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An investigation into the antibacterial mechanism of honey

by

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Summary

The Antimicrobial Resistance, one of the biggest public threats of our times, emerged the development of alternative antibiotics. As such substances/procedures employed historically to cure diseases are now attracting the renewed interest of the scientific community. Honey has been empirically used as a powerful therapeutic since ancient times. However, currently the understanding of the underlying antibacterial mechanism is incomplete and this underestimates the medicinal value of honey.

This study aims to investigate the mechanism underlying the antibacterial activity of hydrogen peroxide (H_2O_2)-producing honeys. To overcome the variability between honey species, a model system was used. This allowed to investigate the antibacterial effect of, and possible synergies between the three main stressors; sugars, gluconic acid and H_2O_2, resulting from the enzymatic conversion of glucose, on honey dilution. The combination of cellular and genetic methods holds the key to understanding the bacterial stress responses towards honey. Multiparametric flow cytometry (FC) and atomic force microscopy (AFM) probed the physiological changes of *E. coli* K-12 while wide-genome transposon mutagenesis (TraDIS) identified genes and pathways which are involved in bacterial pathogenicity and resistance to honey.

The model honey revealed significant synergies occurring on honey dilution. The synergy of H_2O_2 and gluconic acid augmented the antibacterial effect of honey by causing simultaneous cell membrane depolarization and destruction. TraDIS showed that switch to anaerobic metabolism consists the bacterial resistance mechanism to honey. Thus, the mutation of genes implicated in anaerobic metabolic activity, cellular homeostasis and membrane invagination caused a significant growth defect on the respective strains post-exposure to honey. These genes were deemed as “conditionally essential” as encode functions that are absolutely vital for cell viability on honey.

As a proof of concept, the antibacterial effects of natural honeys was tested *in vitro* in a coculture of two pathogenic species, *E. coli* (UPEC)-*C. albicans,* implicated in vulvovaginal infections. Both pathogens were fully susceptible to honey of high acidity and high H_2O_2 accumulation which confirmed the importance of this synergy in antibacterial strength of honey.

Overall this thesis presents a comprehensive and novel approach towards the understanding of the antibacterial mechanism of honey. This is expected to provide an advanced explanation on the mechanistic action and reveal further potential medicinal applications of this natural product.

Dedication

To my family for the unparalleled support

for as long as I can remember

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Abbreviations

Term	Definition
5-HMF	5-(hydroxymethyl)furfural
AF	Artificial honey
AFM	Atomic force microscopy
ARs	Acid response systems
aw	Water activity
BOX	Bis (1,3-dibutylbarbituric acid)
BV	Bacterial vaginosis
cFDA	Carboxyfluorescein diacetate
CFU	Colony forming units
cPMP	Cyclic pyranopterin monophosphate
CR	Carbapenems
CRE	Carbapenems Enterobacteriaceae
DEGs	Differentially expressed genes
ESBLs	Extended-spectrum beta-lactamases
FC	Flow cytometry
FDA	Food and Drug Administration
FSC-A	Forward scatter-area
FSC-H	Forward scatter-height
GO	Gene Ontology
GOX	Glucose oxidase

GTP	Guanosine-5'-triphosphate
H-NS protein	Histone-like nucleoid-structuring protein
H_2DCFDA	2',7'-dichlorodihydrofluorescein diacetate
H2O2	Hydrogen peroxide
IL-1/ IL-6	Interleukin-1/ 6
Kan	kanamycin
KOs	Knockouts
LAB	Lactic acid bacteria
Lac+/-	Lactose positive/negative phenotype
LB	Lysogeny broth
LPS	Lipopolysaccharides
MBC	Minimum bactericidal concentration
MDR	Multidrug resistance (strains)
MFI	Mean fluorescence intensity
MGD	Guanine dinucleotide
MGO	Methylglyoxal
MIC	Minimum inhibitory concentration
MPT	Molybdopterin
MPT-AMP	Adenylated molybdopterin
MRSA	Methicillin-resistant Staphylococcus aureus
MRSE	Methicillin-resistant Staphylococcus epidermidis
NO	Nitric oxide
NS	Not specified

PBP2a	Penicillin binding protein 2a
PBS	Phosphate-buffered saline
PG	Peptidoglycan
PI	Propidium iodide
PMF	Proton motive force
RBCA	Chloramphenicol Agar Base
RFU	Relative fluorescence units
ROS	Reactive Oxygen species
RSM	Response Surface Modelling
RVVC	recurrent Vulvovaginal candidiasis
SEM	Scanning electron microscopy
SSC-A	Side scatter-area
SSC-H	Side scatter-height
ssDNA	Single stranded DNA
TCA	tricarboxylic acid cycle
TCRS	Two-component regulatory system
TEM	Transmission electron microscopy
TNF-a	Tumour necrosis factor-a
TraDIS	Transposon Directed Insertion-site sequencing
TVC	total viable count
UMF	Unique Manuka Factor
UTI	Urinary tract infections
VOCs	Volatile compounds

VRE	Vancomycin-resistant Enterococci
VV	Vulvovaginal
VVC	Vulvovaginal candidiasis
WHO	World Health Organization

CHAPTER 1

Literature review

1.1. History of medical evolution of honey

Honey was empirically acknowledged for its medicinal properties from many civilizations at different periods of time. Regardless of any religious and mythical attributes, it was first identified as a natural sweetener. It was later that many groups of people realized its medicinal properties, suggesting that antimicrobial properties of honey were based on real facts and not hearsay.

Egyptians are among the first civilizations using honey to treat oral, optical, and epidermal ailments, fatigue and vertigo (Jones, 2001, Meda et al., 2004). Ancient Greeks and Romans used honey as a wound-healing agent and multipurpose medical ointment (Lusby, 2002). For many decades, the use of honey was based on empirical evidence rather clinical investigation (Figure 1.1).

In 1892, The Dutch scientist Van Ketel was the first that documented the antimicrobial properties of honey (Dustmann, 1979). Since then, a lot of research initiated on the medicinal and antimicrobial properties of honey culminating in four major publications. In 1919, Sackett's publication was the first which mentioned that antimicrobial activity is increased after the honey is diluted (Sackett, 1919). Almost 25 years later (1937), Dold recognised "inhibine" as an antimicrobial compound in honey, which is light- and heat-sensitive (Dold et al., 1937; Molan, 1992b). The identity of inhibine was unknown by that time. In 1962, Adcock demonstrated that catalase, an enzyme which degrades hydrogen peroxide (H_2O_2), eliminated the antimicrobial activity of honey (Adcock, 1962). Eventually in 1963, White et al., deduced that "inhibine" was the H_2O_2 which is generated on honey's dilution during the oxidation of glucose by the honey innate enzyme glucose oxidase (GOX) (White et al., 1963).

Although honey lost its popularity as a medication between the 1940s and 50s due to the massive use of antibiotics, the research into the antimicrobial activity of honey did not cease. The culmination of 100 years of research was welcomed with the registration of the first honey wound care product (Medihoney) in 1999 (Simon et al., 2009). The following years, research on the physicochemical and microbiological properties of honey provided enough evidence of its antimicrobial capacity (Bijlsma et al., 2006; Souza et al., 2006; De Moura Olivera et al., 2013). The majority of honeys tested induced significant logarithmic reduction of both Gram-negative and positive bacteria, while, *Staphylococcus aureus* proved to be one of the most honey-susceptible bacteria tested (Temaru et al., 2007).

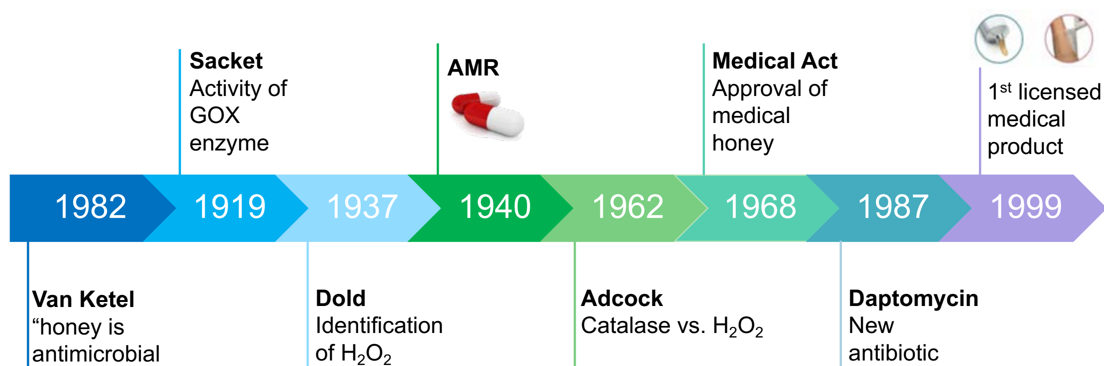


Figure 1.1 Timeline: Major findings on the antimicrobial activity of honey.

1.1.1. Honey in modern medicine

In the last thirty years, the threatening rise of antimicrobial resistance made clinicians and researchers more aware of the medicinal properties of honey and its effectiveness against resistant microbes, such as Gram-negative bacteria (Hannan et al., 2004) and fungal species (Irish et al., 2006). Among the most beneficial and well-characterised features of honey is the broad-spectrum activity against nosocomial pathogens (Molan, 1992a) especially these associated to malignant ulcers (Simon et al., 2005), diabetic foot ulcers (Eddy & Gideonsen 2005) and skin grafts (Schumacher 2004). Since March 2004, over 20 honey medical devices have been approved for clinical use in the UK (Cowan, 2013). Manuka and engineered honey produced in greenhouses under standardized conditions are the two kinds of honey licensed and used in wound management. Different factors were reported to contribute to the antimicrobial effect of each type of honey and they are discussed in detail in § 1.5. However, low pH, high sugar concentration, and the cationic antimicrobial peptide bee defensin-1 are common for both honey types. Extensive research on New Zealand originated Manuka honey led to the registration of patented clinical products, oriented to wound healing.

1.2. The production of honey

The European honey bee (*Apis milifera*) is one of the most widely known bee species of the 16,000 identified (Dunforth et al., 2004). Within a honey beehive, there are three types of bee; the queen, the workers and the drone bees whose collective effort results in the production of honey (Saranraj and Sivasakthi, 2018).

The worker bees travel up to 9 km in one trip to collect nectar, the sugar-rich liquid produced in nectaries, from flowering plants. Once the nectar is in the beehive, worker bees swallow and digest it many times. During the digestion, the processor bees add the enzyme invertase which breaks down the sucrose of the nectar into glucose and fructose (Molan, 2009). The regurgitation process and the wings' fanning ripen the honey and evaporate the water to a concentration of 15-17% which prevents honey from being contaminated by bacteria during storage (Kubota et al., 2004). Further to this, during digestion other bee enzymes (i.e. diastase, invertase, glucosidase, glucose oxidase and catalase) are added to nectar resulting in the supersaturated sugar solution we call honey (Pontoh and Low, 2002). Thus, honey is the by-product of flower nectar which is synthesized in the upper digestive tract of the honeybee once it is processed with stomach enzymes.

1.3. Chemical composition of honey

Honey is a very complex mixture and more than 200 substances have been identified in this. The three main fractions are the sugars (~80% v/v), the water content (~17% v/v) and the minor components fraction (~3%) which includes organic acids, vitamins, metals, protein and phytochemical compounds (White, 1979b) (Table 1.1).

Table 1.1: The major components of honey. Components that make up each of the three honey fractions (White, 1979a).

Fraction	Component	Average weight (%)
Highly conserved sugars	Fructose	38
	Glucose	31
	Maltose	7.3
	Sucrose	1.3
	Other Saccharides	1.5
Water	Water	17
Highly variable compounds	Acidic compounds	0.5
	Nitrogenous compounds	2
	Vitamins and minerals	0.1
	Other compounds	1.3

The principal carbohydrate fraction in honey is composed of fructose and glucose representing 85-95% of the dry weight in honey. Disaccharides such as maltose, sucrose, isomaltose, turanose, melibiose, maltotriose and melezitose comprise the remaining of carbohydrate matter (Eteraf-Oskouei et al., 2013).

Among the constituents of the third fraction, proteins and amino acids are found in a concentration of 0.1-2% (Won et al., 2009) varying according to the pollen source and secondly to honeybee species (Weston, 2000; Escuredo et al., 2013). Honey from *Apis cerana* was reported to contain 0.1-3.3% protein and *Apis milifera* 0.2-1.6% (Won et al., 2009).

Amino acids are responsible for the 1% of the protein content, depending mainly on the honey origin (White et al., 1979). Proline represents a 50-85% of total amino acids, being the most abundant among others such as glutamate, aspartic acid, methionine, phenylalanine, alanine, tyrosine and leucine (Rebane and

Herodes, 2010). Proline is used as an indicator of honey maturation (White et al., 1979). Enzymes make up a small portion of the proteinaceous content and are originated from both nectar and bees (Weston, 2000). Diastase, invertase (glucose oxidase) and catalase are the most common enzymes found in honey. Diastase (α-amylase) is added by worker bees to nectar during the ripening process. Amylase breaks down the starch to simpler compounds once it is activated at pH of 4.6-5 (Babacan, 2002). This enzyme used to be a quality indicator in several European countries, but it was later reported that concentration of amylase in honey varies according to the floral origin, nectar and the foraging pattern of the bees (Oddo et al., 1990).

Glucose oxidase (GOX), which is produced in hypopharyngeal glands of bee, catalyses the oxidation of glucose to gluconic acid and hydrogen peroxide (H_2O_2) in the presence of molecular oxygen (Radwan et al. 1984, Allen et al. 1991) (Figure 1.2). During the oxidation of the glucose, GOX acting as an oxidoreductase, transfers electrons from glucose substrate to O_2 to form H_2O_2 (Raba & Mottola, 1995). GOX is a heat/light-sensitive enzyme, active in nectar and virtually inactive during the ripening process. On honey dilution, when the pH increases (pH>3.5), the activity of GOX is restored. The enzyme demonstrates maximum catalytic activity at pH within 5.5-6.5. The increase of temperature (from 25 to 60°C) was also reported to linearly increase the velocity of the reaction (Odebunmi and Owalude, 2007). Also, the concentration of glucose in honey affects the efficiency of the reaction. At low glucose concentration (<0.05 M) the rate of reaction follows first-order kinetics by linearly increasing with a slope of 1, while, for a higher

concentration of glucose the rate of reaction increases until it reaches a plateau where the reaction follows zero-order kinetics (Owalude, 2004).

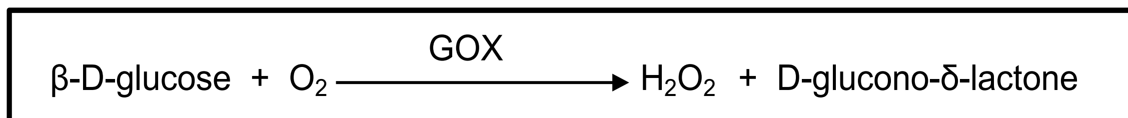


Figure 1.2: Oxidation of glucose by the enzyme GOX (glucose oxidase). GOX is an oxido-reductase that converts 1 mole oxygen and glucose to 1 mole D-glucono-δ-lactone and H_2O_2.

Catalase is found in pollen grains and neutralises the H_2O_2 once it is accumulated in honey (Assia and Ali, 2015).

Trace amounts of vitamins and minerals, with known and unknown biological functions, play a key role in the bioactivity of honey (Bengsch, 1992). Vitamins (riboflavin, niacin, folic acid, pantothenic acid and vitamin B6) and vitamin C (ascorbic acid), coming from the pollen grains, are found in a range of 0.1-0.5 %. The low pH of honey serves as a preservative for the vitamin content (Bonte and Desmouliere, 2013). However, vitamin load is reduced by filtration (which removes the pollen during the honey process) and the accumulation of hydrogen peroxide which oxidises the ascorbic acid (Ciulu et al., 2011).

In dark and light-coloured honeys, the mineral content ranges between 0.4% and 0.2% respectively. Those substances depend on the plant origin, and therefore they reflect the plant that was pollinated and the nectar that was collected (Madejczyk and Baralkiewicz, 2008). Potassium represents one-third of the total mineral content. Sodium, calcium, iron, zinc, phosphorus and magnesium are the

main minerals found in unprocessed honey (Kumar et al., 2010; Rashed et al., 2004; Lachmanet al., 2007).

Volatile compounds (VOCs), constituting the fingerprint of the product, are contained in low concentration and determine the colour and texture of honey. The plant type determines the kind of these compounds while the honey processing, storage conditions, microbial contamination or non-enzymatic browning reaction results in the production or transformation of VOCs (Jerković et al., 2011, Castro-Vázquez et al., 2008). In mono-floral honeys the presence of certain VOCs coming from a certain nectar source give a very distinctive flavour (Castro-Vazquez et al., 2007). From the 600 VOCs already identified in honey, seven major groups are predominant: aldehydes; acids; alcohols; cyclic compounds; esters; hydrocarbons; and ketones. Some of them are biomarkers for certain honey types such as the isophorone, which is found in heather honey (Cuevas-Glory et al., 2007; Alissandrakis et al., 2005), and hexanal and heptanal that are characteristic for lavender honeys (Radovic et al., 2001).

Phenolic compounds are another major group affecting the bioactivity of honey and contribute to its antibacterial, anti-inflammatory and antioxidant properties. Many authors reported the correlation between the phenolic content of honey and its antioxidant properties (Ferreres et al., 1991; Martos et al., 2000) because of their ability to scavenge/reduce the formation of free radicals and prevent the lipids' oxidation in cell membrane (Sghaier et al., 2011). The antioxidant activity is mainly dependent on the presence of certain hydroxyl groups in the flavonoid rings (Sghaier et al., 2011). Flavonoids (quercetin, pinocembrin, pinobanksin, chrysin, galangin, kaempferol and luteolin), are the predominant

phenolic compounds identified in honey followed by the benzoic and cinnamic acids (Michalkiewicz et al., 2008; Truchado et al., 2008; Dong et al., 2013).

The acidic profile of honeys is attributed to the organic acid content (0.57%). Honeybees secrete organic acids during the fermentation of sugars by the enzymes of their stomach (Karabagias et al., 2014; Cherchi et al., 1994). Botanic or geographic factors affect the profile of the organic acids found in honey and this, in turn, affects the colour, flavour, acidity and electrical conductivity of the honey accordingly (Cherchi et al., 1994). Gluconic acid is the predominant organic acid found in honey, produced by the breakdown of glucose by the GOX enzyme (Olaitan, 2007). Acetic, citric, formic, malic, malonic, lactic, quinic, succinic and oxalic acid are also found in honey in lesser amount (Cherchi et al., 1994; Mato et al., 2006). Levulinic and formic acid, produced during the Maillard reaction (when sugars are degraded), increase the total acidity in honey (Cavia et al., 2007). Free acidity in honey was reported to remain constant for a long storage period (12-15 months), with a tendency to increase slightly because of the fermentation of sugars and alcohols from the yeasts in honey (Cavia et al., 2007).

The third fraction of the honey components vary a lot based on the environmental, geographical, temporal, and phylogenetic variables (floral source) (Table. 1.2). These variables have an impact on the colour, density, acidity (pH), electrical conductivity and ultimately the bioactivity of honey (White, 1979b; Molan 1992b, Adams et al., 2008;). Honey colour, smell and taste are the variables serving as identification markers for its floral source (Molan and Betts 2004).

According to Codex Alimentarius and the internationally recognized standards, honey is classified in line with its floral source. Mono-floral honey is

named after a single floral source while poly-floral honeys are derived from the nectar of various floral sources. Many mono-floral honeys, including Manuka (*Leprospermum scoparium*), kanuka (*Kunzea ericoides*), chestnut (*Castanea sativa*), heather (*Calluna vulgaris*), cotton (*Gossypium hirsutum L.*), ulmo (ulmo tree; *Eucryphia cordifolia*) were highlighted for their antimicrobial performance attributed to their composition (Alnaimat et al., 2012; Lu et al., 2013; Sherlock et al., 2010).

Table 1.2: Factors affecting the composition of honey. Abiotic and biotic parameters such as geography, climatic conditions, plant source and seasonality affect the composition of honey (David W. Ball, 2007; Michener, 2007; Michez et al., 2008; Patiny, 2011).

Variable	Example
Geographic	Geography and climatic conditions favour the growth of certain plant types, while these conditions are prohibitive for other species. Manuka bush is exclusively grown in N. Zealand, jelly bush in Australia and vanilla and heather mainly in Greece.
Environmental	Temperature, humidity, wind and land topography favour the flowering of specific plant species. Since bees collect the nectar from different plants this has effect on the type of honey that will be collected.
Temporal	Seasonal plants are only capable of growth during specific time periods, affecting the nectar viability as such the honey composition.
Floral source	The main factor affecting honey's composition is the floral source. Certain phytochemicals found in plants are processed by bees giving particular characteristics to each honey type.

1.4. Factors contributing to the antimicrobial activity of honey

The exact mechanism by which honey inhibits bacterial growth is not yet fully understood. However, to date, high sugar content (low water activity, a_w), acidity, hydrogen peroxide (H_2O_2), phenolic compounds and the antimicrobial peptides bee-defensin-1 and Methylglyoxal (MGO) were found to exert antimicrobial activity in honey (Kwakman and Zaat, 2012).

1.4.1. Sugars

The mixture of polysaccharides occurring in honey creates a high osmolarity (a_w between 0.6-0.9). This environment prevents the spoilage of honey by microorganisms. Although low a_w can inhibit bacterial proliferation, the antimicrobial activity of honey is not a result of the sugar fraction alone (Zumla and Lulat, 1989; Kwakman et al., 2010).

1.4.2. Gluconic acid and H_2O_2

Antimicrobial activity in honey was initiated by GOX enzyme (Figure 1.2). GOX, a flavoprotein which is produced in hypopharyngeal glands of the bee and is added to honey during the processing of the nectar, contributes to the antimicrobial activity of honey (Kretavicius et al., 2010).

Gluconic acid, the predominant organic acid in honey, together with other amino, aromatic and phenolic acids reduce the pH of honey to between 3.4 and 4.5 (Olaitan et al., 2007). The high acidity instils an additional level of antimicrobial

activity, inhibiting the proliferation of bacterial species which commonly grow at more natural pHs (Bogdanov et al., 1997; Daher & Gülaçar, 2008).

H_2O_2 is another antimicrobial compound which also contributes to the acidity and antimicrobial activity of honey. The prooxidant activity of H_2O_2 causes oxidative damage resulting in DNA degradation and bacterial growth inhibition in dose-dependent way (Brudzynski et al., 2011).The role of H_2O_2 on the antibacterial activity of honey is explained later on § 1.5.2.1- 1.5.2.4.

1.4.3. Phenolics

Polyphenols and flavonoids contribute to the antioxidant and antimicrobial activity of honey. As antioxidants, polyphenols and their derivatives exert radical scavenging activity, oxygen quenching and metal chelation (Brudzynski and Miotto, 2011). In addition to antioxidant activity, synergy of phenolics and intermediate products of the Maillard reaction were found to exert antimicrobial activity in honey (Adams et al., 2008; Mavric et al., 2008).

1.4.4. Bee-derived antibacterial peptides

Bee defensins are cysteine-rich cationic peptides, which are produced in salivary glands and fat body cells of bees. They are induced in honey during the digestion of nectar in bees salivary glands (Ilyasov et al., 2012). Defensin 1 and 2, which show 55% similarity between each other, exert antimicrobial activity against both Gram-positive and negative bacteria (Klaudiny J et al., 2005). Both defensins

act synergistically to H_2O_2 by inducing breaks to bacterial membrane and eventually cell lysis (Kwakman et al., 2010).

1.4.5. Methylglyoxal (MGO)

Dihydroxyacetone, a phytochemical substance found in the nectar of certain floral species (i.e. *Leptospermum scoparium*) is the precursor of MGO. During the storage of honey, the natural degradation of dihydroxyacetone through the Maillard reaction generates MGO (Weigel et al., 2004; Adams et al., 2009). MGO was proved to cause oxidative stress by reacting with cellular proteins and DNA (Riboulet-Chavey et al., 2006; Blair et al., 2009), while in combination with bee-defensins and H_2O_2 was proved to have an enhanced activity against a broad spectrum of bacteria (Kwakman et al., 2010).

1.5. Discrimination of honey types based on their antimicrobial activity

Many previous studies were focused on the identification of the antimicrobial components in honey (Allen et al., 1991; Molan, 1992; Cooper et al., 1999, 2002a,b; Lusby et al., 2002; Wilkinson and Cavanagh, 2005; Brudzynski and Kim, 2011). The screening of a broad range of honey types indicated that there are two main categories depending on their ability to produce H_2O_2 or not. These are Manuka and non-Manuka honeys (Kwakman et al., 2011b).

Manuka honeys (*Leptospermum* sp.), in which the MGO is the main antimicrobial factor, demonstrate hydrogen peroxide-independent antimicrobial activity (Adams et al., 2008; Mavric et al., 2008). European and American (non-

Manuka) honeys, originated from any floral source(s), demonstrate H_2O_2-dependent antimicrobial activity. These honeys are also known as catalase-sensitive honeys considering the degradation of H_2O_2 by catalase enzyme (Molan and Russell, 1988; Allen et al., 1991). Although the exact antimicrobial mechanism of honey remains obscure a few studies have elucidated key points of the underlying mechanism in both honey types.

1.5.1. Manuka honey

Manuka honey, exclusively originated from the Australian and New Zealand manuka bush (*Leptospermum scoparium:* Myrtaceae), demonstrates exceptionally high antimicrobial activity. The main antibacterial factor identified in Manuka honey is methylglyoxal (MGO) with concentration being around 83 to 829 mg/kg in contrast to non-Manuka honeys in which the MGO is limited to 1.6-24 mg/kg (Adams et al., 2008). Antimicrobial strength of Manuka medical products is rated by the Unique manuka factor UMF ranging between 5 (83 mg/kg) to 20 (829 mg/kg) (Simon et al., 2006).

MGO arrests cells division by immediate inhibition of protein synthesis and initiation of the DNA replication (Lu et al., 2013; Fraval and Mcbrien, 1980). Lower concentration of MGO can be metabolised by the glyoxalase system of bacteria, therefore, it cannot halt the bacterial growth other than extend their log-phase (Racker, 1951).

Many studies examined the effect of this honey on opportunistic pathogens, clinical isolates, multidrug resistance (MDR) strains and other genera such as *Escherichia, Bacillus, Pseudomonas, Staphylococcus and Streptococcus* in order

to elucidate the mode of Manuka honey's activity (Blair, 2009; Jenkins et al., 2011). *Staphylococcus aureus* and *Pseudomonas aeruginosa* are two opportunistic pathogens well-studied for their susceptibility to Manuka honey. The findings on the antibacterial effect of Manuka honey revealed two distinguishing mechanisms for Gram-positive (*S. aureus*) and negative (*P. aeruginosa)* organisms which will be discussed in the following paragraphs.

Manuka honey was reported to have a bactericidal effect on *S. aureus* and Methicillin-resistant *S. aureus* (MRSA) by inhibiting cell division and the completion of cell cycle, which results in septate non-dividing cells (Kwakman, 2010, 2011). Proteomics and microarrays analysis showed repression of genes involved in cell wall synthesis, cell virulence, communication, adhesion and biofilm formation (Henriques, 2010; Jenkins et al., 2013).

P. aeruginosa was proved to be more tolerant to Manuka honey compared to Gram-positive *S. aureus.* At inhibitory concentrations, Manuka honey caused cell lysis and cell death through the reduced expression of OprF protein, which serves as an osmoregulator between the outer membrane and the peptidoglycan layer that modulates the cell shape (Henriques, 2011; Roberts, 2012). The inhibition of OprF expression leads to membrane blebbing and cell lysis (Roberts, 2012). The same author reported that Manuka honey led to the production of cells lacking flagella hence, the lack of cellular mobility impaired the adhesion and virulence of the pathogen (Roberts, 2015).

Although Manuka honey was shown to demonstrate dissimilar antibacterial activity between Gram-positive and -negative bacteria, inhibition of cell wall

synthesis and membrane destruction was proved to be a mutual mechanism within the two bacterial types.

1.5.2. Non-Manuka honeys

As was initially in 1963 identified, the antimicrobial activity of honeys (poly-floral and mono-floral) is largely attributed to the H_2O_2 produced by glucose oxidase on honey dilution (White et al., 1963). These honeys are known as "H_2O_2-producing" or "catalase sensitive" as their activity is mainly dependent on the final equilibrium within the generation of H_2O_2 by GOX and the degradation of it by the catalase enzyme (Kwakman et al., 2010). Many studies have investigated the factors affecting the accumulation of H_2O_2, the role of this on the antibacterial activity of honey, and the underlying mechanism posed by this component. These findings are discussed in the following paragraphs.

1.5.2.1. H_2O_2 accumulation in honey

Accumulation of H_2O_2 significantly varies within honey species and is dependent on various factors and a cascade of reactions happen after the dilution of honey and the activation of GOX enzyme. Table 1.3 provides a summary of the latest studies which reported the level of H_2O_2 accumulated in various honey types after they were diluted. These concentrations correspond to the highest level of H_2O_2 in each honey tested.

Dilution of honey between 25-50% was proved to cause the highest production of H_2O_2 which varies between 0.04 to 3.5 mM. However, the final H_2O_2 accumulation varies a lot within different honey types and it is basically affected by the level of GOX which in turn depends on nutrition and genetic factors of honey bee (Bucekova et al., 2014).

Table 1.3: Range of H_2O_2 accumulation on honey dilution. These concentrations correspond to the highest value measured in each honey after the dilution.

Honey type	Dilution	H_2O_2 (mM)	Reference
Honeydew honey	50%	0.4	Majtan et al., 2014
Dark (colour)	12.5%,	0.045	Brudzynski et al., 2018
Medium (colour)	12%, 50%	0.055	‘’
Light (colour)	50%	0.033	‘’
Not specified (NS)*	40%	0.025-0.250	Bucekova et al., 2014
Buckwheat	25%	0.2-2.7	Brudzynski et al., 2011
Clover	25%	0.67-2.37	‘’
Blueberry	25%	0.52	‘’
Buckwheat	25%	0.2-2.7	Brudzynski et al., 2012
Blueberry	25%	1.75	‘’
Rewa rewa	30%	1.5	Bang et al., 2003
Pasture	30%	2.8	‘’
Clover	30%	2.5	‘’
Heather	30%	1.2	‘’
Multiflora (NS)	30%	2.5	‘’
NS	25%	4	Grecka et al., 2018
Acacia	25%	0.1	Newby et al., 2018
NS	50%	0.5-3.5	Bucekova et al., 2018
Modified NS	50%	1.2-2.5	Cooke et al., 2015

*NS: honeys whose floral or reginal origin were not specified in the study.

1.5.2.2. Factors affecting the accumulation of H_2O_2 in honey

H_2O_2 is mainly produced by the enzymatic (GOX) conversion of glucose in diluted honey (Figure 1.2). After the dilution, pH rises to 6-6.5 and GOX is activated (White et al., 1963; Schepartz et al., 1964). Maximum H_2O_2 accumulation was reported when honey is diluted within 25-50%. A recent study revealed that dilution induced changes to the conformation of honey macromolecules (i.e. proteins, enzymes, polyphenols, polymerized Maillard reaction products) (Brudzynski et al, 2017). At a certain concentration, which is unique in each honey, these large micron-sized particles are fragmented to nanoparticles. This transition is essential for the antibacterial activity of honey and the production of H_2O_2. However, as was mentioned previously, the peroxide activity of each honey is initiated by the oxidation of GOX enzyme and is eliminated by catalase activity. Thus, the abundance of these enzymes determine the efficiency of the reaction.

The concentration of GOX varies between honeys and depends on the bees state (i.e. health and age) (Brudzynski et al., 2011) and storage conditions (i.e. temperature, light). New honey-bee breeds express higher amount of GOX in their hypopharyngeal glands comparing to older bees. Prolonged exposure of honey to high temperature (>55 °C) and light decreases the level of GOX (Bucekova et al., 2014).

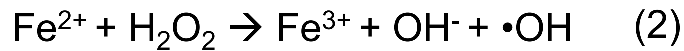
Catalase concentration depends on the amount of pollen grains in honey (Weston, 2000). Hence, this parameter considerably varies even within the same honey species and accordingly affects the accumulation of H_2O_2 (Brudzynski, 2006; Chen et al., 2012).

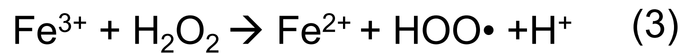
1.5.2.3. Honey compounds which enhance the toxicity of H_2O_2

Polyphenols and transition metals; iron (Fe), copper (Cu) are considered to enhance the oxidative effect of H_2O_2-producing honeys (Imlay et al., 1988; Storz and Imlay, 1999; Cabiscol et al., 2000). Exposure of cells to honey accumulating millimolar of H_2O_2 (>0.1 mM), much lower than its minimum inhibitory concentration (MIC), induces DNA lesions which may mutagenize or kill the bacteria. This was speculated to be by the conversion of H_2O_2 to powerful hydroxyl radicals (•OH) which cause oxidative modifications to DNA, protein and phospholipids (Rojkind et al., 2002). The “free iron” (Fe^{2+}) and (Cu^+) accelerate the production of hydroxyl radicals by transferring electrons to H_2O_2 (Fenton reaction) (Figure 1.3). The resulting hydroxyl radicals can directly damage most biomolecules and especially DNA (this is where the iron is mostly bound) (Imlay, 2003). Moreover, polyphenols were reported to enhance oxidative stress by their autooxidation and the production of reactive oxygen species (ROS), such as H_2O_2, and O_2^-. Autooxidation of polyphenols, catalysed by metal ions (i.e. Cu^{2+}), initially yields in semiquinone and Cu^+. Semiquinone radicals are rapidly oxidised to the corresponding quinone and O_2^- (Akagawa et al., 2003). In absence of metal ions (i.e. Cu^{2+}) the autooxidation of polyphenols yields in H_2O_2 and OH^- (Figure 1.4) however, the rate of the reaction is slower due to the low redox potential of O_2/O_2^-.

Therefore, the abundance of polyphenols and transition metals in honey promotes the formation of ROS by autooxidation and by Fenton reaction respectively. The co-existence of polyphenols and metal ions promotes the

prolonged production of ROS, which causes oxidative bacterial damage (Akagawa et al., 2003).





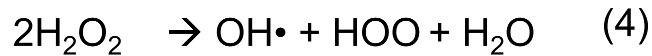


Figure 1.3: Mechanism of free radical production by Fenton reaction. The oxidative stress is caused by Fenton reaction which is part of the Haber-Weiss reaction. The first step is the reduction of ferric ion (Fe^{3+}) into ferrous ion (Fe^{2+}). Second step is the Fenton reaction where Fe^{2+} is oxidized by H_2O_2 to Fe^{3+} forming a hydroxyl radical ($\bullet OH$) and a hydroxide ion (OH^-). The Fe^{3+} is then reduced back to Fe^{2+} by another molecule of H_2O_2 forming a hydroperoxyl radical and a proton. The net reaction is the disproportionation of H_2O_2 to create two oxygen-radical species and water as a by-product.

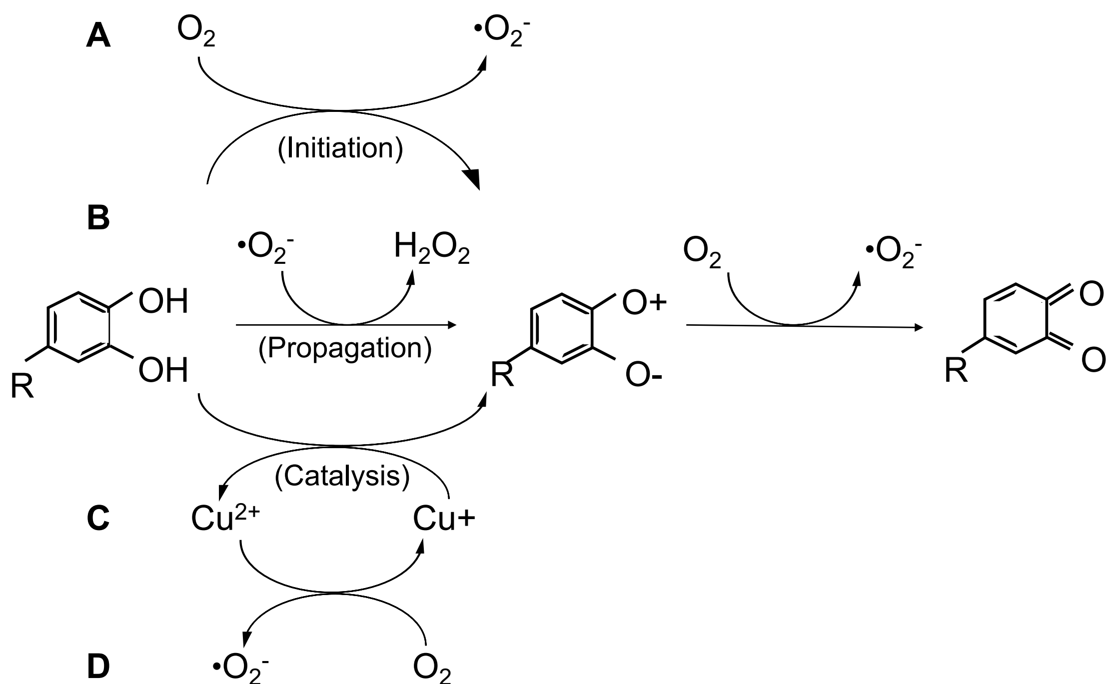


Figure 1.4: Proposed mechanism of autooxidation of polyphenols (catechins). The initial step is the one-electron oxidation of the B ring of catechins by O_2 which generates a superoxide anion ($\bullet O_2^-$) and a semiquinone radical. Superoxide anion is reduced to H_2O_2 which is one of the active oxygen species. At an increase rate of autooxidation, some harmful effects will be caused by the generation of H_2O_2 unless the SOD activity scavenge the $\bullet O_2^-$ and control the oxidation rate of polyphenols. Cupric ion (Cu^{2+}) enhances the overall oxidation rate by one-electron oxidation of catechins. The product Cu^+ can further trigger the Fenton reaction yielding in harmful hydroxyl radicals (Mochizuki et al., 2001).

1.5.2.4. Antimicrobial mechanism induced by H_2O_2-produced honey

The identification of H_2O_2 as an innate compound of honey and the H_2O_2-associated bacterial inhibition brought the assumption that this is the main factor underlying the antimicrobial activity of honey. The study of H_2O_2-induced stress in honey revealed a series of events, which suggest the fundamentals of the oxidative stress mechanism.

Honey was seen to be effective against both Gram-negative and positive bacteria. Regardless the difference in the cell wall arrangement on Gram-negatives (*E. coli*) and Gram-positives (*S. aureus*), H_2O_2 easily penetrated through the outer membrane porins (OMPs) inducing a dose-dependent growth inhibition (Ma and Eaton, 1993; Taormina et al., 2001). Honey-produced H_2O_2 was seen to target bacterial cell wall and lipopolysaccharide (LPS) outer membrane, which resulted in a significant reduction of viable counts. Bacterial cells exposed to honey were seen to form filaments and spheroplasts which eventually lysed. Cell lysis was induced by both cell wall and LPS disintegration as was confirmed by the release of endotoxins post-treatment with honey (Brudzynski and Sjaarda, 2014). H_2O_2-producing honeys induced chromosomal DNA degradation in a dose-dependent way. This effect was abolished when catalase was added to honey and degraded the H_2O_2 confirming the role of this component to honey-derived oxidative stress (Brudzynski et al., 2011).

The effects caused by exogenously added H_2O_2 resembled in most cases these of honey. This confirmed the H_2O_2-directed oxidative stress (inhibition of cell division, cell wall synthesis and phenotypic alterations such as changes from coccoidal to short rod-shaped filaments) (Khan MMT et al., 2010; Oliver, 2010;

Balaban et al., 2004). However, minimum bactericidal concentration (MBC) of exogenously added H_2O_2 was higher than this of the endogenous H_2O_2 suggesting that the oxidizing effect of honey is augmented by other components found in this substrate (Brudzynski et al., 2012).

1.6. A review of the millennium studies on the antimicrobial activity of honey

The upsurge in multidrug-resistant strains urged the need for alternative antimicrobial strategies. The systematic research on the antimicrobial activity of honey started in the late ‘90s. Since then, there has been ongoing research which aims to investigate the antimicrobial mechanism of honey. Gathering a representative volume of publications on antimicrobial activity of honey, a thorough overlook is given on most well-studied kinds of honey, microbial isolates and the methods have been used for this purpose since 2000.

1.6.1. Honey types studied for their antimicrobial activity

Many studies tested (*in vivo* and *in vitro*) the antimicrobial activity of honeys from various floral sources against a range of bacterial species. A review on the publications of this millennium (Figure 1.5, Table 1.6) indicates that the types of honey tested do not fall into a specific floral category, however, certain types were regularly studied. Manuka honey is the most well studied between the species (appeared in 33% of publications) (Figure 1.5). A wide range of indigenous honeys (India, Pakistan, Spain, Greece, etc.) whose floral origin and composition is not specified (NS) represent the second biggest category (26 %) of the most frequently

studied species. Buckwheat (15%), medical honeys (11%) and clover honey (9%) follow (Figure 1.5). “Artificial” or “model” honey, appearing at 11% of the publications, is a rarely used approach and is described in § 1.6.1.1.

Manuka, the monofloral honey (*Leptospermum scoparium*), greatly attracted the attention of researchers for its antimicrobial properties which were discussed previously (§ 1.5.1).

The buckwheat (*Fagopyrum esculentum*), known as one of the most powerful antioxidant honeys, is very rich in phenolics and VOCs (Pasini et al., 2013). Buckwheat honey exhibited powerful bactericidal effect against standard bacteria (*E. coli*, *S. aureus*, *P. aeruginosa*) as well as against MRSA and vancomycin-resistant enterococci (VRE) (Lee et al., 2008; Huttunen et al., 2012; Brudzynski and Sjaarda, 2014). The high content in polyphenols enhanced the efficiency of buckwheat honey, which maintained its antibacterial activity even at higher dilution (12%) in contrast to other honeys which lost their activity at this point (Brudzynski et al., 2012).

The light-coloured clover (*Trifolium repens*) honey, the fourth most well-studied honey, was found to have very low peroxide activity (10%) producing a maximum of 0.029 mM H_2O_2 (Jing Lu et al., 2013). The high concentration of phenolics and other (unspecified) antimicrobial phytochemicals was thought to induce antimicrobial activity equal to this of Manuka honey (Flythet and Kagan, 2010; Halawani and Shohayeb, 2011). Although clover honey completely inhibited the growth of *B. subtilis* (Gram-positive) other Gram-negative species (*E. coli* and *P. aeruginosa*) and *S. aureus* (Gram-positive) were unaffected. This suggests the

low antimicrobial strength of clover honey and a potential species-associated sensitivity (Jing Lu et al., 2013).

Studies on Medical honeys make up for 7% of the publications. Certified honey-based medical products are manufactured either of Manuka or non-Manuka engineered honey produced under standardized conditions in greenhouses (the manufacturers do not disclose any further details) (Kwakman and Zaat, 2012). Although the exact underlying mechanism of medical honey products still remains unknown, their antibacterial activity has been extensively tested mostly against clinical isolates (i.e. Gram-positive; *S. aureus*, *S. epidermidis*, *E. faecium* and Gram-negative; *E. coli*, *P. aeruginosa*, *E. cloacae, S. typhimurium*), antibiotic-resistant strains (i.e. MRSA, Methicillin-Resistant *S. epidermidis* (MRSE) and vancomycin-resistant *E. faecium* (VREF)) and Extended Spectrum Beta-Lactamase bacteria (i.e. ESBL *K. pneumoniae*) (Kwakman et al., 2008; Boorn et al., 2009; Bradsaw, 2011; Jenkins et al., 2011; Muller et al., 2013; Liu et al., 2018). Both types of honey were proved to inhibit the growth of planktonic cells and eradicate the biofilms, which colonize chronic wounds. Active compounds of honey diffuse through the biofilm matrix and disturb the cell cycle, bacterial communication and adhesion (Kwakman et al., 2008; Liu et al., 2018). Moreover, the synergy of certain antibiotics with medical honey was proved to be more effective than the antibiotic itself. The synergistic effect was augmented in case that both; honey and the antibiotic target the same pathway in the bacterial cell (i.e. Manuka and rifampicin which both target the transcription of RNA polymerase). However, honey was seen to have multiple modes of activity, which minimize the risk of bacterial resistance development comparing to other antibiotics (Kwakman et al., 2008).

Finally, other species such as acacia, blueberry, thyme, eucalyptus and heather honey are among the most well-studied species, along with some poly-floral honeys whose exact composition is unknown. Sunflower, Chestnut, Citrus, Levander, Orange, Rape were mentioned in other studies in a lesser extent than the previous (Figure 1.5).

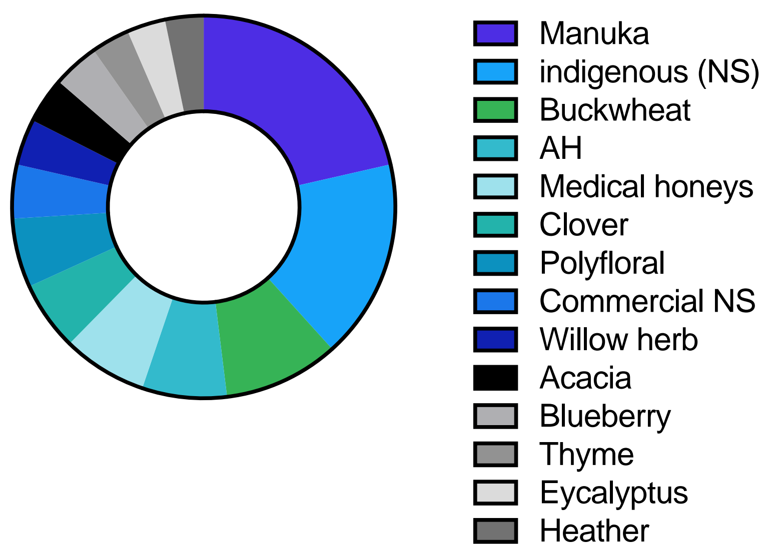


Figure 1.5: **Honey types tested for their antibacterial activity as summarised from Table 1.6.** Manuka honey appeared in 33% of the studies reviewed, with indigenous varieties (26%), Buckwheat (15%) and Model honeys (11%) to follow. NS (honeys with not specified floral source, or composition).

1.6.1.1. Artificial honey: an approach to examine the role of osmotic stress in honey

The use of a “model” or “artificial” honey (AH) is a method obtained from a few researchers as an approach to examine the role of osmotic stress in antimicrobial activity of honey (Sherlock et al., 2010; Brudzynski et al., 2011; Müller et al., 2013; Carnwath et al., 2014; Oinaala et al., 2015; Hammond et al., 2016; Kwakman et al., 2012, 2017). AH is composed of the four main sugars (sucrose, maltose, fructose and glucose) as they were identified in the majority of honeys. This model or AH was first mentioned by Bogdanov (1997) and it was later adopted by other researchers. Fructose is the main sugar and is added in a concentration of 40.5%, glucose at 33.5%, sucrose at 1.5% and maltose at 7.5%, constituting an 83% AH.

Comparisons of the AH against real honeys showed that any real honey was 5-10 times more effective against all bacterial species tested. Pasture (H_2O_2-producing) and Manuka honey were proved to be inhibitory at dilutions down to 3.6 and 3.4% respectively in contrast to AF which failed to inhibit bacterial growth even at 50% of sugars concentration (French et al., 2005; Carnwath et al., 2014). In some cases, bacterial growth was enhanced in the presence of sugars (Müller et al., 2013). Moreover, the neutralisation of three major antimicrobial compounds in honey (MGO, H_2O_2 and bee defensin-1) along with the titration of honey to a more basic pH (7) resulted in antimicrobial activity identical to that of the AH (Kwakman et al.,2012, 2017). These results suggest that osmolality does not fully explain the antimicrobial activity of honey and confirm the importance of the rest of the honey components to its antimicrobial activity.

1.6.2. Most well-studied bacterial species for their susceptibility to honey

Over 60 bacterial species have been reported to be susceptible to honey (Molan, 1992a). *E. coli*, *S. aureus* and *P. aeruginosa*, were seen to be the three species most regularly studied (24%, 20% and 8.4% of reports, respectively) for their susceptibility to honey (Figure 1.6). A recent report (2017) from the World Health Organisation (WHO) categorized those three species as critical for their resistance to antibiotics (Table 1.4) (Tacconelli et al., 2017). Apart from the antibiotic resistance, *Staphylococci*, *E. coli* and *P. aeruginosa* are the most common pathogens isolated from surgical site wounds. These are opportunistic human pathogens which cause significant diseases (i.e. skin infection, pneumonia, urinary tract infections, nausea, diarrhea etc.) (Mangram et al., 1999, Frank et al., 2011). *Pseudomonas* was found to be one of the four predominant species in wound sites, among the 20 phylotypes identified (Frank et al., 2011). In wound biofilms, *P. aeruginosa* acts synergistically with *S. aureus* which grows in the wound surface while the former is distributed in deeper recesses exploiting its facultative anaerobic nature (Fazli et al., 2009; Madsen et al., 2009). Therefore, there is an obvious interest on use of honey as an alternative antibacterial for the eradication of antibiotic-resistant isolates colonizing chronic wounds.

Both *E. coli* and *P. aeruginosa* are Gram-negative bacteria and emerged as a healthcare crisis globally, because of their resistance to carbapenems (CR), the last line of defence against various drug-resistant bacterial infections (Khajuria et al., 2014; Tacconelli et al., 2017). *P. aeruginosa* is a common cause of biofilm-mediated nosocomial infections (bloodstream infections and pneumonia). As a common habitat of moist environments, it is usually found in sink traps and aerators,

health-care devices (i.e. respiratory therapy equipment), aqueous medical solutions (i.e. eye-drops, irrigation fluids, etc.), as well as fruits and vegetables (Paterson, 2006). *P. aeruginosa* counters a range of antibiotics (i.e. carbapenems, aminoglycosides, quinolones and β-lactams) (Hancock and Speert, 2000).

E. coli and *P. aeruginosa* counter the antibiotics by mechanisms, which are located in bacterial chromosome and occur naturally within all cells (intrinsic mechanism) or come from other bacteria usually via plasmid (acquired mechanism) (Reygaert, 2017). The low membrane permeability, expression of efflux pumps and production of enzymes which inactivate antibiotics suggest the intrinsic mechanism of resistance. Further, horizontal transfer of resistant genes or mutational changes and the formation of biofilms which limit the antibiotic diffusion suggest the acquired and adaptive resistance respectively (Drenkard, 2003; Breidenstein et al., 2011).

Except for the non-pathogenic *E. coli* isolates (K-12, ATCC 25922) (Shamala et al., 2002) pathogenic *E. coli* isolates were also tested for their susceptibility to honey. Enterohemorrhagic O157:H7 (Badawy et al., 2009; Lee et al., 2011), β-lactamase producing ATCC 35218 (Sherlock et al., 2010), engineered β-lactamase producing *E. coli* ML-35p (Kwakman et al, 2007) and the sperm-immobilizing MTCC 1687 (Mohapatra et al., 2011) were tested for their susceptibility to H_2O_2-producing and no-producing (Manuka) honeys. Manuka honey prevented biofilm formation by inhibiting quorum sensing and acted as an anti-virulence factor (Hyung et al., 2011). Most of the H_2O_2-producing honeys (Coorg, Ulmo 90, RS Remavil and Indian indigenous) induced a bactericidal effect on pathogenic isolates, however, the mechanism is not known. Enterohemorrhagic *E. coli* O157:H7 was seen to be more

resistant than other strains. Clover honey induced 86.6% morbidity without inducing a bactericidal effect on O157:H7 bacterial population (Sherlock et al., 2010).

In the case of *P. aeruginosa* Manuka honey was found to exert a bactericidal effect on planktonic cells by compromising the cell wall to the point of cell lysis (Robberts et al., 2012). Chestnut honey (H_2O_2-producing honey) combined with a bacteriophage (phage) in a formulation was seen to penetrate the biofilm matrix and damage the bacterial membrane. Thus, the damage of bacterial cell membrane by honey promoted and enhanced the subsequent phage infection (Oliveira et al., 2018).

S. aureus is the main cause of health-care-associated bacteraemia, wound infections and pneumonia (Giacometti et al., 2000; Hoban et al., 2000) and was categorised as a critical threat of level 2 by WHO (Table 1.4). The resistance of *S. aureus* to methicillin (MRSA) was first mentioned in 1961. Since then it is considered as a global epidemic because of the ability to colonise individuals for long time periods due to cross-infection with other species (Wenzel et al.,1991; Grundmann et al., 2006). Enzymatic inactivation, trapping of the antibiotic, alteration of targets for the decreased affinity of the antibiotic, and expression of efflux pumps suggest the resistance mechanism of *S. aureus* to a range of antibiotics (i.e. methicillin, vancomycin, daptomycin, tetracycline and fluoroquinolones) (Pantosti et al., 2007). In particular, MRSA strains acquire a non-native gene (*mecA*), which encodes a penicillin-binding protein (PBP2a) promoting the cell-wall biosynthesis even in the presence of inhibitory antibiotic concentrations (Peacock & Paterson, 2015).

Manuka and monofloral honey types (i.e. sidr, *Nigella sativa*) were moderately effective against both *S. aureus* and MRSA isolates (Almasaudi et al.,

2017). Since Manuka honey was seen to disrupt the regular cell division by inhibiting the activity of murein hydrolase and the cells septation, the antibacterial mechanism is more bacteriostatic rather than bactericidal (Bang et al., 2003). However, the enhancement of engineered medical honey with antimicrobial peptides induced rapid bactericidal activity on *S. aureus* and MRSA proving the limited effect of honey itself on this bacterial species (Kwakman et al 2011).

The rest of the literature is divided among other species associated with nosocomial infections (*K. pneumoniae, E. faecalis*), human diseases (*S. enterica* serovar Typhi; typhoid fever, *S. pneumoniae*, *C. albicans*; candidiasis) and foodborne illness (*B. cereus*, *B. subtilis*).

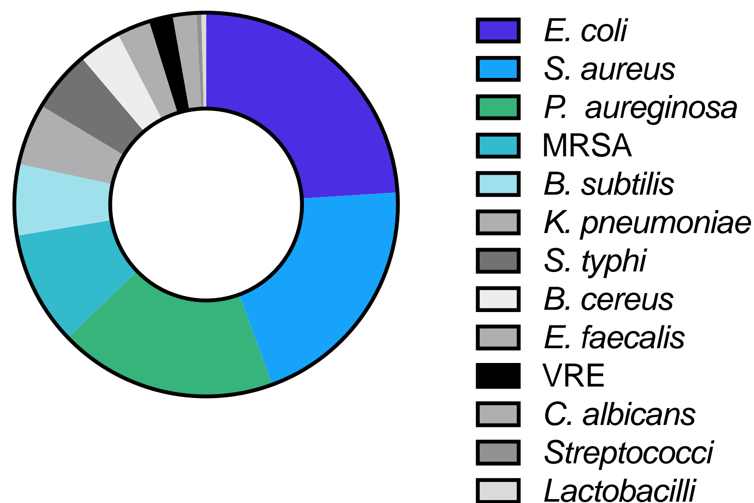


Figure 1.6: Bacterial species tested for their susceptibility to honey as summarised from Table 1.6. *E. coli* (24%) and *S. aureus* (20%) are on the top of the list with *P. aeruginosa* (18.4%) and MRSA (9.6%) to follow.

Table 1.4: Priority pathogens list for their resistance to antibiotics according to WHO.

Priority 1: CRITICAL	Resistant to	Priority 2: HIGH	Resistant to	Priority 3: MEDIUM	Resistant to
A. baumannii	carbapenem	*E. faecium*	vancomycin	*S. pneumoniae*	penicillin (non-susceptible)
P. aeruginosa	carbapenem	*S. aureus*	methicillin, vancomycin	*H. influenzae*	ampicillin
*Enterobacteriaceae**	carbapenem	*H. pylori*	clarithromycin	*Shigella* spp.	fluoroquinolone
Enterobacteriaceae	3rd generation cephalosporin	*Campylobacter*	fluoroquinolone		
		Salmonellae	fluoroquinolone		
		N. gonorrhoeae	cephalosporin, fluoroquinolone		

*Enterobacteriaceae include: *Klebsiella pneumonia*, *Escherichia coli*, *Enterobacter* spp., *Serratia* spp., *Proteus* spp., and *Providencia* spp, *Morganella* spp

1.6.3. Methods used for the assessment of the antimicrobial activity in honey

Although the research on honey has been ongoing for decades it appears that the majority of studies have mostly used the classic analytical methods. In particular, the minimum inhibitory concentration (MIC) and/or minimum bactericidal concentration (MBC) in a wide range of honeys were determined by disk diffusion, CFU counts, and turbidity test. However, these methods cannot explain mechanistically the antimicrobial activity of honey. Thus, further understanding of the cell viability, structural changes and cellular responses to honey were determined by fluorometric methods (indole assay, crystal violet assay) single-cell analysis (flow cytometry), microscopy, and genomic analysis (DNA microarrays, gene sequencing). The review on the methods have been used since 2000 shows the "knowledge gap" on the underlying mechanism of honey.

Agar disk-diffusion and total viable count (TVC) were the most common methods appearing at 49% and 22% of the publications respectively (Figure 1.7). Agar disc-diffusion, developed in 1940, has been used for routine antimicrobial susceptibility testing (Heatley, 1944). The radius of the inhibition zone qualitatively categorizes bacteria as susceptible, intermediate susceptible or resistant (Reller et al., 2009). However, this method cannot distinguish bactericidal and bacteriostatic effects or determine the MIC. In contrast, the TVC, the second most common method used, examines the bactericidal effect by determining the number of living cells as CFU/mL (Konante et al., 2012). Growth inhibition was also studied using the turbidity test to a lesser extent (8%). Turbidity measurement, the oldest susceptibility testing method, involves two-fold dilutions of the respective antibiotic agent (i.e. honey) in a liquid growth medium, inoculated with 1-5x10^5 CFU/mL. After

overnight incubation, the test tubes are tested for growth (turbidity) (Clais et al., 2015).

