School of Molecular and Life Sciences

Investigations into the Molecular, Isotopic and Genomic Mechanisms behind Human Gallstone Disease

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This thesis is presented for the Degree of
Doctor of Philosophy
of
Curtin University

January 2019

Declaration

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This thesis contains no material which has been accepted for the award of any other degree or diploma in any university.

The research presented and reported in this thesis was conducted in accordance with the National Health and Medical Research Council National Statement on Ethical Conduct in Human Research (2007) –updated March 2014. The proposed research study received human research ethics approval from the South Metropolitan Health Service Human Research Ethics Committee (Ref/Approval No: 15–136), the Fiona Stanley Human Research Ethics Committee (Ref/Approval No: 2015–136), the St John of God Health Care Human Research Ethics Committee (Ref/Approval No: 1021), and the Curtin University Human Research Ethics Committee (ref/Approval No: HR229/2015).

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March 26, 2019

Abstract

Gallstone disease is among the most common clinical problems encountered worldwide costing health care systems billions annually. In 2013, Australia had one of the highest rates of hospitalisations related to gallstone disease amongst the Organisation for Economic Co-operation and Development (OECD) nations, with cholecystectomy the second highest procedure after cataract surgery. Gastrointestinal disorders, in which gallstone disease is inextricably linked, accounted for the fourth highest cost to the Australian health care system at an estimated $3.4 Billion during the 2012-2013 financial year alone. Laparoscopic cholecystectomy, the primary treatment option for the disease, is not without complications, and non-invasive treatment options, such as dissolution therapy, are only available to a small percentage of patients. In recent studies, the importance of the gallbladder in digestive system regulation has been highlighted, re-energising efforts to preserve the organ by mitigating gallstone lithification at the onset. Thus far, the leading hypothesis for gallstone lithification has centred on physico-chemical models in which hypersecretion of cholesterol by the liver, or hyposecretion of bile salts, leads to the precipitation of microcrystals. These models, however, are limited in that they are yet to provide a detailed mechanism of formation that accounts for stone growth, all presentations of the disease, i.e. stone formation in the common bile duct, and whether dietary cholesterol or its source plays a role. A secondary hypothesis has implicated bacterial mediation in the formation of pigmented stones alone, with limited data on whether the same hypothesis can be applied to all stone types. Further, questions still remain as to how bacteria may survive in what is the extreme environment of the human gallbladder and whether archaeal, protistal, viral or fungal signatures are present. This research aims to address these gaps in knowledge and provide new insights into both microbial and physico-chemical formation processes with the application of novel tools yet to be systematically applied in this research area.

In **Chapter 2**: The application of shotgun metagenomics analysis and compound specific isotope analysis (CSIA) of cholesterol is applied for the first time on human gallstones. Gallstone disease research is advanced in an unprecedented way by the identification of genes involved in bile stress response, biofilm formation, and anaerobic energy metabolism by Gram-negative *Klebsiella* in the pigmented gallstones of a 76-year-old male patient. *Klebsiella* is also found to be present in one cholesterol stone in a 30-year-old female patient who had additional cholesterol gallstones characterised by a predominance of Gram-positive *Streptococcus*. Pigmented stones further revealed a predominance of genes involved in carbohydrate metabolism, whilst cholesterol stones indicated a profile dominated by protein metabolism possibly reflecting known chemical differences between Gram-negative and Gram-positive biofilm matrices. Further, *Klebsiella* is shown to harbour dimethyl sulfoxide (DMSO) respiration genes, suggesting the reason why this genus may be out-competing other genera in the study. Archaeal, fungal, protistal and viral genes are not detected. Complementary carbon and hydrogen isotopic analyses (CSIA) of cholesterol within the patients' stones reveal homogeneity, suggesting a common diet or cholesterol biosynthesis pathway. This study reveals the strategies that key pathogenic bacteria may be employing to survive in the extreme environment of the human gallbladder. Further, it reveals that strong pathogens like *Klebsiella* are not only equipped to withstand gallbladder toxicity, but their survival strategies, such as the up-regulation biofilm formation genes, may be providing an abundance of glycoproteins hypothesised to lead to stone crystallisation and growth.

