Antimicrobial Mechanism of Action of Ultrasound Technology: A Macroscopic, Genetic and Transcriptomic Perspective for the Disinfection of (Waste)Water.

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DEDICATION

To
my parents,

Godwin and Josephine,

amare et sapere vix deo conceditur

"Most people say that it is the intellect which makes a great scientist. They are wrong: it is character"

Albert Einstein (1879 – 1955).

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ABBREVIATIONS AND ACRONYMS

Abbreviation	Definition
1XTAE	1X Tris base Acetic acid and EDTA Buffer
ABI	Applied Biosystems
API	Active pharmaceutical incipients
BLAST	Basic Local Alignmnt Search Tool
BOD5	5- day Biological Oxygen Demand
C	Celsius
CFU	Colony Forming Unit
COD	Chemical Oxygen Demand
DEG	Differentially expressed Gene
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic Acid
DSB	Double Stranded breaks
EC	European Commission
EC_{50}	half-maximal effective concentration
EPA	Environment Protection Agency
EU	European Union
FAO	Food and Agriculture Organisation
FDR	False discovery rate
GABA	Gamma-aminobutyrate
GAD	Glutamate decarboxylase
GO	Gene Ontology
INRA	Institut national de la recherche agronomique
IPMP	Integrated Predictive Modelling Programme
ISO	International Standard Organisation
KEGG	Kyoto Encyclopedia of Genes and Genomes
MEPA	Malta Environment and Planning Authority
MRA	Malta Resource authority
NCBI	National Center for Biotechnology Information
NGS	Next Generation Sequencing
NIG	National Institute of Genetics
dNTPs	deoxyribonucleotide triposphate
PCA	Principal Component Analysis
PCR	Polymerase Chain Reaction
PPCP	Pharmaceutical and Personal Care Products
PT	Pre-Treated Cell

Abbreviation	Definition
QC	Quality Control
RMSE	Root Mean Square Error
RNA	Ribonuclein acid
RO	Reverse Osmosis
RNA-SEQ	RNA sequencing
SD	Secure Digital
SNP	Single nucleotide polymorphisms
SPRI beads	Solid phase reversiblie immobilisation beads
STP	Sewage Treatment Plant
TCA	The Citric acid Cycle
TE	Tris EDTA Buffer
THM	Trihalomethanes
TSA	Trypticasein Soy Agar
TSB	Trypticasein Soy Broth
TSB-D	Trypticasein Soy Broth without dextrose
UI	Ultrasound Intensity
UK	United Kingdom
UPGMA	Unweighted pair group method with arithmetic mean
US	Ultrasound
US1	Ultrasound Treatment 1
US2	Sequential Ultrasound treatment
USA	United States of America
USB	Univeral Serial Bus
UV	Ultraviolet
WHO	World Health Organisation
WT	Wild Type strain

Bibliography of published material from this research

Peer-reviewed publications

Spiteri D, Scerri C, Valdramidis V (2015) The current situation for the water sources in the Maltese Islands. *Malta Journal of Health Science*, 1(2), 22–25. doi: 10.14614/WATERMALTA.2.1.22

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Spiteri, D., Chot-Plassot, C., Karatzas, K. A., Scerri, C., & Valdramidis, V. P. (2020) Assessing microbial effects of wild-type *Escherichia coli* K-12 using high-power ultrasound by Whole Genome Sequencing (submitted)

Chapter 1 Introduction and thesis structure

1.1 Introduction

Water is essential for life, it is important for the economy and also plays a role in the climate regulation cycle (MOCCAE, 2016). This is why the European Commission has just concluded the re-cast of the Drinking Water Directive (December 2019), which would then be implemented by member states by end of 2020. This new Directive would require EU member states to improve the distribution of safe water, reduce water loss due to leaks, and thus improve consumer opinion on general water quality (Dantin and Hansen, 2020).

Water resources refer to the freshwater available for use in a territory and includes surface water, such as rivers and groundwater (MOCCAE, 2016). Within the European Union, the United Kingdom, Sweden, France and Germany, have the highest amount of fresh water resources, however on the other spectrum of the scale, countries like Poland, Czech Republic, Cyprus and Malta are the most water stressed countries, with an annual water resource of much less than the average 1,700 m^3 of water per inhabitant (WWAP, 2012).

Water stress affects one third of the EU territory all year round (EC, 2012b). Water over-abstraction, particularly for irrigation purposes and industrial use, is one of the main threats to the water environment. Climate change has also played a major role in such water scarcity, and it is expected that by 2030, about half of the EU river basis, will be effected (Vanneuville *et al.*, 2012). As an alternative water supply option, water reuse has emerged as an interesting concept that got EU's attention (EC, 2012b).

To address the water scarcity issue, the European Commission took the initiative to create a report, to highlight actions, that the EU plans to take (EC, 2012a). Among these actions, the EU plans to develop additional water supply options, amongst them water reuse. Thus, this increased the need to understand the mechanisms of water treatment, and the response of bacteria from disinfectants.

Most European countries use chlorination as the primary method of disinfection, as it destroys target organisms by oxidising cellular material (EPA, 1999). Chlorine can easily be produced and is relatively cheap, while also is highly effective in killing pathogens. However, this can also lead to disinfection by-products, which can be highly carcinogenic (Villanueva *et al.*, 2015). For this reason, alternative methods of disinfection have long been considered, such as ultrasound, which has recently received wide attention in water and wastewater treatment and environmental remediation areas, including the application for disinfection purposes (Han *et al.*, 2013; Cesaro and Belgiorno, 2016)

Studies on the mode of action of disinfection technologies such as plasma, ozone (both producing reactive radicals) and nanomaterials have been carried out in the past (Šeputiene *et al.*, 2006; Yamamoto *et al.*, 2009; Groeneveld *et al.*, 2010; Yadav N, 2014). Nonetheless, not much is yet known, on how this mechanism works, and thus it is imperative to unravel further the mechanism of action of such technologies and specifically of ultrasound, to ensure the production of safe and stable ultrasound processed water.

1.1.1 Objectives

The overall objective of this Ph.D thesis is the characterisation of the mechanisms of the microbial responses of *E. coli* K-12 and selected *E. coli* K-12 isogenic mutants under ultrasound treatments. The aim of this work is to identify the genes that play a crucial role on the stress response of wild types and their specific responses incorporated in the ultrasonic mechanism of action. This will allow the optimisation of this technology and its further use on the disinfection of wastewater. The specific objectives of the proposed research project are the following:

- Perform quantitative assessments to fully describe the microbial inactivation and recovery dynamics of selected faecal indicators during ultrasound processing and post-processing.
- Characterise the major effect of sonication stress on *Escherichia coli* mutants that miss important genes involved in general stress tolerance. The role of these genes in the protection or sensitivity against ultrasound generated radicals are also assessed.
- Identify and quantify the specific regulons, i.e., genomic biomarkers, which play an important role in the microbial stress responses during ultrasound processing with transcriptomic analysis.
- Understand the sequence of sonication effects contributing to change in transcription of RNA genes and mutations in the DNA, which may lead to different protein expressions.

A summary of each chapter is detailed below:

Chapter 2: Literature review

Wastewater reuse could be one of the major solutions to water problems, as it can make available large quantities of previously rejected water, for both recreational and irrigation applications. Measures to ensure a sustainable water management process is required to develop integrated approaches to water resource management by wastewater reclamation and reuse implementation. Such reuse implementation will require the application and optimisation of technologies that can be used for the disinfection and purification of sewage and sewage effluents. The current chapter summarises the most common disinfection and purification technologies highlighting their advantages and disadvantages. Additionally, parameters for assessing the efficiency of these technologies, such as microbial indicators, suspended solids, nitrates, emerging pollutants, are showcased. The presented techniques are also described in

relation to their mode of action and their (potential) applications. A comparative commentary of these technologies is provided focusing on issues related to their applicability and efficiency.

Part of this work has been published in a peer-reviewed journal

Spiteri D, Scerri C, Valdramidis V (2015) The current situation for the water sources in the Maltese Islands. *Malta Journal of Health Science*, 1(2), 22–25. doi: 10.14614/WATERMALTA.2.1.22

Chapter 3: Ultrasound processing of liquid system(s) and its antimicrobial mechanism of action

Ultrasound creates cavitation phenomena, resulting in the formation of several free radicals, namely OH• and H•, due to the breakdown of the H_2O molecule. These radicals affect the cellular integrity of the bacteria, causing the inactivation of several processes, and thus it is important to unravel the mechanism of action of this technology. This chapter presented results in the application and mechanism of action of ultrasound technology as a means of disinfection by acoustic cavitation. Sterile water and synthetic waste water inoculated with different mutants of *E. coli* K12 strains containing deletions in genes, affecting specific functional properties of *E. coli*, are studied. These are *dnak soxR, soxS, oxyR, rpoS, gadA/gadB, gadC* and *yneL*. *E. coli* K-12 Δ*oxyR*, appear to be more resistant to the treatment together with *gadW, gadX, gabT and gabD*, whereas the mutant *E. coli* K-12 Δ*dnaK* are more sensitive with approximately 2.5 log (CFU/mL) reduction in comparison to their isogenic wild type *E. coli* K-12. This indicates that the *dnaK* gene participates in general stress response and more specifically to hyperosmotic stress. The other *E. coli* deleted genes tested (*soxS, rpoS, gadB, gadC, yneL*) do not appear to be involved in protection of microbial cells against ultrasound.

Part of this work has been published in a peer-reviewed journal

Spiteri, D., Chot-Plassot, C., Sclear, J., Karatzas, K. A., Scerri, C., & Valdramidis, V. P. (2017). Ultrasound processing of liquid system(s) and its antimicrobial mechanism of action. *Letters in Applied Microbiology*, *65*(4), 313–318. https://doi.org/10.1111/lam.1277

Chapter 4: *E. coli* K-12 transcriptomics for assessing the mechanism of action of high-power ultrasound

Bacterial induced resistance following decontamination/disinfection treatments has become a threat in both industrial and medical fields. For this reason, the mechanisms of action for bacterial evolution involving exposure to stress factors has long been investigated. This chapter aims at assessing the effects of ultrasound on *E. coli* K-12 MG1655 and its isogenic mutant *gadW*, by analysing their transcriptome differences between primary and secondary sequential treatments using RNA-SEQ. Any developed protection for cells between different generations is identified. According to our results, 1825 genes in all conditions, in the control are expressed, playing different roles in the cell. The expression of these genes is associated with DNA damage, cell membrane integrity and also metabolic effects. The studied strains also show different Differential Expressed Genes (DEGs), with some genes being directly responsible for defence mechanisms, while others are playing an indirect effect due to cell damage. A gradual decrease in expression of the genes, as we move from just one cycle of ultrasound treatment to sequential treatment, is evident from a heat map analysis of the results. Overall, *E. coli* K-12 builds a self-protection mechanism by increasing the expression of genes involved in the respiration for increased growth, production of flagellum and pili. It can be concluded that high power ultrasound is a technology that triggers several different defence mechanisms which directly link to Ultrasound.

Part of this work has been submitted for publication in a peer-reviewed journal

Spiteri, D., Chot-Plassot, C., Karatzas, K. A., Scerri, C., Valdramidis, V. P. (2020) *E. coli* k-12 transcriptomics for assessing the mechanism of action of high-power ultrasound (submitted)

Chapter 5: Whole Genome Sequencing of wild type *Escherichia coli* K-12 isolates treated with ultrasound

Ultrasound can stimulate the activity and growth of microorganisms at low intensities and small durations, but at higher intensities, it kills and inactivates microorganisms. The effect of ultrasound treatment on *E. coli* resistance has been previously reported, showing that resistance can be built after repetitive ultrasound treatments. Additionally, ultrasound treatment may cause reversible damage and thus allow cells to recover if the effect ceases under appropriate physical parameters. The current chapter aims at giving further insight into the non-thermal technology of ultrasound on cellular responses. The wild type of *E. coli* K-12 MG1655 and an isogenic mutant Δ*gadW* were treated with ultrasound, and their DNA was analysed via Sanger sequencing and NGS, to assess the direct or indirect effect of ultrasound on DNA. Variations are observed at four different areas of the genome, which indicate that the environmental stresses influence the bacteria. Three of these mutations are substitutions, while another is an insertion just after the IS5 gene. This study forms the basis for further evaluations of specific genes in relation to ultrasound processing environments.

Part of this work has been submitted for publication in a peer-reviewed journal

Spiteri, D., Chot-Plassot, C., Karatzas, K. A., Scerri, C., & Valdramidis, V. P. (2020) Assessing microbial effects of wild-type *Escherichia coli* K-12 using high-power ultrasound by Whole Genome Sequencing (submitted)

Chapter 6: General conclusions and future perspective

Conclusions from the findings in relation to the use of ultrasound as a method of disinfection for water and wastewater, together with the characterisation of such activity on the molecular level are presented. Future research activities, together with potential use of ultrasound in industry are outlined.

Chapter 2: Literature review

2.1 Literature review

2.1.1 Introduction

The human body is made up of more than 60% of water, making water an essential component of a healthy person. Apart from its use as "food", water is needed for many other uses, such as hygiene, irrigation and industry. This demand has put great pressure on water supplies, where the only source of water is dependent on climate factors. Over the years, civilisations tried to solve the problem by building, wells, canals, dams, reservoirs and even altering river courses to bring water near their homes. Unless a suitable distribution system is provided, the supply of good potable water can cause hardship and expense to humans. This expense is mainly due to the cost required for the disinfection and energy needed to make water safe for drinking, which can total to approximately 0.5 Euro of electricity needed per 1 m^3 of water. Higgins *et al.,* mentions, that one of the biggest social and economic problems in many countries is the contradiction between increasing water consumption and water shortage.

Considering the continuous demand for more water, research in devising methods of disinfection for the reuse of sewage effluents is of paramount interest. Wastewater reuse could be one of the major solutions to water problems, as it can make available vast quantities of, previously rejected water, for both recreational, irrigation and industry. All this, however, depends on the water being treated correctly to remove the high amount of bacterial activity found in it. (Huang *et al*. 2011). The European Union Water Framework Directive (98/83/EC) and the United States Clean Water Act, outlines the importance of faecal indicator organisms, which require regulators to manage point and diffuse sources of microbial pollution (Kay *et al.*, 2008). Faecal indicator organisms include the faecal coliform, *Escherichia coli* and intestinal enterococci, *Enterococcus faecalis*. These two organisms generally originate from a

sewage source and are thus suitable indicator parameters for water monitoring or for assessing the efficacy of water disinfection treatments.

Treatment of wastewater has been a decade-long practice for many European countries. Thus, European countries have a long history of water management, particularly in the treatment of sewage. A study published by Bixio *et al.*, summarises the European water reuse practices and sets out the map of the water reclamation technologies and reuse applications (Bixio *et al.*, 2006). The study mentions that almost 70% of the European population is facing water stress (FAO, 2018). This is echoed by a document prepared for the European Commission, which states that water stress affects one-third of the E.U. territory all year round (Deloitte, 2015).

Eurostat reports on the water stress index for all European countries and states that during the past years, several countries remained in the same water exploitation range (EC, 2012a; Deloitte, 2015). Nevertheless, a significant improvement was reported in Bulgaria, Denmark and Lithuania, with a decrease in the water exploitation index from 65%, 18% and 10% to 5%, 4% and 3% respectively. On the other hand, Romania showed an increase from 5% to around 15%, indicating that the country's demand for water is rapidly increasing. A report commissioned by the European Commission in 2015 shows the percentage of the population that is connected to urban wastewater treatment plants. Most European countries have at least 30% of their sewage being treated by a secondary treatment plant. This shows that a large amount of sewage effluent is being produced, which could reduce the stress index of many countries, since most of the water produced is used for irrigation purposes. Furthermore, as of 2018 many European countries have also started to reuse reclaimed water where Cyprus used 100% of its treated effluents and Malta used just under 60% (Deloitte, 2015; MOCCAE, 2016).

It is therefore imperative that national and international measures to ensure a sustainable water management process are implemented, to promote the integrated approach to water resource management by wastewater reclamation and reuse implementation (Bixio *et al.*, 2006; Deloitte, 2015). Nevertheless, the recycling of treated effluent is still not been widely applied, even though a wide variety of treatment methods are available. Such methods may vary from simple disinfection methods, which will allow the reuse of treated water for irrigation purposes to more complex and innovating technologies that use advanced oxidation processes (A.O.P.s), which allow the removal of dissolved organic compounds in wastewater (de Koning, Bixio, Karabelas, Salgot, & Schafer, 2008) and other cases used as a method of disinfection. Nevertheless, wastewater effluent can be reused in several applications such as for industrial operations, irrigation and agricultural purposes. However, specific treatment schemes are needed in order to reach the required water quality (de Koning *et al.*, 2008) to satisfy the criteria listed in the various legislations.

2.1.2 Wastewater treatments

Most wastewater treatment plants are designed to work similarly, with primary and secondary treatments, following the same standard methods in wastewater treatment. The difference would be in the type of tertiary treatment, the plant employs that can vary from simple chlorination, nanotechnology to advanced oxidative methods, such as using ultraviolet, hydrogen peroxide or ozone gas. Table 2-1 presents a summary of different methods for disinfection and their applications (adapted from Hey, (2013).

Table 2-1: Different wastewater treatment methods in different countries.

Treatment	Scale Size	Country	Application
Chlorine Dioxide	Pilot	U.S.A.	Sewer disinfection (Geisser *et al.*, 1979)
Chlorine Dioxide	Pilot	Italy	Wastewater disinfection (Veschetti *et al.*, 2005)
Ozone	Full	U.S.A.	Wastewater disinfection (Rakness and Hegg, 1980; Rakness *et al.*, 1988)
Ozone	Full	Switzerland	Wastewater Pharmaceutical removal (Ternes *et al.*, 2003; Huber *et al.*, 2005a)
Ozone	Full	Japan	Wastewater Pharmaceutical removal (Nakada *et al.*, 2007)
Ozone	Pilot	U.S.A.	Wastewater micropollutant oxidant (Wert *et al.*, 2009)
Ozone	Full	Switzerland	Wastewater micropollutant oxidant (Hollender *et al.*, 2009)
Ozone	Full	Switzerland	Wastewater disinfection (Zimmermann *et al.*, 2011)
Ozone	Pilot	Austria	Tertiary treatment (Altmann *et al.*, 2012)
Ozone / hydrogen peroxide	Pilot	Switzerland	Wastewater Pharmaceutical removal (Ternes *et al.*, 2003)
Ozone / hydrogen peroxide	Pilot	Japan	Wastewater Pharmaceutical removal (Kim and Tanaka, 2010)
Ozone / UV	Pilot	Switzerland	Wastewater Pharmaceutical removal (Ternes *et al.*, 2003)
Fenton	Full	Turkey	Wastewater Pharmaceutical removal (Tekin *et al.*, 2006)
Photo - Fenton	Pilot	Austria	Industrial wastewater treatment (Bauer and Fallman, 1997)

Treatment plants first pre-treat the raw sewage in order to remove bulky materials followed by the addition of a polymer, which aids in the sedimentation of heavy sludge, while the grease and oily material flocculate on the top of the water, which then can be easily skimmed off (Bixio *et al.*, 2006; Brostow *et al.*, 2009; EPA-Ireland, 2013; Sato *et al.*, 2013; Anjum, Al-Makishah and Barakat, 2016). Secondary treatment is aimed to degrade the biological content, whilst the final tertiary treatment is generally about disinfection and can be varied in different plants. Disinfection is carried out mainly through chlorination, as other advanced technologies, although very efficient in disinfection, do not leave any residual chemicals, with most plants

nowadays operating with UV light (Bixio *et al.*, 2006; EPA-Ireland, 2013; Collivignarelli *et al.*, 2018). Other techniques are rarely used, except for two specific cases in Belgium and Italy, which use ozone and peracetic acid, respectively, for industrial and indirect agricultural irrigation (Bixio *et al.*, 2006).

Due to the importance of water and the vast potential in the reuse of wastewater, it is important to examine the studies for the use of different disinfection technologies and discuss with a comparative effect. Henceforth, a comparison between different disinfection technologies based on some efficiency criteria, is outlined. The pros and cons of using these different technologies are presented, while also comparing the use of these methods on a global scale.

2.1.3 Parameters for assessing the efficiency of disinfection methods.

Producing an effluent, in sufficient quantities, possessing no unacceptable risk to the environment, livestock or human health is of paramount importance. For this reason, several parameters are used to assess the efficiency of disinfection methods.

2.1.3.1 Microbial indicators

Disinfection methods must have the capability of inactivating a significant number of microorganisms in order to be effective and suitable for their purpose. Different methods may allow different ways of inactivation. They usually follow the same mechanism by causing damage to the cellular membrane, by means of disruptions to the phospholipid bilayer, making up the cell membrane. Legislation has always looked at the number of bacteria that is found after processing, in order to certify that water is safe for its use. The E.U. Water Framework Directive (98/83/EC) stipulates, that for water to be safe in the distribution network, it must follow an extensive list of chemical parameters, whilst not allowing the growth of *E. coli* and *E. faecalis* in 100 mL of the tested water. On the other hand, for bottled water, due to storage

capacity of the water, the legislation is more stringent with a variety of bacterial species, including *Pseudomonas aeruginosa*, apart from the other already mentioned bacteria, which need to be absent or within a range in 250 mL of tested water. An overview of the microbial pathogens tested in wastewater, and their indicative reported doses is given in Table 2-2.

Table 2-2: Microbial pathogens, commonly tested for in wastewater (adapted from (US EPA, no date; Rose et al., 1996; Toze, 1997)

Microbial pathogen		Indicative reported dose	Infectious dose
Bacteria	*Salmonella* spp.	10,000 – 1,000,000 CFU/	10^4-10^6
	E. coli	100mL	10^6-10^{10} CFU/100mL
Viruses	various	10-10,000 Viruses/L	1-10 Viruses/L
Protozoa	various	10-10,000 Oocytes/L	<10 Oocytes/L
Helminths	various	10-1,000 eggs/L	1-10 eggs/L

2.1.3.2 Suspended Solids

This is a good indication that the primary and secondary treatments are working well and are separating the solid biomass from the liquid medium. The solid mass is normally converted to sludge and then sold off as fertiliser. An overview of the levels of the discussed quality parameters is given in Table 2-3.

2.1.3.3 Biological Oxygen Demand

Biological Oxygen Demand (B.O.D.) is the amount of dissolved oxygen needed by aerobic biological organisms, to breakdown organic materials present at a specific temperature. It is a crucial test, as it can gauge the effectiveness of water treatment plants (refer to Table 2-3 regarding the expected levels).

2.1.3.4 Turbidity

Turbidity is the cloudiness of a fluid caused by particles that are generally invisible to the naked eyes. These particles can be a result of either suspended or dissolved solids. Turbidity can drastically affect water treatment, so it is a crucial test for water quality before and after treatment (Table 2-3).

2.1.3.5 Nitrates and Ammonia

Nitrates are the result of protein degeneration. Proteins, which result from human wastes, are acted upon by bacteria, resulting in ammonia, nitrites and nitrates, depending on the action being carried out. Nitrates are the end product after oxygenation of ammonia, which then be acted upon by anaerobic bacteria, that use up the oxygen ions and release nitrogen gas. Their accepted levels depend on the use, such as in irrigation, second-class industrial water or even further treatment.

2.1.3.6 pH

pH is the measure of acidity or alkalinity of an aqueous solution. It is a measurement of the total amount of H+ ions found in solution. Control of pH is important in wastewater as it directly influences wastewater treatability, being chemical or biological.

Table 2-3: Summary for the quality of the effluent in subsurface discharges depending on treatment (Adapted from, (EPA Victoria, (2002; US Environmental Protection Agency, (2012))

Parameter	Expected value
Total Suspended Solids	30mg/L
Biological Oxygen Demand	25mg/L
Turbidity	<5 N.T.U
Nitrates	Depending on use
Ammonia	<1mg/L
pH	6.0-9.0 pH Units

2.1.4 Toxicity

Disinfectants are used to purify mediums from microbiological pathogens. For this reason, in order for disinfectants to be effective, they must prevent the production of toxic by-products. Apart from this, it must also be safe for the correct and safe disposal in the environment in order not to allow for toxic bioaccumulation of chemicals. Several tests carried out on experimental animals assess these types of toxicity and the levels, which might affect animals or humans (WHO, 2000). Such toxicity tests make use of the water flea *Daphnia magna* and assessing the half-maximal effective concentration (EC_{50}) (ISO 6314: 2012). Most chemicals, if not all, will have a drastic effect on the environment due to the fact that chemicals might not be found naturally.

2.1.5 Emerging Pollutants

Emerging pollutants, such as active pharmaceutical incipients (APIs), drugs, hormones and their metabolites, personal care products etc. often refer to chemicals or compounds that are not commonly monitored in water, but have been documented to have increased, resulting in adverse ecological and human health effects (Xagoraraki and Kuo, 2008). During the years, the development of pharmaceutical technologies, have greatly improved our health and wellbeing. However, this also produced chemicals and compounds, which were identified as "possessing potential environmental threats" (Bolong *et al.*, 2009). Although, these low levels of emerging pollutants may not necessarily cause direct, immediate lethal effects, they may promote long-term effects on human health (Honglan *et al.*, 2012). It is also important to note that several of the active pharmaceutical ingredients, that makeup modern medicine are not metabolised, and are discharged into the sewers, thus ending up into wastewater treatment plants (Bolong *et al.*, 2009). Many studies have reported the presence of pharmaceuticals in

effluents of sewage treatment plants, which act as a primary source of pharmaceuticals and their metabolites into groundwater aquifers, used for drinking water (WHO, 2011).

Sewage treatment plants (S.T.P.s) generally employ conventional sewage treatment with primary sedimentation, secondary treatment and final sedimentation, followed by disinfection. Nowadays, development in alternative water treatment technologies has been made in order to decrease the concentration of such emerging pollutants in sewage waters. Yet their use is not widespread due to the fact that they may result in high-energy costs (Ibáñez *et al.*, 2013).

