake	1.650	-	2.742
	Isochorismatase	EntB		1.655	1.798	2.177
	Enterobactin synthase component E	EntE		1.679	2.366	2.267
	Catecholate siderophore receptor	Fiu		2.595	-	4.715
	putative selenoprotein	YdfZ	Stress reponse	0.309	0.286	0.421
	Autonomous glycyl radical cofactor	GrcA		0.417	-	0.315
	50S ribosomal protein L32	RpmF	Transcription & translation processes	1.448	1.635	-
	Uncharacterized protein	YncE	Uncharacterized	1.651	1.524	1.671

Chemical	Description	Code	Categories	RM109	EC100	DH5alpha
AMPA	Enterobactin synthase component F	EntF	Iron transport & uptake	1.923	-	1.841
	Isochorismatase	EntB		2.300	1.446	2.709
	Enterobactin synthase component E	EntE		2.520	1.746	2.647
	2,3-dihydro-2,3-dihydroDownybenzoate dehydrogenase	EntA		1.848	-	2.555
	Colicin I receptor	CirA		2.240	-	1.668
	Ferrienterobactin receptor	FepA		2.302	-	1.709
	Catecholate siderophore receptor	Fiu		4.406	-	6.384
	Protein tonB	TonB		2.886	-	3.373
	Biopolymer transport protein exbD	ExbD		1.972	-	1.930
	UPF0135 protein	YbgI	Transcription & translation processes	-	1.744	2.345
	Universal stress protein D	UspD	Stress	2.223	-	2.964
	UPF0381 protein	YiiS	Others	2.015	-	2.517
	Uncharacterized protein	YqjH	Uncharacterized	1.635	-	1.555
	Protein	YrdA		2.812	-	1.563
	Uncharacterized protein	YncE		3.992	1.697	2.243

Statistical significance level was set at $p<0.01$ with a minimal fold change of $\pm$ 1.5. $\geq$ + 1.5 fold change indicates up-regulation; $\leq$ - 1.5 fold change indicates down-regulation., (-) = below regulation cut of point.

4.3.5. Response of *E. faecalis* to glyphosate and AMPA

In response to glyphosate, *E. faecalis* differentially expressed 118 proteins (Figure 10) compared to 249 proteins differentially expressed in response to AMPA (Figure 11). Proteins involved in transcription and translational processes constitute the highest (22%; 26 proteins) group of proteins differentially expressed by *E. faecalis* in response to glyphosate apart from the category of hypothetical proteins (27%; 32 proteins), which is a group of all proteins whose functions have not yet been determined (Figure 9). Similarly, proteins involved in transcription and translation processes constituted the highest (29%; 74 proteins) group in the bacteria response to AMPA (Figure 11). It is also remarkable that proteins of carbohydrate metabolism were differentially expressed in high number in response to both glyphosate and AMPA (glyphosate: 18%, 21 proteins; AMPA: 12%, 30 proteins). Five

proteins of the ABC transport functional group were differentially expressed in response to AMPA, but no ABC transport protein was among the identified proteins of *E. faecalis* in response to glyphosate (Figures 10 & 11). This is remarkable given that glyphosate is more toxic than AMPA and the ABC transport proteins function in the extrusion of toxins from the cell, thus, it is expected that the bacterium should also express this group of proteins in response to glyphosate. The number of amino acid metabolism employed by *E. faecalis* in response to both glyphosate (4 proteins) and AMPA (11 proteins) is remarkably few, given that glyphosate is known to impact synthesis of aromatic amino acids. A number of other observed similarities in the response of *E. faecalis* to glyphosate and AMPA are summarized in Table 3.

The figure below represents functional categories of differentially expressed proteins of *E. faecalis*_in response to glyphosate. Refer to Table 1E (Appendix E) for differentially expressed proteins.

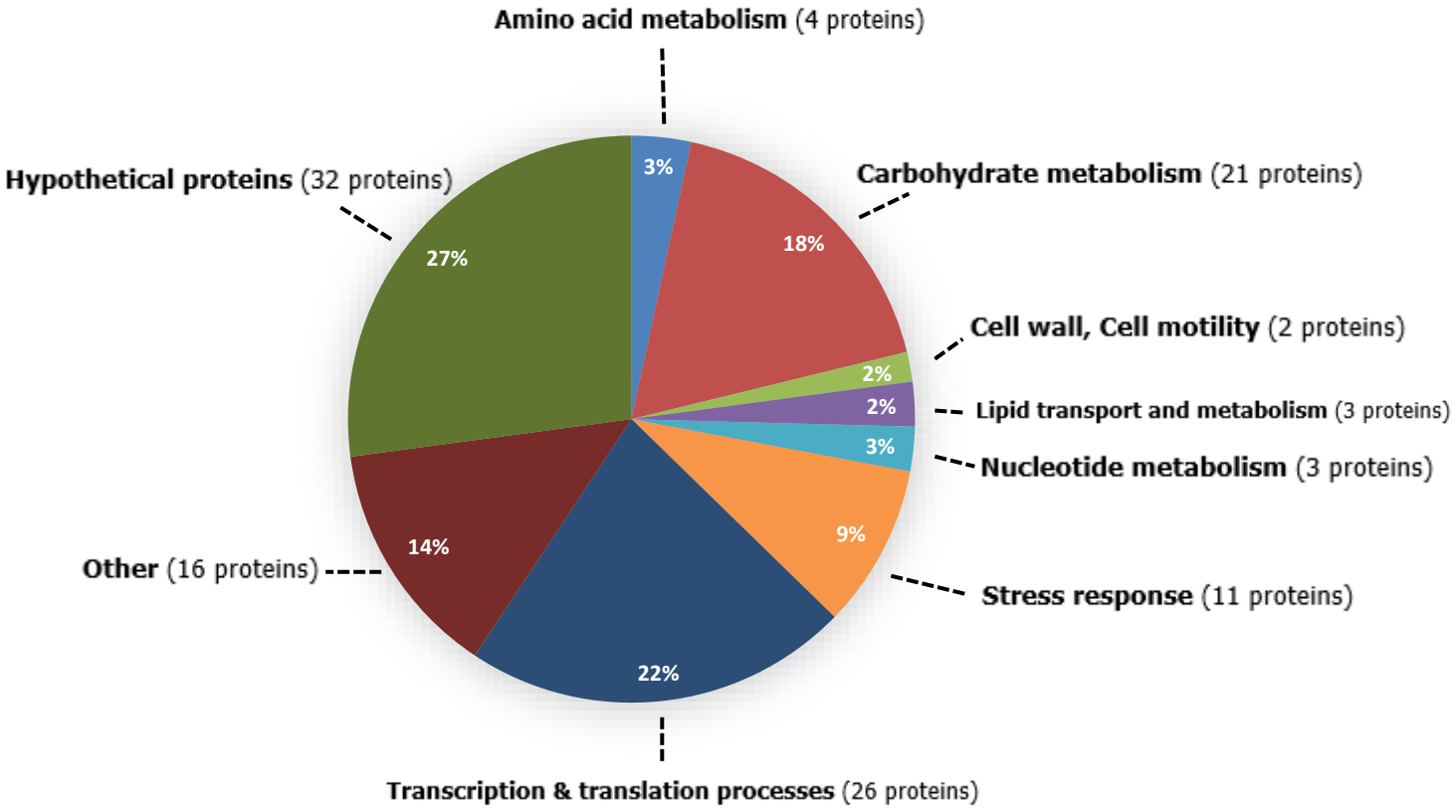


Figure 10: Functional categories of differentially expresses proteins of *E. faecalis* after exposure to glyphosate.

The figure below represent functional categories of differentially expresses proteins of *E. faecalis* in response AMPA. Refer to Table 1F (Appendix F) for differentially expressed proteins.

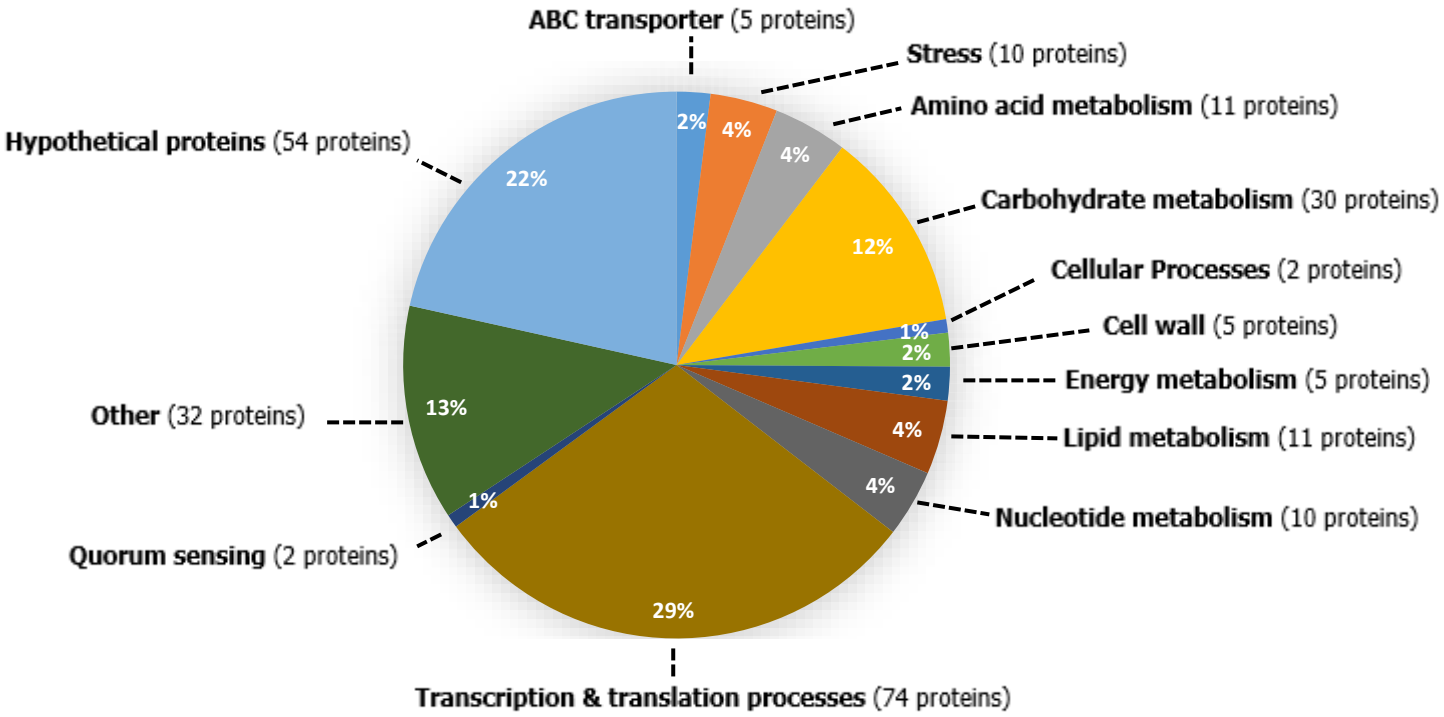


Figure 11: Functional categories of differentially expresses proteins of *E. faecalis* after exposure to AMPA.

The table below represents differentially expressed proteins of *E. faecalis* that are similar after exposure to glyphosate and AMPA over time.

Table 3: Differentially expressed proteins of *E. faecalis* that are similar after glyphosate and AMPA exposure

Description	Code	Categories	Glyphosate Regulation	AMPA Regulation
dipeptidase PepV	EF_0671	Amino acid metabolism	1.807	2.939
thioredoxin reductase	TrxB		1.703	2.046
GcvH family protein	256853854		2.692	1.758
glycosyl hydrolase, family 65	256854201	Carbohydrate metabolism	1.707	1.704
phosphoglycerate kinase	Pgk		1.547	1.700

UTP--glucose-1-phosphate uridylyltransferase	GalU		0.291	7.243
PTS system protein	256617129		2.875	0.142
N-acetylmuramoyl-L-alanine amidase	EF_1583	Cell wall	0.441	0.118
bacteriophage transcriptional regulator	229545375		1.878	0.205
protein phosphatase 2C	EF_3121	Cellular Processes	1.779	1.549
S-ribosylhomocysteinase	LuxS		1.713	1.746
diacylglycerol kinase catalytic subunit	EF_0090	Lipid transport and metabolism	1.808	0.342
uridine kinase	Udk	Nucleotide metabolism	2.388	2.588
adenine phosphoribosyltransferase	Apt		1.465	1.978
cold shock protein CspC	CspC		0.252	0.213
cold shock domain-contain protein	EF_0781		0.344	0.158
cold-shock domain-contain protein	257867741		2.601	0.071
aspartyl/glutamyl-tRNA amidotransferase subunit B	GatB	Transcription & translation processes	1.535	1.613
50S ribosomal protein L14	RplN		0.440	2.630
ribonucleotide reductase stimulatory protein	NrdI		2.405	0.293
MerR-family transcriptional regulator	182419650		2.025	0.079
thioredoxin	229547533		1.542	0.290
UvrC protein	229546360	Stress response	1.665	0.119
Peptide methionine sulfoxide reductase	MsrA		2.484	1.836
DegV family protein	229545703	Other	2.140	1.758
hypothetical protein EF1680	EF_1680		2.461	0.183
hypothetical protein EF3174	EF_3174		2.669	1.479
hypothetical protein EF1014	EF_1014		0.295	0.105
conserved hypothetical protein	229549574		1.474	1.895
hypothetical protein EF1967	EF_1967		1.584	1.504
hypothetical protein EF1315	EF_1315	Hypothetical proteins	1.603	0.087
hypothetical protein EF1324	EF_1324		1.634	1.526
conserved hypothetical protein	256853931		1.704	0.087
conserved hypothetical protein	256853930		1.871	0.222
hypothetical protein EF1915	EF_1915		2.151	0.107
hypothetical protein CBB_1070	168184091		2.168	0.027
hypothetical protein EF2621	EF_2621		2.308	0.277

Statistical significance level was set at $p<0.01$ with a minimal fold change of $\pm$ 1.5. $\geq$ + 1.5 fold change indicates up-regulation; $\leq$ - 1.5 fold change indicates down-regulation., (-) = below regulation cut of point.

4.4. Pathways employed by *E. coli* RM109 and *E. faecalis* in response to glyphosate and AMPA

4.4.1. *E. coli* RM109

To locate the specific metabolic pathways in which the differentially expressed proteins are involved, the KEGG and STRING pathway analysis web resources were used to analyze the differentially expressed proteins of *E. coli* RM109 and *E. faecalis* to glyphosate and AMPA. For RM109 response to both glyphosate and AMPA, the most prominent metabolic pathway for proteins of iron uptake and transport functional group was biosynthesis of siderophore group nonribosomal peptides (Table 4 & 5). Additionally, the beta-lactam resistance, ABC transporter and two-component system pathways featured in the response to glyphosate (Table 4 & 5). For the nitrate metabolism functional group, the notable featured pathways were nitrogen and purine metabolism in response to glyphosate but not AMPA (Tables 4 & 5), No protein of nitrate metabolism functional group was differentially expressed by RM109 in response to AMPA (Figure 6).

In the nucleotide metabolism protein group, pyrimidine metabolism was the only featured pathway in response to glyphosate (Table 4), but not in response to AMPA, because similar to nitrate metabolism, no protein of nucleotide metabolic functional group was differentially expressed by RM109 (Table 4, Figure 7). In the transcription and translation processes, ribosome and phosphotransferase pathways were used in response to glyphosate (Table 4) while the bacterium additionally employed pyrimidine and purine metabolism pathways in response to AMPA (Table 5). For stress response, the autonomous glycyl radical factor (GrcA) that was down-regulated in response to both glyphosate and AMPA is involved in butanoate and pyruvate metabolic pathways (Tables 4 & 5), but other stress response proteins which were additionally expressed in response to AMPA are involved in other pathways namely tryptophan, RNA degradation and biosynthesis of siderophore group non-ribosomal peptides were also involved in response to AMPA (Table 5).

Table 4: Pathway analysis of differentially expressed proteins of RM109 in response to glyphosate after 6 hours exposure

Functional/Metabolic Pathway	Functional Categories	Description	Code	Regulation
Two-component system	Iron uptake and transport	Ferrienterobactin receptor	FepA	1.655
Biosynthesis of siderophore group nonribosomal peptites		Isochorismatase	EntB	1.679
Biosynthesis of siderophore group nonribosomal peptites		Enterobactin synthase component E	EntE	1.682
String: Biosynthesis of siderophore group nonribosomal peptites.		Catecholate siderophore receptor	Fiu	1.650
String: ABC transporter, iron transport,		Fe(3+) dicitrate-binding periplasmic protein	FecB	0.488
String: beta-Lactam resistance		Outer membrane protein W	OmpW	0.476
Nitrogen metabolism	Energy metabolism	Respiratory nitrate reductase 1 beta chain	NarH	0.405
Nitrogen metabolism		Respiratory nitrate reductase 1 alpha chain	NarG	0.460
Nitrogen metabolism		Respiratory nitrate reductase 1 gamma chain	NarI	0.471
Nitrogen metabolism		Nitrite reductase [NAD(P)H] large subunit	NirB	0.481
Nitrogen metabolism		Nitrite extrusion protein 1	NarK	0.500
Two-component system		Nitrogen regulation protein NR(II)	GlnL	0.448
Purine Metabolism	Nucleotide metabolism	Anaerobic ribonucleoside-triphosphate reductase	NrdD	0.446
Pyrimidine metabolism		Aspartate carbamoyltransferase regulatory chain	PyrI	1.611
Pyrimidine metabolism		Carbamoyl-phosphate synthase large chain	CarB	1.561
Pyrimidine metabolism		Aspartate carbamoyltransferase	PyrB	1.697
Pyrimidine metabolism		NAD-dependent dihydropyrimidine dehydrogenase	PreA	0.436
String: Pyruvate metabolism, biological process	Stress response	Autonomous glycyl radical cofactor	GrcA	0.417

String: Ribosome	Transcription & translation processes	50S ribosomal protein L32	RpmF	1.448
String: Ribosome		50S ribosomal protein L33	RpmG	1.770
String: Phosphotransferase system (PTS)		HTH-type transcriptional regulator	AscG	2.554
String: Phosphotransferase system (PTS)	Others	Putative aga operon transcriptional repressor	AgaR	1.507

Statistical significance level was set at $p<0.01$ with a minimal fold change of $\pm$ 1.5. $\geq$ + 1.5 fold change indicates up-regulation; $\leq$ - 1.5 fold change indicates down-regulation., (-) = below regulation cut of point., (-) = below regulation cut of point. Proteins without pathways in Kegg and String were not included in the table.

Table 5: Pathway analysis of differentially expressed proteins of RM109 in response to AMPA after 6 hours exposure

Function/Metabolic Pathway	Functional Categories	Description	Code	Regulation
String: Tryptophan metabolism	Stress response	SuperoDownide dismutase [Mn]	SodA	2.213
String: Tryptophan metabolism, RNA degradation		Superoxide dismutase [Fe]	SodB	0.358
String: Butanoate metabolism, Pyruvate metabolism.		Autonomous glycyl radical cofactor	GrcA	0.468
String: Biosynthesis of siderophore group nonribosomal peptides.		PKHD-type hydroxylase ybiX	YbiX	1.465
Fuctose and mannose metabolism	Carbohydrate metabolism	L-fucose isomerase	FucI	1.630
Fuctose and mannose metabolism		Sorbitol-6-phosphate 2-dehydrogenase	SrlD	2.223
Biosynthesis of siderophore group nonribosomal peptides		Enterobactin synthase component F	EntF	1.923
Biosynthesis of siderophore group nonribosomal peptides.		Enterochelin esterase	Fes	1.909
Biosynthesis of siderophore group nonribosomal peptides		Isochorismatase	EntB	2.300
Biosynthesis of siderophore group nonribosomal peptides		Enterobactin synthase component E	EntE	2.520

Function/Metabolic Pathway	Functional Categories	Description	Code	Regulation
Biosynthesis of siderophore group nonribosomal peptides	Iron transport & uptake	2,3-dihydro-2,3-dihydroDownybenzoate dehydrogenase	EntA	1.848
String: Biosynthesis of siderophore group nonribosomal peptides		Colicin I receptor	CirA	2.240
Two-component system		Ferrienterobactin receptor	FepA	2.302
String: Biosynthesis of siderophore group nonribosomal peptides. neighbourhood		Catecholate siderophore receptor	Fiu	4.406
String: Biosynthesis of siderophore group nonribosomal peptides		Proofreading thioesterase EntH	EntH	1.526
String: Biosynthesis of amino acids	Shikimate pathway	Shikimate kinase 2	AroL	1.491
String: Biosynthesis of siderophore group nonribosomal peptides.	Transcription & translation processes	Uncharacterized protein	YncE	3.992
String: Pyrimidine metabolism, Purine metabolism		Ribonucleoside-diphosphate reductase 2 subunit beta	NrdF	1.515
Pyrimidine metabolism		NAD-dependent dihydropyrimidine dehydrogenase sunbunit	PreT	0.453
Biofilm formation - Vibrio cholerae		RNase E specificity factor	CsrD	0.409
String: Ribosome.		50S ribosomal protein L32	RpmF	1.474
String: Ribosome.		30S ribosomal protein S14	RpsN	1.551
Porphyrin and chlorophyll metabolism	Others	Bacterioferritin	Bfr	0.378
Biosynthesis of amino acids		5-methyltetrahydropteroyltriglutamate--homocysteine methyltransferase	MetE	1.457
Biosynthesis of amino acids		Tryptophan biosynthesis protein TrpCF	TrpCF	1.685

Statistical significance level was set at $p<0.01$ with a minimal fold change of $\pm$ 1.5. $\geq$ + 1.5 fold change indicates up-regulation; $\leq$ - 1.5 fold change indicates down-regulation., (-) = below regulation cut of point., (-) = below regulation cut of point. Proteins without pathways in Kegg and String were not included in the table.