Imaging using Scanning electron (SEM) and Transmission electron (TEM) microscopy were used in a few studies which aimed to investigate further the antimicrobial mechanism of honey. The structural changes of honey-treated cells gave an insight into honey's activity. SEM images revealed a decreased production of curli in *E. coli* O157: H7 honey-treated biofilm (Lee J.H. et al., 2011) and also identified structural changes in the cell wall of *E. coli* and *B. subtilis* post-exposure to glycoproteins isolated from honey (Brudzynski and Sjaarda, 2014). MRSA cells were seen with septations in increasing doses of honey (Jenkins et al., 2011). Also, a significant loss of *S. aureus* cell structure and volume was seen by SEM, post-treatment with Manuka honey (Campeau and Patel., 2014).

Crystal violet assay (5%) was used in order to measure the biofilm-forming activity of cells exposed to increasing honey dose. Crystal violet dye binds to proteins and DNA of living cells while dead cells lose their capacity to take up this dye (Feoktistova et al., 2016). Biofilm forming activity revealed a resistant mechanism of recovered isolates from Manuka honey comparing to the progenitor strain which was more susceptible to honey (Camplin and Maddocks, 2014). The same method showed whether the biofilm-forming ability of antibiotic-resistant strains (MRSA, MRSE, *Klebsiella* and *Pseudomonas*) was affected by Manuka honey (Merckoll et al., 2009).

Other methods such as single-cell analysis (flow cytometry), confocal microscopy, manipulation and analysis of DNA, and gene sequencing were also used in lesser extent (Figure 1.7).

The results between *in vivo* or *in vitro* testing have established the broad-range antibacterial activity of honey that varies a lot within species. For further investigating the medical potential of honey, the research is necessary to determine whether the results of the *in vitro* experiments are also applied to a clinical setting.

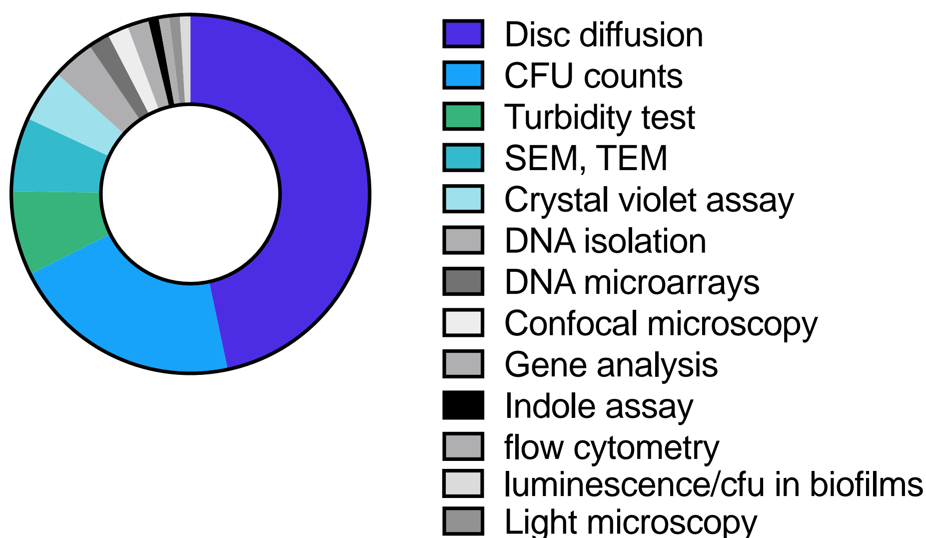


Figure 1.7: Methods used for the examination of the antimicrobial activity in honey as summarised from Table 1.6. Disc diffusion assay (46.6%) is the predominant method, while CFU enumeration (20%) and turbidity test (7.6%) follow.

1.7. Use of licensed honey-based medical products in wound healing

The findings on antimicrobial efficiency of honey against multi-drug resistant bacteria and particularly the isolates of wound biofilms suggested honey as a game-changing solution on wound healing management. The Therapeutic Goods Association in Australia licensed the first sterile honey (Manuka) wound care product on 1999. In 2004, the first honey-impregnated dressing was licensed in the United Kingdom (Cooper and Jenkins, 2009). Since then, an increasing number of honey medical devices were licenced and are available to customers. After 2007, when the Food and Drug Administration (FDA) approved a range of honey-based medical products, companies developed a quickly evolving line of medical honey formulations in form of gels and creams and honey impregnated gauzes and plasters. These products are manufactured with honey only or by a mixture of honey with other antimicrobial compounds (Table 1.5).

Table 1.5. Use of honey in medical devices. List of licenced commercially available medical honeys manufactured from either Manuka or standardised no-Manuka honey. The composition of each medical device is given as disclosed by the manufacturers.

Product name	Composition	Antimicrobial compound/ activity/ target	References
Derma Science- Medihoney			
Antibacterial Wound gel	Gel: 100% medical grade Manuka honey and 20% natural plant waxes	MGO (>355mg/kg)/ Applied to: acute, chronic, infected and malodorous wounds, post-surgical open wounds, diabetic foot ulcers/ Eliminates malodour, autolytic debridement, removal of necrotic tissue, pH adjustment	Simon et al., 2006, George et al., 2007; Blair 2009; Lusby et al., 2005; Wilkinson et al., 2005; Irish et al., 2006; Dunford et al., 2004
Barrier cream **And Derma cream**	Cream 30% medical grade Manuka	MGO/ Applied to: injured skin, inflamed or excoriated areas/ Antimicrobial activity (candida and antifungal) protects from skin breakdown caused by wound exudate.	
Honey Tule 3-ply dressing	Manuka honey	MGO/ Applied to: post-surgical open wounds, diabetic foot ulcers/ The osmotic action removes the bacteria, endotoxins, debris and slough. Enhances the granulation and epithelialisation/ effective against antibiotic resistant strains	
Honey gel sheet	100% medical grade Manuka honey combined with sodium alginate	MGO/ Provides autolytic and enzymatic debridement, removes the necrotic tissue and breaks down the fibrin in the wound site.	
Honey apinate dressing	100% medical grade Manuka honey in calcium alginate fabric	Same as **Honey Tule 3-ply dressing** described above.	
Surgihoney RO			

Product name	Composition	Antimicrobial compound/ activity/ target	References
Surgihoney RO	Natural (no-Manuka) honey with enhanced production of ROS (including H_2O_2)	ROS formation and control delivery provides a consistent level of antimicrobial activity/ effective against wound infected bacteria (incl. MRSA and *Pseudomonas*) and biofilms. ROS promotes wound healing by promoting the repair process and tissue regeneration.	Dryden et al., 2014, 2017; Halstead et al., 2016
		Remavil	
Remavil compress honey wound dressing	Polyacetate compresses impregnated with medical honey (no-Manuka)	Gradual ROS release up to 48 h and ROS activity which promotes the autolytic debridement of the wound and stimulates the fibroblast activity/ Applied to acute and chronic wounds, burns 1st and 2nd degree/ effective against *S. aureus* (Methicillin resistant; SRAM and sensitive; MSSA), Pseudomonas (resistant and sensitive) and Vancomycin -resistant enterococci (VRE).	Kwakman et al., 2011
Remavil healing balm	25% of honey with high GOX content	Manuka honey/ Moderate H_2O_2 production/ creates an antibacterial protective layer once it is mixed with the wound site	
Remavil Medical honey tulle, sterile plasters	Sterile compress with tight-meshed impregnated with healing medical honey	Manuka honey/ Moderate H_2O_2 accumulation/Applied to thermal burns (1st & 2nd degree), radio-induced, traumatic or surgical wounds/ Healing protecting from infections.	
		Advancis Medical	

Product name	Composition	Antimicrobial compound/ activity/ target	References
Activon tube medical grade honey	Medical grade Manuka honey	Manuka honey/ suitable for topping up dressings or directly into cavities/ Intended for any wound: diabetic ulcers, surgical wounds, burns, graft sites, infected wounds, cavity wounds and sinuses.	Reynolds, 2017
Algivon and Algivon Plus	Alginate (Plus: reinforced alginate fibbers) dressing with medical grade Manuka honey	Manuka honey and alginate/ antibacterial effect/ Applied to wetter wounds; small absorbing capacity of the alginate makes honey to remain for longer on the wound bed/ Eliminates odours, removes the harmful tissue and maintains the ideal moisture for healing process.	
Activon Tulle	Knitted viscose mesh dressing with medical grade Manuka honey	Manuka honey/ Applied to granulating or shallow wounds for debriding or de-sloughing of necrotic tissue.	
Actilite	Manuka honey and oil lightly coated dressing	Manuka honey and Manuka oil/ Applied to wounds promoting wound healing, granulation and epithelization / Effective against infectious pathogens including MRSA, VRE and *Providentia stuartii*.	
L-Mesitran			

Product name	Composition	Antimicrobial compound/ activity/ target	References
L-Mesitran Ointment	48% medical grade honey, oils (sunflower, lanolin, cod liver), Aloe, vitamin C, E	Manuka honey and oils/ Applied to superficial and acute wounds (cuts, abrasions and donor sites). Superficial and partial thickness burns (1st and 2nd degree). Chronic wounds (venous, arterial and diabetic ulcers). Colonised acute wounds and surgical wounds/ Aid debridement and reduce bacterial colonisation.	Westgate, 2013; Chatzoulis et al. 2012; Kurahashi & Fujii 2015;
L-Mesitran soft	40% medical grade honey, medical grade lanolin, propylene glycol, PEG400, vitamin C, E	Manuka honey/ Applied to superficial and acute wounds (i.e. abrasions and lacerations and donor sites for skin grafts), burns (1st and 2nd degree), chronic wounds, venous, arterial, diabetic ulcers and oncology wounds.	
L-Mesitran Tulle	Polyethylene dressing impregnated with L-Mesitran soft gel	Manuka honey/ Applied to deep and superficial wounds with high to low exudates, venous, arterial and diabetic ulcers, burns or laser wounds, colonised acute wounds and surgical wounds/ antibacterial and antifungal properties.	
L-Mesitran Hydro (honey) & L-Mesitran Border	30% medical grade honey, acrylic polymer gel and water with a polyurethane film	Manuka honey/ Applied to difficult locations where the normal dressings do not adhere (i.e. chronic wounds (pressure ulcers), partial thickness burns, arterial and diabetic ulcers and fungating wounds).	

Product name	Composition	Antimicrobial compound/ activity/ target	References
L-Mesitran Net (honey)	20% medical grade honey, an acrylic polymer gel and water on a polyester mesh structure	Manuka honey/ Applied to chronic, super -facial and partial thickness burns and fungating wounds/ Allows the passage of exudate to be absorbed by a secondary dressing avoiding the maceration of the surrounding tissue providing a moist environment.	
		Comvita	
Antibacterial Wound Gel	80% Medical grade Manuka honey, natural waxes, and oils	Manuka honey/ Applied to broken and infected skin, cuts, grazes, burns and ulcers/ creates a moist, low pH environment that promotes healing and reduces scaring and the risk of infection.	Gethin et al., 2008
		Others	
Manuka aid skin Patch	Sterile medical grade Manuka honey on a breathable tissue	Manuka Honey UMF 400+	Sherlock et al., 2010; Moussa et al., 2012; Alnaimat et al., 2012; Calderon et al., 2015;
Manuka G Wound Gel	Medical grade Manuka gel	Manuka honey	

1.7.1. Commercially available medical devices

The majority of honey-based medical devices are manufactured using Manuka honey which exerts an antimicrobial activity like it was described earlier (§ 1.5.1.1). Derma Science manufactured and marketed a line of wound and skin care products under the name "MEDIHONEY", chiefly consisting of alginate as an absorbent material covered with Manuka honey. L-Mesitran® company, in 2002, produced its first Manuka-wound care dressings. Advancis Medical® Manuka honey plasters and gels, commercialized by the homonymous company in 2011. The same company launched a new line of honey plasters impregnated with extra antimicrobial compounds which were proved to be more efficient in the management of the exudate in wound sites compared to previously honey-formulations. However, on 2016, SurgihoneyRO (SHRO) marketed for the first time a peroxide- producing bioengineered honey product which demonstrates a prolonged release of ROS that controls/eliminates the growth of bacteria and biofilms in mucosal surfaces and cavities (Cooke et al., 2015). The enhanced and sustained release of ROS (including H_2O_2) was proved to be effective against bacteria (Gram-negative and positive, MRSA) and biofilms (Dryden et al., 2014). Following this product, many other delivery systems which share the same technology have been developed (gel, sprays, nebulizers, and infusions), suitable for administration to other body sites other than superficially.

1.7.2. Healing and antimicrobial mechanism posed by honey-based medical dressings

The anti-inflammatory mechanism of honey is classified into direct and indirect. The antibacterial activity posed by honey components suggests the direct mechanism. Indirect antimicrobial mechanism refers to the immunomodulation posed by honey innate components (Al-Wailli et al., 2011).

1.7.2.1. Direct mechanism: Contribution of honey components to wound healing process

Upon the application of medical plaster to the wound site, exudate aids the dilution of honey and the activation of GOX which oxidises glucose to H_2O_2 and gluconic acid (Stewart et al., 2014). High osmolarity, low pH, H_2O_2 and/or MGO are in the first line of the direct antimicrobial mechanism of honey.

The osmotic effect caused by sugars decreases the optimum a_w needed for bacterial growth. Sugars draw water out of the tissue which cleans the wound surface from debris, necrotic and devitalised cells (Alam et al., 2014). Acidic environment caused mainly by the production of the gluconic acid destructs the habituation of many pathogenic species and the biofilm growth which are favoured by the neutral pH environment (pH 7-7.5) of the wound site (Bittman et al., 2010). The acidic pH inhibits the protease activity, which otherwise is favoured by the wound environment (pH 7) and destroy the growth factors and protein fibres which are necessary for the migration of the epithelial cells. Therefore, the acidic pH (3.5-

5) stimulates the bactericidal action of macrophages and triggers the production of growth factors. The increased activity of fibroblasts, re-epithelisation and oxygenation speed up the healing process (Yaghoobi et al., 2013; Sell et al., 2012).

The interaction of the wound exudate with the glucose of honey results in slow release of H_2O_2. This H_2O_2 is sufficient to be bactericidal yet diluted enough to be nontoxic and cause further inflammation and tissue damage (Lusby et al., 2002). The H_2O_2 was found to enhance the proliferation of fibroblasts and the angiogenesis by leading to blood recruitment (Sell et al., 2012). The breakdown of the H_2O_2 releases reactive oxygen (RO) in low concentration for prolonged period of time (up to 2 days post-dilution). ROS exert bactericidal activity by causing damage to protein, lipids and nucleic acids (Halstead et al., 2016). However, honey disables free ions and poses antioxidant scavenging activity on oxygen radicals produced by H_2O_2, thus, the latter does not cause tissue damage (Manyi-Loh et al., 2011).

Antioxidant activity of honey is massively associated to the presence of flavonoids, phenolic acids, ascorbic acid, peptides, amino acids and Maillard reaction products (Vandamme et al., 2013). These compounds prevent from the high accumulation of ROS produced by neutrophils, macrophages and tumor necrosis factor-α (TNF-α) which induce cytotoxicity in chronic wounds (Tonks et al., 2003). The presence of both lipophilic and hydrophobic antioxidants in honey makes the antiradical activity broader, decreasing the oxidative stress and the inflammatory process (Chepulis, 2007; Tonks, 2007).

1.7.2.2. Indirect mechanism: wound healing promoted by honey and human immune system

Wound healing is a process of a cascade of events arranged in three phases: inflammation, proliferation and remodelling (Majtan, 2010; Tan et al., 2012). In each phase, honey stimulates the immune response of the whole organism inducing the indirect healing mechanism.

During inflammatory phase honey was reported to stimulate B- and T-lymphocytes and promote phagocytosis in the wound site. Honey also stimulates the monocytes to produce cytokines, (TNF-α) and interleukin (i.e. IL-1, IL-6) which all activate the immune response to infection (Molan, 2002; Mandal & Mandal, 2011; Kamaratos et al., 2014). All cells (fibroblast, epithelial cells) involved in wound healing are capable of producing nitric oxide (NO) a gaseous free radical that accelerates angiogenesis, collagen synthesis and re-epithelialization (Witte & Barbul, 2002). The anti-inflammatory effect of honey is based on the scavenging of the free radicals produced during the inflammation and the activation of fibroblasts which promote collagen production. Therefore, honey prevents the extensive inflammatory phase which otherwise leads to hyper-granulation and fibrosis (Tasleem et al., 2011).

During angiogenesis, mainly the low pH of honey stimulates the oxygen release from haemoglobins which aid tissue granulation and wound healing (Rossiter et al, 2010). Wound contraction is accelerated by the fibroblasts, myofibroblasts and collagen deposition (Nakajima et al., 2013). Fibroblasts and epithelial cell proliferation are enhanced by the hydrogen peroxide which also promotes the formation of new capillaries (Boekema et al., 2013).

Re-epithelization and remodelling are the last stages of wound healing. During the re-epithelization, the sugars of honey provide the energy for the epithelial cells to migrate across the wound surface. Trace elements found in honey (i.e. Fe, Cu, Co, etc.) aid the re-epithelization by promoting the proliferation of keratinocytes (Majtan et al., 2010). During the remodelling phase the collagen is realigned across tension lines while the rest of the cells are removed by apoptosis (Subrahmanyam et al., 2001).

As was seen from the previous paragraphs the antimicrobial activity of honey has been tested in a range of (non) pathogenic isolates and many multidrug-resistant bacteria (including these isolated from wound sites). Also, the majority of honey-medical products are oriented to wound healing. Therefore, it became apparent that medicinal use of honey is limited to superficial treatment of open wounds while limited is the knowledge on its effect on polymicrobial infections. This observation shows that honey could have further medicinal properties to be investigated.

1.8. Polymicrobial infections

In their natural habitat, microorganisms exist in close association with other species developing interactions which promote their survival and proliferation. Together these microorganisms form a multispecies biofilm, a complex three-dimensional structure comprising of cell aggregates enhanced by self-produced extracellular matrix (Flemming et al., 2016). The development of biofilm prevents

the pathogenic inhabitants from the antibiotic therapy and the defence of immune system, therefore, polymicrobial infections are still a clinical challenge (Price et al., 2009).

1.8.1. Vulvovaginal Candidiasis (VVC) an example of polymicrobial infection

The most common sites for mixed-species infections are the respiratory tract, the gastrointestinal system, the skin and the urinary tract. Clinically, these sites are common hosts of Gram-negative bacteria and fungi. Among other fungal species (i.e. *Malassezia*, *Cladosporium*, *Saccharomycetales*, *Aspergillus*, *Fusarium*, *Penicillium*, etc.) *Candida albicans* is the most prevalent (Findley et al., 2013).

The greatest manifestation of genitourinary candidiasis in women is the vulvovaginal candidiasis (VVC). After bacterial vaginosis, the VVC is the second most commonly diagnosed in up to 40% of woman with vaginal discomfort (Mendling et al., 2015). *C. albicans* is the prevalent pathogen (90% of the cases) responsible for VVC (Anderson et al., 2004).

Although *Candida* asymptomatically colonises the vaginal environment, under certain conditions such as immunosuppression, damaged mucosa and disturbance of local microbiologic flora, causes inflammation. *Candida* has exceptionally high adaptability in response to host environmental changes via adhesion, hyphal formation, production of extracellular hydrolytic enzymes and biofilm formation (Schaller and Weindl, 2009). However, *Candida* favours the biofilm formation in polymicrobial environments. The bacterial-fungal interaction is

supposed to enhance the virulence of *Candida* which acquires more invasive forms of growth (i.e. hyphae) (Swidsinski et al., 2019).

1.8.2. Bacterial-fungal interactions in VVC

E. coli and *C. albicans* exhibit a co-operative interaction wherein *E. coli* aids the adhesion of the fungi to the mucosal surface enhancing the likelihood of vulvovaginal infections (Levison and Pitsakis, 1987). The interaction between the two species was seen to increase the lethality in infected mice in contrast to mono-infection with either microorganism (Klaerner et al., 1997).

Likewise, *E. coli* and *C. albicans* act synergistically in case of VVC wherein *E. coli* promotes the establishment of *C. albicans* since the latter is not able to attach to bladder mucosal lining. Two of the proposed mechanisms are 1) the attachment of *C. albicans* to the mannose-*E. coli* binding site and 2) the biofilm forming activity of *Candida* in the presence of bacterial polysaccharide (LPS) secreted by *E. coli* (Bandara et al., 2009). Even the exact mechanism is not known, the production of glucocorticoid from LPS is believed to suppress the immune response in the host and aids the proliferation of fungal species (Akagawa et al., 1995). Diverse physical interactions, such as cell to cell contact and bacterial cells aggregation around the fungal hyphae or yeast cells were also seen to promote biofilm formation (Tampakakis et al, 2009).

In contrast, the presence of vaginal microbiota defends the host against the colonisation of the pathogen. Gram-positive *Lactobacilli* are the predominant species of the vaginal environment. In healthy individuals, up to 10^7-10^8 CFU/mL lactobacilli colonise the vaginal mucosa. *Lactobacillus acidophilus* is the prevalent

among other species which were isolated (*L. fermentum*, *L. plantarum*, *L. casei*, *L. salivarius*) (Wylie and Henderson,1969). *Lactobacilli* exert two mechanisms to prevent the colonisation of the pathogens: a) the receptor barrier of the host which prevents from the attachment to epithelial cells; and b) secretion of antimicrobial compounds such as lactic acid, bacteriocin-like substances and H_2O_2 (Boris and Barbés, 2000). *Lactobacillus* was seen to have a better affinity for host receptors which is mediated by the glycoproteins and carbohydrates compared to the pathogens *Gardnerella vaginalis* and *C. albicans* (Boris et al., 1998). Moreover, the production of lactic acid and fatty acids by *Lactobacillus* metabolism acidify the mucosal environment and inhibit the proliferation of pathogens such as *C. albicans* and *E. coli* (Young et al., 1956; Reid et al., 1985). A synergistic effect of an acidic environment, bacteriocin-like compounds and H_2O_2 was seen to inhibit *C. albicans* and a range of Gram-negative and -positive bacteria (Fitzsimmons and Berry, 1994). However, undefined interactions within species of a polymicrobial culture affect the growth of the isolates and their sensitivity to honey (Al-Waili et al., 2011; Oliveira et al., 2018).

1.9. Latest studies and context of this work

As indicated in the introductory sections above, the antimicrobial capacity of honey has been extensively reported against clinical isolates and reference strains. H_2O_2 had been thought to be the predominant antimicrobial component in honey. The latest studies mentioned possible synergies between honey components, however, the high variability within honey species and the complexity of this milieu represent a huge challenge to this investigation. Thus, despite its importance, the understanding of the antimicrobial mechanism in honey is still lacking. This work, aimed to identify the mechanism underlying the activity of honey considering this to be initiated by GOX reaction. In this direction, an adequate model honey was used to investigate the effects of, and possible synergies between, osmotic, acid and oxidative stress occurring in honey after the enzymatic oxidation of the glucose.

The investigation of the synergy posed by the predominant components in honey (i.e. sugars, gluconic acid and H_2O_2) and their mechanism towards *E. coli* K-12 reference strain are described in Chapter 2. Physiological responses and nano-structural changes of honey-treated bacteria were elucidated by a combination of traditional culture methods, single cell analysis and AFM respectively. A greater understanding of the molecular mechanisms that underpin the fitness of *E. coli* to honey was studied in Chapter 3. In particular, a wide-genome transposon mutagenesis method in combination with transposon directed insertion-site sequencing was employed to identify genes which contribute to the ability

of bacteria to survive after treatment with honey. Chapter 4 examines the physiological responses of two pathogenic species, *E. coli* (UPEC) and *C. albicans*, post-exposure to honey. This chapter, aims to relate the previous experimental observations to the clinical context and to identify future applications of honey as an alternative antimicrobial.

Table 1.6: A review of the studies on the antibacterial activity of honey. The studies are sorted in chronological order including the honey type tested, the method of the identification and the target organism.

	Honey type	Method	Target	Reference
1	Acacia, Blossom, Chestnut, Dandelion, Eucalyptus, Levander, Orange, Rape, Sunflower, Honeydew	Turbidity test	*S. aureus*	Bogdanov, 1997
2	Indian commercial (NS*)	CFU counts	*L. acidophilus,* *L. plantarum*	Shamala et al., 2000
3	Manuka, Kanuka, Ling, Kamahi	CFU counts	*E. coli* *S. aureus* *K. pneumoniae* *P. aeruginosa*	Sato & Miyata, 2000
4	Was Bessie, Blue gum, Fynbos	Turbidity test	*C. albicans*	Theunissen et al., 2001
5	Coorg, Standard grade, Indian (NS)	CFU counts	*E. coli* (ATCC 25922)	Shamala, 2002
6	Manuka, Pasture, Artificial Honey (AH*)	Turbidity test	MRSA VRE	Cooper et al., 2002
7	Manuka	Turbidity test	*S. aureus*	Snow & Manley-Harris, 2003
8	Angelita	Disc diffusion	*E. coli* *P. syringae* *A. niger* *P. chrysogenum* *T. viride* *B. subtilis*	Torres et al., 2004
9				
10	natural honey (NS)	Disc diffusion	*E. coli* *S. aureus* *P. aeruginosa* *S. typhi* *S. shiga* *K. aerogenes*	Mulu et al., 2004
11	Malaysian NS	Disc diffusion	*S. aureus* *E. coli* *S. pyogenes* *S. tymphi* *S. sonnei*	Tumini et al. 2005
12	Clover	Disc diffusion	*E. coli* (O157:H7) *S. typhimurium*	Badawy et al., 2005
13	Medihoney (Manuka), Manuka, Rewa-rewa	Agar incorporation	*E. coli* *P. aureginosa* *MRSA* *S. aureus* *Kl. pneumoniae*	George & Cutting, 2005

	Honey type	Method	Target	Reference
14	Levander, Red stringy bark (Eucalyptus macrorrhyncha), Patersons curse (Echium plantagineum), Manuka, Rewa-rewa, Medihoney (Manuka)	Agar incorporation	*E. coli* K-12 *C. albicans* *A. faecalis* *C. freundii* *E. aerogenes* *K. pneumoniae* *M. phlei* *S. enteritidis* *S. tymphimurium* *S. marcesens* *S. sonnei* *S. aureus* *S. epidermidis*	Lusby et al., 2005
15	Levander, Manuka, Medihoney, Rewa- rewa, Rosemary, Red stingy	Disc diffusion	*E. coli* *P. aeruginosa*	Wilkinson & Cavanah, 2005
16	Multifloral (NS)	Disc diffusion	*S. aureus coagulasa*	Tabera et al. 2006
17	Manuka	Disc diffusion Microdilution assay Turbidity test	*E. coli* *B. cereus* *S. aureus* *C. albicans*	Patton et al., 2006
18	NS	Disc diffusion	*E. coli* *P. aeruginosa*	Adeleke et al., 2006
19	Blossom, Clover	Disc diffusion	*H. pylori*	Nzeako & Al-Namaani, 2006
20	White willow, Goat willow, Rape	Disc diffusion	*S. aureus* *S. epidermidis*	Baltrusaityte et al.,2007
21	Chestnut, Great laurel, Thyme	Disc diffusion	*S aureus* *B. stearothermophilus* *A. faecalis* *L. acidophilus* *E .coli*	Küçük et al. 2007
22	NS	Disc diffusion	*P. aeruginosa* *Enterobacter spp.* *K. pneumoniae* *Staphylococci* (Coagulase+)	Abd-El Aal et al., 2007
23	Blossom, Eucalyptus	Disc diffusion	*P. aeruginosa*	Boukraa, 2008
24	Blackberry, Cotton, Buckwheat, Sunflower, Rabbitbrush (Chrysothamnus nauseosus)	Disc diffusion	*S. aureus* *L. monocytogenes*	Lee et al. 2008
25	RS Remavil	Turbidity test	*E. coli* *P. aeruginosa* *E. cloacae* *E. faecium* MRSA	Kwakman et al., 2008
26	Lavender, Thyme, Heather, Rosemary, Eucalyptus	Disc diffusion Turbidity test	*M. luteus* *S. aureus*	Martín et al., 2008
27	natural NS	Disc diffusion	*S. aureus coagulase* *P. aeruginosa* *E. coli*	Tajik and Jalali, 2009

	Honey type	Method	Target	Reference
28	Nigeria indigenous NS	Disc diffusion	*S. aureus* *E.coli* *S. dysenteriae* *P. mirabilis*	Adetuyi et al. 2009
29	Tualang, Manuka	Turbidity test Agar incorporation	*E. coli* *P. aeruginosa* *S. aureus* *MRSA* *S. flexneri*	Tan et al., 2009
30	Medical, AH, NS	Disc diffusion CFU counts	*E.coli* *P. aeruginosa* *E. faecalis* *S. typhimurium* *S. epidermidis* *S. aureus* *B. cereus* *L. monocytogenes* *C. albicans*	Boorn et al., 2009
31	Multiflower (NS), Anzer flower, Lime, Rhododendron, Rhododendron/lime	Disc diffusion	*E. coli* *P .aeruginosa* *B .cereus* *L. monocytogenes* *S. aureus* *C. tropicalis* *E. faecalis*	Ulusoy et al., 2009
32	Manuka, AH	Agar incorporation Microdilution Gene analysis	*S. aureus* (ATCC 9144) *P. aeruginosa* (ATCC 27853) *E. coli* (K-12)	Blair et al., 2009
33	Thyme (commercial), Lemon (commercial), Nabk (commercial)	Disc diffusion	*E. coli* *S. flexneri*	Ali, 2009
34	Thyme (commercial), Lemon (commercial), Nabk (commercial)	Disc diffusion	*E. coli* *P. aeruginosa* *S. aureus*	Al- Naama, 2009
35	Medihoney (Manuka)	Crystal violet assay Turbidity test	MRSA MRSE ESBL *P. aeruginosa*	Merckoll et al., 2009
36	Nigeria indigenous (NS)	Disc diffusion	*S. aureus* *E. coli* *K. pneumoniae* *B. subtillis* *P. aeruginosa*	Osho et al. 2010
37	Malaysia indigenous (NS)	Disc diffusion	*E. coli* *S. aureus*	Rahman et al., 2010

	Honey type	Method	Target	Reference
38	Rhododendron	Disc diffusion	A. hydrophila *E. coli* *B. cereus* *B. subtilis* *L. monocytogenes* *P. aeruginosa* *S. typhimurium* *S. aureus* *C. albicans* *S. cerevisiae* *Y. enterolitica*	Silici et al. 2010
39	India indigenous (commercial)	Agar incorporation CFU counts	*E. coli* *P. aeruginosa* *S. enterica*	Mandal et al., 2010
40	Tualang honey	Disc diffusion	*Acinetobacter* spp. *P. aeruginosa* *K. pneumoniae* Enterobacter cloacae *Streptococcus* spp. *S. aureus*	Nasir et al., 2010
41	Manuka, Ulmo, AH	Disc diffusion	*E. coli* ATCC 35218 *P. aeruginosa* ATCC 27853 MRSA ATCC 43300 MRSA nasal, 01322 & 00745 MRSA surgical (site/wound isolates, 00791 & 28965)	Sherlock et al. , 2010
42	Tualang honey	CFU counts	*P. aeruginosa*	Khoo et al., 2010
43	Clover, Manuka, Buckwheat, Wildflower, Sunflower, Rapeseed, Dandelion	Turbidity test	*E. coli* *B. subtilis*	Brudzynski & Miotto., 2010
44	Citrus, Black cumin, Clover	Disc diffusion	*S. enteritis*	Halawani & Shohayeb, 2011
45	Medihoney (Manuka)	Disc diffusion	*E. coli* *S. aureus* *P. aeruginosa*	Bradshaw, 2011
46	Acacia, Poly-floral (NS), Clover	DNA microarrays SEM/TEM Crystal violet assay Indole assay	*E. coli* O157:H7 *E. coli* K-12	JH Lee et al, 2011
47	Coniferous (NS), Citrus, Thyme, Polyfloral (NS)	Disc diffusion	*S. aureus* *E. coli* *S. enterica* *S. pyogenes* *B. cereus* *B. subtilis*	Voidarou et al., 2011

	Honey type	Method	Target	Reference
48	natural and processed Indian honey	Disc diffusion	*E. coli* MTCC-1687 *P. aeruginosa* MTCC-741 *E. faecalis* MTCC-439 *S. typhi* MTCC-531 *M. luteus* MTCC-2470 *B. subtilis* MTCC-736 *B. cereus* MTCC-430 *S. aureus* MTCC-737	Mohapatra et al., 2011
49	Canadian natural & pasteurised, Buckwheat, Sweet clover, Blueberry, Blackberry	turbidity test	*E. coli* *B. subtilis*	Brudzynski & Kim, 2011
50	Manuka, Sweet clover, Blueberry, Buckwheat, AH	turbidity test DNA isolation	*B. subtilis* *E. coli*	Brudzynski et al., 2011
51	Manuka, Blue-gum, Fynbos, Pincushion, AH	Disc diffusion	*S. aureus* *E. coli* MRSA *P. aeruginosa*	Mandal & Mandal, 2011
52	Manuka, medical honey, AH	CFU counts SEM/TEM DNA isolation	MRSA	Jenkins et al., 2011
53	Manuka (UMF 25), Buckwheat, Sweet clover, Blueberry, Manuka (UMF 20), Clover blend, Buckwheat	CFU counts	*E. coli* *B. subtilis*	Brudzynski et al., 2011
54	RS Remavil	Disc diffusion CFU counts	*B. subtilis* ATCC6633 *S. aureus* 42D *E. coli* ML-35p *P. aeruginosa* PAO-1 (ATCC 15692) MRSA VREF *E. coli* ESBL *P. aeruginosa* ciprofloxacin-resistant (CRPA)	Kwakman et al., 2011
55	Algerian indigenous (NS)	Disc diffusion	*C. albicans* *P. aeruginosa* *S. aureus*	Alzahrani et al., 2012
56	Manuka, Acacia, Lavender, Wild carrot	CFU counts	*E. coli* *S. aureus* *P. aeruginosa* *B. subtilis*	Kwakman & Zaat, 2012
57	RS medical, Manuka, AH	Agar incorporation	*S. aureus* *S. pneumoniae* *S. pyogenes* MRSA	Huttunen et al., 2012

	Honey type	Method	Target	Reference
58	Heather, Willow herb, Buckwheat	Turbidity test	*E. coli* *B. subtilis* MRSA	Brudzynski & Lannigan 2012
59	Blueberry, Buckwheat, Manuka	CFU counts Turbidity test DNA degradation	*B. subtilis* *E. coli*	Brudzynski et al., 2012
60	Dark amber	Turbidity test	*S. aureus* *E. coli* *C. albicans*	Al-Waili et al., 2012
61	Buckwheat, Blueberry, Manuka	CFU counts turbidity test DNA degradation	*E. coli* MRSA VRE *B. subtilis*	Brudzynski et al., 2012
62	Hamadan indigenous (natural)	Microdilution	Streptococci Lactobacillus	Ahmadi–Motamayel et al., 2013
63	Manuka, Medihoney (Manuka), AH	Turbidity test Disc diffusion	MRSA	Muller et al., 2013
64	Iran indigenous (natural)	Disc diffusion	*E. coli* *P. aeruginosa* *C. albicans* *S. aureus*	Khosravi-Darani et al., 2013
65	Manuka Greek & Cypriot indigenous (NS)	Disc diffusion	*S. aureus* *P. aeruginosa*	Anthimidou & Mossialos, 2013
66	Longan flower	Disc diffusion	MRSA	Huttunen et al., 2013
67	Chestnut, Polyfloral (NS), Eucalyptus, Thyme, Erica	Disc diffusion	*E. coli* S. typhimurum	Coniglio et al., 2013
68	Amber, Light amber, Dark amber	Turbidity test	*E. coli* *B. subtilis* *S. aureus* *P. aeruginosa* *K. pneumonia* *S. typhimurum* *B. cereus* *C. albicans*	Al-Wailli et al., 2013
69	Buckwheat, Wildflower	CFU counts SEM/TEM flow cytometry Light microscopy	*E. coli* K-12	Brudzynski & Sjaarda, 2014
70	Manuka	CFU counts	*E. coli* (ATCC-25922) E. *faecalis* (ATCC-29212)	Kumar, 2014
71	Manuka	Crystal violet assay	*P. aeruginosa* (ATCC 9027) *P. aeruginosa* (867 clinical isolate)	Camplin & Maddocks, 2014
72	Natural honey (stingless bees, NS)	Disc diffusion Turbidity test	MRSA	Nishio et al., 2014
73	Manuka	SEM/TEM turbidity test	*S. aureus* *P. aeruginosa*	Campeau & Patel, 2014

	Honey type	Method	Target	Reference
74	Dark yellow multifloral (NS)	CFU counts	*S. aureus* *E. coli* *S. pyogenes* *C. albicans*	Al-Wailli et al., 2014
75	Manuka (UMF*** 10), Manuka (UMF*** 20), Blossom, Vipers bugloss, Floral (NS), Heather, AH	Turbidity test	MRSE *S. aureus* *E. coli* *E. faecalis* *P. aeruginosa* *A. baumanni* MRSA	Carnwath et al., 2014
76	Pakistan indigenous (NS)	Disc diffusion	*P. aeruginosa* *X. campestris* *S. tymphimurium* *K. pneumonia* *E. coli* *E. faecalis* *C. perfigens* *S. aureus* *B. subtilis*	Khalili et al., 2014
77	African indigenous (NS)	Disc diffusion	*E. coli* *E. cloacae* *K. pneumoniae* *C. freundii*	Ramalivhana et al., 2014
78	Wild raspberry, Willow herb, Lingonberry, Bilberry, Clover, AH	Disc diffusion	*C. perfigens*	Oinaala et al., 2015
79	Indian indigenous natural (NS)	Disc diffusion	*E. coli* *P. aeruginosa* *S. aureus* *S. pyogenes* *P. mirabilis*	Mahendran & Kumarasamy, 2015
80	Thailand indigenous (NS)	Disc diffusion	MRSA	Jantakee & Tragoolpua, 2015
81	Natural honey (Scaptotrigona bipunctata & Scaptotrigona postica)	Disc diffusion SEM/TEM turbidity test	*E. coli* *E. faecium* *K. pneumoniae* *P. aeruginosa* *S. enterica* *S. aureus* *S. epidermidis* *S. pyogenes*	Nishio et al., 2016
82	Bulgarian rape (NS)	CFU counts	MRSA	Dinkov, et al., 2016
83	Orange blossom, Cranberry, Wild flower, Buckwheat, AH	MIC Turbidity test	MRSA MSSA VRE *P. aeruginosa* *E. faecalis* *K. pneumoniae*	Hammond et al., 2016
84	Cornflower, Buckwheat	Turbidity test	*S. aureus* PCM 2051	Kuś et al., 2016
85	Ethiopia (longan flower)	Disc diffusion	MRSA	Wasihun & Kasa, 2016

	Honey type	Method	Target	Reference
86	Mexico indigenous (NS)	CFU counts	*E. coli* *P. aeruginosa* *S. typhimurium* *S. aureus* *L. monocytogenes* *B. subtilis*	Pimentel Gonzalez- et al., 2017
87	Manuka (UMF 10,16, & 20), Sidr	CFU counts	MRSA MSSA	Almasaudi et al., 2017
88	Kosovo indigenous (NS)	Disc diffusion	*E. faecalis* *S. aureus* *B. cereus* *E. coli* *S. typhimurium*	Suerdem & Akyalcin, 2017
89	SurgiHoney, Acacia	CFU counts Confocal microscopy	*P. aeruginosa* MRSA *H. infuenza*	Newby et al., 2018
90	Manuka, Kanuka	Turbidity test	*S. aureus* *P. aeruginosa*	Bucekova et al., 2018
91	Poly-floral (NS), Oregano, Acacia, Mint herbs	Disc diffusion	MRSA *P. aeruginosa*	Stagos et al., 2018
92	Nigeria indigenous (NS),Zaria indigenous (NS)	Disc diffusion CFU counts	*S. pyogenes* *S. pneumoniae*	Mshelia et al., 2018
93	Medical (Manuka), Heather, Clover, Raspberry Rapeseed, AH	Disc diffusion	*E. coli* *S. aureus* *P. aeruginosa*	Matzen et al., 2018
94	Nigerian indigenous (NS)	Disc diffusion	*E. coli* *S. aureus* *S. pyogenes*	Onyeka et al., 2018
95	Palestinian indigenous (NS),Moroccan indigenous (NS)	Disc diffusion	*E. coli* *P. aeruginosa* *S. aureus*	Imtara et al., 2018
96	Manuka, Buckwheat, Multiflora (NS)	CFU counts	*S. aureus* *S. epidermidis* *E. coli* *P. aeruginosa*	Grecka et al., 2018
97	Medihoney (Manuka)	Crystal violet assay Confocal microscopy ATP measurement	*S. aureus*	Liu et al., 2018
98	Blossom honey, honeydew, Manuka	Disc diffusion	*S. uberis* *E. coli* *S. agalactiae* *E. faecalis*	Klimesova et al., 2018
99	Agastache, Tea -tree, Manuka, Jarrah honey, Jelly bush	Turbidity test	MRSA	Anand et al., 2019
100	Slovakia indigenous (NS), Manuka, AH	CFU counts	*P. aeruginosa* *S. aureus*	Bucekova et al., 2019

NS* (honey of not specified floral origin and composition)
AH** (artificial honey, composed of the four predominant sugars in honey)
UMF*** (Unique Manuka Factor, represents the antimicrobial strength of Manuka honey based on the concentration in MGO).

CHAPTER 2

Investigation of the antibacterial effect induced by honey innate components

2.1. Introduction

As was discussed in Chapter 1, honey is emerging as an effective antibacterial agent. At present, many studies have acknowledged its efficacy against a range of species, however, its antimicrobial mechanism remains obscure. This is in part because of the complexity in honey composition itself and secondly due to the limited number of methods have been used for this purpose. To bring honey to the forefront of clinical application, it is important to understand the mode of action and the effects of it on bacterial physiology.

It is now established that on honey dilution, the oxidation of glucose results to the accumulation of H_2O_2 and the gluconic acid (Molan, 1992b). The antibacterial strength is determined by honey composition and a cascade of reactions that control the accumulation of H_2O_2 after the initiation of the reaction (Brudzynski et al., 2017).

The literature review showed that the majority of studies investigated the antibacterial activity of various H_2O_2-producing honeys using the classic microbiological methods. Only a few of them focused on the explanation of H_2O_2-induced oxidative stress by using more advanced methods (i.e SEM, TEM, confocal microscopy, flow cytometry and quality assessment of genomic DNA degradation). In particular, it was reported that honey targets a series of events related to growth initiation, cell division and cell wall synthesis. Also, honey was seen to increase permeability of the outer membrane (in Gram-negative bacteria) and cause destruction of the lipopolysaccharide layer (LPS), which in turn caused release of endotoxins at bactericidal concentrations (Blair et al., 2009; Jenkins et al., 2011; Jin-Hyung Lee et al, 2011; Brudzynski et al., 2011; Brudzynski et al., 2012; Campeau & R. Patel, 2014; Nishio et al., 2016; Newby et al., 2018). Advanced microscopy

showed phenotypic alterations on honey-treated bacteria such as changes from coccoidal to short rod-shape which eventually inhibited septation and cell division (Khan et al., 2010; Oliver, 2010; Balaban et al., 2004).

Despite this knowledge and the persistent view that H_2O_2 is in the first line of antimicrobial activity in honey, most studies reached the conclusion that an unidentified synergy underlies the activity of honey, and the complexity of this product complicates further investigations. As such, a full understanding of the source and mechanism of antimicrobial activity is still incomplete.

2.1.1. An overview of this chapter

The aim of this study was to elucidate the importance of the glucose oxidation that initiates the antibacterial activity of honey. In this direction, the physiological consequences of bacterial exposure to the three main honey components, sugars, H_2O_2 and gluconic acid were studied.

Exponentially growing *E. coli* MG1655 K-12 cells were used for this investigation. This allowed to track cellular and molecular stress responses before cells obtain increased sensitivity of the lag phase. Also, the use of a model organism allowed the fundamental understanding of the bacterial stress responses to honey regardless any strain-specific characteristics (i.e. resistance).

To circumvent the effects that arise due to the variability in honey composition, a model honey was employed as a simpler milieu. Two honeys, the acacia (yellow) and the heather (dark brown) were used as reference. The present study was undertaken by combination of a traditional culture method (spot plating assay) with single cell analysis (flow cytometry) and Atomic force microscopy (AFM).

The combination of BOX and PI dual-staining allowed the discrimination of the three sub-populations corresponding to live, dead and injured cells (Freire et al., 2015; Nescerecka et al., 2016). Membrane integrity serves as indicator of “alive” cells, which preserve their internal constituents, potentially capable of metabolic activity/ reproductive growth (Spilimbergo et al., 2010). “Alive” cells possess intact polarised cytoplasmic membranes impermeant to both BOX and PI dyes. In case of membrane potential depletion, “injured” cells are becoming permeable to the lipophilic BOX dye which passes through their polarised inner membrane and binds to positively charged proteins or other hydrophobic regions (Zeng et al., 1999). The PI, a cationic hydrophobic dye (nucleic acid intercalator) is taken up only from compromised membrane cells which are considered as “dead” (Nebe-von-Caron et al., 2000). However, the term “dead” is arbitrary since PI was reported to label highly reproducible cells, presumably during the wall reconstruction which allows the PI to pass through within few mimutes (Shi L et al., 2007). Thus, in this study the combination of dual-staining FCM and CFU counting aims to give a clear discrimination between viable, injured and dead bacterial population (Figure 2.1).

Also, flow cytometry allowed the identification of intracellular ROS accumulation. The intracellular ROS intermediates were tracked by 2’,7’-Dichlorodihydrofluorescein (H_2DCFDA) fluorescence activated dye. H_2DCFDA is cleaved by the intracellular esterase to a relatively polar and membrane impermeable H_2DCFD which is later oxidised by ROS to the highly fluorescent product DCF (Ninganagouda et al., 2014) (Figure 2.1).

AFM provided topographical images of the bacterial surface in real time. The measuring principle of AFM is based on the horizontal scanning of a (bacterial)

surface by a microcantilever. Due to the interaction of the tip with the bacterial surface, the cantilever is deflected and generates a topographic image with a nano-scale resolution (Liu and Yang, 2019).

This chapter starts with a preliminary investigation of the antimicrobial effect induced by each of the three honey stressors on *E.coli* K-12. The effect of sugars, gluconic acid and H_2O_2 was examined in a range of concentration they have been found on honey dilution. Later, potential synergies within these components were identified by using different model honeys. In order to confirm these observations, the susceptibility of single mutants was tested against the parent strain after the exposure to different model honeys. This chapter will conclude with a discussion about the antibacterial mechanism which is induced by the three major honey components.

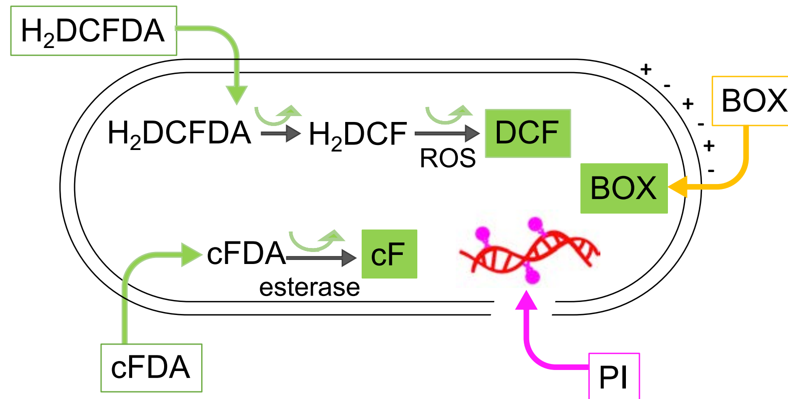


Figure 2.1 Determination of cell physiological state using fluorescent probes. Cells with intact membranes exclude the BOX and PI which penetrate only cells with depolarised and destructed membranes respectively. Non-fluorescent cFDA is permeable through intact membranes and is converted to fluorescent cF by esterase activity. Likewise, H_2DCFDA is diffused into cells, is retained in intracellular level until the cleavage by the esterase and the oxidation by ROS when is converted to a highly fluorescent DCF.

2.2. Materials and methods

2.2.1. Model honey and real honey samples

Acacia and heather honey were purchased from a local retailer (Birmingham, UK). Honey samples were stored in their original packaging, at room temperature (22°C) in the dark. Serial dilutions of real honey in deionized sterile water were prepared in concentrations of 50, 25, 12, 6, 3 and 1.5% w/v. Fresh solutions were prepared before conducting each antibacterial assay.

The stock model honey was prepared by dissolving fructose (2.24 M), glucose (1.85 M), maltose (0.219 M) and sucrose (0.04 M) in deionised sterile water at 37°C as described previously (Bogdanov et al., 1996). The osmolality was measured at room temperature using a refractometer, and expressed in Brix units. Stock solutions of gluconic acid (60, 34 and 8.6 mM) and H_2O_2 (5.6, 3, 0.04 mM) were prepared in deionised sterile water and added immediately before the start of each assay. The compositions of the model honeys as they were made from the initial stock are presented in Appendix 2.1.

2.2.2. Bacterial strains and culture conditions

E. coli K12 (MG1655) was used for all the experiments in this chapter. Two catalase mutants of this strain (Δ*katG::kan* and Δ*katE::kan*) were constructed by P1 transduction from the Keio library and validated using PCR with appropriate gene-specific primers (Appendix 2.2) (Thomanson et al., 2007). The Δ*rpoS::kan* strain was obtained from the School of Biosciences (IMI laboratory, University of Birmingham). For all assays, bacteria were grown in 5 mL of Luria Broth (Sigma-

Aldrich., UK) shaking in 20 mL flask, at 150 rpm/37°C. Overnight cultures were diluted in fresh LB to a starting A_{600} nm of 0.005 and incubated until the A_{600} nm reached 0.5-0.6 McFarland Standard (approx. 1.5 x 10^8-10^9 CFU/mL). Before use, cells were pelleted (3900 g for 3 minutes in an Eppendorf Centrifuge 5810), washed twice in PBS (BR0014, Oxoid Ltd., UK), and resuspended in PBS to a final absorbance of 0.5.

Table 2.1: Strains used in this study and source of reference

Strain	Description	Source
MG1655	Reference strain	Discuva Ltd, Cambridge
MG1655 *katG::kan*	*katG replaced by kan cassette*	This study *
MG1655 *katE::kan*	*katE replaced by kan cassette*	This study *
MG1655 *rpoS::kan*	*rpoS replaced by kan cassette*	This study *

*These strains were obtained from Keio collection by P1 phage transduction.

2.2.3. Time course of antibacterial activity of model and real honeys

The antibacterial activity of real and model honeys was determined using a broth dilution assay in a 96-well microplate format. Exponential phase cultures at a concentration of 10^8 CFU/mL were mixed in a 1:1 ratio with either the honey solutions (50-1.5%), model honey (SGH), or with hydrogen peroxide (H), gluconic acid (G) and sugar solution (S) only at the concentrations shown in Appendix 2.1.

An inoculum in PBS was used as a negative control. Plate counts (CFU) and single cell analysis (flow cytometry) were both used to estimate cell viability. For the killing assay, Kaplan-Meier survival analysis was performed to determine the percentage of *E. coli* survival post-honey treatment. The lower limit of this assay was 10^{-1} CFU/mL. Log-rank (Mantel-Cox) test was conducted in GraphPad Prism 7.0 (GraphPad Software, Inc., San Diego, CA) and a p-value <0.05 was considered to be statistically significant in comparison with the control group or the WT (depending the assay). Each assay was conducted in triplicate with three biological replicates.

2.2.3.1 Plate counting (CFU)

After static incubation at room temperature bacterial growth was measured by serial ten-fold dilution in sterile PBS. Appropriate dilutions (5 µL total volumes) were spotted into square LB plates. The plates were then tilted to spread the spot into a line down the plate. Colonies were scored after overnight incubation at 30°C. Survival was expressed as a percentage of the untreated culture (control).

2.2.4. Flow cytometry

2.2.4.1 Assessment of membrane polarity and integrity

Bacteria were analysed for viability using a BD Accuri C6 flow cytometer (Becton Dickinson Biosciences, Oxford, UK). Samples taken at appropriate time intervals were diluted with 0.2 µm-filtered PBS and stained directly with Propidium iodide (PI) and Bis-(1,3-Dibutylbarbituric Acid) Trimethine Oxonol (BOX) (Sigma,

UK) at final concentrations of 4 μg/mL and 2 μg/mL respectively. Samples were mixed and incubated for 10 minutes in the dark. Untreated bacterial cells, or cells treated with 3 M hydrogen peroxide for 30 minutes, served as "alive" and "dead/injured" controls respectively. Samples were excited using a 488 nm solid-state laser. 25,000 data points were collected at a maximum rate of 2,500 events/sec. Fluorescence was detected using 533/30 BP and 670 LP filters corresponding to BOX and PI fluorescence respectively. Data were analysed using CFlow (BD) software.

2.2.4.2 Intracellular ROS detection

Intracellular ROS accumulation was measured with 2',7'-dichlorofluorescein diacetate (H_2DCFDA) (Sigma- Aldrich, UK). Cell suspensions (10^8 CFU/mL) in PBS were treated with model honey and incubated at RT for 2 h and samples were taken at 15, 30, 60, 90 and 120 min post-exposure. After incubation the cell suspensions were centrifuged and washed in PBS and incubated with H_2DCFDA (10 μL/mL) for 1 h at 37 °C in the dark. Samples were washed and the pellet was resuspended in prewarmed PBS. Untreated cells maintained in PBS served as a negative control. Fluorescence was measured using excitation at 488 nm and a 533/30 BP filter for detection in the BD Accuri flow cytometer.

2.2.4.3 Gating and data plotting

After the data are displayed in the plot and the population of interest is identified (i.e *E. coli* cells), the next step is to define the limits of the this population by creating a region which is called gate. The gating also excludes debris and noise from the sample analysis. This is done by running a sample of filtered deionised water and another with the population of interest. The gating can be performed based on the forward scatter (FSC), side scatter (SSC) or combination of them. Various displays are available for data analysis and plotting depending on the parameters of interest. For instance, forward scatter area (FSC-A) accounts for the cells size and side scatter area (SSC-A) for the cell internal complexity. Fluorescence intensity corresponds to the measurement of the fluorescence emitted by the cell population without showing the percentage of the cells which emit the fluorescence. FC data can also be presented as percentage of cells emitting a fluorescence.

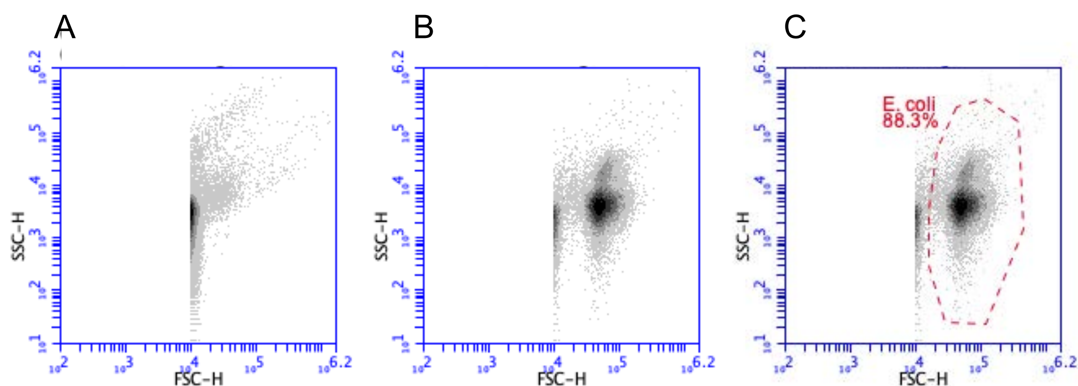


Figure 2.2. An example of gating in FC. (A) Distilled filtered (0.22 μm) water was run in order to detect the noise. (B) cells (*E. coli* MG1655) was run on flow and identified based on their characteristic FSC-H and SSC-H. (C) The population of interest was gated with the polygon.

2.2.5. Hydrogen peroxide assay

Hydrogen peroxide concentration was determined using the Fluorimetric H_2O_2 assay kit (Sigma- Aldrich, UK), according to the manufacturer's instructions. The red fluorescence formed after the reaction of peroxidase and H_2O_2 was measured at 540 nm excitation and at 590 nm emission using the CLARIOstar (BMG Labtech, US) multi-detection microplate reader. Dose–response curves were generated using the MARS software. To calculate the H_2O_2 concentrations in different honeys, a standard curve was generated using dilutions of a fresh 20 mM H_2O_2 stock solution (Appendix 2.3). All determinations were done at least three times.

2.2.6. Atomic force microscopy (AFM)

AFM images of treated bacterial samples were acquired with a BRUKER Innova in dry condition on a ~25 mm^2 p-type silicon wafer (Sigma-Aldrich). Before depositing bacterial suspension onto the substrate, it was cleaned with a CO_2 snow jet while being held on a hot surface at 300°C. Subsequently, 5 µL of *E. coli* (10^5-10^6 CFU/mL) suspended in PBS were deposited onto the substrate and allowed to air dry. Before scanning the sample, the substrate was rinsed with deionised water to reduce build-up of salt during dehydration. The images were acquired in tapping mode with BRUKER RTESPA-300 probe (T: 3.4µm; L: 125µm; W: 40µm; f0: 300kHz; k: 40Nm^{-1}).

2.2.7. Response surface methodology design

A Response surface methodology (RSM) design of experiments approach was used in order to locate the optimum combination of the three stressors (gluconic acid, sugars and H_2O_2) for the highest antibacterial activity of model honey. A central composite design (CCD) was used. The CCD was generated by JMP (version 7.0) software (Statistical DiscoveryTM ,SAS Institute). According to the design, 12 experiments were conducted, in 3 replicates, for estimating the experimental uncertainty (Appendix 2.4). The three variables were analysed at five levels (Table S3).