2.2 Disinfection Methods

Strict World Health Organisation (WHO), Environment Protection Agency (E.P.A.) and European Commission (E.C.) requirements for potable water, allowed several disinfection technologies, ranging from simple physical treatment such as filtration, the addition of chemicals such as ozone or chlorine, or by the method of biological treatment such as in lagoons, to be implemented during the years. Disinfection is the process of the destruction of pathogens in water. This is a process applied both to potable and sewage effluent to protect public health and reduce the risk of disease for recreational users of surface water. Disinfection can be physical (ultraviolet, microfiltration, ultrasound), chemical (chlorination, ozonation, hydrogen peroxide) or biological (detention lagoons) (EPA Victoria, 2002). The choice of the appropriate disinfection technologies is related to several criteria presented hereunder

2.2.1 Criteria for choosing disinfection methods

2.2.1.1 Storage and dispensing properties

Storage and dispensing of the disinfectant is essential, as water suppliers need to have stockpiled in storage, while also be able to quickly dispense, in the most economically feasible

way possible. Storage conditions may vary drastically depending on the state of the disinfectant. The liquid disinfectant may be easier to store than gas, however, be less efficient due to the lower concentration. Nowadays, industry is utilising technologies that allow preparing the disinfectant in-situ, permitting the process to produce only the disinfectant that is required. This is most important, when highly toxic gasses, such as ozone are used (Jo and Mok, 2009).

2.2.1.2 Costs

Cost is one of the essential factors in any industry. Even though some disinfection methods may be safer for use than others, economic feasibility in relation to efficiency plays the most crucial part.

2.2.1.3 Easy analysis by providing residual protection in drinking water

Residual protection is a good way of monitoring the disinfection of water. Water suppliers may indicate deviations in the water quality by looking at the residual amount of disinfectant at the water tap. A variation in the residual amount may indicate bacterial growth due to dead ends, water leaks and also contaminations. One of the known disinfectants that produce residuals is chlorine (or any of its derivatives), which however may produce toxic products (Droste, 1997; Du *et al.*, 2017; World Health Organization, 2017).

Following the description of the criteria for choosing disinfection methods, the type of disinfection methods will be discussed by dividing them into chemical and physical methods.

2.2.2 Disinfection by chemical methods

2.2.2.1 Chlorine

Most European countries use chlorination as the primary method of disinfection, as it destroys target organisms by oxidising cellular material (EPA, 1999). Chlorine can quickly be produced, it is relatively cheap and is highly effective in killing pathogens. In fact, Figure 2-1 summarises the number of E.U. countries that effectively use chlorine or its derivatives as the main disinfectant for water treatment. The Netherlands is one of the only E.U. countries that limit the use of chlorine for water treatment. The Dutch utilise strict guidelines to choose the best source available while using physical process treatment and continuous monitoring for timely detection (Smeets, Medema and van Dijk, 2008). Obviously, this might not be possible in other countries with less groundwater sources, so they will require other measures for treatment.

The United States use another similar alternative, i.e., chloramine, which like chlorine also provides suitable residual protection in drinking water. Major advantages of chlorine are the way it can be supplied, solid (chlorine tablets), liquid (hypochlorite solutions) or even gas (chlorine gas), which makes it:

- penetrable for destroying pathogens
- easily handled and transported
- relatively cheap, when compared to either UV or ozone
- prolonging disinfection even after initial treatment because of its residual activity (EPA, 1999)

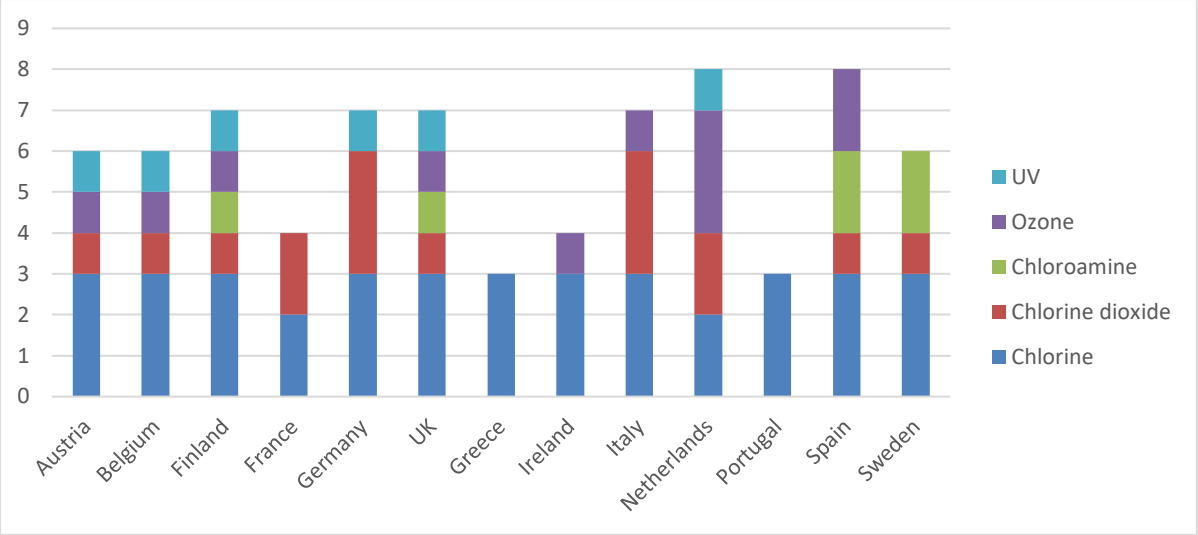

Figure 2-1: Different water treatments used by EU Countries, where 1: Least used, 2 commonly used and 3: mostly used. (Compiled from (Casey & O'Reilly 2009))

On the whole, chlorine is the least costly disinfection agent (Droste, 1997; Du *et al.*, 2017) however, research has identified several by-products, which prove to be carcinogenic. In particular these are the trihalomethanes, commonly referred to THMs, which are produced by the presence of organic material and chlorine in water (Droste, 1997; EPA, 1999; Du *et al.*, 2017). The hazards associated with these are generally small, considering the low concentrations produced in a typical operation. Thus it has been stated that the benefits of chlorination have far outweighed the risks (Droste, 1997; Du *et al.*, 2017; Gheibi, Karrabi and Eftekhari, 2019).

Apart from the toxicity, coliforms, and other enteric bacterial indicators are much more sensitive to free and combined chlorine (Sobsey *et al.*, 1998; Tree, Adams and Lees, 2003; World Health Organization, 2017; Wen *et al.*, 2020). On the other hand, chlorination was found to be ineffective in reducing enteric viruses, bacterial spores and protozoan cysts in sewage (Francy et al. 2012; Huang et al. 2011; Sobsey et al. 1998; Wen et al. 2020). Furthermore, specific parameters in the sewage effluent may negatively affect chlorine disinfection. These include:

- Ammonia – forms chloramines,
- B.O.D. – interference in disinfection,
- Nitrite – reduces effectiveness,
- T.S.S. – reduces disinfection capability,
- pH – greatly affects the effectiveness of chlorine (lower pH, higher disinfection) (EPA, 1999; World Health Organization, 2017).

Thus, alternative methods of disinfection, which indirectly also affect such parameters, would greatly increase the efficacy for methods of disinfection.

2.2.2.1.1 Mode of action of chlorine

On contact with water, chlorine gas reacts to form hypochlorous acid and hydrochloric acid (HOCl and HCl). Hypochlorous acid will immediately dissociate into the hypochlorite ion (OCl^-) and the hydrogen ion (H^+) (Droste, 1997; WHO, 2000; LeChevallier and Au, 2004; World Health Organization, 2017). This dissociation is typically affected by the pH of water, i.e. the concentration of the hydrogen ion. The lower the pH, the less likely that the acid would dissociate (LeChevallier and Au, 2004), since a higher H^+ ion will shift the reaction, allowing for a more significant presence of hypochlorous acid, which would have a larger half-life, than the hypochlorite ion.

The hypochlorite ion is mainly responsible for the disinfection of the water system and is mainly referred to as free chlorine. Baker (1926) theorised that proteins in micro-organisms are destroyed by the action of chlorine to form N-chloro compounds (LeChevallier and Au, 2004). This was later also confirmed with sulfhydryl groups of proteins (Green and Stumpf, 1946; Knox and Stumpf, 1948; Venkobachar, Iyengar and Prabhakara Rao, 1977) and also by the oxidation of several amino acids to their corresponding nitriles and aldehydes (Patton *et al.*, 1972). Thus, chlorine can cause physiological damage to bacterial cell membranes, which may

result in the rupture and eventual lysis of the cells (Virto *et al.*, 2005; WHO, 2011; World Health Organization, 2017; Wen *et al.*, 2020).

However, not all micro-organisms are affected the same. Certain bacteria, especially spore-forming ones such as *Bacillus* or *Clostridium* spp., show a high level of resistance to free chlorine. One particular study concluded that nearly all Gram-positive or acid-fast bacteria survived chlorine disinfection due to the presence of a thick peptidoglycan cell wall and waxy surface, respectively (LeChevallier and Au, 2004).

Enteric viruses are also, generally more resistant to free chlorine, especially more so with viruses being associated with organic debris, like those commonly found in raw sewage (Du *et al.*, 2017; World Health Organization, 2017; Wen *et al.*, 2020). It is common practice that before disinfection, pre-treatment is carried out to improve the turbidity of water. This is because a high level of particles in the water will reduce the chance of contact time of chlorine and bacteria.

Protozoans, like viruses, are also highly resistant due to the formation of cysts. These may require prolonged contact times (C.T.) at higher chlorine concentrations. A case in point is the protozoan *Giardia* which requires a chlorine concentration of 1.5 mg/L with a contact time of at least 10 minutes (Coulon *et al.*, 2010). This, however, may increase the amount of T.H.M.s in the water system, due to an increase in both concentration and thus reaction time with dissolved organic compounds (Du *et al.*, 2017; Wen *et al.*, 2020).

T.H.M.s and other halogenated compounds are the main by-products of disinfection with chlorine. In aqueous solutions, chlorine reacts with ammonia, or amino groups to form several compounds, such as monochloramine, dichloramine and trichloramine. Although these

compounds are mainly dependent on the presence of this amino group, they are also dependent on the pH of the aqueous solution, temperature and contact time (LeChevallier and Au, 2004).

2.2.2.2 Plasma

Plasma technology is one of the most recently used technologies for the treatment of drinking and wastewater (Chang, 2007; Barillas, 2015; Rashmei, Bornasi and Ghoranneviss, 2016; Foster, 2017; Sakudo, Yagyu and Onodera, 2019; Van Nguyen *et al.*, 2019). Three different categories for plasma treatment exist: (i) remote, which involves plasma generation away from the medium, e.g., ozone, (ii) direct in which the medium is treated and (iii) indirect, which involves the generation of plasma near to but not directly in the medium. (Chang, 2007; Sakudo, Yagyu and Onodera, 2019).

Plasma technology usually refers to direct electrohydraulic discharge treatment, which has a much higher potential to treat contaminants effectively, and more economically feasible. However, although plasma was found to be very efficient in the disinfection of micro-organisms, it required a much higher electricity demand when compared with UV light and ozone disinfection technologies. Nonetheless, plasma revealed that the number of by-products produced was much lower, providing plasma technologies with an opportunity to establish in the market (Locke *et al.*, 2006; Jang *et al.*, 2013; Barillas, 2015; Rashmei, Bornasi and Ghoranneviss, 2016; Foster, 2017; Sakudo, Yagyu and Onodera, 2019; Van Nguyen *et al.*, 2019).

2.2.2.2.1 Mode of action of Plasma

The plasma agent, destroy microorganisms, including spores and viruses (Misra *et al.*, 2011; Rashmei, Bornasi and Ghoranneviss, 2016; Van Nguyen *et al.*, 2019) by interacting with the biological material. Low-pressure oxygen plasma has shown to damage lipids, proteins and DNA. This is due to the reactive species in the plasma, which plays a direct oxidative effect on

the outer surface of the microbial cell (Rashmei, Bornasi and Ghoranneviss, 2016).

Oxygen and nitrogen-based species act on unsaturated fatty acids of the lipid bilayer in the cell membrane, by disrupting the double bonds and thereby stopping the transport of biomolecules across it. The same action can be reported on the proteins causing denaturation and cell leakage (Locke *et al.*, 2006; Misra *et al.*, 2011; Boxhammer *et al.*, 2012; Rashmei, Bornasi and Ghoranneviss, 2016; Van Nguyen *et al.*, 2019).

2.2.2.3 Ozone

Ozone is a strong oxidiser that can disrupt the sludge flocs and, in the meantime, also destroy cells. Like chlorine, this is a chemical treatment, resulting in the formation of hydroxyl radicals in situ, that reduce the organic content in the sewage. Nevertheless, although no trihalomethanes are produced, during ozone use one still needs to monitor the production of bromates, which results through the reaction of bromide, found in source water (Lee, Arnot and Mattia, 2011; Vincent *et al.*, 2014). Bromate was found to be highly toxic on prolonged exposure and may cause renal failure on consumption, even though this is usually found at low concentrations. Toxicological tests on mice also resulted in reduced reproductive capabilities in male rats (World Health Organization, 2005).

However, one of the significant advantages of using this method is that the reverse osmosis (R.O.) process reduces or eliminates bromide levels in the water thus preventing the production of bromates. Being highly energy demanding, the R.O. process will drastically increase costs. Apart from costs, filtration technologies are known to occupy vast amount of land area, require substantial capital investment and sophisticated management. Thus, for several years engineers have tried to develop alternative environmentally friendly and cost-effective technologies

(World Health Organization, 2005; Da Silva and Jardim, 2006; Lee, Arnot and Mattia, 2011; Vincent *et al.*, 2014; Martinelli *et al.*, 2017; Ding *et al.*, 2019).

Due to its highly oxidative properties, ozone is one of these alternative methods. In addition, its ability and efficiency in the oxidisation of complex organic molecules, phenols and several other metabolites including pharmaceutical ingredients, makes ozonation a good alternative and less expensive for wastewater effluent treatment and a good alternative and least expensive for water treatment (Da Silva and Jardim, 2006; Tripathi *et al.*, 2011).

2.2.2.3.1 Mode of action of ozone

The mode of action by which ozone inactivates microbes is not well understood. Ozone is bubbled through an aqueous solution and being highly unstable will form highly oxidative radical species, which will act upon the microbes. Apart from this, it is also known that ozone is able to attack electron-deficient bonds, forming aldehydes, ketones and also carbonyl compounds (Nagarkatti, 1991; Rojas-Valencia, 2011; Qi *et al.*, 2016; Martinelli *et al.*, 2017).

Ozone disintegrates in water to form the hydroxyl radicals, which are short-lived, but capable of breaking almost all multiple bonds of both organic and inorganic contents, that are present in water. It reacts via the hydroxyl radical, which reduces the content, resulting in increased biodegradability and inactivation of microorganisms. Ozonolysis has been used for the treatment of sewage discharges since 1970 (Paraskeva and Graham, 1998; Tripathi *et al.*, 2011; Fallis, 2013).

On the other hand, free radicals are generally less effective, as microbial cells can quench the free radical reaction using bicarbonate ions, or through the enzymatic action of several enzymes

such as catalase, peroxidase and superoxide dismutase to control the free radicals produced by aerobic respiration (LeChevallier and Au, 2004; Ding *et al.*, 2019).

Studies have shown that *Escherichia coli* is one of the most sensitive to ozone, unlike *Staphylococcus* sp. and *Streptococcus* sp., which are the most resistant. Furthermore, viruses are generally more resistant to ozone than bacteria (Nagarkatti, 1991; Ding *et al.*, 2019).

2.2.2.4 Ozone in combination with other methods

As explained ozone on its own, is already one of the best options for water treatment, however, it can form a toxic by-product in the form of bromates, resulting in serious health implications. Researchers have shown that when ozone is coupled with the use of ultrasound, the requirement for high ultrasonic energy can be reduced, whilst enhancing the disintegration of wastewater sludge (Yang *et al.*, 2012; Tyagi *et al.*, 2014; Filibeli, Erden and Gunduz, 2018; Le *et al.*, 2018). This is mainly because micro-bubbles generated by ozone increase acoustic cavitation, improving the efficiency for the ultrasound pre-treatment. Similarly, ultrasound also enhances the efficiency of ozone decomposition, allowing for a more significant transfer of ozone into the liquid phase, and thus a final higher concentration of the required hydroxyl radical (Yang *et al.*, 2012; Cuerda-Correa, Alexandre-Franco and Fernández-González, 2020).

Hydrogen peroxide is an oxidant, producing the hydroxide radical. This allows for a highly reactive radical to withdraw atoms from a substrate, allowing the pollutant to become more susceptible to ozone attack. On the other hand, UV (Ultraviolet light), provides energy to break chemical bonds, making any remaining fragment susceptible to ozone attack (Fallis, 2013; Cuerda-Correa, Alexandre-Franco and Fernández-González, 2020).

It can therefore be concluded that ozone, although it plays an essential role in non-residual disinfection, is also essential for the removal of several compounds, commonly found in wastewater. According to Fallis, ozone is capable of disinfecting wastewater with a COD. of > 200mg/L by dosing between 50 to 500g/m^3 of ozone for a retention time of 5-30 minutes (Fallis, 2013).

2.2.3 Disinfection by physical methods

2.2.3.1 Ultraviolet light

Another commonly used form of disinfection is through Ultraviolet (UV) light. UV irradiation is emitted by mercury-amp vapour lamps and is referred to as a physical treatment process, as it leaves no chemical residue (Anastasi *et al.*, 2013; Sharma and Bhattacharya, 2017). One of the main limitations of UV irradiation is the fact that high dosage is required, to ensure that nucleic acid is damaged beyond repair (LeChevallier and Au, 2004; Anastasi *et al.*, 2013; Sharma and Bhattacharya, 2017). This may result in high-energy costs, which may become unfeasible in high volumes of water.

2.2.3.2.1 Mode of action of UV light

Wavelengths of UV light can vary from about 40 to 400 nm, with the most useful spectrum lying in the 200 to 310 nm, i.e. the UV-B and UV-C ranges. The wavelength with the maximum effectiveness lies around 265nm (LeChevallier and Au, 2004; Sharma and Bhattacharya, 2017). UV light can disrupt thymine bases on DNA and RNA to form dimers, which will inhibit transcription and replication of nucleic acids. However, processes called photo-reactivation and dark repair, allow for the repair of these Thymine dimers in the presence or absence of light (LeChevallier and Au, 2004; Anastasi *et al.*, 2013).

Unlike methods previously described, UV can be a very effective disinfectant for both bacteria and viruses, due to its high DNA damage capability. Nevertheless, the effectiveness of UV disinfection can be impaired by the colour and presence of particulate matter, high-suspended solids or high turbidity (LeChevallier and Au, 2004; Barkhudarov *et al.*, 2008; Rubio *et al.*, 2013; Rodriguez *et al.*, 2014; Sharma and Bhattacharya, 2017).

2.2.3.2 Membrane filtration

Membrane filtration utilises a thin semi-permeable membrane of various pore sizes, which acts as a selective barrier to remove contaminants from water. This is one of the widely used methodologies in drinking water treatment, and its use has been growing drastically due to the stringent drinking water regulations and decreasing costs of purchasing and operation of the membrane filters.

Membrane filtration is efficient enough to remove most of the microbial contaminants. Several membrane processes differing in pore size can be used. Such processes include: Microfiltration (0.1 micron), Ultrafiltration (0.01 micron), nanofiltration (0.001 micron) and reverse osmosis (0.0001 micron). Different processes contain different sized pores, allowing membrane filtration to remove pathogens by size exclusion. The fact that membrane filtration can accumulate chemicals, particles and biological growth, requires that several maintenance regimes such as backwash and the complete replacement of the filter are utilised. Certain pre-treatment processes can also be employed to prevent anti-fouling, which may be the result of blocked membrane pores from bacteria and viruses. Such pre-treatment may include surface hydrophilicity, surface charge and surface roughness (Fan *et al.*, 2008; Hakata, Roddick and Fan, 2011; Pramanik, Roddick and Fan, 2014; Sharma and Bhattacharya, 2017).

Surface hydrophilicity offers better fouling resistance, since many foulants, such as proteins are hydrophobic in nature, allowing a natural repulsion. This is further aided by the surface charge of the membrane (Nguyen, Roddick and Fan, 2012; Abdelrasoul, Doan and Lohi, 2013; Gkotsis *et al.*, 2014; Sharma and Bhattacharya, 2017).

The repulsive forces in the feed solution may prevent solute or particle deposition in the membrane surface. If the membrane surface and the foulant molecule contain negatively charged carboxyl groups (COO-.), bridging occurs, allowing divalent calcium ions, in solution to cross-link with the carboxyl of the membrane surface and the fouling molecules. This enhances the attachment of the molecules to the membrane surface. Thus, it is important to have a high ionic strength of the feedwater to minimise electrostatic interactions (Nguyen, Roddick and Fan, 2012; Abdelrasoul, Doan and Lohi, 2013).

Having a smooth topology will decrease the chance for particles getting trapped on the surface of the membrane. Thus, membrane filters are normally highly polished, to avoid uneven surfaces, and thus becoming easily fouled (Pramanik, Roddick and Fan, 2014).

2.2.3.2.1 Mode of action of Membrane filtration

The mode of action of membrane filtration is a simple process, allowing particles to get trapped by decreasing pore size. Different filters have different pore sizes, allowing for different particles to pass through. Membrane desalination by reverse osmosis is the fastest-growing desalination technology (Frenkel, Ph and Wre, 2011; Zotalis *et al.*, 2014). Seawater is pumped under high pressure, through a series of membranes, having a salt rejection of 99.5%. Even though most of the ions and particles are removed, reverse osmosis plants commonly have a

problem of high boron levels, which require second passes to be removed, allowing for higher energy costs (Kabay *et al.*, 2008; Zotalis *et al.*, 2014).

Seawater contains approximately 5 mg/L of boron (Kabay *et al.*, 2008; Gao *et al.*, 2011). In most cases, the rejection of boron by R.O. is not sufficiently high, resulting in about 1.5 mg/L of boron in the permeate, which is 1 mg/L higher than that accepted by WHO standards. Several R.O. operators add NaOH to the feed water, to increase the pH to up to 8.8 as this further improves the system's rejection rate, decreasing the relative boron passage from 0.86 (pH 8.1) to 0.49 (pH 8.8) (Kabay *et al.*, 2008).

2.2.3.3 Microwave

Microwave water disinfection is the use of microwave radiation to heat up the water by means of collisional deactivation. Technological advances, such as smaller magnetrons have, increased microwave input while making the entire process more economically feasible (Fu and Zhang, 2018) because of lower power requirements (Benjamin *et al.* 2009). Apart from this, microwave disinfection is much more advantageous than other conventional radiant heating sources (Lidström *et al.*, 2001; Larhed, Moberg and Hallberg, 2002). Studies have concluded that microwave heating tends to heat more uniformly than other methods, resulting in higher energy efficiency (Barkhudarov *et al.*, 2008; Benjamin *et al.*, 2009; Fu and Zhang, 2018).

Recent studies have also shown that microwave radiation is capable of reducing a significant number of coliforms. Yu, Chan, Liao and Lo (2010), clearly outline the methodology utilised to treat primary and waste activated sludge, using different frequencies of microwaves. In a specific study, a 4.8 log reduction of *E. coli* was reported by just treating sludge to 45 kW of microwave radiation (90 s at 500W). When increasing to 60 kW and 90 kW, complete

inactivation of the faecal coliforms was reported (Woo, Rhee and Park, 2000; Yu *et al.*, 2010; Om, Cardenas and Molof, 2011).

2.2.3.3.1 Mode of antimicrobial actions

Researchers believe that microbial inactivation by microwave irradiation is due to the thermal effect. Several studies suggest that other mechanisms resulting directly from the interaction of the electromagnetic field with organic molecules are playing a role (Vergani, Ribeiro and Dovigo, 2011). Microwaves use high-frequency waves capable of denaturing DNA and also breaking down organic chemical bonds, resulting in the disruption of the phospholipid bilayer, which makes the cell membrane. This was confirmed by Campanha *et al.*, (2007), where it was observed that when microorganisms were submitted to microwave irradiation at 650W for 6 minutes and treated with methylene blue, a distinction could be made between integral and non-integral cells. (Vergani, Ribeiro and Dovigo, 2011).

2.2.3.4 Ultrasound

The application of ultrasonic technology has recently received wide attention for water or wastewater treatment and environmental remediation areas, concerning disinfection procedures (Chen, 2012; Han *et al.*, 2013; Cesaro and Belgiorno, 2016). Such applications include sonochemistry, where ultrasound is used for the acceleration of chemical reactions or extraction of specific chemical compounds; dispersion and disruption of biological cells; removal of trapped gases and other (Madge and Jensen, 2002; Gibson *et al.*, 2008; Naddeo *et al.*, 2009; Oyib, 2010; Sango *et al.*, 2014). These applications are especially important in wastewater plants, where a combination of ultrasound effects for the disruption of bacterial and colloidal particles, or disintegration of algal blooms, can assist in the efficiency of treatments (Madge and Jensen, 2002; Gibson *et al.*, 2008; Oyib, 2010).

2.2.3.4.1 Mode of action of Ultrasound

Ultrasound induces elastic vibrations and waves with a frequency greater than 15-20 kHz. Although ultrasound can stimulate microorganisms' development and growth at low intensities and short durations, it kills and inactivates microorganisms at higher intensities. Long term water treatment by ultrasound of 20 – 100 kHz with a sound intensity of between 10 and 1,000 W/cm^2 can achieve disinfection (Vasilyak, 2011).

The disinfection capacity of sonication in water is due to the phenomenon of acoustic cavitation, which is the formation and collapse of micro-bubbles occurring in milliseconds that produce extreme temperature and pressure gradients (Drakopoulou *et al.*, 2009; Sango *et al.*, 2014). Indeed, the collapse of these micro-bubbles leads to extremely high local temperatures and pressures. These conditions have shown to result in the generation of highly reactive radicals, such as OH· and H·. Ultrasound is, therefore, able to inactivate bacteria and de-agglomerate bacterial clusters through several physical, mechanical, and chemical effects caused by acoustic cavitation (Antoniadis *et al.*, 2007; Broekman *et al.*, 2010; Vasilyak, 2011; Sango *et al.*, 2014).