In **Chapter 3**, this research is extended to include a larger group of 16 patients with diverse backgrounds and gallstones types using 16s rRNA profiling, which provides greater resolution of bacteria present. A predominance of biofilm-producing and bile resistant Gram-negative bacteria are identified in all major stone types, and across a diverse array of patient backgrounds, suggesting traditional risk factors may play a minor role in gallstone pathogenesis. Further, that the predominance and persistence of pathogens from the Enterobacteriaceae family across the majority of patients, most notably *Klebsiella*, may be the key genera associated with gallstone pathogenesis. Complementary carbon and hydrogen cholesterol analysis using CSIA of the patients' stones reveal homogeneity between patients, with a common source of C3 vegetation in the $\delta^{13}C$ values. Further, the δD values show an elevated intake

of richer fats across all 16 patients, suggesting a diet rich in dairy products. The extensive use of antibiotics in the dairy industry is suggested as a potential source for the cholesterol and strong pathogenic signatures identified in this study.

In **Chapter 4**, this research investigates large and solitary stones, an often overlooked stone type, in greater detail using scanning electron microscopy (SEM) and X-Ray diffraction analysis. This is complemented by cholesterol analysis (CSIA) of the stones, including the centre, mid, and outer ring of a particularly large solitary gallstone in order to determine if cholesterol signatures are homogenous in each layer of stone growth. Further, in light of recent finds in this research area which include multiple stone types and morphologies, this study explores biomineralisation processes in nature and proposes a novel theoretical model for gallstone nucleation and growth.

This research provides significant insights into an increasingly complex disease and contributes to previous research efforts by advancing the bacterial mediation hypothesis of gallstone formation. Further, it provides a novel framework to study both microbial and physico-chemical processes in unprecedented detail and gain further insight into stone formation processes across a markedly diverse range of stone types and patient backgrounds. This study also highlights the value of interdisciplinary techniques and how they may aid in elucidating complex medical problems.

Acknowledgements

I'd like to first and foremost thank my supervisors Prof. Kliti Grice and A. Prof. Marco J.L. Coolen for their guidance, care, patience, and generous support over the past four years. You both share a truly joyful enthusiasm for science and research that not only inspires and ignites this within your students but reminds them as to why they chose to pursue a career in science in the first place. I am extremely grateful Kliti, for your generous support, your unwavering faith in the project's success, and for giving me the opportunity to work on such a novel research project. It pushed me to reach beyond my limits, grow as a person and achieve beyond that which I thought I was capable of. Thank you, Marco for introducing me to one of the most fascinating and enthralling areas of science I have ever had the opportunity to pursue. I will always be grateful for your leadership in research, the generous offer of your time, and mentorship in all things molecular biology and genomics related; without which I would not have found my scientific home and this project would be missing its vital half.

My deepest and most sincere thanks go to Dr. Mohammad Ballal, who took time out of his busy practice and family commitments to recruit patients and deliver samples even after long hours of surgery. Without your open-minded enthusiasm for science and research, faith, and selfless assistance, this project would not have been possible.

My sincere thanks to the administrative staff and ethics review boards at the Fiona Stanley and St John of God Hospitals, both for approving the project and for providing invaluable support throughout the application process.

My gratitude and thanks to Elaine Miller from the John de Laeter Centre for her generous assistance and Prof. Andrew Putnis from TIGeR for the generous funding of project materials and conference support throughout the project.

My warmest thanks to all the students and staff at the Department of Chemistry/MLS, with special thanks to the WA-OIGC gang. Thank you for allowing me to escape the grind and gain valuable perspective with your companionship, laughter, and tea. Without you, this PhD would not have been anywhere near as enjoyable as it has been. I wish you all great success in your research and future careers.