2.3. Results

2.3.1. Effect of individual stress factors on exponential phase *E. coli* K-12 cells

Before investigating possible synergies between the three components of model honey, it was necessary to establish the effect of sugars, gluconic acid and H_2O_2 as individual stressors. *E. coli* MG1655 K-12 was subjected to each stress component in concentrations that are found in honey when this is diluted (Appendix 2.1). CFU counts were used to assess bacterial viability, and dual staining (BOX and PI) was used in flow cytometry (FC) for monitoring the effects of each stressor on membrane potential and cellular integrity. Measurements were taken during the first hours of exposure until two days after treatment (Figure 2.4).

Gluconic acid and H_2O_2 had a dose-dependent effect on viable cell counts (Figure 2.3C, D), whereas the sugars had a bacteriostatic effect at all concentrations tested (Figure 2.3A). FC showed that sugars, at all concentrations applied compromised the cell membrane integrity. One day after exposure to sugars, almost 40% of bacteria became permeable to PI/BOX (Figure 2.4). This effect developed more rapidly in higher dosed cells (70%) during the first hours of exposure whereas the same time, lower dosed cells excluded both PI/BOX (Figure 2.4). Within 1 day post-treatment 35-40% of cells were permeable to PI which indicated increased membrane permeability.

Osmotic shock has previously been shown to cause water efflux from the cell interior which eventually decreases the cell volume (Korber et al., 1996). We therefore determined the effects of different sugar concentrations on average cell size over time, using forward scatter measurements in FC (Figure 2.3B). A

significant decrease in the mean of forward scatter area (FSC-A) was seen in populations treated with S50 and S70 models (Figure 2.3B). However, as it is emerged from survival curves, sugar models arrested growth at 20% (or less) of the population (Figure 2.3A). Although all the three models induced similar bacterial killing, higher-dosed cells lost fitness sooner than the others. The results of viable counts and FC suggest that sugars compromise the membrane integrity and polarity. However, the most significant effect is the dose-dependent decrease of bacterial cell volume.

Unlike sugars, both gluconic acid and H_2O_2 had clear dose-dependent bactericidal effects (Figure 2.3C, D). Flow cytometry analysis of cells treated with gluconic acid showed that percentage of depolarised cells (BOX^+/PI^-) increased in a dose-dependent manner during the first hours of exposure. Lower concentrations of gluconic acid induced depolarisation to a lower extent and caused gradual membrane destruction (BOX^+/PI^+) (Figure 2.4). The prolonged exposure to gluconic acid induced loss of cellular integrity as shown by the increased fraction of PI positive cells 24 h later (Figure 2.4). Survival curves showed that the decline in cell counts corresponds quite well to the accumulation of depolarised (BOX^+) cells. This suggest that gluconic acid alters the membrane polarity in dose-dependent manner inducing bactericidal effect. Although, destruction of membrane integrity is believed to be an indicator of cell death, this was not the case here.

Hydrogen peroxide also had a dose- and time-dependent effect on membrane potential. Although higher concentrations of H_2O_2 were bactericidal, the effect on the membrane potential was not as great as that seen with gluconic acid (Figure 2.3E, 2.4). All tested concentrations of H_2O_2 induced a similar extent of

membrane destruction while the depolarisation effect was seen to be dose-dependent and gradually increased over time (Figure 2.4). This suggests that the antibacterial effect of H_2O_2 was associated with the simultaneous loss of membrane potential and integrity. Although H_2O_2 caused depolarisation and disintegration in all the concentrations applied, only higher dosed cells were completely eradicated.

Overall, we conclude from this preliminary analysis that each of the three stressors have clear and distinct effects on cell membrane integrity and bacterial viability. Thus, the mechanism of killing is likely to be different between them. We next went on to investigate synergies between these three stressors.

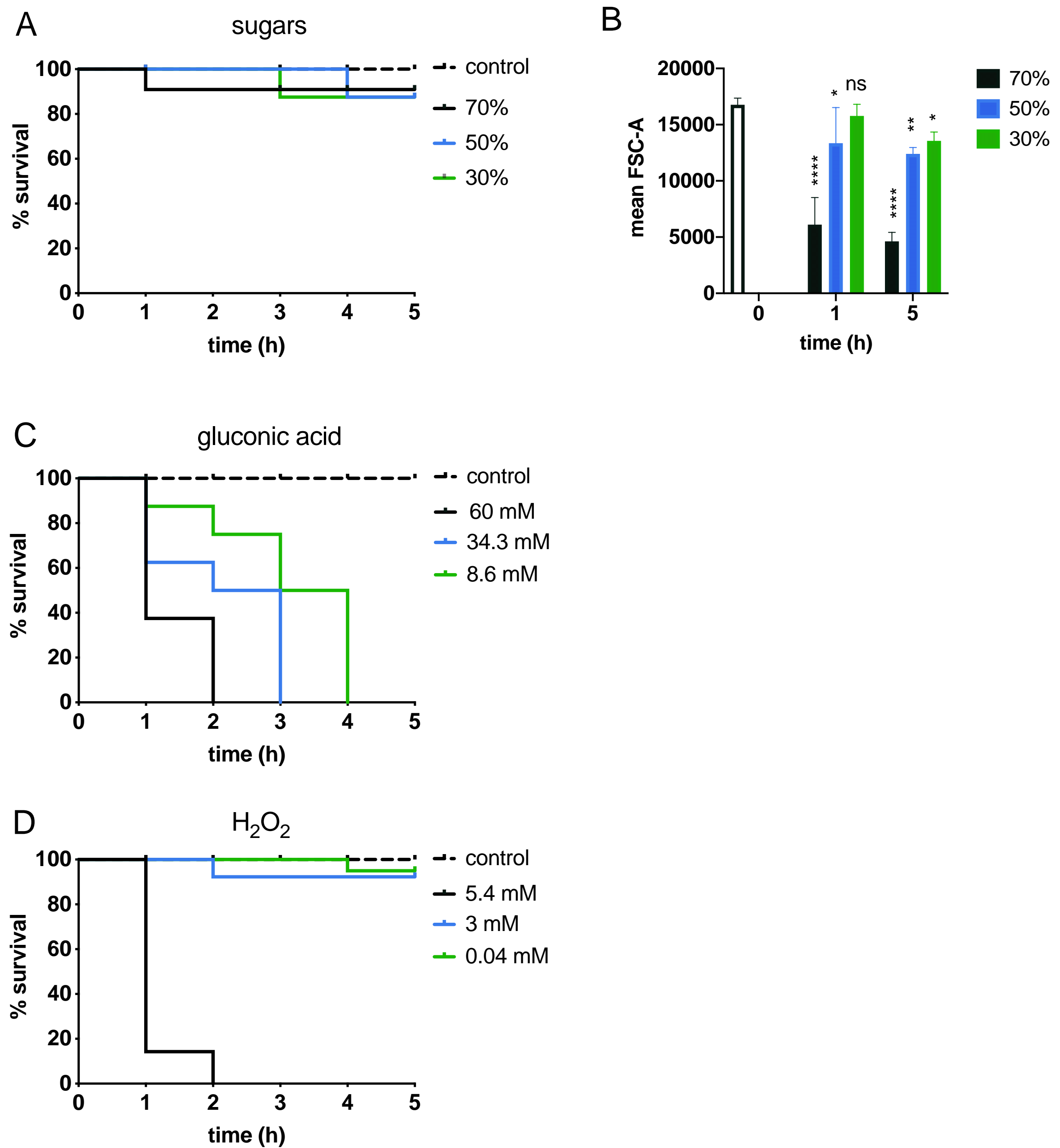


Figure 2.3 Effects of individual stress factors on exponential phase *E. coli* K-12 cells. (A, C, D) Kaplan-Meier survival plots showing percentage survival post- exposure to increasing concentrations of sugar, gluconic acid and H_2O_2 (detection limit: 1 CFU/mL) (average of *n=3*; biological replicates). (B) Changes in mean forward scatter (FSC-A) of bacteria post-challenge with sugar, (2-way ANOVA; asterisks show significance levels of Sidak's multiple comparisons test to the control group (**** p< 0.0001, ns; p> 0.05).

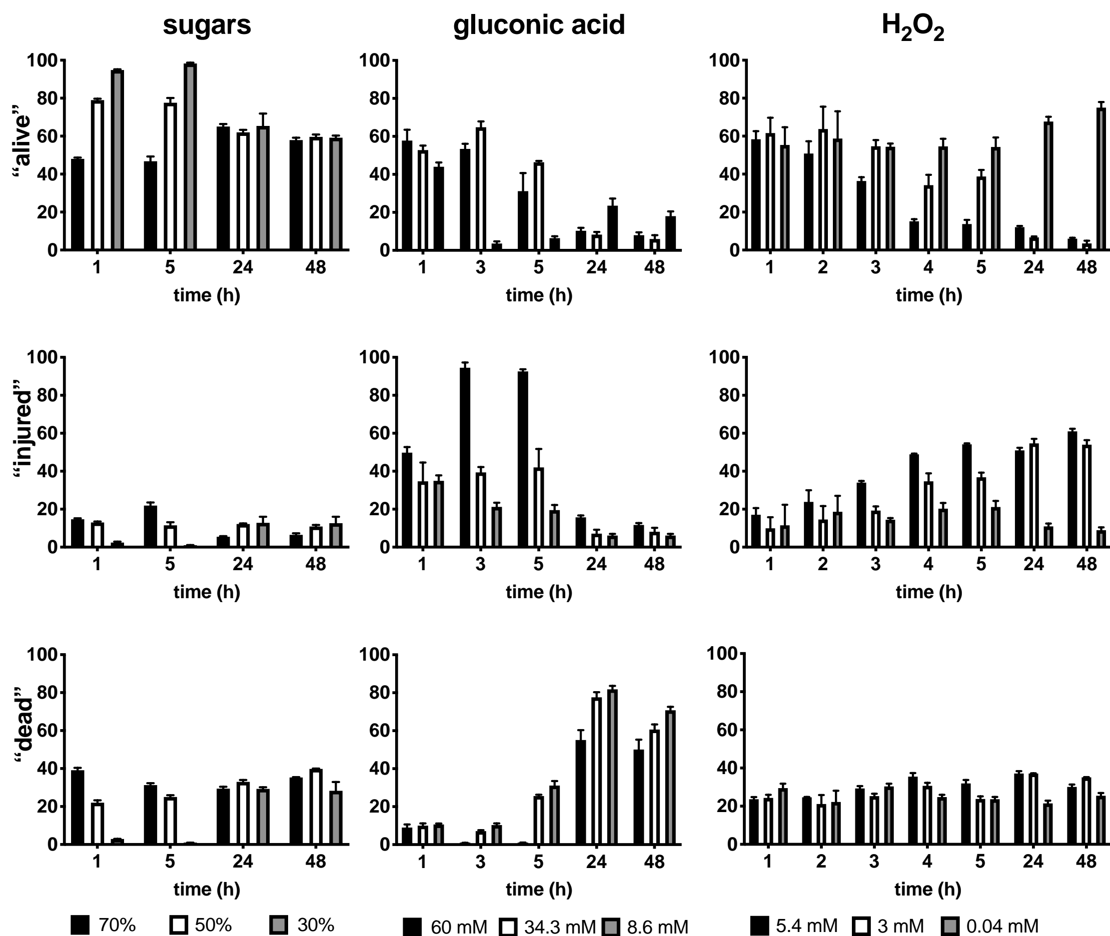


Figure 2.4. Flow cytometry (FC) analysis of *E.coli* K-12 exposed to single stress factor models (sugars, gluconic acid and H_2O_2) at three levels of concentration. Mean percentage of "alive" (PI^-/BOX^-), "injured" (or depolarised) (BOX^+/PI^-), and "dead" (or membrane destructed) (BOX^+/PI^+) are shown for cells exposed to sugars, gluconic acid and H_2O_2. Error bars represent the average ± s.d. (n=3; biological replicates).

2.3.2. Investigation of synergy between sugars, gluconic acid and H_2O_2 as combined in a "model" honey.

To investigate possible synergies between sugars, gluconic acid and H_2O_2, a central composite design (CCD) was used. All the models tested, as proposed by CCD induced a rapid bactericidal effect (Appendix 2.5). Therefore, a model honey composed of the three stressors at the lowest levels (non-inhibitory concentrations) reported in diluted honey (30% sugars, 8.6 mM gluconic acid, 0.04 mM H_2O_2) was used to investigate the underlying mechanism at non-lethal bacterial state. Significant synergies between two of the three stressors were identified by comparing 2-component models to the holistic model (Figure 2.5). The survival of *E. coli* cells was measured at 30, 60, 90 and 120 min after exposure to four combinations of stresses (Figure 2.5A). The rapid changes in bacterial cell membrane integrity and morphology were detected by FC (Figure 2.5B) and AFM respectively (Figure 2.7).

The results showed that synergy of H_2O_2 and gluconic acid (GH) induced significant ($p=0.004$) bactericidal effect 1 h post-treatment. This effect was mitigated in presence of sugars (SGH). Unlike the other models, GH induced simultaneous cell membrane depolarization and disintegration which was a precursor to cell death. Also, the Mean fluorescence intensity (MFI) of PI emitted by cells treated with GH model was equivalent to that of cells treated with 250 times more concentrated H_2O_2 solution (H10) (Figure 2.6A). This suggests that low pH, induced by gluconic acid, accelerates the toxicity of H_2O_2. Also, it was seen that any of the tested models caused higher bacterial damage comparing to this caused by single stressors. This is shown in Figure 2.6B where MFI indicative of membrane destruction (PI^+) and

depolarization (BOX^+), was 10 times less than this caused by the combination of stressors (Figure 2.6A).

As was mentioned previously, the toxicity of the gluconic acid and H_2O_2 was moderated in presence of sugars. Figure 2.5A shows that both SGH and SG models demonstrated identical antibacterial activity (50% survival). However, FC showed significant differences between the populations exposed to these models. The SGH and both H_2O_2-containing models (SH, GH) significantly augmented the fraction of PI/BOX permeable cells, indicating that H_2O_2 causes membrane destruction even when this is at non-inhibitory concentration (Figure 2.5B). The SG which lacking the H_2O_2 is the only that caused significantly lower PI uptake. In agreement to our previous findings (§2.3.1) here we saw that presence of H_2O_2 to model honeys caused destruction of membrane integrity, at any concentrations tested. In contrast to the principle that PI permeable membranes are indication of cell death, here it was seen that PI^+ cells can be culturable under the conditions tested. However, simultaneous membrane depolarisation and destruction are perquisites of cell death.

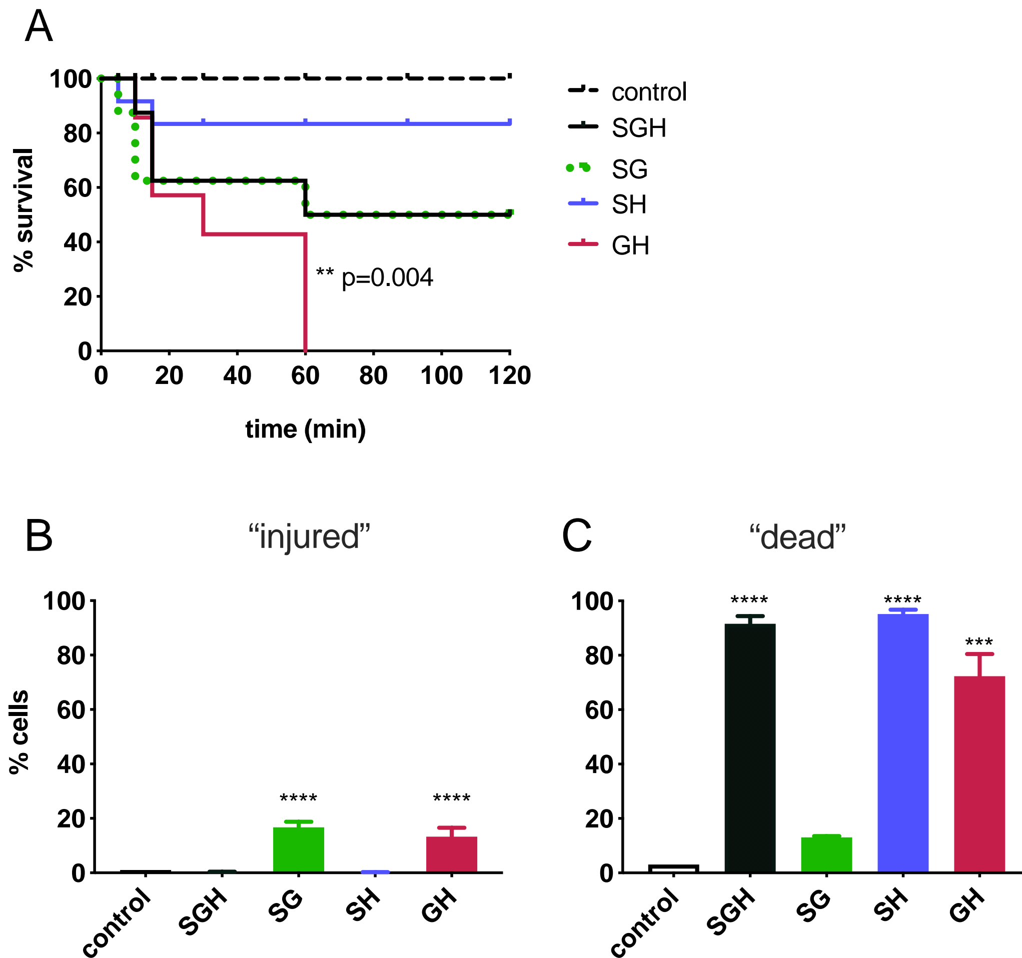


Figure 2.5 Synergistic effect caused by model honeys on exponential phase *E. coli* K-12 cells. (A) Kaplan-Meier survival plots show percentage survival post-exposure to four model honeys (detection limit: 1 CFU/mL). Survival curves were compared to the control using the Log-rank (Mantel-Cox) test (**; p=0.004). (B) FC analysis showing effects on percentage of cells lacking membrane potential (% BOX^+/PI^-) and cell integrity (% BOX^+/PI^+) induced 2h post exposure to 4 model honeys. The significance of relative proportions of BOX^+/PI^- and BOX^+/PI^+ between the model-treated bacteria and the control was tested with One-way ANOVA (Dunnett's multiple comparisons test; 95% CI; *** p=0.0001, **** p<0.0001). Error bars represent the average ± s.d. (*n*=3; biological replicates).

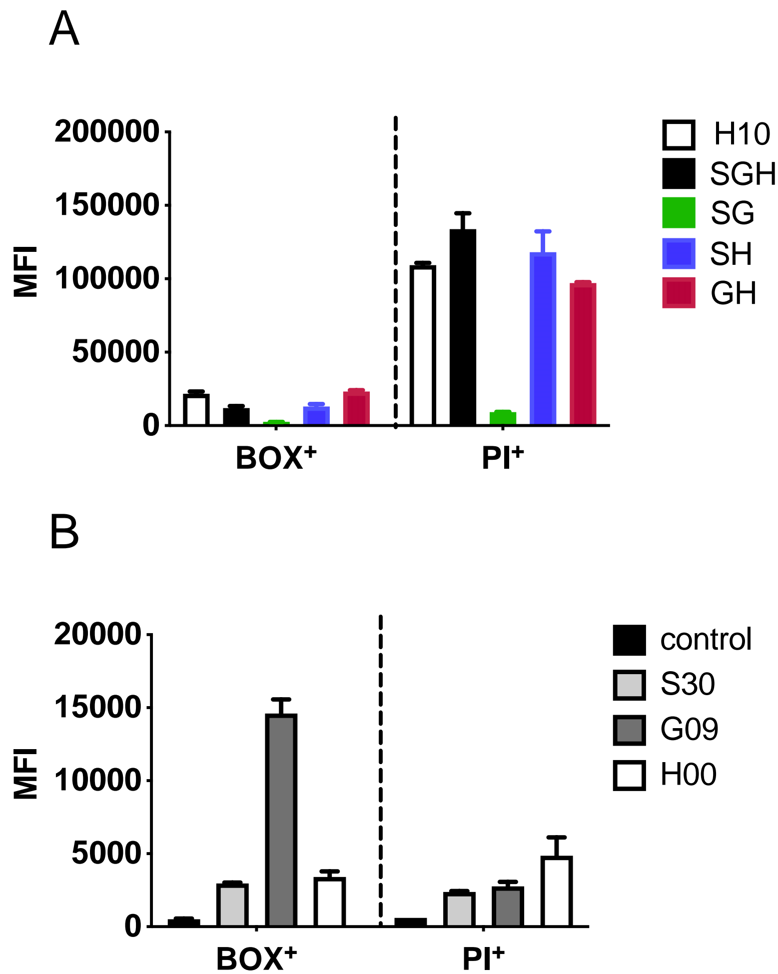


Figure 2.6 Comparison of the effect caused by model honeys and single honey stressors (S, G, H). Mean Fluorescence Intensity units (MFI) of (A) cells exposed to model honeys (SGH, SG, SH, GH) and pure H_2O_2 (10 mM; H10) and (B) to single stressor models (S30, G09, H00) highlights the synergistic effect over the effect of the single stressors on membrane depolarization (BOX^+) and integrity (PI^+); PI (Ex/Em of 493/636 nm) and BOX (490/516 nm). Error bars represent the average ± s.d. (*n*=3; biological replicates).

AFM images showed significant morphological alterations of cell structure following exposure to the SGH. Within 2 h of treatment the cell height declined to less than 300 nm (from 780 nm) (Figure 2.7). The dramatic loss of cellular volume and the destruction of its surface suggests the collapse of the cell wall, and potentially the leakage of intracellular material. Although sugar-treated cells were observed to have a roughened surface, the whole structure did not significantly collapse 2h later as seen with the SGH model. This confirmed the FC results which showed the cell-wall damage caused by the synergy of the 3 stressors comparing to this caused by S, G and H models (Figure 2.5B, 2.6).

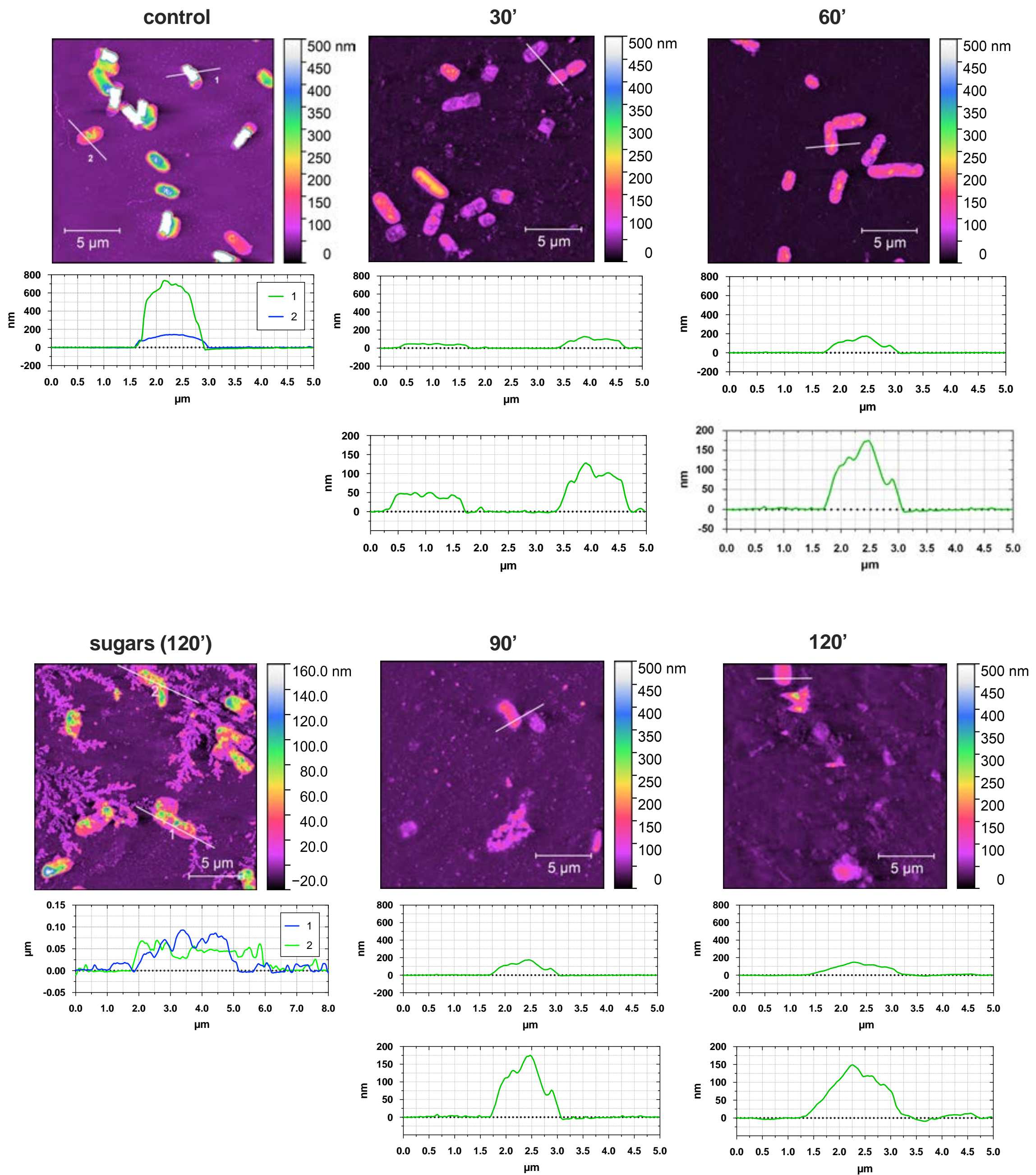


Figure 2.7 AFM images of *E.coli* exposed to sugars and model honey. Topography (upper lane) and cross section images (down lane) show the effect of model honey on structural integrity of bacterial cells. Nanoscale structural changes of cross-sectioned bacteria (cells crossed by white line) are given in the graphs below the AFM images. Control sample is taken from bacteria growing in LB and then transferred in PBS.

In summary, it was seen that H_2O_2 has a particular role in honey stress. This was due to the destruction of bacterial membrane even when this was applied in non-inhibitory concentrations. Gluconic acid enhanced the toxicity of H_2O_2. Their synergy caused depolarisation and destruction of bacterial cell membrane, two events which affected the bacterial viability. The absence of gluconic acid from the model halted its activity. Although sugars attenuated the toxicity of the two stressors, it appeared that osmotic stress have significant effects of cell membrane integrity. However, the osmotic stress could not arrest cell growth at any concentrations tested.

2.3.3. Relationship between H_2O_2 accumulation and ROS-induced antibacterial activity in honey

It was shown that H_2O_2 and gluconic acid are both involved in the antimicrobial effect of honey. Although H_2O_2 can readily cross bacterial membranes and cause oxidative damage far from the site of its formation, it is degraded soon after its generation (Sharma et al., 2012). Thus, it was hypothesized that the formation of other Reactive Oxygen Species (ROS) triggered by H_2O_2 could be implicated in the bactericidal activity of honey.

Among ROS, hydroxyl radicals (•OH) are the most reactive. They are generated by H_2O_2 and superoxide ($•O_2^-$) via the Haber-Weiss and Fenton reactions. The first step of the reaction is the oxidation of ferrous (Fe^{2+}) to ferric iron (Fe^{3+}) which forms a hydroxyl radical and a hydroxide ion (Haber-Weiss reaction). Ferric iron is then reduced back to ferrous producing a hydroperoxyl radical and a proton (Fenton reaction). The net reaction is the disproportionation of H_2O_2 which forms two kinds of radical species and water as by-products (Sharma et al., 2012). Transition metal ions (Fe^{2+}, Cu^{2+}) found in honey are thought to enhance the reaction, increasing the accumulation of •OH (Brudzynski et al., 2012a) (Figure 1.3). Thus, upon dilution of honey, the generation of ROS from H_2O_2 may exerts a prolonged cytotoxic effect in synergy with the rest of its components (sugars and gluconic acid).

To investigate the role of ROS-induced oxidative stress in the antimicrobial effect of honey, *E. coli* K-12 was exposed to serially diluted (50-1.5%) heather (dark colour) and acacia (yellow colour) honey. Dark colour honeys have been reported to exert higher antimicrobial activity, positively correlated with their phenolic content,

which enhances the production of H_2O_2 and phenolic radicals via their auto-oxidation (Sakihama et al., 2002; Brudzysnksi et al., 2017). Model honey and H_2O_2 solution were used to examine whether synergy of the three components, or the H_2O_2 itself, were sufficient to maintain oxidative stress. The intracellular ROS intermediates were tracked by H_2DCFDA fluorescence activated dye. Double-staining with the PI, the DNA intercalating dye, served as an indicator of the membrane damage.

Heather honey (50% and 25%) and acacia (50%) exerted the highest antibacterial activity. These honeys accumulated 3.6 mM, 2.6 mM and 0.9 mM H_2O_2 respectively (Figure 2.8A). At higher dilutions of honey lower H_2O_2 was generated (Appendix 2.6). Overall, acacia honey generated lower H_2O_2 compared to heather for all the dilutions tested here (Appendix 2.6). Consistent with survival rates, bacterial populations exposed to higher H_2O_2-producing honeys were seen to have compromised/depolarised membranes (PI^+/BOX^+) (87-96%) (Figure 2.8B, Appendix 2.7). The same effect was seen previously with all the H_2O_2-containing models (Figure 2.5B). These observations suggest that H_2O_2 attacks the bacterial membrane, inducing irreparable damage, with a dose-dependent bactericidal effect. However, both the killing curves and FC data indicate that the bacterial population continued to decline in viability and did not recover the membrane damage even after degradation of the H_2O_2. This showed that honey exerts an antimicrobial effect after the H_2O_2 is mostly degraded, although the low pH and osmolality are not sufficient to exert significant bacterial killing. Therefore, to examine whether the inhibition of *E. coli* was targeted by other ROS other than H_2O_2, intracellular ROS accumulation was measured with the H_2DCFDA dye.

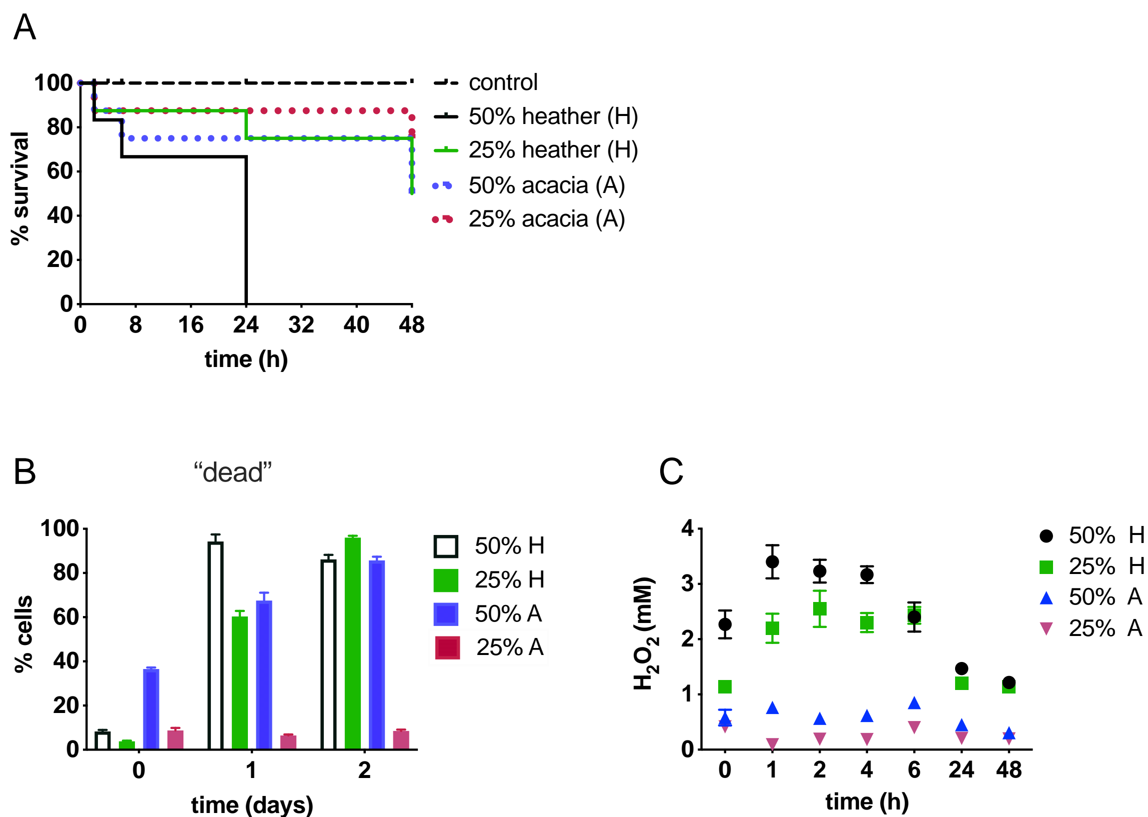


Figure 2.8. Antibacterial activity caused by heather and acacia honeys on *E. coli* K-12. (A) Kaplan Maier survival plots showing percentage of survival, post-exposure to (H) heather and (A) acacia honeys, diluted to 50% and 25%, t=0 corresponds to the initial inoculum (B) FC analysis showing the effect of heather and acacia honey on cell membrane integrity (% BOX+/PI+). (C) Accumulation and degradation of H_2O_2 in each honey sample up to 2 days post-dilution. t=0 corresponds to a few minutes after the dilution of honeys. Error bars represent the average ± s.d. (*n*=3; biological replicates).

The results showed that intracellular ROS level was proportional to the oxidative potential of each of the honeys tested. Heather honey (50%) generating the highest amount of H_2O_2 within 2h post-dilution (Figure 2.8C) caused the highest intracellular ROS accumulation (Figure 2.9A). Although heather (25%) and acacia (50%) caused similar antibacterial effect it appeared that the former, which initially produced double the H_2O_2 concentration, caused higher ROS generation within the cells. The same observation was confirmed with model honeys of increasing H_2O_2 concentration which stimulated ROS production in a H_2O_2 dose-dependent way (Figure 2.9B). These results suggest that H_2O_2 is a precursor of ROS generation in honey. In contrast to model honeys, real honeys caused prolonged ROS generation for 48h post dilution (Figure 2.9A). This is presumably due to other components (metals Fe^{2+}, Cu^{2+}) which are found in honeys and are implicated in the Fenton reaction and ROS formation. The free radicals identified 48 h post dilution are either longer lived, or are newly formed ROS, which are continuously produced as long as the H_2O_2 and transition metals are available in honey.

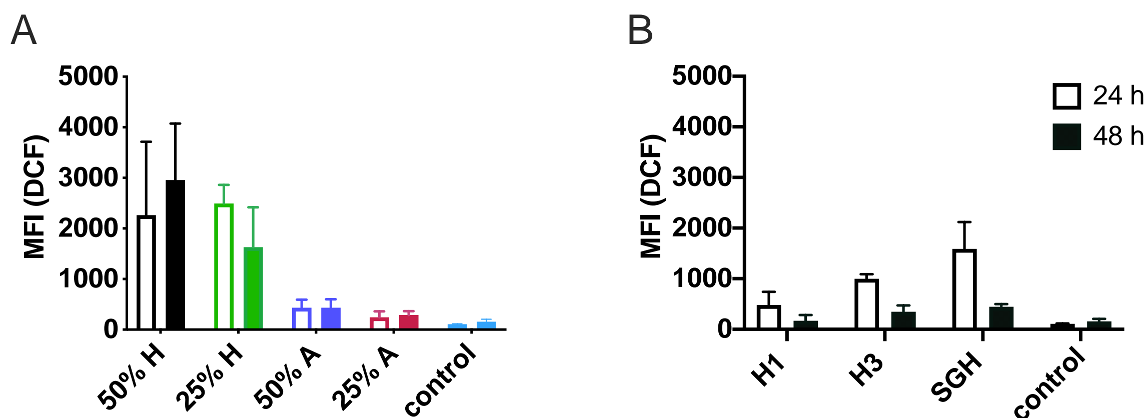


Figure 2.9 Intracellular ROS accumulation in *E. coli* K-12 after the treatment with model and real honey and H_2O_2. Mean fluorescence intensity (MFI) of DCF indicates the ROS accumulation in *E. coli* 24 and 48 h (full bars) of exposure to (A) (H) heather, (A) acacia honeys and (B) model honey (SGH) with H_2O_2 concentration equal to this of heather (3 mM). As controls, H_2O_2 solutions (H1, H3) of 1 and 3 mM H_2O_2 (corresponding to the maximum H_2O_2 found in acacia and heather honey) were used. Error bars represent the average ± s.d. (n=3; replicates).

The relationship between bacterial cell damage (indicated by PI^+ cells) and ROS generation resembled that of H_2O_2 accumulation and bacterial growth inhibition. The H_2O_2 level and the ROS-induced oxidative stress caused cell damage in dose-dependent way (Figure 2.10). Mean PI fluorescence intensity (MFI) showed that honeys of higher oxidative potential caused higher PI uptake from the treated bacteria. Heather and model honey caused 10 and 100 times respectively higher membrane destruction comparing to this caused by H_2O_2 itself (when applied in equal concentrations) (Figure 2.10A,B). This observation confirmed the previous results which showed that toxicity of H_2O_2 was enhanced by other components (gluconic acid and/or sugars).

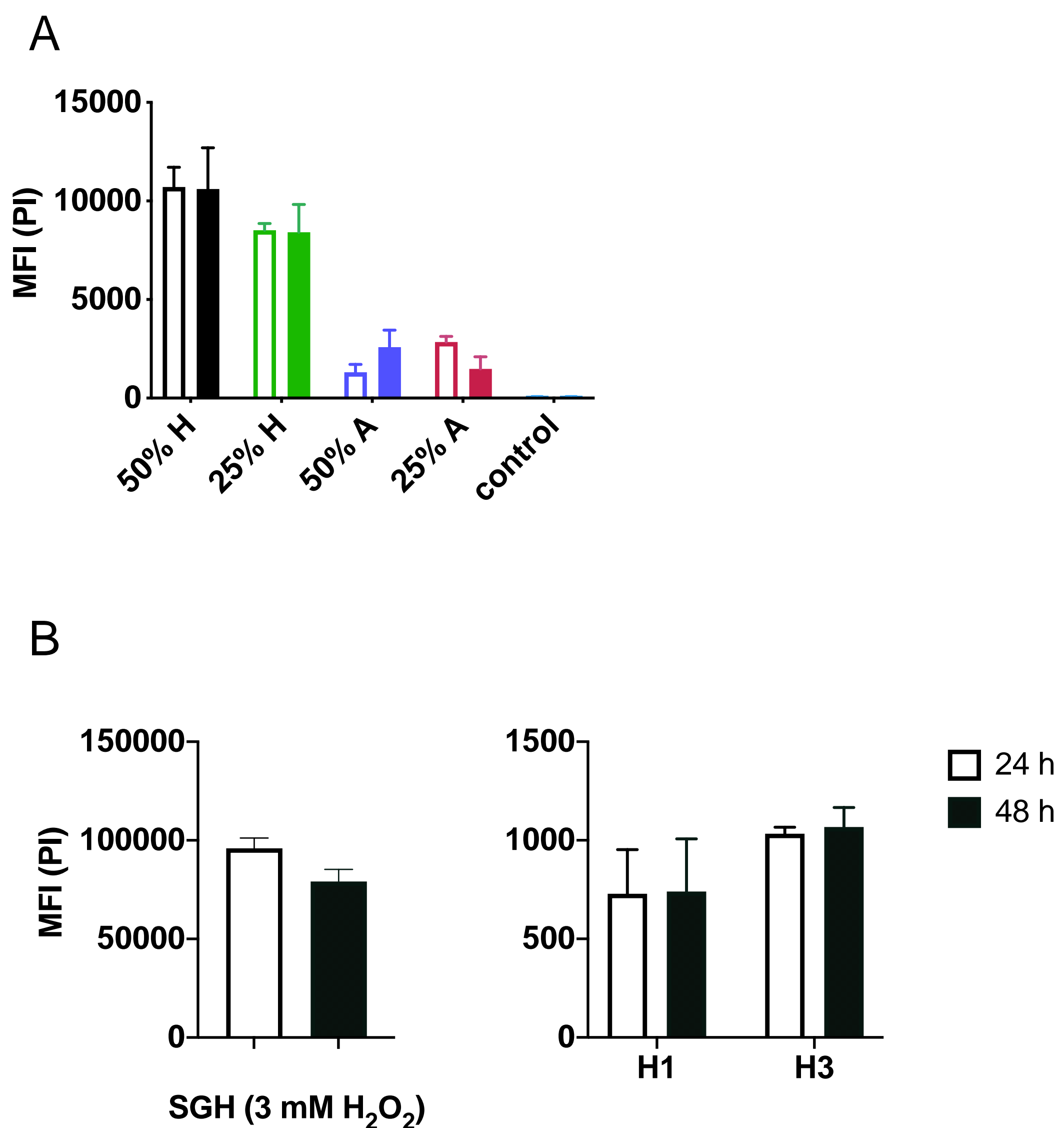


Figure 2.10 Relationship between ROS accumulation and cell membrane destruction. The mean fluorescence intensity (MFI) of PI corresponds to the extent of bacterial damage caused by treatment with real honeys and models (SGH, H1, H3). Each of them was seen in Fig. 2.9 to emit distinct ROS-activated fluorescence signals. Error bars represent the average ± s.d. (n=3; biological replicates).

These findings suggest that (a) H_2O_2 is the main source for ROS formation in honey however, other components contribute to prolonged generation of ROS and the bacterial toxicity. In addition to previous finding (§ 2.3.2) that the antimicrobial effect of honey is a result of the synergy between its major compounds (sugars, gluconic acid and H_2O_2), it was further showed that the level of H_2O_2, initially generated by honey, controls the level of antibacterial strength in honey. This could be explained by the fact that the abundance of H_2O_2 enhance Fenton reaction and the generation of other ROS.

2.3.4. Use of *E. coli* catalase-depleted mutants to confirm the role of oxidative stress in antibacterial mechanism of honey.

As shown in previous paragraph, the bactericidal strength of honey is strongly affected by the available H_2O_2 which stimulates the generation of ROS in a dose-dependent manner. Bacteria employ ROS scavenging and DNA repair mechanisms to overcome the oxidative stress (Imlay, 2015). In *E. coli*, general stress responses are modulated by regulators such as the sigma factor RpoS, and two component systems OxyR and SoxRS (Pomposiello and Demple, 2001). Hydroperoxidase I (*katG*) is transcriptionally induced by OxyR in exponentially growing cells in the presence of low H_2O_2, while Hydroperoxidase II (*katE*) is induced by RpoS in stationary phase. Both *katG* and *katE* catalyse the breakdown of H_2O_2 to water and oxygen (Loewen et al., 1985; Jung et al., 2003). To test the hypothesis that oxidative stress is a predominant in antibacterial mechanism of honey, the susceptibility of mutants lacking catalase activity (Δ*katG* and *ΔkatE)*, and lacking the general stress response regulator (Δ*rpoS*) was tested on model honeys

of increasing H_2O_2 concentrations (0.04, 0.1 and 0.5 mM). Concentration of sugars and gluconic acid was constant (sugars: 30%, gluconic acid: 8.6 mM).

As it is shown in Figure 2.11, *katG* and *katE* mutants were impaired in their ability to survive model honeys with increased H_2O_2 dose. Null *rpoS* mutant and WT were equally susceptible to all three models. This suggests that detoxifying genes regulated by the OxyR compensated for the loss of *rpoS* in the respective strain. However, regardless the catalase activity of *rpoS* mutant and WT, the three models caused 25% reduction in viability of both strains. This is potentially a result of the synergy in model honey components.

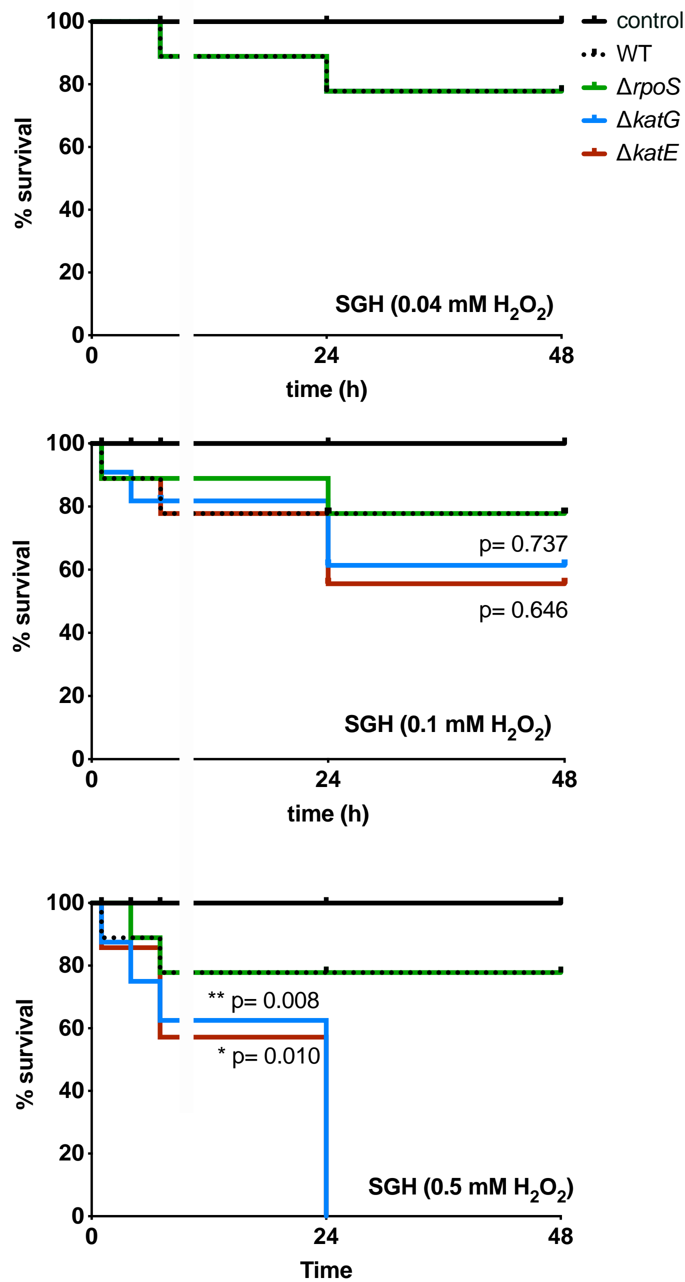


Figure 2.11 Identification of H_2O_2-induced oxidative stress in honey by comparing the susceptibility of catalase deficient (*katG*, *katE*) and *rpoS* mutants. (A) Kaplan Maier survival plots show survival of the knockouts and the WT to model honeys (SGH) of increasing H_2O_2 concentration. Survival curves were compared to the WT with the Log-rank (Mantel-Cox) test (* p=0.02, ** p=0.008). The data represent the average of 3 (*n*=3) biological replicates.

FC showed that the 3 models caused the same extent of membrane damage to all strains. Lower H_2O_2 concentration in the model caused gradual destruction of cell membranes over the 2 days of treatment. When the H_2O_2 concentration increased, more than 90% of the population was PI/BOX permeable soon after the treatment (Figure 2.12). Although all strains showed similar extent of membrane lesions only the growth of catalase mutants was arrested. This suggests that the effect of model honey is mostly relies on the impairment of intracellular functions rather than the membrane permeability. However, this cannot rule out the importance of the membrane integrity for the cell viability.

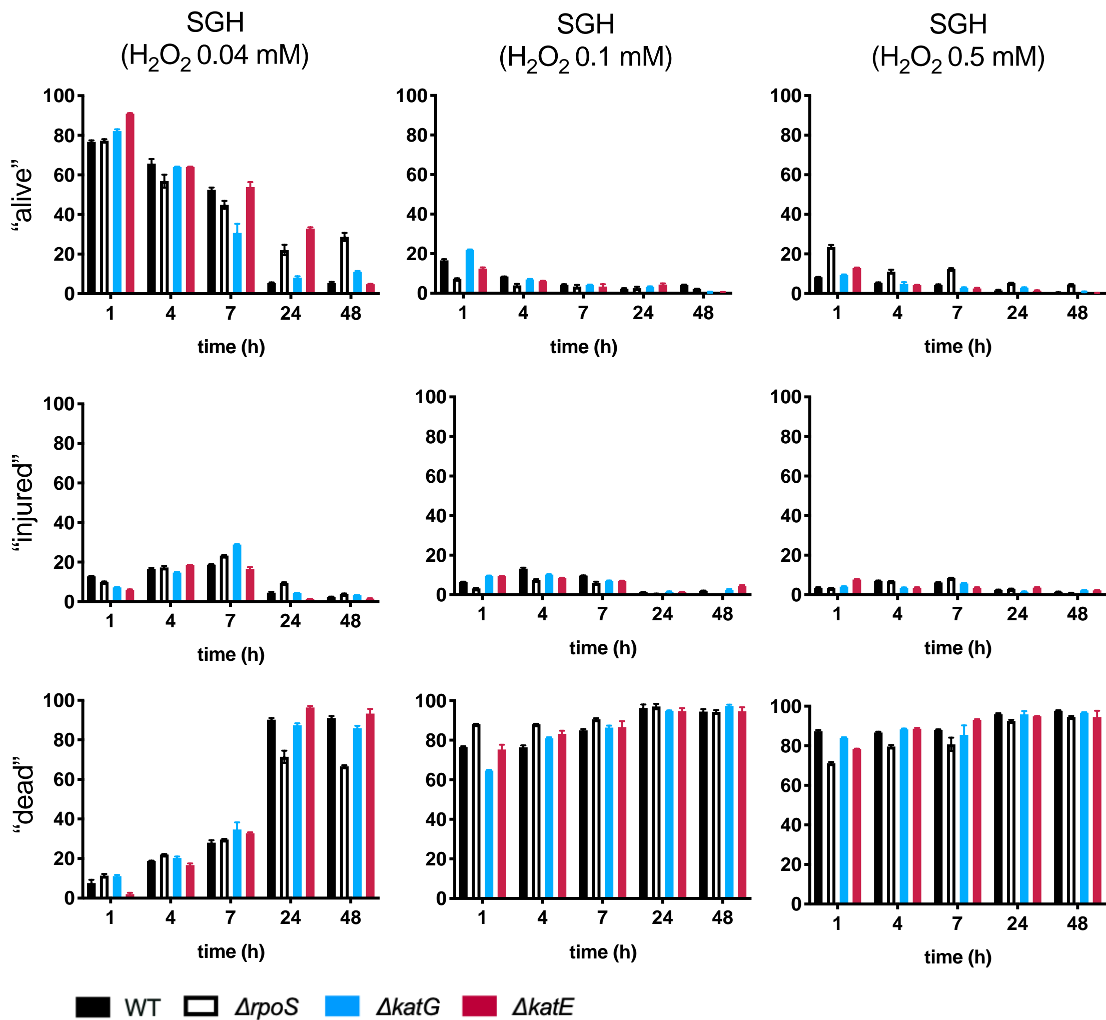


Figure 2.12 FC analysis of *E. coli* WT, *rpoS*, *katG* and *katE* mutants exposed to model honeys of increasing H_2O_2 concentration. Mean percentage of "alive" (PI^-/BOX^-), "injured" (or depolarised) (BOX^+/PI^-), and "dead" (or membrane destructed) (BOX^+/PI^+) are shown for cells exposed to three model honeys (SGH). Error bars represent the average ± s.d. (n=3; biological replicates).

As was seen in previous paragraph, H_2O_2 is a precursor for the generation of other ROS species which maintain the antimicrobial activity of honey while the H_2O_2 is degraded. Thus, we tested whether catalase mutants accumulate higher amount of intracellular ROS comparing to WT and the *rpoS* mutant, which both detoxify the H_2O_2. Results showed that the higher susceptibility of catalase mutants is possibly due to higher ROS accumulation. As seen in Figure 2.13, ROS content was significantly ($p<0.0001$) higher in both catalase mutants 2h after exposure to model honey (0.5 mM H_2O_2). However, the *katE* mutant, which showed higher susceptibility to increasing H_2O_2 compared to Δ*katG*, accumulated significantly ($p<0.0001$) higher ROS 24 h later. The same results were seen post-treatment with acacia honey which generated equal levels of H_2O_2 (1 mM H_2O_2) (Figure 2.13).

Survival curves and the accumulation of ROS in the *rpoS* mutant suggested that this strain has a ROS-scavenging mechanism equivalent to that of the WT which can degrade H_2O_2 faster and prevent from extended oxidative damage. In contrast, the lack of catalase activity in Δ*katG* and Δ*katE* caused the opposite effect. In particular, *katE* mutant demonstrated significantly higher susceptibility to increasing H_2O_2.

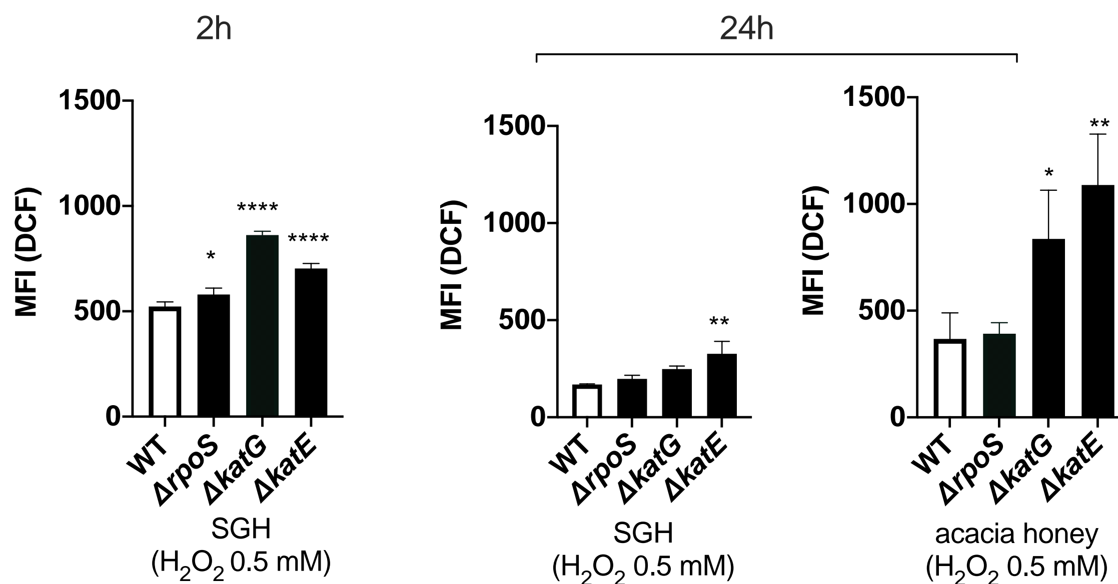


Figure 2.13 Intracellular ROS accumulation in WT, *rpoS* and catalase mutants after the treatment with model and real honey. Mean fluorescence intensity (MFI) of DCF indicates the ROS accumulation within the 4 strains, 2h and 24h post exposure to model (SGH) and real honey (25% acacia, 0.5 mM H_2O_2). One-way ANOVA test was used to compare the significance in ROS accumulation between the WT and the mutants; Dunnett's multiple comparison test (95% CI, significance p<0.05), SGH (2h) (* p=0.0465, **** p<0.0001), SGH (24h) (* p=0.0298, ** p=0.0028), acacia honey (24h) (** p=0.0013). Error bars represent the mean ± s.d. (*n*=3; biological replicates).

These results suggest that catalase activity is important for the bacterial resistance to honey. Interestingly, model honeys (of variable H_2O_2 concentration) induced a moderate antibacterial effect on both catalase-positive WT and *rpoS* mutant. Although this effect was not significant it showed that honey can still exert bactericidal effect even when bacteria possess H_2O_2-degrading mechanisms. It is possible that catalase activity cannot degrade with the same efficiency higher concentration of H_2O_2. Thus, honeys with higher peroxide activity can accelerate the antimicrobial activity either by direct damage of cellular components and/or by the generation of more toxic radicals via the Fenton reaction.

2.4. Discussion

This study aimed to explain the antibacterial activity of H_2O_2-producing honeys. The hypothesis was based on the fact that glucose oxidation, on honey dilution, generates H_2O_2 and gluconic acid. A model honey composed of the three major honey stressors (sugars, gluconic acid, H_2O_2) was used for this investigation.

Each of the stressors exerted a distinct antibacterial mechanism. Sugars did not significantly affect the bacterial growth. This is in agreement with other studies which showed that sugars exert plasmolytic effect without arresting bacterial growth (Csonka., 1989; Korber et al., 1996; Bogdanov, 1984 & 1997; Shamala et al., 2002; Blair et al.,2009; Kwakman et al., 2010; Nishio et al., 2015). However, osmotic stress caused physiological changes such as cell wall destruction, membrane depolarization and reduction of the cell volume. Membrane depolarisation was possibly caused by the binding of solutes to periplasmic proteins which altered their anionic charge (Stock et al., 1977). The same author showed that osmotic upshift caused the collapse of the outer membrane. This collapse and the decrease of cell volume is probably caused by a pressure drop across the cell envelope which results in separation of the inner and outer membrane (Pilizota and Shaevitz, 2012; 2013). Considering these findings it is sensible to speculate that plasmolytic effects made the sugar-treated cells permeable to PI. However, 80% of the population was culturable which showed that sugar-induced stress is not sufficient to arrest cells growth.

In contrast to sugars, both gluconic acid and H_2O_2 induced bactericidal effect on exponentially growing *E. coli*. Treatment with gluconic acid caused a dose-

dependent depolarization of cell membrane, an effect which arrested the cell growth. Prolonged (>3h) exposure to gluconic acid increased also the percentage of PI^+ cells. Considering that gluconic acid is a highly polar molecule, unable to penetrate the cell membrane is sensible to speculate that the antibacterial effect was due to the low pH alone (Breidt et al., 2004). In this case, the antibacterial effect is caused by accumulation of acid anion in the cytoplasm because of the difference between the external and cytoplasmic pH (Russell, 1992). The proton efflux mechanism releases the same protons to maintain the intracellular equilibrium, a mechanism which affects the membrane polarity. In consistence with this, here it was seen that the depolarization effect increased at higher concentrations of gluconic acid (higher protons concentration). Also, the increase of internal anionic strength has been shown to affect the function of cellular metabolic enzymes (i.e. transport proteins, lactate dehydrogenase, chelate ions) and the integrity of the outer membrane (Breidt et al., 2004). Considering this, it was assumed that disruption of cellular functions failed to maintain the membrane integrity, thus, the prolonged exposure to gluconic acid caused increase of PI permeable cells.