2.2.3.5 Lagoons

One of the most advantageous concepts for wastewater lagoons is the small amount of staff hours and low operating costs that are required to successfully run a wastewater lagoon (Schwindamann, 2013). Lagoons are a series of ponds, designed in order for wastewater to enter into the pond by means of inlets and then leave through an outlet pipe, usually placed as far as possible to the inlet. The storage of secondary treated wastewater allows for natural disinfection using sunlight, or even natural microbial die-off. Factors such as temperature, pH, adsorption and sedimentation, all influence and assist in this natural disinfection and inactivation processes occurring in wastewater stored in lagoons. Widely used in Australia, this

method of wastewater treatment is utilised in conjunction with other methods such as UV or chlorination (Benner *et al.*, 2013; Zhang *et al.*, 2013).

2.2.3.5.1 Mode of action of Lagoons

Lagoons are a natural way for wastewater treatment. Lagoons are designed to operate with approximately four feet of water, in order to allow enough sunlight, which is essential for the contribution to the growth of green algae to reach the bottom of the pond. The green algae produce oxygen, which the bacteria use to breakdown the waste found in the lagoon. This results in clean water with no undesirable odours and thus a minimal threat to the environment. An important point to note is that lagoons should not be allowed to get an inflow of surface water or discharge any effluent. Thus, dykes are constructed on all sides of the lagoons (Jupsin and Vasel, 2007).

2.3 Technologies used specifically for emerging pollutant removal

Potential adverse effects on humans by drinking water have attracted, lots of attraction in recent years, however, no specific water treatment plant has been equipped with emerging pollutant removal technology (Honglan *et al.*, 2012). Nevertheless, this has not prevented the development of advanced analytical instrumentation and new analytical techniques, which can detect deficient concentrations of many chemicals.

Several studies have explored the feasibility of using oxide nanomaterials and nano-activated carbon (Grecchi and Turchetti, 1968; Honglan *et al.*, 2012; Fanourakis *et al.*, 2020), ozone (Ibáñez *et al.*, 2013; Fast *et al.*, 2017; Paucar *et al.*, 2019), integrated membrane systems (Dolar *et al.*, 2012; Secondes *et al.*, 2014; Ojajuni, Saroj and Cavalli, 2015; Nghiem and Fujioka,

2016; Tran *et al.*, 2019) and also ultrasound (Secondes *et al.*, 2014; Camargo-Perea, Rubio-Clemente and Peñuela, 2020) to remove several emerging pollutants from the treated effluent or drinking water. These studies have resulted in several different conclusions, with some concluding that advanced oxidative processes are more efficient than direct filtration. However, this is highly dependent on the chemical being removed; as different chemicals are affected differently between each process, namely size exclusion and electrostatic attraction or repulsion, especially with the use of Reverse Osmosis membranes (Dolar *et al.*, 2012; Tran *et al.*, 2019).

The treatments using oxidative processes contrasts well with another study were high removal rates were achieved with reverse osmosis (R.O.) technology when coupled with membrane bioreactor. The R.O. membrane showed removal rates of more than 99% for all the compounds studied (Dolar *et al.*, 2012; Ojajuni, Saroj and Cavalli, 2015; Tran *et al.*, 2019). Similar results were reported with tight nano-filter membranes of 0.9nm pore size, even though tiny organic compounds were not removed (Barceló D, Petrovic M and Radjenovic J, 2009; Yangali Quintanilla, 2010).

Other studies concluded that advanced oxidation processes can remove some of the PPCP compounds (Honglan *et al.*, 2012). Ozone has shown that it is highly efficient for the removal for most of the emerging pollutants even when assisted with ultrasound technology. It was thus concluded that ultrasound had no effect on PPCP compounds and was practically unnecessary, unlike ozone which significantly reduced most of the organic compounds under study (Ibáñez *et al.*, 2013; Fast *et al.*, 2017; Paucar *et al.*, 2019). However, Secondes, M. F. N. *et al.* in 2014 concludes that ultrasound enhanced membrane removal to almost 100%, unlike when utilising ultrafiltration only that was able to remove only 10% of the emerging pollutants and 99% when utilising activated carbon and ultrafiltration. This was due to the fact that ultrasound enhances

the adsorption of the filter membrane and activated charcoal due to cavitation (Secondes *et al.*, 2014).

2.4 Comparison of disinfection methods

Overall, the physical method via filtration is considered one of the most effective disinfection methods in terms of biocidal efficiency. However, costs can be relatively high, and the entire procedure expensive, due to the requirements of building a plant, and monthly maintenance of membranes. On the other hand, UV treatment is also one of the best methods; however, one of the significant drawbacks of this method is the lack of efficiency in the removal of parasites. UV disinfection is not capable of removing helminths, and requires other processes, before disinfection for the efficient removal of parasites (Sinclair 2010). However other studies have concluded that UV is very efficient in killing *Giardia* and *Cryptosporidium* (Liberti, Notarnicola and Petruzzelli, 2003; Adeyemo *et al.*, 2019).

On the other hand, one of the significant limitations of UV disinfection is because wastewater, having a large number of suspended solids, will reduce the efficiency of this method. The EU wastewater directive stipulates that the BOD5 and Total suspended solids should not exceed 25 mg/L and 35 mg/L, respectively. This is higher than the recommended amount of 10 mg/L for suspended solids for ultraviolet disinfection as reported in the report published by E.P.A. Victoria in 2002. However recent publication of the reuse directive has reduced these parameters to 5 mg/L (European Commission, 2020).

Although, most of the parameters mentioned in this report, have minor effects, if any, on the efficiency of ultraviolet disinfection, the total suspended solids, may absorb UV radiation and

thus be able to shield embedded bacteria. Thus it is imperative that flocculated bacteria are first separated, making them more prone for action (Barkhudarov *et al.*, 2008; Nelson *et al.*, 2013).

This may indicate that the bubbling of ozone or chlorine is a more attractive alternative. As explained above, chlorine is not considered one of the safest disinfectants in place, even though it is one of the most economically feasible. Chlorine allows for an increase in toxicity, while also has a tendency to bind with ammonium products to form chloramines. This has the potential to increase the toxicity of the discharge, which may result in a severe environmental impact.

Detention lagoons, as describes earlier, is another option. However, not only does it require large areas of land, but it also requires alternative methods of treatment, as it is not considered to be the best practice where a microbiological quality is assessed. Notwithstanding this, lagoons also tend to suffer from algal blooms and thus require constant maintenance.

2.5 Conclusions

Several treatment methods exist, some of which might be impractical and/or economically unfeasible. Other treatments such as UV light, and or ozone, require long contact times, while methods such as detention lagoons require a large land area. Thus, chlorine, even though being one of the most methods that have a large number of disadvantages, due to by-product production, health and safety requirements etc., is still one of the most sought methods in the world.

CHAPTER 3: Ultrasound processing of liquid system(s) and its antimicrobial mechanism of action

3.1 INTRODUCTION

Europe has large water supplies when compared to other regions of the world, and thus has long been considered an inexhaustible public asset. However, in recent decades this status has been threatened by increasing water stress, both in terms of water scarcity and degradation of water quality. Indeed, roughly half of Europe's nations, comprising nearly 70% of the population, have faced water stress problems in recent years (Wintgens *et al.*, 2006) and have had to turn to alternative sources to bridge the supply-demand gap. Wastewater treatment is one of the alternatives. Wastewater treatment has been a lengthy procedure for many European countries for ten years. Most raw sewage was dumped into the sea before 2011, without being treated, which is now against to the latest EU directive on urban wastewater (91/271/EEC).

The quality specifications for wastewater reuse are primarily geared towards the intended usage and are laid down in country-specific criteria and legal provisions. In addition to the residual concentrations of inorganic nutrients, total suspended solids, dissolved organic matter, and wastewater microbiological contamination are an essential criterion for their beneficial reuse (Haaken *et al.*, 2014). All these parameters make water look appear negatively affected, cause contamination and also allow bacterial to grow. Indeed, many pathogenic microorganisms and parasites are frequently found in domestic wastewater and wastewater treatment plant effluents. In the environment, three types of pathogens are found: bacterial pathogens, including indigenous aquatic bacteria, viral pathogens and protozoan parasites. Wastewater bacteria have been characterized and belong to the following groups: Gram-negative facultatively anaerobic bacteria (e.g. *Aeromonas*, *Vibrio*, *Enterobacter*, *Escherichia*, *Klebsiella*, *Shigella*), Gram-negative aerobic bacteria (e.g. *Pseudomonas*, *Alcaligenes*, *Flavobacterium*, *Acinetobacter*), Gram-positive spore forming bacteria (e.g. *Bacillus* spp) and nonspore-forming Gram-positive

bacteria (e.g. *Arthrobacter*, *Corynebacterium*, *Rhodococcus)* (Bitton, 2005; Machnicka, 2014). *Escherichia coli* is one of the main indicators for assessing the quality of wastewater.

Sonication potential for disinfection in water is due to the acoustic cavitation effect, which is the creation and collapse of millisecond micro-bubbles, creating extreme temperature and pressure gradients (Drakopoulou *et al.*, 2009; Sango *et al.*, 2014). Indeed, the collapse of these micro-bubbles leads to extremely high local temperatures and pressures. These conditions have shown to result in the generation of highly reactive radicals. Ultrasound is therefore able to inactivate bacteria and de-agglomerate bacterial clusters through a number of physical, mechanical, and chemical effects caused by acoustic cavitation (Antoniadis *et al.* 2007; Broekman *et al.* 2010; Vasilyak, 2011). Nevertheless, to the knowledge of the authors, there are no studies focusing on identifying the major effects of sonication stress, and particularly the characterisation of the microbiological responses (e.g., log reductions) of wastewater microorganisms under ultrasound treatment. Several similar studies on the mode of action has been carried out on other novel disinfection technologies such as plasma, ozone and nanomaterials (Laroussi, 1996; Mahapatra, Muthukumarappan and Julson, 2005; Perni *et al.*, 2007; Nath *et al.*, 2014). Unravelling the impact of ultrasound on microbial responses would be essential for fully understanding the sensitivity of *E. coli* and thus its efficient use in industrial applications.

This research aims to examine the antimicrobial mechanisms of ultrasound action on *E. coli* by performing a comparative analysis between wild type bacteria and selected mutants that have important general stress tolerance genes deleted. The outcome aims to address the role of several knock-out genes in the protection or sensitivity against ultrasound generated radicals.

3.2 Materials and Methods

3.2.1 Characterisation of growth properties of E. coli K12

In order to establish pre-culturing conditions to achieve the completion of the exponential growth phase growth curves for *Escherichia coli* K-12 were plotted. An isolated colony from a pure culture was transferred to a 9 mL Tryptic Soy Broth without dextrose (TSB-D) tube with a sterile loop. This first subculture was incubated at 37° C for 25 h. Then, 10 µL from the first subculture was transferred to an Erlenmeyer flask containing 100 mL of TSB-D. This transfer corresponds to time 0 of the bacterial growth, where 1 mL samples was taken and diluted in a 9 mL Ringer's solution tube. Decimal dilutions were prepared and plated on TSA plates. Every hour, a 1 mL sample was taken, and the same procedure was repeated. After 24 h of incubation at 37° C, bacteria on TSA plates were counted. Experiments were ran in order to obtain data until 15 hours of growth.

After collecting the data, the results were expressed for each experimental point by the number of colony-forming units (CFU) per mL in a log scale. The results were then analysed with the software Integrated Predictive Modelling Programme (IPMP 2013) from the United Stated Department of Agriculture, using the Baranyi full growth model (Baranyi and Roberts, 1994). A growth curve and four parameters (Y_0, the initial bacterial population, Y_{max}, the maximum bacterial population, μ_{max}, the specific growth rate and h_0, the physiological state of the microorganisms under consideration) were obtained by performing regression analysis with the selected non-linear model.

$$Y(t) = Y_0 + \mu_{max} A(t) - \ln\left\{1 + \frac{\exp[\mu_{max} A(t)] - 1}{\exp(Y_{max} - Y_0)}\right\}$$

$$A(t) = t + \frac{1}{\mu_{max}} \ln[\exp(-\mu_{max} t) + \exp(-h_0) - \exp(-\mu_{max} t - h_0)].$$

3.2.2 Bacterial strains and preparation of wild type and mutant inocula

In this study, the bacterial strains used were *E. coli* K-12 wild type, and its isogenic mutants Δ*dnaK*, Δ*soxS*, Δ*soxR*, Δ*oxyR*, Δ*rpoS*, Δ*gadA* (Jkl 3485), Δ*gadB* (Jkl 1488) Δ*gadC* (Jkl 1487) and Δ*yneL* (Jkl 5247), all obtained from the National Bio-Resource Project, Japan (NIG, Japan). A description of the mutants and the functions of the knockout gene is given in Table 3-1.

Table 3-1: A description of the *E. coli* mutants and a description of the function of the knockout gene.

Gene	Protein encoded	Function of the knockout gene
dnaK	Chaperone protein DnaK	Essential role in the initiation of phage lambda DNA replication; involved in chromosomal DNA replication; participates actively in the response to hyperosmotic shock.
soxR	Redox-sensitive transcriptional activator SoxR	Activates the transcription of the *soxS* gene which itself controls the superoxide response regulons; contains a 2Fe-2S iron-sulfur cluster that may act as a redox sensor system that recognizes superoxide, the variable redox state of the Fe-S cluster is employed *in vivo* to modulate the transcriptional activity of SoxR in response to specific types of oxidative stress.
soxS	Regulatory protein SoxS	Transcriptional activator of the superoxide response regulon of *E.coli* that includes at least 10 genes such as *43oda*, *nfo*, *zwf* and *micF*; facilitates the subsequent binding of RNA polymerase to the *micF* and the *nfo* promoters.
oxyR	Hydrogen peroxide-inducible genes activator	Hydrogen peroxide sensor; activates the expression of a regulon of hydrogen peroxide-inducible genes; positive regulatory effect on the production of surface proteins that control the colony morphology and auto-aggregation ability
rpoS	RNA polymerase sigma factor RpoS	Master transcriptional regulator of the stationary phase and the general stress response; controls positively or negatively the expression of several hundred genes which are mainly involved in metabolism, transport, regulation and stress management
gadA *gadB*	Glutamate decarboxylase alpha Glutamate decarboxylase beta	Convert glutamate to gamma-aminobutyrate (GABA); the gad system helps to maintain a near-neutral intracellular pH when cells are exposed to extremely acidic conditions.
gadC	Probable glutamate/gamma-aminobutyrate antiporter	Involved in glutamate-dependent acid resistance; imports glutamate inside the cell while simultaneously exporting to the periplasm the GABA produced by GadA and GadB.
yneL	Putative HTH-type transcriptional regulator YneL	A predicted transcriptional regulator which controls the conversion of DNA to RNA and the gene activity.
gadW	Transcriptional regulator	Cellular response to DNA damage
gadX	Transcriptional regulator	Cellular response to DNA damage
gabT	4-aminobutyrate aminotransferase	Involved in the pathway of amino acid degradation induced by general stress conditions
gabD	Succinate semi-aldehyde dehydrogenase	Important for nitrogen metabolism in nitrogen limited conditions

The pure cultures of strains were stored in vials at -80°C with 1% DMSO. Before any experiment, pure cultures with isolated colonies were prepared. Under aseptic conditions, a loop from the frozen vial was streaked on Tryptone Soya Agar (TSA; Oxoid, United Kingdom) plates for *E. coli*. Following overnight incubation at 37°C, these pure culture plates were stored at 5°C, and kept for 3 to 4 weeks the most, until further use.

The first subculture was prepared to transfer one isolated colony from the TSA plates to 10 mL Tryptic Soy Broth without dextrose (TSB-D) tube with a sterile loop and incubated at 37°C for 24h. Then, 10 µL from the first subculture was transferred to a small bottle containing 100 mL of TSB-D and incubated at 37°C for 24 h to reach the stationary phase of growth. After incubation, 10 mL of the culture was taken and centrifuged at 6898 x g rcf using rotor Sigma 12151 (centrifuge Sigma – 2-6) for 20 min. The supernatant was then discarded, and the pellet was rinsed with 9 mL of Ringer's solution and centrifuged once more for 20 min. The washing step was repeated twice. The cells were re-suspended in ringer's solution, and 2 mL were transferred into 298 mL synthetic wastewater, as will be highlighted further down. A similar study was performed for *Enterococcus faecalis* strains but all results and approach are presented in Appendix 3 as the main focus on this chapter were the *E. coli* strains.

3.2.3 Characterisation of ultrasound

In order to standardize the intensity level of ultrasound process due to different ultrasound devices, Ultrasound Intensity (*UI*) for the used ultrasonic horn was calculated. The UI can be determined calorimetrically using the following equations that were described by Mason, T.J., (1990).

$$UI = \frac{4P}{\pi d^2} \qquad \text{(Equation 1)}$$

Where d [cm] is the diameter of the sonotrode.

In the above equation, P [W] represents the absolute ultrasonic power and can be defined as:

$$P = mC_p\left(\frac{dT}{dt}\right)_{x=0} \qquad \text{(Equation 2)}$$

Where, m [g] is the mass, C_p [J/g·K] is the specific heat capacity and dT/dt [K/s] is the ratio of change of temperature during sonication. Where:

m = 100 g

C_p = 4.179 J/g °C

dT/dt = temp change in °C/s

d = 1.4 cm

3.2.4 Ultrasound treatments

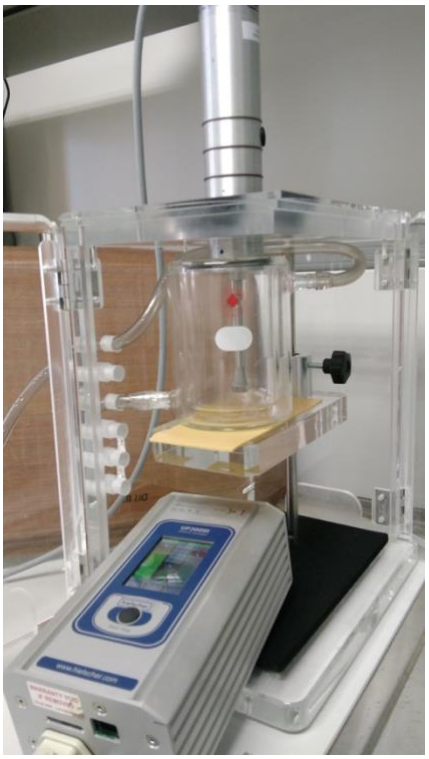

Figure 3-1: Photograph of the ultrasonic device UP200St from Hielscher

The inoculated solution was transferred to a jacketed beaker, which was used to pass cold water, to avoid temperature increase during ultrasound. The ultrasonic equipment used was a UP200St (Hielscher, Germany) comprising an ultrasonic generator UP200St-G (200 W, frequency 26 kHz), and a transducer UP200St-T that could be integrated in a sound protection box. A temperature probe was connected to the transducer and measured the temperature of the solution throughout the ultrasonic treatment and that temperature profile was recorded on an integrated SD/USB ComboCard. A 14 mm diameter sonotrode was used and placed 2 cm deep in the solution to be treated and was carefully cleaned between experiments with 70% ethanol.

The first series of treatments were carried out applying an ultrasound treatment to the working solutions of bacteria during 3 minutes in continuous mode at an intensity of 100% and Ultrasonic Intensity of 31.89 W/cm^2, for all *E. coli* strains using three conditions: (i) controlled temperature I (US-TI): Beaker with SW was surrounded by a cold water bath to keep the temperature lower than 45°C; (ii) non controlled temperature (US): Beaker with SW was not placed in cold water bath in order to study the effect of ultrasound in combination with the generated heat; (iii) Controlled temperature II (US-TII): SyW was placed in a jacketed beaker, which was used to control the temperature preventing it from increasing above 37°C.

After each treatment, 1 mL of the sample was transferred to 9 mL Ringer's solution to perform decimal dilutions which were then plated on TSA plates for plate count enumeration.

3.2.5 Statistical analysis

The performance of the regression analysis for the growth data was evaluated by calculating the RMSE. The RMSE (root mean square error) measures the differences between the values predicted by the model and the values observed. Therefore, it indicates the absolute fit of the model to the data. Lower values of RMSE indicate better fit.

$$RMSE = \sqrt{\frac{\Sigma_{i=1}^{n}(P_i - O_i)^2}{n}}$$

where P is the Predicted value and O is the observed value

An F-test with 99.9% confidence level was used to check significance, within different treatments, whilst a Bonferroni test correction was carried out to assess the significance between each mutant. Where described separately different statistical analysis such as Student T-tests were carried out.

3.3 RESULTS

3.3.1 Establishing of growth curves

The growth curves and parameters obtained for *E. coli* wild type using the Baranyi full growth model (Baranyi and Roberts, 1994) with the software IPMP are presented in figure below. Raw data are reported in Appendix 1.

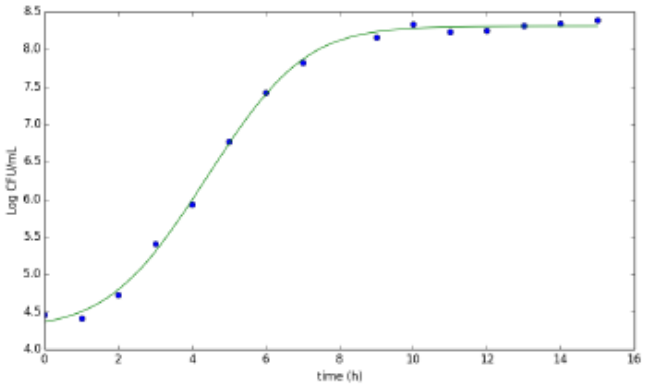

Figure 3-2: Growth curve of *E. coli* K-12 wild type and parameters obtained with the Baranyi full growth model, IPMP

Table 3-2: Root mean square for E. coli growth curve

RMSE = 0.075		
Parameters	Value	Standard Error
Y_0 (log CFU/mL)	4.366	0.059
h_0	2.424	0.272
Y_{max} (log CFU/mL)	8.307	0.030
μ_{max} (h^{-1})	0.988	0.049

The RMSE value obtained is low indicating that the models fit well to the experimental data.

Figure 3-2 show that *E. coli* grows with a specific growth rate of 0.988h^{-1}. *E. coli* reached its stationary phase in around 9 hours. These results confirm that after 16 hours of incubation of the second subculture for *E. coli*, the bacteria will have reached their stationary phase of growth. It is important to note that the growth curves were essential in determining the stationary phase, in order to apply the ultrasound treatment when the bacteria have already

reached it and therefore stopped growing (Rolfe *et al.*, 2012), showing better repeatability for eventual ultrasound treatments.

3.3.2 Characterisation of ultrasound

In order to keep the experiments standard, the Ultrasound intensity and the power of ultrasound were calculated for the three different conditions as shown in Figure 3-3. Using the calculations described previously, the ultrasound intensity and Power for the three methods were as follows (Table 3-3).

Table 3-3: Power and ultrasound intensities of the three methods studied

Treatment	Ultrasound intensity (W/cm^2)	Power (W)
Without cold water	54.51	83.91
With Jacketed Beaker	32.81	50.53
With cold water	27.92	43.00

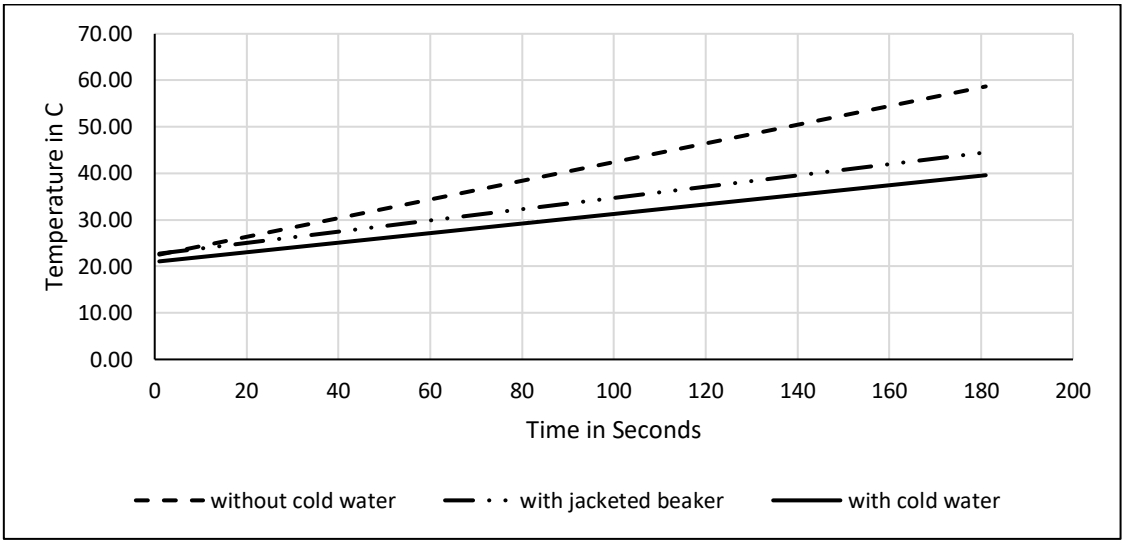

Figure 3-3: Example of temperature profile recorded for the 3 minutes continuous ultrasound treatment

3.3.3 Effects of temperature during ultrasound treatment on E. coli

This study was only made on *E. coli* mutants. Figures 3-4 and 3-5 compare the number of bacteria (in log of CFU/mL) obtained before and after 3 minutes of continuous ultrasound treatment, respectively without and with controlling the temperature with a cold-water bath. Error bars represent the standard deviation between the averages of the results obtained for the 3 replicates. The raw data of this study are shown in appendix I. Examples of temperature profile recorded during one experiment of each series is given in figure 3-3.