4.4.2. *E. faecalis*

In general, majority of the identified pathways of *E. faecalis* were up-regulated in response to glyphosate and AMPA, this is in consonance with the up-regulation of majority of its in response to glyphosate and AMPA (Tables 6 & 7). For example, *E. faecalis* in response to glyphosate up-regulated 12 of the 15 identified proteins involved in several pathways of carbohydrate metabolism. The down-regulation of GalU, Ef-0272 and GalE will result in down-regulation of carbohydrate metabolism at the point where GalU, Ef-0272 and GalE-1 are involved in pentose and glucuronate interconversions, galactose metabolism and glycolysis/gluconeogenesis, however, this will be compensated given that several other proteins involved in the same pathways were up-regulated. Similarly, *E. faecalis* up-regulated 16 out of 21 proteins of different pathways of carbohydrate metabolism. The pathways that were down-regulated are those in which EF-3305, EF-2213, PtsH, EF-0022 and EF-0461 are involved, namely, fructose and mannose metabolism, starch and sucrose metabolism, amino sugar and nucleotide sugar metabolism, and phosphotransferase system, however, several other proteins involved in this specific pathways were also up-regulated, thus, compensating for the down-regulation of carbohydrate metabolism by down-regulating EF-3305, EF-2213, PtsH and EF-0461.

Remarkably, all the identified pathways of nucleotide and energy metabolism were up-regulated by *E. faecalis* in response to AMPA (Table 7). In response to glyphosate, the 3 identified proteins involved in purine and pyrimidine metabolic pathways of nucleotide metabolism, namely Udk, Cdd and Apt, were also up-regulated (Table 6). A similar pattern was observed in pathways involved in transcription and translation processes where *E. faecalis* up-regulated 45 out of 50 proteins involved in the pathways in response to AMPA. The specific down-regulated aspect of the transcription and translation processes are points where the down-regulated EF-1367, EF-2925, EF-0781, CscC, EF-1277, SigA and RpoE are involved in RNA degradation, RNA polymerase, purine and pyrimidine metabolism (Table 7). For the response to glyphosate, transcription and translation processes would be down-regulated also at the points where CspC and EF-0781 are involved in RNA polymerase and RNA degradation (Table 6).

Table 6: Pathway analysis of differentially expressed proteins of *E. faecalis* in response to glyphosate after 3 hours exposure

Functional/Metabolic Pathway	Functional Categories	Description	Code	Regulation
String: Pyruvate metabolism	Stress response	glyoxalase	EF_0745	1,858
String: Alanine, aspartate and glutamate metabolism	Amino acid metabolism	alanine racemase	Q837J0	1,5
Galactose metabolism	Carbohydrate metabolism	aldose 1-epimerase	GalM	2,256
Galactose metabolism		UDP-glucose 4-epimerase	GalE-1	0,431
Amino sugar and nucleotide sugar metabolism		glucosamine-6-phosphate isomerase	NagB	2,279
Carbon metabolism, Biosynthesis of amino acids		triosephosphate isomerase	TpiA	1,683
Glycolysis / Gluconeogenesis		glucose-6-phosphate isomerase	Pgi	1,546
String: Glycolysis / Gluconeogenesis		Glycosyl hydrolase	EF_0272	0,386
String: Glycolysis / Gluconeogenesis		fructose-bisphosphate aldolase	Q836E7	1,493
Carbon metabolism, Biosynthesis of amino acids		phosphoglycerate kinase	Pgk	1,547
Pentose phosphate pathway		phosphopentomutase	DeoB	1,652
String: Phosphotransferase system (PTS), Fructose and mannose metabolism		PTS system mannitol-specific IIBC component	EF_0411	1,917
String: Fructose and mannose metabolism, Phosphotransferase system (PTS)		PTS system transporter subunit IIA	EF_2603	1,836
String: Starch and sucrose metabolism		Beta-glucosidase/6-phospho-beta-glucosidase/beta-galactosid ase	EF_0271	1,964
String: Starch and sucrose metabolism		glycosyl hydrolase	EF_2597	1,967
Pyruvate metabolism		lactoylglutathione lyase	GloA	1,826
Pentose and glucuronate interconversions, Galactose metabolism		UTP--glucose-1-phosphate uridylyltransferase	GalU	0,291

Functional/Metabolic Pathway	Functional Categories	Description	Code	Regulation
String: Pentose phosphate pathway, Purine metabolism	Cell wall	MutT/nudix family protein	EF_2696	1,695
String: ABC transporters	Environmental Information Processing	sugar ABC transporter ATP-binding protein	EF_0938	2,439
ABC transporters		Peptide methionine sulfoxide reductase	MsrA	2,484
String: ABC transporters, Two-component system		phosphate ABC transporter ATP-binding protein	PstB1	2,83
Selenocompound metabolism		thioredoxin reductase	TrxB	1,703
String: Citrate cycle (TCA cycle), Biosynthesis of secondary metabolites		protein phosphatase 2C	EF_3121	1,779
Quorum sensing , Biosynthesis of amino acids	(Cellular Processes)	S-ribosylhomocysteinase	LuxS	1,713
Pyrimidine metabolism	Nucleotide metabolism	uridine kinase	Udk	2,388
Pyrimidine metabolism		cytidine deaminase	Cdd	1,456
Purine metabolism		adenine phosphoribosyltransferase	Apt	1,465
String: Two-component system	Transcription & translation processes	DNA-binding response regulator	293400081	0,356
Two-component system		Transcriptional regulator LytR	LytR	3,154
String: Phosphotransferase system (PTS), Starch and sucrose metabolism		BglG family transcriptional antiterminator	EF_2966	3,952
String: Pyrimidine metabolism		flavoprotein NrdI	EF_1491	1,5
String: RNA polymerase, RNA degradation		cold shock protein CspC	CspC	0,252
String: RNA polymerase, RNA degradation		cold shock domain-contain protein	EF_0781	0,344
String: RNA polymerase, Ribosome		transcription antitermination protein NusG	NusG	1,449
String: RNA polymerase, Pyrimidine metabolism		transcription elongation factor GreA	GreA	1,923
String: RNA polymerase, RNA degradation		Cold-shock domain family protein	EF_1726	1,98
Ribosome		50S ribosomal protein L14	RplN	0,44
Ribosome		Ribosome maturation factor RimM	RimM	0,404

Functional/Metabolic Pathway	Functional Categories	Description	Code	Regulation
String: Ribosome	Transcription & translation processes	translation initiation factor IF-1	InfA	1,782
Aminoacyl-tRNA biosynthesis		aspartyl/glutamyl-tRNA amidotransferase subunit B	GatB	1,535
Aminoacyl-tRNA biosynthesis		Alanine--tRNA ligase	AlaS	1,58
String: Aminoacyl-tRNA biosynthesis		prolyl-tRNA synthetase	229545199	1,849
String: Homologous recombination, Pyrimidine metabolism		DNA gyrase subunit B	GyrB	1,973
String: Pyrimidine metabolism, Purine metabolism		ribonucleotide reductase stimulatory protein	NrdI	2,405
String: Glycine, serine and threonine metabolism	undetermined	lactoylglutathione lyase	227518394	1,693
Microbial metabolism in diverse environments, Quorum sensing		phnA protein	PhnA	2,074
EF_1276 Hypothetical protein		Phage protein	Q835U6	1,701
String: Thiamine metabolism, Sulfur relay system		rhodanese family protein	EF_2988	2,691
Biosynthesis of antibiotic		pyrrolidone-carboxylate peptidase	Pcp	1,647

Statistical significance level was set at $p<0.01$ with a minimal fold change of $\pm$ 1.5. $\geq$ + 1.5 fold change indicates up-regulation; $\leq$ - 1.5 fold change indicates down-regulation., (-) = below regulation cut of point., (-) = below regulation cut of point. Proteins without pathways in Kegg and String were not included in the table.

Table 7: Pathway analysis of differentially expressed proteins of *E. faecalis* in response to AMPA after 3 hours exposure

Function/Metabolic Pathway	Functional Categories	Description	Code	Regulation
Cationic antimicrobial peptide (CAMP) resistance, Two-component system, D-Alanine metabolism.	ABC transporter	D-alanine--poly(phosphoribitol) ligase subunit 2	DltC	0,387
One carbon pool by folate, Aminoacyl-tRNA biosynthesis		Methionyl-tRNA formyltransferase	Fmt	0,408
String: beta-Lactam resistance, ABC transporters		peptide ABC transporter peptide-binding protein	EF_3106	0,413
ABC transporters		Peptide methionine sulfoxide reductase MsrA	MsrA	1,836
String: Citrate cycle (TCA cycle), Biosynthesis of secondary metabolites, Valine, leucine and isoleucine degradation	Amino acid metabolism	protein phosphatase 2C	EF_3121	1,549
Biosynthesis of amino acids, Cysteine and methionine metabolism		S-adenosylmethionine synthetase	MetK	1,565
2-Oxocarboxylic acid metabolism, Biosynthesis of amino acids		Branched-chain-amino-acid aminotransferase	IlvE	2,385
Two-component system		ornithine carbamoyltransferase	ArcB	2,928
Arginine biosynthesis		arginine deiminase	ArcA	3,358
String: Fructose and mannose metabolism, Phosphotransferase system (PTS)	Carbohydrate metabolism	PTS system sorbitol-specfic transporter subunit IIA	EF_3305	0,057
String: Phosphotransferase system (PTS), Starch and sucrose metabolism		PTS system, IIBC components	EF_2213	0,189
Phosphotransferase system (PTS)		phosphocarrier protein HPr	PtsH	0,267
String: Starch and sucrose metabolism , Phosphotransferase system (PTS)		glucan 1,6-alpha-glucosidase	EF_1348	2,927
String: Glycolysis / Gluconeogenesis, Carbon metabolism		phosphoglycerate mutase	GpmA	1,467
Pentose phosphate pathway		deoxyribose-phosphate aldolase	DeoC	1,649
Pentose phosphate pathway		6-phosphogluconate dehydrogenase	Gnd	2,070
Carbon metabolism .Biosynthesis of amino acids		phosphoglycerate kinase	Pgk	1,700
Carbon metabolism, Biosynthesis of amino acids		glyceraldehyde-3-phosphate dehydrogenase	Gap-2	1,854
Streptomycin biosynthesis, Polyketide sugar unit biosynthesis		Glucose-1-phosphate thymidylyltransferase	RfbA	1,880
Streptomycin biosynthesis, Polyketide sugar unit biosynthesis		dTDP-glucose 4,6-dehydratase	RfbB	2,590

Function/Metabolic Pathway	Functional Categories	Description	Code	Regulation
String: Fructose and mannose metabolism , Amino sugar and nucleotide sugar metabolism		PTS system mannose-specific IID component	EF_0022	0,315
Amino sugar and nucleotide sugar metabolism, Fructose and mannose metabolism		mannose-6-phosphate isomerase	ManA	1,481
String: Fructose and mannose metabolism , Amino sugar and nucleotide sugar metabolism		PTS system IIA component	EF_0461	0,308
Starch and sucrose metabolism		beta-phosphoglucomutase	PgmB	2,900
String: Starch and sucrose metabolism		maltose phosphorylase	EF_0957	1,708
Pyruvate metabolism , Citrate cycle (TCA cycle)		pyruvate dehydrogenase complex E1 component subunit alpha	PdhA	1,614
String: Pyruvate metabolism , Microbial metabolism in diverse environments		phosphotransacetylase	Pta	1,487
RNA degradation		6-phosphofructokinase	PfkA	2,442
String: Galactose metabolism		aldolase 1 epimerase Lacx	EF_1644	2,365
Pentose and glucuronate interconversions , Galactose metabolism		UTP-glucose-1-phosphate uridylyltransferase	galU	7,243
Oxidative phosphorylation	Energy metabolism	V-type ATP synthase subunit B	AtpB	1,742
String: Oxidative phosphorylation		V-type ATPase subunit F	EF_1492	1,800
Oxidative phosphorylation		V-type ATPase	AtpA	1,866
String: Oxidative phosphorylation		V-type ATP synthase subunit K	EF_1494	2,153
String: Fatty acid biosynthesis, Fatty acid metabolism	Lipid metabolism	acyl carrier protein	AcpP	0,026
String: Nucleotide excision repair, Base excision repair		autolysin	EF_0799	0,286
Vancomycin resistance, Peptidoglycan biosynthesis		D-alanine--D-alanine ligase	ddl	1,587
Pyrimidine metabolism	Nucleotide metabolism	CTP synthase	PyrG	1,754
Pyrimidine metabolism		cytidylate kinase	Cmk	1,827
Purine metabolism		adenine phosphoribosyltransferase	Apt	1,978
Purine metabolism		GMP synthase	GuaA	1,992
Purine metabolism, Pyrimidine metabolism		purine nucleoside phosphorylase	DeoD-2	1,505
String: Pyrimidine metabolism, Purine metabolism		2,3-cyclic-nucleotide 2'phosphodiesterase	EF_2902	1,682
Cysteine and methionine metabolism		5'-methylthioadenosine/S-adenosylhomocysteine nucleosidase 1	Pfs	1,723

Function/Metabolic Pathway	Functional Categories	Description	Code	Regulation
Pyrimidine metabolism		Uridylate kinase	PyrH	2,497
Pyrimidine metabolism		uridine kinase	Udk	2,588
String: Pyrimidine metabolism		uracil phosphoribosyltransferase	Upp	4,709
Selenocompound metabolism	Stress response	thioredoxin	Trx	0,112
Selenocompound metabolism		thioredoxin reductase	TrxB	2,046
String: RNA degradation, RNA polymerase	Transcription & translation processes	cold-shock domain-contain protein	EF_1367	0,123
String: RNA degradation, RNA polymerase		cold-shock domain-contain protein	EF_2925	0,136
String: RNA degradation, RNA polymerase		cold shock domain-contain protein	EF_0781	0,158
String: RNA degradation, RNA polymerase		cold shock protein CspC	CspC	0,213
RNA degradation		DEAD/DEAH box helicase	CshA	1,558
String: RNA polymerase, Pyrimidine metabolism		RNA polymerase sigma factor RpoD	SigA	0,285
String: Purine metabolism, Pyrimidine metabolism		DNA-directed RNA polymerase subunit delta	RpoE	0,411
Ribosome		30S ribosomal protein S18	RpsR	1,579
Ribosome		30S ribosomal protein S11	RpsK	1,604
Ribosome		50S ribosomal protein L19	RplS	1,606
Ribosome		30S ribosomal protein S13	RpsM	1,708
Ribosome		30S ribosomal protein S12	RpsL	1,919
Ribosome	Transcription & translation processes	30S ribosomal protein S2	RpsB	1,983
Ribosome		50S ribosomal protein L21	rplU	1,518
Ribosome		30S ribosomal protein S19	RpsS	1,526
Ribosome		50S ribosomal protein L23	RplW	1,538
Ribosome		50S ribosomal protein L10	RplJ	1,607
Ribosome		50S ribosomal protein L15	RplO	1,615
Ribosome		50S ribosomal protein L3	RplC	1,649
Ribosome		30S ribosomal protein S3	RpsC	1,659
Ribosome		50S ribosomal protein L4	RplD	1,783
Ribosome		50S ribosomal protein L20	RplT	1,800

Function/Metabolic Pathway	Functional Categories	Description	Code	Regulation
Ribosome	Transcription & translation processes	30S ribosomal protein S7	RpsG	1,925
Ribosome		30S ribosomal protein S4	RpsD	1,964
Ribosome		50S ribosomal protein L22	RplV	1,976
Ribosome		50S ribosomal protein L17	RplQ	2,031
Ribosome		50S ribosomal protein L1	RplA	2,056
Ribosome		30S ribosomal protein S10	RpsJ	2,186
Ribosome		50S ribosomal protein L14	RplN	2,630
Ribosome		30S ribosomal protein S16	RpsP	2,499
Ribosome		30S ribosomal protein S8	RpsH	3,695
String: Ribosome		elongation factor G	FusA	2,825
String: Ribosome		translation initiation factor IF-2	InfB	1,559
String: Ribosome		30S ribosomal protein S1	EF_1548	1,784
String: Ribosome		translation initiation factor IF-3	InfC	2,253
String: Ribosome		Peptide chain release factor 1	PrfA	2,493
Aminoacyl-tRNA biosynthesis		aspartyl/glutamyl-tRNA amidotransferase subunit B	GatB	1,613
Aminoacyl-tRNA biosynthesis		glutamyl-tRNA(Gln) amidotransferase subunit A	GatA	1,630
Aminoacyl-tRNA biosynthesis		Tyrosine--tRNA ligase 2	TyrS2	2,099
Aminoacyl-tRNA biosynthesis		arginyl-tRNA synthetase	ArgS	2,186
Aminoacyl-tRNA biosynthesis		isoleucyl-tRNA synthetase	IleS	2,482
Aminoacyl-tRNA biosynthesis		glycyl-tRNA synthetase subunit alpha	GlyQ	2,105
Aminoacyl-tRNA biosynthesis		glutamyl-tRNA synthetase	gltX	1,633
Aminoacyl-tRNA biosynthesis		phenylalanyl-tRNA synthetase subunit beta	PheT	1,483
String: D-Glutamine and D-glutamate metabolism, Peptidoglycan biosynthesis		cell division protein FtsZ	FtsZ	1,877
Two-component system		DNA-binding protein HU	Hup	2,556
Carbon metabolism, Biosynthesis of amino acids		glyceraldehyde 3-phosphate dehydrogenase	Gap-1	2,745
String: Homologous recombination, Base excision repair		S-adenosylmethionine--tRNA ribosyltransferase-isomerase	QueA	1,989
String: Aminoacyl-tRNA biosynthesis, Selenocompound metabolism		seryl-tRNA synthetase	SerS2	2,321
One carbon pool by folate		peptide deformylase	Def	2,701

Function/Metabolic Pathway	Functional Categories	Description	Code	Regulation
Quorum sensing, Protein export	Quorum sensing	Protein translocase subunit SecA	SecA	0,403
Quorum sensing, Biosynthesis of amino acids		S-ribosylhomocysteinase	LuxS	1,746
Microbial metabolism in diverse environments	Other	fumarate reductase flavoprotein subunit	EF_2556	0,110
String: Glycine, serine and threonine metabolism, Glyoxylate and dicarboxylate metabolism		GcvH family protein	EF_2500	0,231
String: Peptidoglycan biosynthesis, beta-Lactam resistance		penicillin-binding protein 1A	EF_1148	0,306
Alanine, aspartate and glutamate metabolism, Amino sugar and nucleotide sugar metabolism		glucosamine--fructose-6-phosphate aminotransferase	GlmS	0,306
String: Pyrimidine metabolism, Purine metabolism		flavoprotein NrdI	EF_1491	0,349
String: Pyrimidine metabolism, Purine metabolism		NrdI protein	NrdI	0,293
String: ABC transporters		endocarditis specific antigen	EF_2076	1,639
Glycerolipid metabolism		Glycerol kinase	GlpK	1,672
Ubiquinone and other terpenoid-quinone biosynthesis		1,4-Dihydroxy-2-naphthoyl-CoA synthase	MenB	1,795
Nicotinate and nicotinamide metabolism		NAD synthetase	NadE	2,112
Fructose and mannose metabolism		fructokinase	CscK	2,123
String: Pyruvate metabolism, Carbon metabolism		malate dehydrogenase, decarboxylating	EF_1206	2,239
String: Pyruvate metabolism, Citrate cycle (TCA cycle)	Other	NADH oxidase	Nox	2,733
String: Alanine, aspartate and glutamate metabolism, Arginine and proline metabolism		carbamate kinase	ArcC1	2,989
String: Pantothenate and CoA biosynthesis, Biosynthesis of secondary metabolites,		2-dehydropantoate 2-reductase	EF_2445	3,615
Fatty acid metabolism, Fatty acid biosynthesis		ACP S-malonyltransferase	FabD	2,318
Carbon metabolism, Biosynthesis of amino acids		serine hydroxymethyltransferase	GlyA	1,479
String: Phenylalanine, tyrosine and tryptophan biosynthesis		Phospho-2-dehydro-3-deoxyheptonate aldolase	EF_1562	1,606
Cationic antimicrobial peptide (CAMP) resistanc, Two-component system		D-alanine--poly(phosphoribitol) ligase subunit 1	DltA	1,639
Quorum sensing, Protein export		signal recognition particle protein	Ffh	1,667
String: Pentose phosphate pathway, Purine metabolism		ribose-phosphate pyrophosphokinase	Prs2	1,747
String: Chloroalkane and chloroalkene degradation, Butanoate metabolism		NADH-dependent butanol dehydrogenase	EF_0194	1,978
String: Chloroalkane and chloroalkene degradation, Naphthalene degradation		oxidoreductase, zinc-binding	EF_1671	2,108

Statistical significance level was set at $p<0.01$ with a minimal fold change of $\pm$ 1.5. $\geq$ + 1.5 fold change indicates up-regulation; $\leq$ - 1.5 fold change indicates down-regulation., (-) = below regulation cut of point., (-) = below regulation cut of point. Proteins without pathways in Kegg and String were not included in the table.

CHAPTER 5

DISCUSSION

5.1. Introduction

Studies have shown that residues of glyphosate and AMPA accumulate in some genetically modified (GM) food crops and extend to water. As a consequence, glyphosate and AMPA are inadvertently being introduced into the food chains of man and animal. Resident gut bacteria will be exposed, to glyphosate and AMPA at varying sub-lethal concentrations. Studies have shown that glyphosate modulates gut microbial communities in chickens and cows, through eliminating members of beneficial bacteria as *Enterococcus faecalis* and other non-pathogenic commensals that are highly susceptible to glyphosate (Shehata *et al*., 2013 & Krüger *et al*., 2013). Common chemicals like glyphosate that are used in agriculture and domestic areas can induce a multiple-antibiotic resistance phenotype in potential pathogens (Kurenbach *et al*., 2015). Additionally, studies have suggested that glyphosate could act as antibiotics in mammalian gut microbiome (Lozano *et al*., 2018). Changes in the gut microbial community may have detrimental health impacts on the host organism, more so hosts with poor diet and compromised immune system (Nielsen *et al*., 2018).