Treatment with H_2O_2 caused a dose-dependent bactericidal effect by simultaneous depolarization and destruction of the bacterial membrane. As has been shown, H_2O_2 up to 30 μM does not affect the bacterial membrane integrity (Uhl and Dukan, 2016) while at higher concentrations H_2O_2 diffuses relatively easy within cells (Bienert et al., 2006). During H_2O_2 diffusion, ROS readily attack the polyunsaturated fatty acids of cell membrane which results in significant tissue damage by proliferation of oxidative chain reactions (Vatansever et al., 2013). This can possibly explain the irreparable membrane lesions observed during all the

H_2O_2-treatments (0.04-5.4 mM). Here, it was also demonstrated that H_2O_2 caused membrane depolarisation in dose-dependent manner which gradually increased over the time of exposure. This could be explained by the fact that transport channels control the permeability of H_2O_2 across the membrane and protect this from a permanent damage (Yost and Joshi, 2015). The same authors showed that increasing ROS concentration caused dose-dependent depolarization with eventual membrane destruction. Considering the above it was speculated that both depolarization and destruction of the membrane are related to the diffusion of H_2O_2 within the cell. However, the concentration of H_2O_2 applied controls the cell damage and viability. Therefore, higher H_2O_2 arrested cell growth by depolarization, destruction of cell wall hence DNA damage and oxidation of other macromolecules (proteins, enzymes, DNA etc.) (Imlay and Linn, 1986).

The synergy of the gluconic acid and H_2O_2 caused a significant bactericidal effect soon after the treatment. This is explained by increased toxicity of the H_2O_2 in presence of low pH (Rafellini et al., 2007). According to Van Haute (2015), decomposition of H_2O_2 produces OH radicals which become more toxic in presence of organic acids. In agreement to this, a recent study showed that synergy of H_2O_2 and organic acid caused significant logarithmic reduction of *E. coli* O157:H7 (Valiolahi et al., 2019) while this effect was mitigated after the addition of NaCl. Likewise, here it was seen that presence of sugars in the model mediated the toxicity of the other two components. However, AFM images showed that the synergy of the three stressors significantly reduced the cell volume causing severe destruction of cell envelop.

The effects of model honey were confirmed by heather and acacia honeys. All honey dilutions which yielded higher than 1mM of H_2O_2 caused membrane depolarisation and destruction likewise it was seen with the model honey. However, only honeys which accumulated up to 2 mM H_2O_2 arrested cells growth. This suggests that low H_2O_2 can only cause a transient bacterial damage which is accelerated in presence of low pH. However, bacterial catalase activity can degrade the H_2O_2 hence treated cells can resume growth using the available carbon sources of honey sugars. In contrast, for higher H_2O_2 concentration is possible that catalase cannot exert the same degradation mechanism (Imlay, 2015). Thus, the remaining H_2O_2 can be used by Fenton reaction which produces more reactive and toxic radical species. In agreement to these findings, this study showed that lower H_2O_2 (<1 mM) is quickly degraded while the prolonged exposure of bacteria to honeys with higher peroxide activity increased the intracellular accumulation of ROS. As was mentioned previously, H_2O_2 serves as active intermediate of Fenton reaction that promotes the ROS generation (Sharma et al., 2012). Transition metal ions (Fe^{2+}, Cu^{2+}) found in honey enhance the Fenton reaction, increasing the accumulation of •OH (Brudzynski et al., 2012a). The abundance of honey in polyphenols and transition metals (i.e. Cu(I), Fe(II)) drive the generation of ROS enhancing the antibacterial strength of honey (Bogdanov et al., 2007; Brudzynski & Lannigan, 2012; Imlay, 2015). Therefore, the higher antimicrobial activity of heather honey is may due to its composition. However, since the composition of both honeys is uncharacterised, no further assumptions can be made.

Susceptibility of catalase mutants (*katG*, *katE*), to models of increasing H_2O_2 concentration, confirmed the H_2O_2-induced oxidative stress in honey. As it was

shown in previous study, the H_2O_2 concentration is controlled by the balance between H_2O_2 production and its degradation by Ahp (78%), catalase (12%) and membrane permeability (10%). However, at H_2O_2 concentrations higher than 30 μM, bacteria only employ the catalase activity to defend the exogenously applied oxidative stress (Uhl and Ducan, 2016). Null *rpoS* strain demonstrated similar sensitivity to the WT which is explained either by the low expression of *rpoS* in exponentially growing cells or by the existence of OxyR regulon which activates the *katG* and *ahpC* for the detoxification of H_2O_2 in logarithmic growing cells (Jung et al., 2003). FC analysis showed that increased H_2O_2 caused immediately membrane destruction to all strains. In contrast to catalase mutants, it appeared that detoxifying activity of Δ*rpoS* and WT prevented cells from further oxidative damage (i.e. DNA/protein damage) (Dong et al., 2008). These observations suggest that prolonged presence of H_2O_2 within the cells causes further oxidative damage, rather the membrane destruction, which arrests cells growth.

Overall this chapter, by successfully using a simulation model, gave an explanation of the bacterial physiological changes induced by H_2O_2-producing honeys. It was demonstrated that the synergy of the H_2O_2 and the gluconic acid is responsible for the antibacterial activity of honey. This combination caused simultaneous membrane disintegration and depolarization, two events perquisites for cell death. However, *E. coli* was able to resume growth unless H_2O_2 was enough to cause sustain ROS generation which further caused irreparable cell damage. This was confirmed by catalase mutants in which the excess of H_2O_2 increased the bactericidal effect of honey by the sustained presence of ROS within the cell.

Although the phenotype of two mutants has been quiet informative for the role of oxidative stress in honey, it cannot explain the synergies underlying its activity. Thus, in Chapter 3 a high-throughput technique is used in order to measure the survival dynamics within the whole *E. coli* K-12 genome and acquire a better understanding of the antibacterial mechanism of honey.

CHAPTER 3

Molecular targets of honey: Use of TraDIS to identify conditionally essential genes required for resistance of *E. coli* K-12 to honey

Declaration:

The focus of this chapter is the design of TraDIS experiment and the analysis of the generated data. The initial TraDIS experiment (design, optimization, growth and sequencing of the library was completed by me. The construction of the transposon library, the mapping of TraDIS data to the MG1655 genome and the enrichment analysis were conducted by Mat Milner. The subsequent analysis presented in this chapter was completed by me.

3.1. Introduction

The previous chapter described the fundamental investigation of the antimicrobial activity of honey focusing on the phenotypic responses of *E. coli* K-12 to osmotic, acid, oxidative stress and their synergy. The significant susceptibility of catalase mutants compared to the WT proved the role of honey-induced oxidative stress. However, the antimicrobial effect was seen to be a result of the synergy induced by honey components rather than the H_2O_2 itself.

A possible way to have a better understanding of how bacteria are “killed” by honey would be to screen mutants of different stress-responsive regulons against the model honey. Generation and screening of several single-deletion mutants would be a time-consuming and laborious process which potentially would overlook some of honey’s targets. Although a few assumptions on the antimicrobial mechanism of honey was made, we still do not know all the possible targets. For this reason, here Transposon Directed Insertion-site sequencing (TraDIS) is used, a negative selection assay which sequences in parallel a massive number of mutants thereby identifying putative gene functions under specific conditions.

Exploration of the genome-wide responses holds the key to understand the bacterial capacity to adapt and survive in honey. This will undoubtedly yield additional understanding on the antibacterial mechanism of honey and will reveal other unidentified potential uses as antimicrobial compound.

3.1.1. Transposon Directed Insertion Sequencing (TraDIS) method

Transposon Directed Insertion Sequencing is a genome-wide mutagenesis method by which every non-essential gene in the genome can be analysed in terms of its contribution to fitness in a particular environment. Fitness is a measure of how well a cell competes with others to reproduce. A cell with lower or very low fitness will be outcompeted or completely disappeared from the population. The genome-wide mutagenesis allows the screen of millions of single-transposon mutants for a multitude of conditions.

Single gene deletion libraries are considered the gold standard for the identification of genes essentiality. Transposon insertion library contains a set of mutants which collectively have transposon insertions in all non-essential genes. These mutants are created by the insertion of transposon within a gene. Then the function of this gene will be disrupted (Hayes, 2003). Each gene can potentially contain hundreds of individual transposon insertions. This is dependent on the density of the library. The increased insertion number (compared to the control) indicates increased survival rate of the mutant while the opposite indicates that the mutant has low fitness to the specific growth conditions. The quantitative analysis of TraDIS data determines the positions and the frequencies of transposons in every gene of the whole population which results in a list of essential genes. Essential genes are defined these genes required for cell growth, proliferation and survival. Therefore, a deletion of an essential gene eventually leads to cell death or severe proliferation defect (Liu et al., 2015). Also, there are genes required for growth under specific environmental and nutritional conditions (i.e. a gene that codes for a protein essential at a specific growth phase or

nutrient availability). These are known as conditionally essential genes. In contrast to essential genes, deletion of a conditionally essential gene may cause loss of fitness under a tested condition only. If the exposure to certain condition causes killing of the mutant, then the respective gene is defined as "conditionally essential". In contrast, slower growing rate or faster killing of the mutants after the exposure to the condition, suggests the "loss of fitness" genes. Likewise, in this study, we defined as "loss of fitness" genes, the ones whose deletion caused a growth defect when *E. coli* is exposed to honey. Also, there are genes whose deletion confers resistance or increases the fitness under the same condition. These genes are the resistome of honey. In fact, resistome is a set of genes whose functions directly or indirectly block the activity of an antimicrobial (i.e. antibiotic) (Wright, 2007). In this experiment, we considered as resistome the mutants which cause a growth advantage on honey.

In this study, we adapted TraDIS in order to identify the "loss of fitness" genes. This analysis is expected to show the resistome of honey. The method also provides a system-level view of the independent gene networks that respond to honey stress.

3.1.2. An overview of the TraDIS experiment

TraDIS simultaneously screens thousands of transposon mutants and maps the location and the frequency of the transposon insertions using next generation sequencing technology (Langridge et al., 2009).

The overview of the TraDIS method is given in Figure 3.1. The first step is the construction of the library. As previously mentioned, Transposon insertion library contains a group of mutants which collectively have transposon insertions in all non-

essential genes. Electro-transformation by a mini-Tn*5*, usually used for the construction of *E. coli* library, results in the random insertion of a single transposon to each bacterial cell (Nováková et al., 2014). Tn*5* transposes at a high frequency into a wide range of Gram-negative bacteria with low target/specificity resulting in dense transposon mutant libraries. Transposition occurring by Tn*5* transposon is a “cut and paste” mechanism where the engineered DNA fragment is inserted into a host chromosome (Figure 3.2). This nucleic acid sequence is flanked by the inverted repeats (IR) of a transposon. The transposition is catalysed by the transposase, an enzyme encoded within the Tn*5* sequence or provided exogenously. Transposase recognises these inverted repeats (IRs) flanking the transposon sequence and mobilizes the IR-IR structure to another DNA region or replicon. The transposase is not mobilised with the transposon, thus once inserted it cannot be re-transposase elsewhere (Christie-Oleza et al., 2013). The transposon inserts via a staggered cut in the target DNA and the targeted gene is deactivated. Both sides of DNA, in the spot of inserted transposon, are duplicated by 9 bp (Berg et al., 1983; Reznikoff, 1993). Successful transformants are selected on a supplemented medium and the resulted colonies are pooled to form a library of transposon mutants. For the identification of the transposon insertion sites, gDNA is extracted from the pooled mutants and the transposon junction is sequenced. The data obtained are mapped to a reference genome and the presence or absence of insertions sites within the genome is used for the identification of essential and nonessential genes. In this study, we used the MG1655 transposon library generated by Mathew Milner (School of Biosciences). This transposon library was created by transformation of a mini-Tn*5* transposon with a kanamycin resistance cassette into competent cells before grow overnight into a

selective medium. The pooled colonies created a library composed of ~700,000 million mutants corresponding to 450,581 unique insertions.

The identification of “loss of fitness” genes is an extension of the TraDIS method described above, which also involves the exposure of the transposon library to a “condition” or environment (i.e. honey). The quantitative analysis of the location and the frequency of transposon insertions allows for the identification of genes that confer an advantageous or disadvantageous fitness after the treatment of library with honey.

Here, the transposon mutant library was exposed to the model honey (condition tested) (Figure 3.3). Early bacterial responses to antimicrobial compounds (i.e. honey) is a key step to consider in the treatment of infectious diseases (Dupont et al, 2007). Thus, based on previous findings (Chapter 2) which showed that the major phenotypic changes are induced during the 2 hours of exposure to model, the mutant library was exposed to model honey for 30 and 90 minutes and then allowed to grow in LB broth for two hours (~5 to 6 generations). After the exposure of library to honey, there are mutants that may either have lost fitness or may have grown at different rates (i.e. slower growth or overgrowth). The outgrowth step allows the growth of honey-resistant and elimination honey-sensitive mutants respectively (Phan et al., 2013). Also, this allows the recovery of gDNA enough for the TraDIS procedure. The gDNA from the control and treated samples was sequenced using the Illumina multiplexed TraDIS procedure as described in § 3.2.2.3-3.2.2.5 (Figure 3.4).

The TraDIS analysis resulted in a list of “loss of fitness” genes which in their majority are common between the two time-points tested. Only genes which appeared in both lists (30’ and 90’) and the genes appeared in the 90’ list were considered as “loss of function” genes. Apart from the genes which came up as significant for their

decrease in fitness, genes in which the transposon insertion led to the strain having a growth advantage were also identified. As was previously mentioned, these genes consist the resistome of honey. On that basis, we focused on the roles of individual genes within the cell and then we performed a functional enrichment analysis in order to identify whether genes of interest are more often (statistical significantly) associated to certain biological functions.

This chapter starts with a preliminary optimization step aiming to the identification of the suitable dose of model honey (SGH) that induces a logarithmic reduction, without a complete elimination of the population during the treatment. Later, the 2h-outgrowth step aims to examine the phenotypic changes that happen after the stressed population was supplemented and grown in rich medium. The analysis of TraDIS data results in a list of genes in which transposon insertions caused a significant growth advantage or disadvantage to *E.coli* after exposure to honey. Their function within the cell and their involvement in biological pathways is extensively reported. In order to confirm the TraDIS results, single mutants were constructed and competed against the WT under the same conditions as applied to TraDIS experiment. This chapter will conclude with a discussion on *E. coli* stress responses to honey and a proposal on the antibacterial underlying mechanism.

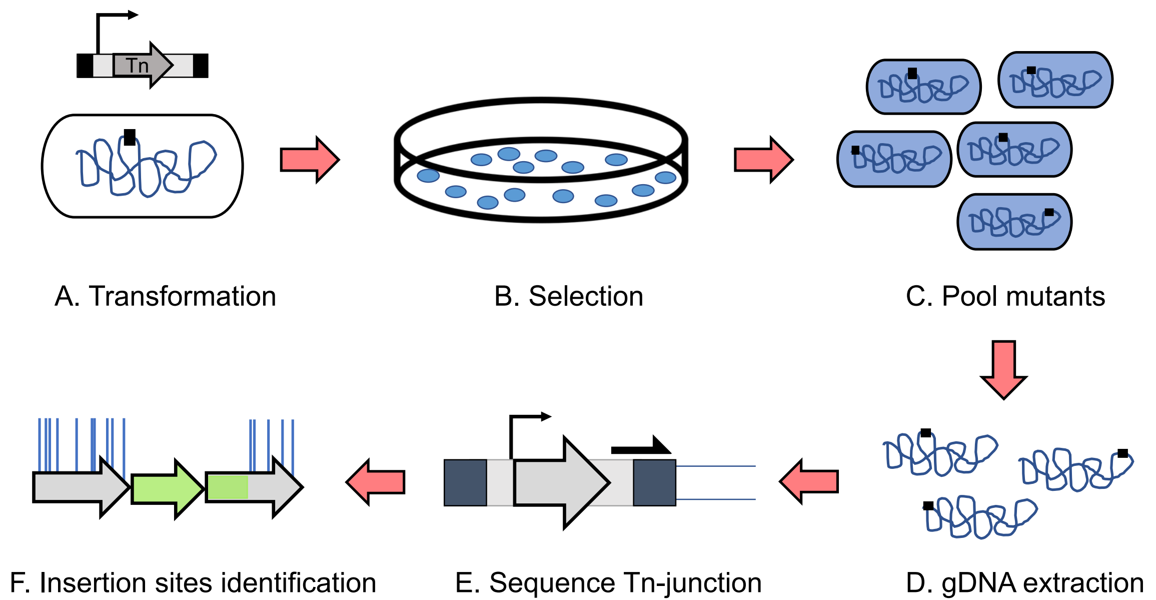


Figure 3.1 Construction of the transposon library and overview of TraDIS experiment. The steps of a general TraDIS experiment start with (A) the construction of the mini-Tn5 transposon library by transformation of a single Tn-5 transposon per cell followed by (B) the selection of successful transformants on an agar plate. (C) Transformed mutants are pooled and (D) gDNA is extracted. (E) Sequencing of the transposon junction is to identify the position of the transposon insertion site. (F) Insertion data are compared to a reference genome and the presence and absence of insertion sites indicates the nonessential (grey) and essential genes or genes with essential regions (green) respectively.

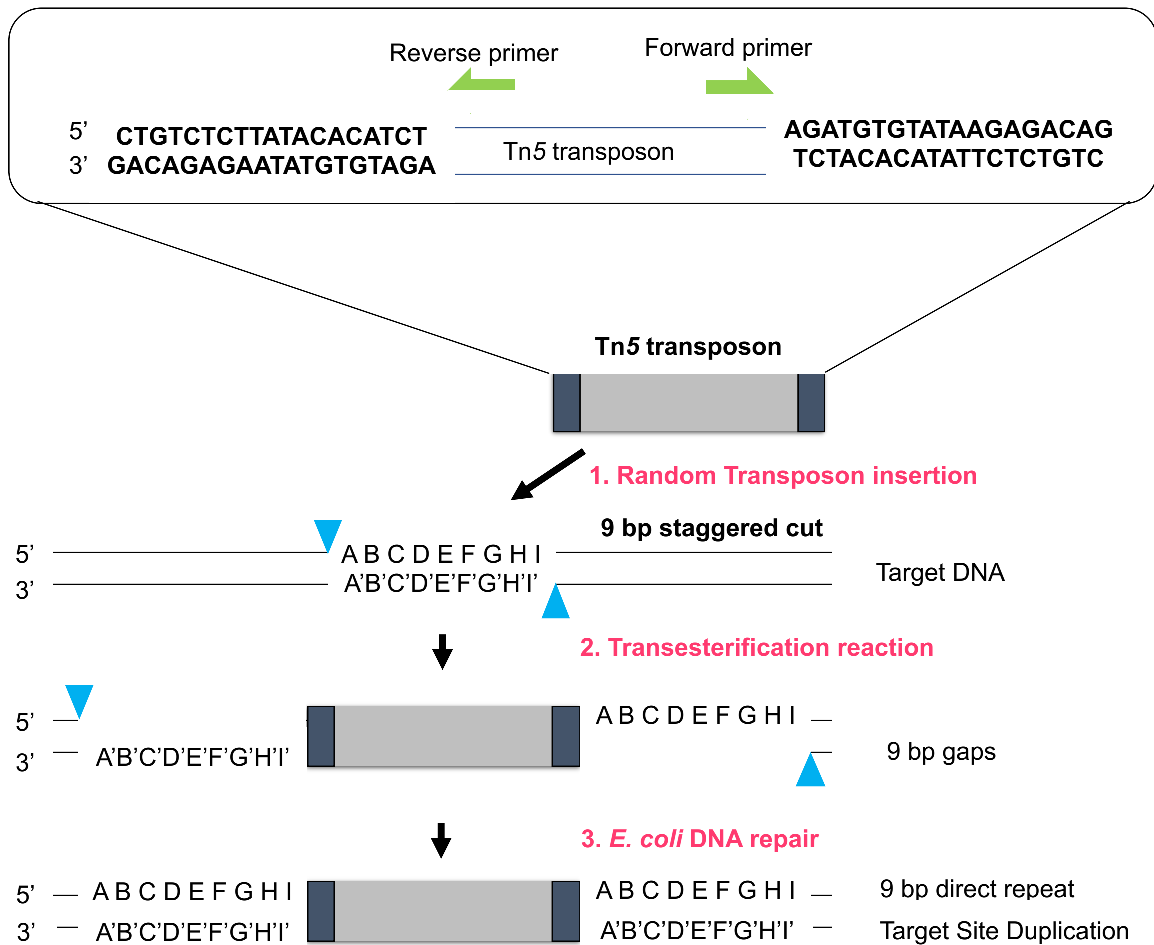


Figure 3.2 Tn*5* transposon insertion mechanism. Tn*5* transposon structure consists of 19 bp end sequence in left and right flanking regions. The insertion starts with the transposase nicking the 3' and 5' end of the transposon on transposon's DNA strands. (1) Transposase nicks also the 5' and 3' end of the target DNA. (2) The 5' end of the transposon is ligated to the 3' of the target sequence and the 3' end of the transposon is ligated to the 5' end of the target sequence. (3) The ssDNA is filled by DNA replication on both strands resulting in a direct repeat of the flanking target sequences.

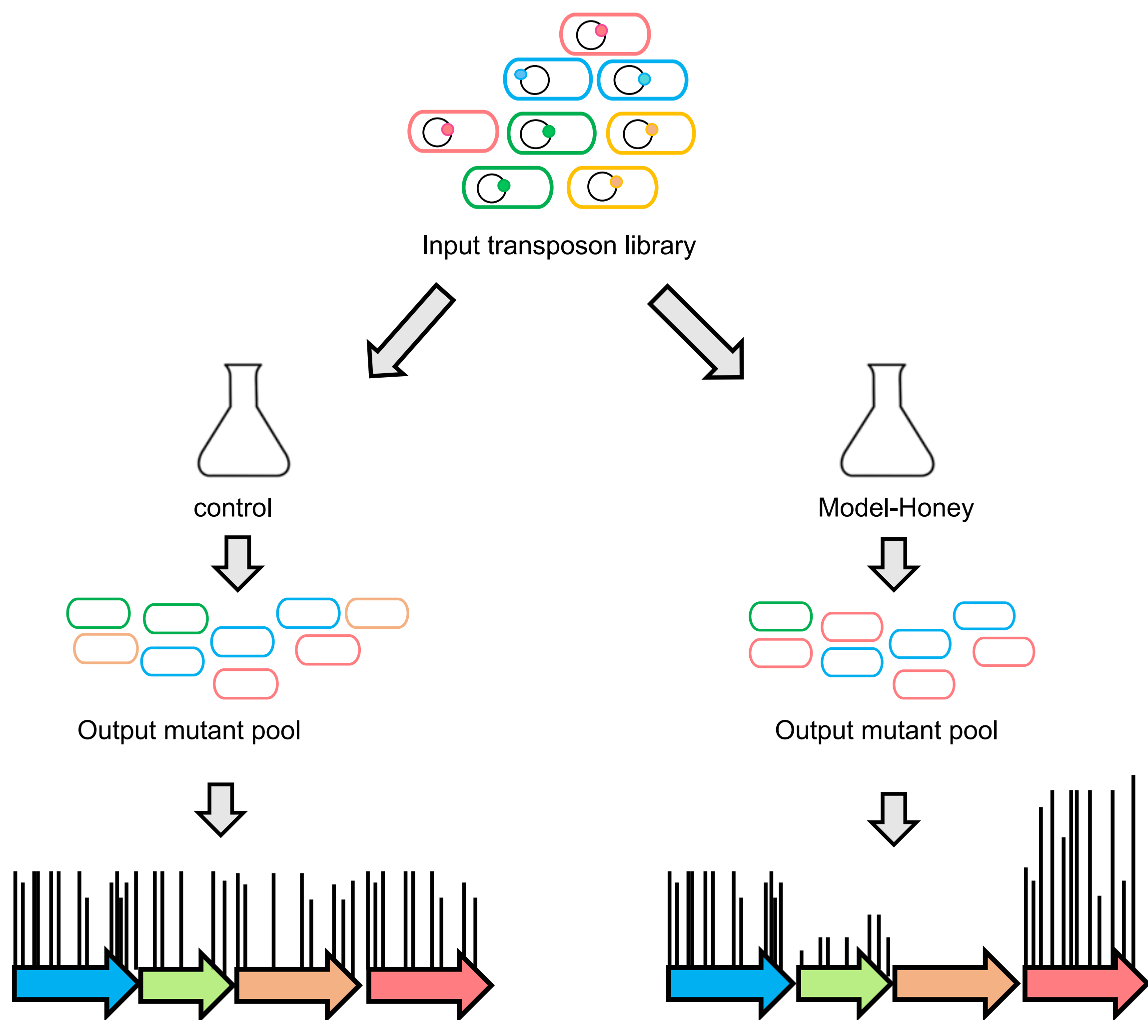


Figure 3.3 TraDIS for the identification of conditionally essential genes. Differences in insertion sequence abundance are compared between the unexposed (left) and model honey exposed library (right). Selection posed by model honey results in the loss of some mutants (orange) and enrichment of others (red) within the bacterial population. Also, honey had a neutral effect on some mutants (blue). Insertion frequency can be used as a measure of mutant abundance. Comparison of the input and output pools, between control and honey treated library, identifies “loss of fitness” genes (green, orange), genes that confer a selective advantage under the tested condition (red) and genes with no significant effect on the fitness of the population (blue).

3.2. Materials and Methods

3.2.1. Bacterial strains

Bacterial strains used in this study are shown in Table 3.1. *E. coli* MG1655 was used for the TraDIS experiment and the construction of the knockouts (KOs), as a recipient strain, from the Keio library collection. *E. coli* K-12 strain BW25113 is the parent strain for the Keio collection and the donor strain in the P1 phage transduction.

Table 3.1: Strains used in this study and source of reference

Strain	Description	Source
BW25113	Parent strain of the Keio collection	Datsenko & Wanner, 2000
MG1655	Reference strain	Discuva Ltd, Cambridge
MG1655 *fdoH::kan*	*fdoH replaced by kan cassette*	This study *
MG1655 *fdhD::kan*	*fdhD replaced by kan cassette*	This study *
MG1655 *moaA::kan*	*moaA replaced by kan cassette*	This study *
MG1655 *moaB::kan*	*moaB replaced by kan cassette*	This study *
MG1655 *moaC::kan*	*moaC replaced by kan cassette*	This study *
MG1655 *wecA::kan*	*wecA replaced by kan cassette*	This study *
MG1655 *wecC::kan*	*wecC replaced by kan cassette*	This study *
MG1655 *wecG::kan*	*wecG replaced by kan cassette*	This study *
MG1655 *gor::kan*	*gor replaced by kan cassette*	This study *
MG1655 *prc::kan*	*prc replaced by kan cassette*	This study *
MG1655 *ompR::kan*	*ompR replaced by kan cassette*	This study *

*These strains were obtained from Keio collection by P1 phage transduction.

3.2.2. TraDIS experiment and DNA isolation

DNA was extracted from the transposon library post-treatment with the model honey and post-activation in LB for 2 h. 10 µL of the initial library (~2.4 x 10^11 CFU/mL) were inoculated in 50 mL LB (OD_{600} ~0.05) and 5 mL of culture (OD_{600} ~1) was harvested by centrifugation at 4,000 x g for 10 min at 4°C (activation step). 50 mL LB were inoculated with 500 µL of the activated library (OD_{600}~0.05) and 10 mL of exponentially grown culture was harvested by centrifugation at 4,000 x g (10 min at 4°C) and washed twice in PBS. The OD_{600} was adjusted to 1. The library (1 mL) was mixed 1:1 with the model honey (SGH; sugars: 30%, gluconic acid: 8.6 mM, H_2O_2: 3 mM) and incubated for 30 and 90 min (in duplicates). The procedure was performed in parallel with control samples where model honey was replaced by PBS. Honey-treated library and control was harvested by centrifugation at 3,000 x g for 3 min, resuspended in equal volume (2 mL) of fresh LB and were grown at 37°C for 2 h at 150 rpm. Genomic DNA (gDNA) was extracted from the cell pellets using the STRATEC RTP Bacteria DNA Mini kit following the protocol for Gram-negative bacteria.

3.2.2.1. DNA fragmentation

To process gDNA for TraDIS sequencing a series of steps are followed. Quantification of gDNA was done using the Qubit method as described in § 3.2.2.2. The gDNA was diluted in Nuclease-Free water (Ambion) (1:500) and incubated on ice for 10 min. A bioruptor (Diagenode) was used to shear DNA samples mechanically by pulses of ultrasound waves through an ice-cold water bath. The shearing profile used was 30 s ON, 90 s OFF at low intensity to fragment DNA to an average length of ~300

bp. Following shearing, samples were condensed using a vacuum concentrator (Eppendorf, concentrator 5301) to a final volume of 55.5 μL.

3.2.2.2. Qubit quantification of DNA

DNA was quantified using Qubit^{TM} dsDNA HS Assay kit (Invitrogen) following the product protocol at a 1:1 ratio of DNA to dye using 1 μL of the sample.

3.2.2.3. Preparation of transposon library for TraDIS sequencing

Steps followed for TraDIS sequencing are outlined in Figure 3.4. NEBNext Ultra I kit (New England Biolabs) was used to repair the ends of the fragmented DNA and ligate an adaptor to the newly prepared DNA fragments end, according to kit instructions. AMPure XP SPRI beads (Beckman Coulter) were used for purifying the gDNA by two-sided selection of fragments (250 bp). At the final elution step samples were eluted in 16 μL of nuclease free water. Next was the enrichment of the transposon junction fragments. The first PCR step amplified DNA between a custom forward primer that annealed to the transposon end and a reverse primer that annealed to the ligated adaptor, to amplify the transposon-gDNA junction. The PCR reaction contained 25 μL 2x KAPA HiFi DNA Polymerase (Kapa Biosystems), 2.5 μL TnC/K_P1.F1 (10 μM), 2.5 μL TnC_P1.R (10 μM), 15 μL sample and 5 μL nuclease-free water and was amplified using the following cycle conditions: 98°C, 48 s; (98°C, 15 s; 65°C, 30 s; 72°C, 30 s) x10; 72°C, 1 min; 4°C hold. The PCR product was then purified using SPRI beads at a ratio of 0.9:1 beads:sample. The second PCR step prepared the sample for

TraDIS sequencing by the addition of flow cell adaptors. This step allows the sample to bind to the MiSeq flow cell and provide a priming site for the sequencing primers (Figure 3.4: all primers aligned to gDNA sequence). The PCR reaction contained 25 µl 2X HiFi DNA Polymerase (Kapa), 2.5 µL custom forward primer (TnC/K_.X.Y; 10 µM), 10 µM NEBNext Index Reverse primer, 15 µL sample made up to a total reaction volume of 50 µL with nuclease-free water. The PCR product was purified using SPRI beads at 0.9:1 beads:sample ratio and eluted in 33 µL nuclease-free water. Finally, 32 µL sample was transferred to a new 1.5 mL microcentrifuge tube and stored at -20°C for future sequencing.

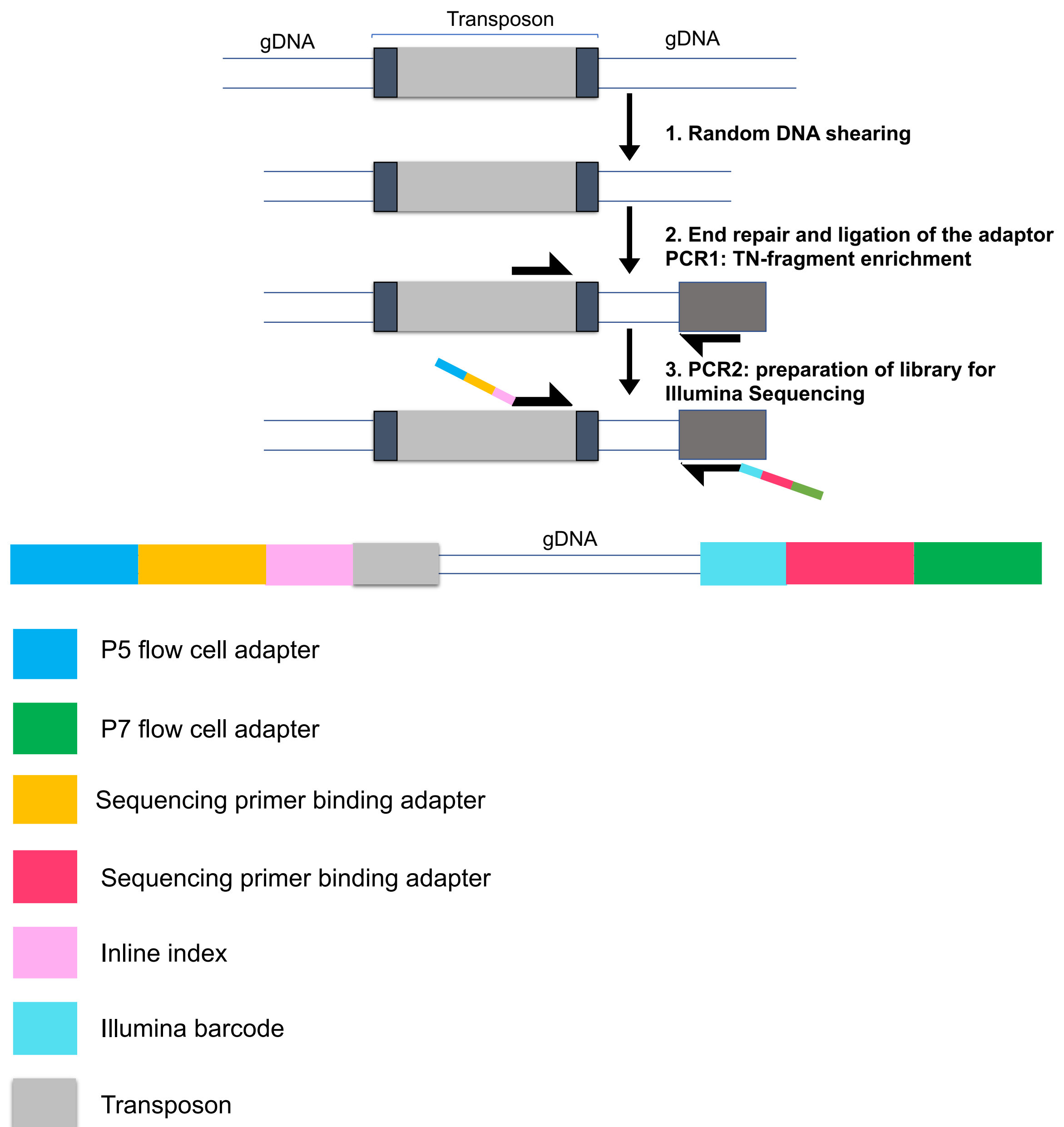


Figure 3.4 Steps for the preparation of DNA fragment for sequencing. The transposon gDNA is amplified first with primers specific to the transposon (PCR1). The second amplification step (PCR2) uses sequencing specific adapters and barcodes which allows the sample to bind to the MiSeq flow cell.

3.2.2.4. Quantification of sequencing libraries using qPCR

Genomic libraries ready for Illumina sequencing were quantified using the Library Quant Kit (Illumina) Universal qPCR Mix (Kapa Biosystems), following the kit instructions for 10 µL volume reactions. As standard, 3 independent replicates of the library were quantified at 2 different dilutions: 1/50,000 and 1/500,000, and a no template control was used as a blank control. Samples were quantified using a Mx3005P qPCR system (Agilent Technologies) and the thermal profile used was: 95°C for 5 min, then 35 cycles of 95°C for 30 sec followed by 60°C for 30 sec, including a dissociation melt curve from 65°C to 95°C to assess sample quality.

3.2.2.5. MiSeq sequencing of the transposon junction

The library was diluted to 8 nM stock concentration. Equal volumes (1.5 µL) of each sample were combined to a nuclease-free 1.5 mL microcentrifuge tube. The library was denaturated, diluted (according to Illumina instructions) and inoculated with 5% denaturated ΦX174 library (20 pM). The sample was load to the MiSeq (Illumina) flow cell. The optimal cluster density aimed is 1,000 clusters/mm^2.

3.2.2.6. TraDIS data processing and analysis

ESSENTIALS: the software for high throughput Transposon Insertion Sequencing data was used to identify conditionally essential genes with a significant Log2 fold change in total reads when compared to the control (Figure 3.3). Transposon insertion sites were mapped on *E. coli* K-12 (reference genome) and a measurement

of fitness for every KO was generated after comparison between the challenged and the control condition. ESSENTIALS use the EdgeR, a negative polynomial model distribution model. EdgeR models count data using an over-dispersed Poisson model and a Bayesian procedure to moderate the degree of over-dispersion within the genes (Zomer et al., 2012). The required input data are the table of counts and two vectors which annotate the experimental data (condition tested) and the library size. Figure 3.5 describes the steps followed in the processing and analysis of TraDIS results.

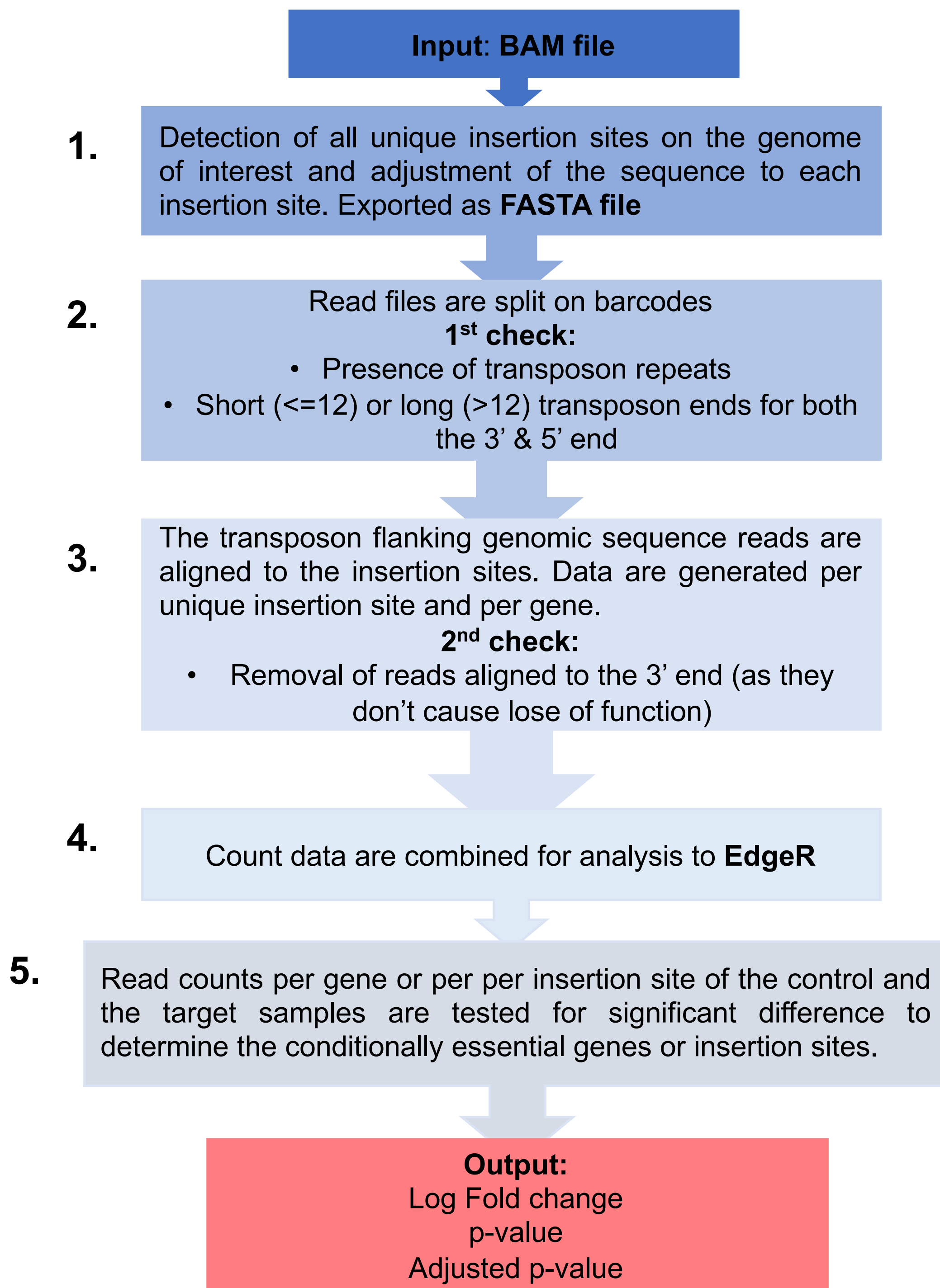


Figure 3.5 Flow graph of the TraDIS data processing. The consecutive steps of filtering (2, 3), normalisation (4) and statistical tests (5) performed by ESSENTIALS which results in identification of conditionally essential genes and the insertion sites.

3.2.3. P1 phage transduction

P1 transduction was used to transfer the kanamycin resistance cassette from the Keio collection of donor strains into *E. coli* K-12 like it was described in Chapter 2.

3.2.3.1. Validation of P1 transduction by colony PCR

The deletion of the desired gene in each of the KOs strains was validated by colony PCR as was described in Chapter 2. The oligonucleotides used in this chapter are listed in Table 3.2.

Table 3.2: Oligonucleotides used in this study

Gene name	Primer
fdoH	3’ CCTTGTGAATGTGGAAAAGGTG 5’ 5’ ATGAAGCAGAAGGCGGTGAT 3’
fdhD	3’ CAGAATACCATAATGTTGGTGTGTG 5’ 3’ AGTCCTGCAATTTAATATATTTCGCAG 5’
moaA	3’ CCAGGGCGAAGGAAGAAATGAC 5’ 5’ GATAAATTCAGTGCTTACCTGACTCATCTG 3’
moaB	3’ GCGCTGGAAGATGGCAAATTACTG 5’ 5’ GACTTGAGCCATATAAGTTTGCAAG 3’
moaC	3’ AACAAAACGCTGATTTTCGCCATG 5’ 5’ AAATCCGCAGCCACTTCGGTTG 3’
wecA	3’ GAATAAAGGTCTTCGTGGTTATACTTCTG 5’ 5’ CTCCACTCCTGACGAGCAAAAAACGTATA 3’
wecC	3’ ATAAGCAGCGAATTGTCGAGGAAGTGACG 5’ 5’ GATCGCAGATATCAACTTTCTCAAAGGC 3’
wecG	3’ GGCGCATGTCTGATGATCGCAAAACTGTTG 5’ 5’ GTGTTACCGGTTATCGTTATGGGTACATCG 3’
gor	3’ GTAATCAACGATAAGGACACTTTG 5’ 5’ TTCTTATCTAACAATGAGACATGCG 3’
prc	3’ GATGCCACCGTATTAGAAATCACCAAAG 5’ 5’ GTAGCATCTGATTTACGGCATCTTGTCGCT 3’

3.2.4. Susceptibility of single KOs to model honey

Exponentially grown KOs were tested for their susceptibility to model honey followed the method as described previously in Chapter 2.

3.2.5. Competition experiments

The selected KOs (LacZ+ phenotype) were competed against WT MG1655 marked with a l*acZ* mutation for visualisation on indicator plates (MacConkey agar plates). Following overnight growth, the strains were grown to the exponential phase (OD_{600} ~0.5), washed twice in PBS and the OD_{600} was adjusted to 0.8-0.9. Each knockout was combined with the *E. coli* LacZ- in 1:1 ratio and was mixed with equal volume (1 mL) of model honey (SGH). Thirty (30) and ninety (90) minutes post-treatment with model honey, cell pellet was harvested by centrifugation at 4,000 x g for 3 min and was resuspended in equal volume of PBS (2 mL). A 100 µL sample of the honey-treated samples (for 30' (t30) and 90' (t90) min respectively) and the control (KOs/ LacZ-) were plated on MacConkey Lactose agar and incubated overnight at 37°C. The equation to measure fitness was adapted by Lenski et al., (1991) and modified to the following:

$$\mathbf{W = (R_{tx}/V_{tx})/(R_{t0}/V_{t0})}$$

where W is defined as the relative competitive index, R (mutant) and V (WT) represent the two competing populations, t0 is the colony counts at timepoint zero (control) and tx is the colony counts at timepoint 30 and 90 min respectively. The R_{t0}/V_{t0} was normalised to the V_{tx} value for each time point (tx) tested.

3.3. Results

3.3.1. Optimization of the model honey used in TraDIS experiment

As was seen in the preliminary findings (Chapter 2), bacteria undergone significant phenotypic changes during the first 2 h of exposure to model honey. During that time, H_2O_2 is more abundant and the synergy of the model honey components more effective. The MH used in previous chapter had been the right substrate to investigate the antibacterial mechanism of honey at non-lethal bacterial state. Here, by increasing the H_2O_2 concentration (model A: 2 mM H_2O_2, model B: 3 mM H_2O_2), it was aimed to define a MH capable of inducing higher logarithmic reduction without completely eradicating the bacterial population. The bacterial viability was determined by colony counts and the phenotypical changes induced in *E. coli* cells were examined by FC using the PI and BOX dyes (procedure as was described in Chapter 2).

Although none of the models tested induced significant logarithmic reduction within the time of exposure, model B (3 mM) caused almost 3 logs bacterial reduction within 90 min of exposure. By that time, model A caused 1 log reduction (Figure 3.6 A). However, FC has been more informative with respect to the phenotypical changes induced in bacterial cell during the 2 h treatment.

As was seen by the MFI of PI both models induced immediately membrane destruction (Figure 3.6 B). This effect is basically associated with the presence of H_2O_2 which instantly penetrates the bacterial membranes as was shown in Chapter 2. In case of lower dosed cells mean PI fluorescence was fluctuating within the 2h of treatment which is potentially due to the self-repair mechanism and the catalase activity of *E. coli* which degrades the H_2O_2. In contrast, higher dosed population

accumulated constant levels of PI by 90 min of treatment. This observation suggests the highest the H_2O_2 concentration the longer time the model exerts disrupting effects to bacterial cell or the longer it gets for bacteria to degrade it.

The second optimisation step was to define the outgrowth time of the transposon library post-treatment with the model honey. This step aimed to ensure that the bacterial generations resulting from the outgrowth step yield enough gDNA for TraDIS sequencing. Moreover this method, as adapted by Phan et al (2013), was expected to allow the growth of honey-resistant mutants while eliminating or reducing the mutants that were sensitive to honey.

Two hours outgrowth of honey-treated *E. coli* cells was enough for them to repair the membrane damage. As was seen by FC data, within this time cells repaired their organisation (Figure 3.6 D, 3.7). However, colony counts clearly showed that the cultivability of *E. coli* decreased post-treatment with model B (higher H_2O_2 dose) (Figure 3.6 C). This is explained by the fact that higher doses of H_2O_2 arrests cells growth by irreparable membrane damage and ROS accumulation (Chapter 2). These cells can hardly recover and be metabolically active within 2 h outgrowth. Therefore, only intact and moderately injured cells are expected to grow in LB rich medium during the activation step.

Considering the observations from batch and single-cell analysis it was concluded that both models induced membrane destruction while model B caused a higher log reduction within this time. Also the outgrowth step was sufficient for the intact and mild injured cells to restore their structure and resume their growth.

Considering these results, transposon library was exposed to a model honey composed of H_2O_2 (3mM), sugars (30%) and gluconic acid (8.6 mM) for 30 and 90 min.

Given that *E. coli* quickly degrades the H_2O_2, the comparison of bacterial responses between the two timepoints (30' and 90') is to give insight on the synergy between honey components when H_2O_2 is more abundant (30') and when this is mostly degraded (90'). The phenotypic changes observed by that time are expected to be seen as a molecular fingerprint in the analysis of TraDIS data.

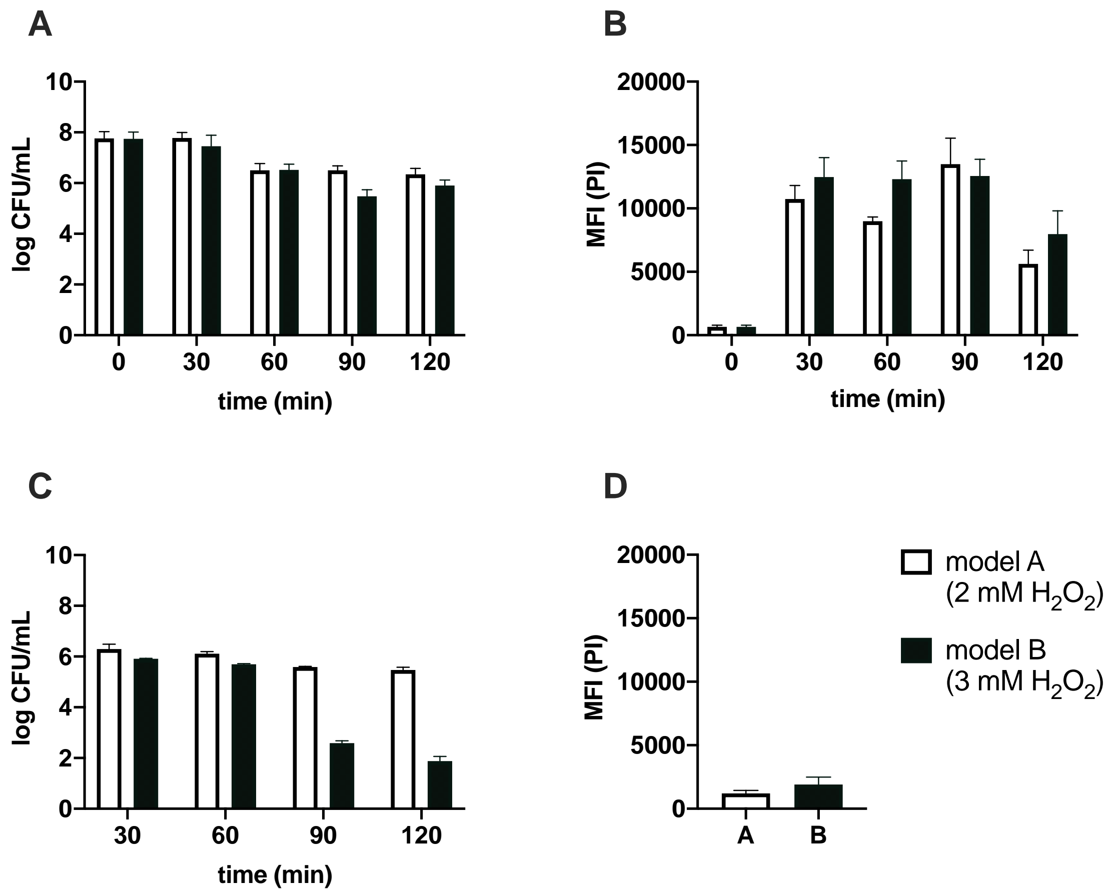


Figure 3.6 Antibacterial effect caused by the 2 model honeys on exponentially growing *E. coli* K-12. Colony counts show the survival of *E. coli* (A) post-exposure to 2 models (A: white bar, B: black bar) for 30, 60, 90 and 120' and (C) after 2h outgrowth of previously treated library in LB. Mean fluorescence intensity (MFI) of PI shows the extend of bacterial membrane damage (B) post-exposure to models and (D) after the 90' treated sample was harvested and inoculated in fresh LB for growing 2h. Error bars represent the average ± s.d. (*n*=3; biological replicates).

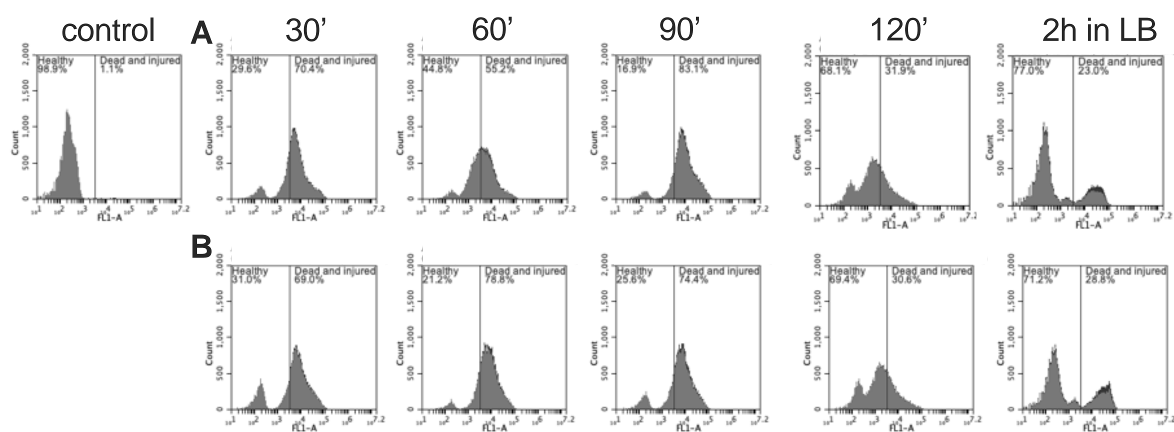


Figure 3.7 FC: Progression of bacterial damage during the 2h exposure to model honey. The progression in accumulation of PI positive cells (defected membranes) detected by FC (FL1-A channel) is shown for treatment with (A) (upper lane) 2 mM H_2O_2 and (B) (lower lane) 3 mM H_2O_2 model honey respectively. The last column (on the right) shows the extent of bacterial damage after outgrowth experiment. Upper left graph shows the control whereas the whole bacterial population is impermeable to PI (PI^-).

3.3.2. Sequencing of the Keio library

A modified TraDIS method was used in order to identify genes whose mutation causes a significant loss of fitness in *E. coli* K-12 MG1655 post-exposure to model honey (Figure 3.3). The library was exposed to model honey for 30' and 90' and then allowed to grow in LB medium for 2 hours. The procedure was performed in parallel with a control where the model honey was replaced by PBS. The gDNA extracted both from test and control samples (two biological replicates), was processed as described in § 3.2.2.3. It was then sequenced by Illumina MiSeq to obtain TraDIS data.

Before the analysis of TraDIS data, for the identification of the significantly affected mutants, some "filtration" steps were done. Firstly, the raw data were checked for any inline index barcode. Independently processed samples and short sequence reads were removed. The data were then combined in order to increase the coverage resulting in 4,423,778 million transposon-tagged reads, which were aligned to the MG1655 chromosome resulting in 450,581 unique insertion sites. The average of insertion site is 10.1 bp per base. Reproducibility of biological replicates were checked by a typical R^2 (Pearson) correlation of the individual insertion indexes within the two replicates. The correlation coefficient fell between 0.97-0.98 indicting the high reproducibility of the individual replicates (Figure 3.8).

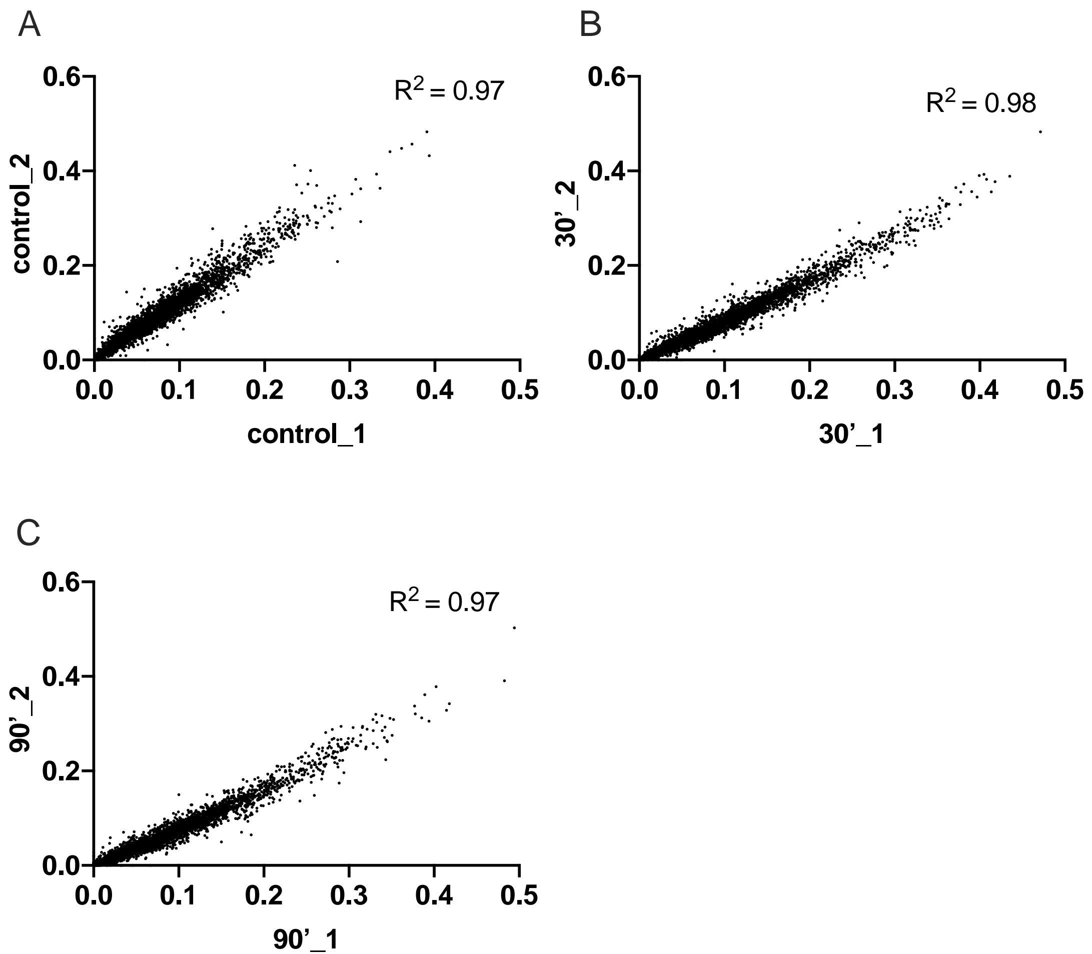


Figure 3.8 Comparison of gene insertion index scores between two biological replicates. The correlation coefficient of gene insertion index scores of (A) the control and two independently treated samples of transposon library exposed to model honey for (B) 30' and (C) 90'.