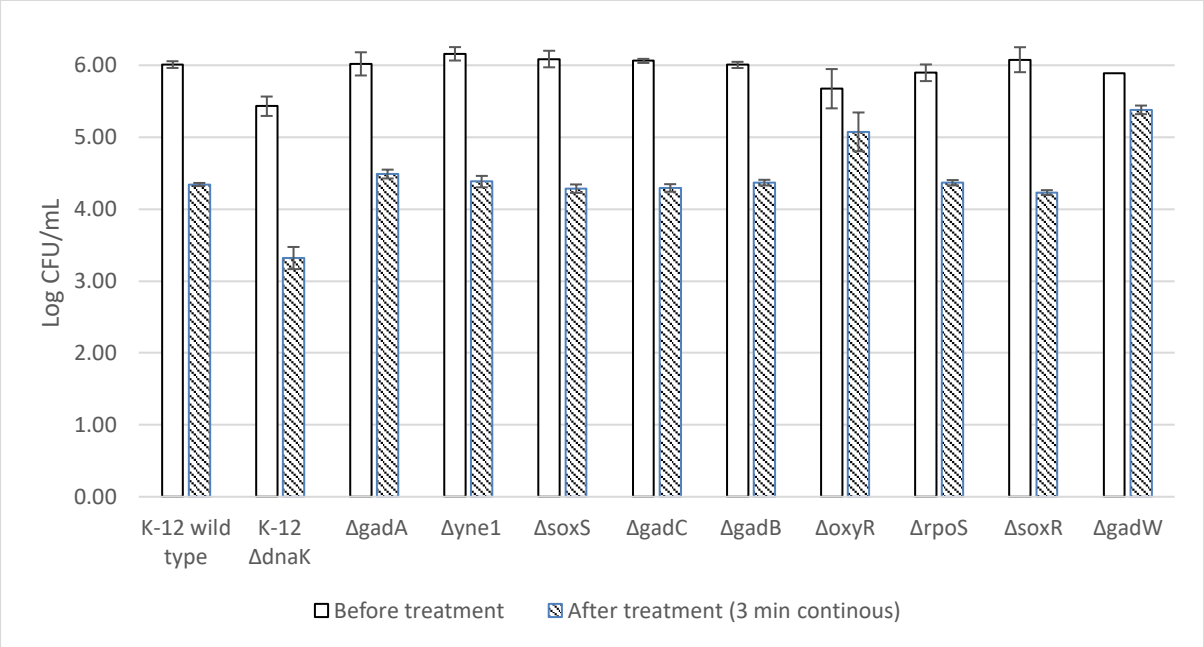

Figure 3-4: Log bacterial counts, before and after ultrasound treatment using cold water bath

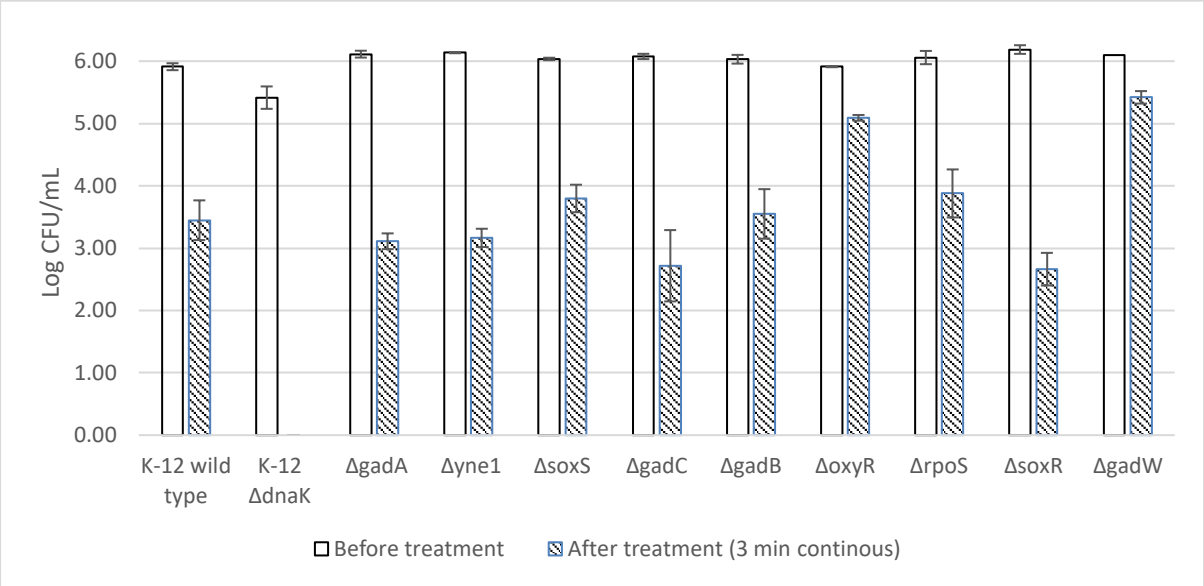

Figure 3-5: Log bacterial counts, before and after ultrasound treatment without temperature control

The reduction noticed in figure 3-5 is both due to temperature and ultrasound effects whereas in figure 3-4, the reduction can be assigned only due to ultrasound effects. This is because the temperature during the treatment, reaching a maximum of 45ºC at the end, is not considered lethal for the *E. coli*. Moreover, the experimental variation is smaller when the temperature is controlled with the cold-water bath. As can be observed by the values of standard deviations, represented by the error bars, the variation is indeed higher in figure 3-4 than in figure 3-5.

From the results collected, it is therefore possible to make a distinction between the reduction due to ultrasound and the reduction due to the increase in temperature (deduced from experimental results), as presented in figure 3-6. It is noticeable that temperature had a great impact on the K-12 *dnaK* mutant.

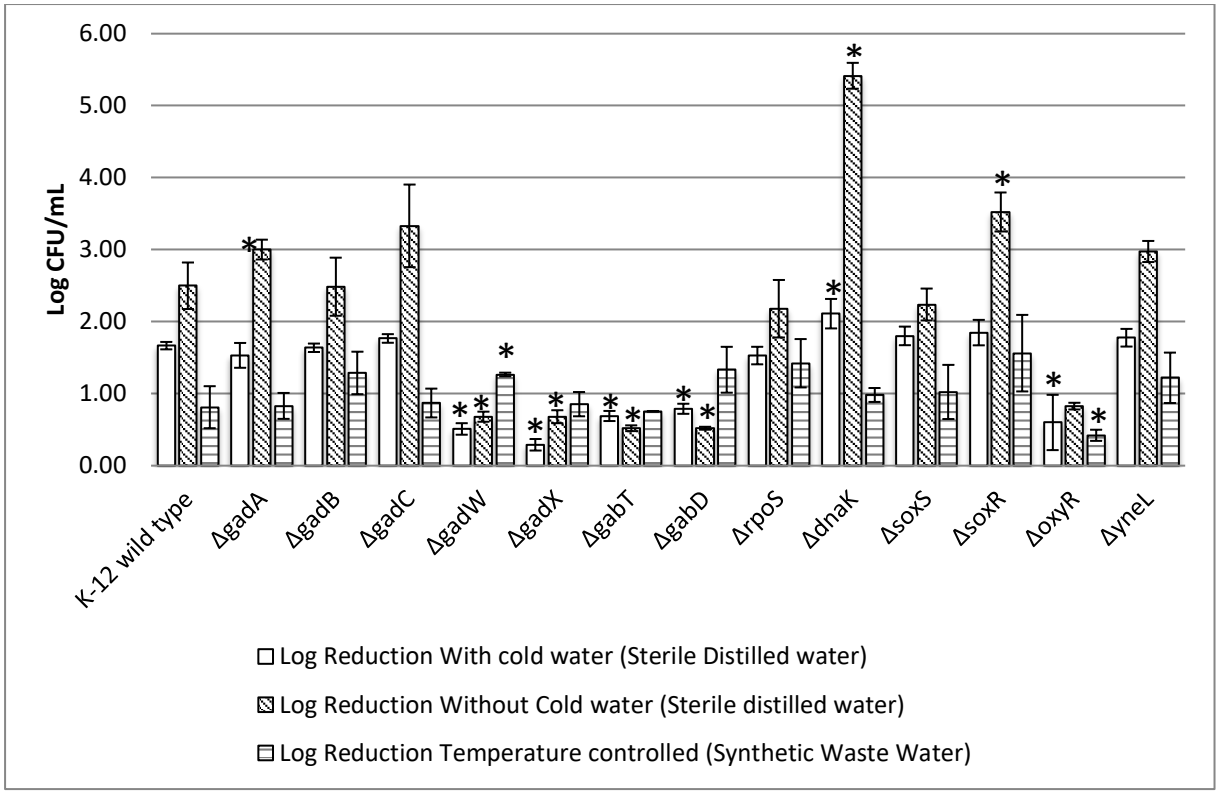

Figure 3-6: Log reduction of different mutants under both controlled and freely increasing temperature. Asterisk shows significantly different mutants.

3.4 DISCUSSION

The medium effect on microbial reactions during ultrasound treatments was examined during this study. The findings suggest that the only significant difference between the media was found in the *dnaK* mutant. It should be pointed out that, in this case, the *dnaK* mutant was mainly influenced by temperature. Table 3-1 displays the behaviour of all mutant strains relative to their *E. coli* K-12 wild type isogenic strain. It appears that the mutant Δ*oxyR* was more resistant to the treatment (reduction of 0.60 log) whereas Δ*dnaK* was almost as sensitive as the wild type after 3 minutes of continuous treatment, even though temperature was controlled. The reduction was identical to that of *E. coli* K-12 wild type for all other mutants. On average, most of the mutants, similarly to the wild type, showed a 1 log reduction.

The temperature profiles obtained suggest that, of the three different procedures, all showed a noticeable difference in the rate of heating between the three different configurations. The controlled temperature treatment resulted in 0.1029°C/s and a final temperature 39.5°C, non-temperature-controlled treatment with a heating rate of 0.2008°C/s and a final temperature of 58.3°C and with just cold water 0.1209°C/s with a maximum temperature of 44.5°C. It is therefore obvious that the log reduction observed in some of the mutants is related to ultrasound activity rather than temperature as shown in table 3-2. In fact, according to Patil *et al.* (2011), the *soxR*, *soxS*, *oxyR*, *rpoS* and *dnaK* genes have been reported to play an important role in the protection against reactive oxygen radicals. One of the phenomena caused by cavitation is the formation of radicals $H^{\bullet}$ and $OH^{\bullet}$ and of H_2O_2 (Joyce *et al.*, 2003), which are known to cause oxidative stress in bacteria. The experimental results indicate that not all mutants were affected in the same way by the ultrasonic treatment.

Two of the most affected mutants were found to be Δ*oxyR* and Δ*dnaK* (temperature sensitive) mutants. The OxyR subunit of RNA polymerase is the master regulator of hydrogen peroxide genes in *E. coli* as it positively regulates the production of surface proteins that govern the morphology and auto-aggregation ability of the colony. Among other things, the DnaK protein is essential for growth at high temperatures and plays a role in the regulation of the heat shock response. The heat shock response is an inducible cellular response to a variety of stresses such as heat, exposure to ethanol, oxidants, and DNA-damaging agents, production of abnormal proteins, viral infections, and starvation for nutrients (Bukau and Walker, 1989). The deletion of the *dnaK* gene can explain the sensitivity of the corresponding mutant. It is particularly sensitive to heat in the ultrasound studies where the temperature during the treatment was not controlled. It can also be an explanation to the fact that this mutant which was more susceptible to change by the ultrasonic treatment than the K-12 wild type of *E. coli*, as ultrasounds lead to

an oxidative stress on bacteria. Deletion of *dnaK* resulted in a phenotype sensitive, to ultrasound, although the bacterial populations were not completely inactivated with the applied treatment. This *dnaK* gene would therefore play a role in the protection against ultrasound treatment of the bacteria.

Under the conditions tested, the mutant K-12 Δ*oxyR* appeared to be more resistant to the treatment, while the K-12 Δ*dnaK* was more sensitive in comparison with the wild type strain. As a result *dnaK* would therefore play a role in the protection against ultrasound treatment of the bacteria, and the corresponding mutant also shows a great sensitivity to the heat generated during the ultrasonic treatment. A significant finding that needs to be noted is that of Δ*oxyR*. The *oxyR* regulates the expression of a group of genes that make up the *oxyR* regulon. The OxyR protein is constitutively formed and is oxidized by H_2O_2. The oxidized form of OxyR binds to promoter regions of target genes and activates transcription by protein–protein contact with RNA polymerase. The OxyR-activated genes have direct and indirect antioxidant functions in the defence of the cell, such as removal of H_2O_2 by catalase and the protection of DNA from oxidative attack by the Dps protein (Pomposiello and Demple, 2001). The current results show that this mutant was more resistant to ultrasound suggesting that the produced H_2O_2 during ultrasound treatments is not stable, as the OxyR mutant would have been more sensitive to the effect of peroxide.

In addition, the study also evaluated mutants in genes associated with the GAD system (Table 3-2) and found a potential role in ultrasound treatment. The GAD system is known to play an important role in acid tolerance of bacteria (Smith *et al.*, 1992; Feehily and Karatzas, 2013; Paudyal and Karatzas, 2016) but it has been shown to play a role in oxidative stress only in *Saccharomyces cerevisiae* (Coleman *et al.* 2001) and *Francisella tularensis* (Ramond *et al.*,

2014) but not in other organisms. This is the first report showing a possible involvement of the GAD system in oxidative stress in *E. coli*. Here we show that the lack of decarboxylase *gadB* did not affect survival (Table 3-2). However, the lack of *gadA* and *gadC* resulted in sensitivity when treatment occurred without cold water and in resistance in synthetic wastewater. This might suggest a differential role of the GAD system in different temperatures/conditions, or the upregulation of alternative mechanisms that protect against oxidative stress under specific conditions (e.g. synthetic wastewater).

We also assessed the role of other genes linked to the regulation of the GAD system and the GABA shunt. Deletion of the GAD system regulators *gadW*, *gadX*, (Tramonti *et al.*, 2006; Sayed, Odom and Foster, 2007) resulted in resistance to ultrasound in sterile water enhancing the role of the GAD system in oxidative stress. Similarly deletion of *gabT* and *gabD* that encode for the GABA shunt that catabolises intracellular GABA pools formed by the intracellular GAD system (Feehily *et al*. 2013), resulted in resistance in sterile water but not in wastewater. It has been suggested that the GAD system coupled with the GABA shunt feed into the TCA cycle affecting the levels of succinate and oxoglutarate that have anti-oxidant properties and can confer resistance to oxidant species (Ramond *et al.*, 2014) that might be produced during ultrasound treatment. However, further work is required to confirm the above hypothesis and other possible links between the GAD system and oxidative stress.

3.5 Conclusion

In conclusion, this study looked into the application and process of ultrasound technology as a means of disinfection by acoustic cavitation. Sterile water and synthetic wastewater inoculated with various mutants of *E. coli* K12 strains containing deletions in genes affecting specific functional properties of *E. coli*. *E. coli* K-12 Δ*oxyR*, tended to be more resistant to the treatment

together with *gadW, gadX, gabT and gabD*. In contrast, the mutant K-12 Δ*dnaK* was more sensitive with a reduction of approximately 2.5 log (CFU/mL) relative to its isogenic wild type *E. coli* K-12. This suggests that the *dnaK* gene contributes to the general stress response and more specifically to hyperosmotic stress. The other *E. coli* deleted genes tested (*soxS*, *rpoS,*, *gadB, gadC, yneL*) did not appear to be involved in defence of microbial cells against ultrasound. Furthermore, the study reveals for the first time, the possible role of the GAD system in ultrasound treatment and oxidative stress that requires further investigation, as these have shown that they are essentially crucial in the protection from oxidative stress.

In the context of the wastewater recycling and reuse, the aim is to find a treatment capable of eliminating or substantially reducing all the pathogens to reduce pollution of the receiving waters and to provide public health protection. Ultrasound treatments can be a potential technology for this type of treatments.

CHAPTER 4: *E. coli* K-12 transcriptomics for assessing the mechanism of action of high-power ultrasound

4.1 Introduction

As described in chapter 3, recent research on the mechanism of action of ultrasound technology as a means of disinfection by acoustic cavitation has shown the possible role of the GAD system in ultrasound treatment and oxidative stress as well as that of *dnaK* gene of *E. coli* in general stress response. Similar studies on the mode of action have been carried out on other novel disinfection technologies such as plasma, ozone (both producing reactive radicals) and nanomaterials (Laroussi 1996; Mahapatra *et al*. 2005; Perni *et al*. 2007; Nath *et al*. 2014). The most common methods that have been applied included biochemical tests that assess the destruction of food toxins (Mahapatra, Muthukumarappan and Julson, 2005) and physiochemical techniques using optical emission spectroscopy that look at bacterial inactivation kinetics in conjugation with radicals produced in situ (Perni *et al.*, 2007). Nevertheless, it is important to further unravel the mechanism of action of such technologies and specifically of ultrasound to ensure the production of safe and stable ultrasound processed water.

Ultrasound is known to have an impact on the acidity of the treated medium, which may affect bacterial resistance. Previous reports have shown that the pathogenic *E. coli* may survive in acidic environments for long periods (Patil *et al.*, 2009), and an acid-adaptive response in *E. coli* O157:H7 has been previously reported (Leyer, Wang and Johnson, 1995; Berry and Cutter, 2000; Šeputiene *et al.*, 2006; Tosun, 2014). Inducible resistance mechanisms could increase the resistance of bacteria to acidic conditions (Tosun, 2014). As such, ultrasound at high amplitudes of 37.5µm has already been shown to enhance survival under acidic conditions, however further studies are required to fully understand the underlying mechanism (Patil *et al.*, 2009).

As of recent, transcriptomics has been used to study the effect of different microbial stresses. This was referred to as the "age of omics" by Wecke and Macher in 2011. Study of gene expression by looking at RNA transcripts present in cells has increased our knowledge of cell resistance mechanisms and/or regulatory networks that coordinate bacterial stress responses (Chueca, Pagán and García-Gonzalo, 2015). Several works studying the transcriptome of *E. coli* during such stress responses have increased our knowledge on modes of action of stress (e.g., Chueca et al., 2015; Harcum & Haddadin, 2006; King, Lucchini, Hinton, & Gobius, 2010; Li et al., 2018; Royce et al., 2014; Yung et al., 2016; Zheng et al., 2001). These studies have described, among others, mode of actions of many antibiotics, mechanisms of bacterial adaptation and inactivation by heat or by high hydrostatic pressure (Wecke and Mascher, 2011; Chueca, Pagán and García-Gonzalo, 2015).

Furthermore, transcriptional profiling has also shown induction of general stress responses and proteins after specific methods of due to specific cross-resistance phenomena (Carruthers and Minion, 2009; Shin *et al.*, 2010; Chueca, Pagán and García-Gonzalo, 2015). Hence, the first heat-shock response in *E. coli* was first described by Neidhardt and Yura groups in 1978 (Harcum and Haddadin, 2006). They observed that 20 proteins were responsive to heat and that they were controlled by transcription levels. This discovery gave rise to several others showing that many heat-shock proteins promote protein folding while others are proteases (Harcum and Haddadin, 2006). Further analysis with RNA-Seq methods that can capture the global transcriptional response during particular conditions of any organism could allow the simultaneous analysis of all the regions within the genome, unlike other methods such as RT-PCR, which are still limited to analysing specific and known genomic regions.

In the current study presented in this chapter, the main aim was to assess the mRNA differentiation of *E. coli* K-12 wild type and its isogenic mutant Δ*gadW* following the exposure to singe or sequential cycles of high-power ultrasound treatments. High-throughput RNA-sequencing (RNA-Seq) was used to perform a transcriptomic analysis and understand the mechanism of action of ultrasound treatments.

4.2 Material and Methods

4.2.1 Bacterial strains and preparation of inoculum

In this study, the bacterial strains used were *E. coli* K-12 wild type MG1655 strain (WT) which is one of the most commonly used laboratory strains with minimal genetic manipulation (Blattner *et al.*, 1997), and its isogenic mutant Δ*gadW* obtained from the National Bio-Resource Project, Japan (Baba *et al.*, 2006; Yamamoto *et al.*, 2009). Δ*gadW* was used, as this gene was found to have a higher resistance to ultrasound treatment when compared with the wild type and other mutants, possibly playing a possible role in ultrasound treatment (refer to Chapter 3).

The pure cultures of strains were stored in vials at -80°C in a freezer using 1% DMSO Solution. Before any experiment, pure cultures with isolated colonies were prepared. Under aseptic conditions, a loop from the frozen vial of *E. coli* was streaked onto Tryptone Soya Agar (TSA; Oxoid, United Kingdom) plates. Following overnight incubation at 37°C, these pure culture plates were stored at 5°C and kept for a maximum of 3 to 4 weeks before further use.

4.2.2 Preparation of the working culture

A first subculture was prepared by transfering one isolated colony from the TSA plates to 10 mL Tryptic Soy Broth without dextrose (TSB-D) tube with a sterile loop and incubated at 37°C for 24h. Then, 10 µL from the first subculture was transferred to a small bottle containing 100 mL of TSB-D and incubated at 37°C for 24 h to reach the stationary phase of growth. After

incubation, 10 mL of the culture was taken and centrifuged at 7000 x g using rotor Sigma 12151 (centrifuge Sigma - 2-6) for 20 min. The supernatant was then discarded, and the pellet was rinsed with 9 mL of Ringer's solution and centrifuged once more for 20 min. The washing step was repeated twice. The cells were suspended, and 2 mL were transferred into 298 mL model enriched water. The model enriched water was used to simulate high nutrient load in the water, to mimic wastewater. This was the sample for the ultrasound treatment. After the first cycle of ultrasound, five colonies from post-treatment samples were isolated and treated for the second cycle of ultrasound treatment to see if resistance was induced. The inoculum was also grown without ultrasound treatment, and this served as control.

4.2.3 Ultrasound treatments

As described in section 3.2.4, a Hielscher UP200St Ultrasonifier operating at a constant frequency of 26 kHz was used. It was equipped with a generator UP200St-G 200W and a transducer UP200St-T which could be integrated in a sound protection box. A 14 mm sonotrode was used, which was suitable to transmit the ultrasound smoothly across a relatively large surface. Ultrasound treatments were applied for 5 minutes on a continuous mode at 100 % intensity Conditions were chosen based on previous studies (see Chapter 3) which resulted in a microbial reduction of 0.8 log for the WT and 1.27 log for the *gadW* strain.

The model enriched water was produced as described by Antoniadis (2007) and Ayyildiz. (2011), that is, peptone 64.0g/L; Meat Extract 44.0g/L; Urea 12.0g/L; K_2HPO_4 11.2g/L; NaCl 2.8g/L; $CaCl_2.2H_2O$ 1.6g/L; $MgSO_4.7H_2O$ 8g/L. For each sample, the working solution, consisting of the inoculated enriched water which was transferred into a 500 mL big jacketed beaker attached to a water pump. The big beaker was carefully disinfected with alcohol between each experiment. Hereafter, the tip of the sonotrode was placed in the centre of the

beaker, containing 300 mL of bacterial suspension (refer above) with a submerged depth of 2 cm.

Sequential treatment was then applied. After the first treatment, colonies were isolated and re-cultured before a second treatment was run to assess the impact of ultrasound on microbial induced resistance. The conditions of the second treatment were the same as the first treatment. Experiment groups that were non treated (i.e. Pre-Treatment of ultrasound) or treated with ultrasound were abbreviated as PT or US, respectively. US2 refers to the sequential ultrasound treatment on the same biological replicate.

After each treatment, 1 mL of the sample was transferred to 9 mL Ringer's solution to perform decimal dilutions which were then plated on TSA plates for plate count enumeration. Colonies were then stored in a -80ºC freezer for future genetic analysis.

4.2.4 RNA Extraction

RNA extraction was carried out by following the E.Z.N.A.® Bacterial RNA Kit Centrifugation Protocol (Omega BIO-TEK V.5.0, 2018). In summary, the bacteria were cultured from cryovials by inoculating a 10 mL TSB-D broth with a 10 µL sterile loop. The culture was then incubated for 4 h at 37°C. After incubation, the culture was aspirated and dispensed well with a 1mL micropipette tip, and 3 mL of culture was transferred into a sterile centrifuge tube. The culture was then centrifuged at 4,000 x g for 10 min at 4°C. The media was aspirated and discarded. For each pellet, 100 µL lysozyme solution was added, and the tube vortexed at maximum speed for 30 seconds. Manufacturer instructions were followed.

4.2.5 RNA-SEQ

RNA-SEQ was carried out at Omega Bioservices, Georgia, USA, and thus the standard methods of RNA-SEQ on Illumina machines were applied. Briefly, the method is as follows:

A QC check for RNA was carried out by using a bioanalyser (Thermo Scientific Nanodrop™, 2018) to achieve the total concentration. The recommended concentration for RNA ≥1µg for RNA-SEQ; however, Illumina kits can successfully amplify RNA from lower amounts starting from 100 ng, provided that more amplification cycles are performed during PCR.

As soon as QC was completed, and the samples were considered free from DNA contamination or RNA degradation, the RNA library was prepared. This was carried out by attaching the samples to oligo-dT probes, which were joined to magnetic beads. This allows the poly-A RNA to be selected that produces the mRNA. These fragments were then broken down with high temperatures, to 200 bp long fragments, which were then used to prepare the cDNA libraries. This cDNA had adaptors ligated and was then QC checked once again via a bioanalyser, while in this case an Agilent TapeStation was used. At this point, the QC results revealed a peak of products at the expected size based on the average fragment size and length of the adaptors. Once the QC was completed, and all samples showed a sufficient number of reads, data analysis was performed.

4.2.6 Bioinformatics

The analysis was carried out by using Geneious prime 2019. The raw data files from RNA-SEQ analysis were imported into the bioinformatics software. Each data file was then assembled with the latest reference genome, downloaded from the NCBI website: https://www.ncbi.nlm.nih.gov/genome/?term=e%20coli%20k%2012. Once the contig was created, this was repeated for all the samples. All contigs were then annotated from the reference, and expression levels for all different RNA genes were calculated and compared, to export the volcano and PCA plots. The RNA genes present were then exported to MS Excel, where the genes were sorted depending on their expression levels. Venn diagrams were also

plotted to compare transcribed genes between different treatments, while a heat map (generated by https://software.broadinstitute.org/morpheus) of all the expressed genes was produced to show patterns between the treatments. Finally, the genes obtained were put in Gene Ontology databases (GO) to obtain the mechanisms and KEGG pathways. GO is an international standardised gene functional classification system, which used three ontologies: molecular function, cellular component and biological process. KEGG is one of the accessible pathway analysis databases, which allows researchers to understand further biological functions related to gene expression (Kanehisa *et al.*, 2008).

4.2.7 Principal component analysis

Principal component analysis (PCA) is a statistical tool, capable of analysing patterns from large and complex datasets (Marini and Binder, 2019). PCA plots will provide a visual representation of the distances between samples from which the characteristics of gene expression can be inferred. However one of the major limitations of this would be that PCA plots would only be able to discriminate the quality, and not the sequencing quality of the RNA-SEQ (Son *et al.*, 2018). The analysis was carried out by Geneious Prime 9 software.