Lastly, I’d like to thank my closest friends and family, in particular, my mother Jan. Only due to your love, support, encouragement and generosity am I able to finish this work. You made a difficult road, at a difficult time, that much easier, and for that I will be forever grateful.

Primary Publications

The chapters integrating this thesis correspond to papers published or accepted with minor changes, submitted, under review or in preparation, and are listed below.

Chapter 2

Kose, S.H, Grice, K, Orsi, W.D, Ballal M, Coolen M.J.L. (2018). Metagenomics of pigmented and cholesterol gallstones: the putative role of bacteria.
Scientific Reports, 8:11218, 1-13.

Chapter 3

Kose, S.H, Grice, K, Ballal, M, Suthananthan, A, Wuchter, C, Coolen M.J.L. (2018). Biofilm-producing bacteria identified in all major human gallstone types. *PLOS One (under preparation).*

Chapter 4

S.H. Kose, M.J.L. Coolen, M. Ballal, K. Grice. Characterisation of large and solitary human gallstones: A case study for a natural biomineralisation model. *(under preparation).*

Statement of Contribution of Others

The work presented in this thesis was primarily designed, experimentally executed, interpreted, and individual manuscripts were prepared by the first author (Sureyya H. Kose). Contributions by colleagues are described below. See **Appendix 4** for co-author acknowledgement statement.

Chapter 2

K.G & M.J.L.C assisted in experiment design. M.J.L.C, W.D.O & K.G assisted in analyses and data interpretation. M.B organised patient consent, sample extraction and delivery. M.J.L.C assisted in writing the main manuscript text with contributions from K.G, W.D.O and M.B.

Chapter 3

K.G & M.J.L.C assisted in experiment design. K.G, M.J.L.C, and C.W. assisted in data analysis and interpretation. M.B and A.S organised patient consent, sample extraction and delivery. M.J.L.C assisted in writing the manuscript text with contributions from K.G and C.W.

Chapter 4

K.G & M.J.L.C assisted in experiment design. K.G, M.J.L.C assisted in the analyses and data interpretation. M.B organised patient consent, sample extraction and delivery.

Secondary Publications

The following correspond to manuscripts based on research conducted during the preparation of this thesis and abstracts for conference presentations.

Conference abstracts:

Kose, S.H., Grice, K., Orsi, W., Ballal, M., Coolen, M.J.L. Molecular, Isotopic and Genomic Composition of Human Gallstones. 19th Australian Organic Geochemistry Conference (AOGC), Perth, Australia, November 2016, oral presentation.

Kose, S., Grice, K., Ballal, M., and M.J.L. Coolen. Molecular, Isotopic and Genomic Composition of Human Gallstones. 7th International Conference on Medical Geology, Moscow, Russia, Aug 2017, oral presentation.

Kose, S., Grice, K., Ballal, M., and M.J.L. Coolen. Molecular, Isotopic and Genomic composition of Human Gallstones. 28th International Meeting on Organic Geochemistry (IMOG), Florence, Italy, Sep 2017, poster presentation.

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Chapter 1: Introduction

1.1 Overview

Gallstone disease or Cholelithiasis from the Greek *chole* = bile and *lithos* = stone is defined as '…a small, hard crystalline mass formed abnormally in the gallbladder or bile ducts from bile pigments, cholesterol, and calcium salts.' (Goad and Akihisa, 1997, p.1-3; Stevenson, 2010, p. 718).

In this chapter, a general overview of the anatomy of the human digestive system, in which gallstone disease pathology is directly linked, will be discussed. This will begin with the GI tract, and progress to the accessory digestive organs where particular focus will be given to the liver, gallbladder, pancreas and associated bile ducts that are central to bile secretion and transportation. Subsequently, a literature review comprising disease pathophysiology, diagnosis and treatment options, traditional risk factors, and previous interdisciplinary research in the area will be explored. Finally, the aims of this thesis will be outlined, followed by the methodology, approaches and new technology that was utilised in this research in an attempt to answer some of the most pressing questions that have arisen in this research area over past three decades.