In this study *E. coli* and *E. faecalis* were exposed to sub-lethal concentrations of 0.1 mM and 10 mM glyphosate respectively, and 50 mM AMPA to investigate the physiology and metabolism of gut bacteria. These selected concentrations allowed for minimum inhibition of growth as compared to other tested concentrations. At sub-lethal exposure to AMPA, *E. coli* early log-phase seemed to be longer as compared to its response to glyphosate. Additionaly, under glyphosate stress *E. coli* enters the stationary phase sooner than when exposed to AMPA (Figure 2A & 2B). This could be due to the more potent nature of glyphosate. *Enterococcus faecalis* is more susceptible to glyphosate as compared to *E. coli* (Shehata *et al*., 2013). In the current study *E. faecalis* undergoes substantial stress under 0.1 mM glyphosate as compared to the 10 mM used on *E .coli* (Figure 3A).

5.2. Regulated physiological and metabolic functions of *E. coli* and *E. faecalis* in response to glyphosate and AMPA

The general pattern of protein modulation by both the *E. coli* strains and *E. faecalis* was up-regulation (Table 1). Such skewness towards up-regulation could also have occurred if, during

the experimentation, greater amount of the control sample (bacteria peptides unexposed to glyphosate/AMPA) were used. This situation is unlikely given that relative (as opposed to absolute) quantitation was employed in this study in which equivalent amounts of total proteins were used from each sample in order to compensate for potential loss in bacterial cells. More so, the use of a greater amount of one sample type would have resulted in all identified proteins being either up- or down-regulated. This was not the case with the results in this study where appreciable number of proteins across several protein species were either not differentially expressed or down-regulated not withstanding that a greater number of proteins were up-regulated by the bacteria (Table 1) as a response mechanism to glyphosate and AMPA. The majority of differentially expressed proteins for *E. coli* strains after exposure to glyphosate and AMPA are involved in iron uptake and transport, transcription and translation processes, and stress response; however, proteins of energy response were identified in glyphosate but not AMPA response.

5.2.1. Nitrate metabolism

The down-regulation by RM109 of all the identified proteins of nitrate metabolism is in consonance with the fact that glyphosate affects energy production. Literature reported energy drainage in *E. coli* responding to glyphosate shock through repression of nuo genes encoding NADPH dehydrogenase of the respiratory chain (Lu *et al*., 2013). In the current study energy production was affected through impairment of nitrate assimilation which is observed by down-regulation of the respiratory nitrate reductase complex (NarGHI) and nitrite extrusion protein (NarK). This observation may also indicate that glyphosate affects the synthesis of amino acid through non-shikimate pathways, because nitrate is reduced to nitrite followed by reduction to ammonia. Ammonia is then assimilated into cellular organic material (Moir & Wood, 2001). Interestingly, there is evidence that nitrogen assimilation is strongly inhibited by feedback from starvation for shikimate pathway products (Vivancos *et al*., 2011).

Dissimilatory nitrate reductases catalyse the first step of the catabolic, anaerobic respiration pathway (Argandoña *et al*., 2006). The membrane-bound nitrate-reductase enzyme is composed of three sub-units encoded by NarG, NarH, and NarI. NarG and NarH are situated in the cytoplasm but anchored to the membrane through NarI (González *et al*., 2006). Nar accepts electrons from substrate oxidation to reduce nitrate (NO3-) to nitrite (NO2). The resulting electrochemical gradient is in turn used for ATP generation and thus energy conservation (Argandoña *et al*., 2006). The nitrate-reductase complex function with NarK transporter which imports nitrate and exports nitrite. NarL activates transcription of narGHJI and narK operons

(Stewart *et al*., 1989). The narGHJI operon is expressed in response to nitrate being available as an electron acceptor (Argandoña *et al*., 2006). Down regulation of these genes stops/reduce the transport of nitrogen into the cell, making nitrogen unavailable as an electron acceptor and micro molecule thus leading to energy and growth starvation. Down regulation of glnL of the two-component system which codes for Nitrogen regulation protein NR (II) was also observed in this study.

5.2.2. Nucleotide metabolism

Glyphosate has been shown to induce DNA damage (Mañas *et al*., 2009; Kwiatkowska *et al*., 2017; Townsend *et al*., 2017). Up-regulation of nucleotide metabolism by RM109 may be an indication thereof. Aspartate carbamoyltransferase (PyrB), Aspartate carbomyltransferase regulatory chain (PyrI), and Carbamoyl-phosphate synthase large chain (CarB) were upregulated almost 2-fold under the influence of glyphosate stress, an indication of increase rate of pyrimidine syntheses. This may suggest that RM109 is compensating for glyphosate stress on DNA, by producing more pyrimidines needed to repair and synthesis new nucleic acids. NAD-dependent dihydropyrimidine dehydrogenase (PreA) was downregulated which may suggest that pyrimidine degradation is not taking place, allowing uracil and thymine to be used in RNA and DNA synthesis.

Surprisingly, nucleotide metabolism was not affected for EC100 and DH5-Alpha by glyphosate and a similar response was observed for AMPA. In *E. faecalis*, glyphosate affected purine and pyrimidine metabolism by up-regulating proteins (Udk and Cdd) participating in uridine synthesis. Under AMPA exposure a similar response was observed. The results show a reflection of the extent at which glyphosate and AMPA increased regulation of proteins involved in the ribosome pathway under transcription and translation processes. Increased expression of pyrimidine syntheses in this study may also be a sign to induced virulence in the presence of glyphosate and AMPA, since some bacteria must be able to perform pyrimidine synthesis to be virulent (Fields et al., 1986).

The pyrBI operon of *E. coli* encodes the catalytic (PyrB) and regulatory (PyrI) subunits of the allosteric enzyme aspartate transcarbamylase and expression of the operon is negatively regulated by pyrimidine availability. The carAB operon encodes the two subunits of carbamylphosphate synthetase. The above mentioned enzyme catalyses the formation of carbamylphosphate, which is an intermediate in both the pyrimidine nucleotide and arginine biosynthetic pathways. Its expression is repression by the end products (Turnbough & Switzer,

2008). Anaerobic ribonucleoside-triphosphate reductase (NrdD) is activated by NrdG under anaerobic conditions. The enzyme catalyzes the conversion of ribonucleotides into deoxyribonucleotides, which are required for DNA synthesis and repair. NAD-dependent dihydropyrimidine dehydrogenase (PreA) involved in pyrimidine base degradation, it catalyzing the conversion of uracil to 5,6-dihydrouracil.

Degradation of uracil and thymine allows for them to be used as nitrogen and carbon sources for growth (Hidese *et al*., 2011). The decrease in nucleotide derivatives which serve as direct indicators of nucleotide metabolism, DNA replication, cell division and growth state, expose reduction in cell growth (Bhat *et al*., 2015).

5.2.3. Iron uptake and transport

Iron is an essential element for the growth and development of living organisms, and plays a crucial part in bacterial virulence and pathogenicity. Iron participates in a large number of cellular processes, the most important of which are oxygen transport, ATP generation, cell growth, and detoxification (Symeonidis & Marangos, 2012). Upregulation of enterobactin synthase complex and iron transport proteins (TonB, FepA, ExbD) by *E. coli* under glyphosate and AMPA exposure may indicate iron depletion in the cell, because these genes are expressed during iron-limiting conditions, and may have implications on the bacterial virulence (Chu *et al*., 2010; Symeonidis & Marangos, 2012). Considering the competition between host and pathogen for iron serves an important feature influencing the course of an infection (Ferreira *et al*., 2016). Surprisingly, *E. faecalis* did not exhibit a similar profile in response to the two chemicals.

Enterobactin synthase is a multienzyme complex formed by proteins EntB, EntD, EntE, and EntF respectively. The enzyme participates in the biosynthesis of the bacterial siderophore enterobactin from chorismic acid and serine (Rusnak *et al*., 1990). The enterobactin molecule chelates Fe^{3+} ion, which is then taken up into the cell by the outer membrane transport protein FepA of the two-component system (Raymond *et al*., 2003). Their biosynthesis is confined to bacterial and fungal cells, and expression increases the virulence of these species (Neilands, 1995). Protein TonB interacts with outer membrane receptor proteins that carry out high-affinity binding and energy dependent uptake of various iron compounds. Catecholate siderophore receptor Fiu is TonB-dependent and it is involved in iron transport. The expression of this protein is repressed by iron (Niehaus *et al*., 1991). ExbB and ExbD proteins function as part of

the TonB-dependent energy transduction system to support transport of iron-siderophore complexes across the outer membrane.

5.2.4. Stress response

Bacterial response to stress is vital for coping with the stress and for adapting to the new environment (Ron, 2013). To overcome the stress the bacteria needs to enhance its protective potential. This can be done by permeability of cellular membranes to reactive species and up-regulation of antioxidant and associated enzymes (Lushchak, 2011). Many stressors like glyphosate and AMPA might be toxic to bacteria due to their acceleration of endogenous ROS formation. Elevation of intracellular levels of superoxide and hydrogen peroxide can produces enough enzyme damage and DNA damage that can stall growth (Imlay, 2015). The gene regulatory response of *E. coli* to superoxide and peroxide stress is largely mediated through the induction of superoxide dismutases MnSOD (sodA), FeSOD (sodB), CuZnSOD (sodC) and catalases, respectively.

Our results are consistent with the fact that glyphosate causes oxidative stress (Pham *et al*., 2004; Vivancos *et al*., 2011; Lozano *et al*., 2018). Regulation of PerR, Sod, and other defense proteins by *E. coli* strains and *E. faecalis* is in agreement with literature indicating that accumulation of antioxidant enzymes superoxide dismutase may result in oxidative stress (Ahsan *et al*., 2008). Even though AMPA is less toxic than glyphosate (Vivancos *et al*., 2011), it is interesting to observe that AMPA may also induce oxidative stress at sub-lethal concentrations. *E. coli* up-regulated proteins involved in superoxide removal and protection like SodA and universal stress proteins (Usp). While, *E. faecalis* had a similar response but employed different defence proteins like thrioredoxin. Glyphosate and AMPA seem to induce oxidative stress through superoxide production, leading to protein and DNA damage. This is observed by the proteins regulated across *E. coli* and *E. faecalis*. These proteins are mainly involved in superoxide removal and protection against DNA damage.

5.2.5. Transcription and translation processes

Bacteria constantly adjust gene expression in response to perturbation from their environment. Regulation of gene expression involve transcription factors that sense metabolic signals and specifically activate or inhibit target genes (Berthoumieux *et al*., 2013). Transcription and translational play an essential role in expression of the genetic information encoded in cells. In this study 50S ribosomal protein L32 (rpmF) expression was increased almost 2-fold in

response to glyphosate and AMPA for *E. coli*. This protein respond to reactive oxygen species. *E. faecalis* seem to down-regulate RNA degradation and up-regulate Ribosome pathway in response to glyphosate and AMPA, seen by down-regulation of cold shock proteins (eg: CspC) and up-regulating 30S and 50S ribosomal proteins which are structural constituents of the ribosome. It seems *E. coli* and *E. faecalis* compensate the stress caused by glyphosate and AMPA by guarding genetic information from damage by reactive oxygens species. These results are in accord to results obtained in section 5.2.4.

5.2.5.1. Aminoacyl-tRNA biosynthesis

Aminoacyl-tRNA synthetases form part of a family of enzymes that catalyse the aminoacylation reaction by covalently joining an amino acid to its respective tRNA in the first step of protein translation (Grube & Roy, 2018; Rajendran *et al*., 2018). These enzymes play an important role in several metabolic and signalling pathways that are vital for cell viability (Rajendran *et al*., 2018). In response to glyphosate *E. faecalis* up-regulated all proteins involved in Aminoacyl-tRNA biosynthesis. A similar response was observed under AMPA exposure, although the number of proteins regulated was higher. In addition, to the 7 cytoplasmic aminoacyl-tRNA synthetases, Tyrosine--tRNA ligase 2 (TyrS2) and seryl-tRNA synthetase (SerS2) of the mitochondrial aminoacyl-tRNA synthetases were up-regulated.

Mitochondrial aminoacyl-tRNA synthetases parttakes in facilitating protein synthesis. Thus glyphosate and AMPA interfere with translation processes, because aminoacyl-tRNA synthetase warrants accurate transfer of information from nucleic acid to amino acid. Disruption of aminoacyl-tRNA biosynthesis pathway may interrupt cell proliferation (Zhao *et al*., 2014), since these enzymes are crucial for growth and survival of an organism. Furthermore AMPA behaves as an antimicrobial given that most antibiotics that target protein translation interact with microbial ribosomes (Rajendran *et al*., 2018). For example, study on *E. faecalis* response to chloramphenicol treatment indicated up-regulation of genes encoding ribosomal proteins, and repression of genes for tRNA synthetases which may indicate reduced protein synthesis (Aakra *et al*., 2010).

5.2.6. Carbohydrate metabolism

Glyphosate and AMPA effects on *E. faecalis* extend to several pathways of carbohydrate metabolism. The two chemicals seem to simultaneously up-regulate and down-regulate proteins within the same pathway; an observation similar to a study by Lu indicating involvement of

opposing mechanisms (Lu *et al*., 2013). Interestingly AMPA induced up-regulation of 6-phosphogluconate dehydrogenase (Gnd), glyceraldehyde-3-phosphate dehydrogenase (Gap-2), and deoxyribose-phosphate aldolase (DeoC) which are involved in carbohydrate degradation pathway (PP pathway), indicating that AMPA favours carbohydrate degradation as opposed to glyphosate (Lu *et al*., 2013). Similarly proteins of the phosphotransferase system (EF_3305, EF_2213, EF_0022, and EF_0461) were down-regulated, suggesting that the phosphotransferase system is not active under the influence of AMPA.

Reduced phosphotransferase system metabolites are a good indication for loss of coupling between glycolysis, the TCA cycle and oxidative phosphorylation (Escalante *et al*., 2012). Through repressing energy intensive pathways, such as cellular respiration, cell division and nucleotide metabolism (Bhat *et al*., 2015), *E. coli* and *E. faecalis* are able to adapt to glyphosate and AMPA. All V-type ATPase (AtpB, AtpA, EF_1492, and EF_1494) were up-regulated following AMPA exposure in *E. faecalis*. The up-regulation of these proteins signals the significance of optimal redox conditions in bacteria experiencing stress (Solheim *et al*., 2007; Aakra *et al*., 2010). Interestingly ribosomal proteins, V-type ATPase, and pyrimidine biosynthesis were up-regulated in this study similar to *E. faecalis* response chloramphenicol treatment (Aakra *et al*., 2010).

5.3. Glyphosate and AMPA effects on bacterial virulence and influence on antibiotics

Exposure to glyphosate and AMPA induce a cascade of response pathways that can result in detrimental or beneficial changes in bacterial gene expression and physiology. *Escherichia coli* and *E. faecalis* differ in their responses to glyphosate and AMPA but share some similarities, which can be expected given the inherent difference of the microbes. Major perturbed categories from the current study include nitrate metabolism/energy metabolism, nucleotide metabolism, iron uptake and transport, stress response, transcription and translation processes, carbohydrate metabolism, and hypothetical proteins, with nitrate metabolism and iron uptake and transport absent in *E. faecalis*. In addition AMPA affected amino acid metabolism, lipid metabolism/cell wall, ABC transporters, and quorum sensing in *E. faecalis*.

These results share similarities with studies on transcriptional response of *E. faecalis* to erythromycin (Aakra *et al*., 2005) and transcriptional response of *E. coli* to glyphosate (Lu *et al*., 2013), in terms of the general functional categories influenced by AMPA and glyphosate. For example energy production and conversion was affected in both studies but different genes were targeted, respiratory nitrate reductase complex (NarGHI) was repressed in the current

study, while nuo genes were repressed in the other study (Lu *et al*., 2013). Distinct differences observed were iron uptake and transport regulated in the current study, and cell motility being regulated in the latter.

Glyphosate and AMPA heavily affected transcription and translation processes in *E. faecalis*, with AMPA having a pronounced effect on this group compared to glyphosate. Seventy four proteins involved in this category were regulated in response to AMPA, 29 proteins of the ribosome consisting of 30S and 50S ribosomal proteins were up-regulated [these proteins serve as target for erythromycin and other antibacterial agents, more specifically the 50S ribosomal subunits (Aakra *et al*., 2005)], 9 aminoacyl-tRNA biosynthesis proteins also up-regulated, and 6 RNA polymerase/RNA degradation proteins were down-regulated (Table 7). Additionally ABC transporter proteins were down-regulated which are involved in cationic antimicrobial peptide (CAMP) resistance (DltC), and beta-Lactam resistance (EF_3106, EF_1148). Other ABC transporter proteins including peptide methionine sulfoxide reductase (MsrA), vancomycin resistance (Ddl), and virulence factor (EF_2076) were up-regulated. The endocarditis specific antigen (EF_2076) is involved in endocarditis, a complication of *Enterococcal* bacteraemia that usually occurs on damaged or prosthetic heart valves. Factors leading to the development of enterococcal endocarditis appear to be hospitalisation and multiple antibiotic therapies (Baldassarri *et al*., 2004). This may suggest that AMPA exposure may induce virulence in *E. faecalis* similar to antibiotics. Similarly, glyphosate has the potential to induce virulence in *E. coli* through expression of enterobactin synthase complex proteins and iron transport proteins, increasing the bacterial competitive ability for iron with the host (as described in section 5.1.3).

Additionally, MsrA and MsrB forms part of the msr-system which plays a role in bacterial resistance to oxidative stress (Denkel *et al*., 2011), and confers an increased resistance to macrolides through MsrC (Singh *et al*., 2001). Methionine sulfoxide reductase (MsrC) is essential for growth and survival of an intracellular pathogen in macrophages in combination with MsrB (Denkel *et al*., 2011). Taken together, ribosomal proteins, RNA polymerase, and ABC transporters play a vital role in survival mechanism of *E. faecalis*, not only in adopting to AMPA but eventually have an influence on antibiotic susceptibility or resistance.

Even though glyphosate and AMPA induced proteins dealing with oxidative stress and some DNA protecting proteins pointing towards DNA damage, an SOS response was not induced, possibly because DNA damage was not high enough to elicit SOS or simply the bacteria mitigated DNA damage through SOD, universal stress proteins, or other pathways. Given that

important genes in the SOS response such as recA and soxS were not differential regulated in the current study.

Commercial herbicides can lead to an increased or decreased adaptive nature in bacterial susceptibility to antibiotics, leading to adaptive resistance due to altered bacteria fitness caused by the selective pressure at low concentrations (Kurenbach *et al*., 2018). Herbicides such as glyphosate not only increase the concentration of antibiotics (Kurenbach *et al*., 2015; Kurenbach *et al*., 2017), but also has the potential to change the MIC of certain antibiotics (such as tetracycline) when exposure is simultaneous (Kurenbach *et al*., 2018). Considering increased survival or adaptive resistance of bacteria is triggered by differential induction of efflux pumps (Kurenbach *et al*., 2015; Kurenbach *et al*., 2017), and with a large number of these efflux pumps not being substrate specific and able to confer cross protection (Elkins & Mullis, 2007). Differential expression of ABC transporters (DltC, EF_3106, EF_1148, MsrA, and Ddl) in the current study may indicate the adaptive nature of bacteria. These findings are of importance because bacteria can be exposed to herbicides and antibiotics in environments such as the gut of animals and human.

5.4. Glyphosate and AMPA implication on gut bacteria

Animals and humans can encounter low concentrations of herbicides through residues in food or feed, and water. Reported residues rang from 3.3 mg kg^{-1} in soybean (Bøhn *et al*., 2014), 308 ng g^{-1} in corn (Reddy *et al*., 2008), and 0.23 µg/L up to 700 µg/L in water (Pesce *et al*., 2008, Peruzzo *et al*., 2008). Taking into account that in South Africa the basic staple food crop is GM, glyphosate is in heavy usage. In 2012, 46% of glyphosate was applied on maize, 13% on wheat, and 6% on soybean from a total of 23 million litres (Gouse, 2014). The community heavily rely on maize and alternative grains such as wheat and sorghum (with limited fruits and vegetables), especially in low income household and poorer communities (Ekpa *et al*., 2018), were by maize is sometimes served for breakfast, lunch, and dinner because it is prepared in a variety of ways such as maize meal porridge, snacks, and beverages (Ekpa *et al*., 2018). At times maize is alternated with other grains, meaning that commensal bacteria of the gut are continuously exposed to low glyphosate and AMPA concentrations through residual contamination in a staple food.

At sub-lethal concentrations glyphosate can modulate microbial communities (Krüger *et al*., 2013; Shehata *et al*., 2013; Newman *et al*. 2016; Nielsen *et al*., 2018), leading to an imbalance favouring an overgrowth of resistant pathogens over susceptible commensal bacteria (Shehata

et al., 2013). Not only are commensal bacteria important for assisting the host with part-taking in immune system homeostasis and gastrointestinal tract permeability (Littman & Pamer, 2011), but serve an antagonist effect on pathogens such as *C. botulinum* (Shehata *et al*., 2013, Krüger *et al*., 2013), over growth of which could lead to disease. Glyphosate also has the potential to modulate susceptibility of certain antibiotics in cases of simultaneous exposure (Kurenbach *et al*., 2015; Kurenbach *et al*., 2017; Kurenbach *et al*., 2018).

Thus, when bacteria are in the presence of antibiotics, adaptive resistance may occur from the selective trigger glyphosate and AMPA causes in the environment. Not only may this allow bacteria to overcome low levels of glyphosate, AMPA, or antibiotics and subsequently result in resistance to higher concentrations (Händel *et al*., 2014). It can also speed up the frequency of resistance evolution within the host (Kurenbach *et al*., 2017), which may predispose the public to detrimental health effects with continuous exposure, especially in individuals with poor diets and compromised immune systems.