4.3 Results

Using the Illumina sequencing platform, the raw reads of Table 4-1 were collected. No poor-quality reads were obtained as a paired score of >30 was achieved for all runs. The reads were subjected to the reference transcriptome to map to the existing gene annotations and were found to have a total of 4497 known genes.

Table 4-1: Summary of the raw reads for each sequencing run per sample.

Strain with Treatment	Replicate 1		Replicate 2	
	Unique reads	Duplicate reads	Unique reads	Duplicate reads
WT PT	1,907,618	9,782,951	1,313,674	10,376,895
WT US	1,638,411	8,729,411	2,259,703	8,107,832
WT US2	2,510,105	7,998,899	2,896,851	7,612,563
ΔgadW PT	1,903,140	8,570,279	2,348,814	8,124,605
ΔgadW US	2,247,793	8,586,961	2,727,471	8,107,283
ΔgadW US2	2,466,568	7,854,287	2,819,178	7,501,677

4.3.1 Differentially expressed genes (DEGs) and function enrichment analysis

In this study, the criteria of the false discovery rate (FDR) were set as 0.01 and $|\log_2 FC| > 1$ was used to screen DEGs. The following DEGs were found following comparative analysis between different treatments of different genes. For the Wild Type PT vs US strain, the majority of genes (35.4%) obtained were up-regulated after treatment. This went down to just 1.8% for the Wild Type PT vs US2, while for the Wild Type US vs US2 the majority of genes (41.9%) were down-regulated. On the other hand, for the Δ*gadW* strain, US versus US2 showed a majority of down-regulated genes: 37.2%. The genes observed are summarised in Figures 4-2 and 4-3 via a volcano plot, which demonstrates the different clusters of expression of the genes, depending on the fold changes vs the absolute confidence.

Venn diagrams revealed unique and common DEG patters between different treatments, as seen in Figure 4-1

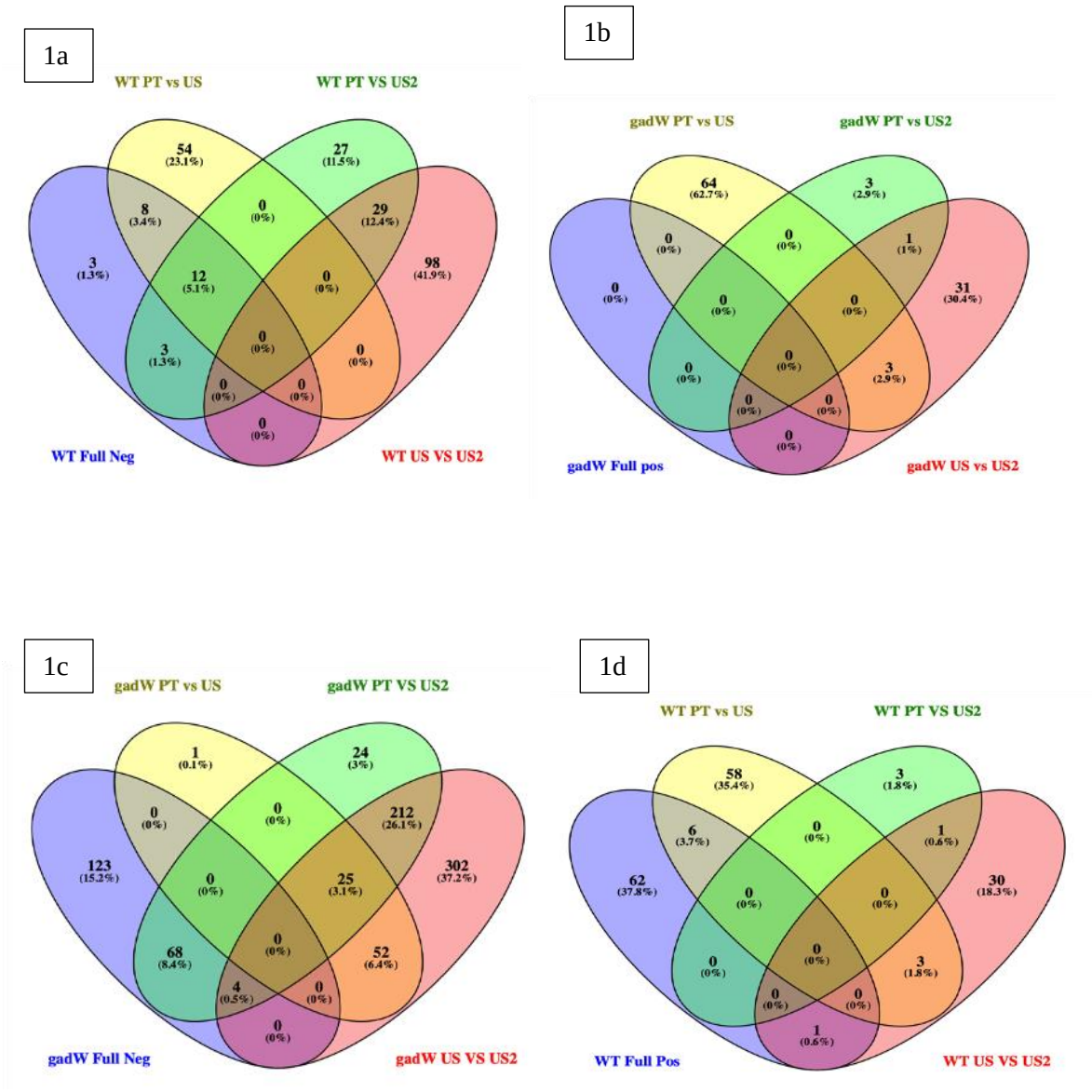

Figure 4-1: Venn diagrams for the negatively expressed DEGs of *E. coli* wild type over all treatments (1a), *E. coli* ΔgadW (1b) and of positively expressed DEGs of *E. coli* K-12 wild type (1c), *E. coli* gadW (1d). The diagrams show the common DEGs between each treatment, together with the overall percentage.

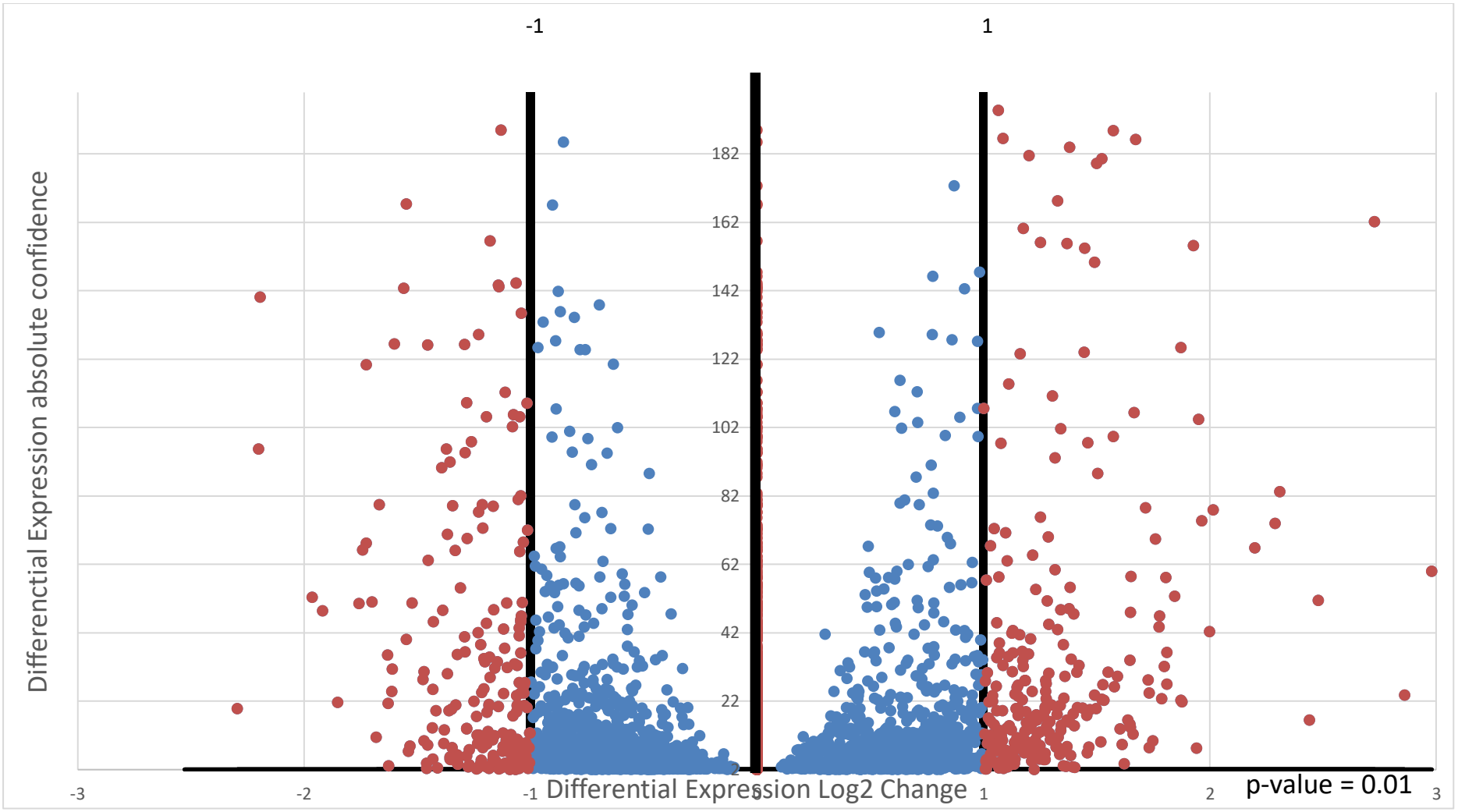

Figure 4-2: Volcano plot for DEG for Wild Type showing gene expression with significant genes in red colour

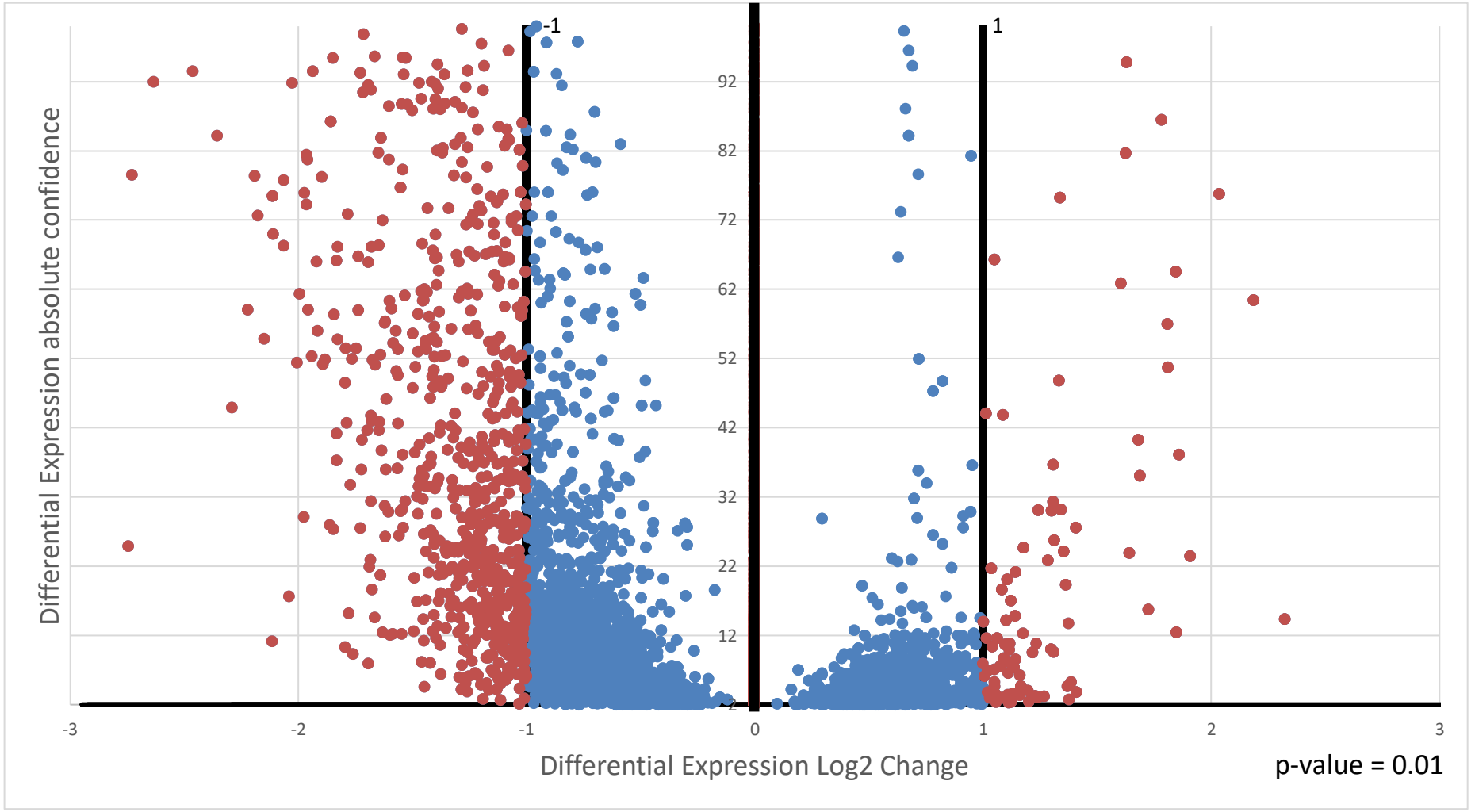

Figure 4-3: Volcano plot for DEG for Δ*gadW* showing gene expression with significant genes in red colour

Venn diagrams are visual representations comparing the different genes obtained under each treatment. The expression of DEGs was found to be significantly different when comparing between the first ultrasound and the second ultrasound treatment, with several genes being affected both positively and negatively by the respective treatment (see Table 4-2). The different strains, i.e., the WT and Δ*gadW* mutant also showed different DEGs, indicating that the response of the cells to ultrasound treatment was different, with some genes being directly responsible for defence mechanisms, while others are maybe playing an indirect effect due to cell damage. Table 4-2 shows that most of the listed genes become negatively expressed, as soon as the cell is treated with ultrasound. These genes, initially positively expressed, indicate that ultrasound has a negative impact on the expression.

Table 4-2: Summary of DEGs expressed. All DEGs listed were significant at P < 0.05

		Biological process		**Molecular function**		**Cellular component**	
Strain	Treatment	Positive expression	Negative expression	Positive expression	Negative expression	Positive expression	Negative expression
WT	PT vs US	396	N/A	N/A	N/A	N/A	N/A
	PT vs US2	301	N/A	11	N/A	52	85
	US vs US2	41	220	14	N/A	15	90
gadW	PT vs US	104	281	N/A	75	N/A	481
	PT vs US2	N/A	2585	N/A	1186	N/A	2582
	US vs US2	N/A	4714	N/A	1634	N/A	4122

A heat map of all the expressed genes was then plotted in Figure 4-4, where hierarchical clustering summarises the differences between the transcription of each gene and each sample. This shows a gradual decrease in expression of the genes as the cells are treated with ultrasound as we move from PT to US1 and from PT to US2, which is less pronounced in the gadW then the WT. On the other hand, the clustering groups together several groups of genes, which are

being expressed similarly, indicating patterns that the US treatment has the highest impact on the expression when compared to the sequential US application.

Furthermore, Principal Component Analysis (PCA), was carried out for both treatments in order to show the differences between biological replicates and the different strain. These are shown in figures 4-5 to 4-7. As explained before, PCA is a statistical procedure which uses linear combinations. In such case the gene expression values of each biological replicate, were plotted on the same graph in order to tabulate similarities. In fact, it can be denoted that all replicates showed similar overall expression. Figure 4-7 demonstrates an overlap of the PCA for both the wild type and the gadW mutant, indicating that under different treatments, similar action was observed. This grouping concludes the similarity in the analysis of both different mutants under the treatments.

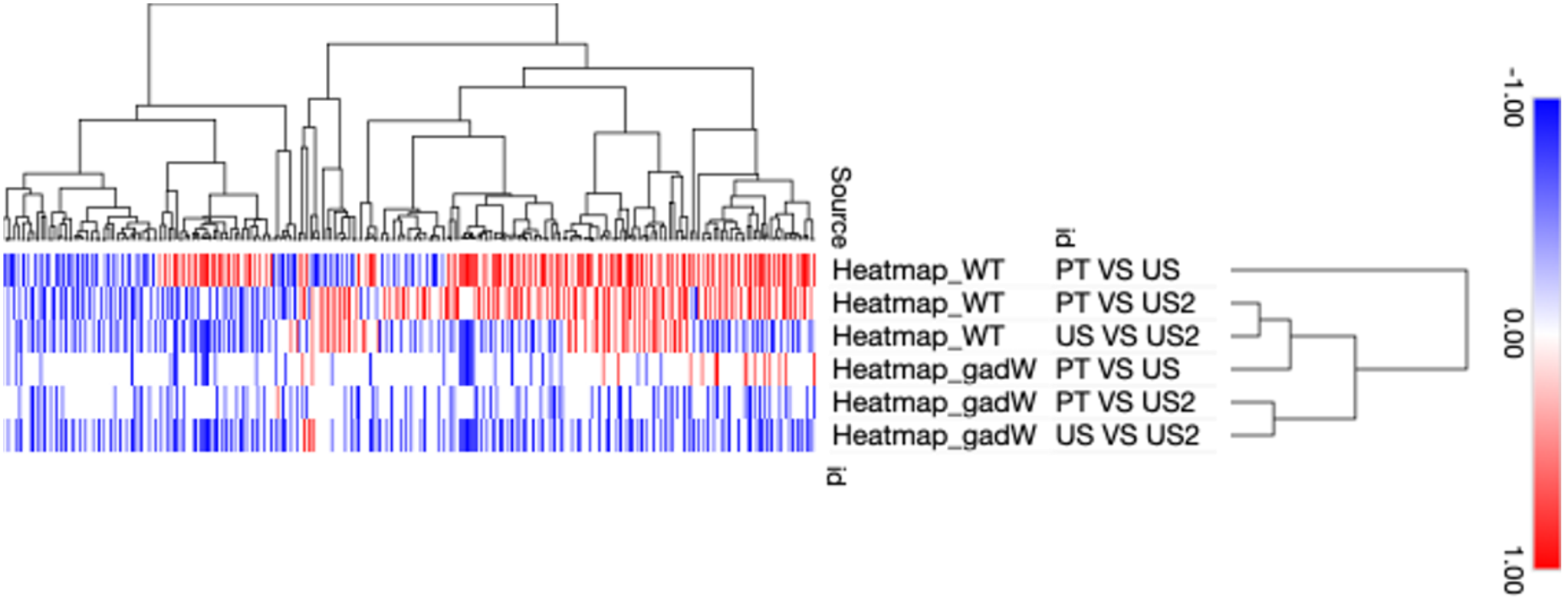

Figure 4-4: Heat map showing the expression and phylogenetic tree of all the genes in relation to pre-treatment (PT), first ultrasound treatment (US1), and sequential ultrasound treatment (US2). Blue denotes down-regulation and red up-regulation

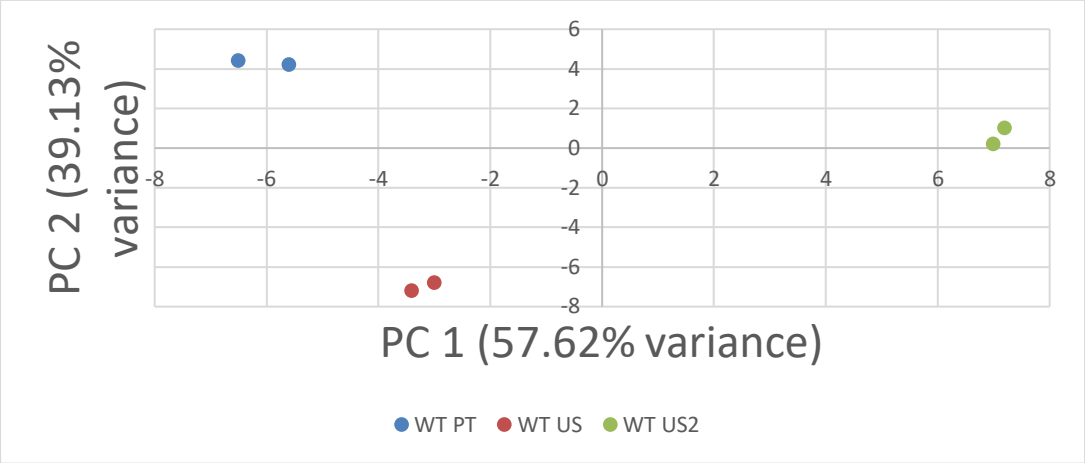

Figure 4-5: Principal Component Analysis for the three treatment types for Wild Type

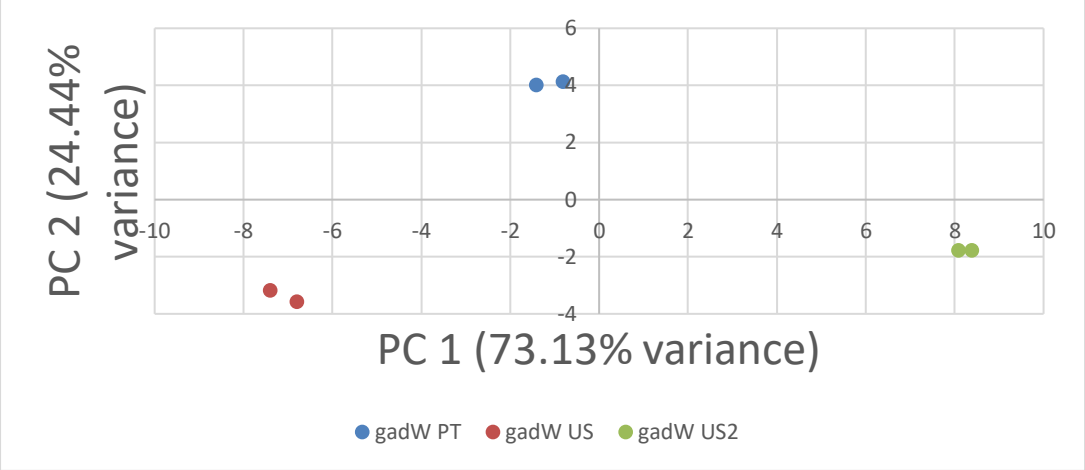

Figure 4-6: Principal Component Analysis for the three treatment types for gadW

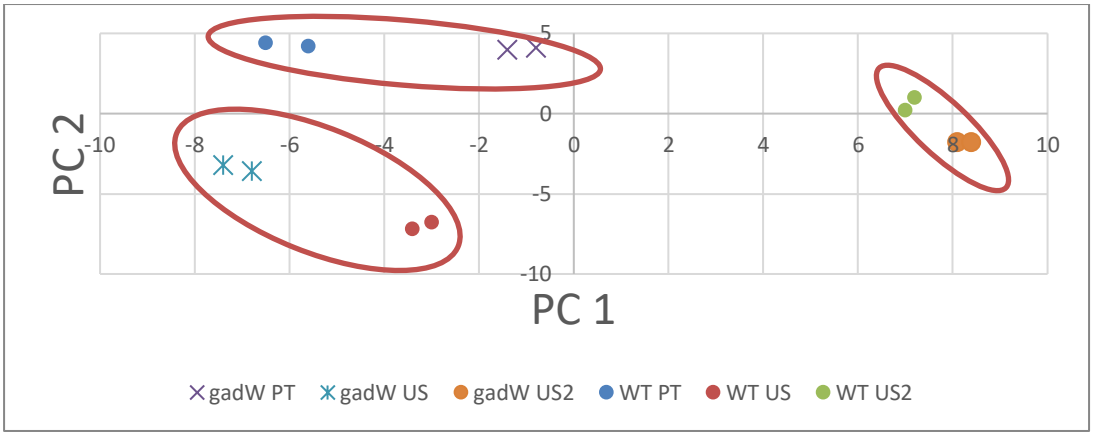

Figure 4-7: Super imposition of both PCA plots showing grouping of treatment type

4.3.2 GO analysis of DEGs

To highlight the categories of the DEGs, all DEGs were annotated to terms in the GO database and were assigned to at least one of the three primary GO categories; biological process, molecular function, cellular component. In this case, all the DEGs obtained were used, irrespective of the criteria of the false discovery rate (FDR), which was set as 0.01 while $|\log_2 FC|$ as $>\pm 1$. A Fisher's exact test was then carried out to standardise the results. In the PT vs US for the WT, 396 DEGs were annotated in the biological process, however in the case of Δ*gadW*, 385 DEGs for the biological processes, 75 DEGs for the molecular function and 481 DEGs for the cellular component. All the DEGs expressed for all treatments are summarised in Table 4-3.