1.2 Anatomy

1.2.1 The GI Tract

The gallbladder, where the majority of gallstones are formed, is a part of the greater human digestive system which comprises the gastrointestinal (GI) tract and the accessory digestive organs (Tortora and Derrickson, 2009, p. 921-949). The gastrointestinal tract (or *alimentary canal*) comprises a continuous tube that extends from the mouth to the anus and is approximately 7-9 meters long (Tortora & Derrickson, 2009, p. 921-949; Van De Graaf, 2000). The GI tract is seen to be the most exposed section, per surface area, of the human body to the outside environment, and includes the major digestive organs of the mouth, pharynx, oesophagus, stomach, small intestine, and large intestine (Betts et al., 2017). The accessory digestive organs are the teeth, tongue, salivary glands, liver, gallbladder and pancreas (Betts et al., 2017).

The major function of the GI tract and accessory organs is: ingestion, secretion, mixing, propulsion, digestion (which includes mechanical and chemical digestion), absorption and defecation (Tortora & Derrickson, 2009, p. 921-949).
The physiology of the GI tract from the oesophagus to the anal canal comprises, with slight variation, the same four structural and functional layers termed (from internal to external) the mucosa, submucosa, muscularis, and serosa (Betts et al., 2017; Figure 1.1).

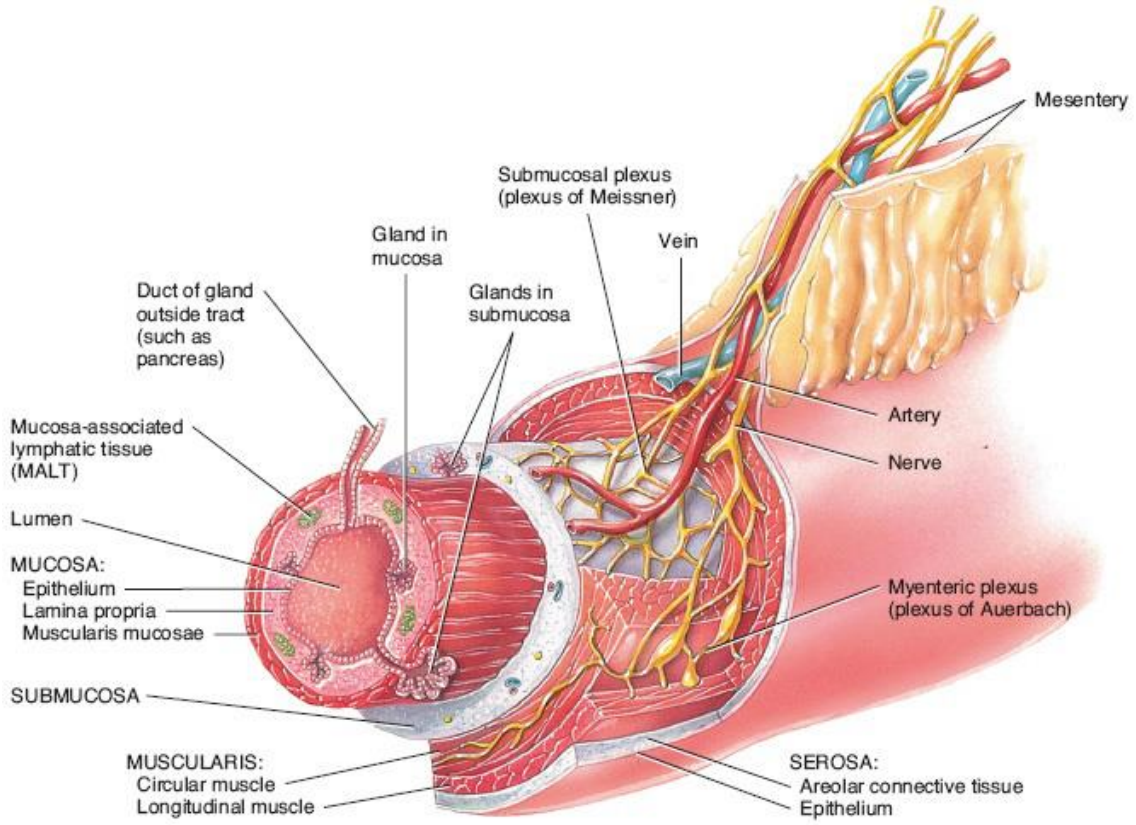

Figure 1.1 The four layers of the GI tract; Mucosa, Submucosa, Muscularis and Serosa (Tortora and Derrickson, 2009).

These four layers are lined with a framework of cells that are vital to the digestive process. These include epithelium cells that perform absorption and secretion functions, enteroendocrine cells that secrete various hormones to aid in the digestive process, exocrine cells that secrete mucus and fluid into the lumen (G.I cavity) for lubrication, immune cells that provide protection against disease, and associated blood and lymphatic vessels that provide routes for nutrients absorbed in the GI tract to reach various tissues in the body (Betts et al., 2017). Further, a neural network comprising approximately 100 million neurons extends throughout the tract. These include motor neurons, which control the involuntary muscle movement or *peristalsis* that aids in the breakdown, mixing, and propulsion of digested food throughout the tract, and sensory neurons acting as stretch receptors and chemoreceptors. Chemoreceptors are activated when certain food molecules are present in a given GI tract organ and require particular chemical secretions to aid in its digestion or absorption (Tortora & Derrickson, 2009, p. 921-949).