3.3.3. Processing of TraDIS data for the identification of conditionally essential genes

Prior to the analysis of TraDIS data transposon insertion frequency data were loaded to ARTEMIS, a genome annotation tool, in order to visualize the alignment of the sequencing reads mapped against the reference genome (MG1655) (Rutherford et al., 2000).

Figure 3.9A shows the genome-wide transposon insertion site mapped to *E. coli* MG1655. The outermost track marks the MG1655 genome in base pairs. The 3 inner circles, corresponding to the control (black), 30’ (blue) and 90’ (pink) samples, depict the frequency and the location of transposon insertion sequences mapped to the MG1655 genome. Some of the insertion sites appeared highly enriched (i.e. *envZ*) and some others (i.e. *fdoH*) lost insertions within the time of exposure to honey (Figure 3.9 B). However, in order to identify the genes whose mutation caused a significant change of fitness (increase or decrease) in honey further analysis was done.

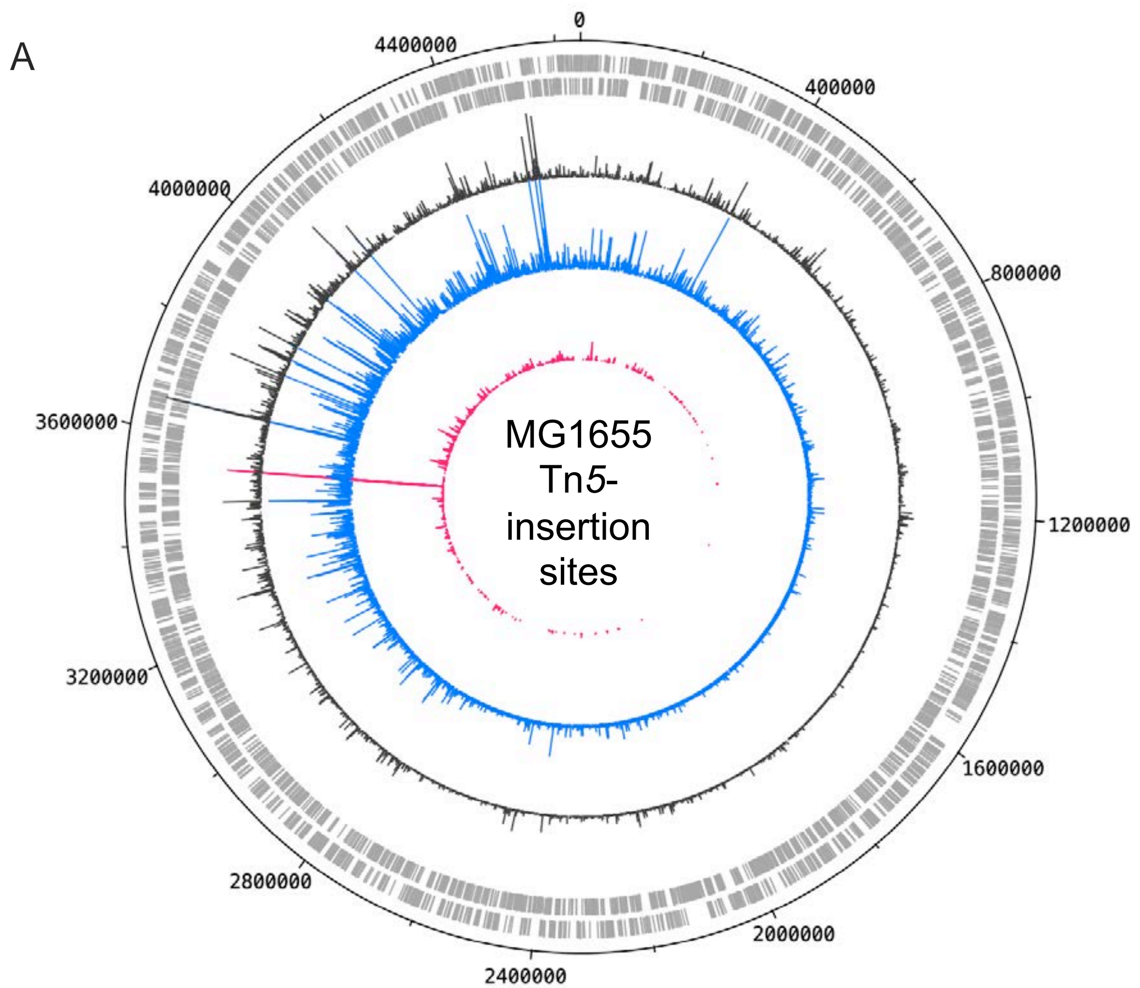


Figure 3.9 Genome-wide transposon insertion sites mapped to *E. coli* strain MG1655. (A) The outermost track marks the MG1655 genome in base pairs. The 3 inner circles depict the frequency & location of transposon insertion sequences for the control (black), the 30' (blue) and 90' (pink) sample as mapped to the MG1655 genome after the identification of a transposon sequence. The figure was created by DNAploter in ARTEMIS software.

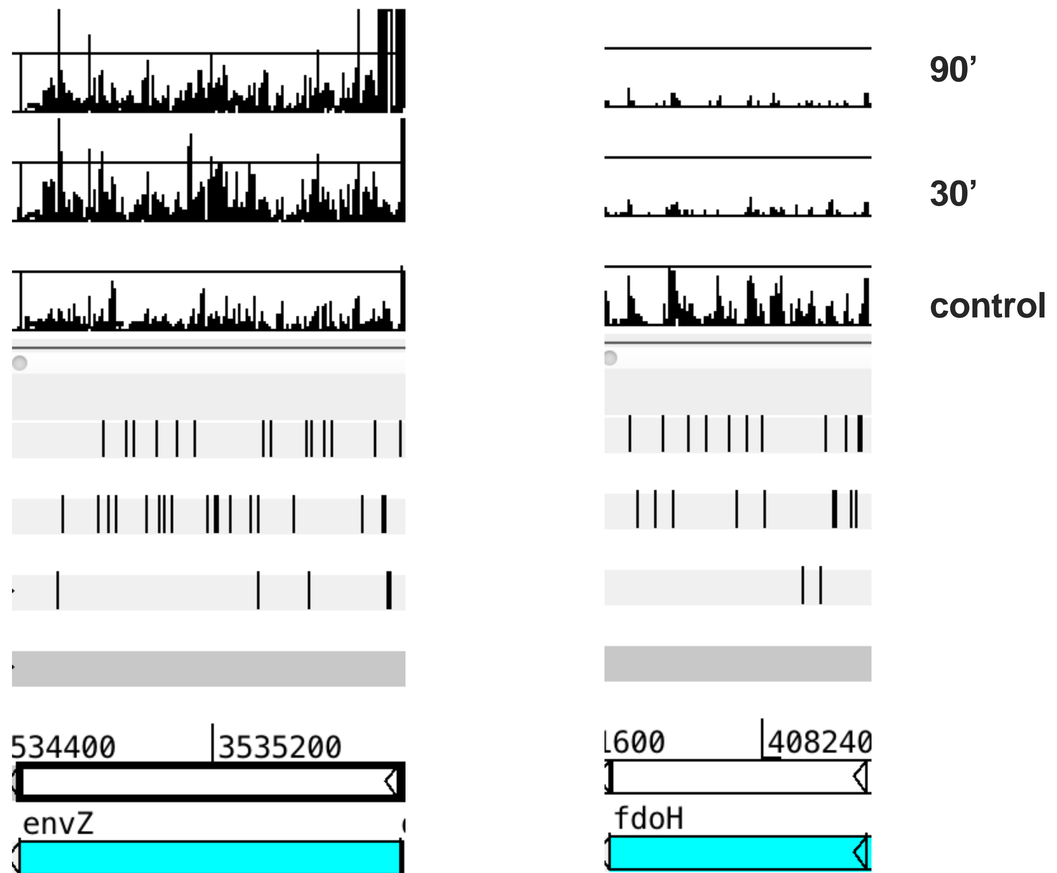


Figure 3.9 Genome-wide transposon insertion sites mapped to *E. coli* strain MG1655. (B) Comparison of transposon insertion frequency between the control and (30’ and 90’) treated samples for *envZ* and *fdoH* KOs. The *envZ* KO gained transposon insertions by the time of exposure while the opposite happened with the *fdoH* KO.

TraDIS data were processed and analysed by ESSENTIALS as was described previously (§ 3.2.2.6). ESSENTIALS uses a non-parametric density estimation approach which does not make any assumptions about the underlying form of distribution. Thus, the estimation approach is only based on how the data are related to each other (Duda et al., 2012). After removing the insertions at the 3' end of genes, which are not supposed to have an impact on mutant fitness, LOESS regression was used to correct insertion counts. LOESS regression corrects the read counts per insertion site and per gene considering the genomic location. The corrected value for each gene is summed into a numeric value: the insertion count per gene (Zomer et al., 2012). The main output of ESSENTIALS is the Log fold change between the expected and the measured insertion counts which were calculated by EdgeR internal package (Robinson et al, 2010). Essential genes of the *E. coli* genome, as recently identified by Goodall (2018), were excluded from the ESSENTIALS output list. These genes are critical for the growth and the survival of *E. coli* K-12. Thus, they should not be confused with genes that when mutated affect the bacterial fitness particularly to honey (or any condition tested).

Later, we investigated the genes whereas the frequency of transposon inserts has significantly changed compared to the control sample. The fold change was calculated as stress/control. This resulted in a positive number when the insertion frequency increases post-exposure to model and in a negative number for decrease in insertion frequency. Figure 3.10 shows the Log2 fold change curves for the whole transposon library post exposure to model honey for 30' (A) and 90' (B). Most of the genes fall across the middle of the graph around or at zero indicating that these genes have no impact on the fitness of the population. The genes falling above or under zero

correspond to the mutants that respectively show an increased or decreased fitness post exposure to the model honey. Genes with increased fitness suggest the resistome of honey or the “gain of fitness” genes while the others are defined as the “loss of fitness” genes.

In order to set a threshold on the significance of Log2 score, 6 p-values (0.05, 0.005, 0.0001, 0.001 and 0.01) were tested. Transposon mutants were classified as significantly under- or over-represented in the final population on the basis of a p-value of 0.005, once adjusted for a false discovery rate (FDR) of 10%.

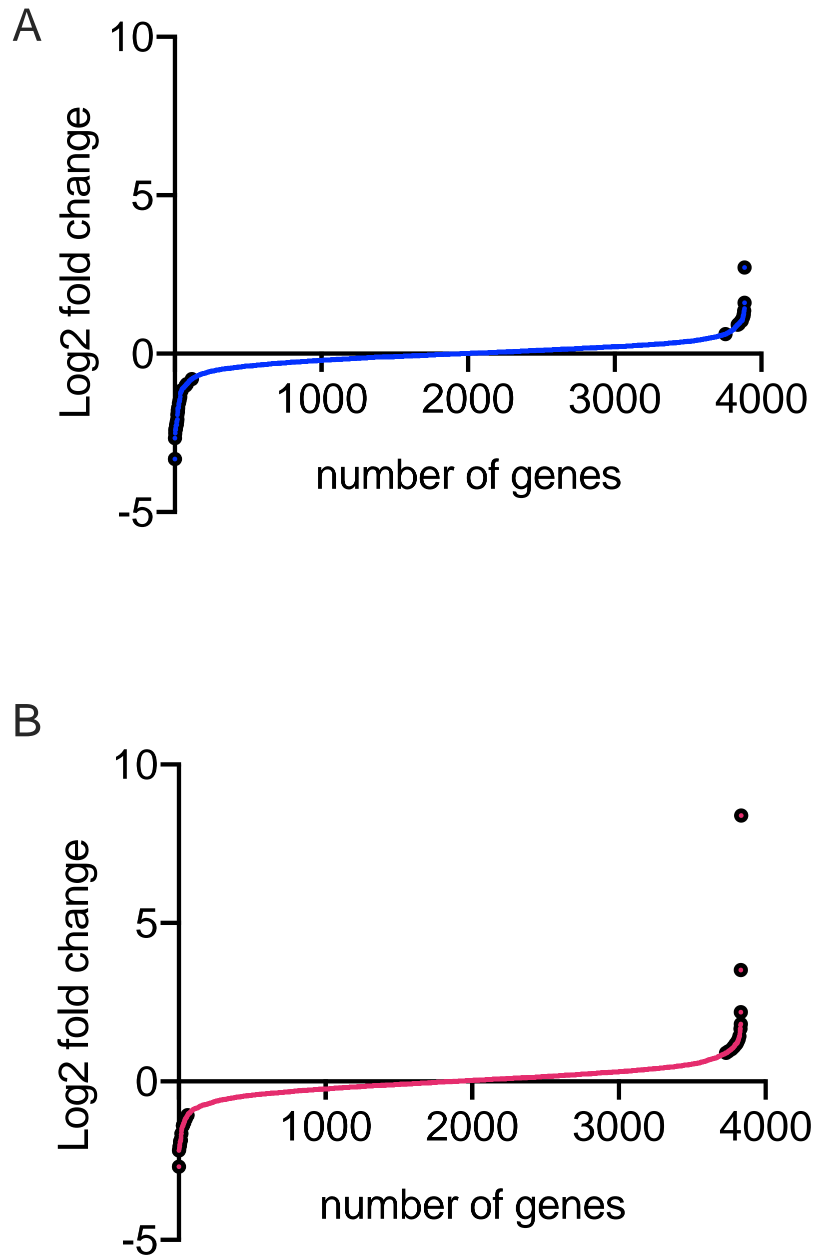


Figure 3.10 Log2 fold change of transposon inserts frequency for all the genes in the genome compared to the control. The fold change was calculated as (A) 30' stress/control and (B) 90' stress/control. For the whole genome, Log2 scores were ranked from lowest to highest and were plotted in a log transformed axis. Annotated genes (black) are the ones with significant ($p<0.005$) Log 2 score in transposon inserts compared to control as was emerged by analysis on ESSENTIALS. The genes with significant Log2 score are listed in Appendix 3.1.

3.3.4 Comparison between datasets of genes with significant Log2 scores

First, we ranked the genes, according to the Log2 score calculated by the ESSENTIALS. By setting a p-value<0.005 as threshold we identified the genes whose mutation caused a significant impact on the fitness of the population (a full list of the significant genes in both conditions is provided in the supplementary data, Appendix 3.1). At 30' and 90' samples, 37 and 43 mutants respectively were found to have significant changes in their fitness. Twenty-six (26) of them are common between the two time-point samples (Table 3.4, Ven diagram, Figure 3.11A) and they are described in detail in the following paragraphs.

For the rest of the "non-common" genes, 1 by 1 inspection was done in order to assess whether any of them should be also categorized as "loss of fitness" gene. As was emerged from this comparison (Table 3.3 A & B), genes appeared with significantly low Log2 score at 90' only were considered as significant "loss of fitness" genes (coloured in green). This case is a typical example of mutants which gradually lost fitness with the time of exposure to the model. Although these genes cannot be recognised as "conditionally essentials" for survival in honey, they can definitely be considered as "loss of fitness" genes. This inspection showed that 4 genes (*yfjV*, *gnd*, *yfjQ, yfeX*) had low transposon insertions at both conditions (30' and 90'). However, the Log2 score was significant only at 90' list which shows that longer time of exposure to the model induced significant loss of fitness for these 4 KOs. Therefore, the *yfjV*, *gnd*, *yfeX* and *yfjQ* can also be considered as "loss of fitness" genes.

The opposite was not applied. Mutants which increased in fitness, from 30’ to 90’, cannot be considered as mutants with significant growth defect and they are coloured in red. These mutants are likely to develop alternative mechanisms which allow them to recover the initial stress. Thus, for the time-course tested here, these mutants cannot be considered as significantly “loss of fitness” KOs. Some of these KOs are subunits of an operon (i.e. *atp*, *moa*, *mod*). As it is shown in the following paragraphs, other genes of the same operons were identified as “loss of fitness” genes. Therefore, for reasons that we have not identified, is possible that honey caused the differential response of the KOs within the same operon.

Table 3.3 List of non-common genes between the 2 conditions tested. Table A shows the genes with significant Log2 score at 30', the respective score at 90' is listed on the right. Table B shows the genes with significant Log2 score at 90' and the respective score at 30' is listed on the right. Green annotated genes can be only considered as significant "loss of fitness" genes as their Log2 score significantly decreased with the longer exposure to honey. This is not the case with red labelled genes.

A	30'		90'		B	90'		30'	
gene	Adj. p-value	Log2 score	Adj p-value	Log2 score	gene	Adj. p-value	Log2 score	Adj. p-value	Log2 score
atpA	1.78E-12	-2.662060113	0.0433814	-1.6774499	*ompC*	6.59E-07	1.061466897	0.50985554	0.62404085
fabF	1.08E-16	-2.405290212	1	-0.8765553	*yciF*	1.05E-05	1.817741584	0.10304367	-0.9001392
moaC	1.41E-08	-2.253630134	1	-0.8544341	*plsX*	2.73E-05	1.208870308	1	0.52048653
gntR	3.37E-13	-2.155764877	0.20305583	-1.0793376	*ydcC*	3.08E-05	1.165021122	0.02415971	0.89290436
moaE	1.26E-09	-2.115695948	0.12923149	-1.32484	*qseC*	0.000117722	1.112440505	0.01034458	0.88328554
modB	4.38E-06	-1.845157599	0.03397776	-1.4253783	*yfjV*	0.00029052	-1.173435848	0.06417631	-0.8085127
mog	2.40E-06	-1.777804628	1	-0.8373218	*gnd*	0.00080456	-1.172275889	1	-0.5383162
mrp	5.88E-09	-1.712620164	0.12638442	-0.9644307	*ycfQ*	0.000919182	1.262971422	1	0.42219549
lon	0.000260751	-1.562394766	1	-0.4758279	*mgrR*	0.000965818	2.186930637	1	0.16572384
ppk	3.71E-08	-1.363313252	0.29049956	-0.9501145	*tig*	0.001003391	0.953095093	0.98249629	0.64894751
glnA	0.000799183	-1.224506163	1	-0.0312399	*mepS*	0.001503633	1.013291204	1	0.61880851
gor	0.003018973	-1.006743572	0.27906378	-0.9404822	*yfjQ*	0.001584836	-1.253874048	0.06713597	-0.8297109
mfd	0.004504012	-0.804016568	0.18297743	-0.7059844	*manA*	0.002288279	1.429445338	1	0.52303473
rapA	0.00397333	0.616105716	1	0.40555728	*ydcT*	0.002624382	1.288511797	1	0.32894404
phnI	1.47E-05	1.200326592	1	-0.3978838	*dosC*	0.003509038	0.903291278	1	0.57191509
phnF	5.67E-05	1.244969554	1	0.13630222	*yejG*	0.003726169	1.66120879	1	1.00331094
yagF	2.83E-11	1.35362792	1	-0.4282574	*yfeX*	0.003741649	-1.39580683	1	-0.9199903
ymjC	0.002237258	2.72224638	N/A	N/A					

3.3.5. Genes with a decrease in insertion frequency after exposure to model honey

Table 3.4 shows genes shared between the 2 time points and considered as significant in terms of frequency of transposon insertions before and after treatment with the model honey (Figure 3.11A). All genes with a Log2 score below 0 are the ones that when mutated caused loss of fitness to the respective strain after this was exposed to honey (Figure 3.11B). Based on that we defined these genes as “loss of fitness” genes.

The *prc* and *selD* have the greatest negative Log2 score in the frequency of transposon insertions (Table 3.4). The three of the 4 selenocysteine genes (*selDABC*), the membrane-bound formate dehydrogenases (*fdoGHI*), molydenumtransferases (*moeAB*), molybdenum cofactor (*moaA*) and the required for dehydrogenase activity (*fdhED*) are gene clusters which were listed as ”loss of fitness” genes in both conditions because of the low insertion frequency post-selection with honey. These graphs also show that *ompR* and *envZ* mutants demonstrated the greatest fitness advantage on honey. In the two following paragraphs the function of each of these genes is discussed considering observations from previous studies along with some hypothesis based on our results.

Table 3.4 Genes with significant Log2 score shared across the 2 datasets (30' and 90'). The genes are ranked according to Log2 score.

30'			90'		
Gene	Adj. p-value	Log2-score	Gene	Adj. p-value	Log2-score
prc	1.13E-27	-3.3228998	*selD*	5.57E-09	-2.6846038
moeB	4.26E-23	-2.5010238	*prc*	7.70E-13	-2.1804702
moaA	3.54E-15	-2.4188103	*selA*	1.51E-09	-2.1361767
fdoH	6.72E-20	-2.3928778	*fdhE*	2.15E-06	-2.04681
selB	4.13E-24	-2.3603875	*fdoG*	1.85E-15	-2.0354739
selA	5.02E-18	-2.2744317	*yohD*	0.00166114	-1.9574062
fdoI	4.04E-11	-2.2685012	*selB*	8.01E-12	-1.9014615
atpD	3.99E-11	-2.2572989	*fdoI*	4.39E-05	-1.8894201
yohD	2.25E-07	-2.2454598	*fdoH*	5.10E-09	-1.8883053
fdhE	1.16E-11	-2.1282873	*fdhD*	7.38E-06	-1.8690168
fdhD	4.48E-12	-2.1027986	*atpD*	0.00034103	-1.8367194
fdoG	1.26E-25	-2.0702544	*moeB*	0.00113266	-1.6458777
selD	1.45E-06	-1.9307232	*glnG*	3.41E-05	-1.3905399
moeA	6.25E-09	-1.4674787	*moaA*	0.00373388	-1.2884631
glnG	8.43E-05	-1.2152076	*moeA*	0.00091976	-1.1997349
fetA	0.00019444	-1.1486974	*fetA*	5.61E-05	-1.1724089
fetB	0.00261712	-0.9609117	*fetB*	0.00412931	-1.0612796
rep	0.00017567	0.91260619	*wecA*	0.00015225	1.0916747
acnB	0.00229511	0.93990081	*tolA*	0.00086849	1.17426211
wecC	5.14E-05	0.94691221	*acnB*	1.39E-05	1.18168313
envZ	7.95E-06	1.03020014	*wecG*	6.74E-05	1.20291296
wecB	2.23E-12	1.03466846	*wecC*	6.58E-06	1.21168436
wecG	1.59E-05	1.04357666	*rep*	6.97E-10	1.2630543
wecA	8.17E-07	1.04473038	*wecB*	1.11E-11	1.33535514
tolA	0.00309428	1.14776095	*envZ*	1.70E-44	3.52185648
ompR	6.99E-12	1.61321646	*ompR*	5.06E-134	8.39233298

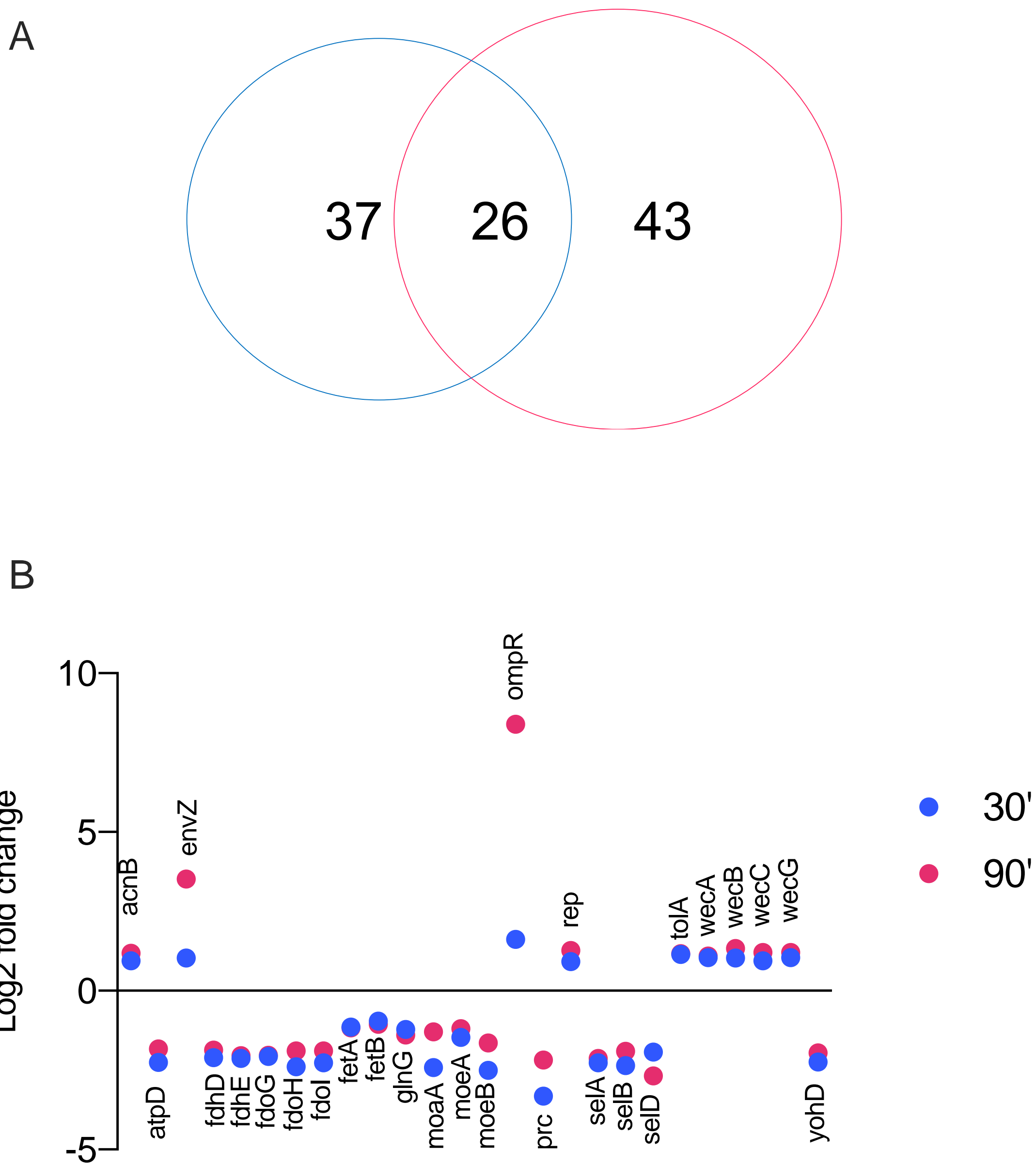


Figure 3.11 Genes with significant changes of transposon insertion frequency compared to the control. (A) Ven diagram shows number of genes which overlap between the two conditions. (B) Among the 26 common genes there are these which have significantly lower (Log2 score<0) and higher (Log2 score >0) insertion frequency compared to the control.

The prc is a periplasmic space protease contributing to the pathogenicity of *E. coli* (Hara et al., 1991). Here, *prc* KO had the highest negative Log2 score 30' post-treatment the model honey treatment. Under low osmotic and oxidative stress, *prc* acts as a modulator of the cell-envelope stress response exhibiting membrane permeability and periplasmic protein leakage. Inactivation of the *prc* caused susceptibility to a range of hydrophobic and hydrophilic antibiotics (tetracycline, chloramphenicol, erythromycin, spectinomycin, norfloxacin etc.) (Deng et al., 2014; Singh et al., 2015). According to our previous findings, honey causes oxidative-induced membrane damage. This can be lethal in case of higher H_2O_2 dose. Thus, it is sensible to speculate that null *prc* strain, will be more susceptible to honey because of the increased membrane permeability and leakage that it causes as result of the synergy of acid and oxidative stress mainly.

The fetAB membrane proteins encode for the iron homeostasis by removing the excess of Fe^{2+} so this cannot get involved in Fenton reaction and produce more ROS. The null *fetAB* strains were seen to be more sensitive because the accumulation of intracellular iron accelerated the Fenton reaction in presence of H_2O_2 (Nicolaou et al., 2013). In agreement to this study, here it was seen that *fetAB* significantly lost fitness upon exposure to honey. The loss of iron homeostasis may stimulated the Fenton reaction and the generation highly toxic ROS. This is one of the main stressors in honey as we identified in Chapter 2. Although *E. coli* WT exerted detoxifying activity the increased and prolonged accumulation of ROS caused cell death.

The deletion of *atpD* had also a significant growth defect on treatment with honey. *atpD* encodes the β-subunit of the F-ATPase (known as F_oF_1 ATPase). This uses a proton gradient to drive ATP synthesis by allowing the passive flux of protons

across the membrane. Manipulation of calcium anions is especially important for the regulation of biological processes (i.e. cell movement, gene expression etc). As has been shown previously, null *atpD* strain was defective in Ca^{2+} efflux and showed reduced levels of intracellular ATP. Also, it was observed that prolonged high levels of intracellular Ca^{2+} caused cell injury or death (Naseem et al., 2009). In agreement to this, the significant drop in fitness of *atpD* mutant was observed post-model honey treatment. Considering the above, it was speculated that decreased intracellular ATP levels impaired the pH homeostasis which caused a growth defect in the cell under the acid stress of honey.

At 90' of exposure to the model, *selD* had the highest negative Log2 score while *selA* and *selB* mutants were also seen to be significantly underrepresented post-exposure to honey (Table 3.4). *Sel* genes are constituents of 2 unlinked operons and are needed for the translation of selenoproteins. The *selD* is the central gene in the operon and the other two, *selA* and *selB*, form the transcriptional unit (Sawers et al., 1991). Selenoproteins have multiple functions such as the regulation of redox activity, signalling, synthesis of selenocysteines, storage of selenium and protein folding. With respect to their redox signalling/regulation, they protect cells in different compartments (depending on their cellular localisation) against the oxidative stress (Watson, 2015). In *E. coli* there are three selenocysteine containing enzymes (FDH-H, FDH-O and FDH-N) whose formate dehydrogenase (FDH) activity is controlled by *selAB*. Deletion of the *selAB* induced susceptibility of *E. coli* to menadione (a redox cycling drug) because of the inactivation of FDH activity (Iwadate et al., 2017). As was seen in Chapter 2, honey stimulates the ROS accumulation within the cell in a H_2O_2-dependent way. Here, it appeared that the destruction of the redox homeostasis rendered *sel*

mutants incapable to withstand the honey-induced oxidative stress, because of the impairment of FDH activity. However, this is contradictory to the fact that *E. coli* possesses OxyR and SoxR/S systems to respond to H_2O_2-induced stress. Thus, the enrichment analysis in the following paragraph aims to reveal a biological pattern of the response of *E. coli* to honey which potentially can explain this observation.

Formate dehydrogenase genes, *fdoHGI*, encode subunits of the FDH-O which are located in bacterial inner membrane. They are responsible for the redox balancing activity by electron transfer which represses the endogenous ROS production (Sharma et al., 2009; Iwadate et al., 2017). Similarly, *fdhD* and *fdhE* are two genes essential for the maturation and function of all formate FDHs (FDH-O, FDH-N, and FDH-H) (Schlindwein et al., 1999). The activity of *fdhE* is only required by the periplasmic located FDH-N and FDH-O while *fdhD* is an essential cofactor for all FDHs (Lüke et al., 2008; Arnoux et al., 2015). Deletion of the *fdhD* made *E. coli* more susceptible to menadione drug by completely inactivating the FDH activity of FDH-O (Abaibou et al., 1995). In the same way as it was mentioned in the previous paragraph, it appeared that the loss of FDH activity make *fdOGI* and *fdhDE* mutants more susceptible to model honey. The same phenotype was reported after exposure to redox cycling drugs (i.e. menadione). Menadione, likewise honey, increased the permeability of cell membrane which eventually causes cell death (Andrade et al., 2017). Thus, here it was hypothesized that the uncontrolled generation of ROS and the destruction of cell membrane made these mutants to significantly lose fitness to honey.

Moa and *moe* are 2 of the 6 *E. coli* loci which are directly involved in the biosynthesis of moco (molybdenum cofactor) (Coughlan, 1993). Molybdenum is the second most essential transition metal in biological systems. It is involved in redox

activities and has a key role in nitrogen, sulphur and carbon metabolism (Hille, 1996) and the maintenance of proton motive force (Leimkühler, 2014). The *moa* locus, the biggest operon being involved in biosynthesis of moco, contains the *moaABCDE* genes and the *moeAB* is belonged to *moe* locus (Nichols and Rajagopalan, 2002; Leimkühler et al., 2001). The deletion of molybdoprotein synthesis genes caused increased susceptibility to *E. coli* when this was treated with HAP (6-N-hydroxylaminopurine) and chlorate because of the reduced reductase activity which further caused imbalance to proton motive force (PMF) (Kozmin and Schaaper, 2013). As it has been reported, reduced reductase activity caused destruction of pH homeostasis and PMF. This changed the polarity of membrane proteins and eventually increased membrane permeability to protons (Riondet et al., 1999). In consistence to these observations, in Chapter 2 it was seen that synergy of low pH and oxidative stress in honey changed the membrane polarity and destructed its integrity. Thus, it was speculated that *moa* and *moe* mutants lost fitness to honey in a way similar to this described above.

Null *glnG* strain significantly lost fitness after exposure to honey. The product of *glnG* is both a positive and a negative regulator of a number of genes which are involved in the uptake and degradation of nitrogen-containing compounds (Pahel et al., 1982). Nitrogen is an essential element for most macromolecules in cell (i.e. proteins, nucleic acid, cell wall components) and supports the fast growth of bacteria. Nitrogen depletion activated the products of *glnG* so that they scavenge nitrogen from any other alternative sources (Brown et al., 2014). As it has been reported, ROS inhibit the nirtogenase activity which in turn affects the growth rates for the bacteria (Alquéres

et al., 2010). Considering the oxidative capacity of model honey, it would be expected that growth of nitrogenase mutant would be significantly affected.

Finally, *yohD* mutant was seen with significant growth defect post-honey treatment. YohD is a member of the DedA family of inner membrane proteins. These proteins have important roles on the membrane homeostasis. The respective mutants display phenotypes such as cell division defects, altered membrane lipid composition, loss of PMF and increased envelop-related stress responses. For these important functions the members of DedA family have been studied as potential drug targets (Doerrler et al, 2013). Considering the effects of honey on PMF and the effects on membrane polarity and integrity, we assumed that the synergy of these events caused the loss of fitness in *yohD* KO.

Within the 4 genes found to significantly lose fitness after 90' of exposure to honey, *yfjQ* and *qfjV* are both uncharacterised *E. coli* proteins. YfeX protein is a cytoplasmic peroxidase able to provide iron from exogenous heme sources to *E. coli*. Since acquisition of iron from the host is major source for bacterial pathogenesis, the deletion of *yfeX* inhibits the ability of *E. coli* to obtain iron from the host which affects the bacterial growth (Dailey et al., 2011). The deletion of oxidoreductase encoded by *gnd* also caused loss of fitness after the treatment with honey. This gene is involved in the last step of oxidative Pentose Phosphate Pathway (oxPPP). Thus, disruption of *gnd* function was reported to result in metabolic rearrangements by recharging the generation of NADPH (McCloskey et al., 2018). Considering these, is possible that honey caused also perturbations of the central metabolism. However, the enrichment analysis is expected to show whether any other of the “loss of fitness” genes are implicated in the regulation of metabolic pathways.

As emerged from the above-mentioned, the genes whose deletion caused severe loss of *E. coli* fitness to honey, are involved in redox balancing activity (*selABD*, *fdoHGI*, *fdhED*), proton-motive force balance (*moaA*, *moeAB*) and organisation/protection of OM integrity (*prc, yohD*). The next paragraph will consider these KOs which showed fitness advantage post-model treatment.

3.3.6. Genes with an increase in insertion frequency after exposure to model honey

Figure 3.11 A shows also genes with (significant) Log2 score higher than 0, shared between the 2 conditions tested (30 and 90 min). In these genes, the presence of transposon insertion provided a growth advantage to the cells compared to the control condition. These genes were deemed as the resistome of honey.

The core resistome of *E. coli* when it is exposed to honey comprises 9 genes shared between the two datasets. The *ompR* and *envZ* were the two genes with exceptionally high Log2 score when they are both compared to the rest 7 genes and the control sample. The aconitate hydratase (*acnB*), DNA helicase (*rep*), *tolA* (protein of Tol-Pal system) and the *wec* operon comprise the resistome of *E. coli* to honey. The role of each of these genes is described in the following paragraphs.

The *acnB* is the major exponential phase aconitase in *E. coli* which protects from the endogenous oxidative stress during the aerobic growth by the release of iron. The coupling of iron with the H_2O_2 induces highly reactive hydroxyl radicals which promotes the oxidative stress. In *acnB* mutants the enhanced expression of *sodA*

serves as a defence mechanism against the oxidative stress. Under low oxidative stress the *acnB* serves both as repressor and activator of the *sodA*, whereas, in higher stress level the *acnA* takes over the activity of *acnB* (Nunoshiba et al., 1999; Tang et al., 2002). Here, *acnB* KO was seen to significantly increased in fitness after exposure to honey. This might imply that *acnB* inactivation stimulated the expression of *sodA* which compensated for the detoxifying activity of the former.

Rep is the DNA helicase, being involved in DNA replication (although is not essential for the replication of the chromosome) (Lohman, 1992). The three mutants being carried in *rep* determine its growth rates. The mutation at -44 which increases the DNA amount per cell, the 142 codon (opal) mutant determines the growth rates and prevents from fast growing, and the 414 stop codon (G414S) mutant which grow in slower rates than the WT) (Laneand and Denhardt, 1975). The mbrA4 mutation, an allele of the leading strand of *rep*, conferred resistance to oxidative stress. The resistant phenotype observed by the G414S mutant was associated with increased DNA content per cell (Trun, 2003). Considering these observations, the increased fitness of *rep* KO could be explained by the locus of the mutation. However, the present data are not enough to support this assumption.

Wec operon (*wecABCG*) and both *wzxE*, *wzyE* genes encode for the synthesis of the Enterobacterial Common Antigen (ECA) and the translocation of this within transmembrane (Danese et al., 1998). ECA is a surface glycolipid, consisting of four trisaccharide repeat units which are localized exclusively in the periplasm. In particular, *wecA* catalyses the first step in ECA biosynthesis which is the transfer of N-acetylglucosamine- 1-phosphate onto undecaprenyl phosphate (Und-P) to form ECA-lipid. As it has been reported, ECA null mutants and mutants with variable ECA lengths

are viable. However, viable *wec* mutants were seen to have elongated and swollen cell morphology. Only when ECA biosynthetic pathway is disrupted (mid-pathway mutants) causes lethal accumulation of ECA-lipid intermediates (Jorgenson et al., 2016). Also, another study showed that every gene of the *wec* operon exposed to vancomycin, a cell-wall active antibiotic, had a resistant phenotype (Leimkühler, 2014). The same phenotype was observed in this study when *wecABCG* single KOs were exposed to model honey. Considering these observations, it was speculated that a single ECA mutant cannot arrest the cell growth. This might suggest that the other two genes, *wzxE*, *wzyE*, and the rest of *wec* genes are also capable of ECA synthesis even in absence of another subunit. In support to this speculation, it has been reported that both *wzyE* and *wzxE* are required for the synthesis of ECA (Kajimura et al., 2005).

Tol genes are constituents of the well-conserved Tol-Pal system which maintain the integrity of the outer membrane (OM) on Gram-negative bacteria. Null *tolA* and *tolB* mutants were seen to cause permeability of the OM, release periplasmic proteins and form OM vesicles when they were exposed to harmful compounds (Lazzaroni et al., 1999). *E. coli tolA* mutant treated with streptonirgin (iron-activated antibiotic) and H_2O_2 was seen with perturbations in OM and leakage of intracellular proteins. The extend of membrane damage was dependent on the extent of oxidative stress and perturbations in iron homeostasis (Sikora et al., 2009). However, highly streptonirgin resistant bacteria employs mechanisms for the elimination of ROS formation (i.e. reduction of intracellular ion) or alter the catalysis of radicals at the cell surface (Cohen et al., 1988). Although this study has extensively mentioned and proved the role of oxidative stress in honey, surprisingly, *tolA* mutant showed increased fitness. This might show that an alternative system compensates for the possible effects of honey's oxidative stress. Is

possible that this system employs a resistant mechanism like this described above. It is worth to mention that all the individual KOs of the Tol-Pal system showed increased fitness (although p-value>0.005) or unchanged fitness post-model treatment. Thus, it is possible that interruption of any of the subunits of the Tol-Pal operon induces higher activity of the rest operon or does not to affect the fitness of the strain.

Both *ompR* and *envZ* transposon mutants demonstrated significantly high resistance post-exposure to model honey. Their fitness increased from 30 to 90 min of treatment. The two-component system, *ompR*-*envZ*, regulates bacterial response to acid and osmotic stress which both induce acidification of the cytoplasm. The histidine kinase membrane-located *envZ* senses the cytoplasmic signals and activates the downstream target *ompR* which in turn acidifies the cytoplasm. The acidification of the cytoplasm is done by repression of the *cadCBA* operon and s*peF* which normally allows the recovery from the acid stress (Stincone et al., 2011; Quinn et al., 2014). As it was seen, *envZ* deletion does not prevent from the cytoplasm acidification in contrast to what happens when the *ompR* is mutated (Srividhya and Krishnaswamy, 2004). This is might implies that acidification of the cytoplasm causes a growth defect under the conditions tested here. In support of this, our data showed that *ompR* deletion which prevents from the acidification of the cytoplasm was fitter than *envZ* KO which still support this stress response. Also, both KOs were fitter than the WT which acidifies the cytoplasm in response to osmotic/acid stress induced by model honey. In case that this hypothesis is true it can be assumed that regulation of *cad* system compensates for cell response to acid stress which gives a growth advantage in the strain. However, this speculation could only be verified by the comparison of intracellular pH when the three strains are challenged with model honey. Also, another possible explanation

relies on the role of OM porins regulated by *ompR*. Phosphorylated *ompR* regulates (repress or activate) the transcription of *ompF* and *ompC* the most abundant outer membrane proteins (OMPs). OMPs allow the passive diffusion of small hydrophilic molecules and hydrophilic antibiotics (i.e. b-lactams and fluoroquinolones (Ceccarelli & Ruggerone 2008). In low osmotic stress *ompF* is the major porin and when osmolality is increased *ompF* is repressed and *ompC* becomes the major OM porin (Chakraborty and Kenney, 2018). According to a recent study, deletion of OM porins can prevent from re-entry of passively diffused molecules which can disrupt the intracellular pH and oxidative balance (Tan et al., 2017). As we showed, model honey affected pH and redox homeostasis. Thus, it is possible that deletion of OM porins prevented from the influx of these molecules. This in turn, possibly caused a fitness advantage to mutant compared to WT which employed stress response mechanism to maintain the cell homeostasis.

3.3.7. "Loss of fitness" genes: Identification of significantly enriched pathways by GO

After the identification of the mutants with significant loss of fitness in honey, the function of the respective genes within the cell was discussed. Further, Gene Ontology (GO) enrichment analysis was used to identify the significantly enriched biological pathways in which these genes are involved. The identification of the biological pathways involved in the pathogenicity of *E. coli* can help to unravel the molecular targets and eventually the underlying mechanism of honey. GO analysis contains 3 components: biological process, molecular functions and cellular components.

Ultimately, a biological process is accomplished by a set of molecular functions performed in specific genes (or cellular components).

Figure 3.12 shows the enriched GO terms for the list of genes whose mutation caused significant growth defect post-honey treatment. The most enriched biological terms according to GO analysis are: tRNA seleno−modification, translational readthrough, selenocysteinyl−tRNA biosynthetic process, selenocysteine incorporation, selenocysteine metabolic process, selenocysteine biosynthetic process, molybdenum incorporation into molybdenum−molybdopterin complex and metal incorporation into metallo−molybdopterin complex, formate oxidation and formate metabolic process. This implies that selenocysteine, Mo cofactor biosynthesis and the activity of formate dehydrogenases (FDHs) are the most significantly affected pathways. A thorough description of the three individual pathways is given in the following paragraphs in order to identify whether these categories are functionally related and how key processes in *E. coli*'s biochemistry are targeted by honey.

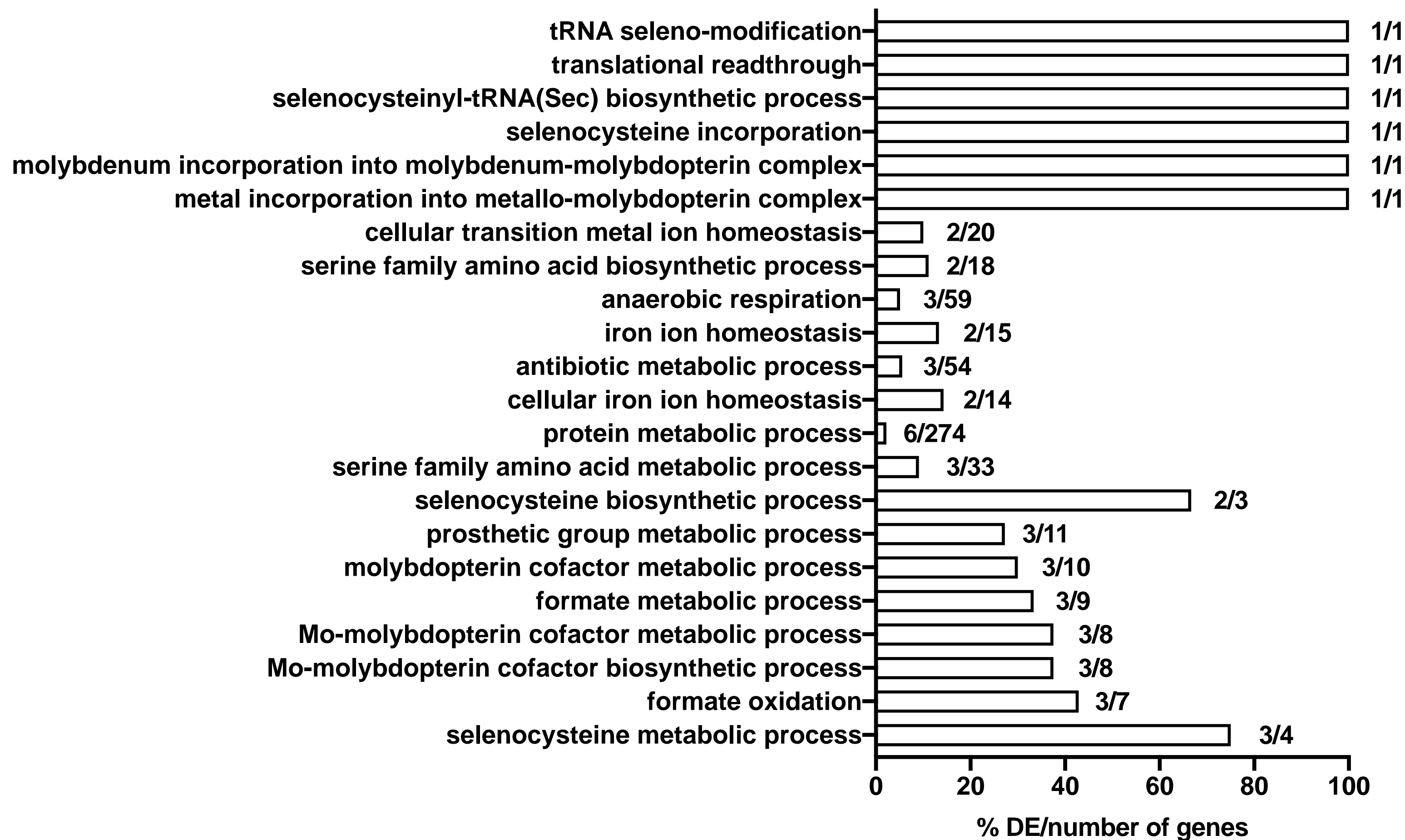


Figure 3.12: Significantly enriched gene ontology (GO) terms for the 26 essential genes. Genes included in the analysis were these whose corresponding mutants had a Log2 fold change bellow 0 when compared to control library. TopGo test was used in R with a $p < 0.005$. Comparisons were made using the number of genes associated with each term in the gene set versus the total number of genes associated with each term in the (*E. coli* MG1655) genome. The respective percentage is given in x axis.

In *E. coli* there are 3 formate dehydrogenases (FDHs): FDH-N, FDH-O and FDH-H. All 3 FDH are redox-active and molybdenum cofactor-selenocysteine (moco-sec) containing enzymes which share the same mechanism of formate cleavage. Since honey inhibited the biosynthesis and incorporation of moco and sec, the function of the 3 FDH enzymes was severely impaired. Figure 3.13 gives a description of the biological pathways targeted by honey and these are discussed in the following paragraphs.

FDH-N and FDH-O are located in inner membrane, are selenocysteine-, moco, and iron-sulfur ([4Fe-4S])- containing enzymes which oxidise formate into CO_2 and H_2. The maturation of both enzymes is absolutely dependent of the FdhE and FdhD proteins (Lüke et al., 2008).

FDH-N is mostly activated during the anaerobic respiration (Wang and Gunsalus, 2003) whereas FDH-O is essential constitutive and its presence in low amounts reduces the toxic effect of the formate (Abaibou et al.,1995). Both oxidoreductases are multi-subunit enzymes composed of a large catalytic subunit α (*FdnG* or *FdoG*), an electron transfer subunit β (*FdnH* or *FdoH*) and a membrane subunit γ (*FdnI* or *FdoI*). The latter has a quinone binding which allows the electron transfer into the respiratory chain during the formate oxidation. After the oxidation of the formate, electrons are transferred across the membrane to *fdnG* (α subunit) to the site of *fdnI* (γ subunit) and are bound to menaquinone (MQ) which is further reduced to menaquinol (MQH_2). MQH_2 diffuses across the membrane transferring 2 e^- to the reduction cytoplasmic site of the nitrate reductase. Nitrate is reduced to nitrite with the consumption of 2 cytoplasmic protons. Ultimately, the energy is conserved in the system by the net movement of negative charge and protons across the membrane

(Berks et al., 1995; Jormakka et al., 2002). Model honey did not affect the FDH-N system and did not cause a significant effect on the fitness of *fdnGHI* mutants (Log2 scores: 0.052875849, 0.04489367 and -0.539470628 respectively). However, the function of FDH-N is disrupted by the inhibition of biosynthesis and incorporation of moco and selenocysteines into the respective subunits. Thus, the redox loop mechanism operated by the FDH-N and nitrate reductase (Nar) for the generation of PMF was compromised.

FDH-O is a respiratory molybdoenzyme which catalyses the oxidation of formate to a carbon dioxide which in turn results in electron donation to quinone pool for the reduction of nitrate. This formate to nitrate electron transport is operated in cells shifted from aerobic to anaerobic conditions enabling them to survive better although FDH-O is also increased under aerobic conditions (Abaibou et al., 1995). FDH-O is encoded by *fdoGHI* subunit. The subunit α (*fdoG*) functions with the moco which are necessary for the FDH activity, whereas the other two subunits are membrane-bound electron transferases (Iwadate et al., 2017). When the moco and sec biosynthesis and incorporation was impaired in the respective mutants, the maturation and function of the FDH-O was also compromised. The maturation of FDH-O is also controlled by FdhDE proteins. TraDIS analysis showed a significantly defective growth of FdhDE mutants after they exposed to honey. Thus, the FDH-O lost its hydrogen oxidizing activity and preservation of PMF. In agreement with these results another study showed that overproduction of *fdoGI* conferred tolerance to *E. coli* cells when treated with the ROS-producing antibiotic menadione. The same study proved that FDH-O serves as an electron transfer element in the metabolism of glucose under aerobic growth (Iwadate et al., 2017).

In contrast to FDH-N and FDH-O, FDH-H is localised in the cytoplasm and it is synthesized under fermentative condition. FDH-H is encoded by the *fdhF* and it is induced under formate disproportionation (in absence of external electron acceptors). FDH-H oxidizes the formate to CO_2 and H_2 (Mandrand-Berthelot et al., 1988). FDH-H contains selenium as the sec is incorporated at the UGA stop codon position in the *fdhF* open reading frame. In turn, the *fdhF* is induced by formate but repressed in presence of nitrate and oxygen (Boyington et al., 1997). Here, after exposure to honey, null *fdhF* mutant lost fitness, however, this gene is not considered as important for honey resistance (Log2 score, p-value>0.005). *fdhF* plays a role under fermentative conditions and it is not actively involved in electron transfer in either of the respiratory nitrate reductases (Chaudhry et al., 1983).

The deletion of genes encodes for the activity of FDH-N (*fdhGHI*) and FDH-H (*fdhF*) did not induce significant loss of fitness of *E. coli* to honey. However, two other regulators (*fdhDE*) required for the FDH activity, were seen to cause significant growth defect when mutated. Specifically, *fdhD* acts as sulphur carrier for the moco before it is inserted into the FDH while the *fdhE* is involved in maturation of the FDH and both *fdhE, fdhD* are required for the its activity. In terms of stress response, under low pH and oxidative stress, *fdhD* was upregulated in order to accelerate the acid consumption and the proton export (Barker et al., 2000; Maurer et al., 2005). Therefore, is possible that the deletion of *fdhD* caused significant loss of fitness of the respective mutants because of the impairment of the proton efflux mechanism.

As previously mentioned FDHs are moco-sec enzymes. The products of 4 *sel* genes have been identified as essentials for the biosynthesis and incorporation of selenocysteine (sec) into selenoenzymes (Leinfelder et al., 1998a). Other 6 loci (moa,

mob, moc, mod moe and mog) in *E. coli* are also implicated in moco biosynthesis. These pathways are described in the following paragraphs.

For the incorporation of the selenium (Se) into macromolecules *sel* genes (*selABCD*) are required. Selenium is an essential trace element which is co-translationally incorporated into the polypeptide chain as part of the sec amino acid following a series of events (Santesmasses et al., 2017). Initially, Se is reduced in hydrogen selenide (H_2Se) which in turn is metabolized into selenophosphate ($SePO_3$) (mediated *selD*). Selenophosphate reacts with the t-RNA molecule ($tRNA^{sec}$) which has an anticodon that recognises UGA specific codon and allows the incorporation of the sec into the selenoproteins. The $tRNA^{sec}$ allows also to be charged with serine and after it is converted to selenocysteyl-$tRNA^{sec}$, by the action of *selA*, it recognises only certain UGAs where it inserts the selenocysteine. This insertion is dependent on a special elongator factor (product of *selB*) and the adjacent sequence in the mRNA named SECIS (selenocysteine insertion sequence). This complex recognizes the certain UGAs where the insertion occurs (Turner et al., 1998). Null *selD* strain significantly lost fitness in honey, with *selA* and *selB* to follow, showing that the abovementioned steps for the biosynthesis of selenoproteins are essentials for resistance in honey. As was previously reported sec-containing proteins trap the H_2O_2 and produce selenic acid before the former can react with other targets. Therefore, in selenoproteins KOs H_2O_2 is not readily consumed and can be transmitted faster to potential targets inducing DNA and proteins damage (Hawkes and Alkan, 2010).

Molybdenum is the only 4rd transition metal required for biological systems as it is implicated in nitrogen, sulphur and carbon compounds (Hille, 1996). Once within the cell it is inserted into metal cofactors which in turn are incorporated into enzymes

known as molybdoenzymes. Molybdoenzymes are found in nearly all prokaryotes (Zhang et al., 2011). There are 2 types of molybdoenzymes: (a) the nitrogenases which have a molybdenum-iron-sulphur cluster (known as FeMoco) and (b) the oxidoreductases which have a molybdenum cofactor (moco) associated with a dithiolene group of pterin (molybdopterin) (Hille, 1996).

Mo is transported in form of oxyanion molybdate (MoO_4^{2-}) through 3 transport systems: (a) high affinity *modABC* (Maupin-Furlow et al., 1995) (b) low affinity cys-PTWA sulphate-thiosulfate permease and (c) a non-specific anion transport system (this also transfers sulphate, selenite and selenite) (Rosentel et al., 1995). The high affinity transport system *modABC* consists of *modA*, a periplasmic binding protein which captures the substrate, and 2 integral membrane proteins *modB* and *modC* which transport the molybdate through the membrane and translocate it to cytoplasm by ATP hydrolysis (Imperial et al., 1998).

The biosynthesis of moco is divided in 4 general steps (Figure 3.13). Initially, in *E. coli* two proteins MoaA and MoaC convert the GTP to the cyclic pyranopterin monophosphate (cPMP). Secondly, is the formation of molybdopterin (MPT) after the dithiolene group is introduced into the cPMP by the *moaD*, *moaE* and *moeB* gene products. Later step is the insertion of molybdenum into molybdopterin to form Moco. The molybdenum atom is inserted into dithiolene sulphurs via an intermediate product (MPT-AMP) which is synthesized by the *mogA* gene product. In the final step the MPT-AMP can be modified by the *mobA* gene which yields in the a molybdopterin guanine dinucleotide form (MGD). This MGD is utilized by at least ten *E. coli* molybdoenzymes, including three nitrate reductases and three formate dehydrogenases (Iobbi-Nivol and Leimkühler, 2013). Among the 6 genes being implicated in moco biosynthesis, the KOs

of *moaA* (cMP precursor), *moeA* (Molybdopterin molybdenumtransferase) and *moeB* (Molybdopterin-synthase adenylyltransferase) significantly lost fitness post-exposure to model honey. The KOs of the rest of the genes encoding for moco biosynthesis were also seen to be underrepresented after honey treatment (although the Log2 scores were not significantly lower). Thus, the inhibition of moco biosynthesis and incorporation of moco factor to the respective molybdoenzymes is required for the bacterial resistance to honey.

Our results showed that there are two biological functions associated to these differentially expressed genes (DEGs). Selenocysteine and moco synthesis and incorporation were the most enriched pathways. This result suggests that these pathways, which eventually affect the function of the FDHs contribute to the pathogenesis of *E. coli* to honey. Also, disruption of genes encoding for maintenance of redox potential, iron, PMF homeostasis (*fetAB*) and membrane integrity (*prc*, *yohD*) contributed also to bacterial pathogenesis in honey.