Table 4-3: Table summarising specific DEGs over three treatments. P-values in bold are significantly different

	WT						
	Fold change log_2FC			P-value			
	PT vs US	PT vs US2	US vs US2	PT vs US	PT vs US2	US vs US2	
rpoS	0.510157732	0.465587140	-0.075631600	**$2.63x10^{-12}$**	**$2.98x10^{-10}$**	$1.90x10^{-01}$	General stress
sodC	1.182132369	1.012699790	-0.16510600	**$1.17x10^{-08}$**	**$4.34x10^{-06}$**	$3.10x10^{-01}$	Oxidase gene
sodA	1.164036767	-0.169843600	-1.36500300	**0.000**	**$5.64x10^{-04}$**	**0.000**	Oxidase gene
SodB	0.438066141	0.269851670	-0.194582800	**$1.99x10^{-04}$**	$3.00x10^{-02}$	$5.00x10^{-02}$	Oxidase gene
yifE	0.381227525	-0.958895300	-1.366673800	**$1.56x10^{-03}$**	**$1.28x10^{-10}$**	**$5.52x10^{-32}$**	Stress-induced mutagenesis
nuoG	0.520768054	0.449158840	-0.098598900	**$6.81x10^{-05}$**	**$7.45x10^{-04}$**	$3.30x10^{-01}$	Stress-induced mutagenesis
hemL	0.654061544	-0.201538300	-0.882732600	**$1.04x10^{-10}$**	$9.00x10^{-2}$	**$5.09x10^{-22}$**	Stress-induced mutagenesis
hdfR	0.713193000	0.495771380	-0.250165800	**$8.45x10^{-06}$**	**$2.60x10^{-03}$**	$5.00x10^{-02}$	negatively expresses flagellar master
cspC	0.811330215	-0.246460900	-1.101264200	**$9.00x10^{-14}$**	$5.00x10^{-02}$	**$7.22x10^{-34}$**	Stress-induced mutagenesis
hfq	0.854999033	-0.151303600	-1.039051700	**0.000**	**$5.31x10^{-03}$**	**0.000**	Stress-induced mutagenesis
sdhB	1.552462064	0.333895190	-1.213704800	**$1.14x10^{-32}$**	$5.00x10^{-02}$	**$1.65x10^{-26}$**	Stress-induced mutagenesis
lpxD	0.265231458	0.246972250	-0.060005700	$5.00x10^{-02}$	$8.00x10^{-02}$	$6.00x10^{-01}$	lipid biosynthesis
dctA	1.253518972	0.522407840	-0.761345200	**0.000**	**$2.38x10^{-12}$**	**$8.62x10^{-46}$**	required for dicarboxylate transport

Further analysis under each category indicates that specific biological processes were positively expressed throughout the US, US2 treatments, while others were negatively expressed. When looking at the WT strain, the biological processes for carbohydrate (GO: 0008643) and polyol transport (GO: 0015791) were positively expressed, whilst other biosynthetic and metabolic processes were negatively expressed (such as siderophore biosynthetic processes (GO: 0019290), secondary metabolite biosynthetic processes (GO: 0044550) and others. On the other hand, when looking at Δ*gadW* most of the DEGs were negatively expressed. However, the few biological processes that were consistent were responses to various stress factors such as Hyperosmotic response (GO: 0006972), osmotic stress (GO: 0006970), temperature stimulus (Go: 0009266), abiotic stimulus (GO: 0009628), stress (GO: 006950) and stimulus (GO: 0050896).

The WT strain only showed upregulation in both treatments, in the biological process, and to a lower extend molecular function, and cellular component with all of the DEGs expressed, being related to the respiration cycle (Citrate cycle), construction of flagellum bodies and transporter activity related to the nutrient transfer, respectively. This was not evident in the *gadW* mutant.

4.3.3 KEGG analysis

For this type of analysis of particular interest was the positive expression of cellular component and various genes related to respiration or flagellum proteins that occurred in the WT strain. From the 67 DEGs in the cellular component group, 55 were related to the respiration cycle while the other 12 are associated with the production of the flagellum. These are summarised in Table 4-3.

4.4 Discussion

In this study, the mechanism of action of ultrasound on *E. coli* cells, involving various genes were transcribed, with a total of 1825 genes expressed. These genes are responsible for mechanisms involved in biological, metabolic and biosynthetic processes. The isogenic mutant Δ*gadW* was used, as this gene was found to play a possible role in ultrasound treatment as described in chapter 3. The GAD system is known to play an important role in acid tolerance of bacteria (Smith *et al.*, 1992; Feehily and Karatzas, 2013; Paudyal and Karatzas, 2016). However, it has been shown to have an impact on oxidative stress only in *Saccharomyces cerevisiae* (Coleman *et al.*, 2001) and *Francisella tularensis* (Ramond *et al.*, 2014) but not in other organisms. In fact, the GAD system was reported as playing a role in oxidative stress in *E. coli* for the first time in a recent study (Spiteri *et al.*, 2017; Boura, Brensone and Karatzas, 2020).

The transcriptomic analysis provided evidence that *E. coli* K-12 and its isogenic *gadW* mutant responded to ultrasound treatment by altering their gene expression to maintain cellular homeostasis and hence facilitate its survival. As previously reported by in chapter 3, the GAD system coupled with the GABA shunt feeds into the TCA cycle by affecting the succinate and oxoglutarate levels, both of which have anti-oxidant properties and thus can confer resistance to oxidant species (Ramond *et al.*, 2014). This was evident in the DEGs expressed in the WT, as from the 67 genes in the cellular component, 55 were related to respiration cycle, while the other 12 were related to the production of the flagellum. These genes were all positively expressed and are known to be involved in the production of the flagellum, which could easily be as a form of repair mechanism, or else are involved in facilitating cell movement. One must keep in mind that the production of flagella, may be quite an energy-intensive process, and may not be promoted under stress conditions. This shows that *gadW* is involved in the regulation of

various cellular functions apart from that of the GAD system. Seo *et al.* (2015) observed that the repression of the *speG* gene protects the cell from acidic damage, which is in line with the current study in which the *speG* gene of the WT of both PT/US2 and US/US2 was also negatively transcribed. This correlated well with the fact that the *gadEWX* regulation network must be playing a role in resistance. On the other hand, in the isogenic mutant, the *speG* gene was not significantly expressed in any of the treatments, corroborating the findings mentioned in the study above.

As mentioned in several studies, succinate transport is carried out by dicarboxylic acid transport (Lo, T.C.Y.; Khalil, M; Sanwal, 1972; Janausch *et al.*, 2002; Groeneveld *et al.*, 2010; Sá-Pessoa *et al.*, 2013; Karinou *et al.*, 2017). This is regulated by the gene *dctA*, which was positively expressed in the WT when treated with ultrasound. However, it has to be noted that this was only significantly different at the single treatment, rather than the double treated cells. This was also evident when comparing the expression of the US1 treatment with the US2 treatment, which gave a negative expression of this gene.

Furthermore, since specific oxidase genes, *sodA* and *sodC* were positively transcribed, this may indicate that the bacterial cells were under oxidative stress (Gottesman, 2019). Both genes were positively expressed when the treated cells were compared with the non-treated cells, and however, when compared with the cells treated for the second time, with the non-treated cells, only *sodC* was overexpressed.

As mentioned in the Ecocyc, *sodA*, *SodB* and *sodC* are part of a three-enzyme system that is implicated in response to a large number of environmental changes that lead to the generation of superoxide radicals. The three enzymes differ in their metal cofactor requirement, allowing

them to work simultaneously. *SodC* uses copper or zinc, while *SodA* uses manganese and *SodB* uses iron. Further to this, *SodC* is induced by *RpoS*, which helps to increase resistance to hydrogen peroxide (Karp *et al.*, 2014).

The sigma factor RpoS can be activated and when activated it can regulate the expression of genes in response to general stress (Li *et al.*, 2018; Gottesman, 2019). An interesting note to point out is that under stress, RpoS induces the mutagenic repair of DNA breaks through a network of genes (Amar *et al.*, 2012), some of which were positively expressed in this study: *yifE, nuoG, hemL, hdfR, cspC, hfq* and *sdhB*. This suggests that ultrasound treatment induces similar effects to those elicited by superoxide groups, while giving rise to DNA damage. The *hfq* is particularly interesting as it was found to play an essential role in stress response as it regulates two major transcription factors σS and σE (Brown and Elliott, 1996; Muffler, Fischer and Hengge-Aronis, 1996; Guisbert *et al.*, 2007). These genes sense stress and promote repair DNA of double-stranded breaks (DSBs). The σS controls general or starvation stress response, while σE response promotes membrane protein stress responses (Amar *et al.*, 2012). Regulator genes such as *rpoS* exhibit the capability of controlling several pathways under stress by regulating many proteins involved in the central metabolic system, indicating that the cell would be trying to promote resources for the continued survival of the cell. This was consistent with other studies, where transcriptomic changes were studied under different stresses (Carruthers and Minion, 2009; King *et al.*, 2010; Chueca, Pagán and García-Gonzalo, 2015; Li *et al.*, 2018).

In *E. coli* cells, the activity of *CpxA* is influenced by the composition of the membrane lipids (Li *et al.*, 2018). In our results, it is evident, that both CPX and LPX systems, do not seem to be playing a significant role in the WT strain, as the systems come in play at a bigger

significance in the *gadW* mutant. In all cases for the isogenic mutant, both the *cpxP* and *lpxC* systems were down-regulated. However, the study of Li et al. (2018) showed that these systems were up-regulated in cases where cells were treated with prolonged cold stress. The down-regulation, in the ultrasound treated cells, in the *CpxP* provides a negative feedback regulator for the pathway and thus an effector of the stress response. This results in the displacement of *CpxP* from *CpxA* and thus activating the Cpx response (Karp *et al.*, 2014).

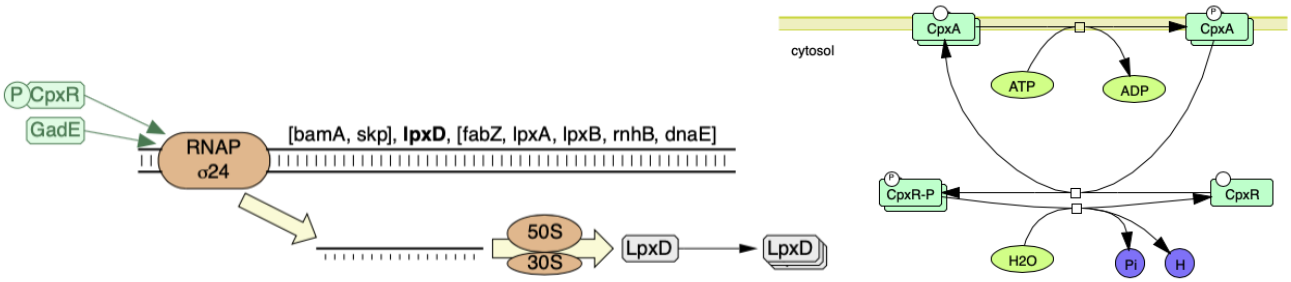

Figure 4-8: A pathway of the cpx system and its regulatory genes, together with the activation process for lpxD on the right, showing the responsible genes and it's activation. (extracted from: https://biocyc.org/gene?orgid=ECOLI&id=PHOSPHO-CPXR)

The cpx system is a complex system, as shown in figure 4-8. The *cpxA* serves as a stress response and also the assembly of pili. Moreover, the system is also a periplasmic chaperone, which could be induced directly in response to damage to pili (Allen, White and Morby, 2006) caused by ultrasound treatment. On the other hand, another gene *lpxA*, which is required for cell division, growth under hypoosmotic conditions and viability, in general, seem to have a role, in conjunction with the cpx system in cell integrity on the loss of outer membrane impermeability (Audrain *et al.*, 2013; Raivio, Leblanc and Price, 2013; Karp *et al.*, 2014; Hews *et al.*, 2019).

The Cpx response, together with a *gadE* transcriptional activator, gives rise to a *lpx* response. The *lpxD* gene is generally activated by the presence of antibiotics, in the formation of lipids and is an essential gene (Kelly *et al.*, 1993; Dwyer, Kohanski and Collins, 2009). However, for this to be transcribed, the *lpxD* needs an activated Cpx response together with the

transcriptional activator, which was absent in the *gadW* mutant, thus showing a negative transcription. This process was however not found to be crucial in the protection of the WT strain from ultrasound as no significant expression was observed in this strain, unlike that found by Li. et al. (2018) in the protection of cold shock.

4.5 Conclusion

In conclusion, this study identified several DEGs, with many playing a role in stress protection in *E. coli* K-12 by using throughput transcriptome sequencing technology. *E. coli* K-12 also appears to have gene expression involved in the protection from prolonged harm, in the respiration for increased growth, production of flagellum and pili, and also expression in various protection mechanisms such as σ S and E. Furthermore, changes in membrane responses also indicate the widespread effect of ultrasound treatment on the cell. Although this study indicated that ultrasound has an impact on the bacteria that in turn is changing its mechanisms for enhanced resistance, further work would provide a better understanding of what resistance is being permanently shown in future generations of the bacteria, providing additional light in the use of disinfection in industry. Furthermore, one may also look at other mutants in the Keio collection to test various other global stress regulators and identify if other stress responses were involved. Other studies may also use the above responses to replicate conditions using biofilms and other suspended particles, to identify differences in responses.

Chapter 5: Whole genome sequencing of Wild Type *Escherichia coli* K-12 isolates treated with ultrasound

5.1 Introduction

A major limitation of studies using ultrasound or other non-thermal alternatives is that the presence of intermediate state cells remains undetectable by routine methods (Li, Suo, *et al.*, 2017). For example, culture-based methods can be time-consuming and cannot be solely used to detect viable but non-culturable cells. This will typically bias the results as it shows a significant underestimation of the actual numbers of surviving bacteria, as the bacteria may remain virulent and will eventually recover under appropriate conditions (Aurass, Prager and Flieger, 2011; Li, Ding, *et al.*, 2017). Studies have also concluded that ultrasound treatment may cause reversible damage and thus allow cells to recover if the treatment ceases under appropriate physical parameters (Ananta *et al.*, 2005; Yeo and Liong, 2013). In a study, *E. coli* treated by ultrasound was able to recover the following storage at 4°C for up to 5 days, indicating that the membrane permeabilisation was reversible upon treatment at low intensities (Cruz-Cansino *et al.*, 2016). The authors suggested that the damage to the cell membrane probably increased the transport of nutrients increasing cell metabolism and thus enhancing bacterial viability

With the use of whole-genome sequencing, direct measurements of mutation rates have become possible (Wielgoss *et al.*, 2011). This is especially true for *E. coli* were 12 populations were reported to propagate independently for over 40,000 generations (Philippe *et al.*, 2007; Batt, 2014). Owing to such short generation times, large population size, the accuracy of experiment results, and the ability to preserve ancestor strains, microorganisms have been used to study adaptive evolution mechanisms (Elena and Lenski, 2003).

In 2013, Pavlov and Ehrenberg came up with a model in which bacteria manipulate their gene expression to quickly adapt to changes in the environment (Pavlov and Ehrenberg, 2013). Their

studies showed that bacteria have the ability to focus their resources on cell growth by increasing pathways to activate other sources of energy to increase metabolism instead of multiplying under environmental stress (Berney, Weilenmann and Egli, 2007; Wu *et al.*, 2020). This was further studied by Ferenci (2019) who suggested a very imbalanced relationship between stress and mutations specifically in *E. coli* (Ferenci, 2019). Thus, it is possible for cells in certain physiological states or carrying out certain mutations to survive non-thermal physical treatments (Li, Suo, *et al.*, 2017; Wu *et al.*, 2020). Studying the mechanisms of the responses of microorganisms to non-thermal treatments may help to understand the possible risks and prevent any potential safety incidents which may result, e.g., induced resistance, uncontrolled mutations, enhanced recovery. Further studies are required to understand the response mechanisms of microorganisms under non-thermal physical environmental stress, such as systems that combat oxidative stress, cell repair, and resistance regulation (Wu *et al.*, 2020). Furthermore, the maintenance of genetic systems, which till now, appear to have no selective advantages, has confused many scientists. Such a genetic system includes 'cryptic' genes that have no function in wild type organisms and require mutational activation for expression (Harwani, 2014).

The study of this Chapter aims at giving a further insight into the non-thermal technology of ultrasound and relevant cellular responses. Here, the wild type of *E. coli* K-12 MG1655 and an isogenic mutant Δ*gadW* were treated with ultrasound, and DNA analysed via Sanger sequencing and NGS, to get an overview of the direct or indirect effect, if any, of ultrasound on DNA.

5.2 Material and Methods

5.2.1 Bacterial strains and preparation of inoculum

In this study, the bacterial strains used were *E. coli* K-12 wild type, and its isogenic mutants Δ*gadW* and Δ*oxyR* obtained from the National Bio-Resource Project, Japan (Baba *et al.*, 2006; Yamamoto *et al.*, 2009). The pure cultures of strains were stored in vials at -80°C in a freezer using a 1% DMSO solution. Before any experiment, single colonies were isolated. Under aseptic conditions, a loop from the frozen vial of *E. coli* was streaked on Tryptone Soya Agar (TSA; Oxoid, United Kingdom) plates. Following overnight incubation at 37°C, these pure culture plates were stored at 5°C and kept for a maximum of 3 to 4 weeks before further use.

5.2.2 Preparation of the working culture

The first subculture was prepared by transfering one isolated colony from the TSA plates to a 10 mL Tryptic Soy Broth without dextrose (TSB-D) and incubation at 37°C for 24 h. Then, 10 µL from the first subculture was transferred to a small bottle containing 100 mL of TSB-D and incubated at 37 °C for 24 h to reach the stationary phase. After incubation, 10 mL of the culture was taken and centrifuged at 9,000 xg using rotor Sigma 12151 (centrifuge Sigma - 2-6) for 20 minutes. The pellet was rinsed with 9 mL of Ringer's solution and centrifuged once more for 20 minutes. The washing step was repeated twice. The cells were suspended, and 2 mL was transferred to 298 mL synthetic wastewater. The synthetic wastewater was prepared as described by Antionadis *et al.* 2007 and Ayyildiz *et al.* 2011 (i.e., peptone 64.0g/L; Meat Extract 44.0g/L; Urea 12.0g/L; K_2HPO_4 11.2g/L; NaCl 2.8g/L; $CaCl_2.2H_2O$ 1.6g/L; $MgSO_4.7H_2O$ 0.8g/L) (Antoniadis *et al.*, 2007; Ayyildiz, Sanik and Ileri, 2011). This medium was used to increase the nutrients in the water to closely mimic wastewater. This liquid was considered the sample for the ultrasound treatment. After the first cycle of ultrasound, five colonies from post-treatment samples were isolated and treated as samples for the second cycle

of ultrasound treatment to see if resistance was induced. As control, the bacteria under study was also grown before the treatment of ultrasound.

5.2.3 Ultrasound treatment and antimicrobial assessment

A Hielscher UP200St Ultrasonicator operating at the constant frequency of 26 kHz was used. It was equipped with a generator UP200St-G 200W and a transducer UP200St-T which can be integrated with a sound protection box. A 14-mm sonotrode was used, which was suitable to transmit the ultrasound smoothly across a relatively large surface as described in section 3.2.4.

For each sample, the working solution was transferred into a 500-mL big jacketed beaker attached to a water pump. The big beaker was carefully disinfected with alcohol between each experiment. Then, the tip of the sonotrode was placed in the centre of the beaker, containing 300 mL of bacteria suspension (see above) with a submerged depth of 2cm. In this study, sequential treatment was performed. The first treatment for each strain consisted of sonication for 5 minutes on the continuous mode and at an intensity of 100 %. After the first treatment, colonies were isolated and cultured for a second treatment to see if resistance is developed. The conditions of the second treatment were the same as the first treatment.

After each treatment, 1 mL of the sample was transferred to 9 mL Ringer's solution, serially diluted and then plated on TSA plates. Counts of survivors were obtained and an F test was used to see if there was any significant difference between the results.

5.2.4 DNA Extraction

DNA extraction was carried out by following the PureLink™ Pro genomic DNA Protocol (Invitrogen, June 2009). In summary, a loop of cells from the TSA plate was suspended in 200μL Genomic Digestion Buffer with Proteinase K. The tube was vortexed well and incubated at 55°C for 60 minutes, with occasional vortexing. The tubes were then briefly centrifuged at

2,100 x g for 2 min. 20μL of RNase A was added to each tube, and again vortexed. The tubes were again briefly centrifuged, followed by a 2-minute incubation at room temperature. 400μL of PureLink™ Pro genomic Lysis/Binding buffer with ethanol was added, followed by vortexing and brief centrifugation. The lysate was then transferred to a PureLink™ gDNA Filter Plate, which was also placed in a 2 mL tube. The filters were centrifuged at 2,100 x g for 5 min. A further 2 min centrifugation was carried out in the case that not all lysate was filtered through. The filtrate was discarded, and 500 µL of Wash Buffer 2 prepared with ethanol was added to each filter. These were then centrifuged again at 2,100 x g for 15 min. The filtrate was again discarded. The filter was then transferred to a clean 1.5mL centrifuge tube, and 100 µL of PureLink™ Pro Genomic Elution Buffer was added to the centre of the membrane. The tubes were left for 1 minute at room temperature and centrifuges for 3 min at 2,100 x g. The purified gDNA was stored at -20 ℃ for further downstream applications.

5.2.5 16S rRNA polymerase chain reaction (16S-PCR)

The PCR reaction mix was prepared by mixing adequate quantities of the following; blend master mix buffer (5µL), 1µL dNTPs (A, G, T, and C), 2µL 100 pmol/µL forward and reverse primers:

ECR – 5'-CTTGTGCGGGCCCCCGTCAATTC-3'

ECF – 5'-AGAGTTTGATCCTGGCTCAG-3' (HVD Life Sciences, Austria),

0.5µL DNA TOP polymerase (Solis Biodyne, Estonia) and water (water for injection). 23 µL of the prepared PCR reaction mix was mixed with 2 µL of bacterial DNA in a clean reaction tube. The 25 µL sample was placed in a thermal cycler (ABI 2720TC, USA). The thermal cycler (ABI 2720TC, USA) was set to perform the PCR cycles with initial denaturation of 95 for 5 min, followed by 35 cycles of 95°C for 45 sec, 44°C for 30 sec and 72°C for 45 sec, finishing off with a final elongation of 72°C for 5min.

5.2.6 Gel electrophoresis

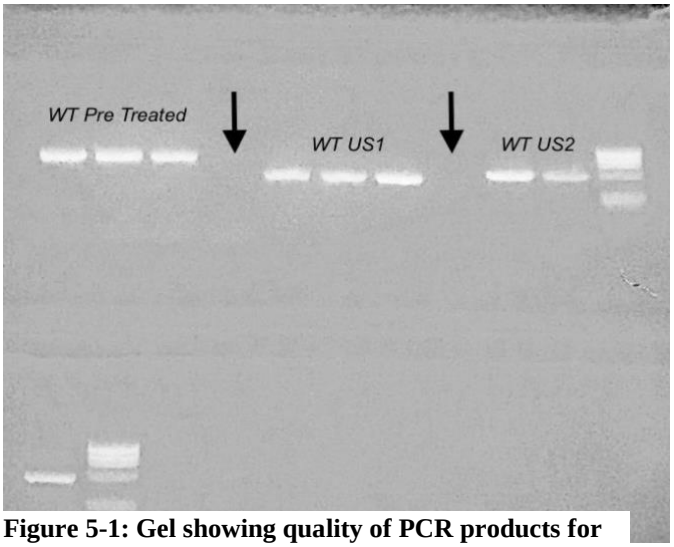

Figure 5-1: Gel showing quality of PCR products for the three different treatment methods

After the PCR, gel electrophoresis was carried out, to check the quality of the PCR reaction. For the electrophoresis, a 2% agarose gel was prepared by adding a small amount of ethidium bromide. In a separate 96-well tray, 2 µL of loading dye was mixed with 4µL of water and 4 µL of PCR product and mixed well by pipetting. The whole mixture was aspirated using a micropipette and gently loaded into each well of the gel, which was in the electrophoresis chamber filled with 1XTAE buffer. 6 µL of 100 base pair ladder was loaded into the first well of each row of the gel. The lid of the electrophoresis tank was closed, and the electrical current was turned on to 100 Volts for 90 minutes (or 15 min for the smaller gels). After 90 min, the electrical current was switched off, and the gel was viewed under the ultraviolet trans-illuminator.

5.2.7 PCR product purification

In order to prepare the PCR product for cycle sequencing, the unused PCR reagents would need to be removed. 100 µL binding buffer and 100 µL isopropanol were mixed with the 25 µL PCR product. The mixture was placed on a PureLink ™ PCR purification filter, and the filter was centrifuged at 10,000 x g for 1 min, after which the filtrate was discarded. 100 µL of wash buffer 1 was added to the filter, and again, the mixture was centrifuged at 10,000 x g for 1 min. The filtrate was removed, and 100 µL of wash buffer 2 was added to it, followed by another centrifugation step (10,000 x g for 1 min). The filtrate was again discarded and again

centrifuged at 10,000 x g for 1 min to remove the excess alcohol, which may denature the subsequent PCR reaction. The filter was then placed in a sterile reaction tube, and 50 µL of elution buffer was added. The mixture was then left to stand for 5 min at room temperature under a laminar airflow. The tubes were then centrifuged at max speed for 1 min. The tubes were then stored at -20 ℃ until used.

5.2.8 Cycle sequencing

Prior to preparation for the cycle sequence reaction, all tubes from the BigDye™ Terminator 3.1 kit were vortexed for 3 seconds, followed by brief centrifugation. The components were added as recommended by the manufacturer. The tubes were vortexed and transferred to an ABI 2720 thermal cycler, to run a PCR reaction, as recommended by the manufacturer. When ready to process the samples were purified by using MagSi-DNA cleanFIX kit to remove extra unused reagents, which may mask the DNA reads due to the presence of dye blobs.

5.2.9 Cycle sequencing product clean-up

10 µL MagSi-DNA clean^{FIX} kit reagent (amsbio, UK), was placed in the tube consisting of the cycle sequencing PCR product. 29 µL of alcohol mix, which includes 42.5% isopropanol, 42.5% ethanol & 15% ddH20, was then added to the tube with the cycle sequencing product and magnetic beads. The tubes were mixed well by aspiration of a pipette. The mixture in the 96-well plate was left to stand for 3 min at room temperature. The 96-well plate was then placed on a magnetic separator plate (amsbio, UK), and left to stand for another 3 min. Hereafter, the supernatant in each well was removed and discarded, and 100 µL of the alcohol mix was added to each of the wells. The magnetic beads were resuspended in the alcohol mix, and the 96-well plate was placed again on the magnetic separator (amsbio, UK). This washing step with alcohol mix was performed twice, after which the supernatant was removed, and the plate was removed from the magnetic separator (amsbio, UK). The contents

in the well were then left to air dry for 5 min, and 40 μL of water was added to each tube (the mixture was again mixed well by pipetting for 2 min). The 96-well plate was placed again on the magnetic separator (amsbio, UK).

5.2.10 Sanger sequencing

The nucleotide base sequence of the 16S rRNA gene was primarily determined by Sanger sequencing. The resulting base-pair sequences were compared to establish the similarity between replicates. The 3500 Genetic Analyser (ABI, USA), was used to perform Sanger sequencing. The 3500 Genetic Analyser (ABI, USA) produces sequences, bypassing DNA through a polymer (pop-7™) separating them via size, and passing through them a laser, which electronically transfer to a computer where the computation of the sequence is performed. This analysis involved the inputting of the sequences produced into an NCBI BLAST database, to give the final definitive identification of the bacterium.