Ingestion involves taking food and drink into the mouth where it is masticated by teeth. The process of secretion involves cells within the GI tract, and accessory organs, secreting a daily supply of approximately 7 litres of water, acid, buffers, and enzymes into the lumen (Betts et al., 2017; Tortora & Derrickson, 2009, p. 921-949). The mixing and propulsion function constitutes digested food being pushed through the GI tract and accessory organs by peristalsis. This process is part of the function behind mechanical digestion, in which food is broken down by mastication, swallowed, and then further broken down mechanically by the involuntary movement of smooth muscles in the stomach and small intestine. Chemical digestion begins in the mouth, where salivary glands produce saliva, a solution that is composed of 99.5% water and 0.5% solutes. The solutes contain sodium, potassium, chloride, bicarbonate, phosphate ions, urea, uric acid, mucus, immunoglobulin A, lysozyme and salivary amylase (Betts et al., 2017; Tortora & Derrickson, 2009, p. 921-949). This composition enables the partial digestion and decontamination of the food being ingested. Saliva, alongside the voluntary skeletal muscle motion of the tongue, produces a smooth partially digested ball of food called a *bolus* (Betts et al., 2017). The bolus is then swallowed and pushed down the oesophagus to the stomach where it mixes with gastric juices. Gastric juice is composed of HCl to kill bacteria and denature protein, pepsin for protein digestion, intrinsic factor for absorption of B_{12} and gastric lipase which aids but does not completely digest triglycerides. Together these actions form a liquid called *chyme* in the stomach, which is deposited into the duodenum of the small intestine for further chemical digestion and absorption (Betts et al., 2017). The final aim of mechanical and chemical digestion is to break down large food molecules such as carbohydrates, lipids, and proteins into glucose, fatty acids/glycerol, and amino acids respectively for the body's use (Sears, 1992). The liver, gallbladder and pancreas are vital to this process.

1.2.2 The Liver, Gallbladder and Pancreas

The Liver

The liver is located in the upper right quadrant of the abdomen where it is connected to the gallbladder and pancreas via a system of biliary ducts or biliary tree (Figure 1.2). It is subdivided into left and right lobes, where hepatocytes, the major functional cells of the liver, comprise approximately 80% of its volume (Springhouse, 2002; Betts et al., 2017; Figure 1.2). Bile canaliculi (small canals), are interspersed in the grooves between hepatocyte cells, which merge into the larger right and left hepatic ducts. The right and left hepatic ducts combine to form the common bile duct extending to the duodenum (small intestine), with this connection point termed the ampullar of Vater, and the opening and closure point termed the sphincter of Oddi (Springhouse, 2002; Tortora & Derrickson, 2009, p. 921-949).