Glyphosate is linked with increased anxiety and depression-like behaviours in mice (Aitbali *et al*., 2018) and a role in the epidemic of intestinal disorders (Lozano *et al*., 2018), paralleled with decreased total bacterial count and altered gut microbial composition of *Firmicutes*, *Bacteroidetes* and *Lactobacillus*. If this observation translates to the human host it can stir up major public health concerns. Even though, Nielsen *et al*. (2018) reported very limited effects of pure glyphosate and glyphosate-based herbicide on gut microbial community composition of rats, attributed to alleviation of antimicrobial effect of glyphosate due to supplementation of aromatic amino acid in the gut environment. The possibility still remains for detrimental effects of glyphosate in people suffering from malnutrition or individuals on special diets (Nielsen *et al*., 2018).

CHAPTER 6

CONCLUSION AND RECOMMENDATIONS

Extensive usage of glyphosate may lead to resident gut bacteria like *E. coli* and *E. faecalis* being exposed to glyphosate and AMPA albeit at sub-lethal concentrations, due to a widespread presence of residues in water (Battaglin *et al*., 2014), dairy products and food crops (Baig *et al*., 2003; Duke *et al*., 2003; Lorenzatti *et al*., 2004; EFSA, 2015). Information is sparse on how sub-lethal concentrations of glyphosate and AMPA alter the physiology and metabolism of gut bacteria. To tackle this we assessed metabolic pathways perturbed by glyphosate and AMPA through proteomic approaches. The results indicate *E. coli* strain differences in response to glyphosate and AMPA. Glyphosate seems to exhibits greater stress on *E. coli* as compared to AMPA. In contrast AMPA affected more proteins than glyphosate in *E. faecalis* even though glyphosate is more potent (Vivancos *et al*., 2011), suggesting that AMPA is less specific as compared to its parent compound.

At lethal doses glyphosate causes starvation for aromatic amino acids, energy drain, and other off-target effects (Fischer *et al*., 1986; Lu *et al*., 2013). In this study sub-lethal glyphosate and AMPA affected nitrate metabolism, indicating impaired energy production and to some degree reduced amino acid production due to impaired nitrate assimilation. *Escherichia coli* also experienced iron depletion, which in turn induced acquiring of iron by up-regulation of iron uptake and transport proteins. Suggesting glyphosate and AMPA may play a role in bacterial pathogenesis due to the key role played by iron in progression of pathogenesis. In line with literature, glyphosate affected carbohydrate metabolism both in *E. coli* and *E. faecalis*. Glyphosate and AMPA also caused expression of an array of defence proteins in *E. coli* and *E. faecalis*, pointing towards oxidative stress which is detrimental to micromolecules and growth. Oxidative stress could result in selective pressure enabling the bacteria to withstand glyphosate or AMPA (Lozano *et al*., 2018).

Glyphosate and AMPA seemed to severely affect transcription and translation processes in *E. faecalis* as this was the functional category with the most differentially expressed proteins in both accounts. The ribosome pathway which plays a pivotal role in translations was mostly affected in the transcription and translation processes functional category. This may be a result of the oxidative stress induced under glyphosate and AMPA exposure, as such this may lead to mutations that may prove to be beneficial or detrimental to the bacteria. The changes in

expression of hypothetical genes indicate that some physiological responses following glyphosate exposure remain uncharacterized (Lu *et al*., 2013). These proteins may serve as potential targets for non-toxic herbicides and anti-microbial compounds (Li *et al*., 2015).

The overall result presents an intricate set of interactions which promote adaptation of bacteria to the stressful environment. Each of the perturbed pathways serve an important role in bacterial fitness and survival, some of which serve as a key point for adaptive resistance and to some degree stimulate virulence. Consequently it raises the possibility that environmental stimuli such as glyphosate and its breakdown product AMPA may cause phenotypic antibiotic resistance variation between individuals which could serve as hotspots for evolution of acquired antibiotic resistance (Kurenbach *et al*., 2018). As such hosts of gut microbiota exposed to herbicide residue through the food chain, may encounter negative effects especially when their diet is deficient of aromatic amino acids (Nielsen *et al*., 2018)

In conclusion these results suggest that sub-lethal concentrations of glyphosate and AMPA induces changes in energy metabolism, iron uptake and transport, carbohydrate metabolism, transport and stress response in bacteria. This serves as an indication that, the stress endured by bacteria under glyphosate stress is not only due to a lack of certain amino acids (Armendáriz *et al*., 2016), but due to several mechanisms of adaptation to metabolic inhibition (Lozano *et al*., 2018). Glyphosate and AMPA exhibit similar perturbation of metabolic pathways but differ in number and type of proteins regulated. This suggests that the two chemicals differ in their mode of action (WANG, 2001). Hence, AMPA unknown mode of action is yet to be elucidated. Furthermore, Glyphosate and AMPA at sub-lethal doses may serve as environmental cue for antibiotic resistance, virulence expression and habitat adaptation of *E. coli* and *E. faecalis*.

Recommendations

- Further pathway analysis may be performed with the help of transcriptomic approaches to provide a full map of the response of bacteria exposed to sub-lethal glyphosate and AMPA concentrations.
- With the limited studies conducted on the response of gut bacteria to glyphosate and AMPA at sub-lethal concentrations, a study on long term in-vivo effects of glyphosate and AMPA would be of interest, given that gut microbiota will be exposed to

accumulative glyphosate and AMPA residues over time. Additionally, studies have suggested that glyphosate could act as antibiotics in mammalian gut microbiome (Lozano *et al*., 2018).

- Given that AMPA mode of action has not been identified it would be beneficial to investigate, as this will help explain why AMPA perturbate such a large number of protein, especially in gut commensals that are more susceptible to glyphosate like *E. faecalis*.

REFERENCES

Aakra, A., Vebø, H., Indahl, U., Snipen, L., Gjerstad, Ø, Lunde, M., & Nes, I.F. 2010. The response of *Enterococcus faecalis* V583 to chloramphenicol treatment. *International Journal of Microbiology.* 2010:1-7.

Aakra, A., Vebø, H., Snipen, L., Hirt, H., Aastveit, A., Kapur, V., Dunny, G., Murray, B.E., & Nes, I.F. 2005. Transcriptional response of *Enterococcus faecalis* V583 to erythromycin. *Antimicrobial agents and chemotherapy.* 49(9):2246-2259

Aebersold, R. & Mann, M. 2003. Mass Spectrometry-Based Proteomics. *Nature*. 422:198–207

Ahsan, N., Lee, D.G., Lee, K.W., Alam, I., Lee, S.H., Bahk, J.D., & Lee, B.H. 2008. Glyphosate-induced oxidative stress in rice leaves revealed by proteomic approach. *Plant Physiology and Biochemistry*. 46(12):1062–1070.

Aitbali, Y., Ba-M'hamed, S., Elhidar, N., Nafis, A., Soraa, N., & Bennis, M. 2018. Glyphosate based- herbicide exposure affects gut microbiota, anxiety and depression-like behaviors in mice. *Neurotoxicology and Teratology.* 67:44–49.

Anadón, A., Martínez-Larrañaga, M.R., Martínez, M.A., Castellano, V.J., Martínez, M., Martin, M.T., Nozal, M.J., & Bernal, J.L. 2009. Toxicokinetics of glyphosate and its metabolite aminomethyl phosphonic acid in rats. *Toxicology Letters.* 190(1):91–95.

Argandoña, M., Martínez-Checa, F., Llamas, I., Arco, Y., Quesada, E., & Del Moral, A. 2006. A membrane-bound nitrate reductase encoded by the narGHJI operon is responsible for anaerobic respiration in *Halomonas maura*. *Extremophiles*. 10(5):411–419.

Armendáriz, O., Gil-Monreal, M., Zulet, A., Zabalza, A., & Royuela, M. 2016. Both foliar and residual applications of herbicides that inhibit amino acid biosynthesis induce alternative respiration and aerobic fermentation in pea roots. *Plant Biology.* 18(3):382–390.

Avarshinah. A Modest Desire, Surely. *All poetry*. https://allpoetry.com/poem/7378992-A-Modest-Desire--Surely-by-avarshinah. Date Accessed: 10 July 2018.

Bai, S.H. & Ogbourne, S.M. 2016. Glyphosate: environmental contamination, toxicity and potential risks to human health via food contamination. *Environmental Science and Pollution Research. Res. Environmental Science and Pollution Research*. 23(19):18988–19001.

Baig, M.N., Darwent, A.L., Harker, K.N., & O'Donovan, J.T. 2003. Preharvest applications of glyphosate affect emergence and seedling growth of Field Pea (*Pisum sativum*). *Weed Technology*. 17(4):655–665.

Baldassarri, L., Creti, R., Arciola, C.R., Montanaro, L., Venditti, M., & Di Rosa, R. 2004. Analysis of virulence factors in cases of *Enterococcal endocarditis*. *Clinical Microbiology and Infection. European Society of Clinical Infectious Diseases*. 10(11):1006–1008.

Bantscheff, M., Lemeer, S., Savitski, M.M., & Kuster, B. 2012. Quantitative mass spectrometry in proteomics: Critical review update from 2007 to the present. *Analytical and Bioanalytical Chemistry.* 404(4):939–965.

Battaglin, W.A., Kolpin, D.W., Scribner, E.A., Kuivila, K.M., Sandstrom, M.W., William, A., Kolpin, D.W., Scribner, E.A., & Kuivila, K.M. 2005. Glyphosate, other herbicides, and transformation products in midwestern streams, 2002. *Journal of the American Water Resources Association.* 41(2):323–332.

Battaglin, W.A., Meyer, M.T., Kuivila, K.M., & Dietze, J.E. 2014. Glyphosate and its degradation product AMPA occur frequently and widely in U.S. soils, surface water, groundwater, and precipitation. *Journal of the American Water Resources Association.* 50(2):275–290.

Benbrook, C.M. 2016. Trends in glyphosate herbicide use in the United States and globally Background. *Environmental Sciences Europe.* 28(3):1-15.

Berg, G.M. & Jørgensen, N.O.G. 2006. Purine and pyrimidine metabolism by estuarine bacteria. *Aquatic Microbial Ecology.* 42(3):215–226.

Bergström, L., Börjesson, E., & Stenström, J. 2011. Laboratory and lysimeter studies of glyphosate and aminomethylphosphonic acid in a sand and a clay soil. *Journal of environmental quality.* 40(1):98–108.

Bertani, G. 2004. Lysogeny at Mid-Twentieth Century: P1, P2, and Other Experimental Systems. *Journal of Bacteriology.* 186(3):595–600.

Berthoumieux, S., De Jong, H., Baptist, G., Pinel, C., Ranquet, C., Ropers, D., & Geiselmann, J. 2013. Shared control of gene expression in bacteria by transcription factors and global physiology of the cell. *Molecular Systems Biology.* 9(634):1–11.

Bhat, S. V., Booth, S.C., Vantomme, E.A.N., Afroj, S., Yost, C.K., & Dahms, T.E.S. 2015. Oxidative stress and metabolic perturbations in *Escherichia coli* exposed to sublethal levels of 2,4-dichlorophenoxyacetic acid. *Chemosphere*. 135:453–461.

Bøhn, T., Cuhra, M., Traavik, T., Sanden, M., Fagan, J., & Primicerio, R. 2014. Compositional differences in soybeans on the market : Glyphosate accumulates in Roundup Ready GM soybeans. *Food Chemistry*. 153:207–215.

Borggaard, O.K. & Gimsing, A.L. 2008. Fate of glyphosate in soil and the possibility of leaching to ground and surface waters: areview. *Pest Management Science.* 64:441–456.

Carvalho, L.B., Duke, S.O., Messa, J.R., Costa, F.R., & Bianco, S. 2016. Plant growth responses of apple and pear trees to doses of glyphosate. *Planta Daninha*. 34(4):815–822.

Cerdeira, A.L. & Duke, S.O. 2006. The current status and environmental impacts of glyphosate-resistant crops:A Review. *Journal of Environmental Quality.* 35:1633–1658.

Chauzat, M. & Faucon, J. 2007. Pesticide residues in beeswax samples collected from honey bee colonies (*Apis mellifera L*.) in France. *Pest Management Science.* 1106:1100–1106.

Chiang, S.M. & Schellhorn, H.E. 2012. Regulators of oxidative stress response genes in *Escherichia coli* and their functional conservation in bacteria. *Archives of Biochemistry and Biophysics.* 525(2):161–169.

Chu, B.C., Garcia-Herrero, A., Johanson, T.H., Krewulak, K.D., Lau, C.K., Peacock, R.S., Slavinskaya, Z., & Vogel, H.J. 2010. Siderophore uptake in bacteria and the battle for iron with the host; a bird's eye view. *BioMetals*. 23:601–611

Cilia, M., Fish, T., Yang, X., Mclaughlin, M., Thannhauser, T.W., & Gray, S. 2009. A comparison of protein extraction methods suitable for gel-based proteomic. *Journal of Biomolecular Techniques.* 20:201–215.

Cuhra, M., Bøhn, T., & Cuhra, P. 2016. Glyphosate : Too much of a good thing ?. *Fontiers in Environmental Sciences*. 4(28):1–14.

Cuhra, M., Traavik, T., & Bøhn, T. 2013. Clone- and age-dependent toxicity of a glyphosate commercial formulation and its active ingredient in *Daphnia magna*. *Ecotoxicology*. 22(2):251–262.

Cuhra, M., Traavik, T., Dando, M., Primicerio, R., Holderbaum, D.F., & Bøhn, T. 2015. Glyphosate-residues in roundup-ready soybean impair *Daphnia magna* life-cycle. *Journal of Agricultural Chemistry and Environment*. 4:24–36.

Dallegrave, E., Mantese, F.D., Oliveira, R.T., Andrade, A.J.M., Dalsenter, P.R., & Langeloh, A. 2007. Pre- and postnatal toxicity of the commercial glyphosate formulation in Wistar rats. *Archives of Toxicology.* 81(9):665–673.

Dayon, L., Pasquarello, C., Hoogland, C., Sanchez, J.C., & Scherl, A. 2010. Combining low- and high-energy tandem mass spectra for optimized peptide quantification with isobaric tags. *Journal of Proteomics*. 73(4):769–777.

de Hoffmann, E. & Stroobant, V. 2007. Mass Spectrometry - Priniples and Applications. 3rd ed. , Chichester, West Sussex. John Wiley & Sons Ltd.

De Mey, M., Lequeux, G.J., Maertens, J., De Muynck, C.I., Soetaert, W.K., & Vandamme, E.J. 2008. Comparison of protein quantification and extraction methods suitable for *E. coli* cultures. *Biologicals*. 36(3):198–202.

Delorenzo, M.E., Scott, G.I., & Ross, P.E. 2001. Toxicity of pesticides to aquatic microorganisms: A review. *Environmental Toxicology and Chemistry.* 20(1):84–98.

Denkel, L.A., Horst, S.A., Rouf, S.F., Kitowski, V., Böhm, O.M., Rhen, M., Jäger, T., & Bange, F.C. 2011. Methionine sulfoxide reductases are essential for virulence of *Salmonella typhimurium*. *PLoS One*. 6(11):1-12.

Dill, G.M. 2005. Glyphosate-resistant crops : history , status and future. *Pest Management Science.* 61:219–224.

Dill, G.M., Sammons, R.D., Feng, P.C.C., Kohn, F., Kretzmer, K., Mehrsheikh, A., Bleeke, M., Honegger, J.L., Farmer, D., Wright, D., & Haupfear, E.A. 2010. Glyphosate: Discovery, Development, applications, and properties. *Glyphosate Resistance in Crops and Weeds: History, Development, and Management.*1–33.

Domon, B. & Aebersold, R. 2006. Mass spectrometry and protein analysis. *Science.* 312(577):212–217.

Dorigo, U., Leboulanger, C., Bérard, A., Bouchez, A., Humbert, J.F., & Montuelle, B. 2007. Lotic biofilm community structure and pesticide tolerance along a contamination gradient in a vineyard area. *Aquatic Microbial Ecology.* 50(1):91–102.

Duke, S.O. 2014. Perspectives on transgenic , herbicide-resistant crops in the United States almost 20 years after introduction. *Pest Managagemnet Science*. 71: 652–657.

Duke, S.O., Lydon, J., Koskinen, W.C., Moorman, T.B., Chaney, R.L., & Hammerschmidt, R. 2012. Glyphosate Effects on Plant Mineral Nutrition, Crop Rhizosphere Microbiota, and Plant Disease in Glyphosate-Resistant Crops. *Journal of Agricultural and Food Chemistry*. 60: 10375−10397

Duke, S.O. & Powles, S.B. 2008. Glyphosate : a once-in-a-century herbicide. *Pest Management Science*. 64:319–325.

Duke, S.O., Rimando, A.M., Pace, P.F., Reddy, K.N., & Smeda, R.J. 2003. Isoflavone, glyphosate, and aminomethylphosphonic acid levels in seeds of glyphosate-treated, glyphosate-resistant soybean. *Journal of Agricultural and Food Chemistry.* 51(1):340–344.

Duncan, K., Lewendon, A., & Coggins, J.R. 1984. The purification of 5-enolpyruvylshikimate 3-phosphate synthase from an overproducing strain of *Escherichia coli*. *FEBS Letters.* 165(1):121–127.

EFSA Panel on Generically Modified Organisms (GMO). 2014. Scientific Opinion on application (EFSA-GMO-NL-2009-64) for the placing on the market of herbicide-tolerant genetically modified soybean BPS- CV127-9 for food and feed uses , import and processing under Regulation (EC) No 1829 / 2003 from BASF Plant S.*EFSA Journal* 12(1):3505.

Einsle, O. & Kroneck, P.M.H. 2004. Structural basis of denitrification. *Biological Chemistry.* 385(10):875–883.

Ekpa, O., Palacios-Rojas, N., Kruseman, G., Fogliano, V., & Linnemann, A.R. 2018. Sub-Saharan African maize-based foods: Technological perspectives to increase the food and nutrition security impacts of maize breeding programmes. *Global Food Security.* 17:48–56.

Elkins, C.A. & Mullis, L.B. 2007. Substrate competition studies using whole-cell accumulation assays with the major tripartite multidrug efflux pumps of *Escherichia coli*. *Antimicrobial Agents and Chemotherapy.* 51(3):923–929.

Escalante, A., Cervantes, A.S., Gosset, G., & Bolívar, F. 2012. Current knowledge of the *Escherichia coli* phosphoenolpyruvate-carbohydrate phosphotransferase system: Peculiarities of regulation and impact on growth and product formation. *Applied Microbioliogy and Biotechnology.* 94(6):1483–1494.

European Food Safety Authority, E.F.S.A. 2015. Conclusion on the peer review of the pesticide risk assessment of the active substance glyphosate. *EFSA Journal.* 13(11):4302.

Farr, S.B. & Kogoma, T. 1991. Oxidative stress responses in *Escherichia coli* and *Salmonella typhimurium*. *Microbiol Reviews*. 55(4):561–85.

Fei, Y.Y., Gai, J.Y., & Zhao, T.J. 2013. Identification of regulated genes conferring resistance to high concentrations of glyphosate in a new strain of *Enterobacter*. *FEMS Microbiology Letters.* 349(2):135–143.

Ferreira, D., Seca, A.M.L., Pinto, D.C.G.A., & Silva, A.M.S. 2016. Targeting human pathogenic bacteria by siderophores: A proteomics review. *Journal of Proteomics*.145:153-166

Fischer, R.S., Berry, A., Gaines, C.G., & Jensen, R.A. 1986. Comparative action of glyphosate as a trigger of energy drain in eubacteria. *Journal of Bacteriology.* 168(3):1147–1154.

Ganesh, A. & Lin, J. 2011. Comparisons of protein extraction procedures and quantification methods for the proteomic analysis of Gram-positive *Paenibacillus sp*. strain D9. *World Journal of Microbiology and Biotechnology.* 27(7):1669–1678.

Ghassemi, M., Fargo, L., Painter, P., Quinlivan, S., Scofield, R., & Takata, A. 1981. Environmental fate and impacts of major forest used pesticides. *Environmental Protection Agency*.

Giesy, J.P., Dobson, S., & Solomon, K.R. 2000. Ecotoxicological risk assessment for roundup® herbicide. *Reviews of Environmental Contamination and Toxicology*. 167:35-120

González, P.J., Correia, C., Moura, I., Brondino, C.D., & Moura, J.J.G. 2006. Bacterial nitrate reductases: Molecular and biological aspects of nitrate reduction. *Journal of Inorganic Biochemistry.* 100:1015-1023

Gouse, M. 2014. Assessing the value of glyphosate in the South African Agricultural sector. (November). Working Papers 206520, University of Pretoria, Department of Agricultural Economics, Extension and Rural Development.

Gouse, M., Pray, C.E., Kirsten, J., & Schimmelpfennig, D. 2005. A GM subsistence crop in Africa: the case of Bt white maize in South Africa A GM subsistence crop in Africa. *International Journal of Biotechnology.* 7(3):84–94.

Grube, C.D. & Roy, H. 2018. A continuous assay for monitoring the synthetic and proofreading activities of multiple aminoacyl-tRNA synthetases for high-throughput drug discovery. *RNA Biology.* 15(4–5):659–666.

Gustavsson, N., Diez, A.A., & Nyström, T. 2002. The universal stress protein paralogues of *Escherichia coli* are co-ordinately regulated and co-operate in the defence against DNA damage. *Molecular Microbiology.* 43(1):107–117.

Guyton, K.Z., Loomis, D., Grosse, Y., El Ghissassi, F., Benbrahim-Tallaa, L., Guha, N., Scoccianti, C., Mattock, H., Straif, K., Blair, A., Fritschi, L., McLaughlin, J., Sergi, C.M., Calaf, G.M., Le Curieux, F., Baldi, I., Forastiere, F., Kromhout, H., 't Mannetje, A., Rodriguez, T., Egeghy, P., Jahnke, G.D., Jameson, C.W., Martin, M.T., Ross, M.K., Rusyn, I., & Zeise, L. 2015. Carcinogenicity of tetrachlorvinphos, parathion, malathion, diazinon, and glyphosate. *Lancet Oncology*. 16(5):490–491.