For the genetic analysis, a sample size of 70 samples was used. The samples consisted of 39 wild type, 24 GadW samples, and seven oxyR samples in triplicate, all of which were pooled from 5 clones, to increase diversity. The samples were sequenced and run through a BLAST system with the use of the software *Geneious 8.*

To identify the strains, the E value was checked. The E value represents the number of alignments found, and if the E value is 0 or close to 0, it means that the sequence is close to the results from the database in the software. The E value is dependent on the length of the sequence, and if the sequence is trimmed, the E value will be influenced. If the E value is around 0, the percentage of similarity can be calculated with the software. It will compare the results of the sequence to the results of the genome to find a match.

5.2.11 Next-Gen Sequencing

NGS analysis was carried out at the University of Birmingham using the Illumina method. Three beads were washed with extraction buffer containing lysozyme and RNase A and incubated for 25 min at 37 ℃. Proteinase K and RNaseA were added and incubated for a further 5 min at 65 ℃. The genomic DNA was purified using an equal volume of SPRI beads and resuspended in the elution buffer. Genomic DNA libraries were then prepared using Nextera XT Library Prep Kit (Illumina, San Diego, USA) following the manufacturer's protocol with a slight modification to the use of a longer PCR elongation time (1min compared to 30 sec) and use of double the amount of DNA. The libraries were then sequenced on the Illumina HiSeq using 250bp paired-end protocol.

5.2.12 Bioinformatics – Variant calling

NGS analysis was carried out by Geneious Prime 2019. The reads were adapter trimmed using BBDuk with a sliding quality cut-off of Q20. The trimming impacts the results, because the sequence is shortened, and this might cause a problem when the sequences are compared to each other and the genome. After trimming, the reads were mapped to reference, mentioned below, using the in-built Geneious mapper, with a medium sensitivity. The reference sequence used was the same as the one used in RNA-SEQ and assembled with the latest reference genome, downloaded from the NCBI website:

https://www.ncbi.nlm.nih.gov/genome/?term=e%20coli%20k%2012

The respective new contig files for each sample was produced. Various settings were made available to graphically represent better the results, also showing the annotating tracks. Using these files, the Geneious variant finder was used to find SNPs in the mapped data. Default settings were used and the data was exported to MS Excel sheets. The SNPs were then

compared to filter SNPs based on their overlap with another annotation track or annotation type.

5.2.13 Validation – Motility test

Motility testing was carried out in order to assess difference in the movement of the bacteria. Such difference could be related to damage to the flagella, or other physiological effects, resulting from ultrasound treatment. Sub-cultures from different treated cultures were transferred by a nichrome wire to the motility agar, prepared by adding 20.0g of Pancreatic digest of casein, 6.1g of Peptic digest of animal tissue, 3.5 g of agar, 0.2g of $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$ and 0.2g of $Na_2S_2O_3 \cdot 5H_2O$. The bacteria were then incubated for up to 3 days, with readings taken every 24 hours. Positive clouding was marked as 1, while negative clouding was described as 0.

5.3 Results

5.3.1 Sanger sequencing

Preliminary data collection from Sanger sequencing, allows the investigators to see if any mutations are being selected in the 16s rRNA gene. Examples of the results obtained from the sequencing software is displayed in Figure 5-2. Some of the sequences were trimmed to remove unwanted parts of the sequence to improve the quality of the sequence. The trimming was performed near the sequencing primer site and towards the end of the sequence where the quality is poor. The smaller the sequence, the lower the chance of finding a match in the genome of over 4 million base pairs.

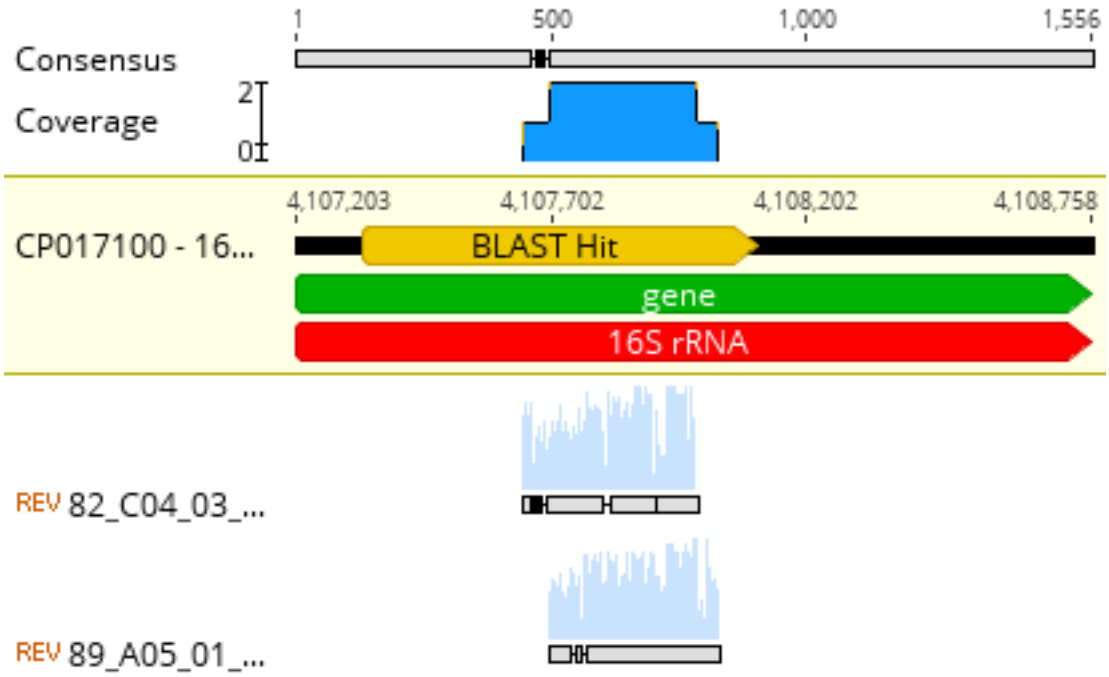

Figure 5-2: 16s rRNA amplified gene, when compared with the extracted gene from the wild type K-12 E. coli

The sequence of the samples was compared to sequences of the whole genome, which was found with the use of the software, and the results were aligned with the genome to find the percentage identity of the bacteria. In this study, some samples had an identification of 80 to 90 % which is not sufficient to fully identify the bacteria at the serotype level.

Results showed that a significant shift (t-test; T_{calc}: 4.4398 p-value: 0.007068 for two tail at 95% confidence limit) was reported in the after-treatment wild type strain % similarity when compared with the original database gene (Table 5-1). Extracted DNA in the after-treatment samples, had a less similarity with the reference 16s rRNA gene, signifying that the treatment is having a negative effect on the DNA. This could be a result of DNA damage or DNA mutation.

Table 5-1: Student T-test for wild type, before and after treatments (* significant difference)

Strain	p-value

***E. coli* K-12 WT**	0.07064(*)
E. coli* K-12 *GadW	0.16907
E. coli* K-12 *OxyR	0.10082

In both the Δ*oxyR* and Δ*gadW* strains, no further difference was found. To assess this further, the Wild type and the Δ*gadW* strains were selected for NGS analysis, to further understand any direct or indirect differences due to Ultrasound treatment.

5.3.2 *Next-Gen sequencing – DNA variants*

Table 5-2 summarises the differences obtained when comparing the different methods of treatment for the WT strain. A total of four significant various changes occurred with a minimum of 6 base pair changes. All, except one of these changes, were substitutions, but one insertion of 11 bps was present in the US1 sample. Furthermore, most of the changes were common for both treatments, indicating that these might be due to other factors apart from the US. However, two variations in the US1 and two in the US2 were evident, as summarised below

Table 5-2: Summary of variant calling due to NGS for both treatments

US 1

Name	Change	Coverage	Polymorphism Type	Variant Frequency	Variant P-Value	
GGTAGAGGAAAGTG	TCGCACCTTCC -> GGTAGAGGAAAGTG	6 -> 9	Insertion	66.7% -> 100.0%	6.30E-20	IS5 transposase and trans-activator
GATAAA	CGGCCT -> GATAAA	5	Substitution	100.00%	1.00E-15	transcription site of araBAD

US2

Name	Change	Coverage	Polymorphism Type	Variant Frequency	Variant P-Value	
GCCGTTTGCAC	ATGAAGAAATA -> GCCGTTTGCAC	14 -> 16	Substitution	43.8% -> 56.3%	4.30E-23	Side tail fibre
CGCCAGCTAA	TTATGTAGTC -> CGCCAGCTAA	3 -> 7	Substitution	28.6% -> 80.0%	5.30E-07	Intragenic valX

5.3.3 Validation – Motility test

E. coli k-12 MG1655 exhibits a vigorous flagella-driven swarming migration, developing a robust colonial pattern associated with a motile collective behaviour. A mutation in the side tail fibre may assist in the swarming of the cells, which could easily be observed by phylogenetic methods. Table 5-3 summarises the motility response of the bacteria, after 72 h of incubation. The binary responses are tabulated in which the value of 1 represents motility in the medium, while 0 represents no motility after a particular time. Each strain was repeated five times, using different colonies, to increase the statistical power of the test. The results show that although the motility is decreasing, following an ultrasound treatment (US1) of the cells, this change is not overall uniform and most probably occurs randomly. The reduction in motility seems to be more evident during the sequential ultrasound treatment (US2), which indicates that the cell is being affected by the treatment but is easily recovering by the third day.

Table 5-3: Summary of motility tests over 24, 48, 72 hours for Wild type strain

		WT										
	Sample code	24h	48h	72h	PT	24h	48h	72h	PT	24h	48h	72h
PT	101	1	1	1	101	1	1	1	101	1	1	1
	102	0	1	1	102	0	1	1	102	0	0	1
	103	1	1	1	103	1	1	1	103	1	1	1
	104	0	1	1	104	0	1	1	104	0	0	1
	105	1	1	1	105	1	1	1	105	1	1	1
US	106	1	1	1	106	1	1	1	106	1	1	1
	107	1	1	1	107	1	1	1	107	1	1	1
	108	1	1	1	108	1	1	1	108	1	1	1
	109	1	1	1	109	1	1	1	109	1	1	1
	110	1	1	1	110	1	1	1	110	0	0	1
US2	36	1	1	1	36	1	1	1	36	0	0	1
	37	1	1	1	37	1	1	1	37	1	1	1
	38	1	1	1	38	0	0	0	38	0	0	1
	39	1	1	1	39	1	1	1	39	1	1	1
	40	1	1	1	40	1	1	1	40	0	0	1

5.4 Discussion

This study shows the impact of the direct or indirect damage to *E. coli* cells by ultrasound treatment. Previously, it was observed that ultrasound technology is effective in water disinfection, with the Glutamate Decarboxylase (GAD) system possibly playing a role in ultrasound treatment and oxidative stress. To further elucidate the mechanism of action, whole-genome sequencing was carried out to identify variations within the DNA strand. The quantification of spontaneous mutation rates is crucial for unravelling the mechanism of the evolutionary process

(Wielgoss *et al.*, 2011). In bacteria, traditional estimates using experimental or comparative genetic methods are prone to statistical uncertainty. These errors, although having been reduced drastically by Sanger sequencing (99.99%), are mostly limited to PCR fidelity, which can vary from different brands of polymerase (McInerney, Adams and Hadi, 2014). According to Huxlet, Helps, and Bradley (2004), sequencing of the 16S rRNA gene is considered the main reference technique used to differentiate related genera and species (especially in bacteria which have high 16S rRNA gene divergence rates). 16S rRNA sequencing is also capable of identifying the genus, species, and strain bacteria that do not have a particular biochemical profile and for ones that give a "low likelihood" with BLAST databases (Janda and Abbott, 2007). Nowadays, with the use of next-gen sequencing, more accurate estimates are now possible. In a study published by Wiegloss *et al*. (2011), the resulting estimate of mutation was found to be 8.9 $X10^{-11}$ base-pair per generation. The authors also found significant bias toward increased AT-content (Wielgoss *et al.*, 2011).

The effect of ultrasound treatment on resistance development has been reported in literature for *E.coli* (Rajkovic *et al.*, 2009; Garcia-Gonzalez *et al.*, 2010). These studies concluded that bacteria were able to develop resistance after repetitive treatment. Furthermore, deleted genes, such as in the case of the *GadW* mutant, may affect the metabolism of the bacteria, impacting negatively or positively, the cells' function on ultrasound treatment. The GAD system is known to play an

important role in acid tolerance of bacteria (Smith *et al.*, 1992; Feehily and Karatzas, 2013; Paudyal and Karatzas, 2016) but it has been shown to respond to oxidative stress only in *Saccharomyces cerevisiae* (Coleman *et al.*, 2001), *Francisella tularensis* (Ramond *et al.*, 2014) and lately in *Listeria monocytogenes* (Boura, Brensone and Karatzas, 2020) but not in other organisms. On the other hand, other studies show that when the *oxyR* gene is deleted, and the bacteria are stressed, it will activate the expression of defensive mechanisms (Storz and Tartaglia, 2018). This potentially explains why the Δ*oxyR* strain in the current study showed less reduction compared to the wild-type, even though no genotypic difference could be related.

The results indicate that in both biological samples of the wild type bacteria with the first step of the US, a single mutation just outside the transcription site of *araBAD* site is being affected. This site regulates the sigma 70 factor, which is essential for the normal growth of the cell, especially during exponential growth (MacIąg *et al.*, 2011). This may correlate well with a phenotypic observation, where the expression of motility of the bacteria cells was delayed in the wild-type bacteria. Even though slower growth was observed in the control cells, some replicates showed a faster expression of motility in the cells treated with ultrasound. This was, however again reverted with US2, were some replicates showed delayed motility. The observed phenomenon could also be related to the fact that a mutation in the part coding for the side tail fibre was reported. This gene was mentioned to alter

pathogenic colonisation (da Re *et al.*, 2013). This mutation could indicate that a change in the coding, is affecting the bacterium to trigger differential mechanisms to aggregate more or less due to a response from the ultrasound.

Previous studies (Schoner, 1983; Rak and von Reutern, 1984; Chernak, Schlaffer and Smith, 1988; Birkenbihl and Vielmetter, 1989, 1991; Umeda and Ohtsubo, 1990; Schnetz and Rak, 1992; Jeong *et al.*, 2017) have shown that the inactivation of genes by insertion of mobile DNA elements into coding and control regions have been quite frequent, but yet still not understood much in both eukaryotic and prokaryotic organisms (Schnetz and Rak, 1992). *IS5* is an 1195-bp mobile DNA element that was found to be present in the genome of various *E. coli* K-12 strains (Rak and von Reutern, 1984; Chernak, Schlaffer and Smith, 1988; Schnetz and Rak, 1992; Jeong *et al.*, 2017). This comprises three genes, one of which, *ins5A* encodes *IS5* transposase (Schnetz and Rak, 1992). The sequence *IS5* contains three open reading frames (ORFs), associated with functional promoters and terminator signals (Chernak, Schlaffer and Smith, 1988). Furthermore, *IS5* was shown to behave as a transcriptional enhancer of the otherwise cryptic *E. coli* bgl operon (Schnetz and Rak, 1992). In our results, an insertion mutation in the *IS5* seems to be occurring just outside the gene *IS21*, just upstream the *oppA* gene. This may indicate that no major effect on the genes is occurring. However, one must point out that the *oppA* gene, is an essential gene for survival under heat stress (Krisko *et al.*, 2014). This mutation may be triggered because of the thermosonication phenomena occurring

during the applied US treatments. *OppA* is an oligonucleotide peptide transporter subunit encoded by the *oppABCDF* operon whose regulation is by the *bgl* operon via regulators *gcvA* and *gcvB* (Harwani, Zangoui and Mahadevan, 2012). It is important for the uptake of oligo-peptides from the growth medium and supplying a nitrogen source by recycling the cell wall peptides for the synthesis of new peptidoglycan (Lee *et al.*, 2004).

5.5 Conclusion

This study attempted to assess the mutation effects of ultrasound treatment on the *E. coli* K-12 bacteria. Variations were observed at four different areas of the genome, which although may not be having a significant effect on the bacteria, indicate how the environmental stresses influence the bacteria. This study will form the basis for further evaluations on specific genes, and may also be linked to transcriptomic studies on particular RNA-SEQ.

CHAPTER 6: General conclusions and future perspective

2.1 Overall conclusions

Innovative disinfection technologies such as ultrasound are expected to increase public safety and better consumer perception to water and food quality. This will allow potable water suppliers, sewage treatment plant operators and also food technologists to introduce efficient and safe disinfection methods in order to respond to the continuous increase in demand in water consumption. This is also compounded with the pressures established by authorities and also end-users who are looking at technologies which use less chemicals.

Climate change, increase in anthropogenic recreational and industrial activities, together with an increase in population, have increased the possibility of water stress in several countries, especially those where freshwater resources are very limited. For this reason several countries within the EU have recently started using re-use water, i.e. water coming from the effluent of sewage treatment plant, for industrial, recreational and agricultural purposes. Still, such purified water is still disinfected by means of chlorine, which gives rise to several by-products.

Several alternative treatment methods for wastewater exist nowadays, some of which are impractical, inefficient and not economically feasible. Treatments such as UV light, and/or ozone, require long contact times, while other methods such as detention lagoons and membrane technology require a large land area and capital and maintenance investment. Therefore, chlorine, even though being one of the

methods that have a large number of disadvantages, due to by-product production, health and safety requirements etc., is still one of the most sought methods in the world.

It is thus imperative that the mechanisms during disinfection processes, such as ultrasound, are elucidated in order to be able to develop rapid and reliable methods. For such purposes, this research looked into the application and mechanism of action of ultrasound technology as a means of disinfection by acoustic cavitation. Sterile water and synthetic waste water were inoculated with different mutants of *E. coli* K12 strains containing deletions in genes affecting specific functional properties of *E. coli*. *E. coli* K-12 Δ*oxyR,* appeared to be more resistant to the treatment together with Δ*gadW,* Δ*gadX,* Δ*gabT and* Δ*gabD*, whereas the mutant K-12 Δ*dnaK* was more sensitive with approximately 2.5 log (CFU/mL) reduction in comparison to their isogenic wild type *E. coli* K-12. This indicated that the *dnaK* gene participates in general stress response and more specifically to hyperosmotic stress. The other *E. coli* deleted genes tested (*soxS, rpoS, gadB, gadC, yneL*) did not appear to be involved in protection of microbial cells against ultrasound. This study was able to show for the first time a possible role of the GAD system in ultrasound treatment and oxidative stress that was further investigated by RNA-SEQ and WGS.

Whole genome sequencing (WGS) is a comprehensive method for analysing entire genomes. This allows researchers to investigate a base-by-base view of the entire genome, in order to capture large and small variants that might be missed with targeted approaches such as RNA-SEQ. These variants can be further assessed by studies such as gene expression and regulation mechanisms. The mutation effects of ultrasound treatment on the *E. coli* K-12 bacteria observed, were at four different areas of the genome IS5 transposase area, araBAD transcription site, side tail fibre and intragenic valX site, which although may not be having a significant effect on the bacteria, indicate how the environmental stresses influence the bacteria.

In both biological replicates of the wild type bacteria with the first step of the treatment, a single mutation just outside the transcription site of *araBAD* site was observed. This site was reported to regulate the sigma 70 factor, which is essential for the normal growth of the cell, especially during exponential growth. This was also found to correlate well with a phenotypic observation, where the expression of motility of the bacteria cells was delayed in the wild type bacteria. The results also showed that this change was again reverted with the second treatment, were some replicates showed delayed motility. The observed phenomenon could also be related to the fact that a mutation in the part coding for the side tail fibre was reported, which was found to alter the microbial colonisation. This may indicate that single US treatment should be sufficient for reducing the viability of bacterial cells. Further sequential treatment should only be advocated if bacterial cells need

to be totally killed off, in order to avoid cell response and repair. On the other hand, an insertion mutation in the *IS5* was observed just outside the gene *IS21*, just upstream the *oppA* gene. This is an indication that no major effect on the genes is occurring. However, as explained in chapter 5, one must point out that the *oppA* gene, is an essential gene for survival under heat stress and as such could be a defence mechanism, which merits further investigation.

RNA-SEQ analysis identified several DEGs, which play a role in stress of *E. coli* K-12. Upon ultrasound treatment *E. coli* K-12 appears to have had gene expressions involved in (i) the protection from prolonged harm, (ii) in the respiration for increased growth, (iii) the production of flagellum and pili, and (iv) some protection mechanisms such as sigma S and E. Furthermore, responses in membrane responses also indicate the widespread effect of ultrasound treatment on the cell. This shows the widespread effect that ultrasound treatment has on the cell, with both molecular and morphological effects related to ultrasound treatment.

2.2 Limitations and Future research

Although the current research outputs indicated that ultrasound is having an impact on the bacteria, which in turn is changing its mechanisms for enhanced resistance, further work on resistance mechanisms being triggered would provide a better understanding of which mechanisms are being permanently expressed in future generations of the bacteria. This will provide additional light in the use of

disinfection in the water and food industry where ultrasound can be considered for disinfection. Furthermore, one may also look at other mutants in the Keio collection to test various other global stress regulators and identify if other stress responses were involved. Potential candidates could include the *OxyR*, *SodC*, *RpoS* genes, which have shown sensitivity to oxidation processes. Other studies may also use the above responses to replicate conditions using biofilms and suspended particles such as colloids, which may be found naturally in environmental waters, to identify differences in responses. This can be further complemented by looking at the membranes with Scanning electron microscopy to check if any physical damages is occurring, which may lead to further defence mechanisms.

Furthermore, throughout this study, all mutants and bacterial work was limited to using colonies at the stationary phase. When the bacteria enters the stationary phase, morphological and genetic alternations occur that lead to an increase resistance to a variety of stress conditions (Velliou *et al.*, 2011). Thus, using bacterial at a different phase, such as exponential phase may potentially influence the stress adaptation which may see enhanced effects, that will amplify mutations or RNA transcription.

Further studies in the gene expression may also validate or enhance the work performed in this research. RNA-SEQ is a very strong tool in the identification of gene expression, however such expression will easily revert back once the

environmental stress conditions are not present anymore. For this reason, RT-qPCR tests can be carried out to confirm the significant transcriptional difference between genes in both treatment samples, using different strains. Specific genes can be selected to calculate fold changes between treatment and control samples as described by Gallup and Ackermann, 2006.

The current research identified several genes which are involved in several pathways, which play a role in metabolic and biological pathways. Growth studies could be carried out to identify positive expression in metabolic pathways. Such pathways of interests include *rpoS* and *cpxA* (Li *et al.*, 2018; Gottesman, 2019), *oxyR* and *sodC* (Karp *et al.*, 2014). These pathways would allow the bacteria to become more efficient in their growth under different environmental conditions, which might allow them to grow faster, or use less resources in their metabolic pathways.