3.3.8. Why does loss of function of some genes improve fitness in honey

Insertion sequencing data and Log2 score analysis identified genes in which the transposon insertion caused a growth advantage after treatment with honey. They are of particular importance as they are related to maintenance of growth and effective stress response to honey. Among the 9 genes of honey resistome, *ompR*, *envZ* consist the EnvZ-OmpR two component system (TCS), while the *tolA* is constituent of the Tol-Pal system. Figure 3.14 shows the localization of the genes within *E. coli* cell and their function was described in § 3.3.7. TCS is composed of histidine kinase and the

response regulator which regulates the gene expression under different environmental conditions. TCSs are required for growth in environments of infection sites, therefore, they could be described as essential for pathogenicity. For these reason TCSs have been regarded as potential drug targets.

A successful infection requires sensing of environmental conditions and transferring of signals to regulatory systems which in turn regulate virulence genes. These genes produce virulence factors or assembly of macromolecules. The TCS, EnvZ-OmpR, employs this mechanism in response to certain environmental conditions (acid/osmotic stress) and this is described in the following paragraphs.

The EnvZ-OmpR, has a role in the regulation of the outer membrane (OM) porins (OmpF and OmpC) in response to osmotic and acid stress (Walthers et al., 2005; Chakraborty et al., 2015, 2017). The sensor kinase *envZ* (localised in inner membrane) is triggered by the osmolyte concentration (and/or low pH) in cytoplasm and activates via phosphorylation its downstream target *ompR* (Wang et al., 2012). Phosphorylated *ompR* regulates (repress or activate) the transcription of ompF and ompC the most abundant outer membrane proteins (OMPs). OMPs allow the passive diffusion of small hydrophilic molecules and hydrophilic antibiotics (i.e. b-lactams and fluoroquinolones (Ceccarelli & Ruggerone 2008). In low osmotic stress ompF is the major porin and when osmolality is increased ompF is repressed and *om*pC becomes the major OM porin (Chakraborty and Kenney, 2018). In addition, in response to osmotic and/or mild and strong acid stress, *ompR* promotes acidification of the cytoplasm by repressing the *cadABC* operon which otherwise contributes to acid resistance. A recent study showed that, under osmotic/acid stress, *ompR* programs the transcriptional switch from aerobic to anaerobic growth. This was seen to be a

strong requirement for survival in acid stress (Stincone et al., 2011). The same study showed that *ompR* also activates other genes which are involved in acid response. Thus, *ompR* contributes to acid adaptation.

In contrast to these findings, an older study showed that the mutation of the whole TCS may affects the virulence of the strain, however, deletion of one of the components it was not reported to cause a growth defect (Hsing et al., 1998). In support of this it was recently reported that TCSs are capable of employing resistant mechanisms such as alteration of outer membrane permeability. This results to the limited uptake of the stressor or upregulation of efflux pumps which leads to increase efflux of antimicrobial molecules (i.e antibiotics) (Lingzhi et al., 2018).

Considering these observations is sensible to speculate that deletion of any of the *envZ* and *ompR* KOs is not definitely lethal. In contrast to *envZ*, *ompR* is implicated in many regulatory pathways with any of them being exclusively controlled by *ompR*. However, phosphorylation of the *ompR*, under osmotic/acid stress, repress the *cad* operon which otherwise compensates for the acid stress. Thus, likewise we mentioned in previous paragraph, this could be one of the reasons that *ompR* KO showed a significant fitness to honey. Also, considering the resistance mechanism employed by the TCS is possible that the deletion of any of the components to stimulate a resistant mechanism which leads to cell stress adaptation. Also, given that change of outer membrane conformation gave a fitness advantage to parent strain, is also possible that deletion of *ompR* that failed to regulate OMPs (ompC, ompF), eventually caused the resistance of the KO to honey stress possibly by limiting the outer membrane permeability.

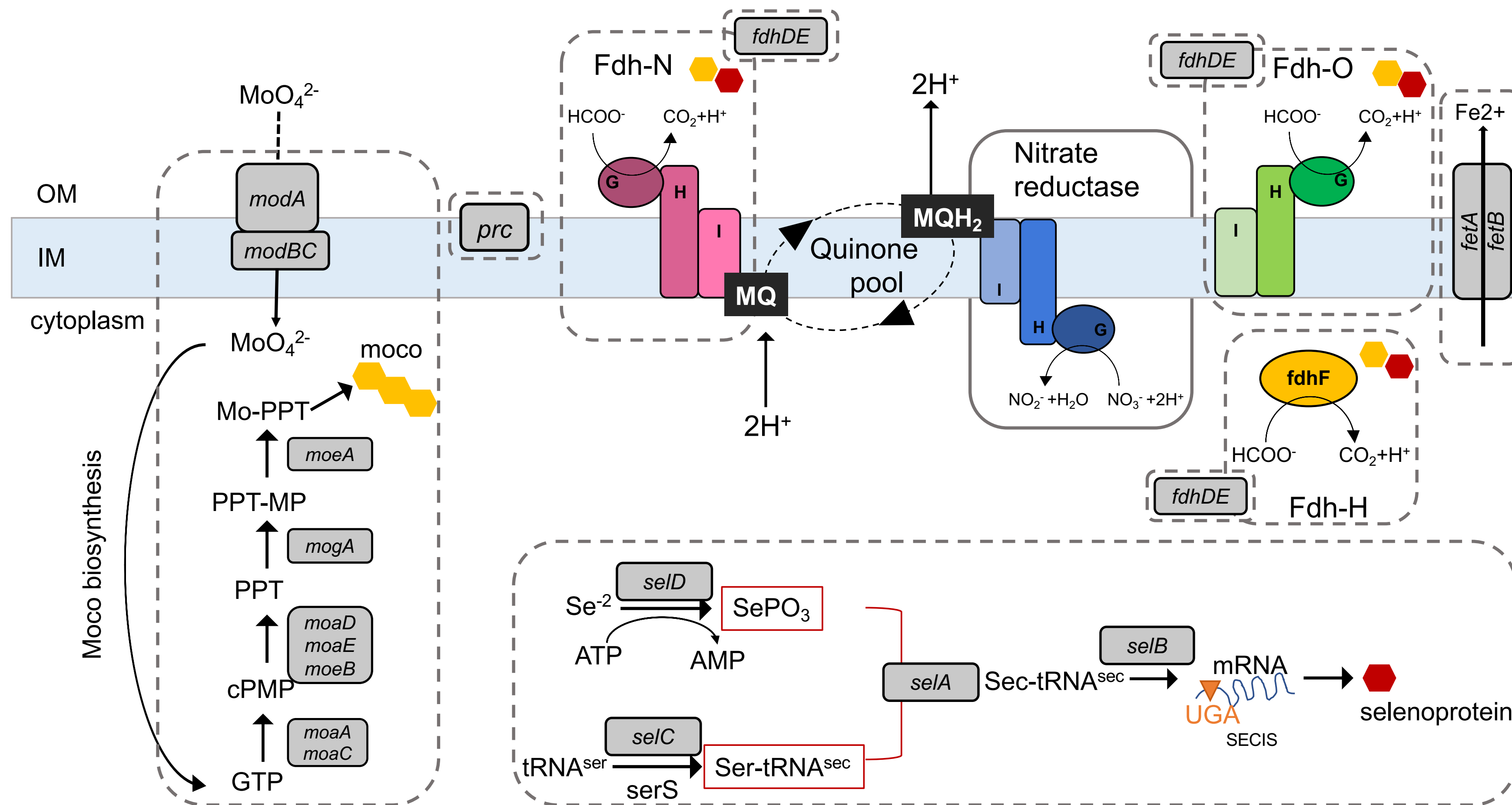


Figure 3.13 Biological pathways where the "loss of fitness" genes are involved. Dashed boxes include biological pathways in which "loss of fitness" genes are involved. Mo cofactor and selenocysteine, being incorporated into the 3 FDHs after the biosynthesis, are denoted by yellow and red polygons respectively.

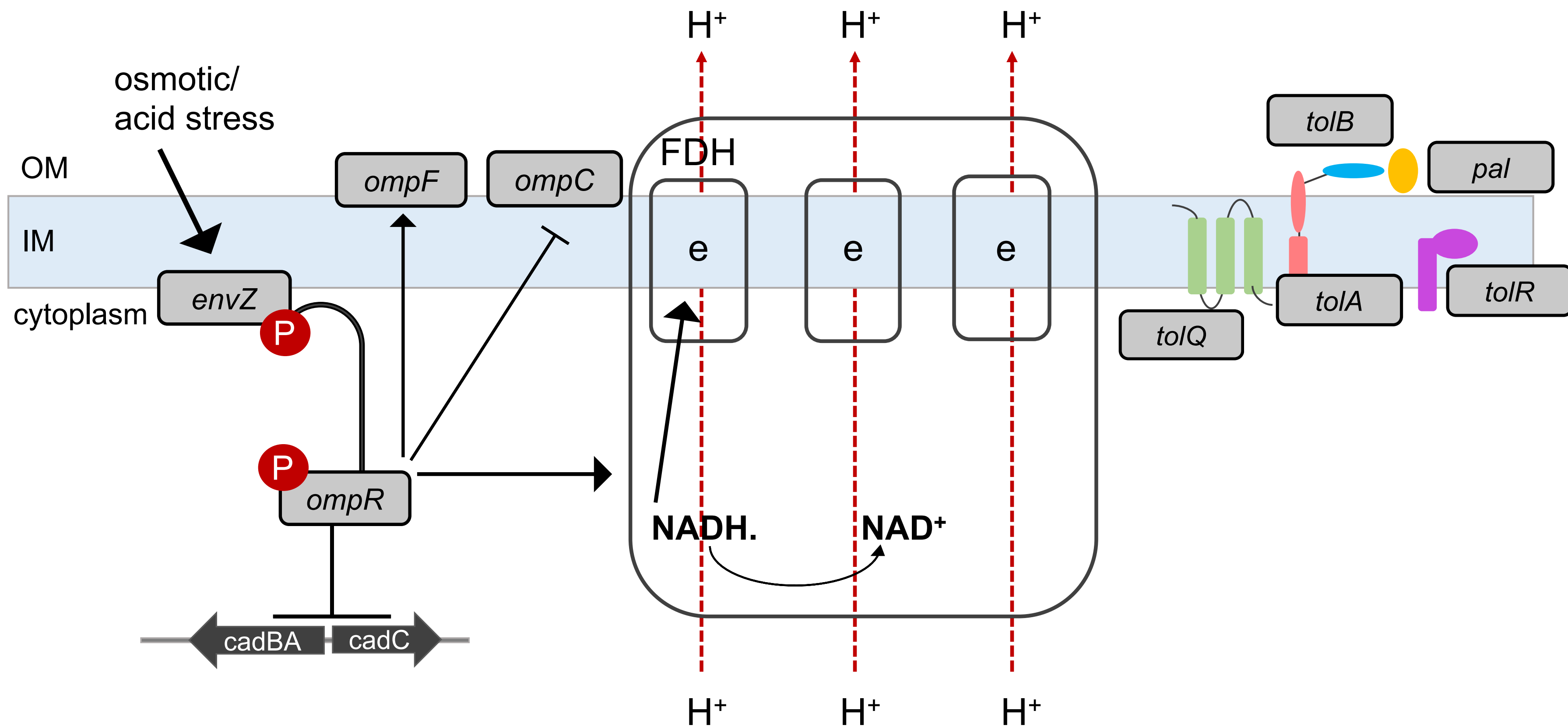


Figure 3.14. Localization of "gain of fitness" genes. The TCS EnvZ-OmpR and the Tol-Pal are regulated in response to osmotic and acid stress. The phosphorylation of the *ompR* results in stimulation of the transcription of OMPs encoded by *ompF* and *ompC* genes, repression of acid resistance and activation of anaerobic metabolism. Tol-Pal system regulates the membrane invagination during cells division and the uptake of bacteriocins.

3.3.9. Explanation of the antimicrobial mechanism of honey as revealed by TraDIS experiment

TraDIS analysis provided an understanding of the molecular mechanisms underlying the fitness of *E. coli* to honey. The identification of biological pathways where “loss of fitness” or “gain of fitness” genes are involved helped us to define a potential resistance mechanism developed by *E. coli* when is exposed to model honey.

As was concluded in Chapter 2, H_2O_2 is not the main stressor in honey since the maximum level that it is generated is much lower than the bactericidal and also H_2O_2 has a very short lifespan. However, as was shown H_2O_2 is essential for the antimicrobial activity of honey since it is implicated in Fenton reaction and the generation of more reactive and long-living ROS. These results were confirmed by TraDIS experiment which showed that *oxyR* (the regulator of antioxidant genes) mutant did not significantly lose fitness. As has been reported, in presence of micromolar H_2O_2, *oxyR* transcriptionally activates a number of genes which are involved in detoxification and repair mechanisms. These are catalase genes (*katEG*), alkyl hydroperoxide-NADPH oxidoreductase (*ahpCF*) which directly degrade the H_2O_2, proteins which restore the intracellular thiol balance (*gorA*, *grxA*, *trxC*), and the ferric uptake regulation protein (*fur*) which prevents transport of Fe^{2+} in order to eliminate the efficiency of Fenton reaction (Zheng et al., 2001a, Zheng et al., 2001b). Although the deletion of most of these genes (*katE*, *ahpF*, *gor, trxC*) caused a growth defect under honey treatment, none was significant considering

the p-value threshold (p-value<0.005). Therefore, this result questioned again the predominance of H_2O_2-induced oxidative stress in honey.

Low pH, mainly controlled by gluconic acid, is the other stressor in honey. In response to acid stress, *E. coli* regulates the 4 acid response systems (ARs). Glutamate, arginine and lysine shared the same mechanism by reductive decarboxylation of the amino acid, export of the product from the cytoplasm and import of the original amino acid. The fourth AR system encodes subunits of the F_0F_1 synthase which transports H^+ into the cell with the consumption of ATP (Richard, and Foster, 2003; Johnson et al., 2011). Among the regulators of AR systems only deletion of the *atpD* caused a significant defect on bacterial growth to honey. This is possibly explained by the sudden drop of intracellular pH which in turn causes uncoupling of oxidative phosphorylation and alteration of the energy metabolism (Richard and Foster, 2004). Thus, neither acid stress alone accounts for the antibacterial effect of honey.

As it turns out from TraDIS results and the enrichment analysis, a large proportion of genes which were important for bacterial fitness in honey, are involved in maintenance of respiration, PMF, redox potential and membrane integrity. These genes are mostly related to the maintenance of cellular homeostasis and membrane integrity rather than defence of oxidative, osmotic or acid stress exclusively. Also, as it was seen by the enrichment analysis, most of these genes are implicated in bacterial stress response under anaerobic metabolism. Considering this, is possible that in presence of gluconic acid and ROS (generated by H_2O_2) which cause the influx of H^+ and anions, cells elaborate the above-mentioned homeostatic mechanisms. Thus, the transposon insertion into vital subunits of these pathways,

reduced their ability to exert homeostasis or other detoxifying mechanisms which caused significant loss of fitness to the respective mutants.

Particularly, the destruction of structural subunits implicated in the function of Formate dehydrogenases (FDHs) had a great growth defect on cellular function. The function of FDHs was impaired by the deletion of genes implicated in synthesis and incorporation of selenoproteins (*selAB*) and moco (*moaA*, *moeAB*) and also deletion of FDHs structural subunits (*fdhED*, *fdoGHI*). As it has been reported FDHs have a particular role in resistance to oxidative stress. In the presence of H_2O_2, the FDH-N/Nar complex is responsible for the function of PMF redox pool by effectively controlling the translocation of ions across the membrane (Simon et al., 2008). Also, in the WT, selenoproteins trap H_2O_2 before it reacts with other targets (Winterbourn and Hampton, 2008) and *fetAB* operon displaces the excess of Fe^{2+} out of cell so as to prevent the Fenton reaction in presence of H_2O_2 (Nicolaou et al., 2013). Therefore, it is sensible to speculate that the loss of *sel* and *fet* genes caused greater impact on cellular function because of the unrestrained ROS generation and the prompt damage of DNA and proteins by H_2O_2.

Mutations of genes which are implicated on invagination of the outer membrane (*prc*) and the inner membrane homeostasis (*yohD*) were also seen to decrease in fitness. This implies severe lesions of cell envelop and inner membrane under honey stress and this is in agreement with the findings of Chapter 2.

Genes whose mutation caused a fitness advantage are diverse. However, the presence of TCSs (EnvZ-OmpR) encoding for outer membrane porins is of particular interest. Although the explanation of the resistant phenotype of these mutants needs further investigation a few possible explanations were given. One

explanation suggests that deletion of one of the TCS subunits accelerates the resistance mechanism of the rest. The resistance could be possibly due to the change in the conformation of the bacterial membrane. This change suggests decrease of the membrane permeability or acceleration of the bacterial efflux mechanism in order to maintain intracellular homeostasis. Also, the significant phenotype of *ompR* mutant could be explained by the regulation of *cad* operon in response to acid stress induced by model honey. As was previously mentioned, *ompR* repress the expression of *cad* operon in presence of acid/osmotic stress. Thus, in *ompR* KO, *cad* operon is possible to be positively regulated. The *cad* operon encodes two genes which are basically induced under conditions of low pH, anaerobiosis, and excess lysine (Stincone et al., 2011). Considering the significant fitness of *ompR* mutant, it is sensible to speculate that, under the conditions tested here, the *cad* operon confers adaptation and growth of the mutant.

The results discussed in this section will be considered again in the next paragraph which aims to confirm these observations by competition experiments and testing of single mutants susceptibility to model honey.

3.3.10. Validation of gene essentiality

In the previous paragraphs the TraDIS results were discussed focusing on the identification of the genes whose deletion conferred significant growth defect/advantage in model honey and the pathways in which they are involved. TraDIS is considered as “a large” competition experiment in which millions of single

transposon mutants compete with each other after treatment with the model honey. In order to confirm the phenotypes caused by the loss of function in individual genes, single mutants were constructed and exposed to model honey by applying the same conditions as previously (TraDIS experiment).

The validation experiments provide two lines of evidence for the significant KOs as they were identified by TraDIS analysis. First, the phenotype of certain transposon library KOs were compared to individual mutants when they compete to the WT. Second, this investigation will go on to test the susceptibility of the selected mutants on a killing assay as it was performed in Chapter 2. In doing so it may be possible to confirm the TraDIS data and distinguish between KOs that lose, gain fitness (or not affected) or completely eradicated. Ultimately, this investigation aims to confirm the molecular target(s) of honey providing with a proposal on the underlying mechanism of it.

To validate the TraDIS results, individual gene KOs were constructed for some of the significant genes identified before. Kanamycin resistant mutants from the Keio library (*E. coli* BW131) were transferred into MG1655 strain by P1 transduction. For 4 of the "loss of fitness" genes (*atpD*, *fdhE*, *fdoG*, *fdoI*), and 3 of "gain of fitness" genes (*tolA*, *rep*, *envZ*) we were unable to create mutants from the Keio library. Although *gor* does not belong to the list of the "loss or gain fitness" genes shared between the two datasets, it was tested as it controls the electron transfer activity and cell redox homeostasis (Zheng et al., 2001a). In particular, *gor* mutant showed a significant growth defect after 30' exposure and up to 90' the 2Log fold change got a positive value. This is might relevant to the higher oxidative potential of honey during the first minutes of exposure before the H_2O_2 get

degraded. Also, this is another typical example of KOs that grow slower on honey stress.

Prior to the competition experiment, the *lacZ* mutant was competed against the WT in order to determine whether the former has any competitive advantage or disadvantage. Surprisingly, when the two strains mixed in a 1:1 ratio, *LacZ* KO grew slightly better (1 Log higher) than the WT. Thus, the strains were mixed in a 1:3 (KO:WT) ratio which yielded in equal growth. Then, each of the mutants was competed against the *lacZ*- strain in model honey (for 30' and 90'). In contrast to TraDIS experiment, the outgrowth step was skipped as a preliminary experiment showed that implementation of this step resulted in no detectable fitness defect in any of the KOs tested.

The Figure 3.15 shows the relative fitness of 11 mutants when they competed to the *lacZ*- strain (considering here as the WT). In agreement with TraDIS results, most of the mutants which were significantly underrepresented post-honey treatment also lost fitness in the competition experiment. Within 90', *fdhD*, *gor*, *moaA* and *moaB* mutants significantly lost fitness. The *prc* mutant was completely eradicated which validated the TraDIS data showing the importance of this gene in resistance to honey. Among the *moaABC* operon, essential for the moco biosynthesis, *moaC* mutant was the less affected. Also, the null *fdoH* strain gained fitness from 30' to 90' minutes being in agreement with TraDIS data which showed increase of the Log2 score within this time of exposure. Null *gor* strain significantly lost fitness from 30' to 90' of treatment while the opposite was seen in TraDIS analysis. This might showes that *gor* mutant is in viable but not culturable state which implies low metabolic activity without ability to divide.

Although TraDIS analysis showed *wecACG* KOs to be significantly enriched, null *wecACG* strains significantly lost fitness comparing to the control. This implies that *wec* operon, encoding for the ECA synthesis, is possibly important for the survival of *E. coli* to honey. This observation is in agreement with a study which claimed that the enrichment of these mutants is due to polarity effects that cause insertion bias (Manna et al., 2007). *OmpR*, the most significantly enriched mutant, showed a resistant phenotype for the time of exposure. In fact, *ompR* KO showed a relative fitness higher than 1 (average fitness 1.2). Although this was not seen to be significant comparing to the *lacZ*- strain it is obvious that *ompR* has a growth advantage post-exposure to model.

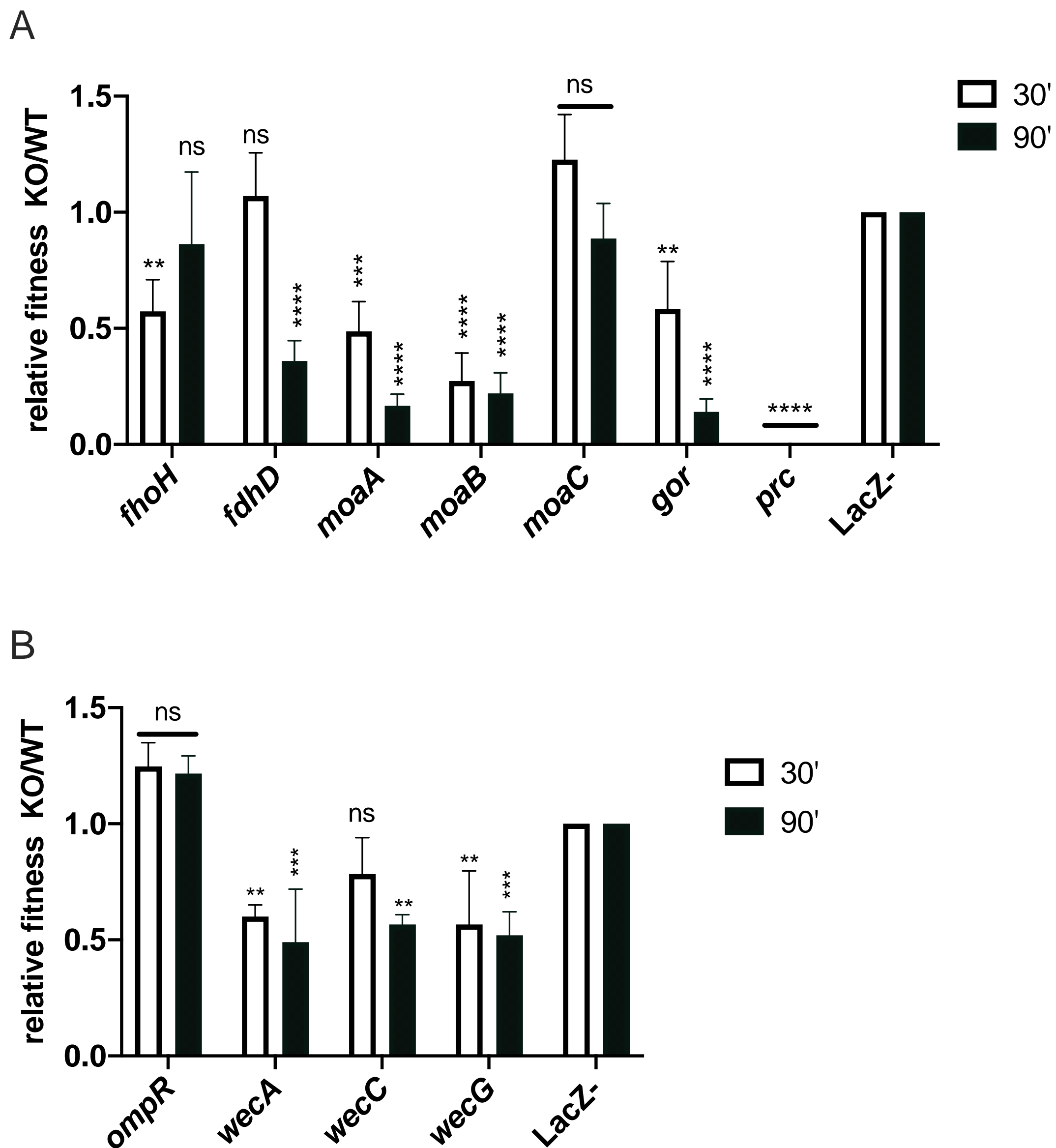


Figure 3.15 Relative fitness of single KOs when they are competed to WT (LacZ- phenotype). (A) underrepresented and (B) over-represented mutants were competed the LacZ- phenotype (*E. coli* MG1655) in model honey for 30' and 90'. LacZ+ (WT) was also competed the LacZ- phenotype. Two-way ANOVA: asterisks show significance levels of Sidak's multiple comparisons test to the control (LacZ-) (**** p< 0.0001, ns; p> 0.05). Error bars represent the average ± s.d. (*n*=3; biological replicates).

A second line of evidence of the importance of these genes in honey resistance was given by testing their susceptibility to model honey. This was done likewise was described for the catalase mutants in Chapter 2. The susceptibility of the mutants was tested 24 h post exposure to model as no significant reduction was seen by 2 hours (Appendix 3.2). In order to define a severity of the phenotype, a threshold was set in order to cluster the mutants in 3 groups as having strong/weak/intermediate phenotype. Considering the more and the less weak mutants we defined 3 phenotypes: (a) strong phenotype for mutants showing less than 2 logs reduction, (b) intermediate weak for those showing 3-4 logs reduction and (c) weak those with higher than 5 logs reduction.

As was seen in Figure 3.16 *fdhD*, *gor* and *prc* mutants were the most susceptible to model honey. In agreement with TraDIS and competition killing assay *prc* mutant demonstrated a weak phenotype followed by *gor* and *fdhD* which both had an intermediate weak phenotype. The rest of mutants (*moaABC* and *fdoH*) had an intermediate weak phenotype excepting the *ompR* and *wecC* which both were seen to be resistant. In agreement to our previous findings *ompR* mutant demonstrated almost equal survival to control (untreated cells) and higher survival comparing to the WT.

Overall, most of the phenotypes observed in previous experiments are in agreement with TraDIS analysis. As it turns out, *prc*, gor and *fdhD* are the genes conditionally essential for bacterial resistance while the *ompR* KO conveys selective advantage and increase of the bacterial fitness in honey.

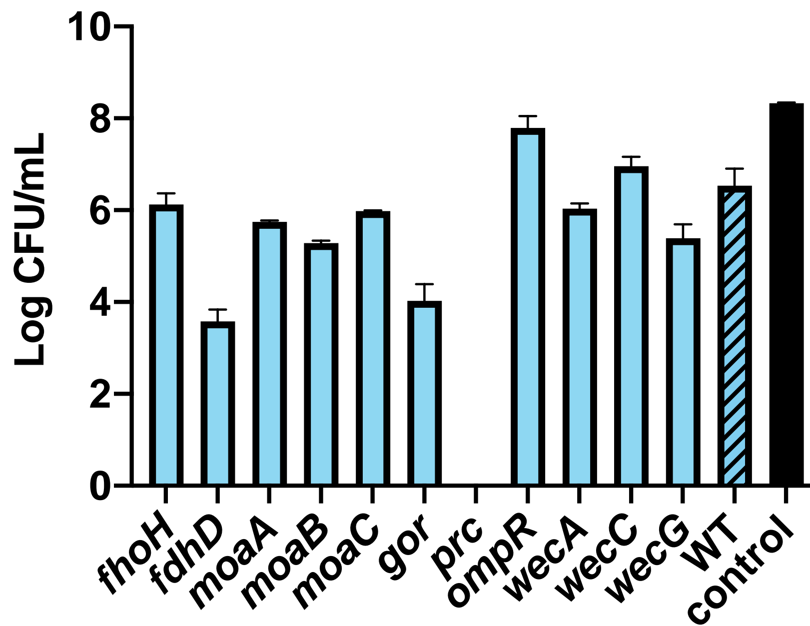


Figure 3.16 Effect of model honey on *E. coli* KOs. Single KOs of genes previously shown (Table 3.4) as “loss of fitness” and “gain of fitness” were treated with model honey for 24h. Error bars represent the average ± s.d. (*n*=3; biological replicates).

3.4 Discussion

In summary, this chapter demonstrated that a modified TraDIS method can be used for the identification of genes whose mutation confer significant phenotype (resistance/susceptibility) to *E. coli* when exposed to model honey. The high correlation coefficient within the biological replicates provided reliable data for the identification of these genes within the MG1655 genome. The identification of the pathways where these genes are involved enables the better understanding of underlying mechanism of honey which was the scope of this thesis.

In agreement with the findings of the previous chapter, TraDIS results demonstrated that antimicrobial effect of honey results from the synergy of the three stressors: osmolality, low pH and oxidative stress. Under these stress conditions is possible that bacteria swift from aerobic growth to anaerobic respiration. This is explained by the fact that a large proportion of genes essential for the bacterial fitness in honey was seen to get involved in anaerobic metabolism and maintenance of proton motive force. Below is provided a hypothesis on the antibacterial mechanism of honey which is based on TraDIS results and a recent study which suggested the role of the *ompR* in response to osmotic and acid stress (Stincone et al., 2011).

As it was reported the switch from aerobic to anaerobic respiration, controlled in part by the *OmpR* regulator, is key for survival in osmotic/acid stress conditions. In anaerobic metabolism Formate dehydrogenases (FDHs) and Nitrate reductase (Nar) control the cellular homeostasis maintaining the PMF. TraDIS data showed that inactivation of genes encoded for the formation and maturation of FDHs caused

a growth defect, thus, the cell homeostasis was disrupted post-treatment with honey. In this case the influx of H^+, brought about by the gluconic acid, is possible that decreased the intracellular pH. In addition, it was seen that deletion of *fetAB* encoding for iron homeostasis conferred growth defect to the mutants. As it was discussed before, impairment of iron homeostasis results in the abundance of Fe^{2+} within the cytoplasm. This in turn enhances the ROS production, via the Fenton reaction which is initiated by the honey-induced H_2O_2. Also, impairment of selenoproteins synthesis (sel*AB* KOs*)*, augments the toxic effect of H_2O_2. Therefore, it was speculated that by mutation of these defence mechanism cells are unable to maintain the internal pH and the redox balance. This effect was potentially augmented by the irreparable cell membrane damage. Indeed, *prc* KO encoding for the PG enlargement, membrane invagination and integrity, showed the most significant growth defect. In accordance to previous studies, *prc* KO has been very defective in osmolality and redox-active antibiotics. Likewise, the synergy of the stressors in honey is possible to cause increased membrane permeability and the periplasmic protein leakage. These events is very likely to cause such a strong phenotype to *prc* null strain. This phenotype was also observed in FC data and AFM pictures of after the exposure of the WT to model honey.

Moreover, TraDIS analysis showed that that deletion of the TCS OmpR-EnvZ induced growth advantage which also confirmed by further experiments. This is might due to the fact that mutation of TSC prevents from further acidification of the cytoplasm and decrease the outer membrane permeability. As has been showed the decrease of intracellular pH, in response to acid/osmotic stress, induces further ROS generation by increase cellular respiration activity (Levicán et al., 2019). Thus,

prevention of this activity may confers a growth advantage of *ompR* and *envZ* mutants. The data acquired here are not enough to make further conclusions. However, these genes are of particular interest as they potentially employ unexploited mechanisms which disarm the effect of the antimicrobial and enable a sustained bacterial stress response.

Overall, in this Chapter were provided two layers of evidence for the role of genes significant for the pathogenicity/resistance of *E. coli* towards honey: by simultaneously assaying the whole transposon library and by the generation of defined mutants for independent phenotypic testing. The agreement of data shows that the modified TraDIS method employed here is suitable for the identification of mechanistic action of any kind of antimicrobial compound. Metabolomics would be a useful tool to elucidate the metabolic pathways in mutants which were significantly affected post-honey treatment. Also, RNA-seq could further be used in order to define the global gene expression under the same conditions. Thus, the combination of these data will give a complete characterization of *E. coli*'s cellular and molecular responses to honey.

In conclusion, two major findings turn out from this study. The first relies on the deeper explanation of the antibacterial mechanism in honey confirming our initial hypothesis of the synergy posed by three main stressors in honey. Secondly, these results showed that *prc*, a candidate which has been previously reported as a major target of antibiotics, is one of the major targets of honey as well. This holds further premises for the potential use of honey as an antibiotic.

CHAPTER 4

The effect of honey in mixed microbial population

4.1. Introduction

In the previous chapters, the model organism *E. coli* K-12 MG1655 was used to probe the phenotypic and genotypic changes induced by honey stress. This bacterium serves as a reference to explain the biochemistry and molecular biology of many prokaryotic cells. However, in their natural environment, microorganisms exist in close association with other species (bacteria-bacteria, bacteria-fungi/yeast etc.). The interactions between the taxonomically diverse microorganisms are highly dynamic and depend on the microorganisms themselves and the host factors. Particularly, polymicrobial biofilm formation requires a range of physical interactions and molecular communication between the cells which confers a selective advantage on their growth. They are usually tolerant to antimicrobial therapy at concentrations higher than these required for eradication of planktonic cells (Stewart and Costerton, 2001; Frey-Klett et al., 2011). In clinical cases, biofilms are associated with persistent chronic infections (i.e. foot ulcers, anaerobic soft tissue infections, cystic fibrosis lung infection etc.).

As was seen in the literature review of Chapter 1, the majority of publications studied the effect of various honey types on a range of microorganisms. Only some of these studied (*in vitro* or *in vivo*) the effect of medicinal honey products on single species or mixed microbial community isolated from wound sites. Although the results were encouraging for the use of honey in the wound healing, limited is the knowledge about the potential of honey to treat other kinds of polymicrobial infections other than the epidermal. In particular, although fungal infections account

for the 40-75% of lethal diseases less than 5% of the publications studied the susceptibility of yeast species to honey (Figure 1.6).

Therefore, this chapter aims to investigate the effect of honey on a bacterial-yeast community *in vitro*. A vaginal simulation fluid (VSF) was developed in order to study *in vitro* the interaction of two pathogenic species, *E. coli* (UPEC) and *C. albicans*, during the bacterial vaginosis (BV). The goal of this study is to give further insight on the honey-induced physiological changes on a BV-associated microbial community. Thus, this may unravel a new potential medical application of honey other than the wound healing.

4.1.1. *Candida albicans*

Candida albicans is one of the most prevalent pathogens among the yeast species and is common in mucocutaneous diseases (Richards et al., 2000). Fungal infections account for the 40-75% of lethal diseases and candidemia in particular accounts for the 25-38% of the cases (Tortorano et al., 2004). This dimorphic yeast adheres to mucosal cells, competes with commensal bacteria and thrives as a biofilm (Nobile and Johnson, 2015). *Candida albicans* is the most infectious agent which colonizes the skin, and the mucocutaneous regions of the gastrointestinal and reproductive tracks (Sobel, 2006). Among the vulvovaginal (VV) infections, bacterial vaginosis (BV) and vulvovaginal candidiasis (VVC) are the most frequent and are diagnosed in up to 40% of woman with vaginal discomforts (Anderson et al., 2004).

4.1.2. The two common causes of Vaginitis

Bacterial vaginosis (BV) and vulvovaginal candidiasis (VVC) or yeast infection are the two most common forms of vaginitis in women. Although the predominant causative agent in each case is different, both types of vaginitis are examples of mucosal polymicrobial infections (Figure 4.1).

BV is the clinical condition associated with the loss of *Lactobacillus* species and the replacement by a mixed microbiota community where *Gardnerella vaginalis* is predominant. Other anaerobic bacteria (i.e. *Atopobium* spp., *Prevotella* spp. and *Peptostreptococcus* spp.) are also present on the mixed microbial community (Spiegel, 1991). Vaginal yeast infection, the second most common vaginitis, is caused by the excessive growth of *Candida albicans* in conditions where *Lactobacilli* are decreased (dysbiosis). In this case, *C. albicans* is transitioning from a commensal to opportunistic pathogen and causes symptoms like pain and discharge (Marrazzo, 2003). Although *C. albicans* accounts for the 80% of the VVC cases, there are also other species (i.e. *C. glabrata*, *C. krusei*, *C. parapsilosis*) commonly associated with the non-*C. albicans* VVC.

Standard test consisting of vaginal pH measurement, amine, saline and potassium hydroxide (10%) microscopy are done in order to discriminate between BV and VVC. In general, VVC does not affect the vaginal pH, which remains between 4-4.5. However, cases such as BV, trichomoniasis or some sort of mixed infection increase the vaginal pH. Saline microscopy is used for the identification of clue cells indicative of BV. Potassium hydroxide (KOH) microscopy, is used to

differentiate fungal elements form bacteria and other cells in vagina which are killed by KOH (Marrazzo, 2003).

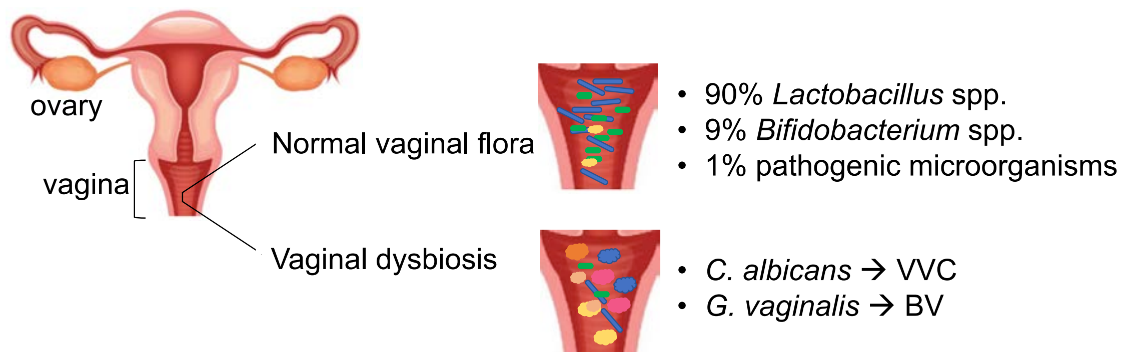


Figure 4.1 The two types of vulvovaginal infection. *C. albicans* and *G. vaginalis* are the main causative agents of VVC and BV respectively which overgrow the vaginal microbiota (i.e. *Lactobacillus*) and cause inflammation (Marrazzo, 2003).

4.1.3. Polymicrobial interactions involving *Candida* and Gram-negative bacteria

Interaction of *Candida* with *E. coli* increases colonisation and infection compared to mono-infection with either microorganism (Klaerner et al., 1997; Ikeda et al., 1999). In cases of fungal VV infections, *E. coli* enhances the adhesion of *C. albicans* to mucosal surfaces (Levison and Pitsakis, 1987) as in general bacteria have always higher binding affinities for the vaginal cellular receptors. After bacterial binding to epithelial cells, *Candida* can attach to bacterial surface by protein-protein interaction or by recognition of salivary proteins (Morales and Hogan, 2010). Because of these interactions, ventilator-associated pneumonia is more persistent when *P. aeruginosa* co-colonises with *C. albicans* (Nseir et al., 2007) and

inflammation of the oral mucosa is greater when oral bacteria (i.e Streptococus, Actinomyces etc.) co-exist with *C. albicans* (Bamford et al., 2009).

A characteristic interaction between *E. coli*, *C. albicans* and *Lactobacilli* is presented in Figure 4.2. *C. albicans* agglutinates to the mannose-binding site of *E. coli* which contributes to the attachment of the yeast to VV mucosa (Morales and Hogan, 2010). Also, *In vitro*, it was seen that formation of lipopolysaccharide (LPS) by *E. coli* promoted biofilm growth of *Candida* (Bandara et al., 2009). The biofilm formation is enhanced by quorum-sensing signals that promote the communication between bacteria and fungi. In particular, *Candida*, secretes a quorum-sensing molecule called farnesol which controls also the hyphae growth. This molecule can modulate bacterial behaviour, by making them more virulent. Also, farnesol promotes the generation of ROS through effects on electron transport chain components. This process plays important role in competition of the yeast with the bacteria (Machida and Tanaka, 1999; Davis-Hanna et al., 2008). Eventually, mechanisms such as adhesion, phenotypic switching (hyphae formation), production of hydrophilic enzymes and biofilm formation, contribute to virulence of *Candida* (Dhamgaye et al., 2016).

In contrast, *Lactobacillus* spp., the lactic acid bacteria consisting the microbiological flora of female reproductive tract, compete with *Candida* for adhesion to the mucosal surface. This is done by (a) the formation of a barrier which competes for receptors on the epithelial cells and (b) the production of antimicrobial compounds (i.e. H_2O_2, lactic acid and bacteriocin-like compounds) (Figure 4.2) (Boris, S. and Barbés, 2000). This mechanism is explained in paragraph 4.1.4.1.

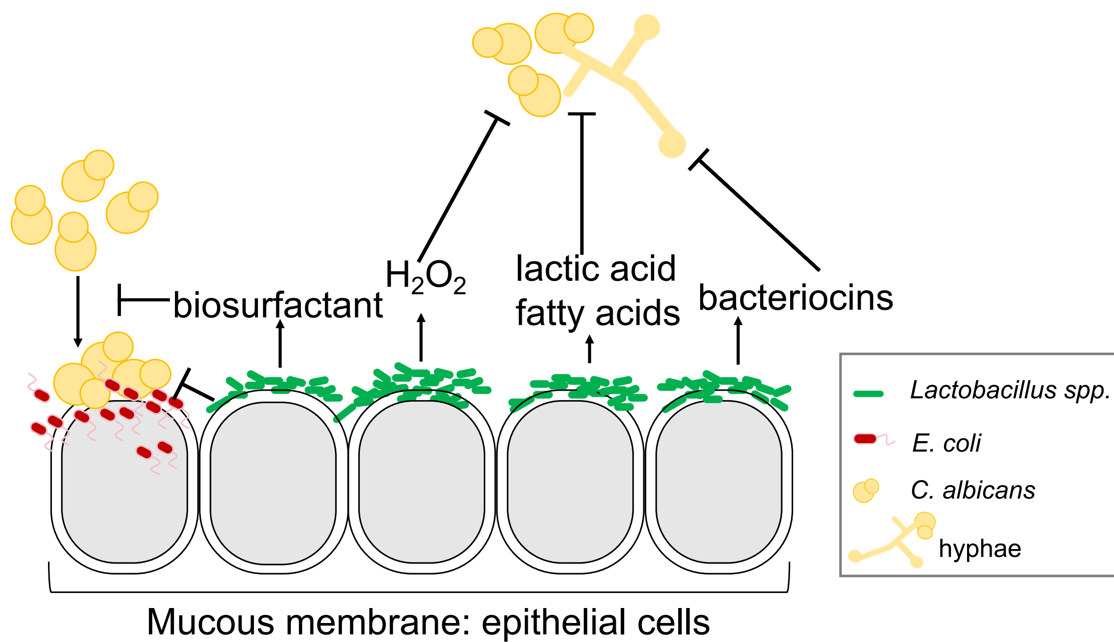


Figure 4.2. Interactions of *C. albicans*, *Lactobacillus* spp. and *E. coli* on epithelial cells of female reproductive tract. Coaggregation of *E. coli* and *C. albicans* contributes to biofilm formation and cell to cell communication. Biofilm forming *E. coli* secretes LPS which facilitates the attachment and colonisation of *C. albicans* to vaginal mucosa. *Lactobacillus* spp., which normally inhabits the female reproductive tract, defends the host against colonization of *C. albicans* by competitive exclusion from binding sites, and by secretion of lactic acid, bacteriocin-like compounds, biosurfactant and H_2O_2 which inhibit the proliferation and invasive hyphae formation of the yeast.

4.1.4. Treatment of VVC

Bacterial-fungal mixed infections increase the frequency and severity of diseases because of biofilm formation. This promotes the resistance of the species to both the host defence and antimicrobial agents. A typical example is the increased resistance of *S. aureus* to vancomycin when it is co-cultured with *C. albicans,* because of the formation of a viscous matrix which restricts the penetration of drugs (Xu et al., 2008).

Likewise 75% of all women develop at least one episode of VVC and 50% of them will have more than 1 recurrent VVC in their lifetime which is a result of a persisting single *Candida* strain or introduction of new strains (or even species) (Xie et al., 2017). Some studies suggest that while antibiotics initially relieve symptoms, they eventually increase the frequencies of recurrent VVC (Spinillo et al., 1995; 1999; Leegaard, 1984) while others reached the opposite conclusion (Geiger and Foxman, 1996; Reed et al., 1989).

Most of the fungistatic drugs (i.e. azole), which inhibit cell wall metabolism, are used in non-complicated VVC cases. They inhibit the synthesis of β-glucan (a cell wall molecule), synthesis of membrane lipids and DNA replication. Butoconazole, clotrimazole, fluconazole, miconazole, terconazole and tioconazole are used in oral and topical therapies demonstrating long-term cure and short-term mycologic cure rates. In case of recurrent VVC (RVVC), ketoconazole, clotrimazole, and fluconazole of a different dose/intake frequency are suggested (Nyirjesy, 2008). Other approaches such as use of yogurt and *Lactobacilli* therapy (Hilton et al., 1992), “inactivation” of *Candida* antigen (Rigg et al., 1998) and hormonal

manipulation (Dennerstein, 1986) were also suggested in treatment of RVVC. However, these methods are not officially recommended because of the limited experimental data and clinical studies.

4.1.4.1. Use of probiotics in VVC treatment

It has been shown that 96% of healthy women are colonised by H_2O_2-producing *Lactobacillus* spp. a major part of the vaginal microflora. As was mentioned before, lactobacilli, that make up the vaginal microbiota, produce bacteriostatic/bactericidal compounds, maintain the pH within the range of 4-4.5 and create a protective layer which prevents the host by VV infections. Lactobacilli have higher binding efficiency to epithelial cells receptors compared to *C. albicans* and *G. vaginalis* thus they have the ability to displace these pathogens from vaginal cells. The metabolic activity of *Lactobacillus* spp. controls the acidification of the vagina, by the conversion of glycogen to fatty acids and lactic acid which in turn lowers the pH and inhibits the proliferation of pathogens (i.e. *C. albicans*, *E. coli*, *G. vaginalis* etc.). Also, LABs produce antimicrobial compounds such as H_2O_2, bacteriocins, biosurfactants and lactic acid. The H_2O_2 contributes to non-specific antimicrobial host defence in the vagina. This is used by peroxidase which forms hypohalous acid or halogens with toxic properties. Bacteriocin-like compounds, secreted by LABs, inhibit the pathogenic species and promote the growth of microbiota. Biosurfactants facilitate the uptake of water-immiscible substrates inducing antibiotic activity in a range of microbes (Boris and Barbés, 2000).

L. acidophilus, *L. crispatus*, *L. gasseri*, *L. iners*, *L. vaginalis* and *L. jensenii* are the predominant *Lactobacillus* spp. in the vagina. Administration of medical formulations combining vaginal microbiota with a microbicide, has been proposed for the treatment of BV and VVC (Martinez et al., 2009; Larsson et al., 2011; Köhler et al., 2012). The majority of these supplements contain lactobacilli at 10^{8-9} CFU/dose along with the respective active ingredient (i.e. antibiotic, excipients etc.) (Van de Wijgert and Verwijs, 2019). These studies showed that probiotics can suppress the growth or kill the VVC-causing *C. albicans* while a recent review supported that probiotics were efficient against BV but not VVC (van de Wijgert et al., 2019). The same review reported that *Candida* species often co-exist with *Lactobacillus*, thus use of probiotics for the treatment of BV does not guarantee the elimination of increasing VVC incident after treatment. However, this should not be a reason to discontinue the development of the probiotic supplements for the treatment of vaginitis, given the rising risks of antimicrobial resistance.

4.1.4.2. Honey enhances the growth of probiotic bacteria

Honey contains oligosaccharides which can be utilized by fermentative lactic acid bacteria to yield metabolites that promote the probiotic effect of honey. Previous studies investigated the role of honey on the enhancement of lactic acid bacteria when this was added to milk. Specifically, addition of 5% honey in cow and camel milk enhanced the growth of *L. acidophilus*, and *S. thermophillus* (Varga et al., 2014). Other three *Lactobacillus* spp. (*L. plantarum, L. casei, L. helveticus*) maintained their viability in presence of honey and they were significantly increased

only in presence of the inulin (the oligosaccharide fructan) (Nagpal and Kaur, 2011). The same was observed for *L. acidophilus* and *B. bifidum* when both were exposed to honey (diluted at 5%), sucrose and inulin (Popa and Ustunol, 2011). Also, *L. acidophilus* maintained its viability after exposure to three honeys (clover, Jordan and India indigenous) while the growth and the production of lactic acid was enhanced by the addition of sucrose in honey (Shamala et al., 2000; Chick et al., 2001, Haddadin et al., 2007).

Although these findings encourage the use of honey as a way to increase or maintain the viability of probiotics, further research is needed in order to define the criteria that increase the functionality of probiotics (i.e. types of honey, optimal concentration, composition in oligosaccharides etc.).

4.1.5. Overview of this experiment

Considering all the above in the management of VVC and the contribution of probiotics to the host defence mechanism it was speculated that the ideal VVC medication should be applied in such a way that it causes the inhibition of *C. albicans* (and any other opportunistic pathogens) while preserving or enhancing the viability of *Lactobacillus* spp. Therefore, this Chapter aims to investigate the effect of honey on the eradication of a polymicrobial infection comprising of two species occurring in vulvovaginal infections: *E. coli* ST131 (UPEC) and *C. albicans*.

Although *Candida* is implicated in VVC and *E. coli* in UTI, symbiosis of the two species in vaginal mucosa has been recorded in prepubescent girls (Tibaldi et al., 2009; Hall and Noverr, 2017). Following the methodology of Chapter 2, it was

first investigated the susceptibility of the two species, as monocultures, to both real and model honey. In this case, model honey was used as reference while the real honeys were tested for their antimicrobial efficiency against the mixed microbial community. Subsequently, a vaginal fluid simulant was used to evaluate *in vitro* the effect of honey on eradication of the two species growing in this environment. This simulant has been used before for testing of liposome gels in therapy of vaginitis (Pavelić et al., 2005a, 2005b) and the effect of other agents on the reregulation of cervical mucus fertility (Lee et al., 2002). Later, the susceptibility of *L. acidophilus* to the same honeys was tested. This aimed to identify whether any of the honeys that have a high antimicrobial activity could also promote the growth of *L. acidophilus*.

Eventually, this chapter will conclude with a discussion on the physiological changes happen in the two pathogenic species post- honey treatment and will give a further prospective on the medicinal use of honey for the treatment of infections occur in body cavities other than epidermally.

4.2. Materials & Methods

4.2.1. Honey samples

Heather honey purchased from a local market (Birmingham, UK), natural (unprocessed) Indonesian stingless bee honey (*Trigona* spp.) and the model honey (as was prepared previously: 30% sugars, 8.6 mM gluconic acid and 3 mM H_2O_2) were used in this study.

4.2.2. LAPTg broth

LAPTg broth and LAPTg agar was used for the culture and enumeration of *L. acidophilus* (Tomás and Nader-Macías, 2007). LAPTg broth consists of peptone (15 g/L), tryptone (10 g/L), glucose (10 g/L), yeast extract (10 g/L), Tween 80 (1 g/L) and agar (13.7 g/L).

4.2.3. Vaginal simulation fluid

Vaginal simulation fluid (VSF) was made as described by Owen and Katz (1999). VSF consists of NaCl (3.5 g/L), KOH (1.4 g/L), $Ca(OH)_2$ (0.22 g/ L), bovine serum albumin (18 mg/L), 90% lactic acid (2.2 g/L), glacial acetic acid (1 g/L), 50% glycerol (0.32 g/L), urea (0.4 g/L), and glucose (5 g/L). The pH of VSF was adjusted to pH 4.2 using HCl (Pavelić et al., 2005a). Before use, VSF was sterilised through a 0.22 µm filter and stored at 4°C until use.

4.2.4. Bacterial strains and culture conditions

E. coli ST131 (UPEC strain), *C .albicans* SC5314/ ATCC MYA-2876 and *L. acidophilus* were used for the experiments in this Chapter. *E. coli* was grown overnight in 5 mL of Luria Broth (Sigma-Aldrich., UK) shaking in 20 mL flask (150 rpm/ 37°C). *C .albicans* was grown overnight in 5 mL of Yeast Peptone Dextrose (YPD: 1% yeast extract, 2% peptone, 2% glucose) broth (Sigma-Aldrich., UK) shaking in 20 mL flask (200 rpm/ 37°C). Activation of the stock *L. acidophilus* culture preceded the preparation of the working culture. For the activation a 30 µL aliquot of *L. acidophilus,* stored in LAPTg/glycerol stock at -80°C, was revitalized twice in 20 mL of LAPTg broth in 20 mL flask at 37°C for 48 hours without agitation. Then, 30 µL aliquot of the activated culture was sub-cultured in 20 mL of LAPTg and was grown for 48h under the same conditions. Before use, cells were centrifuged (3900 g/ 3minutes) washed twice and resuspended in Ringer's solution (2.25% NaCl, 0.105% KCl , 0.12% CaCl hexahydrate and 0.05% $NaHCO_3$) (Oxoid Ltd., UK) to a final absorbance of 1. All strains were stored long term at -80 °C in stocks containing 50% glycerol.

Coculture experiments were conducted in filter sterilized YPD liquid medium. One loopful of overnight *C. albicans* and *E. coli* respectively were inoculated in 5 mL of YPD in 20 mL flask and allowed to grow overnight (170 rpm/ 37°C). Overnight cultures were centrifuged (3900 g /3 minutes), washed twice and resuspended in same volume of Ringer's solution. 5 mL of mixed culture were inoculated to 15 mL of VSF in a 20 mL conical culture flask and grown statically at 37°C. Over the next five days of inoculation, CFUs were measured by plating 5 serial dilutions on

MacConkey lactose agar base (peptone 17 g/L, Proteose peptone 3 g/L, Lactose monohydrate 10 g/L, bile salts 1.5 g/L, sodium chloride 5 g/L, neutral red 0.03 g/L, crystal violet 0.001 g/L, agar 13.5 g/L) (CM0007; Oxoid, UK) and Rose Bengal Chloramphenicol Agar Base (RBCA: mycological peptone 5 g/L, glucose 10 g/L, Dipotassium phosphate 1 g/L, Magnesium sulphate 0.5 g/L, Rose-Bengal 0.05 g/L, agar 15.5 g/L) (CM0549; Oxoid, UK), selective media for *E. coli* and *C. albicans* respectively. The plates were incubated overnight at 37°C and then the CFU/mL were measured.

MacConkey lactose agar plates for competition experiments were made by combining 40 g/L MacConkey agar base with 10 g/l lactose.

4.2.5. Time course of antibacterial activity of honey.

The antimicrobial effect of honey on the three species was tested as was described before (§ 2.2.3). Exponential phase *E. coli* and *Candida* growing in monocultures were treated with model and heather honey (diluted at 50%). Stationary phase *E. coli* and *C. albicans* growing in monocultures and co-culture were treated with heather (50%) and Indonesian (50%) honey. Stationary phase *L. acidophilus* was treated with heather and Indonesian honey at 50%, 25% and 12.5% final concentration. CFU counts were measured daily up to 5 days of incubation by plating 5 serial dilutions into MacConkey and RBCA and LAPTg agar palates for *E. coli*, *C. albicans* and *L. acidophilus* respectively.

4.2.6. Flow cytometry: Monitoring of esterase activity, membrane integrity and intracellular ROS accumulation within bacterial and yeast cells

Mixed cultures were analysed using a BD Accuri C6 flow cytometer (Becton Dickinson Biosciences, Oxford, UK) as described previously (Chapter 2). *E. coli* and *C. albicans* were stained with Carboxyfluorescein diacetate (cFDA) and PI at final concentrations of 4 µg/mL and 2 µg/mL respectively. After incubation in the dark for 10', samples were excited using a 488 nm solid-state laser. Fluorescence was detected using 530 BP and 670 LP filters corresponding to cFDA and PI fluorescence respectively. Data were analysed using CFlow (BD) software. Detection of intracellular ROS accumulation within the polymicrobial cultured was monitored by staining with H_2DCFDA as was described in § 2.2.4.2.

4.2.6.1. Gating

Gating boundaries were positioned based on the forward scatter (FSC-H) and side scatter (SSC-H) plot. A monoculture of *E. coli* (UTI) strain, washed in filtered PBS, was examined and the gate was adjusted to the respective population (Figure 4.3A). Then, a coculture of *E. coli*- *C. albicans* was examined and the second gate was adjusted to yeast cells (Figure 4.3B). The percentage of yeast cells was lower because *Candida* in coculture grows less well than *E. coli*. These gating boundaries remained identical during the experiments and the analysis.