Händel, N., Schuurmans, J.M., Feng, Y., Brul, S., & Ter Kuile, B.H. 2014. Interaction between mutations and regulation of gene expression during development of de novo antibiotic resistance. *Antimicrobial Agents and Chemotherapy.* 58(8):4371–4379.

Hidese, R., Mihara, H., Kurihara, T., & Esaki, N. 2011. *Escherichia coli* dihydropyrimidine dehydrogenase is a novel NAD-dependent heterotetramer essential for the production of 5,6-dihydrouracil. *Journal Bacteriology.* 193(4):989–993.

Hove-Jensen, B., Zechel, D.L., & Jochimsen, B. 2014. Utilization of glyphosate as phosphate source: biochemistry and genetics of bacterial carbon-phosphorus lyase. *Microbiology and Molecular Biology Reviews.* 78(1):176–97.

Huang, J., Ellis, C., Hauge, B., Qi, Y., & Varagona, M.J. 2015. Herbicide tolerance. *Recent Advancements in Gene Expression and Enabling Technologies in Crop Plants*. :213–237.

Imlay, J.A. 2015. Diagnosing oxidative stress in bacteria: not as easy as you might think. *Current Opinion in Microbiology*. 24:124–131.

Isolauri, E., Arvilommi, H., & Salminen, S. 2001. Probiotics, effects on immunity. *American Journal of Clinical Nutrition* 73:444–450.

Johnson, W.G., Davis, V.M., Kruger, G.R., & Weller, S.C. 2009. Influence of glyphosate-resistant cropping systems on weed species shifts and glyphosate-resistant weed populations. *European Journal of Agronomy.* 31(3):162–172.

Kilstrup, M., Hammer, K., Jensen, P.R., & Martinussen, J. 2005. Nucleotide metabolism and its control in lactic acid bacteria. *FEMS Microbiology Reviews.* 29(3):555–590.

Kirkwood, R.C., Hetherington, R., Reynolds, T.L., & Marshall, G. 2000. Absorption, localisation, translocation and activity of glyphosate in barnyardgrass (*Echinochloa crus-galli* (L) *Beauv*): Influence of herbicide and surfactant concentration. *Pest Management Science.* 56(4):359–367.

Kittle, R.P., McDermid, K.J., Muehlstein, L., & Balazs, G.H. 2018. Effects of glyphosate herbicide on the gastrointestinal microflora of Hawaiian green turtles (*Chelonia mydas*) Linnaeus. *Marine Pollution Bulletin*. 127(2018):170–174.

Kolpin, D.W., Thurman, E.M., Lee, E.A., Meyer, M.T., Furlong, E.T., & Glassmeyer, S.T. 2006. Urban contributions of glyphosate and its degradate AMPA to streams in the United States. *Science of the Total Environment.* 354(2–3):191–197.

Kraft, B., Strous, M., & Tegetmeyer, H.E. 2011. Microbial nitrate respiration - Genes, enzymes and environmental distribution. *Journal of Biotechnology.* 155(1):104–117.

Krüger, M., Ali, A., Schrödl, W., & Rodloff, A. 2013. Anaerobe Glyphosate suppresses the antagonistic effect of *Enterococcus spp* . on *Clostridium botulinum*. *Anaerobe*. 20:74–78.

Kurenbach, B., Gibson, P.S., Hill, A.M., Bitzer, A.S., Silby, M.W., Godsoe, W., & Heinemann, J.A. 2017. Herbicide ingredients change *Salmonella enterica* sv. *Typhimurium* and *Escherichia coli* antibiotic responses. *Microbiology*. 163(12):1791–1801.

Kurenbach, B., Hill, A.M., Godsoe, W., van Hamelsveld, S., & Heinemann, J.A. 2018. Agrichemicals and antibiotics in combination increase antibiotic resistance evolution. *PeerJ*. 6:e5801.

Kurenbach, B., Marjoshi, D., Amábile-cuevas, C.F., Ferguson, G.C., Godsoe, W., Gibson, P., & Heinemann, J.A. 2015. Sublethal exposure to commercial formulations of the herbicides changes in antibiotic susceptibility in *Escherichia coli* and *Salmonella enterica serovar Typhimurium*. *Mbio*. 6(2):1–9.

Kwiatkowska, M., Huras, B., & Bukowska, B. 2014. The effect of metabolites and impurities of glyphosate on human erythrocytes (in vitro). *Pesticide Biochemistry and Physiology.* 109(1):34–43.

Kwiatkowska, M., Reszka, E., Woźniak, K., Jabłońska, E., Michałowicz, J., & Bukowska, B. 2017. DNA damage and methylation induced by glyphosate in human peripheral blood mononuclear cells (in vitro study). *Food and Chemical Toxicology.* 105:93–98.

Li, X.Z., Plésiat, P., & Nikaido, H. 2015. The challenge of efflux-mediated antibiotic resistance in Gram-negative bacteria. *Clinical Microbiology Reviews.* 28(2):337–418.

Littman, D.R. & Pamer, E.G. 2011. Role of the commensal microbiota in normal and pathogenic host immune responses. *Cell Host and Microbe*. 10(4):311–323.

Lorenzatti, E., M.I. Maitre, L. Argelia, R. Lajmanovich, P. Peltzer, and M. Anglada. 2004. Pesticide residues in immature soybeans of Argentina croplands. *Fresenius Environmental Bullitin*. 13:675–678.

Lozano, V.L., Defarge, N., Rocque, L.M., Mesnage, R., Hennequin, D., Cassier, R., de Vendômois, J.S., Panoff, J.M., Séralini, G.E., & Amiel, C. 2018. Sex-dependent impact of Roundup on the rat gut microbiome. *Toxicology Reports*. 5:96–107.

Lu, W., Li, L., Chen, M., Zhou, Z., Zhang, W., Ping, S., Yan, Y., Wang, J., & Lin, M. 2013. Genome-wide transcriptional responses of *Escherichia coli* to glyphosate, a potent inhibitor of the shikimate pathway enzyme 5-enolpyruvylshikimate-3-phosphate synthase. *Molecular Biosystems*. 9(3):522–530.

Lushchak, V.I. 2011. Adaptive response to oxidative stress: Bacteria, fungi, plants and animals. *Comparative Biochemistry and Physiology. - Part C Toxicology and Pharmacologyl.* 153(2):175–190.

Macwilliams, M.P. & Liao, M. 2006. Luria Broth (LB) and Luria Agar (LA) Media and Their Uses Protocol Resource Type : Publication Date : (May 2017):7–9.

Mamy, L., Barriuso, E., & Gabrielle, B. 2005. Environmental fate of herbicides trifluralin, metazachlor, metamitron and sulcotrione compared with that of glyphosate, a substitute broad spectrum herbicide for different glyphosate-resistant crops. *Pest Management Science.* 61(9):905–916.

Mañas, F., Peralta, L., Raviolo, J., Ovando, H.G., Weyers, A., Ugnia, L., Cid, M.G., Larripa, I., & Gorla, N. 2009. Genotoxicity of glyphosate assessed by the comet assay and cytogenetic tests. *Environmental Toxicology and Pharmacology.* 28(1):37–41.

Moir, J.W.B.M. & Wood, N.J. 2001. Nitrate and nitrite transport in *Escherichia coli*. *CMLS Cellular Molecular Life Sciences.* 58(1):215–224.

Murray, P. R., Baron, E. J., Jorgensen, J.H., Landry, M.L. and Pfaller, M. A. 2007. Manual of clinical microbiology, 9th ed. ASM Press, D.C.

Neilands, J.B. 1995. Siderophores : Structure and function of microbial iron transport compounds siderophores : Structure and function of microbial iron transport compounds *. *Journal of Biological Chemistry.* 270(45):26723–26726.

Newman, M.M., Lorenz, N., Hoilett, N., Lee, N.R., Dick, R.P., Liles, M.R., Ramsier, C., & Kloepper, J.W. 2016. Changes in rhizosphere bacterial gene expression following glyphosate treatment. *Science of the Total Environment*. 553:32-41.

Niehaus, F., Hantke, K., & Unden, G. 1991. Iron content and FNR-dependent gene regulation in *Escherichia coli*. *FEMS Microbiology Letters.* 68(3):319–323.

Nielsen, L.N., Roager, H.M., Casas, M.E., Frandsen, H.L., Gosewinkel, U., Bester, K., Licht, T.R., Hendriksen, N.B., & Bahl, M.I. 2018. Glyphosate has limited short-term effects on commensal bacterial community composition in the gut environment due to sufficient aromatic amino acid levels. *Environmental Pollution.* 233:364–376.

Niemann, L., Sieke, C., Pfeil, R., & Solecki, R. 2015. A critical review of glyphosate findings in human urine samples and comparison with the exposure of operators and consumers. *Journal fur Verbraucherschutz und Lebensmittelsicherheit.* 10:3-12

Novagen. 2008. BugBuster® Protein Extraction Reagent. User Protocol TB245 Rev. E 0304. 1–8.
http://wolfson.huji.ac.il/purification/PDF/Protease_fusion_cleavage/NOVAGEN_HRV_3C_Protease.pdf. Date Accessed: 02 Nov 2018.

Nygaard, P. & Saxild, H.H. 2009. Nucleotide Metabolism. , in: Schaechter, M. (Ed.), Encyclopedia of Microbiology (3rd ed.). Academic Press: Oxford. . Academic Press: Oxford, pp. 296–307.

Okoli, A.S., Wilkins, M.R., Raftery, M.J., & Mendz, G.L. 2010. Response of *helicobacter hepaticus* to bovine bile. *Journal of Proteome Research.* 9(3):1374–1384.

Paulo, J. A., Gaun, A., & Gygi, S. P. (2015). Global Analysis of Protein Expression and Phosphorylation Levels in Nicotine-Treated Pancreatic Stellate Cells. *Journal of proteome research*, *14*(10), 4246-56.

Pérez, G.L., Vera, M.S., & Miranda, L.A. 2011. Effects of herbicide glyphosate and glyphosate-based formulations on aquatic ecosystems. *Herbicide and the Environment.* :343–368.

Peruzzo, P.J., Porta, A.A., & Ronco, A.E. 2008. Levels of glyphosate in surface waters, sediments and soils associated with direct sowing soybean cultivation in north pampasic region of Argentina. *Environmental Pollution.* 156(1):61–66.

Pesce, S., Fajon, C., Bardot, C., Bonnemoy, F., Portelli, C., & Bohatier, J. 2008. Longitudinal changes in microbial planktonic communities of a French river in relation to pesticide and nutrient inputs. *Aquatic Toxicology.* 86(3):352–360.

Pham, C., Min, J., & Gu, M. 2004. Pesticide induced toxicity and stress response in bacterial cells. *Buletin of. Environmental Contamination.* 72:380-386

Landrigan, P.J., & Benbrook, C.B. 2015. GMOs, Herbicides, and Public Health. *New Englend Journal of Medicine.* 373(8):691–693.

Pollegioni, L., Schonbrunn, E., & Siehl, D. 2011. Molecular basis of glyphosate resistance-different approaches through protein engineering. *FEBS Journal.* 278(16):2753–66.

Portier, C.J., Armstrong, B.K., Baguley, B.C., Baur, X., Belyaev, I., Bellé, R., Belpoggi, F., Biggeri, A., Bosland, M.C., Bruzzi, P. *et al*. 2016. Differences in the carcinogenic evaluation of glyphosate between the International Agency for Research on Cancer (IARC) and the European Food Safety Authority (EFSA). *Journal of Epidemiology and Community Health*. 70(8).

Quinn, L.P., Vos, J. De, Roos, C., Bouwman, H., Kylin, H., Pieters, R., & Berg, J. Van Den. 2011. Pesticide Use in South Africa : One of the largest importers of pesticides in Africa. *Pesticides in the modern World-Pesticides Use & Management.*1–48.

Rajendran, V., Kalita, P., Shukla, H., Kumar, A., & Tripathi, T. 2018. Aminoacyl-tRNA synthetases: Structure, function, and drug discovery. *International Journal of Biological Macromolecules.* 111:400–414.

Ranum, P., Peña-Rosas, J.P., & Garcia-Casal, M.N. 2014. Global maize production, utilization, and consumption. *Annals of the New York Academy of Sciences.* 1312 (2014): 105–112.

Rauniyar, N., Gao, B., McClatchy, D.B., & Yates, J.R. 2013. Comparison of protein expression ratios observed by sixplex and duplex TMT labeling method. *Journal of Proteome Research.* 12(2):1031–1039.

Raymond, K.N., Dertz, E.A., & Kim, S.S. 2003. Enterobactin: An archetype for microbial iron transport. *Proceedings of the National Academy of Sciences.* 100(7):3584–3588.

Reddy, K.N., Rimando, A.M., Duke, S.O., & Nandula, V.K. 2008. Aminomethylphosphonic acid accumulation in plant species treated with glyphosate. *Journal of Agricultural and Food Chemistry.* 56(6):2125–2130.

Roberts, D.M., Buckley, N.A., Mohamed, F., Eddleston, M., & Daniel, A. 2010. A prospective observational study of the clinical toxicology of glyphosate-containing herbicides in adults with acute self- poisoning. *Clinical toxicology (Philadelphia).* 48(2):129–136.

Ron E.Z. (2013) Bacterial Stress Response. In: Rosenberg E., DeLong E.F., Lory S., Stackebrandt E., Thompson F. (eds) The Prokaryotes. Springer, Berlin, Heidelberg Rubio, F., Guo, E., & Kamp, L. 2014. Survey of glyphosate residues in honey, corn and soy products. *Journal of Environmental and Analytical Toxicology.* 05(01):1–8.

Rueppel, M.L., Brightwell, B.B., Schaefer, J., & Marvel, J.T. 1977. Metabolism and degradation of glyphosate in soil and water. *Journal of Agricultural and Food Chemistry.* 25(3):517–528.

Rusnak, F., Liu, J., Quinn, N., Berchtold, G.A., & Walsh, C.T. 1990. Subcloning of the enterobactin biosynthetic gene entB: expression, purification, characterization and substrate specificity of isochorismatase. *Biochemistry*. *American Chemical Society*. 29(6):1425–1435.

Salman, J.M., Abdul-adel, E., & Alkaim, A.F. 2016. Effect of pesticide Glyphosate on some biochemical features in cyanophyta algae *Oscillatoria limnetica*. *International Journal of PharmTech Research.* 9(8):355–365.

Samsel, A. & Seneff, S. 2013. Glyphosate, pathways to modern diseases II : Celiac sprue and gluten intolerance.*Interdisciplinary Toxicology. International Journal of Pharm TechResearch*. 6(4):159–184.

Saunders, L. & Pezeshki, R. 2015. Glyphosate in runoff waters and in the root-zone: A Review. *Toxics*. 3(4):462–480.

Schönfeldt, H.C., Hall, N., & Pretorius, B. 2018. The important role of food composition in policies and programmes for better public health: A South African case study. *Food Chemistry*. 238:94–10

Schuette, J. 1998. Environmental fate of glyphosate. *Environmental Monitoring & Pest Management - Department of Pesticide Regulation.* Sacramento:13 p.

Scribner, E. a., Battaglin, W. a., Gilliom, R.J., & Meyer, M.T. 2007. Concentrations of glyphosate, its degradation product, aminomethylphosphonic acid, and glufosinate in ground- and surface-water, rainfall, and soil samples collected in the United States, 2001–06. *U.S. Geological Survey Investigations Report 2007-5122*. 111.

Sezonov, G., Joseleau-Petit, D., & D'Ari, R. 2007. *Escherichia coli* physiology in Luria-Bertani broth. *Journal of Bacteriology.* 189(23):8746–8749.

Shaner, D.L., Lindenmeyer, R.B., & Ostlie, M.H. 2012. What have the mechanisms of resistance to glyphosate taught us?. *Pest Management Science.* 68(1):3–9.

Shehata, A.A., Kühnert, M., Haufe, S., & Krüger, M. 2014. Neutralization of the antimicrobial effect of glyphosate by humic acid in vitro. *Chemosphere*. 104(2014):258–261.

Shehata, A.A., Schrödl, W., Aldin, A.A., Hafez, H.M., & Krüger, M. 2013. The effect of glyphosate on potential pathogens and beneficial members of poultry microbiota in vitro. *Current Microbiology.* 66(4):350–358.

Shipitalo, M.J. & Owens, L.B. 2006. Comparative losses of glyphosate and selected residual herbicides in surface runoff from conservation-tilled watersheds planted with corn or soybean. *Journal of environmental quality.* 40(4):1281–9.

Sigma-Aldrich. 2013. Brain Heart broth product information. https://www.sigmaaldrich.com/content/dam/sigma-aldrich/docs/Sigma-Aldrich/Datasheet/1/53286dat.pdf. Date accessed: 02 Nov 2018

Singh, K. V., Malathum, K., & Murray, B.E. 2001. Disruption of an *Enterococcus faecium* species-specific gene, a homologue of acquired macrolide resistance genes of *staphylococc*i, is associated with an increase in macrolide susceptibility. *Antimicrobial Agents and Chemotherapy.* 45(1):263–266.

Skark, C., N. Zullei-Seibert, U. Schottler, and C. Schlett, 1998. The occurrence of glyphosate in surface water. *International Journal of Environmental Analytical Chemistry*. 70(1-4):93-104.

Solheim, M., Aakra, Å., Vebø, H., Snipen, L., & Nes, I.F. 2007. Transcriptional responses of *Enterococcus faecalis* V583 to bovine bile and sodium dodecyl sulfate. *Applied and Environmental Microbiology.* 73(18):5767–5774.

Stewart, V., Parales, J., & Merkel, S.M. 1989. Structure of genes narL and narX of the nar (nitrate reductase) locus in *Escherichia coli* K-12. *Journal of Bacteriology.* 171(4):2229–2234.

Struger, J., Thompson, D., Staznik, B., Martin, P., McDaniel, T., & Marvin, C. 2008. Occurrence of glyphosate in surface waters of southern Ontario. *Bulletin of Environmental Contamination and Toxicology.* 80(4):378–384.

Symeonidis, A. & Marangos, M. 2012. Iron and microbial growth. *Insight and Control of Infectious Disease in Global Scenario.* 16:289–330.

Taj, M., Samreen, Z., Ling, J., Hassani, I., Hassani, T., & Yunlin, W. 2014. *Escherichia coli* as a model organism. *International journal of Engineering Research and Science & Technology*. 3(2):1-8.

Tan, S.C. & Yiap, B.C. 2009. DNA, RNA, and protein extraction: The past and the present. *Journal of Biomedicine and Biotechnology.* 2009:1-10.

Testbiotech E.V. 2013. High levels of residues from spraying with glyphosate found in soybeans in Argentina. *TESTBIOTECH Background.* (April).

Then, C. 2014. Golden Lies : No credibility for Golden Rice campaign. https://www.foodwatch.org/uploads/media/golden_lies_golden_rice_project_2012_01.pdf. Date Accessed: 02 Nov 2018

ThermoFisher. 2014. Thermo Scientific pierce products for mass spectrometry sample preparation. https://tools.lifetechnologies.com/content/sfs/brochures/1602773-Mass-Spec-Sample-Prep-Handbook.pdf. Date Accessed: 02 Nov 2018.

Townsend, M., Peck, C., Meng, W., Heaton, M., Robison, R., & O'Neill, K. 2017. Evaluation of various glyphosate concentrations on DNA damage in human Raji cells and its impact on cytotoxicity. *Regulatory Toxicology and Pharmacology.* 85:79–85.

Turnbough, C.L. & Switzer, R.L. 2008. Regulation of pyrimidine biosynthetic gene expression in bacteria: Repression without repressors. *Microbiology and Molecular Biology Reviews.* 72(2):266–300.

van der Veen, S. & Abee, T. 2011. Bacterial SOS response: A food safety perspective. *Current Opinion in Biotechnology.* 22(2):136–142.

Van Stempvoort, D.R., Roy, J.W., Brown, S.J., & Bickerton, G. 2014. Residues of the herbicide glyphosate in riparian groundwater in urban catchments. *Chemosphere*. 95:455–463.

Vandenberg, L.N., Blumberg, B., Antoniou, M.N., Benbrook, C.M., Carroll, L., Colborn, T., Everett, L.G., Hansen, M., Landrigan, P.J., Lanphear, B.P., Mesnage, R., vom Saal, F.S., Welshons, W. V, & Myers, J.P. 2017. Is it time to reassess current safety standards for glyphosate-based herbicides? *Journal of Epidemiology and Community Health*. 0:1-6.

Villeneuve, A., Larroudé, S. & Humbert, J.F. 2011. Pesticides - formulations, effects, fate. herbicide contamination of freshwater ecosystems: Impact on microbial communities. *Margarita Stoytcheva*. 201:285–312.

Vivancos, P.D., Driscoll, S.P., Bulman, C.A., Ying, L., Emami, K., Treumann, A., Mauve, C., Noctor, G., & Foyer, C.H. 2011. Perturbations of amino acid metabolism associated with glyphosate-dependent inhibition of shikimic acid metabolism affect cellular redox homeostasis and alter the abundance of proteins involved in photosynthesis and photorespiration. *Plant Physiology.* 157(1):256–268.

Walker, J.M. 2002. The Bicinchoninic Acid (BCA) Assay for Protein Quantitation BT - The Protein Protocols Handbook. , in: Walker, J.M. (Ed.), . Humana Press: Totowa, NJ. . Humana Press: Totowa, NJ, pp.11–14.

Wang, A.C. 2001. Effect of Glyphosate on Aromatic Amino Acid Metabolism in Purple Nutsedge (*Cyperus rotundus*). *Weed Technology*. 15(4):628–635.