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Spiteri, D., C., Karatzas, K. A., Scerri, C., & Valdramidis, V. P. (2020) *E. coli* k-12 transcriptomics for assessing the mechanism of action of high-power ultrasound (submitted)

Spiteri, D., C., Karatzas, K. A., Scerri, C., & Valdramidis, V. P. (2020) Assessing microbial effects of wild-type *Escherichia coli* K-12 using high-power ultrasound by Whole Genome Sequencing (submitted)

Conferences attended (abstracts)

Spiteri D, Valdramidis V., Scerri C. (2014) Reviewing the water resources management of the Maltese Islands: current practices and perspectives. National Symposium of Health Sciences University of Malta 24th April 2014

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Awards and grants

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Appendix 1

Table A1-1: Raw data for *E. faecalis* EF1027 wild type growth study

Time (h)	Log CFU/mL				Average	Std deviation
0	4.38	4.19	4.10	4.07	4.19	0.1225
4	4.51	4.51	-	-	4.51	0.0000
8	5.73	5.51	-	-	5.62	0.1132
12	7.05	6.84	-	-	6.94	0.1077
16	8.18	8.31	-	-	8.24	0.0684
20	8.59	8.43	-	-	8.51	0.0764
24	8.68	8.49	8.65	8.66	8.62	0.0766
28	8.69	8.63	-	-	8.66	0.0309
32	8.67	8.68	-	-	8.67	0.0076
40	8.73	8.61	-	-	8.67	0.0598
44	8.65	8.39	-	-	8.52	0.1320
48	8.79	8.48	-	-	8.63	0.1559

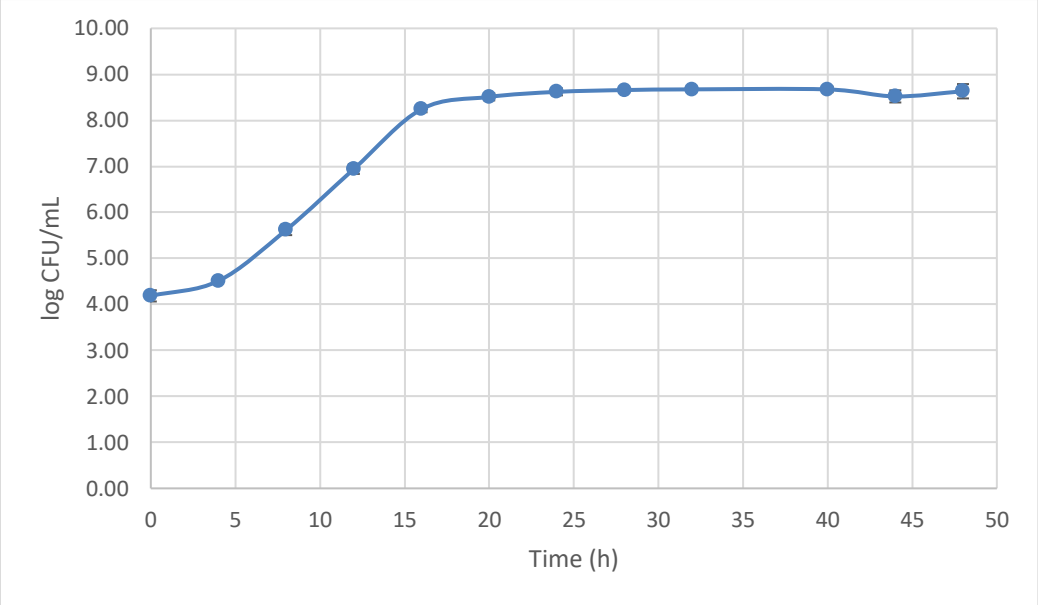
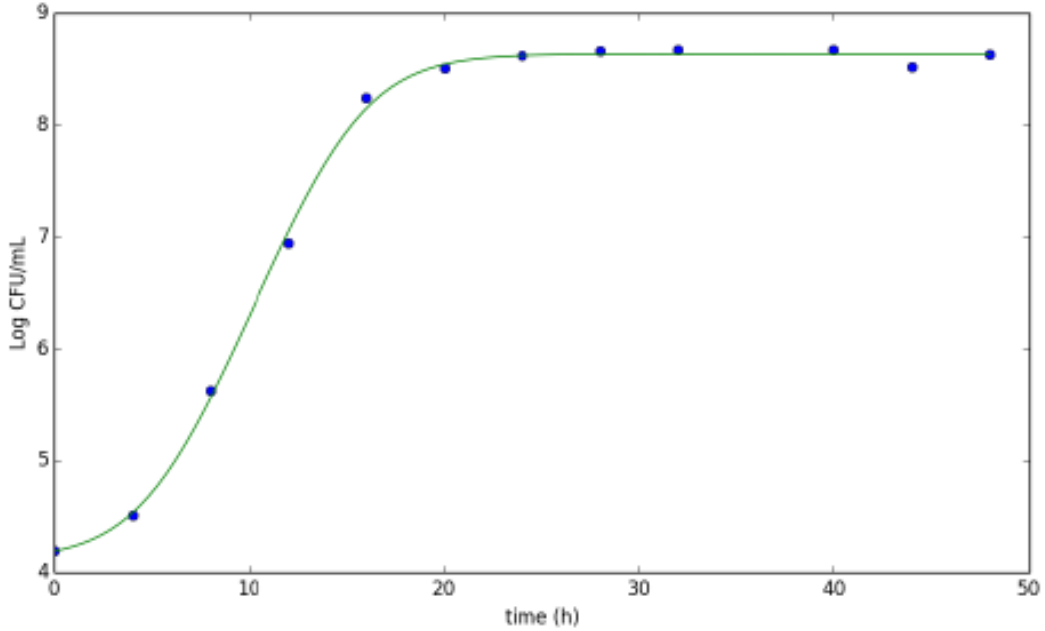


Figure A1-1: Growth curve for E. faecalis EF1027 wild type growth study



RMSE = 0.071

Parameters	Value	Std-Error
Y_0 (log CFU/mL)	4.188	0.064
h_0	2.582	0.286
Y_{max} (log CFU/mL)	8.634	0.028
μ_{max} (h^{-1})	0.467	0.023

Figure A1-2: Growth curve of E. faecalis mutant EF1027 and parameters obtained with the Baranyi full growth model, IPMP

Table A1-2: Raw data for *E. coli* K-12 Wild type growth study

Time (h)	Log CFU/mL			Average	Std deviation		Max-average
0	4.46	4.57	4.35	4.46	0.09083	0.04541	0.11
1	4.43	4.57	4.23	4.41	0.13936	0.06968	0.16
2	4.66	4.79	4.71	4.72	0.05344	0.02672	0.07
3	5.45	5.51	5.26	5.40	0.10777	0.05389	0.11
4	5.64	6.06	6.09	5.93	0.20445	0.10222	0.16
5	6.64	6.69	6.97	6.77	0.14646	0.07323	0.21
6	6.93	7.47	7.87	7.42	0.38624	0.19312	0.45
7	7.49	7.67	8.30	7.82	0.34866	0.17433	0.48
9	8.20	8.12	-	8.16	0.03987	0.01993	0.04
10	8.28	8.39	-	8.34	0.05426	0.02713	0.05
11	8.26	8.20	-	8.23	0.02976	0.01488	0.03
12	8.24	8.27	-	8.25	0.01753	0.00876	0.02
13	8.29	8.35	-	8.32	0.02809	0.01405	0.03
14	8.34	8.35	-	8.35	0.00293	0.00147	0.00
15	8.37	8.41	-	8.39	0.02087	0.01043	0.02

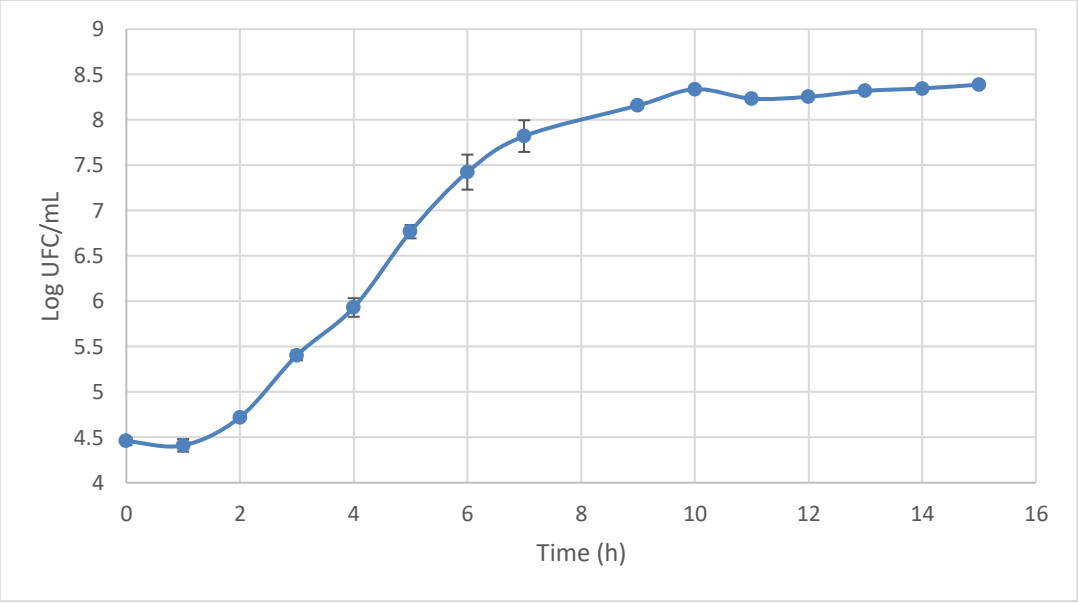


Figure A1-3: Growth curve for *E. coli* K-12 Wild type growth study

Table A1-3: Raw data for experiments applying a 3 min continuous ultrasonic treatment to the *E. coli* mutants

Strain	3 min cont with cold water			3 min cont without cold water			Temp controlled		
	Before treatment	After treatment	Reduction	Before treatment	After treatment	Reduction	Before treatment	After treatment	Reduction
K-12 wild type	6.01	4.34	1.67	5.91	3.45	2.50	6.16	5.22	0.81
Delta gad kl	6.42	5.91	1.67	6.26	5.52	0.68	6.39	5.12	1.27
K-12 ΔdnaK	5.43	3.32	2.11	5.42	0.00	5.42	6.27	5.28	0.98
Jkl 3485 ΔgadA	6.02	4.49	1.53	6.11	3.11	3.00	6.47	5.64	0.73
Delta Gab T	6.20	5.52	0.69	6.36	5.38	0.52	6.18	5.42	0.75
Jkl 5247 ΔyneL	6.16	4.38	1.78	6.14	3.17	2.97	6.54	5.51	1.02
K-12 ΔsoxS	6.09	4.28	1.80	6.04	3.80	2.24	6.54	5.51	1.02
Jkl 1487 ΔgadC	6.06	4.29	1.77	6.08	2.72	3.33	6.59	5.72	1.02
Jkl 1488 ΔgadB	6.01	4.37	1.64	6.03	3.55	2.49	6.92	5.63	1.29
Delta Gab D	6.45	5.64	0.79	6.30	5.82	0.52	7.04	5.71	1.33
K-12 ΔoxyR	5.67	5.07	0.60	5.91	5.09	0.83	6.43	5.97	0.42
K-12 ΔrpoS	5.90	4.37	1.53	6.06	3.88	2.18	5.92	4.58	1.42
K-12 ΔsoxR	6.08	4.23	1.85	6.19	2.66	3.52	5.53	4.26	1.56
Jkl 3484 delta gad X	6.09	5.80	0.29	6.60	5.92	0.68	6.23	5.40	0.85

Table A1-4: Raw data for experiments applying a 5 min and 20 min continuous ultrasonic treatment with temperature controlled for the *E. faecalis mutants*

	20 min controlled temperature			5 min uncontrolled temperature		
	Before	After	Reduction	Before	After	Reduction
EF0071	6.6360	6.2260	0.4099	6.5850	6.3628	0.2222
EF0073	6.5463	6.0511	0.4952	6.3001	6.1121	0.1880
EF0079	6.6981	6.3057	0.3906	6.8421	6.3916	0.4975
EF1027	6.5281	6.3893	0.1388	6.8229	6.4570	0.5829
EF1597	6.8614	6.3256	0.5357	6.7460	6.0243	0.7038
EF1864	6.4799	6.1021	0.3778	6.4551	6.1793	0.2840
EF2195	6.5141	6.1464	0.3677	6.2909	5.9890	0.2798
Wild Type	6.4906	5.9753	1.4164	6.2730	5.5246	0.8733

Table A1-5: Averaged results for the *E. coli* mutants after 3 minutes treatment, showing significant different reductions between mutants using comparison by T-test

Strain	Log Reduction		
	US with cold water (W)	US without cold water (W)	Temperature controlled US (SyW)
K-12 wild type	1.67±0.05[a]	2.50±0.32[a]	0.81±0.29[a]
ΔgadA	1.53±0.17 [a]	3.00±0.14[b]	0.83±0.18[a]
ΔgadB	1.64±0.06 [a]	2.49±0.40[abc]	1.29±0.29[ab]
ΔgadC	1.77±0.06 [a]	3.33±0.57[abcd]	0.87±0.20[a]
ΔgadW	0.51±0.08 [b]	0.68±0.07[e]	1.27±0.03[b]
ΔgadX	0.29±0.08 [b]	0.68±0.09[e]	0.85±0.17[a]
ΔgabT	0.69±0.07 [c]	0.52±0.04[ef]	0.75±0.00[a]
ΔgabD	0.79±0.07 [c]	0.52±0.02[ef]	1.33±0.32[ab]
ΔrpoS	1.53±0.12 [a]	2.18±0.40[a]	1.42±0.34[ab]
Δ*dnaK*	2.11±0.20 [d]	5.42±0.18[h]	0.98±0.10[a]
Δ*soxS*	1.80±0.13 [ad]	2.24±0.22[ac]	1.02±0.38[ab]
ΔsoxR	1.85±0.18 [ad]	3.52±0.27[d]	1.56±0.53[ab]
ΔoxyR	0.60±0.38 [bc]	0.83±0.05[e]	0.42±0.08[c]
ΔyneL	1.78±0.12 [ad]	2.97±0.15[abcd]	1.22±0.35[ab]

Appendix 2

Table A2-1: List of the mutated genes, JCVI role categories - Enterococcus faecalis

Code Number	Locus	Protein function	JCVI role category
1	EF0031	membrane protein, putative	Cell envelope
2	EF0055	adhesion lipoprotein	Cellular processes
3	EF0071	lipoprotein, putative	Cell envelope
4	EF0073	transcriptional regulator, Cro/CI family	Regulatory functions
5	EF0079	gls24 protein	Cellular processes
6	EF0080	gls24 protein	Cellular processes
7	EF0082	major facilitator family transporter	Transport and binding proteins
8	EF0086	conserved domain protein	Hypothetical proteins
9	EF0089	conserved domain protein	Hypothetical proteins
10	EF0091	conserved hypothetical protein	Hypothetical proteins
11	EF0100	seryl-tRNA synthetase	Protein synthesis
12	EF0107	transcriptional regulator, Crp/Fnr family	Regulatory functions
13	EF0146	surface exclusion protein, putative	Cellular processes
14	EF0169	lipase/acylhydrolase	Fatty acid and phospholipid metabolism
15	EF0176	basic membrane protein family	Cell envelope
16	EF0177	basic membrane protein family	Cell envelope
17	EF0201	translation elongation factor Tu	Protein synthesis
18	EF0252	N-acetylmuramoyl-L-alanine amidase, family 4	Cell envelope
19	EF0280	cation efflux family protein	Transport and binding proteins
20	EF0362	chitin binding protein, putative	Cell envelope
21	EF0384	hypothetical protein	No Data
22	EF0386	carbamate kinase	Energy metabolism
23	EF0389	membrane protein, putative	Cell envelope
24	EF0392	hypothetical protein	No Data
25	EF0403	transcriptional regulator, MarR family	Regulatory functions

Code Number	Locus	Protein function	JCVI role category
26	EF0420	drug resistance transporter, EmrB/QacA family protein	Transport and binding proteins
27	EF0465	transcriptional regulator	Regulatory functions
28	EF0502	membrane protein, putative	Cell envelope
29	EF0516	membrane protein, putative	Cell envelope
30	EF0541	PTS system component, authentic frameshift	Signal transduction
31	EF0553	PTS system, IID component	Signal transduction
32	EF0559	polysaccharide biosynthesis family protein	Cell envelope
33	EF0567	potassium-transporting ATPase, subunit A	Transport and binding proteins
34	EF0573	hypothetical protein	No Data
35	EF0577	adhesion lipoprotein	Cellular processes
36	EF0579	transcriptional regulator, putative	Regulatory functions
37	EF0583	ABC transporter, ATP-binding protein/permease protein	Transport and binding proteins
38	EF0590	polysaccharide deacetylase family protein	Energy metabolism
39	EF0600	transcriptional regulator, TetR family	Regulatory functions
40	EF0601	transcriptional regulator, TetR family	Regulatory functions
41	EF0604	gls24 protein	Cellular processes
42	EF0609	conserved hypothetical protein	Hypothetical proteins
43	EF0617	membrane protein, putative	Cell envelope
44	EF0644	transcriptional regulator, LysR family	Regulatory functions
45	EF0746	penicillin-binding protein, putative	Cell envelope
46	EF0779	glycerophosphoryl diester phosphodiesterase family protein	Fatty acid and phospholipid metabolism
47	EF0785	drug resistance transporter, EmrB/QacA family protein	Transport and binding proteins
48	EF0786	tributyrin esterase, putative	Fatty acid and phospholipid metabolism
49	EF0797	conserved domain protein	Hypothetical proteins
50	EF0814	transcriptional regulator, GntR family	Regulatory functions
51	EF0818	polysaccharide lyase, family 8	Cellular processes

Code Number	Locus	Protein function	JCVI role category
52	EF0876	hypothetical protein, MGA Helix-turn-helix domain	No Data
53	EF0887	glycosyl transferase, group 2 family protein	Cell envelope
54	EF0889	conserved hypothetical protein	Hypothetical proteins
55	EF0892	amino acid ABC transporter, ATP-binding protein	Transport and binding proteins
56	EF0906	conserved hypothetical protein	Hypothetical proteins
57	EF0929	amino acid permease family protein	Transport and binding proteins
58	EF0962	transcriptional regulator, AraC family	Regulatory functions
59	EF0994	UDP-N-acetylglucosamine--N-acetylmuramyl-(pentapeptide) pyrophosphoryl-undecaprenol N-acetylglucosamine transferase	Cell envelope
60	EF1027	membrane protein, putative	Cell envelope
61	EF1042	multidrug resistance protein, putative	Transport and binding proteins
62	EF1076	streptomycin 3-adenylyltransferase, putative	Cellular processes
63	EF1100	ABC transporter, ATP-binding/permease protein	Transport and binding proteins
64	EF1156	transcriptional regulator, GntR family	Regulatory functions
65	EF1157	peptidase, M20/M25/M40 family	Protein fate
66	EF1172	teichoic acid biosynthesis protein B, putative	Cell envelope
67	EF1173	glycosyl transferase, WecB/TagA/CpsF family	Cell envelope
68	EF1175	glycerol-3-phosphate cytidylyltransferase	Cell envelope
69	EF1193	DNA-binding response regulator VicR	Signal transduction
70	EF1211	NADH peroxidase	Energy metabolism
71	EF1212	transcriptional regulator	Regulatory functions
72	EF1224	transcriptional regulator, Cro/CI family	Regulatory functions
73	EF1238	glycosyl hydrolase, family 3	Energy metabolism
74	EF1288	conserved hypothetical protein	Hypothetical proteins
75	EF1347	glycosyl hydrolase, family 13	Energy metabolism

Code Number	Locus	Protein function	JCVI role category
76	EF1408	ABC transporter, ATP-binding protein	Transport and binding proteins
77	EF1413	msrC protein, putative	Cellular processes
78	EF1420	hypothetical protein	No Data
79	EF1493	V-type ATPase, subunit I	Transport and binding proteins
80	EF1516	PTS system, IIABC components	Signal transduction
81	EF1525	transcriptional regulator, Fur family	Regulatory functions
82	EF1583	N-acetylmuramoyl-L-alanine amidase, family 4	Cell envelope
83	EF1585	transcriptional regulator, Fur family	Regulatory functions
84	EF1592	ABC transporter, ATP-binding/permease protein	Transport and binding proteins
85	EF1597	catalase/peroxidase	Cellular processes
86	EF1599	TPR domain transcriptional regulator, Cro/CI family	Regulatory functions
87	EF1606	glycosyl hydrolase, family 1	Energy metabolism
88	EF1656	transcriptional regulator, LysR family	Regulatory functions
89	EF1663	branched-chain phosphotransacylase	Fatty acid and phospholipid metabolism
90	EF1681	peptide methionine sulfoxide reductase	Protein fate
91	EF1705	phosphate-binding protein	Transport and binding proteins
92	EF1709	sugar-binding transcriptional regulator, GntR family	Regulatory functions
93	EF1741	catabolite control protein A	Regulatory functions
94	EF1743	proline dipeptidase	Protein fate
95	EF1746	UTP-glucose-1-phosphate uridylyltransferase	Cell envelope
96	EF1759	phosphate ABC transporter, phosphate-binding protein	Transport and binding proteins
97	EF1760	cell division ABC transporter, permease protein FtsX, putative	Transport and binding proteins
98	EF1798	hypothetical protein	No Data
99	EF1814	drug resistance transporter, EmrB/QacA family protein	Transport and binding proteins

Code Number	Locus	Protein function	JCVI role category
100	EF1824	glycosyl hydrolase, family 31/fibronectin type III domain protein	Cell envelope
101	EF1851	glycosyl hydrolase, family 35	Energy metabolism
102	EF1864	DNA-binding response regulator	Signal transduction
103	EF1869	permease, putative	Transport and binding proteins
104	EF1904	glycerophosphoryl diester phosphodiesterase family protein	Fatty acid and phospholipid metabolism
105	EF1920	C4-dicarboxylate anaerobic carrier	Transport and binding proteins
106	EF1943	drug resistance transporter, Bcr/CflA family protein	Transport and binding proteins
107	EF2020	hypothetical protein	No Data
108	EF2068	multidrug resistance protein, putative	Transport and binding proteins
109	EF2076	endocarditis specific antigen	Cellular processes
110	EF2144	lipoprotein, putative	Cell envelope
111	EF2163	glycerophosphoryl diester phosphodiesterase, putative	Fatty acid and phospholipid metabolism
112	EF2167	glycosyl transferase, group 2 family protein	Cell envelope
113	EF2170	glycosyl transferase, group 2 family protein	Cell envelope
114	EF2180	glycosyl transferase, group 2 family protein	Cell envelope
115	EF2181	glycosyl transferase, group 2 family protein	Cell envelope
116	EF2190	glycosyl transferase, group 2 family protein	Cell envelope
117	EF2195	glycosyl transferase, group 2 family protein	Cell envelope
118	EF2196	glycosyl transferase, group 2 family protein	Cell envelope
119	EF2197	glycosyl transferase, group 2 family protein	Cell envelope
120	EF2198	glycosyl transferase, group 4 family protein	Cell envelope
121	EF2221	ABC transporter, substrate-binding protein	Transport and binding proteins
122	EF2248	hypothetical protein	No Data

Code Number	Locus	Protein function	JCVI role category
123	EF2250	conserved domain protein	Hypothetical proteins
124	EF2268	conserved hypothetical protein	Hypothetical proteins
125	EF2276	hypothetical protein	No Data
126	EF2318	peptidase, M23/M37 family	Protein fate
127	EF2347	cell wall surface anchor family protein	Cell envelope
128	EF2417	transcriptional regulator, Fur family	Regulatory functions
129	EF2425	phosphoglucomutase/phosphomannomutase family protein	Energy metabolism
130	EF2442	phosphate transporter family protein	Transport and binding proteins
131	EF2464	conserved domain protein	Hypothetical proteins
132	EF2490	conserved hypothetical protein	Hypothetical proteins
133	EF2491	glycosyl transferase, group 2 family protein	Cell envelope
134	EF2492	glycosyl transferase, group 2 family protein	Cell envelope
135	EF2505	cell wall surface anchor family protein	Cell envelope
136	EF2525	cell wall surface anchor family protein	Cell envelope
137	EF2556	fumarate reductase flavoprotein subunit precursor, putative	Energy metabolism
138	EF2618	carboxylesterase precursor, putative	Fatty acid and phospholipid metabolism
139	EF2662	choline binding protein	Cellular processes
140	EF2671	conserved hypothetical protein	Hypothetical proteins
141	EF2682	conserved hypothetical protein	Hypothetical proteins
142	EF2703	transcriptional regulator	Regulatory functions
143	EF2709	glycosyl hydrolase, family 2	Energy metabolism
144	EF2746	dltD protein	Cell envelope
145	EF2748	peptidase, U32 family, putative	Protein fate
146	EF2750	conserved hypothetical protein	Hypothetical proteins
147	EF2772	drug resistance transporter, putative, authentic frameshift	Transport and binding proteins
148	EF2773	major facilitator family transporter	Transport and binding proteins
149	EF2795	LysM domain lipoprotein	Cell envelope

Code Number	Locus	Protein function	JCVI role category
150	EF2796	hypothetical protein	No Data
151	EF2889	2-hydroxy-3-oxopropionate reductase	Energy metabolism
152	EF2890	glycosyl transferase, group 1 family protein	Cell envelope
153	EF2891	glycosyl transferase, group 1 family protein	Cell envelope
154	EF2932	AhpC/TSA family protein	Unknown function
155	EF2958	transcriptional regulator, LysR family	Regulatory functions
156	EF2963	esterase, putative	Unknown function
157	EF2992	major facilitator family transporter	Transport and binding proteins
158	EF2997	peptidase, M20/M25/M40 family	Protein fate
159	EF3012	membrane protein, putative	Cell envelope
160	EF3023	polysaccharide lyase, family 8	Cellular processes
161	EF3034	transcriptional regulator, GntR family	Regulatory functions
162	EF3041	pheromone binding protein	Transport and binding proteins
163	EF3060	secreted lipase, putative	Unknown function
164	EF3103	membrane protein, putative	Cell envelope
165	EF3156	transcriptional regulator, GntR family	Regulatory functions
166	EF3157	glycosyl hydrolase, family 65	Energy metabolism
167	EF3164	PilB family protein	Unknown function
168	EF3180	RNA polymerase sigma-70 factor, ECF subfamily	Transcription
169	EF3191	lipase, putative	Fatty acid and phospholipid metabolism
170	EF3206	adhesion lipoprotein	Cellular processes
171	EF3245	cell-envelope associated acid phosphatase	Unknown function
172	EF3255	thiamin biosynthesis lipoprotein ApbE, putative	Biosynthesis of cofactors, prosthetic groups, and carriers
173	EF3272	zinc-binding transcriptional regulator, Cro/CI family	Regulatory functions
174	EF3279	peptidase, U32 family	Protein fate
175	EF3280	peptidase, U32 family, putative	Protein fate

Code Number	Locus	Protein function	JCVI role category
176	EF3281	conserved domain protein	Hypothetical proteins
177	EF3294	membrane protein, putative	Cell envelope

Appendix 3

The same procedure was followed as described in chapter 3, however for *Enterococcus faecalis*, the antibiotic tetracycline (1.8 μg/mL) was used with the media, in order to avoid the cells from mutating from its original state.

Table A3-1: A description of the *Enterococcus faecalis* mutants and their proteins' functions. All these mutants were provided by the mutant library of INRA, AgroParisTech UMR1319 Micalis, Jouy-en-Josas, France

Gene	Missing encoded protein	Protein function
EF0071	Trehalase	Catalysis of the reaction: α,α-trehalose + H2O ⇌ β-D-glucose + α-D-glucose
EF0073	Transcriptional regulator, Cro/CI family	Any molecular function by which a gene product interacts selectively and non-covalently with DNA
EF0079	Gls24 protein	Involved in virulence, bile salts stress response and glucose starvation stress response
EF1027	Membrane protein (putative gene)	It's a mprF-like gene from *Staphylococcus aureus* which codes for phosphatidylglycerol lysyltransferase that transfers the lysyl group from L-lysyl-tRNA(Lys) to membrane-bound phosphatidylglycerol
EF1597	Catalase/Peroxydase	Catalysis of the reaction: 2 H_2O_2 → O_2 + 2 H_2O Interacting selectively and non-covalently with any metal ion and with heme.
EF1864	DNA-binding regulator	The cellular synthesis of RNA on a template of DNA. Any process that modulates the frequency, rate or extent of cellular DNA-templated transcription.
EF2195	Glycosyl transferase	Catalysis of the transfer of a saccharide moiety from one compound to another compound

Experiments were performed in two types of liquid systems: (i) sterile water (SW) and (ii) synthetic wastewater (SyW). The working solution to be treated was prepared by diluting 2 mL of the working culture in 298 mL in SW or SyW in a 500 mL sterile beaker. The SyW was prepared by mixing peptone 64.0g/L; Meat Extract 44.0g/L; Urea 12.0g/L; K_2HPO_4 11.2g/L; NaCl 2.8g/L; $CaCl_2.2H_2O$ 1.6g/L; $MgSO_4.7H_2O$ 0.8g/L) (Antoniadis *et al.*, 2007; Ayyildiz, Sanik and Ileri, 2011).

Table A3-2: Log reductions on Enterococcus faecalis mutants and wild type for 5 mins and 20 mins respectively

		5 min treatment		20 min treatment	
		Average	St deviation	Average	St deviation
3	EF0071	0,21[a]	0.14	0,41[a,b]	0.14
4	EF0073	0,16[a]	0.10	0,56[a]	0.14
5	EF0079	0,48[b]	0.08	0,46[a,b]	0.12
60	EF1027	0,36[b,e]	0.07	0,21[c]	0.14
85	EF1597	0,69[c]	0.10	0,54[a]	0.10
102	EF1864	0,26[d]	0.11	0,48[a,b]	0.15
117	EF2195	0,2[a,d]	0.09	0,38[b]	0.11
Wild Type		0,97[c,e]	0.12	1,42[d]	0.09