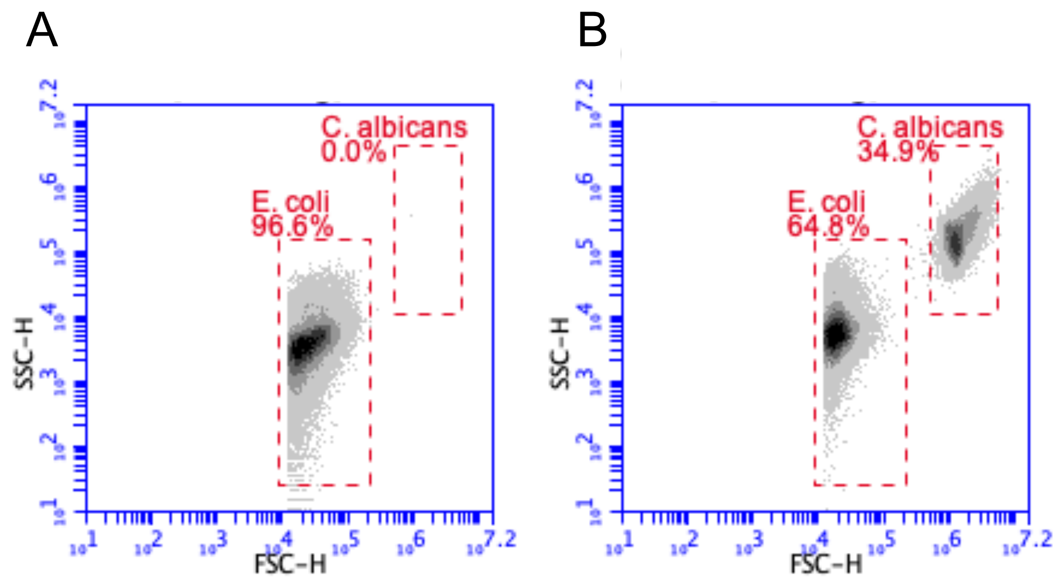


Figure 4.3 Gating of *E. coli* and *C. albicans* growing in coculture. (A) Monoculture of *E. coli* was run in flow and the gate was adjusted to the respective population. (B) Gating of *C. albicans* was done after the 2-species coculture was examined in flow.

4.2.7. Fluorescent microscopy

Cell suspensions labelled with cFDA/PI were visualised with an epifluorescence LSM 700 Laser Scanning Microscope equipped with the ZEN 2011 software. 5 μL were loaded onto slide and a piece of 0.1% agarose gel was applied on top in order to fix cells on the surface. Slides were imaged using GFP and PI filter sets (for visualizing cFDA/PI stained cells respectively) at a final optical magnification of 100X (and 20X). Microscopy was carried out at room temperature.

4.2.8. Monitoring of H_2O_2 and pH within infected VSF

The concentration of the H_2O_2 was determined by daily sampling of the infected VSF model and mixing with the reagents as was described in § 2.2.5. The pH of the same sample was measured using pH meter METTLER TOLEDO SevenCompact (Mettler Toledo, UK).

4.3. Results

4.3.1. Susceptibility of exponentially and stationary growing *E. coli* and *C. albicans* cultures to honey.

In chapter 2, model and natural honey were shown to have antibacterial effect towards exponentially growing *E. coli* K-12, due to low pH and intracellular ROS accumulation that caused subsequent membrane damage. This Chapter, which studies a potential "real life "application" of honey, investigates the susceptibility of two pathogenic strains. To do so, the effect of honeys was tested on stationary growing cells. Exponentially growing cultures were used as reference of bacterial/fungi susceptibility. Culturable cells were detected by standard plate counting. Dual cFDA/PI staining was used to detect physiological changes on esterase activity and membrane integrity in both species.

Exponentially growing *E. coli* ST131 and *C. albicans* were more susceptible to honey compared to stationary phase growing cells. The susceptibility of both species was monitored during the first hours of treatment until 2 days later. Heather

honey reduced both species below the level of detection and model honey caused significant reduction within 24 h of treatment (Figure 4.4A, B). In contrast, the same honeys were less effective when applied to stationary phase cell cultures. In particular, model honey caused 2 Logs reduction in *E. coli* 48 h post-treatment, while no growth defect was seen with heather. *C. albicans* was reduced by 1 Log during the first 6 h of exposure to both honeys and no further reduction monitored up to 2 days of exposure (Figure 4.4C, D). Following that, FC was used to track physiological changes of stationary *E. coli* and *C. albicans* when exposed to model honey.

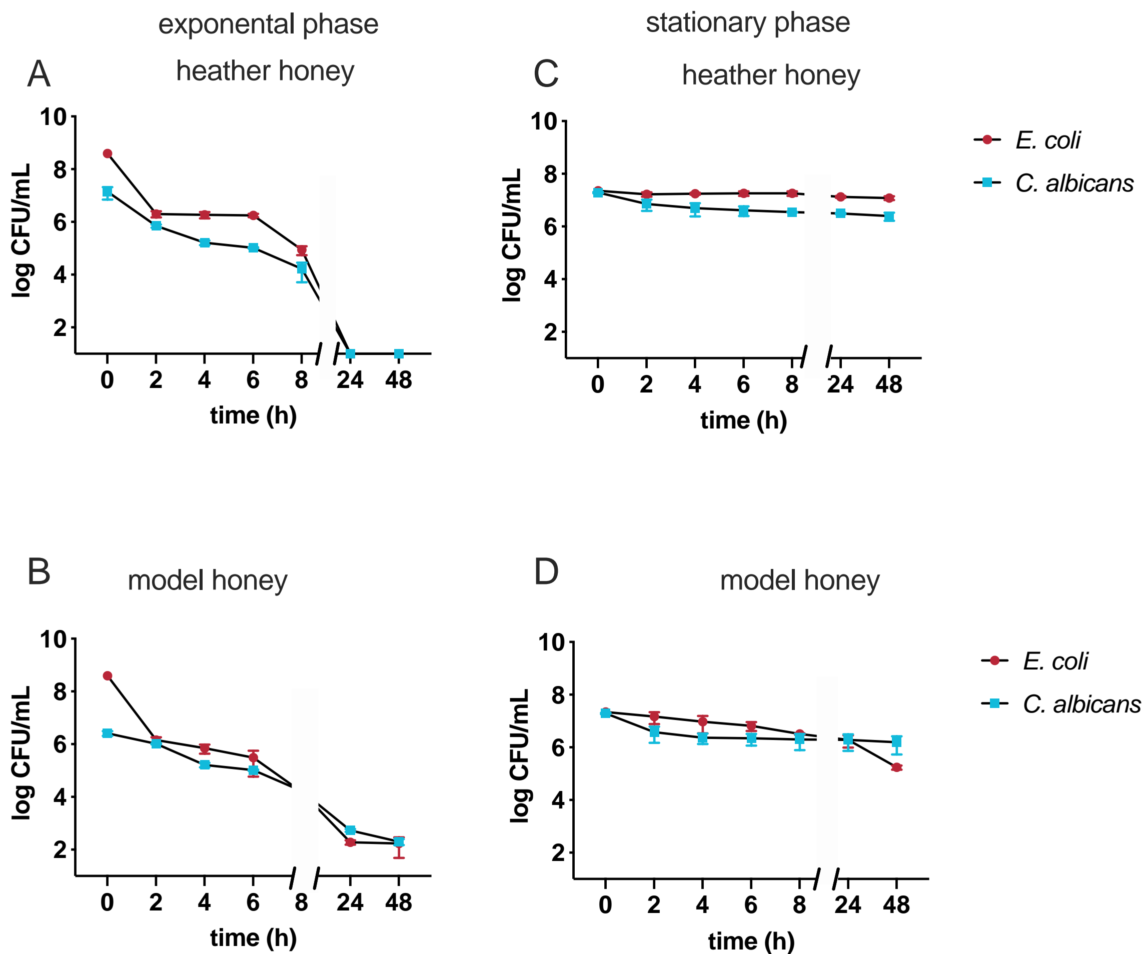


Figure 4.4 Effect of honey on *E. coli* ST131 and *C. albicans*. The antimicrobial effect of heather and model honey was tested on (A, B) exponential and (C, D) stationary growing cells. Both honeys caused elimination of both exponentially growing species within a day of treatment. Stationary phase *E. coli* and *C. albicans* were more resistant to both honeys. Error bars represent the average ± s.d. (*n*=3; biological replicates).

4.3.1.1. Physiological changes induced by model honey on stationary growing *E. coli* and *C. albicans*

Model honey has been more effective against both species. Thus, physiological changes caused by model honey of *E. coli* and *Candida* harvested from stationary phase were monitored by FC. Cell viability was assessed by simultaneously analysing the membrane integrity using PI and the intracellular esterase activity using carboxyflorescein diacetate (cFDA). The cFDA is an esterified fluorogenic dye which diffuses through cell membrane to cytoplasm and hydrolysed by non-specific esterase producing a green fluorescent product, the carboxyfluorescein (cF) (Morono et al., 2004, Chan et al., 2012).

As it is shown in Figure 4.5A during the first hours of treatment more than 90% of *E. coli* cells excluded the PI DNA-binding dye. This effect is might be attributed to the increased thickness of peptidoglycan layer of stationary cells (Jaishankar and Srivastava, 2017). Also at this growth phase, general stress-related proteins are already increased in a RpoS-controled way, thus, cells are less prone to H_2O_2 and the medium/high osmolality (Allen and Griffiths, 2012). Within a day of treatment, 60% of the bacterial population was permeable to PI, showing that prolonged exposure to model honey induced gradual cell damage/rupturing. This is in agreement to findings of Chapter 2.

By the time of exposure the percentage of *E. coli* cells with esterase activity also increased (Figure 4.5B). There are two possible reasons for this observation: (a) the limited permeability of cFDA within bacterial cells which gradually increased

by the time and (b) the oxidative stress which may cause transient cease of the esterase activity in *E. coli* during the first hours of exposure. According to previous findings, lipophilic probes (i.e. cFDA) have low ability to diffuse across the cytoplasmic membrane of Gram-negatives because of the LPS layer and the abundance of restrictive porins (Hewitt et al., 1998; Nikaido, 2003). Considering this, it was assumed that the rest of cells with intact membranes (40% of population impermeable to PI) are also metabolically active. In support of this hypothesis, one day post-treatment the percentage of PI^+ cells was equal to this of cFDA positive (Figure 4.5B). However, two days post-exposure the percentage of PI^+ remained the same (50%) while less than 20% were esterase positive. This suggests that prolonged exposure to model honey compromised the bacterial membrane and affected the esterase activity, however, this was not capable to arrest cells growth.

Another explanation on the decrease of the esterase activity during the first hours of exposure is the bacterial response mechanism to oxidative stress. As was reported previously, under oxidative stress, bacterial cells decrease metabolic rates of glycolysis and TCA cycle. Although ROS attack a range of metabolic enzymes, a decrease of these pathways was seen to cause metabolic perturbations in ROS-stressed cells (Charbon et al., 2017).

Physiological changes in *Candida* differed from these in *E. coli*. Esterase activity of *Candida* was higher compared to the respective of *E. coli* monoculture (Figure 4.5 E, F). Membrane damage and fluctuations on the esterase activity of the yeast evolved in parallel (Figure 4.5 D, E). In *Candida*, the percentage of PI^+ cells were gradually increasing up to 48 hours of exposure when almost 90% of cells

were PI labelled. By that time, 60-80% of yeast cells were seen to be cF labelled. This percentage was fluctuating during the first hours of exposure and up to 24 h of exposure 66% of the population was seen to be esterase positive. This implies that the gradual loss of membrane integrity during the first hours affects the esterase activity. This is in agreement with a previous study showing that loss of membrane integrity affects the function of intracellular molecules (i.e. enzymes, proteins) and the esterase activity (Kwolek-Mirek and Zadrag-Tecza., 2014). However, in yeast cells, respiration activities are not supported by plasma membrane. Instead they function in mitochondria, thus, the loss of plasma integrity does not imply the loss of viability unless the respective intracellular components are targeted (Guillard et al., 2014). Thus, according to these results heather honey in sub-inhibitory concentration caused destruction in yeast cells membrane without affecting its viability. In contrast to FC, fluorescence image shows an aggregate of cF labelled yeast cells (Figure 4.5F).

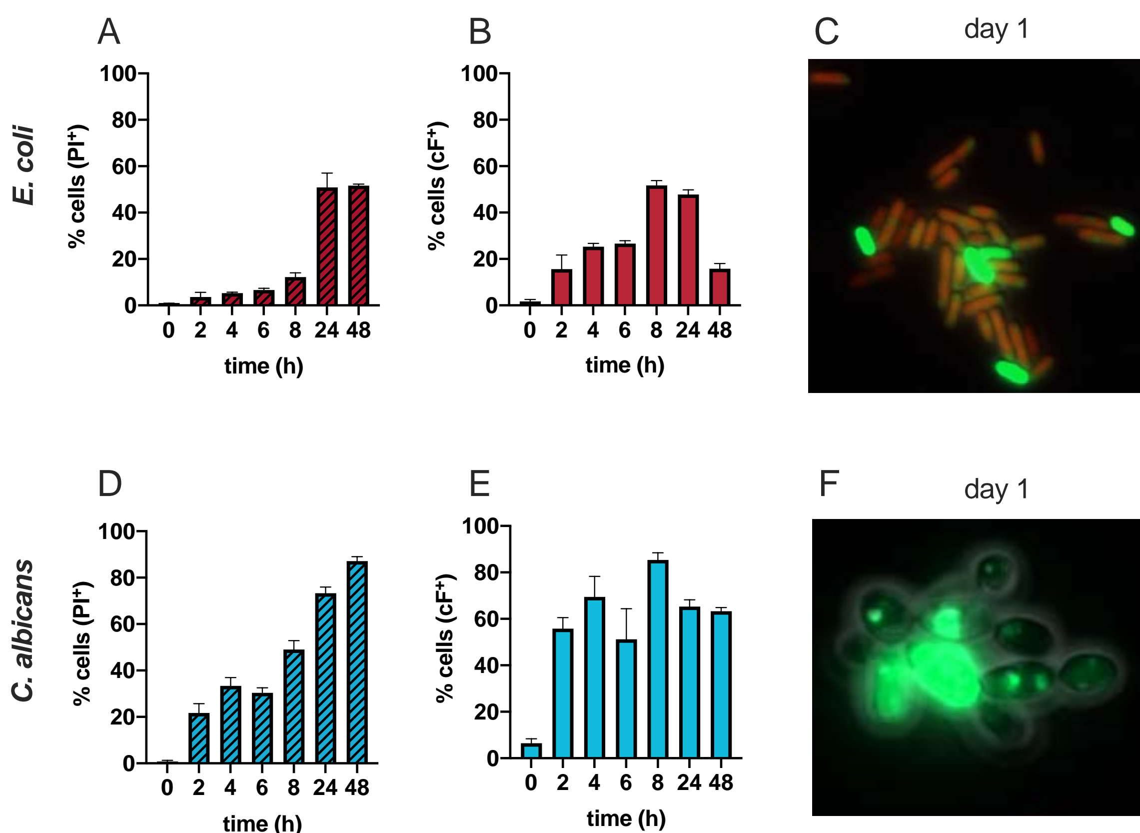


Figure 4.5 Effect of model honey on *E. coli* and *C. albicans* monocultures. Changes in (A, D) membrane integrity and (B, E) esterase activity in *E. coli* (red) and *Candida* (blue) stationary phase cultures were monitored by FC. Time course following treatment with model honey shows the percentage of PI^+ and cF^+ cells. Time zero corresponds to untreated cells (physiological state of cells kept in PBS, before the honey application). A capture of fluorescence images shows dual labelled (C) *E. coli* and cF labelled (F) *Candida* cells after treatment with model honey. Error bars represent the average ± s.d. (n=3; biological replicates).

Overall, from this preliminary analysis we concluded that that (a) as expected, stationary phase cells were significantly more resistant comparing to exponentially growing cultures and (b) bacterial susceptibility to honey differs from this of the yeast. Although model honey caused only slight reduction of culturable *E. coli* and *Candida*, FC showed distinct physiological changes between the two species. During the first hours of exposure higher percentage of *Candida* cells, compared to *E. coli*, were permeable to PI. However, both species retained their esterase activity and cultivability. These observations suggest that a honey below the inhibitory concentrations can cause membrane destruction without affecting any other vital cell function. Assuming that those bacterial and yeast cells permeable to PI were also culturable, it was speculated that honey of low activity increases the membrane permeabilization rather rupturing it. This is in agreement with another study claiming that non-inhibitory concertation of honey causes permeabilization, rather than destruction of membrane integrity and perturbation of key biological functions (Wei et al., 2008). After this preliminary findings the effect of honey on *E. coli* -*C. albicans* coculture was investigated.

***4.3.2. E. coli* and *C. albicans* in a microbial co-culture**

Before evaluate the effect of honey on 2-species co-culture, growing in the vaginal fluid simulant, the interactions happen between the two pathogens were monitored. As was found previously, in a co-culture, *E. coli* (MG1655) outcompetes *Candida* (SC5314) by limiting the available magnesium which is essential for toxicity (Cabral et al., 2017). By growing the two pathogens as monocultures and co-

cultures, it was tested whether UPEC strain has a competitive advantage over *Candida* and later how honey affects this interaction.

4.3.2.1. Growth competition between *E. coli* and *C. albicans* during in vitro growth

To study the interaction between *E. coli* and *C. albicans in vitro*, both organisms were grown as monocultures (in LB and YPD respectively) and as co-culture (YPD medium). Growth of *E.coli* was unaffected by the presence of *Candida*, but *Candida* grew less well when *E. coli* was present (Figure 4.6). The reduction in *Candida* growth in the presence of *E. coli* was approximately 2 logs. This is consistent with previous studies (Gibson et al., 2009; Cabral et al., 2018).

The identification of any interactions happen in the bacterial-fungi coculture is not within the scope of this study. Thus, we went on the identification of phenotypical changes induced by honey when this is applied in a mixed microbial community.

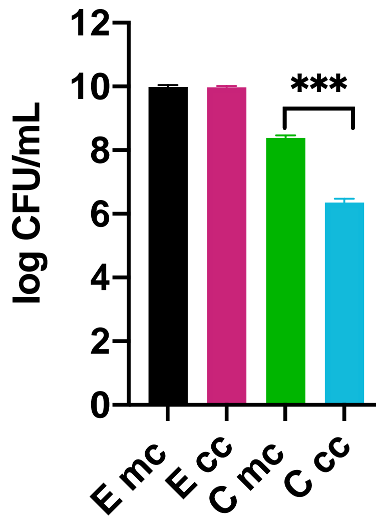


Figure 4.6 Comparison of *C. albicans* CFU counts after 24 hours growing in monoculture and co-culture with *E. coli*. *Candida* growing alone (C mc) yielded in significantly (p =0.0008) higher CFU counts than *Candida* (C cc) growing in co-culture with *E. coli* (E cc). *E. coli* grew equally well either case. Measurements were taken after overnight growth. Error bars represent the average ± s.d. (*n*=3; biological replicates). The "mc" and "cc" stand for the monoculture and co-culture respectively.

4.3.2.2. Comparing the effect of model and heather honey on *E. coli*-*C. albicans* mono and co-culture

As was seen previously, *Candida* grows significantly better in monoculture than in co-coculture with *E. coli*. This suggest that the two species in a co-culture develop chemical and physical interactions that potentially affect their growth. Thus, firstly was tested whether honey has a distinct effect on each of the species when they grow as monocultures or co-cultures. To do so, the three cultures (*E. coli*,

Candida, *E. coli*-*Candida*) were treated with (50%) heather and the model honey (same as previously). CFU measurements were taken during the first hours of exposure (0-8h where we previously found that honey has the maximum activity) up to 2 days.

As it is shown from the initial CFU counts (t=0), *Candida* in co-culture grew less well than in monoculture (Figure 4.7). In contrast, *E. coli* was not affected by the presence of *Candida* as it is shown by initial counts (t=0).

In agreement to previous observations, stationary phage *E. coli* and *Candida* were not severely affected by heather and model honey. In particular, *Candida* growing in co-coculture, demonstrated a resistant phenotype. During the first hours of exposure, heather and model honey caused a gradual decrease of 2 Logs of yeast viable counts. By 48 h of exposure *Candida* in coculture increased to the initial CFU counts (Figure 4.7 A, B). In contrast, both honeys caused 1 log reduction to monoculture of *Candida* and no further effect was seen up to two days of exposure. This suggests a relationship between the cells density and the antimicrobial efficacy of honeys. In this case, honeys were more effective in lower density of yeast cells. Also, as was previously reported, *Candida* develops osmoadaptation strategy which renders the yeast able to grow or survive in hyper or hypo-osmotic stress (Hohmann, 2002). In consistence to this, another study showed that in osmotic environment, *Candida* adapts its metabolism, uses the available carbon sources and develops virulence mechanisms such as adhesion, morphogenesis, biofilm formation and invasion (Van Ende et al., 2019). These facts suggest that *Candida* can recover the stress caused by a honey of low fungicidal activity. Thus, is possible that yeast cells

recovered from the effect of heather and model honey and the presence of carbon source enhanced their adaptation and virulence.

E. coli growing in the co-culture, demonstrated a different phenotype upon honey stress. Model honey caused 2 logs reduction in *E. coli* while heather honey caused less than 1 log reduction. This can be explained by the higher efficiency of model honey. Monoculture of *E. coli* was not affected by heather and model honey. These results suggest that *E. coli* in co-culture with *Candida* is more susceptible to honey stress.

These preliminary findings showed that both honeys were more effective when applied to *E. coli* and *Candida* coculture. Also, each organism demonstrated distinct phenotypes after the treatment with these honeys. *Candida* showed recovery and adaptation within 2 days of honey treatment whereas this was not observed in *E. coli*. These results suggest that antimicrobial effect of honey is also species-related. Considering the distinct phenotypes within the two pathogens further physiological changes were studied by FC.

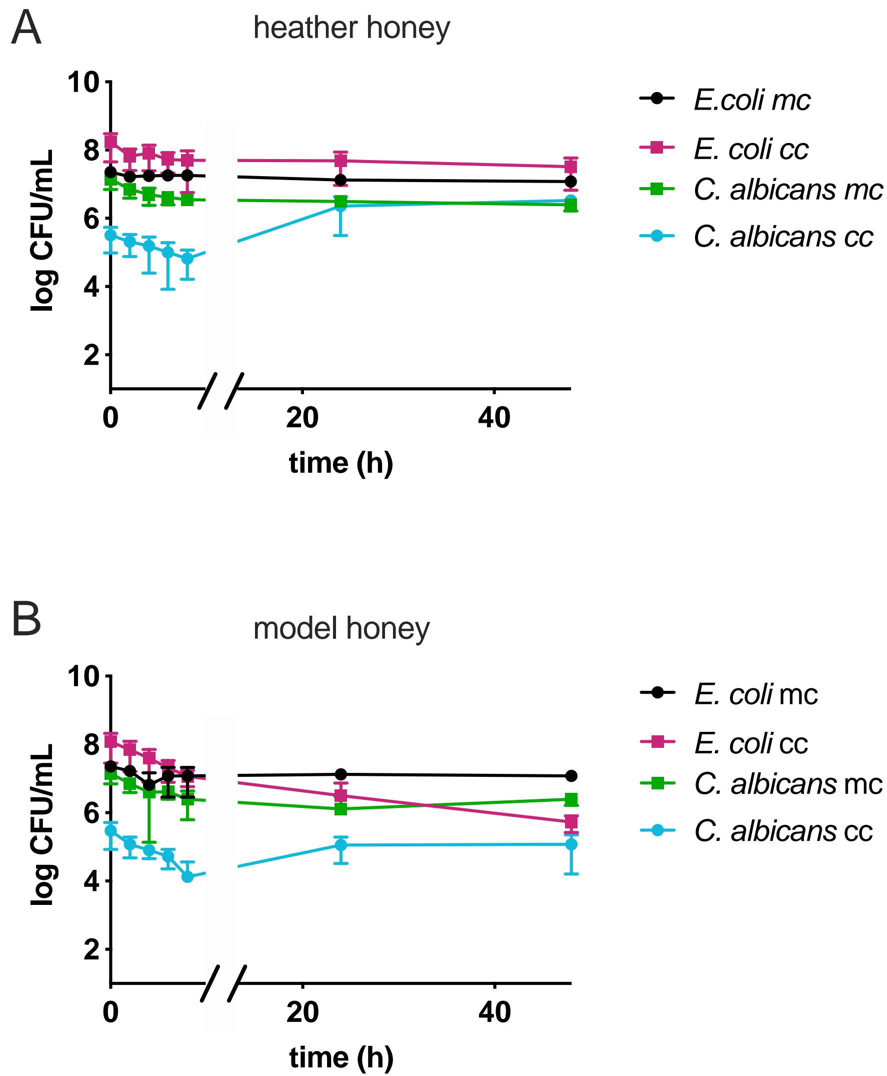


Figure 4.7 Effect of heather and model honey on *E. coli* and *C. albicans* cultures. The effect of (A) heather honey and (B) model honey was tested on *E. coli* and *C. albicans* when they were grown in co-culture (E cc, C cc) or they were mixed after they grew as monocultures (E mc, C mc). Error bars represent the average ± s.d. (*n*=3; biological replicates).

4.3.2.3. Physiological changes caused by heather honey on *E. coli-C. albicans* coculture.

Cell viability was assessed as previously by dual PI/cFDA staining indicative for membrane integrity and esterase activity. By using the H_2DCFDA intracellular probe, we also monitored the ROS accumulation within the two species during the treatment with heather honey. Samples stained with PI/cFDA were also visualised in fluorescence microscope. The results are shown in Figure 4.8 and 4.9.

Heather honey did not cause high accumulation of intracellular ROS to any of the two species in co-culture. During the first hours of treatment, less than 10% of *E. coli* cells were detected with ROS and within a day the percentage decreased to levels of unexposed cells (t=0) (Figure 4.8 A). Likewise, less than 25% of *Candida* cells were gradually accumulating ROS during the first hours of treatment (up to 8h) and this percentage declined after 1 day of treatment (Figure 4.8 B). Both species can degrade H_2O_2 within an hour or less (Pradhan et al., 2017). This implies that catalase activity of *E. coli* and *Candida* can detoxify H_2O_2 and suppress the uncontrolled accumulation of ROS. However, the catalase activity of *Candida* is affected by the presence of combination of stresses, such as oxidative stress and high osmolality. The cations inhibit or delay the catalase activity which in turn affects the adaptation of the yeast to oxidative stress (Kaloriti et al., 2014). Thus, it was speculated that the synergy of honey stressors, delayed the detoxifying mechanism of *Candida* and that caused higher ROS accumulation during the first hours of exposure to honey.

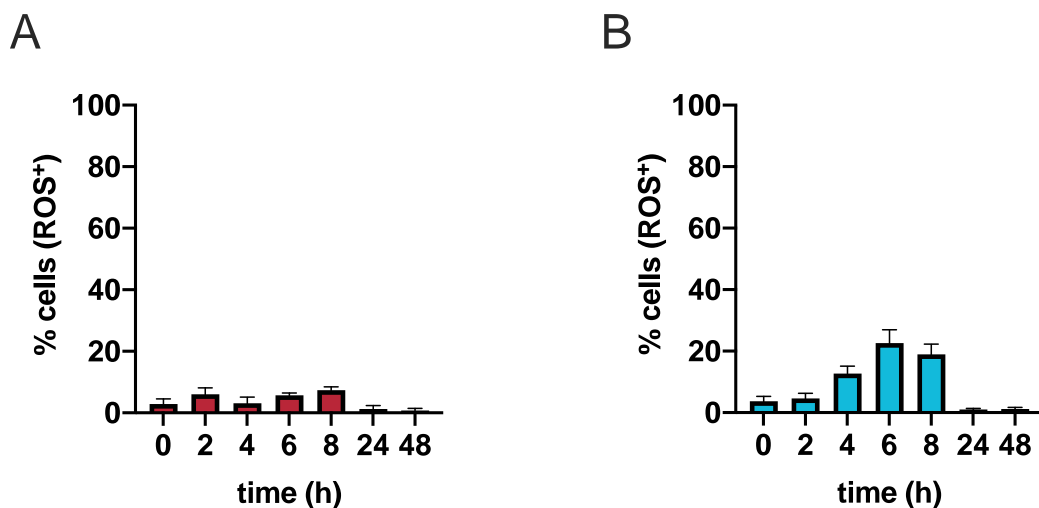


Figure 4.8 Intracellular ROS detection in *E. coli* and *Candida* after treatment with heather honey. Intracellular ROS was assessed by measuring the percentage (A) *E. coli* and (B) *Candida* cells positive to FDA fluorescence. A control/no-treated sample is shown as t=0. Error bars represent the average ± s.d. (*n*=3; biological replicates).

Figure 4.9 shows the effect of heather honey on two indicators of cell viability; the membrane integrity and esterase activity. In *E. coli* cells, membrane permeability was gradually increasing up to 2 days of treatment (44% *E. coli* cells PI+) (Figure 4.9A). In agreement with our previous findings, increasing of membrane permeability by honey increased the uptake of cFDA dye by bacterial cells (Figure 4.9C). Thus, in agreement to our previous results we saw that although honey caused gradual destruction of cell membrane it did not affect the esterase activity of cells. These phenotypes are also consistent with the viable counts that did not decrease post-honey treatment (Figure 4.7).

In *Candida* percentage of PI positive cells increased up to 4 hours of exposure and then declined (Figure 4.8B). The percentage of cells with esterase

activity increased within two to four hours of exposure when almost 90% of the population were cF labelled (Figure 4.8D). As has been reported in a previous study, under non-inhibitory H_2O_2 concentration and presence of sugars, *Candida* overcome the oxidative stress, uses the available carbon sources and becomes more virulent (Van Ende et al., 2019). Thus, it is possible that honey-induced oxidative stress which affected the esterase activity during the first hours of exposure. For longer exposure it appeared that *Candida* adapted to honey and increased the esterase activity. Also, during the adaptation is possible that *Candida* elaborates membrane repair mechanisms as it was shown by the exclusion of PI from 85% of the yeast population (Figure 4.9B).

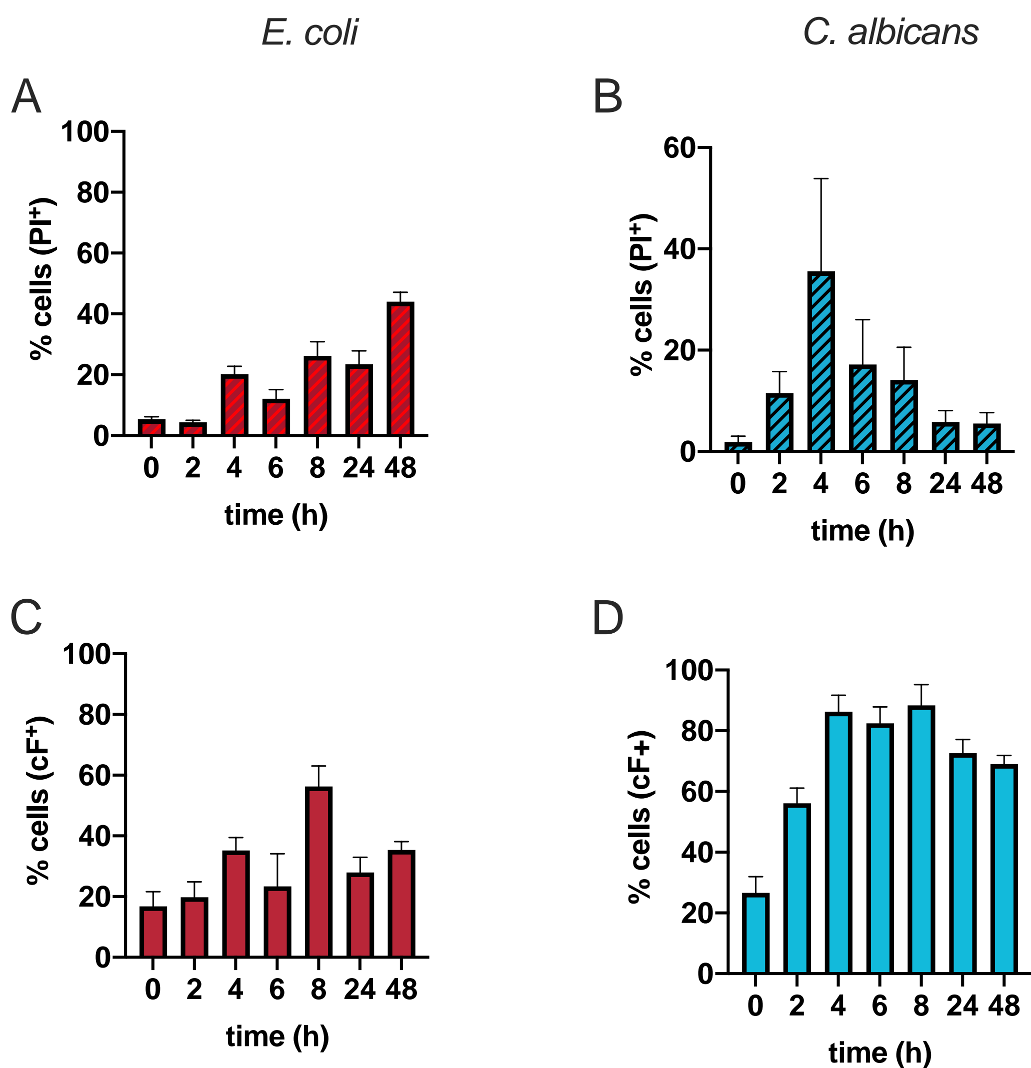


Figure 4.9 Phenotypical changes induced in *E. coli* and *Candida* co-culture after treatment with heather honey. (A, B) Membrane integrity and (C, D) esterase activity was assessed by capturing the percentage of *E. coli* and *Candida* cells positive to PI and cFDA fluorescence respectively. T=0 corresponds to no treated cells. Error bars represent the average ± s.d. (*n*=3; biological replicates).

In summary, it was found that stationary phase cells (either as mono or co-culture) are moderately sensitive to honeys tested here. Considering the viable plate counts (VPC) and FC results it was showed that model and heather honey, at sub-inhibitory concentration, caused low growth defect to both species. *Candida* showed adaptation and overgrowth after a certain time of treatment which might be explained by its osmoadaptation mechanism. These observations suggest that heather honey destabilized the yeast membrane rather than rupturing it and affecting other intracellular functions. Likewise, it was seen that heather honey caused membrane destruction to *E. coli* without affecting the esterase activity or arresting the bacterial growth.

The experiments presented in this paragraph were performed by adding the natural honey or model honey to PBS-washed *E.coli* and *Candida* cells. Thus, is possible that some effects were underrated. In order to further investigate the effect of honey on *E. coli* and *Candida* coculture in conditions mimicking the vaginal environment, a vaginal simulation fluid (VSF) that simulates the pH and nutrients within the cavity was used.

4.3.3. Effect of honey on of *E. coli* and *C. albicans* growing on a VSF

Following the initial experiments, we moved to the investigation of the antimicrobial effect of honey on 2-species microbial community which had been grown in VSF for 5 days. For this study, another highly antibacterial natural

Indonesian honey was used. Figure 4.10 shows the workflow of this experimental set up. The culture flasks were incubated statically at 37°C for 5 days.

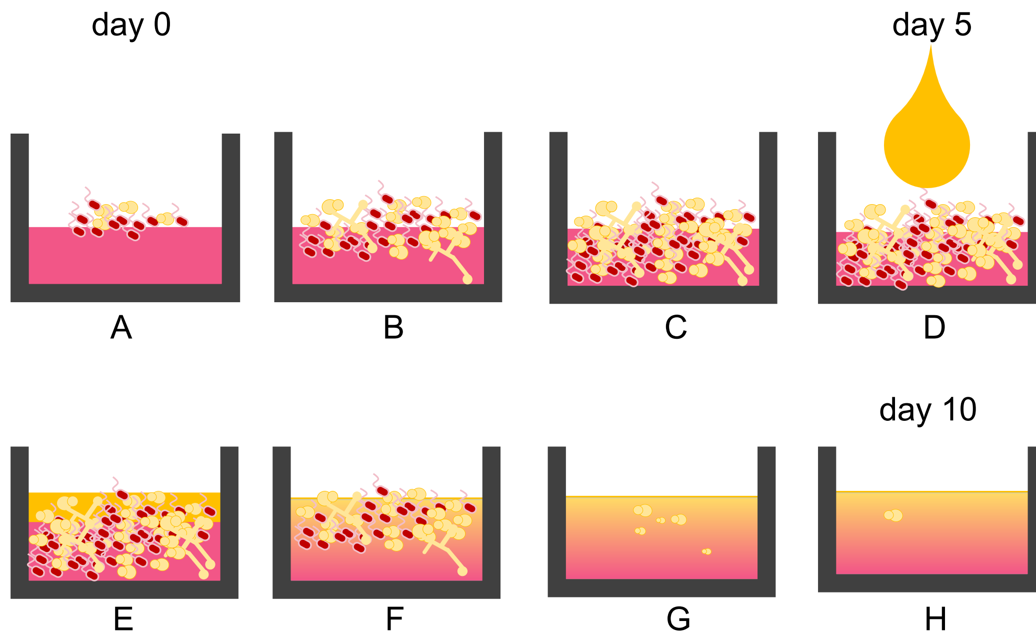


Figure 4.10 Workflow of the *in vitro* infection of VSF model. A cell culture flask was filled with 15 mL of pre-warmed VSF. (A) Coculture of *E. coli* and *C. albicans* was inoculated in VSF and let grow into a biofilm for 5 days (A-D). (D) On day 5, honey (heather or Indonesian) was applied directly on the culture into the culture flask. Honey started exerting the antimicrobial activity within a day (D-F). The biofilm was gradually destroyed by eradication of *E. coli* first and *Candida* later. Viable counts were counted daily before and after application of honey.

4.3.3.1. Inoculation of VSF with *E. coli* and *C. albicans*

E. coli and *C. albicans* cells growing in coculture, were harvested, washed in Ringer's solution and inoculated in the VSF model into a static culture flask. The VSF, adapted by Owen and Katz (1999), simulates the pH, nutrients composition

and osmolality of vaginal fluid Linhares et al., 2011). The pH of VSF was adjusted to 4.2 by adding acetic and lactic acid, mimicking the natural acidification process mediated by vaginal Lactobacilli (Linhares et al., 2011). Viable counts of both species were monitored for 5 days and are shown in Figure 4.11. Timepoint 0 is considered the day when the VSF was inoculated with *E. coli*-*Candida* coculture, before the honey was introduced.

As it was seen in preliminary experiments *Candida* grows less well than *E. coli* in coculture. After the first day of inoculation the viable counts of *Candida* dropped below detection limit (1 CFU/mL). This is maybe due to the low pH and the presence of acetic and lactic acid in VSF. The presence of two acids in vagina inhibits the growth and colonisation of *C. albicans* (Goncalves et al., 2016). However, a recent study, ruled out the synergy of two acids. Specifically, acetic acid had a toxic effect on *Candida* (at pH 4 or lower) while lactic acid did not augment this toxicity. The lactic acid can be rapidly metabolised by *Candida* (Lourenço et al., 2018) while the acidic environment (pH 4-4.5) was seen to induce cell wall perturbation which in turn activated the fungal stress response (Sherrington et al., 2017). This enhanced the adaptation and virulence of the pathogen likewise it happens during the colonization of vaginal mucosa by *Candida*. In agreement with the abovementioned, *Candida* was seen to have a prompt stress response to low pH and presence of weak acids in VSF. However, within a day yeasts cells demonstrated an adaptive phenotype and overgrow the initial inoculum size.

In contrast, *E. coli* was unaffected by the low pH of VSF model and the number of viable counts were constant during that time. These observations were in agreement with a previous study (Presser et al., 1997).

During the 5-days growth, the pH of the medium was increased (Figure 4.11B). On day 2, pH raised from 4.2 to 4.8 and the maximum value of 5.8 was monitored on day 3. In case of vaginal candidiasis, the pH is typically less than 5, while, overgrowth of bacteria (i.e. *G. vaginalis*) that cause bacterial vaginosis usually raises the pH above 5 (Jeanmonod et al., 2019). Here, the VSF model was inoculated with yeast and bacteria and lacks the pH regulators (*Lactobacillus* spp.). Thus, pH change is mainly controlled by the growth of the two species and any change cannot be correlated to any type of vaginitis (BV or VVC) (Tietz and Klein, 2018). As was previously reported, growth of *Candida* in presence of carbon sources and aeriation increase the pH of the medium from 4 to 7 or higher by the production of amonia (Danhof et al., 2016). Here, the adaptation of *Candida* in low pH on day 2 coincided with the increase of pH within the VSF. However, the growing conditions (lack of agitation and symbiosis with *E. coli*) might have affected the levels of pH which had a maximum of 5.8. Also, growth of *E. coli* may contribute to the raise of the pH. Considering that the optimum pH for *E. coli* growth is 5.5-7, is possible that cells adapted in VSF model (pH 4.2) by slowly increasing the pH. Metabolism of carbon sources of VSF (i.e. glucose) usually results in acidification of the growing media. However, as it has been reported growth of *E. coli* in more acidic environment regulates mechanisms (i.e. production of less acidic metabolites) for

pH homeostasis which results in slow increase of pH during the growth (Šeputienė et al., 2006; Sánchez-Clemente et al., 2018).

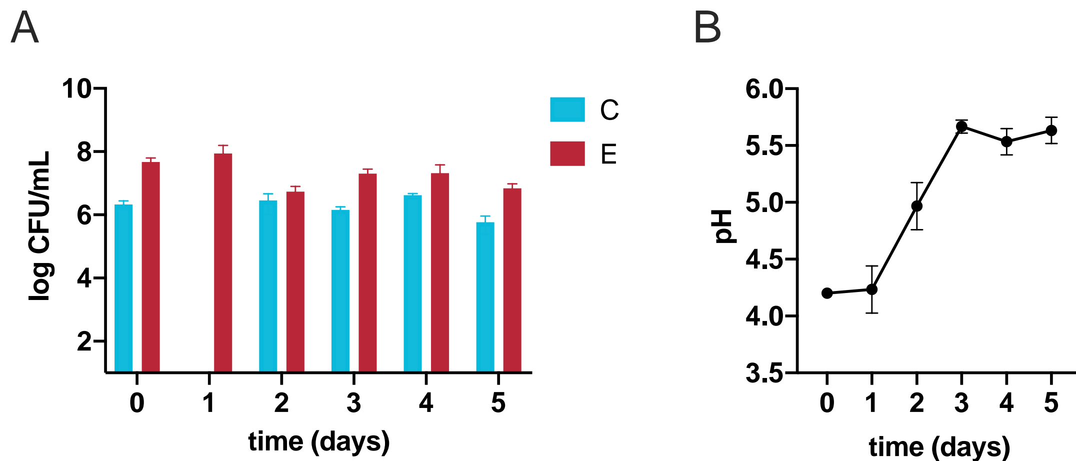


Figure 4.11 Monitoring of *E. coli- Candida* viability and pH changes during the 5 days growth of bacteria-yeast in VSF. *E. coli-Candida* co-culture was inoculated in VSF on day0. (A) CFU counts and (B) pH changes within the VSF were measured daily. Error bars represent the average ± s.d. (*n*=3; biological replicates and 3 pH measurements in the respective culture batches).

As shown the fluorescence images (Figure 4.12), during the 5-days growth in VSF, there are cells which are both PI/cF labelled, or PI and cF labelled only. Although, both *E. coli* and *Candida* remained culturable (with exception the *Candida* on day 1), perturbations in membrane integrity (PI^{+}) and esterase activity were observed in fluorescence microscope. On day 1, *E. coli* cells were labelled by PI/ FDA or PI only. This shows that inoculation of *E. coli* in VSF caused destruction of the membrane integrity, however this did not affect esterase activity of the cells. There were also *E. coli* cells impermeable to PI and positive to esterase activity.

However, they did not emit strong green fluorescence because of the limited dye permeability through intact bacterial membranes. Brightfield and fluorescence images show that on day 1 *Candida* cells were impermeable to both dyes. Loss of the esterase activity can be explained by the loss of yeast cultivability during this time. As was previously mentioned, the acids of VSF is possible to cause perturbation in cell wall integrity. Considering this we would expect to see PI labelled yeast cells. However, here it was not the case. This is in agreement to a previous study showing that low concentrations of acetic acid made the yeast cells permeable to PI, however, conformational changes on DNA did not allow the efficient binding of the PI (Phillips et al., 2003). Based on this finding it was assumed that acidic pH of VSF caused a “ghost-like” phenotype to yeast cells.

On day 3, when *Candida* viable counts were already increased, esterase positive yeast cells impermeable to PI were observed. This showed that *Candida* recovered the acid stress, repaired cellular functions (i.e. esterase activity, membrane integrity) and became culturable. On day 5, aggregates of green labelled *Candida* cells were observed to be surrounded by intact *E. coli*. Although there are also PI labelled yeast and bacterial cells the viable counts did not change for either organisms. This suggest that changes on cells physiology may arise from physical or chemical interactions between them without these affecting their growth (Hall and Noverr, 2017). According to previous study, *Candida* exerts antagonistic effect to some Gram-negatives by secretion of chemical mediators such as candydalycin or by selective pressure to maintain itself among a polymicrobial population. Candydalycin, predominately formed by hyphae, is a pore-forming peptide which

possibly cause membrane damage of bacterial cells (Moyes et al., 2016). *E.coli* was also reported to secrete fungicidal factors and deplete the available magnesium which is essential for the toxicity of the yeasts (Cabral et al., 2018). Considering these reports and the results presented here, it was spculated that both *Candida* and *E. coli* are gradually adapted to VSF while the antagonism between the species (secretion of antimicrobial compounds) and the presence of weak acids (in VSF) cause destructions on cells' membrane. However, the uptake of PI does correspond to "dead" or non-culturable cells. The next paragraphs examine the effect of heather and natural Indonesian honey on *E. coli* and *Candida*.

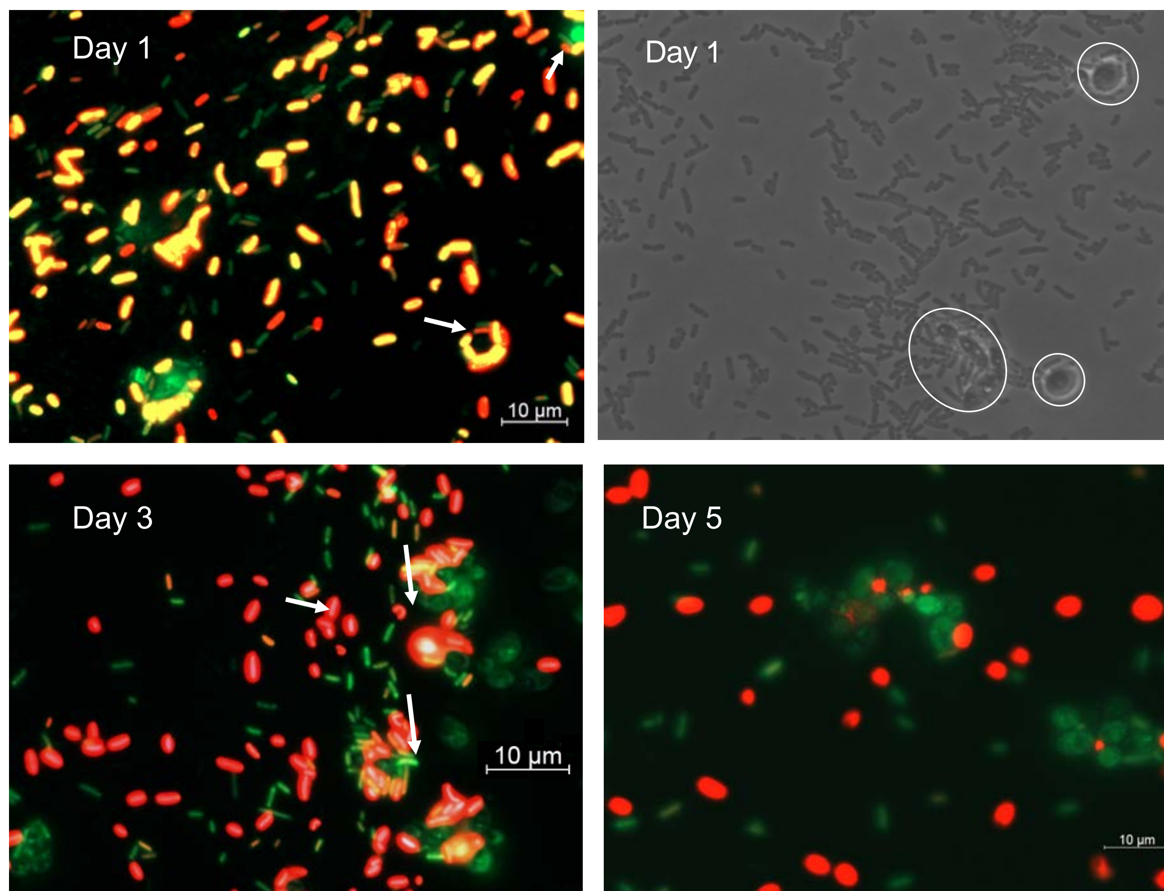


Figure 4.12 Physiological changes during the growth of *E. coli* and *Candida* in VSF. Fluorescence images show the *E. coli-Candida* growing in VSF. PI/cFDA dual staining tracks the changes on membrane integrity and esterase activity in both species. White arrows show *Candida* cells impermeable to PI/cFDA (day 1) and cFDA labelled (day 3).

4.3.3.2. Effect of heather and natural Indonesian honey on eradication of *E. coli* and *C. albicans* growing in VSF.

Although heather honey did not significantly affected the growth of *E. coli* and *C. albicans*, physiological changes were observed for both species. Here, another H_2O_2-producing honey (Indonesian stingless bees' honey; *Trigona* spp.) was tested.

In preliminary testing, we found that Indonesian honey had significantly higher antimicrobial efficacy compared to other two honeys tested before (heather and acacia). Following the 5-days incubation, dual-species microbial community growing in VSF into the culture flask, was treated with heather and Indonesian honey at 50% final concentration. To examine the effect of each honey on *E. coli* and *Candida*, CFU counts were tested daily. As previously, physiological responses of cells to each honey were tested by CFU counts and FC. Also, pH changes in VSF medium and generation of H_2O_2 by honey were monitored throughout the treatment.

Figure 4.13 shows the cFDA-stained polymicrobial culture on day 5 before the application of honey. As it is shown in panel A (Taken by magnification 20X), within the culture, there are cell aggregates or premature biofilm which locally demonstrated high esterase activity. Panels B and D show that *E. coli* cells (white arrows) surrounded single yeast cells, yeast aggregates and hyphae. As has been previously mentioned cFDA cannot diffuse easily through rigid bacterial membranes thus the green fluorescence signal is much weaker in *E. coli* compared to *Candida*. However, the CFU counts showed that both pathogens were culturable during that time (Figure 4.11).

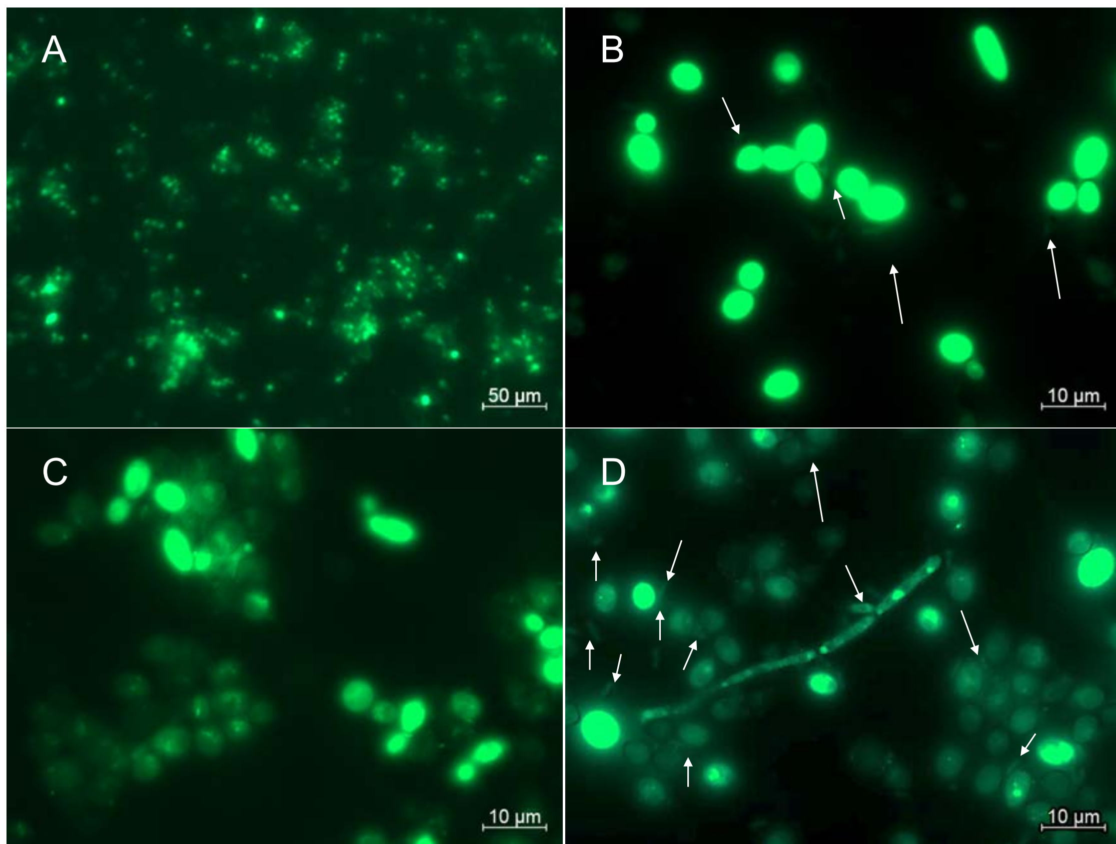


Figure 4.13 Fluorescence images showing the esterase activity of *E. coli* and *Candida* growing in the VSF for 5 days. Picture on panel A, taken by 50X magnification shows the polymicrobial culture consisting of cells with high esterase activity and others with less strong green fluorescence. (B,C,D) *Candida* cells and hyphens are surrounded by (low fluorescent) *E. coli* cells (white arrows).

As it is shown in Figure 4.10, honey was added on day 5 and cells cultivability was tested daily by measuring the viable counts in selective media. The results are shown in Figure 4.14. Indonesian honey demonstrated significantly higher antimicrobial activity comparing to heather honey, however, the efficacy of each honey was also species-related.

E. coli was significantly more susceptible to both honeys comparing to *Candida* which was only eradicated by Indonesian honey. Both honeys caused total eradication of *E. coli* within a day. Growth of *Candida* was enhanced after it was exposed to heather honey and completely arrested after the exposure to Indonesian honey (Figure 4.14A, B).

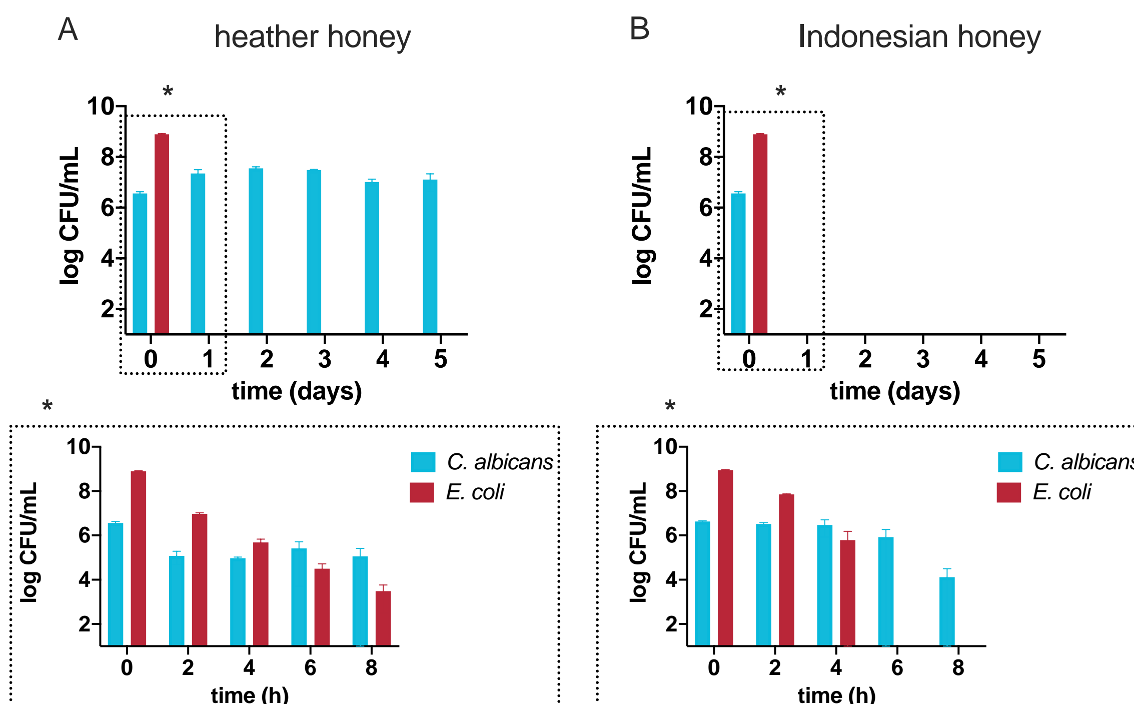


Figure 4.14 Effect of heather and Indonesian honey on *E. coli* and *C. albicans* colonising the VSF. (A) heather and (B) Indonesian honey were applied to bacterial-yeast coculture. Antimicrobial effect of both honeys was monitored for 5 days. CFU counts were also measured up to 8 h since the honey administration (Asterix). Error bars represent the average ± s.d. (*n*=3; biological replicates).

Fluorescence images (Figure 4.15) show the effect of heather honey on the polymicrobial community. *E. coli* cells were permeable to PI only (white arrows). Membrane destruction caused by heather honey was lethal for *E. coli* and this confirmed by CFU counts (Figure 4.14). These results agree with previous finding that Gram-negative bacteria start to die 4-6 hours after treatment with honey and complete killing takes up to 24-48 hours (Molan, 1992a). In contrast, *Candida* was

more resistant to heather honey. Panel D (Figure 4.15) shows yeast cells aggregates and hyphae to emit strong cF fluorescence one day post exposure to heather honey. Panels A, B and C show that the majority of *Candida* cells emitted strong cF fluorescence while only some of them were positive to PI. This suggests that heather honey increased the permeability of yeast plasma membrane without affecting vital cellular functions.