Williams, G.M., Kroes, R., & Munro, I.C. 2000. Safety evaluation and risk assessment of the herbicide Roundup and its active ingredient, glyphosate, for humans. *Regulatory Toxicology and Pharmacology.* 31(2):117–165.

Zabaloy, M.C., Gómez, E., Garland, J.L., & Gómez, M.A. 2012. Assessment of microbial community function and structure in soil microcosms exposed to glyphosate. *Applied Soil Ecology.* 61:333–339

Zhao, Y., Meng, Q., Bai, L., & Zhou, H. 2014. In Silico discovery of aminoacyl-tRNA synthetase inhibitors. *International Journal of Molecular Sciences.* 15(1):1358–1373.

Zheng, H., Wisedchaisri, G., & Gonen, T. 2013. Crystal structure of a nitrate/nitrite exchanger. *Nature*. *Nature Publishing Group*. 497(7451):647–651.

Zhou, X.L., Chen, Y., Fang, Z.P., Ruan, Z.R., Wang, Y., Liu, R.J., Xue, M.Q., & Wang, E.D. 2016. Translational quality control by bacterial threonyl-tRNA synthetases. *Journal of Biological Chemistry.* 291(40):21208–21221.

LIST OF APPENDIX

Appendix A

Table A: Protein expression profiles of *E. coli* (RM109) in response to glyphosate after 2 and 8 hours

Time (hours)	Functional Categories	Description	Code	Regulation
2	Adaptation to aberrant conditions	Thioredoxin-1	trxA	↓
		33 kDa chaperonin	hslO	↓
		Alkyl hydroperoxide reductase subunit C	ahpC	↓
		Probable Fe(2+)-trafficking protein	yggX	↓
		Putative peroxiredoxin bcp	bcp	↓
		Undecaprenyl-diphosphatase	uppP	↑
	Amin acid metabolism	Elongation factor P	efp	↓
		N-acetylneuraminate lyase	nanA	↓
		3-isopropylmalate dehydratase small subunit	leuD	↓
		RutC family protein yjgF	yjgF	↓
		Alanine racemase, biosynthetic	alr	↓
		Dihydrodipicolinate reductase	dapB	↑
		Histidinol-phosphate aminotransferase	hisC	↑
		Acetolactate synthase isozyme 1 small subunit	ilvN	↑
		Betaine aldehyde dehydrogenase	betB	↑
		Ribose-5-phosphate isomerase A	rpiA	↓

Time (hours)	Functional Categories	Description	Code	Regulation
2	Carbon metabolism	Phosphocarrier protein HPr	ptsH	↓
		Phosphopentomutase	deoB	↓
		2,3-bisphosphoglycerate-independent phosphoglycerate mutase	gpmI	↓
		Transaldolase B	talB	↓
		Enolase	eno	↓
		Phosphoenolpyruvate-protein phosphotransferase	ptsI	↓
		Glucose-specific phosphotransferase enzyme IIA component	crr	↓
		Maltose transport system permease protein malF	malF	↑
	Cellular Processes	Malonyl CoA-acyl carrier protein transacylase	fabD	↓
		D-alanine--D-alanine ligase A	ddlA	↓
		Uncharacterized fimbrial-like protein ElfG	elfG	↑
		Virulence factor mviN homolog	mviN	↑
		UPF0118 inner membrane protein ydiK	ydiK	↑
		Major outer membrane lipoprotein	lpp	↑
		Pilin	traA	↑
		Chaperone protein AggD	aggD	↑
		Phosphatidate cytidylyltransferase	cdsA	↑
		Undecaprenyl-phosphate alpha-N-acetylglucosaminyl 1-phosphate transferase	wecA	↑
		Glycerol-3-phosphate dehydrogenase [NAD(P)+]	gpsA	↑
	Cofactor biosynthesis; molybdopterin biosynthesis	Thiol:disulfide interchange protein DsbA	dsbA	↓
		Dihydrofolate reductase	folA	↓
		Molybdopterin synthase catalytic subunit	moaE	↓
		GTP cyclohydrolase 1	folE	↑
	Iron transport & uptake	Fe(3+) dicitrate-binding periplasmic protein	fecB	↓
		Colicin I receptor	cirA	↑
		Fe(3+) dicitrate-binding periplasmic protein	fecB	↓
		Iron-binding protein iscA	iscA	↓

Time (hours)	Functional Categories	Description	Code	Regulation
2		2,3-dihydro-2,3-dihydroxybenzoate dehydrogenase	entA	↓
	nitrate assimilation	Nitrogen regulation protein NR(II)	glnL	↑
		Nitrogen regulatory protein	ptsN	↓
		Nitrite extrusion protein 2	narU	↑
	Pyrimidine metabolism; UMP biosynthesis	Uridine phosphorylase	udp	↑
		Anti-adapter protein iraP	iraP	↓
		Adenylate kinase	adk	↓
		Hypoxanthine phosphoribosyltransferase	hpt	↓
	Transcription & translation processes	Cold shock protein CspA	cspA	↓
		Elongation factor Ts	tsf	↓
		50S ribosomal protein L7/L12	rplL	↓
		Ribosomal-protein-alanine acetyltransferase	rimI	↓
		Transcription elongation factor greA	greA	↓
		UPF0133 protein ybaB	ybaB	↓
		Cold shock-like protein CspB	cspB	↓
		Protein traI	traI	↓
		UPF0350 protein ygfY	ygfY	↓
		DNA gyrase inhibitor YacG	YacG	↓
		Peptide chain release factor 2	prfB	↓
		UPF0082 protein yebC	yebC	↓
		DNA-directed RNA polymerase subunit omega	rpoZ	↓
		UPF0082 protein yeeN	yeeN	↓
		Regulator of ribonuclease activity B	rraB	↓
		Chaperone protein DnaK	dnaK	↓
		Uncharacterized protein yceD	yceD	↓
		GTP-dependent nucleic acid-binding protein engD	engD	↓
		Ribosome maturation factor rimM	rimM	↓

Time (hours)	Functional Categories	Description	Code	Regulation
		Sulfurtransferase TusA		↓
		Cell division topological specificity factor	minE	↓
		Preprotein translocase subunit secE	secE	↑
		Ribosomal-protein-alanine acetyltransferase	rimJ	↑
		30S ribosomal protein S18	rpsR	↑
		30S ribosomal protein S21	rpsU	↑
		30S ribosomal protein S19	rpsS	↑
		tRNA 5-methylaminomethyl-2-thiouridine biosynthesis bifunctional protein MnmC	mnmC	↑
		30S ribosomal protein S14	rpsN	↑
		30S ribosomal protein S20	rpsT	↑
		Uncharacterized protein yaeJ	yaeJ	↑
		Hemolysin expression-modulating protein Hha	hha	↑
		50S ribosomal protein L34	rpmH	↑
		50S ribosomal protein L35	rpmI	↑
		Glycine betaine-binding periplasmic protein	proX	↓
		Multidrug export protein EmrA	emrA	↓
		4-alpha-L-fucosyltransferase	wecF	↑
2	Transport	Outer membrane protein C	ompC	↑
		Maltoporin	lamB	↑
		Calcium/proton antiporter	chaA	↑
		Putative transport protein YidE	yidE	↑
		Inner membrane protein ybjJ	ybjJ	↑
		Long-chain fatty acid transport protein	fadL	↑
		Inner membrane protein YccS	yccS	↑
		LPS-assembly lipoprotein	lptE	↑
		Protein phnA	phnA	↓
		Protein iscX	iscX	↓
		Protein yciN	yciN	↓

Time (hours)	Functional Categories	Description	Code	Regulation
2	Others	Cold shock-like protein CspG	cspG	↓
		Lactoylglutathione lyase	gloA	↓
		NifU-like protein	nifU	↓
		Pyrazinamidase/nicotinamidase	pncA	↓
		Acyl carrier protein	acpP	↓
		Protein-export protein secB	secB	↓
		Thiosulfate sulfurtransferase glpE	glpE	↓
		UPF0352 protein yejL	yejL	↓
		Oxygen-insensitive NAD(P)H nitroreductase	nfnB	↓
		Cold shock-like protein CspE	cspE	↓
		S-(hydroxymethyl)glutathione dehydrogenase	frmA	↓
		Shikimate kinase 1	aroK	↓
		Peptidyl-prolyl cis-trans isomerase B	ppiB	↓
		Glutaredoxin-3	grxC	↓
		Co-chaperone protein hscB	hscB	↓
		Protein mioC	mioC	↓
		Protein yihD	yihD	↓
		FKBP-type 16 kDa peptidyl-prolyl cis-trans isomerase	fkpB	↓
		FKBP-type 22 kDa peptidyl-prolyl cis-trans isomerase	fklB	↓
		Elongation factor P--(R)-beta-lysine ligase	epmA	↓
		UPF0434 protein ycaR OS=Escherichia coli O139:H28 (strain E24377A / ETEC) GN=ycaR PE=3 SV=1 - [YCAR_ECO24]	ycaR	↓
		Inorganic pyrophosphatase	ppa	↓
		GTP cyclohydrolase-2	ribA	↓
		Cold shock-like protein CspC	cspC	↓
		Fe/S biogenesis protein nfuA	nfuA	↓
		UPF0337 protein yjbJ	yjbJ	↓
		Glutaredoxin-4	grxD	↓
		Protein syd	syd	↓
		Phosphatase YidA	yidA	↓
		Hydrogenase-4 component G	hyfG	↓

Time (hours)	Functional Categories	Description	Code	Regulation
2	Others	Chaperone surA	surA	↓
		Elongation factor P-like protein	yeiP	↓
		Protein CyaY	cyaY	↓
		Putative prophage CP4-6 integrase	intF	↑
		Adenosylcobalamin/alpha-ribazole phosphatase	cobC	↑
		Na(+)/H(+) antiporter nhaA	nhaA	↑
		Quinone oxidoreductase 2	qorB	↑
		Sucrose operon repressor	cscR	↑
		Probable adenine permease PurP	purP	↑
		Maltodextrin glucosidase	malZ	↑
		Outer membrane protein X	ompX	↑
		Formate dehydrogenase, cytochrome b556(fdo) subunit	fdoI	↑
		MltA-interacting protein	mipA	↑
		Chaperone protein fimC	fimC	↑
		Glycine dehydrogenase [decarboxylating]	gcvP	↑
		UPF0270 protein yheU	yheU	↑
		Glycolate oxidase subunit glcE	glcE	↑
	Uncharacterized	Uncharacterized protein	ychH	↑
		UPF0253 protein	yaeP	↓
		Uncharacterized protein	YihF	↑
		UPF0033 protein	yeeD	↓
		Uncharecterized	yecJ	↓
		Uncharacterized	yqcC	↓
	Uncharacterized	Uncharacterized	yebO	↓
		Uncharacterized	ygeR	↑
		Uncharacterized	yjiN	↑
		Uncharacterized	ychH	↑
		Uncharacterized	yihM	↑
		Uncharacterized	yibQ	↑

Time (hours)	Functional Categories	Description	Code	Regulation
		Uncharacterized	ygbI	↑
		Uncharacterized	ycbJ	↑
		Uncharacterized	yegW	↑
		Uncharacterized	ydcY	↑
	Cell motility	Flagellar brake protein YcgR	YcgR	↑
	Environmental Information Processing	UPF0409 protein ycdO	ycdO	↑
		Stationary-phase-induced ribosome-associated protein	sra	↑
8	Iron transport & uptake	Ferrienterobactin receptor	fepA	↑
		Isochorismatase	entB	↑
		Enterobactin synthase component E	entE	↑
		Protein	tonB	↑
		Biopolymer transport protein	exbD	↑
		Catecholate siderophore receptor	fiu	↑
		Outer membrane protein W	ompW	↓
		Colicin I receptor	cirA	↑
	Metabolism	Nicotinate phosphoribosyltransferase	pncB	↑
		Cytochrome bo(3) ubiquinol oxidase subunit 3	cyoC	↑
		Phosphatidylglycerophosphatase B	pgpB	↑
		Betaine aldehyde dehydrogenase	betB	↑
8	Nitrate metabolism/Energy metabolism	Respiratory nitrate reductase 1 beta chain	narH	↓
		Nitrogen regulation protein NR(II)	glnL	↓
		Respiratory nitrate reductase 1 alpha chain	narG	↓
		Respiratory nitrate reductase 1 gamma chain	narI	↓
		Nitrite reductase [NAD(P)H] large subunit	nirB	↓
		Nitrite extrusion protein 1	narK	↓

Time (hours)	Functional Categories	Description	Code	Regulation
8				
	Transcription & translation processes	Rac prophage repressor	racR	↑
		Probable Sec-independent protein translocase protein TatE	TatE	↑
		Cold shock-like protein CspD	CspD	↑
	Uncharacterized protein	Uncharacterized protein	yncE	↑
		UPF0253 protein	yaeP	↑
		Uncharacterized protein	YihF	↑
		UPF0033 protein	yeeD	↑
	Other	Autonomous glycyl radical cofactor	grcA	↓
		Anaerobic ribonucleoside-triphosphate reductase	nrdD	↓
		HTH-type transcriptional regulator	ascG	↓
		LPS-assembly lipoprotein	lptE	↑
		Sigma-E factor negative regulatory protein	rseA	↑
		Glycerol uptake facilitator protein	glpF	↑
		TraT complement resistance protein	TraT	↑

↑= Upregulation, ↓=Downregulation, - =Below regulation cut of point

Appendix B

Table B: Protein expression profile of *E.coli* (RM109) after 2 and 8 hours of AMPA exposure

Time (hours)	Functional Categories	Description	Code	Regulation
2	Adaptation to aberrant conditions	HTH-type transcriptional regulator gadX	gadX	↑
	Carbohydrate metabolism	Release factor glutamine methyltransferase	prmC	↓
		Sucrose operon repressor	cscR	↑
	Iron transport & uptake	Enterobactin synthase component F	entF	↑
		Enterochelin esterase	fes	↑
		Isochorismatase	entB	↑
		Enterobactin synthase component E	entE	↑
		2,3-dihydro-2,3-dihydroDownybenzoate dehydrogenase	entA	↑
		Colicin I receptor	cirA	↑
		Ferrienterobactin receptor	fepA	↑
		Catecholate siderophore receptor Fiu	fiu	↑
		Protein FecR	fecR	↑
	Metabolism of other amino acids	Alanine racemase, biosynthetic	alr	↑
		Acetolactate synthase isozyme 1 small subunit	ilvN	↑
	Transcription & translation processes	Uncharacterized protein YncE	yncE	↑
		Ribonucleoside-diphosphate reductase 2 subunit beta	nrdF	↑
		Protein smg	smg	↓
	Transport related	Arsenical pump-driving ATPase	arsA	↑
		Multidrug eDownport protein EmrA	EmrA	↓
	Others	Putative outer membrane porin BglH	bglH	↓
		Protein HdeD	hdeD	↑
		Protein YffB	yffB	↑
		L-2-hydroxyglutarate oxidase LhgO	lhgO	↑
	Others	Uncharacterized protein YdiZ	ydiZ	↑

Time (hours)	Functional Categories	Description	Code	Regulation
2		OriC-binding nucleoid-associated protein	cnu	↑
		Potassium-transporting ATPase B chain	kdpB	↓
		Protein smg	smg	↓
		Isochorismate synthase entC	entC	↑
	Uncharacterized	Uncharacterized protein YjiN	YjiN	↓
		Uncharacterized protein yodD	yodD	↓
		UPF0263 protein yciU	yciU	↑
		Uncharacterized protein ybgJ	ybgJ	↑
		Protein syd	syd	↑
8	Adaptation to aberrant conditions	Protein HdeD	hdeD	↑
		SuperoDownide dismutase [Mn]	sodA	↑
	Amino acid metabolism	Carbamoyl-phosphate synthase small chain	carA	↑
		Carbamoyl-phosphate synthase large chain	carB	↑
		Tryptophan synthase beta chain	trpB	↑
		Aspartate carbamoyltransferase regulatory chain	pyrI	↑
		Tryptophan biosynthesis protein TrpCF	trpCF	↑
		Dipeptide and tripeptide permease A	dtpA	↑
	Iron transport & uptake	Enterobactin synthase component F	entF	↑
		Enterochelin esterase	fes	↑
		Isochorismatase	entB	↑
		Enterobactin synthase component E	entE	↑
		Colicin I receptor	cirA	↑
		Ferrienterobactin receptor	fepA	↑
		Catecholate siderophore receptor Fiu	fiu	↑
		Protein tonB	tonB	↑

Time (hours)	Functional Categories	Description	Code	Regulation
8	Shikimate pathway	Shikimate kinase 2	aroL	↑
	Transcription & translation processes	Uncharacterized protein YncE	yncE	↑
		RNase E specificity factor	csrD	↓
		Stationary phase-inducible protein	csiE	↑
		Universal stress protein D	uspD	↑
		Trp operon repressor	trpR	↑
		50S ribosomal protein L34	rpmH	↑
		Transposase insH for insertion sequence element IS5Y	insH5	↑
	Transport related	Arsenical pump-driving ATPase	arsA	↑
		Multidrug eDownport protein EmrA	EmrA	↓
	Others	Putative outer membrane porin BglH	bglH	↓
		5-methyltetrahydropteroyltriglutamate--homocysteine methyltransferase	metE	↑
		UPF0381 protein yiiS	yiiS	↑
		UPF0324 inner membrane protein YeiH	yeiH	↑
		Electron transport complex subunit RsxC	rsxC	↑
		Regulator of sigma E protease	rseP	↓
		Anaerobic glycerol-3-phosphate dehydrogenase subunit C	glpC	↑
		Lipid II flippase FtsW	ftsW	↑
		Farnesyl diphosphate synthase	ispA	↑
8	Uncharacterized	Uncharacterized protein yfgJ	yfgJ	↓
		Uncharacterized protein ymdF	ymdF	↓
		Uncharacterized protein YuaX	yuaX	↑
		Uncharacterized lipoprotein ygdI	ygdI	↑

↑= Upregulation, ↓=Downregulation, - =Below regulation cut of point

Appendix C

Figure C: Common differentially expressed proteins of *E. coli* (RM109) over time, after glyphosate exposure

Description	Functional Categories	Code	Time		
			2	6	8
Nitrogen regulation protein NR(II)	Energy metabolism	glnL	1.497	0.448	0.410
Respiratory nitrate reductase 1 beta chain		narH	-	0.405	0.360
Respiratory nitrate reductase 1 alpha chain		narG	-	0.460	0.390
Respiratory nitrate reductase 1 gamma chain		narI	-	0.471	0.425
Nitrogen regulation protein NR(II)		glnL	-	0.448	0.410
Nitrite reductase [NAD(P)H] large subunit		nirB	-	0.481	0.357
Nitrite extrusion protein 1. Nitrate/nitrite transporter NarK		narK	-	0.500	0.468
Fe(3+) dicitrate-binding periplasmic protein	Iron uptake & transport	fecB	0.342	0.488	-
Ferrienterobactin receptor		fepA	-	1.650	1.749
Isochorismatase		entB	-	1.655	1.893
Enterobactin synthase component E		entE	-	1.679	1.579
Protein		tonB	-	1.682	2.786
Biopolymer transport protein		exbD	-	1.735	1.968
Catecholate siderophore receptor		fiu	-	2.595	2.494
Colicin I receptor		cirA	2.225	-	1.538
Outer membrane protein W		ompW	-	0.476	0.327
LPS-assembly lipoprotein	Not sure of cluster function	lptE	1.555	-	1.586

Autonomous glycyl radical cofactor	threonine catabolic process, Stress response	grcA	-	0.417	0.353
Anaerobic ribonucleoside-triphosphate reductase	DNA synthesis and repair	nrdD	-	0.446	0.443
HTH-type transcriptional regulator	regulation of transcription, DNA-templated	ascG	-	2.554	0.382
Uncharacterized protein	Uncharacterized protein	ychH	1.587	1.602	
Uncharacterized protein		yncE	-	1.651	2.414

Regulation levels: ≤0.5 =protein expression is down regulated, ≥1.5=protein expression is up regulated.

Appendix D

Figure D: Common differentially expressed proteins of *E. coli* (RM109) over time, after AMPA exposure

Description	Categories	Code	Time		
			2	6	8
Enterobactin synthase component F	Iron uptake and transport	entF	1.596	1.923	1.465
Isochorismatase		entB	1.716	2.300	1.777
Enterobactin synthase component E		entE	1.764	2.520	1.873
2,3-dihydro-2,3-dihydroDownybenzoate dehydrogenase		entA	1.970	1.848	-
Ferrienterobactin receptor		fepA	2.476	2.302	2.033
Enterochelin esterase		fes	1.604	1.909	1.604
Colicin I receptor		cirA	2.388	2.240	2.096
Catecholate siderophore receptor		fiu	3.333	4.406	3.162
Protein FecR		fecR	3.761	2.667	-
Protein tonB		tonB	-	2.886	2.656
Uncharacterized protein	Trancription and translation processes	yncE	1.570	3.992	2.758
Ribonucleoside-diphosphate reductase 2 subunit beta		nrdF	1.688	1.515	-
RNase E specificity factor		csrD	-	0.409	0.352
Stationary phase-inducible protein		csiE	-	1.452	1.590
Shikimate kinase 2	Metabolic intermediate biosynthesis	aroL	-	2.034	2.034
Arsenical pump-driving ATPase	Stress response	arsA	2.164	4.332	-
Multidrug export protein		EmrA	0.321	0.485	-
Universal stress protein D		uspD	-	2.223	2.906
Superoxide dismutase [Mn]		sodA	-	2.213	1.543

Putative outer membrane porin BglH	others	bglH	0.368	0.389	0.202
Protein HdeD		hdeD	1.559	-	2.655
5-methyltetrahydropteroyltriglutamate--homocysteine methyltransferase		metE	-	1.457	1.489
Tryptophan biosynthesis protein TrpCF		trpCF	-	1.685	1.993
UPF0381 protein yiiS		yiiS	-	2.015	2.755
UPF0324 inner membrane protein YeiH		yeiH	-	2.703	3.539

Regulation levels: ≤0.5 =protein expression is down regulated, ≥1.5=protein expression is up regulated.

Appendix E

Table 1E: Protein expression profiles of *E. faecalis* in response to glyphosate after 3 hours

Time (hours)	Functional Categories	Description	Code	Regulation
3	Adaptation to aberrant conditions	glyoxalase	EF_0745	↑
		AhpC/TSA family protein	255970839	↑
		structural protein	EF_1284	↑
		AhpC/TSA family protein	EF_2932	↑
		OsmC/Ohr family protein	EF_0453	↑
	Amino acid metabolism	proline dipeptidase	pepQ-2	↑
		thioredoxin reductase	trxB	↑
		alanine racemase	29375437;Q837J0	↑
	Carbohydrate metabolism	aldose 1-epimerase	galM	↑
		glucosamine-6-phosphate isomerase	nagB	↑
		PTS system	255972095	↑
		glycosyl hydrolase, family 65	256854201	↑
		triosephosphate isomerase	tpiA	↑
		glucose-6-phosphate isomerase	pgi	↑
		phosphoglycerate kinase	pgk	↑
		phosphopentomutase	deoB	↑
		PTS system mannitol-specific IIBC component	EF_0411	↑
		Beta-glucosidase/6-phospho-beta-glucosidase/beta-galactosid ase	295112789	↑
		glycosyl hydrolase	EF_2597	↑
		lactoylglutathione lyase	gloA	↑
		UTP--glucose-1-phosphate uridylyltransferase	galU	↓

Time (hours)	Functional Categories	Description	Code	Regulation
3		Glycosyl hydrolase	EF_0272	↓
		UDP-glucose 4-epimerase	galE-1	↓
		fructose-bisphosphate aldolase	29375743;Q836E7	↑
		PTS system transporter subunit IIA	EF_2603	↑
	Cellular Processes	S-ribosylhomocysteinase	luxS	↑
	Cell wall	N-acetylmuramoyl-L-alanine amidase	EF_1583	↓
		MutT/nudix family protein	EF_2696	↑
	Environmental Information Processing	superoxide dismutase	sodA	↑
		sugar ABC transporter ATP-binding protein	EF_0938	↑
		Peptide methionine sulfoxide reductase	msrA	↑
		phosphate ABC transporter ATP-binding protein	pstB1	↑
	Lipid transport and metabolism	diacylglycerol kinase catalytic subunit	29377128	↑
		diacylglycerol kinase catalytic subunit	EF_0090	↑
	Nucleotide metabolism	uridine kinase	udk	↑
		cytidine deaminase	cdd	↑
		adenine phosphoribosyltransferase	apt	↑
	Transcription and translation processes	DNA-binding response regulator	293400081	↓
		Transcriptional regulator LytR	LytR	↑
		BglG family transcriptional antiterminator	EF_2966	↑
		flavoprotein NrdI	EF_1491	↑
		cold shock protein CspC	cspC	↓
		cold shock domain-contain protein	EF_0781	↓
		transcription antitermination protein NusG	nusG	↑

Time (hours)	Functional Categories	Description	Code	Regulation
3	Transcription and translation processes	HSP70 [Weissella confusa]	86169521	↑
		ATP-dependent Clp protease, ATP-binding protein ClpE	clpE	↑
		transcription elongation factor GreA	greA	↑
		DNA gyrase subunit B	gyrB	↑
		Cold-shock domain family protein	EF_1726	↑
		CoA-binding domain-containing protein	EF_1616	↑
		translation initiation factor IF-1	infA	↑
		antirepressor	EF_2037	↑
		tRNA (uracil-5-)-methyltransferase Gid	trmFO	↑
		aspartyl/glutamyl-tRNA amidotransferase subunit B	gatB	↑
		peptide methionine sulfoxide reductase	229545706	↑
		Alanine--tRNA ligase	alaS	↑
		50S ribosomal protein L14	rplN	↓
		Ribosome maturation factor RimM	rimM	↓
		prolyl-tRNA synthetase	229545199	↑
	Undetermined	lactoylglutathione lyase	227518394	↑
		phnA protein	phnA	↑
		Phage protein	Q835U6	↑
		protein phosphatase 2C	EF_3121	↑
		dipeptidase PepV	EF_0671	↑
		cold-shock domain-contain protein	257867741	↑
		flavoprotein NrdI	EF_1491	↑
		HMG-CoA synthase	256853000	↑
		possible N-isopropylammelide isopropylaminohydrolase	229547733	↑
		cyclophilin type peptidyl-prolyl cis-trans isomerase	29377362;Q82ZZ9	↑
		acetyltransferase	EF_0945	↑
		bacteriophage transcriptional regulator	229545375	↑
		rhodanese family protein	EF_2988	↑
		ribonucleotide reductase stimulatory protein	nrdI	↑

Time (hours)	Functional Categories	Description	Code	Regulation
3		pyrrolidone-carboxylate peptidase	pcp	↑
		glyoxalase	29377078	↑
	Unknown function	dihydrodipicolinate reductase, N-terminal domain protein	300861612	↑
		thioredoxin	229547533	↑
		Uncharacterized protein	EF_0686	↑
		UvrC protein	229546360	↑
		aldose 1-epimerase	227517875	↑
		transcriptional regulator	116492380	↑
		3-oxoacyl-(acyl-carrier-protein) synthase II	229547772	↑
		pyruvate kinase	422867784	↑
	Unknown function	MerR-family transcriptional regulator	182419650	↑
		DegV family protein	229545703	↑
		GcvH family protein	256853854	↑
		PTS system protein	256617129	↑
		aspartyl/glutamyl-tRNA amidotransferase subunit B	261418611	↑
		Penicillin-binding protein 4	H7C6V7	↑
		conserved hypothetical protein	257087405	↓
		hypothetical protein EF1680	EF_1680	↑
		hypothetical protein EF0857	EF_0857	↑
		hypothetical protein EF1180	EF_1180	↑
		hypothetical protein EF1359	EF_1359	↑
		hypothetical protein EF3174	EF_3174	↑
		hypothetical protein HMPREF9475_03583	323694553	↓
		hypothetical protein EF1014	EF_1014	↓
		hypothetical protein EF0742	EF_0742	↓
		hypothetical protein EF1560	EF_1560	↑
		hypothetical protein EF1918	EF_1918	↑
		conserved hypothetical protein	229549574	↑

Time (hours)	Functional Categories	Description	Code	Regulation
3	Hypothetical proteins	hypothetical protein EF2888	29377353;Q830A8	↑
		hypothetical protein EF0665	29375264;Q838A7	↑
		hypothetical protein EF0890	29375475;Q837F2	↑
		hypothetical protein EF1933	EF_1933	↑
		hypothetical protein EF1967	EF_1967	↑
		hypothetical protein EF1247	EF_1247	↑
		hypothetical protein EF1315	EF_1315	↑
		hypothetical protein EF1324	EF_1324	↑
		hypothetical protein EF1772	EF_1772	↑
		conserved hypothetical protein	256853931	↑
		hypothetical protein EF1708	EF_1708	↑
		hypothetical protein EF1490	EF_1490	↑
		hypothetical protein EF2672	EF_2672	↑
		conserved hypothetical protein	256853930	↑
		hypothetical protein EF2909	EF_2909	↑
	Hypothetical proteins	conserved hypothetical protein	256854366	↑
		hypothetical protein EF1915	EF_1915	↑
		hypothetical protein CBB_1070	168184091	↑
		hypothetical protein EF2621	EF_2621	↑
		hypothetical protein EF62_2625	EF62_2625	↑
		hypothetical protein EF2483	EF_2483	↑

↑= Upregulation, ↓=Downregulation, - =Below regulation cut of point

Table 2E: Protein expression profiles of *E. faecalis* in response to glyphosate after 5 hours

Time (hours)	Functional Categories	Description	Code	Regulation
5	Amino acid metabolism	Chorismate synthase	aroC	↓
		serine/threonine protein kinase	EF_3120	↑
		arginine deiminase	arcA	↑
		glutamate racemase	murI	↑
	Environmental Information Processing	iron ABC transporter substrate-binding protein	EF_3082	↑
		Peptide methionine sulfoxide reductase	msrA	↑
	Carbohydrate metabolism	aldose 1-epimerase	galM	↑
		glucosamine-6-phosphate isomerase	nagB	↑
		PTS system	255972095	↑
		glycosyl hydrolase, family 65	256854201	↑
		triosephosphate isomerase	tpiA	↑
		ribose-5-phosphate isomerase A	rpiA	↑
		glyceraldehyde-3-phosphate dehydrogenase	gap-2	↑
		regulatory protein pfoR	EF_0097	↑
	Cellular Processes	major tail protein	EF_1285	↑
		ornithine carbamoyltransferase	arcB	↑
	Cell wall/membrane/envelope biogenesis, Cell motility	N-acetylmuramoyl-L-alanine amidase	EF_1583	↓
		MutT/nudix family protein	EF_2696	↑
		sulfatase	EF_1264	↓
	Transcription & translation processes	DNA-binding response regulator	293400081	↓
		Transcriptional regulator LytR	LytR	↑
		BglG family transcriptional antiterminator	EF_2966	↑

Time (hours)	Functional Categories	Description	Code	Regulation
		flavoprotein NrdI	EF_1491	↑
		DNA-binding response regulator	EF_1050	↑
	Undetermined	diacylglycerol kinase catalytic subunit	EF_0090	↓
		lactoylglutathione lyase	227518394	↑
		phnA protein	phnA	↑
		glyoxalase	EF_0745	↑
		Phage protein	Q835U6	↑
		peptide methionine sulfoxide reductase	229545706	↑
		protein phosphatase 2C	EF_3121	↑
		dipeptidase PepV	EF_0671	↑
		AhpC/TSA family protein	255970839	↑
		cold-shock domain-contain protein	257867741	↑
		flavoprotein NrdI	EF_1491	↑
		N-acetylmuramoyl-L-alanine amidase	EF_0252	↓
		FemAB family protein	EF_2150	↓
5		LysM domain-containing protein	EF_0443	↓
		Glycosyl hydrolase, family 20	EF_0114	↓
		Protease synthase and sporulation negative regulatory protein pai 1	EF_3001	↑
		iron-sulfur-dependent L-serine dehydratase subunit alpha	sdhA-1	↑
		amidase	EF_0737	↑
		cell-envelope associated acid phosphatase	EF_3245	↑
		short chain dehydrogenase/reductase family oxidoreductase	EF_1690	↑
		endoribonuclease L-PSP	EF_0030	↑
		16S rRNA methyltransferase GidB	rsmG	↑
		carbamate kinase	arcC1	↑
		Branched-chain-amino-acid aminotransferase	ilvE	↑
		pheromone binding protein	EF_3041	↑
		dihydroxyacetone kinase	EF_1360	↑

Time (hours)	Functional Categories	Description	Code	Regulation
5		Ferredoxin	fer	↓
		RinA family transcriptional regulator	EF_1283	↑
		enoyl-ACP reductase	fabI	↑
		dihydrofolate reductase	folA	↑
		Holin, putative	EF_1292	↑
	Unknown function	dihydrodipicolinate reductase, N-terminal domain protein	300861612	↑
		secreted lipase	229547510	↓
		lipase	EF_3060	↓
		phosphoenolpyruvate-protein phosphotransferase	422867979	↑
		glyoxalase	227517592	↑
		nucleoside deoxyribosyltransferase	229545228	↑
		PTS system fructose IIA component	300860349	↑
		ROK family sugar kinase	229549746	↑
		cold-shock DNA-binding protein family protein	366052576	↑
		triosephosphate isomerase	229545455	↑
		ethanolamine utilization protein eutM	323489766	↑
		bacterial microcompartments family protein	386317821	↑
		microcompartments protein	158321406	↑
	Hypothetical proteins	conserved hypothetical protein	257087405	↓
		hypothetical protein EF1680	EF_1680	↑
		phnA protein	phnA	↑
		hypothetical protein EF0857	EF_0857	↑
		hypothetical protein EF1180	EF_1180	↑
		hypothetical protein EF1359	EF_1359	↑
		hypothetical protein EF3174	EF_3174	↑
		hypothetical protein EF2965	EF_2965	↑
		hypothetical protein EF0003	EF_0003	↑
		PTS system, hypothetical pentitol phosphotransferase enzyme IIB component	227518189	↓

Time (hours)	Functional Categories	Description	Code	Regulation
		conserved hypothetical protein	227517945	↑
		hypothetical protein EF1135	EF_1135	↑
		conserved hypothetical protein	256854944	↑
		hypothetical protein EF0819	EF_0819	↑

↑= Upregulation, ↓=Downregulation, - =Below regulation cut of point

Appendix F

Table 1F: Protein expression profiles of *E. faecalis* in response to AMPA after 3 hours

Time (hours)	Functional Categories	Description	Code	Regulation
3	ABC transporter	copper transport protein CopZ	copZ	↓
		D-alanine--poly(phosphoribitol) ligase subunit 2	dltC	↓
		Methionyl-tRNA formyltransferase	fmt	↓
		peptide ABC transporter peptide-binding protein	EF_3106	↓
		Peptide methionine sulfoxide reductase MsrA	msrA	↑
	Adaptation to aberrant conditions	flavodoxin	EF_2201	↓
		thioredoxin	trx	↓
		Flavodoxin	EF_2562	↓
		thioredoxin	229547533	↓
		Oxidoreductase, short chain dehydrogenase/reductase family	EF_0076	↑
		thioredoxin reductase	trxB	↑
		chaperonin, 33 kDa	hslO	↑
		Thioredoxin	EF_0730	↑
		NADH oxidase	256853150	↑
		Dps family protein	EF_3233	↑
		chaperonin, 10 kDa	groS	↑
		gls24 protein	EF_0080	↑
		NADH oxidase	nox	↑
		gamma-glutamylcysteine synthetase	384516887	↑

Time (hours)	Functional Categories	Description	Code	Regulation
3	Amino acid metabolism	protein phosphatase 2C	EF_3121	↑
		S-adenosylmethionine synthetase	metK	↑
		methionine sulfoxide reductase B	msrB	↑
		Branched-chain-amino-acid aminotransferase	ilvE	↑
		ornithine carbamoyltransferase	arcB	↑
		arginine deiminase	arcA	↑
		Aminopeptidase C	pepC	↑
		HIT family protein	EF_0687	↑
		aminotransferase AlaT	EF_1314	↑
		cysteine synthase A	229545799	↑
		carbamate kinase	arcC1	↑
		serine hydroxymethyltransferase	glyA	↑
	Carbohydrate metabolism	PTS system sorbitol-specfic transporter subunit IIA	EF_3305	↓
		PTS system protein	256617129	↓
		PTS system, IIBC components	EF_2213	↓
		phosphocarrier protein HPr	ptsH	↓
		phosphoglycerate mutase	gpmA	↑
		deoxyribose-phosphate aldolase	deoC	↑
		phosphoglycerate kinase	pgk	↑
		glyceraldehyde-3-phosphate dehydrogenase	gap-2	↑
		Glucose-1-phosphate thymidylyltransferase	rfbA	↑
		6-phosphofructokinase	pfkA	↑
		dTDP-glucose 4,6-dehydratase	rfbB	↑
		PTS system mannose-specific IID component	EF_0022	↓
		mannose-6-phosphate isomerase	manA	↑
		beta-phosphoglucomutase	pgmB	↑
		glucan 1,6-alpha-glucosidase	EF_1348	↑
		pyruvate dehydrogenase complex E1 component subunit alpha	pdhA	↑
		PTS system IIA component	EF_0461	↓

Time (hours)	Functional Categories	Description	Code	Regulation
3		phosphotransacetylase	pta	↑
		maltose phosphorylase	EF_0957	↑
		6-phosphogluconate dehydrogenase	gnd	↑
		Glycerol kinase	glpK	↑
		aldolase 1 epimerase Lacx	EF_1644	↑
		glycosyl hydrolase, family 65	256854201	↑
		phosphoglucomutase/phosphomannomutase	256763117	↑
		fructokinase	cscK	↑
		malate dehydrogenase, decarboxylating	EF_1206	↑
		Phospho-2-dehydro-3-deoxyheptonate aldolase	EF_1562	↑
		UTP-glucose-1-phosphate uridylyltransferase	galU	↑
		aldose 1-epimerase	229546800	↑
		glucosamine--fructose-6-phosphate aminotransferase	glmS	↓
	Cell growth and death-Cellular Processes	rod shape-determining protein MreC	mreC	↓
		cell division protein [Enterococcus faecalis]	2149908	↑
	Energy metabolism	V-type ATP synthase subunit B	atpB	↑
		V-type ATPase subunit F	EF_1492	↑
		V-type ATPase	atpA	↑
		V-type ATP synthase subunit K	EF_1494	↑
		NAD synthetase	nadE	↑
		NADPH-dependent FMN reductase domain-containing protein	EF_1698	↓
	Lipid metabolism	acyl carrier protein	acpP	↓
		pheromone cAD1 lipoprotein	EF_3256	↓
		secreted lipase	229547510	↓
		lipase	EF_3060	↓
	Lipid metabolism	extracellular protein	EF_0944	↓
		Lipoprotein	EF_1596	↓

Time (hours)	Functional Categories	Description	Code	Regulation
3		autolysin	EF_0799	↓
		diacylglycerol kinase catalytic subunit	EF_0090	↓
		Lipase/acylhydrolase	EF_0169	↑
		D-alanine--D-alanine ligase	ddl	↑
		ACP S-malonyltransferase	fabD	↑
		DegV family protein	EF_1684	↑
	Nucleotide metabolism	CTP synthase	pyrG	↑
		cytidylate kinase	cmk	↑
		adenine phosphoribosyltransferase	apt	↑
		GMP synthase	guaA	↑
		purine nucleoside phosphorylase	deoD-2	↑
		2,3-cyclic-nucleotide 2'phosphodiesterase	EF_2902	↑
		5'-methylthioadenosine/S-adenosylhomocysteine nucleosidase 1	pfs	↑
		Uridylate kinase	pyrH	↑
		uridine kinase	udk	↑
		uracil phosphoribosyltransferase	upp	↑
	Transcription & translation processes	cold shock protein B		↓
		transcriptional regulator	EF_0465	↓
		cold-shock DNA-binding protein family protein	366052576	↓
		cold-shock domain-contain protein	EF_1367	↓
		cold-shock domain-contain protein	EF_2925	↓
		cold shock domain-contain protein	EF_0781	↓
		bacteriophage transcriptional regulator	229545375	↓
		cold shock protein CspC	cspC	↓
		Phage repressor	EF_1277	↓
		RNA polymerase sigma factor RpoD	sigA	↓
		cold shock protein (beta-ribbon, CspA family)	270291153	↓
		DNA-directed RNA polymerase subunit delta	rpoE	↓

Time (hours)	Functional Categories	Description	Code	Regulation
3	Transcription & translation processes	peptidyl-tRNA hydrolase SpoVC	212637895	↓
		cold shock protein [Lactobacillus casei ATCC 334]	116494613	↓
		cold-shock domain-contain protein	257867741	↓
		DNA replication protein, putative	EF_1279	↓
		30S ribosomal protein S18	rpsR	↑
		30S ribosomal protein S11	rpsK	↑
		50S ribosomal protein L19	rplS	↑
		aspartyl/glutamyl-tRNA amidotransferase subunit B	gatB	↑
		Cell division protein ZapA	EF_1402	↑
		glutamyl-tRNA(Gln) amidotransferase subunit A	gatA	↑
		30S ribosomal protein S13	rpsM	↑
		cell division protein FtsZ	ftsZ	↑
		30S ribosomal protein S12	rpsL	↑
		30S ribosomal protein S2	rpsB	↑
		transcription antitermination protein NusB	nusB	↑
		DNA-binding protein HU	hup	↑
		DNA-binding response regulator	EF_1050	↑
		50S ribosomal protein L14	rplN	↑
		glyceraldehyde 3-phosphate dehydrogenase	gap-1	↑
		50S ribosomal protein L21	rplU	↑
		30S ribosomal protein S19	rpsS	↑
		50S ribosomal protein L23	rplW	↑
		DEAD/DEAH box helicase	cshA	↑
		translation initiation factor IF-2	infB	↑
		50S ribosomal protein L10	rplJ	↑
		50S ribosomal protein L15	rplO	↑
		50S ribosomal protein L3	rplC	↑
		30S ribosomal protein S3	rpsC	↑
		Chaperone protein DnaJ	dnaJ	↑
		50S ribosomal protein L4	rplD	↑

Time (hours)	Functional Categories	Description	Code	Regulation
3	Transcription & translation processes	30S ribosomal protein S1	EF_1548	↑
		50S ribosomal protein L20	rplT	↑
		50S ribosomal protein L13 [Streptococcus infantis SK1302]	309798666	↑
		30S ribosomal protein S7	rpsG	↑
		30S ribosomal protein S4	rpsD	↑
		50S ribosomal protein L22	rplV	↑
		S-adenosylmethionine--tRNA ribosyltransferase-isomerase	queA	↑
		50S ribosomal protein L17	rplQ	↑
		50S ribosomal protein L1	rplA	↑
		Tyrosine--tRNA ligase 2	tyrS2	↑
		30S ribosomal protein S10	rpsJ	↑
		arginyl-tRNA synthetase	argS	↑
		translation initiation factor IF-3	infC	↑
		seryl-tRNA synthetase	serS2	↑
		transcriptional repressor CodY	codY	↑
		ATP-dependent protease ATP-binding protein HslU	hslU	↑
		isoleucyl-tRNA synthetase	ileS	↑
		30S ribosomal protein S16	rpsP	↑
		elongation factor G	fusA	↑
		Probable transcriptional regulatory protein EF_0663	EF_0663	↑
		30S ribosomal protein S8	rpsH	↑
		glycyl-tRNA synthetase subunit alpha	glyQ	↑
		glutamyl-tRNA synthetase	gltX	↑
		phenylalanyl-tRNA synthetase subunit beta	pheT	↑
		Hemolysin A	tlyA	↓
		chaperonin, 60 kDa	groL	↑
		Endoribonuclease YbeY	ybeY	↑
		Peptide chain release factor 1	prfA	↑
		ribosomal subunit interface protein	yfiA	↑
		NrdI protein	nrdI	↓