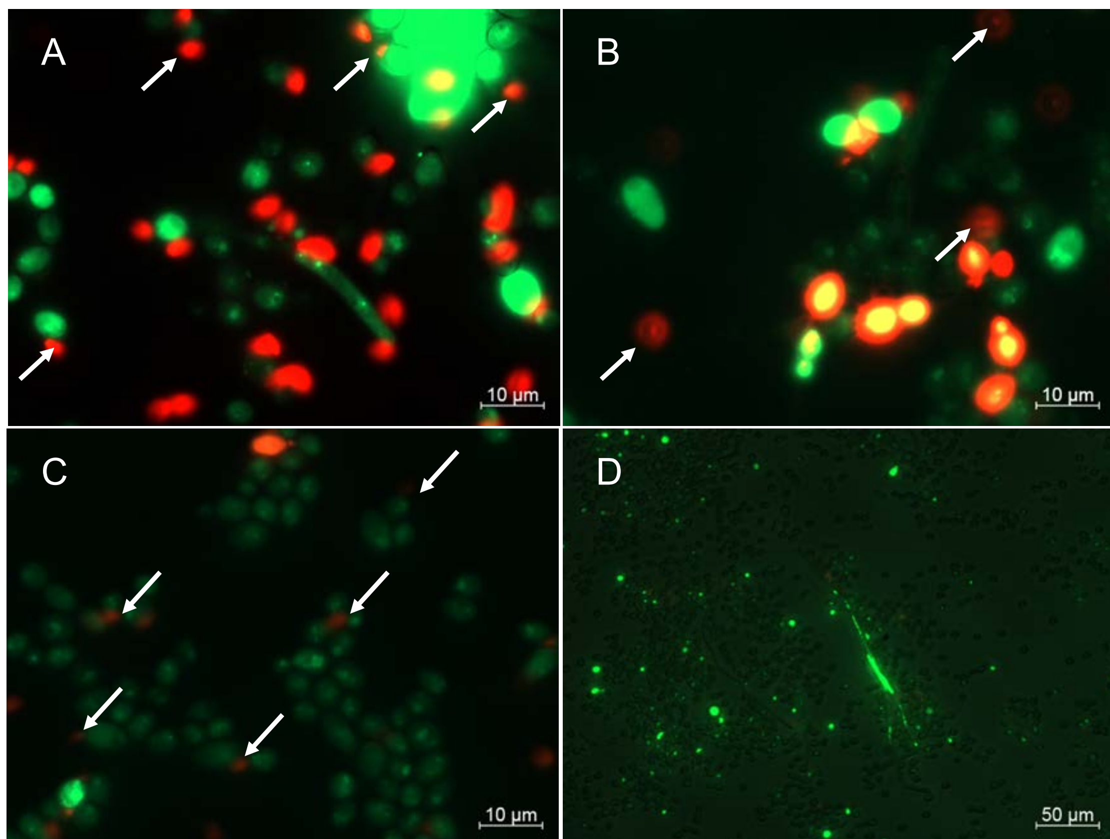


Figure 4.15 Fluorescence image showing the effect of heather honey on *E. coli* and *Candida*. Picture on panel D, taken by 20X configuration shows the polymicrobial culture consisting of cells aggregates and hyphae with high esterase activity. *Candida* cells and hyphae were either intact/ metabolically active (PI^{-}/cF^{+})

or (B) membrane destructed/metabolically active (PI^+/cF^+). (A, B, C) *E. coli* cells (white arrows) were permeable to PI only.

Figure 4.16 shows a snapshot of the *E. coli*-*Candida* in VSF one day after treatment with heather honey: bacterial cells surrounding single yeast cells an early formed hyphae. Fluorescence labelling could be correlated to the susceptibility of each species to heather honey treatment. PI permeable *E. coli* was the most susceptible to honey and the membrane destruction was lethal. *Candida* cells are shown to be either PI^+/cF^+ or PI^-/cF^+. This is in agreement with previous findings of this study which showed *Candida* to perform a resistant phenotype after the exposure to 50% of heather honey regardless the increased membrane permeability of cells. Hyphae, the most virulent yeast form, was impermeable to PI and emitted strong cF signal.

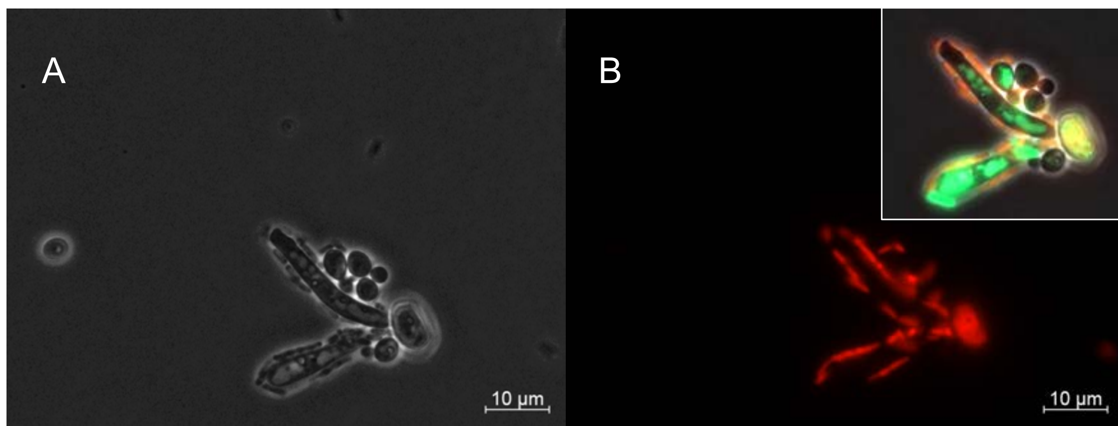


Figure 4.16 Snapshot of the bacterial and yeast cells 1 day post-treatment with heather honey. The aggregation of single yeast cells, hyphens and *E. coli* cells shows 3 types of fluorescence labelling 1 day post-treatment with 50% heather honey (*E. coli* :PI^+, *Candida* cells: PI^+/cF^+, hyphae: cF^+).

In agreement to these results, *Candida* has been reported to be resistant to many honeys species and in a range of concentration they were tested. Indigenous Arabic honey and stingless bees honey (unknown origin) were more effective against Gram-negative and positive bacteria comparing to the *C. albicans* (Al-Waili, 2004; Boorn et al., 2010). Also, Nigerian indigenous honey (Anyanwu, 2012), commercially available, natural unprocessed (applied between 0.1-25%) (Lusby et al., 2005) and commercial Nigerian honey (applied between 25-100%) (Omafuvbe and Akanbi, 2009) did not arrest the growth of *C. albicans*. Turkish and Iranian indigenous honeys, applied at 80% concentration, inhibited the growth of the yeast (Banaeian-Borujeni et al., 2013; Ansari et al., 2013). Only one of the previous studies showed that undiluted honey, that demonstrated very low pH, was the most effective against *Candida (*Anyanwu, 2012). Also, any H_2O_2-producing varieties tested were more effective towards *C. albicans* comparing to high antimicrobial *Leptospermum* spp. (Manuka honey that lacks the peroxide activity)(Al-Waili, 2004).

As emerged from these observations, it was established that heather honey cannot eradicate the polymicrobial community. It inhibited the growth of pathogenic *E. coli* and enhanced the growth of *C. albicans*. These results agree with the above-mentioned studies which mentioned the increased resistance of *Candida* to honey compared to other species tested. Thus, it was assumed that honeys of low acidity and high H_2O_2 activity could potentially demonstrate higher fungicidal effect. Thus, considering the high antimicrobial effect and high peroxide activity of Indonesian honey it was expected that both species will be more susceptible.

Looking at Figure 4.17, it is apparent that Indonesian honey has an exceptionally high H_2O_2 activity. This honey generated 5.8 mM H_2O_2 within the culture flask 4h after it was applied. This concentration is higher than the usual range of H_2O_2 accumulated in honeys (Table 1.3). Also, the same honey was consistently generating H_2O_2 up to 8 hours in contrast to heather whereas the H_2O_2 started to decline 2 hours post dilution. This suggests that sustained release of H_2O_2, in exceptionally high concentrations, was a factor that increased the toxicity of the Indonesian honey.

Low acidity is the other factor which contributes to antimicrobial activity of honey. Oxidation of glucose generates gluconic acid which lowers or maintains the low pH of honey and enhances the toxicity of H_2O_2 (Raffellini et al., 2007). Here, it was showed that both honeys caused a significant decrease to pH of the VSF. Although Indonesian honey is significantly more antimicrobial than heather, both acidified the medium from 5.8 to 3.5 (Indonesian) and 3.7-3.9 (heather) (Figure 4.17 B). Thus, honey administration acidified the vaginal fluid in level lower than this corresponding to a healthy vaginal mucosa (pH 4.2) when dominated by lactic acid bacteria (Boris, S. and Barbés, 2000).

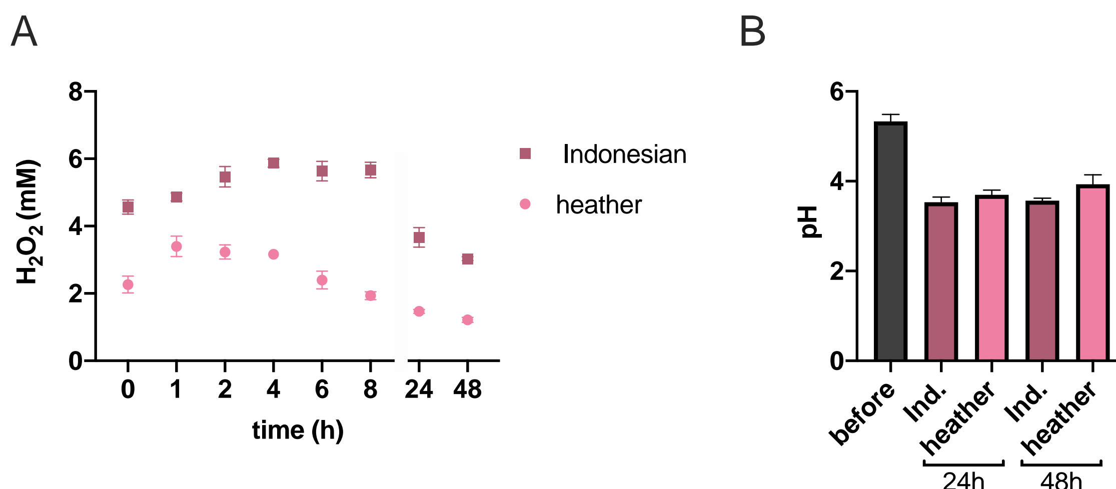


Figure 4.17 Factors affecting the antimicrobial strength of heather and Indonesian honeys. (A) H_2O_2 accumulation and (B) acidification of VSF was monitored up to 2 days of treatment with heather and Indonesian honeys. Error bars represent the average ± s.d. (*n*=3 measurements).

Considering the above it appears that the exceptionally high and sustained generation of H_2O_2 accounts for the high antimicrobial activity of Indonesian honey. Although both honeys caused the acidification of the media only Indonesian honey eradicated both species (Figure 4.14). Heather honey caused transient damage to yeast cells and eventually enhanced the growth of *Candida*. Since, Indonesian honey caused sustained release of H_2O_2, we speculated that it would also promote the generation of intracellular ROS. Thus, ROS accumulation was examined in both pathogens. H_2DCFDA is an intracellular probe which is oxidised by ROS. Dual staining of the culture with PI/H_2DCFDA showed the physiological changes on membrane integrity and ROS accumulation after *Candida* and *E. coli* were treated

with Indonesian honey. The respective samples were stained with PI/cFDA and were visualised in fluorescence microscope.

Figure 4.18 shows that within a day, almost 80% of yeast and 50% of bacterial cells were permeable to PI. For *Candida*, the percentage of PI permeable cells (PI^+) was equal to this of DCF^+, a tantamount to ROS formation. This observation implies that the ROS accumulation in yeast possibly caused the destruction of membrane integrity. Although *E. coli* was more susceptible than *Candida*, only 50% of the population was permeable to PI and the equal percentage was also detected with intracellular ROS. This percentage is possible that corresponds to *E. coli* cells with destructed cell envelop which still maintain a cell structure rather to cell fragments. Oxidative stress causes cells bursting. In case of cells lysis, DNA and intracellular product are released to the respective growing media (Newton et al., 2016). Thus, is expected that only cells with compromised membranes that are not lysed will retain the PI.

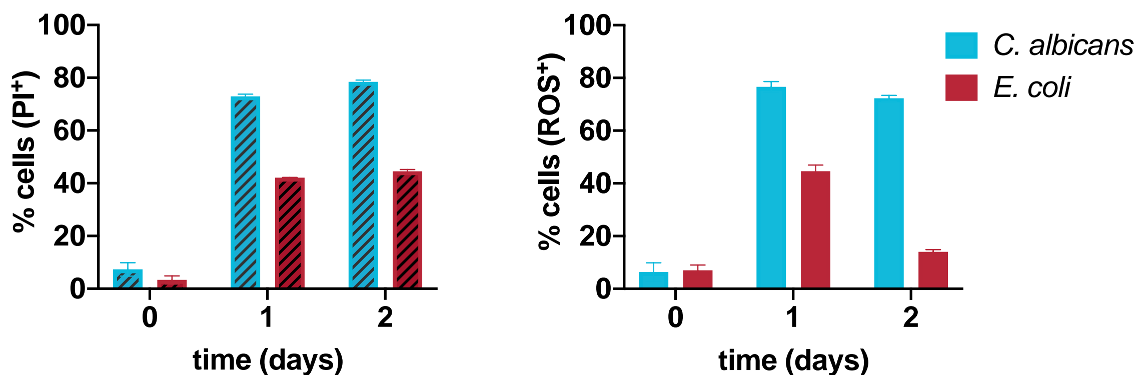


Figure 4.18 Physiological changes induced by Indonesian honey on *E. coli* and *Candida* coculture growing in VSF. Membrane integrity and intracellular ROS accumulation were assessed by capturing the percentage of *E. coli* and *Candida* cells positive to PI (left) and FDA (right) fluorescence respectively. The t=0 corresponds to control/no-treated samples. Error bars represent the average ± s.d. (*n*=3; biological replicates).

Fluorescence images show the cellular damage of *Candida* and *E. coli* cells after treatment with the Indonesian honey (Figure 4.19). By comparing bright field and fluorescence images, it is showed that red labelled cells correspond to the individuals with damaged membranes and not to cell fragments which do not uptake any dye. This result confirms the hypothesis that lysed cells or cell fragments cannot retain the PI as such they did not appear as PI labelled. Brightfield images show destruction, shrinkage and cell lysis of yeast and bacteria. These images showed the significant bactericidal effect of Indonesian honey compared to heather (Figure 4.15).

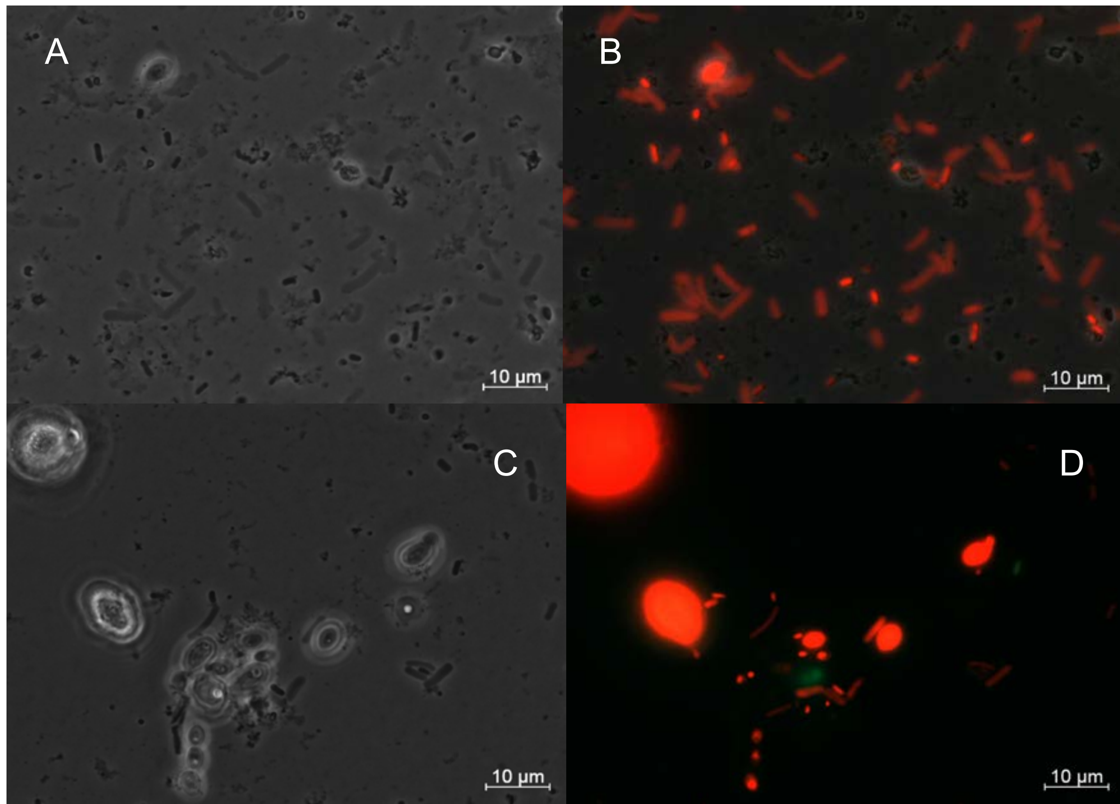


Figure 4.19 Fluorescence images showing the effect of Indonesian honey on *E. coli* and *C. albicans* 1 day post-treatment. (A, C) Brightfield images show compromised yeast and bacterial cells. (B, D) only cells that retain their organisation are PI labelled. Cell fragments corresponding to burst yeast or bacteria are not labelled with any dye.

4.3.4. Effect of heather and Indonesian honey on viability of *L. acidophilus*.

Considering the effect of heather and Indonesian honey on *E. coli*-*C. albicans* coculture, it was next questioned their effect on *L. acidophilus*. As was mentioned previously, the ideal treatment for VV infections would eradicate the pathogens while enhancing the growth of lactic acid bacteria (LAB). Thus, here it was examined whether heather and Indonesian honeys at concentrations they were applied for the eradication of pathogens are also beneficial for *L. acidophilus.* Honeys diluted at 50% or less were tested towards *L. acidophilus*. The viability of *L. acidophilus* was assessed by viable counts as was performed previously. The generation of H_2O_2 was also monitored for each of the concentrations of honey tested above.

The inoculum size was adjusted to 10^{8-9} CFU/mL. This was adapted by other studies whereas the lactic acid bacteria were used in medical formulations for VVC treatment (Van de Wijgert et al., 2019).

Indonesian honey, at all concentrations tested, decreased the viable counts of *L. acidophilus* below the detection limit: 1 CFU/mL (data are not shown). Heather honey caused a dose-dependent effect. As it is shown in Figure 4.20, honey diluted at 50% caused 4 logs reduction while honey diluted to 25% caused 2 logs reduction. Viability of *L. acidophilus* increased within a day post-exposure to 12% honey and then decreased by day 2.

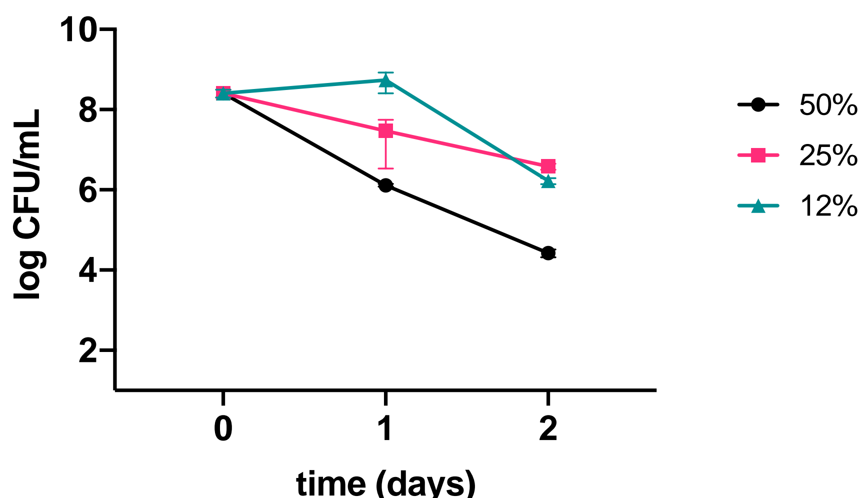


Figure 4.20 Effect of heather honey on the viability of *L. acidophilus*. Susceptibility of LAB was tested after heather honey was added to 3 individual cultures of *L. acidophilus* (kept in PBS). Heather honey was added in final concentration of 50%, 25% and 12% respectively. Viable counts of day 0 correspond to the initial *L. acidophilus* inoculum before honey was added. Error bars represent the average ± s.d. (*n*=3; biological replicates).

Monitoring of H_2O_2 three cultures, showed that as expected production of H_2O_2 was dependent on honey dilution (t=0). Heather diluted at 50% initially generated the highest amount of H_2O_2, followed by 25% and 12% honeys (Figure 4.21). Within 2 days after dilution concentration of H_2O_2 was measured in the *L. acidophilus* cultures. The H_2O_2 produced by 50% honey was degraded from 2.2 to 1.4 mM while this produced by 12% honey increased from 0.5 to 1.16 mM. As it was shown previously (Figure 2.8C) H_2O_2 started to get degraded within 1-2 hours post-dilution. Specifically, H_2O_2 produced by 25% and 12% heather honey degraded to

less than 0.8 mM concentration. Thus, here it was speculated that honey, when applied in high dilutions (i.e. 25% or higher), allows the growth of *L. acidophilus* and subsequently allows their H_2O_2-producing activity. In this case *L. acidophilus* retains its metabolic activity and grow in presence of honey. However, the concentration of this honey is far below this which caused bactericidal/fungicidal effect.

In agreement to this, it has been reported that 0.4 mM H_2O_2 slowed down the growth while higher concentration (>1.2 mM) arrested the growth of *L. acidophilus* (Calderini et al., 2017). As was seen here, *L. acidophilus* was more susceptible to 2.2 mM H_2O_2 while could moderately survive to lower H_2O_2 concentration. This result could be possibly due to the honey type applied here and also due to the defence mechanism employed by *Lactobacillus*. In support of this, it has been known that *L. acidophilus* possess manganese superoxide dismutase and pseudo-catalase to convert hydrogen peroxide in harmless compounds (Strus et al., 2006). This mechanism promotes the consumption of oxygen species (Guerzoni et al., 2001), promotes intracellular homeostasis (Toledano et al., 2007) and employs DNA repair mechanism (Miyoshi et al., 2003). Also, it was seen that oligosaccharides of honeys give a fitness advantage on the *Lactobacillus* spp. (Machado et al., 2017). These findings suggest, that honey causes a H_2O_2-dependent damage to lactobacilli cells, however, the detoxifying of this species mechanisms and the sugar fraction promotes their growth in honey.

Monitoring of H_2O_2 concentration within bacterial cultures exposed to three concentrations of honey, showed that H_2O_2 was not degraded as was expected. In contrast, *Lactobacillus* increased the H_2O_2 within the culture. This is in agreement with other studies which reported the H_2O_2-producing activity of some lactobacilli

species (among them *L. acidophilus*). As has been shown, in aerated cultures, accumulation of H_2O_2 started after 30 min of incubation and increased up to 3 mM within 3 hours growth while supplementation of the cultures with Fe^{3+} caused degradation of H_2O_2 (Martín and Suárez, 2010). Therefore, it is sensible to speculate that heather honey and especially its oxidative strength affected the respective H_2O_2-activity of *L. acidophilus*. Honey diluted at 50% generated higher H_2O_2 which was bactericidal considering the low detoxifying activity of LABs. Also, in honey diluted at 50%, Fe^{3+} is presumably higher compering to this in higher dilutions of honey. Thus, in higher honey dilution (lower H_2O_2 dose and potentially lower Fe^{3+} concentration), whereas the antibacterial activity of honey is low, *L. acidophilus* exerted H_2O_2-producing activity. This activity could be potentially higher under different growth conditions (i.e. aeriation).

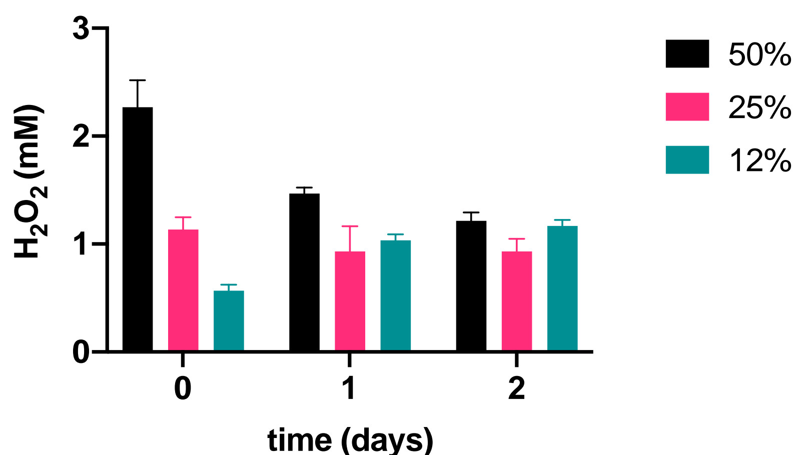


Figure 4.21 Monitoring of H_2O_2 within *L. acidophilus* cultures when exposed to different concentrations of heather honey. Concentration of H_2O_2 was measured in each of the 3 *L. acidophilus* cultures after honey treatment. H_2O_2 level shown in day 0 corresponds to a measurement taken a few minutes after honey was added to the respective cultures. Error bars represent the average ± s.d. (*n*=3; replicates).

Overall, this study showed that viability of *L. acidophilus* is effected by honey in H_2O_2 manner. At low honey concentration (<25%), is possible that *L. acidophilus* can grow and exert H_2O_2-producing activity in presence of carbon sources (i.e sugars). As it has been claimed previously, there are factors which affect the generation of H_2O_2 by lactobacilli when these bacteria are growing in honey. As it appears growth of lactobacilli and metabolic activity in honey is a complicated process which is affected predominately by honey composition. The uncharacterised composition of the honeys used here puts a limitation to make further conclusions. From these findings it was deduced that Indonesian honey can be effective against the two pathogenic species however, it cannot promote the growth of *L. acidophilus* under the conditions tested here.

4.4. Discussion

After investigating stress responses of a model organism to honey, it was questioned whether this could be extended to a real life scenario. Thus, this study examined the effect of honey on two pathogenic species implicated in vulvovaginal infections, *E. coli* UPEC and *C. albicans*. To do so, a few parameters such as the growth phase of the tested organisms and their growth behaviour when are found in coculture were considered initially.

The results of this work showed that both species growing in stationary phase were more resistant compared to log-phase cultures. Also, *Candida* was seen to grow less well in coculture with *E.coli*. This is potentially due to unknown interactions developed between the two microorganisms. Besides that, *Candida* showed an adaptive phenotype post-exposure to heather honey. In particular, *E. coli* was completely eradicated while growth of *Candida* was enhanced in presence of heather honey. This is in agreement with previous findings mentioning the increased resistance of *C. albicans* compared to other species and its adaptation phenotype when exposed to honeys of sub-inhibitory concentrations (Ansari et al., 2013). The ability of *Candida* to counteract oxidative attack and its osmoadaptation presumably aid fungal resistance and invasiveness by hyphae formation (Almeida 2008).

However, natural Indonesian, a high antimicrobial honey caused the eradication of both species growing in VSF. It was speculated that the synergy of the low acidity with the high and sustained generation of H_2O_2 of this honey caused the irreparable damage to both species. As have been discussed in this thesis, H_2O_2

enhances the generation of other intracellular ROS which in synergy with the low pH enhance the antimicrobial strength of honey. Although *Candida* has been shown to be osmotolerant and to survive up to 20 mM of H_2O_2 (Brown et al., 2014), is also susceptible to honeys of low acidity and high oxidative potential (Boorn et al., 2009). Thus, it was assumed that synergy of low pH and peroxide activity in Indonesian honey account for the strong fungicidal effect. The data obtained by FC and fluorescence microscopy proved that Indonesian honey caused significant cell damage or even lysis which eventually halted cellular growth. This is in agreement with previous data showing that treatment of *C. albicans* with a high antimicrobial honey caused irreparable cell damage and significant eradication of biofilm (Ansari et al., 2013). The same study showed that formation of pores in yeast cell walls, bursting and shrinkage of cells, were linked to oxidative and osmotic stress respectively.

In contrast to both pathogens, *L. acidophilus* was completely eradicated by both Indonesian and heather honey while it grew only in subinhibitory concentration (12%) of the latter exerting H_2O_2-producing activity. These observations agree with previous reports which showed specific honey oligosaccharides (i.e. isomaltose, melezitose, raffinose etc.) to enhance the growth of LABs (Shamala et al., 2000; Chick et al., 2001; Haddadin et al., 2007; Nagpal and Kaur, 2011; Saran et al., 2011; Varga et al., 2014). This effect is determined by the composition of each honey and the concentration it was applied.

Overall, it was demonstrated that a honey which combines high peroxide activity and low pH is capable of eradicating both pathogenic species growing in VSF. This honey could also modify the pH within the vaginal cavity and possibly

provide prolonged protection from reoccurrence which is a promising finding. Indonesian honey had been bactericidal for the LABs which is undesired for the current application. This confirms that antimicrobial effect of honey is also species-related. Thus, another challenge is the discovery of a honey which selectively arrests the growth of pathogenic species while it does not affect or enhance the variability of LABs. However, before we rule out the use of such a honey as a potential VVC treatment, further investigation is needed on the physiological changes of LAB under a range of honey types or even a broader testing on the susceptibility of various LAB strains. This is expected to provide with a better understanding on the honey components that promote the growth and accelerate the H_2O_2-producing activity of lactobacilli.

CHAPTER 5

Conclusions & Discussion

Nature has served as human's pharmacy for millennia. Before the golden era of antibiotics (1950) natural medicine represented almost 22% of all the molecular entities in medicine. However, since the emergence of antimicrobial resistance natural products and their derivatives make up 70% of all FDA-approved drugs. Although medical use of honey is traced to some 5,000 years ago, medical Act approved its medical application on 1999 (Cooper and Jenkins, 2009). Medical honey is extensively used in wound healing and It has been shown to combat antibiotic-resistant strains isolated from wound sites. Thus, there is ongoing interest to develop our understanding on the antimicrobial mechanism underlying the activity of honey. This is believed to unravel the full potential use of honey as antimicrobial and indicates new medicinal applications. The understanding of the underlying antimicrobial mechanism has been challenging at time due to the highly complex composition and the variability of this substrate. Also, research used to focus more on the proof of antimicrobial activity rather than the understanding of this mechanism.

Throughout this Ph.D. project *E. coli* K-12 MG1655, a model organism with well characterized genome, was used in order to investigate and understand bacterial stress responses to all H_2O_2-producing honeys. Applied microbiology and high throughput sequencing were combined to give the cellular and molecular fingerprint of honey's antibacterial activity. In order to avoid discrepancies that could arise from the complexity of honey composition and variability, a model honey was used. This novel approach was based on the principle that dilution of honey results on the enzymatic production of two antibacterial compounds: H_2O_2 and gluconic acid. Thus, the model honey allowed to identify the antibacterial effect caused by

the synergy of the main honey stressors, which cannot be done using any natural honey.

In Chapter 2 the combination of multiparametric flow cytometry, AFM and viable counts provided valuable information on the changes of bacterial physiology upon honey treatment. Distinct antibacterial mechanisms were induced by the individual honey stressors. In particular, gluconic acid and H_2O_2, caused membrane lesions and depolarisation. This study showed for first time that the effect of H_2O_2 was significantly augmented by the presence of gluconic acid. Synergy of the two components caused membrane destruction analogous to that caused by 150 times more concentrated H_2O_2. This finding provided also a logical explanation to the fact that honey-induced H_2O_2 can be bactericidal even this is accumulated in no-bactericidal concentration (Brudzynski et al., 2011b). Although sugars caused plasmolytic effects their presence in model honey mediated the toxicity of the other two components. This also explains the fact that honey is antibacterial without being toxic when applied to open wounds (Martinotti et al., 2019). AFM provided evidence for the effects of the model on bacterial cell envelope which were caused within short time of treatment.

The effects of the model were confirmed by two types of natural honey (heather and acacia). It was found that presence of H_2O_2 in concentration higher than 0.8 mM caused the same physiological changes to bacteria (membrane destruction and depolarisation) while concentrations up to 2 mM H_2O_2 arrested bacterial growth. This confirmed the abovementioned observations that synergy of honey components caused bacterial cell damage rather the H_2O_2 itself. However,

the balance between H_2O_2 generation by honey and degradation by bacterial catalase activity determined the efficacy of each honey applied (Brudzynski et al., 2012).

Two other findings of this study support this assumption. First, the peroxide activity of each honey controlled the intracellular ROS accumulation. Single cell analysis showed that honeys with high peroxide activity caused higher ROS accumulation 48 h post-exposure, despite the fact that H_2O_2 was degrading a few hours after the dilution. Thus, it was assumed that prolonged presence of H_2O_2, even in sub-inhibitory levels, promotes ROS generation by Fenton reaction (Brudzynski et al., 2012). However, composition of both honeys used here is uncharacterised thus, no further hypothesis can be made on this mechanism. Second, catalase mutants were significantly more susceptible to model honeys with increased H_2O_2 and the ROS accumulation was higher in these mutants compared to the WT. This suggested that excess of H_2O_2, which cannot get degraded by bacteria, causes cell killing probably by damage of DNA and other macromolecules (Blair et al., 2009). It is also possible that oxidative stress inhibited other cellular functions and the bacterial repair mechanism (Giroux et al., 2017). Interestingly WT and *rpoS* mutant, that possess catalase activity, were moderately affected by low peroxide producing honey which confirmed again the synergy of acid/oxidative stress (Lowen et al., 1985; Jung et al., 2003).

Further, in order to obtain a greater understanding of the molecular mechanisms underpinning the survival of *E. coli* K-12 to model honey a whole-genome sequencing method was employed. TraDIS, the wide-genome

mutagenesis, combined with directed insertion-site sequencing, was employed to define the genes whose mutation significantly cause a growth defect post-exposure to honey. This method allowed also the identification of genes that when mutated lead to an increased survival of the respective strains.

TraDIS data and enrichment analysis showed that in response to honey stress bacteria potentially switch from aerobic to anaerobic metabolism. As was discussed in Chapter 3, in aerobiosis, Formate dehydrogenases (FDHs) and Nitrate reductase (Nar) control the cellular homeostasis maintaining the PMF (Iwadate et al., 2017). Given the effect of the model honey on the membrane integrity and polarity it is possible that the H^+ accumulating in the cytoplasm increased the toxicity. Thus, mutation of biological pathways that control the cell homeostasis caused significant growth defect to the respective strains.

In particular, genes involved in cell homeostasis (*fetAB*, *gor*), metabolism under anaerobiosis (FDHs genes, *selABD, moaAC, moeBDE*) and membrane invagination (*prc*) were found to be conditionally essential for bacterial survival in model honey. Among them the periplasmic protease, *prc*, has been mentioned as a favourable antibiotic target because of its role in bacterial pathogenesis (Wang et al.,2012). Also, TraDIS analysis showed that the mutation of any of the subunits in Tol-Pal and EnvZ-OmpR two components systems (TCS) significantly increased bacterial fitness to model honey. This could be explained either by conformational changes in outer membrane porins (Tan et al., 2017), or by the transcription of other operons such as *fur* and *cad* which otherwise are repressed by this TCS in response to acid/osmotic stress (Jaworska et al., 2018). The validation of TraDIS data, by the

construction of single mutants, enhanced the prospects of this method. However, the use of metabolomics could provide a better understanding on the system-wide metabolism and metabolic pathways that are employed by respective mutants. Also, the use of RNA-seq could measure the gene and transcript abundance under the conditions tested here.

Looking forward from research in model organisms, it would be highly informative to identify the effect of honey on pathogenic species when growing *in vitro* in coculture. Thus, the effect of two natural honeys with distinct antimicrobial potency was tested on two pathogens implicated in VVG infections: *E. coli* (UPEC strain)-*C. albicans* growing in vaginal simulation fluid. The results showed that both species were more resistant when growing in monocultures. Once in coculture, pathogenic *E. coli* became more susceptible to honey of medium peroxide activity while *Candida* developed an adaptive mechanism. The viability of both species declined below the detection limits when treated with honey which combined high acidity and significantly higher H_2O_2 concentration. This confirmed the importance of the synergy of acid and oxidative stress and showed the potential use of honey for eradication of this infection. However, a detailed analysis of the composition in both honeys is needed to affirm this hypothesis. Also, the use of a 3D simulation model could allow a better understanding of the interactions developed within opportunistic pathogens and the vaginal microbiota (i.e. biofilm formation) in a VV infection.

Overall, this thesis presents a systematic study on the antibacterial mechanisms of honey. The advantage compared to previous studies is the

combination of standard microbiological and genetic methods which allowed the advanced understanding of the bacterial stress responses towards honey. Also, the use of the model honey allowed the identification of significant synergies induced by the enzymatic reaction which applies in all H_2O_2-producing honeys. As a limitation of this approach remains the unidentified reactions which may promote or regulate the efficiency of the GOX enzymatic activity during honey dilution. To answer this, a thorough compositional analysis of many honey varieties should be carried out.

With regard to the benefit of these findings for the medical applications of honey there are two questions to be answered: (a) Is there any particular honey component(s) which can accelerate the efficiency of GOX reaction and (b) whether any natural honey can be engineering modified in order to fortify its antibacterial strength. Considering these questions, what is still lacking is the identification of all honey components implicated or affect the efficiency of GOX reaction. The major consideration, when applying this to a medical formulation, is the mechanism which controls the sustained accumulation of H_2O_2 in inhibitory, yet non-toxic levels.

In conclusion, the scientific society is tasked with solving one of the most challenging problems of our times, that of antibiotic resistance. The methodology applied in this study gave insights on the effects of honey on the model organism *E. coli* K-12, and the molecular pathways implicated bacterial stress responses. One of the most promising findings of TraDIS experiment was the identification of *prc* (bacterial periplasmic protease) as one of the honey targets. This has been reported as a favourable antibiotic target since the inactivation of *prc* exhibits increased outer

membrane permeability and leakage of the pathogenic *E. coli* RS218. Thus, a more "real world" approach would be the use of this method for the identification of molecular responses of critical and resistant isolates when are exposed to honey. This is expected to improve the scientific communication on this topic and stimulate further research as a step towards the acceptance of honey as an alternative antimicrobial.

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APPENDICES

Supplementary Information

The individual chapters contain references to the supplementary figures and tables listed below

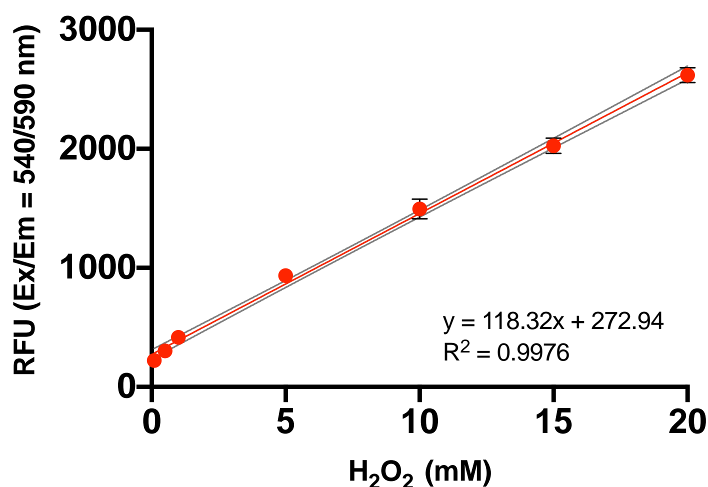
Chapter 2

Appendix 2.1. Composition of model honeys tested in the antibacterial assays. The "S", "G" and "H" stands for the sugars, gluconic acid and hydrogen peroxide (H_2O_2) respectively. The "SGH, SG, SH, GH" stands for the models combining two or three stressors in the concentrations stated bellow. In these 4 models, the concertation of gluconic acid and H_2O_2 correspond to the lowest level have been found in honey. Concentration of sugars (30%) corresponds to the honey dilution which demonstrates low antimicrobial activity (Table 1.3).

	Components / concentration						
Model	**Sugars (%)**	**Fructose (M)**	**Glucose (M)**	**Maltose (M)**	**Sucrose (M)**	**Gluconic acid (mM)**	**H_2O_2 (mM)**
S70	70%	1.88	1.56	0.184	0.033	-	-
S50	50%	1.34	1.11	0.131	0.024	-	-
S30	30%	0.8	0.66	0.07	0.014	-	-
G60	-	-	-	-	-	60	-
G34	-	-	-	-	-	34	-
G09	-	-	-	-	-	8.6	-
H54	-	-	-	-	-	-	5.6
H03	-	-	-	-	-	-	3
H00	-	-	-	-	-	-	0.04
SGH	30%	0.8	0.66	0.07	0.014	8.6	0.04
SG	30%	0.8	0.66	0.07	0.014	8.6	-
SH	30%	0.8	0.66	0.07	0.014	-	0.04
GH	-	-	-	-	-	8.6	0.04

Appendix 2.2. Gene primers designed for the validation of P1 transduction of mutants from *E. coli* BW25113 to MG1655.

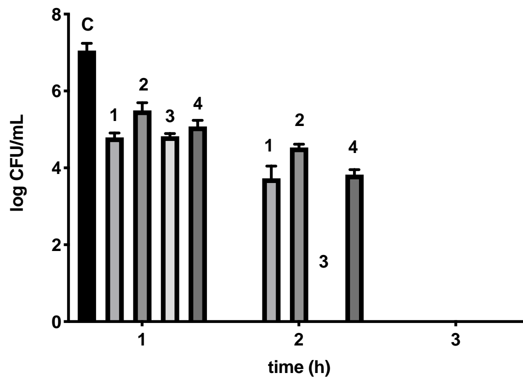
Gene name	Primer
katG (F)	5'- TGCCCGTTCCATCAGG -3'
katG (R)	5'- TACAGCAGGTCGAAACGG -3'
katE (F)	5'- ATGTCGCAACATAACGAAAAGAACC -3'
katE (R)	5'- TCAGGCAGGAATTTTGTCAATCTTAG -3'

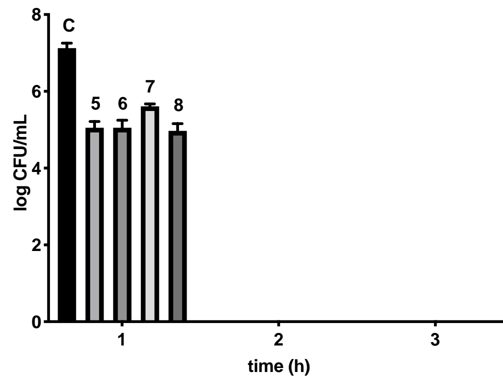


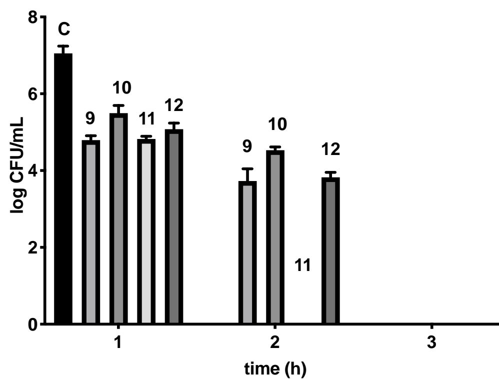
Appendix 2.3. H_2O_2 fluorometric standard curve for a range of concentration 0-20 mM. Standards H_2O_2 solutions (0.1, 0.5, 1, 5, 10, 15 and 20 mM) were prepared and mixed with peroxidase substrate. The reaction generates a red fluorescent product (Ex=540 nm/ Em=590 nm) which was analysed by the fluorescent microplate reader (CLARIOstar; BMG Labtech, US).

Appendix 2.4. Composition of the model honeys as proposed by CCD (Central Composite Design) experiment.

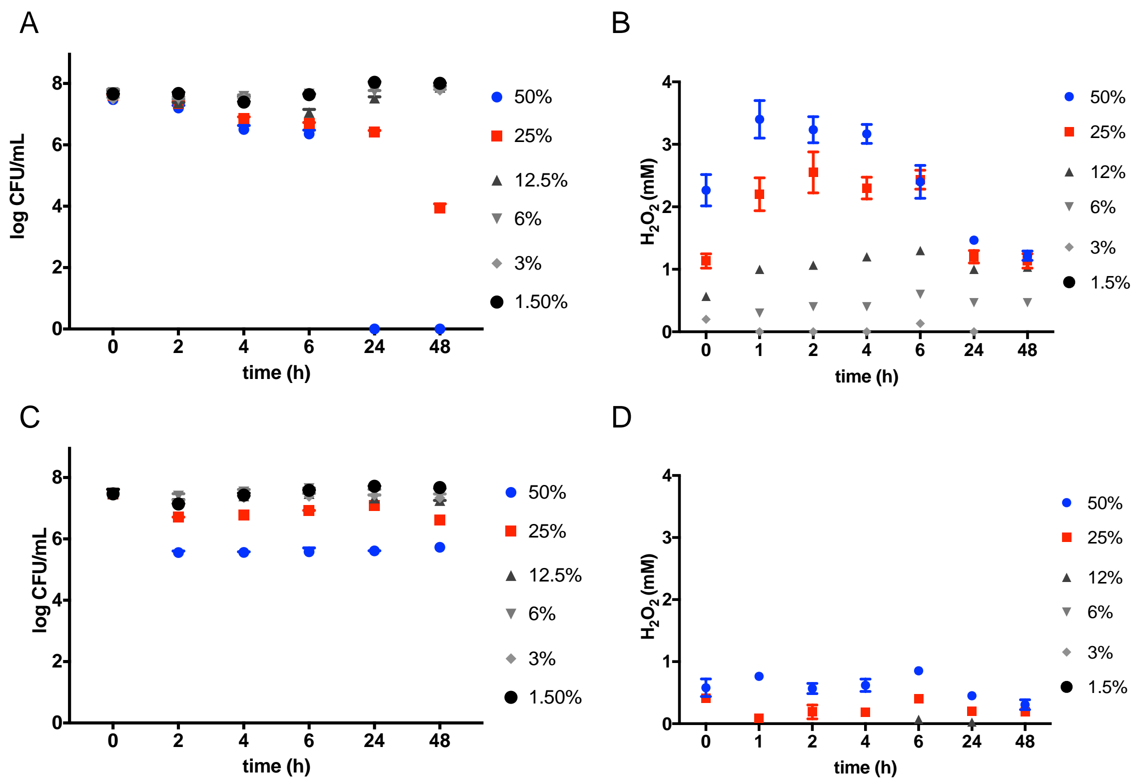
Concentration of components			
TEST	**Sugars (%)**	**Gluconic acid (mM)**	**H_2O_2 (mM)**
1	62.5	34.3	0.6
2	62.5	8.6	4.7
3	62.5	34.3	4.7
4	70	34.3	4.7
5	67	49.6	7.1
6	62.5	60	4.7
7	58	19	7.1
8	67	49.6	2.26
9	67	19	2.26
10	58	49.6	7.1
11	62.5	34.3	4.7
12	62.5	34.3	8.8



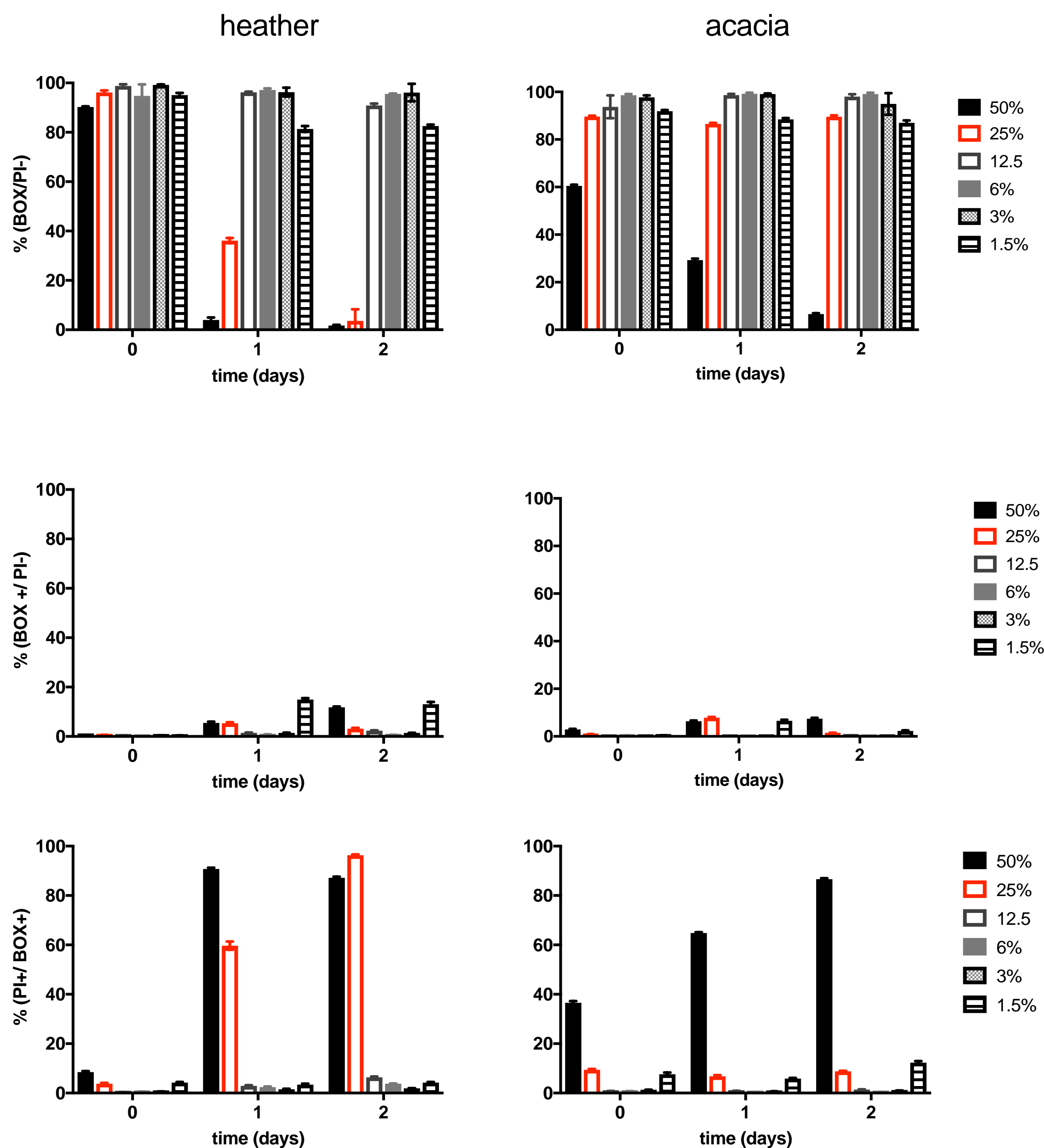




Appendix 2.5. Susceptibility of exponential growing *E. coli* K-12 to (12) models composed of the three stress factors in concentrations as proposed by the CCD experiment (Appendix 2.4). Antibacterial assay was conducted up to 48 h, however, cells' viability was reduced lower than the detection limit (1 CFU/mL) soon after the exposure. No resuscitation was reported for any of the challenged bacterial populations shown above. Error bars represent the average ± s.d. (n=3; biological replicates).



Appendix 2.6. Correlation between antimicrobial strength of honey and H_2O_2 accumulation. (A) Heather and (C) acacia honey were diluted (50-1.5%) and tested for their antimicrobial activity on *E. coli* up to 48 hours of exposure. (B, D) The H_2O_2 accumulation was measured for the same time course in order to identify the correlation between honeys' antimicrobial effect and the kinetics of H_2O_2. Error bars represent the average ± s.d. of three (n=3) biological replicates and 3 individual measurements of H_2O_2 accumulation in the respective honey samples.



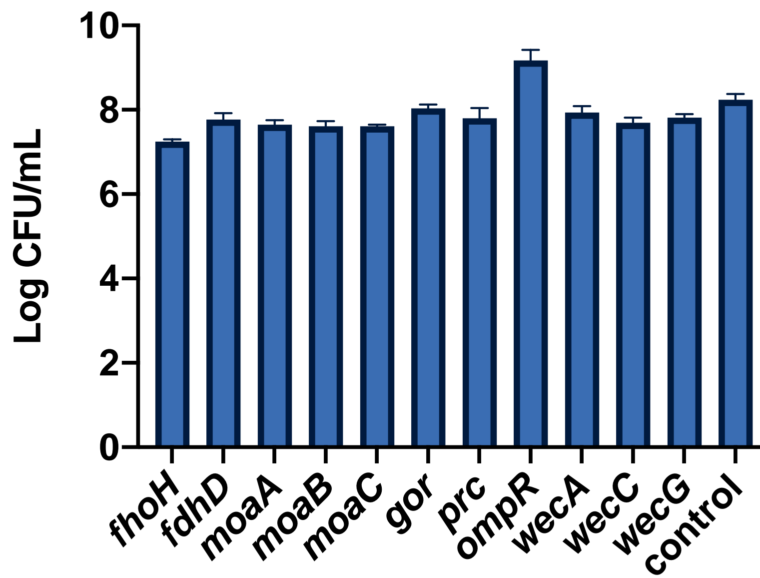
Appendix 2.7: FC analysis of *E. coli* exposed to heather and acacia honeys diluted at 50-1.5%. Double staining (PI/BOX) discriminates three populations; "live"/viable (PI^-/BOX^-), "injured "(or depolarised) (PI^-/BOX^+), and "dead" (or membrane destructed) (PI^+/BOX^+). Error bars represent the mean ± s.d. of three (n=3) biological replicates.

Chapter 3

Appendix 3.1 Genes with significant Log2 score across the 2 datasets (30min and 90min). The genes are ranked according to Log2 score.

30' sample			90' sample		
Gene	**adj. p-value**	**LogFC**	**Gene**	**adj. p-value**	**LogFC**
prc	1.13E-27	-3.3228998	*selD*	5.57E-09	-2.6846038
atpA	1.78E-12	-2.6620601	*prc*	7.70E-13	-2.1804702
moeB	4.26E-23	-2.5010238	*selA*	1.51E-09	-2.1361767
moaA	3.54E-15	-2.4188103	*fdhE*	2.15E-06	-2.04681
fabF	1.08E-16	-2.4052902	*fdoG*	1.85E-15	-2.0354739
fdoH	6.72E-20	-2.3928778	*yohD*	0.00166114	-1.9574062
selB	4.13E-24	-2.3603875	*selB*	8.01E-12	-1.9014615
selA	5.02E-18	-2.2744317	*fdoI*	4.39E-05	-1.8894201
fdoI	4.04E-11	-2.2685012	*fdoH*	5.10E-09	-1.8883053
atpD	3.99E-11	-2.2572989	*fdhD*	7.38E-06	-1.8690168
moaC	1.41E-08	-2.2536301	*atpD*	0.00034103	-1.8367194
yohD	2.25E-07	-2.2454598	*moeB*	0.00113266	-1.6458777
gntR	3.37E-13	-2.1557649	*yfeX*	0.00374165	-1.3958068
fdhE	1.16E-11	-2.1282873	*glnG*	3.41E-05	-1.3905399
moaE	1.26E-09	-2.1156959	*moaA*	0.00373388	-1.2884631
fdhD	4.48E-12	-2.1027986	*yfjQ*	0.00158484	-1.253874
fdoG	1.26E-25	-2.0702544	*moeA*	0.00091976	-1.1997349
selD	1.45E-06	-1.9307232	*yfjV*	0.00029052	-1.1734358
modB	4.38E-06	-1.8451576	*fetA*	5.61E-05	-1.1724089
mog	2.40E-06	-1.7778046	*gnd*	0.00080456	-1.1722759
mrp	5.88E-09	-1.7126202	*fetB*	0.00412931	-1.0612796
lon	0.00026075	-1.5623948	*dosC*	0.00350904	0.90329128

30' sample			90' sample		
moeA	6.25E-09	-1.4674787	*tig*	0.00100339	0.95309509
ppk	3.71E-08	-1.3633133	*mepS*	0.00150363	1.0132912
gltA	0.00079918	-1.2245062	*ompC*	6.59E-07	1.0614669
glnG	8.43E-05	-1.2152076	*wecA*	0.00015225	1.0916747
fetA	0.00019444	-1.1486974	*qseC*	0.00011772	1.11244051
rep	0.00017567	0.91260619	*ydcC*	3.08E-05	1.16502112
wecC	5.14E-05	0.94691221	*tolA*	0.00086849	1.17426211
envZ	7.95E-06	1.03020014	*acnB*	1.39E-05	1.18168313
wecB	2.23E-12	1.03466846	*wecG*	6.74E-05	1.20291296
wecG	1.59E-05	1.04357666	*plsX*	2.73E-05	1.20887031
wecA	8.17E-07	1.04473038	*wecC*	6.58E-06	1.21168436
phnI	1.47E-05	1.20032659	*ycfQ*	0.00091918	1.26297142
phnF	5.67E-05	1.24496955	*rep*	6.97E-10	1.2630543
yagF	2.83E-11	1.35362792	*ydcT*	0.00262438	1.2885118
ompR	6.99E-12	1.61321646	*wecB*	1.11E-11	1.33535514
			manA	0.00228828	1.42944534
			yejG	0.00372617	1.66120879
			yciF	1.05E-05	1.81774158
			mgrR	0.00096582	2.18693064
			envZ	1.70E-44	3.52185648
			ompR	5.06E-134	8.39233298



Appendix 3.2 Effect of model honey on *E. coli* KOs. Survival of KOs after 2h exposure to model honey. Error bars represent the mean ± s.d. (n=3; biological replicates).