Time (hours)	Functional Categories	Description	Code	Regulation
		peptide deformylase	def	↑
	Quorum sensing	Protein translocase subunit SecA	secA	↓
		S-ribosylhomocysteinase	luxS	↑
	Others	fumarate reductase flavoprotein subunit	EF_2556	↓
		major tail protein	EF_1285	↓
		N-acetylmuramoyl-L-alanine amidase	EF_1583	↓
		acyl carrier protein	229547469	↓
		ErfK/YbiS/YcfS/YnhG family protein	EF_2860	↓
		acylphosphatase	255972128	↓
		GcvH family protein	EF_2500	↓
		Clpx	256961587	↓
		N-acetylmuramoyl-L-alanine amidase	EF_2367	↓
3		penicillin-binding protein 1A	EF_1148	↓
		LysM domain protein	300860323	↓
		flavoprotein NrdI	EF_1491	↓
		flotillin	227518585	↓
		LysM domain-containing protein	EF_1546	↓
		endocarditis specific antigen	EF_2076	↑
		DegV family protein	229545703	↑
	Others	malate dehydrogenase (oxaloacetate-decarboxylating) (NADP(+))	229546116	↑
		1,4-Dihydroxy-2-naphthoyl-CoA synthase	menB	↑
		transketolase, partial	256762519	↑
		oligoendopeptidase F,plasmid	pepF	↑
		dipeptidase PepV	EF_0671	↑
		2-dehydropantoate 2-reductase	EF_2445	↑
3	Others	cation transporter E1-E2 family ATPase	EF_1938	↓
		D-alanine--poly(phosphoribitol) ligase subunit 1	dltA	↑
		signal recognition particle protein	ffh	↑

Time (hours)	Functional Categories	Description	Code	Regulation
3		40.1 kDa homolog, partial [Streptococcus pyogenes]	6456493	↑
		ribose-phosphate pyrophosphokinase	prs2	↑
		cyclophilin type peptidyl-prolyl cis-trans isomerase	EF_2898	↑
		NADH-dependent butanol dehydrogenase	EF_0194	↑
		oxidoreductase, zinc-binding	EF_1671	↑
		DegV family protein	EF_1191	↑
	Hypothetical proteins	conserved hypothetical protein	229545479	↓
		PTS system, hypothetical pentitol phosphotransferase enzyme IIB component	227518189	↓
		hypothetical protein EF1318	EF_1318	↓
		hypothetical protein EF1934	EF_1934	↓
		hypothetical protein EF2965	EF_2965	↓
		conserved hypothetical protein	256853931	↓
		hypothetical protein EF1315	EF_1315	↓
		Uncharacterized protein	EF_1014	↓
		hypothetical protein EF1915	EF_1915	↓
		hypothetical protein EF1286	EF_1286	↓
		Uncharacterized protein	EF_1689	↓
		hypothetical protein EF1680	EF_1680	↓
		hypothetical protein EF2923	EF_2923	↓
		hypothetical protein EF0708	EF_0708	↓
		conserved hypothetical protein	229546500	↓
	Hypothetical proteins	hypothetical protein EF0394	EF_0394	↓
		hypothetical protein EF2621	EF_2621	↓
		Uncharacterized protein	EF_0830	↓
		hypothetical protein EF3173	EF_3173	↓
		hypothetical protein EF0998	EF_0998	↓
		hypothetical protein CBB_1070	168184091	↓
		conserved hypothetical protein	229547401	↓
		conserved hypothetical protein	256853930	↓

Time (hours)	Functional Categories	Description	Code	Regulation
3	Hypothetical proteins	conserved hypothetical protein	229545698	↓
		conserved hypothetical protein	256852892	↓
		hypothetical protein EF0681	EF_0681	↑
		hypothetical protein EF1324	EF_1324	↑
		hypothetical protein EF2458	EF_2458	↑
		conserved hypothetical protein	229549574	↑
		hypothetical protein EF1580	EF_1580	↑
		Conserved domain protein	EF_2697	↑
		hypothetical protein EF0665	EF_0665	↑
		Uncharacterized protein	EF_0798	↑
		hypothetical protein HMPREF0345_1298	229549086	↑
		hypothetical protein EF2866	EF_2866	↑
		hypothetical protein EF1753	EF_1753	↑
		hypothetical protein EF3174	EF_3174	↑
		hypothetical protein EF1967	EF_1967	↑
		conserved hypothetical protein	256619247	↑
		PspC domain-containing protein	EF_1752	↑
		hypothetical protein EF1047	EF_1047	↑
		cold-shock domain-contain protein	257869999	↓
		MerR-family transcriptional regulator	182419650	↓
		cold shock protein	336396270	↓
		sex pheromone cAD1	256854906	↓
		UvrC protein	229546360	↓
		GcvH family protein	256853854	↓
		basic membrane protein family	256855139	↓
		ribosomal protein L13	282883113	↓
		Uncharacterized protein	EF_1250	↓
		DNA-3-methyladenine glycosylase 1	312870167	↓
		Uncharacterized protein	EF_1947	↑
	Hypothetical proteins	cystathionine gamma-synthase/cystathionine beta-lyase	255970905	↑

Time (hours)	Functional Categories	Description	Code	Regulation
		Uncharacterized protein	EF_2215	↑
		Uncharacterized protein	EF_2904	↑

↑= Upregulation, ↓=Downregulation, - =Below regulation cut of point

Table 2F: Protein expression profiles of *E. faecalis* in response to AMPA after 5 hours

Time (hours)	Functional Categories	Description	Code/Accession nr	Regulation
5	Adaptation to aberrant conditions	flavodoxin	EF_2201	↓
		thioredoxin	trx	↓
		Flavodoxin	EF_2562	↓
		thioredoxin	229547533	↓
		Oxidoreductase, short chain dehydrogenase/reductase family	EF_0076	↑
		thioredoxin reductase	trxB	↑
		chaperonin, 33 kDa	hslO	↑
		Thioredoxin	EF_0730	↑
		NADH oxidase	256853150	↑
		alkyl hydroperoxide reductase subunit C	ahpC	↓
	Amino acid metabolism	protein phosphatase 2C	EF_3121	↑
		S-adenosylmethionine synthetase	metK	↑
		methionine sulfoxide reductase B	msrB	↑
		Branched-chain-amino-acid aminotransferase	ilvE	↑
		ornithine carbamoyltransferase	arcB	↑
		arginine deiminase	arcA	↑
		threonine synthase	thrC	↑
		aminotransferase	226313771	↓
5	Carbohydrate metabolism	PTS system sorbitol-specfic transporter subunit IIA	EF_3305	↓
		PTS system protein	256617129	↓
		PTS system, IIBC components	EF_2213	↓
		phosphocarrier protein HPr	ptsH	↓
		phosphoglycerate mutase	gpmA	↓
		phosphoglycerate kinase	pgk	↓
		phosphoglycerate kinase		↓

Time (hours)	Functional Categories	Description	Code/Accession nr	Regulation
5	Carbohydrate metabolism	phosphoglycerate mutase		↓
		pyruvate kinase	pyk	↓
		glucosamine--fructose-6-phosphate aminotransferase	29376660;Q832R7	↓
		1-phosphofructokinase	fruK-2	↓
		formate acetyltransferase	pflB	↓
		HAD superfamily hydrolase	EF_3158	↓
		PTS system transporter subunit IIA	EF_2438	↓
		PTS system sorbitol-specfic transporter subunit IIA		↓
		PTS system transporter subunit IIA	29377088;Q831B1	↓
		ABC transporter ATP-binding protein	EF_0178	↓
		deoxyribose-phosphate aldolase	deoC	↑
		glyceraldehyde-3-phosphate dehydrogenase	gap-2	↑
		Glucose-1-phosphate thymidylyltransferase	rfbA	↑
		6-phosphofructokinase	pfkA	↑
	Carbohydrate metabolism	dTDP-glucose 4,6-dehydratase	rfbB	↑
		maltose phosphorylase	EF_0957	↑
		6-phosphogluconate dehydrogenase	gnd	↑
		aldolase 1 epimerase Lacx	EF_1644	↑
		UTP-glucose-1-phosphate uridylyltransferase	galU	↑
		acetate kinase	ackA	↑
		glucose-6-phosphate isomerase	pgi	↑
		PTS system transporter subunit IIABC	EF_0958	↑
		phosphoenolpyruvate-protein phosphotransferase enzyme I	ptsI	↑
	Cell motility(Cellular Processes)	major tail protein	29375854;Q835T8	↓
		N-acetylmuramoyl-L-alanine amidase	29376145;Q834Q8	↓
		N-acetylmuramoyl-L-alanine amidase	29376865;Q831X9	↓
		Adapter protein MecA	mecA	↓
		UTP-glucose-1-phosphate uridylyltransferase	29376295;Q834C3	↑
		major tail protein	EF_1285	↓

Time (hours)	Functional Categories	Description	Code/Accession nr	Regulation
5				
	Lipid metabolism	acyl carrier protein	acpP	↓
		pheromone cAD1 lipoprotein	EF_3256	↓
		secreted lipase	229547510	↓
		lipase	EF_3060	↓
	Lipid metabolism	extracellular protein	EF_0944	↓
		Lipoprotein	EF_1596	↓
		autolysin	EF_0799	↓
		Lipase/acylhydrolase	EF_0169	↓
		acyl carrier protein		↓
		enoyl-ACP reductase	fabI	↓
		D-alanine--D-alanine ligase	ddl	↑
		DegV family protein	EF_1684	↑
		diacylglycerol kinase catalytic subunit	EF_2644	↑
	Nucleotide metabolism	5'-methylthioadenosine/S-adenosylhomocysteine nucleosidase 1	pfs	↑
		Uridylate kinase	pyrH	↑
		uridine kinase	udk	↑
		uracil phosphoribosyltransferase	upp	↑
		peptidase T	pepT	↓
		dihydroorotate dehydrogenase 1A	pyrDA	↑
	Oxidative phosphorylation/energy metabolism	V-type ATP synthase subunit B	atpB	↑
		V-type ATPase subunit F	EF_1492	↑
		V-type ATPase	atpA	↑
		V-type ATP synthase subunit K	EF_1494	↑
		pyridine nucleotide-disulfide family oxidoreductase	EF_2055	↓
		F0F1 ATP synthase subunit alpha	atpA	↓
		alpha-glycerophosphate oxidase	EF_1928	↑

Time (hours)	Functional Categories	Description	Code/Accession nr	Regulation
		cold shock protein B	347522027	↓
		transcriptional regulator	EF_0465	↓
		cold-shock DNA-binding protein family protein	366052576	↓
	Transcription & translation processes	cold-shock domain-contain protein	EF_1367	↓
		cold-shock domain-contain protein	EF_2925	↓
		cold shock domain-contain protein	EF_0781	↓
		bacteriophage transcriptional regulator	229545375	↓
		cold shock protein CspC	cspC	↓
		Phage repressor	EF_1277	↓
	Transcription & translation processes	RNA polymerase sigma factor RpoD	sigA	↓
		cold shock protein (beta-ribbon, CspA family)	270291153	↓
		DNA-directed RNA polymerase subunit delta	rpoE	↑
		30S ribosomal protein S18	rpsR	↑
		30S ribosomal protein S11	rpsK	↓
		50S ribosomal protein L19	rplS	↑
		aspartyl/glutamyl-tRNA amidotransferase subunit B	gatB	↑
		Cell division protein ZapA	EF_1402	↑
		glutamyl-tRNA(Gln) amidotransferase subunit A	gatA	↑
		30S ribosomal protein S13	rpsM	↓
		cell division protein FtsZ	ftsZ	↑
		30S ribosomal protein S12	rpsL	↓
		30S ribosomal protein S2	rpsB	↓
		transcription antitermination protein NusB	nusB	↑
		DNA-binding protein HU	hup	↑
		DNA-binding response regulator	EF_1050	↑
		50S ribosomal protein L14	rplN	↓
		glyceraldehyde 3-phosphate dehydrogenase	gap-1	↑
		Peptide chain release factor 1	prfA	↑
5	Transcription & translation processes	peptide deformylase	def	↑
		Phage repressor		↓

Time (hours)	Functional Categories	Description	Code/Accession nr	Regulation
	Transcription & translation processes	30S ribosomal protein S12		↓
		transcriptional regulator		↓
		cold-shock domain-contain protein		↓
		cold shock protein B	347522028	↓
		recombinase A	recA	↓
		cold-shock domain-contain protein		↓
		cold shock domain-contain protein		↓
		DNA-directed RNA polymerase subunit omega	rpoZ	↓
		DNA-directed RNA polymerase subunit beta	rpoB	↓
		cold shock protein CspC		↓
		dnak protein	dnaK	↓
		30S ribosomal protein S13		↓
		30S ribosomal protein S2		↓
		50S ribosomal protein L14		↓
		30S ribosomal protein S18		↑
		50S ribosomal protein L30	rpmD	↑
	Others	fumarate reductase flavoprotein subunit	EF_2556	↓
		N-acetylmuramoyl-L-alanine amidase	EF_1583	↓
		acyl carrier protein	229547469	↓
		ErfK/YbiS/YcfS/YnhG family protein	EF_2860	↓
		acylphosphatase	255972128	↓
		GcvH family protein	EF_2500	↓
		Clpx	256961587	↓
		N-acetylmuramoyl-L-alanine amidase	EF_2367	↓
		penicillin-binding protein 1A	EF_1148	↓
		glucosamine--fructose-6-phosphate aminotransferase	glmS	↓
		LysM domain protein	300860323	↓
		flavoprotein NrdI	EF_1491	↓
		flotillin	227518585	↓

Time (hours)	Functional Categories	Description	Code/Accession nr	Regulation
5		LysM domain-containing protein	EF_1546	↓
		aldose 1-epimerase	229546800	↑
		endocarditis specific antigen	EF_2076	↑
		Aminopeptidase C	pepC	↑
		Glycerol kinase	glpK	↑
		glycosyl hydrolase, family 65	256854201	↑
		HIT family protein	EF_0687	↑
		DegV family protein	229545703	↑
		malate dehydrogenase (oxaloacetate-decarboxylating) (NADP(+))	229546116	↑
		phosphoglucomutase/phosphomannomutase	256763117	↑
		1,4-Dihydroxy-2-naphthoyl-CoA synthase	menB	↑
		aminotransferase AlaT	EF_1314	↑
		chaperonin, 10 kDa	groS	↑
		transketolase, partial	256762519	↑
		gls24 protein	EF_0080	↑
		oligoendopeptidase F,plasmid	pepF	↑
		NAD synthetase	nadE	↑
		fructokinase	cscK	↑
		ribosomal subunit interface protein	yfiA	↑
		malate dehydrogenase, decarboxylating	EF_1206	↑
		ACP S-malonyltransferase	fabD	↑
		cysteine synthase A	229545799	↑
		NADH oxidase	nox	↑
		dipeptidase PepV	EF_0671	↑
		carbamate kinase	arcC1	↑
		2-dehydropantoate 2-reductase	EF_2445	↑
		pheromone cAD1 lipoprotein		↓
		Flavodoxin	29377699;Q82Z23	↓
		autolysin	Q831E9;29377049	↓
		acetyltransferase	29375389;P37710	↓

Time (hours)	Functional Categories	Description	Code/Accession nr	Regulation
5	Others	GcvH family protein	29375529;Q836Z8	↓
		fumarate reductase flavoprotein subunit	29376992;Q831K4	↓
		dihydroxyacetone kinase	29377044;Q831F4	↓
		PTS system, IIBC components	Q832L4	↓
		Lipase/acylhydrolase	Q839J7	↓
		LysM domain-containing protein	EF_0443	↓
		pheromone binding protein	EF_0063	↓
		flavodoxin	29376709;Q832M5	↓
		Lipoprotein	Q834P7	↓
		dihydrolipoamide acetyltransferase	aceF	↓
		penicillin-binding protein 1A	29375724;Q836G5	↓
		thioredoxin	29375972;Q835H3	↓
		phosphocarrier protein HPr	29375306;P07516	↓
		Uridylate kinase	Q831V2	↓
		30S ribosomal protein S11	Q839E0;29374877	↓
		ErfK/YbiS/YcfS/YnhG family protein	29377328;Q830C9	↓
		Aminopeptidase C	Q838Y2	↓
		flavoprotein NrdI	29376058;Q834Y7	↓
		Dihydrofolate reductase	folA	↓
		endoribonuclease L-PSP	EF_0030	↓
		LysM domain-containing protein	29376109;Q834T8	↓
	Others	glyceraldehyde 3-phosphate dehydrogenase	29376091;Q834V6	↑
		ATP-dependent Clp protease, ATP-binding protein ClpB	clpB	↑
		D-alanine--D-alanine ligase		↑
		thermostable carboxypeptidase 1	EF_1153	↑
		Conserved domain protein	Q830S6	↑
		large conductance mechanosensitive channel protein	mscL	↑
		Metallo-beta-lactamase	EF_2432	↑
		conserved hypothetical protein	229545479	↓

Time (hours)	Functional Categories	Description	Code/Accession nr	Regulation
5	Hypothetical proteins	PTS system, hypothetical pentitol phosphotransferase enzyme IIB component	227518189	↓
		hypothetical protein EF1318	EF_1318	↓
		hypothetical protein EF1934	EF_1934	↓
		hypothetical protein EF2965	EF_2965	↓
		conserved hypothetical protein	256853931	↓
		hypothetical protein EF1315	EF_1315	↓
		Uncharacterized protein	EF_1014	↓
		hypothetical protein EF1915	EF_1915	↓
		hypothetical protein EF1286	EF_1286	↓
		Uncharacterized protein	EF_1689	↓
		hypothetical protein EF1680	EF_1680	↓
		hypothetical protein EF2923	EF_2923	↓
		hypothetical protein EF0708	EF_0708	↓
		conserved hypothetical protein	229546500	↑
		hypothetical protein EF0394	EF_0394	↓
		hypothetical protein EF2621	EF_2621	↓
		Uncharacterized protein	EF_0830	↓
		hypothetical protein EF3173	EF_3173	↓
		hypothetical protein EF0998	EF_0998	↑
		hypothetical protein EF0681	EF_0681	↑
		hypothetical protein EF1324	EF_1324	↑
		hypothetical protein EF2458	EF_2458	↑
		conserved hypothetical protein	229549574	↑
		hypothetical protein EF1580	EF_1580	↑
		Conserved domain protein	EF_2697	↑
		hypothetical protein EF0665	EF_0665	↑
		Uncharacterized protein	EF_0798	↑
		hypothetical protein HMPREF0345_1298	229549086	↑
		hypothetical protein EF2866	EF_2866	↑
		hypothetical protein EF1753	EF_1753	↑

Time (hours)	Functional Categories	Description	Code/Accession nr	Regulation
5	Hypothetical proteins	conserved hypothetical protein	ahpC	↓
		hypothetical protein EF2965		↓
		hypothetical protein EF1915		↓
		conserved hypothetical protein	229545480	↓
		hypothetical protein EF1934		↓
		hypothetical protein EF1315		↓
		conserved hypothetical protein	256853932	↓
		Uncharacterized protein	EF_2157	↓
		Uncharacterized protein	Q837K7;29375419	↓
		hypothetical protein EF3173	29377623;Q82Z98	↓
		hypothetical protein EF1286	29375855;Q835T7	↓
		hypothetical protein EF2923	29377387;Q82ZX5	↓
		hypothetical protein EF2621	29377106;Q830Z4	↓
		hypothetical protein EF1680	29376234;Q834H3	↓
		hypothetical protein EF1318	29375886;Q835Q8	↓
		Uncharacterized protein	Q834G5	↓
		hypothetical protein EF0394	29375030;H7C6X7	↓
		hypothetical protein EF0708	29375305;Q837W8	↓
		hypothetical protein EF1135	EF_1135	↓
		Uncharacterized protein	Q836U3;29375595	↓
		hypothetical protein EF2909	EF_2909	↓
		conserved hypothetical protein	257087406	↓
		hypothetical protein EF1794	EF_1794	↑
		hypothetical protein EF0998		↑
		hypothetical protein EF1180	EF_1180	↑
		hypothetical protein EF1560	EF_1560	↑
	Hypothetical proteins	hypothetical protein EF0742	EF_0742	↑
		secreted lipase	229547510	↓

Time (hours)	Functional Categories	Description	Code/Accession nr	Regulation
5	Unknown function	cold-shock DNA-binding protein family protein	366052576	↓
		lipase	29377518;H7C6Z1	↓
		PTS system protein	256617129	↓
		PTS system, hypothetical pentitol phosphotransferase enzyme IIB component	227518189	↓
		extracellular protein	29375528;Q836Z10	↓
		flotillin	227518586	↓
		cold shock protein (beta-ribbon, CspA family)	270291154	↓
		acylphosphatase	255972128	↓
		LysM domain protein	300860323	↓
		ClpX	256961588	↓
		Dihydroxyacetone kinase, phosphotransfer subunit	EF_1359	↓
		glycosyl hydrolase, family 65	256854826	↓
		copper transport protein CopZ	229548283	↓
		phnA protein	phnA	↑
		thioredoxin	229547533	↓
		PTS system, IIA component	227519845	↓
		acyl carrier protein	229547469	↓
		glyoxalase	227517592	↓
		Glucose-1-phosphate thymidylyltransferase	H7C715	↑
		bacteriophage transcriptional regulator	229545376	↓

↑= Upregulation, ↓=Downregulation, - = Below regulation cut of point.