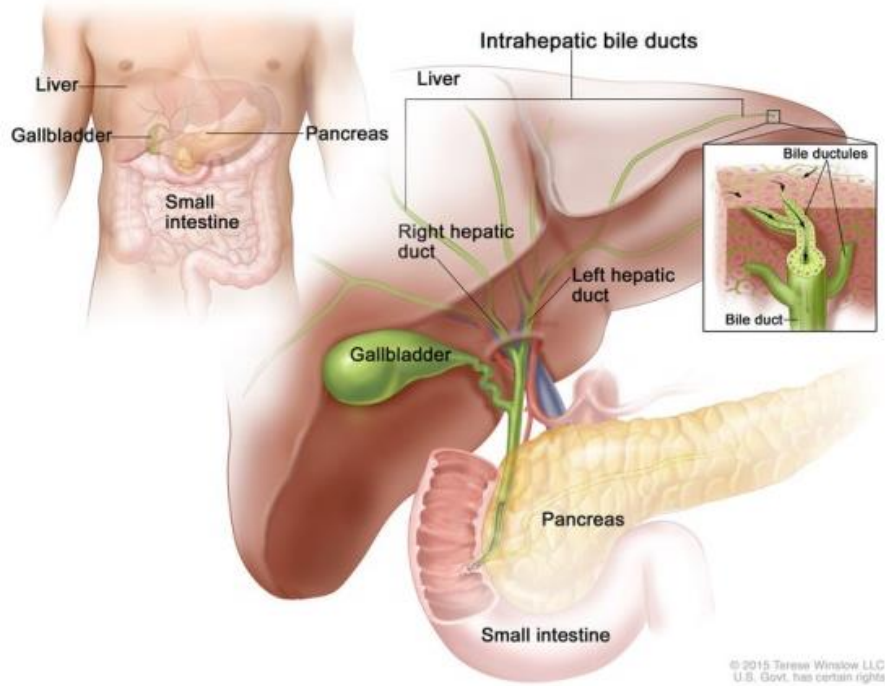

Figure 1.2. Overview of the Liver, Gallbladder, Pancreas, and Intrahepatic bile duct anatomy (obtained with permission from Terese Winslow LLC, for the National Cancer Institute © 2018).

The gallbladder is connected to the common bile duct via the cystic duct, with the pancreas connecting to the common bile duct via the accessory and pancreatic ducts (Figure 1.5). Hepatocytes have a number of vital functions in the liver that include carbohydrate metabolism, lipid metabolism, protein metabolism, processing of drugs and hormones, excretion of bilirubin by phagocytosis (recycling of aged red blood cells), and activation of vitamin D (Springhouse, 2002; Tortora & Derrickson, 2009, p. 921-949). One of the most important processes is lipid metabolism, which includes the synthesis of cholesterol and lipoproteins. Lipoproteins are proteins that have a core containing cholesterol esters and triglycerides, with a shell of non-esterised cholesterol, phospholipids and apolipoproteins (Feingold et al., 2018). The inner non-polar region is able to attach to fatty acids, triglycerides and cholesterol particles, whilst the outer polar region is able to solubilise in blood plasma (Feingold et al., 2018). Low-density lipoproteins (LDLs) are used to transport cholesterol to various sites in the body, whilst High-density lipoproteins (HDL) are used to transport unused cholesterol back to the liver to be degraded into bile acids or excretion into feces (Brown, 2000). The liver is the primary source of cholesterol in the human body, with dietary cholesterol accounting for approximately 25% (Blesso & Fernandez, 2018). Cholesterol is fundamental to the regulation of cell membranes in mammalian cells and is a precursor for bile and the synthesis of steroid hormones, such as progesterone, oestrogen, cortisol and testosterone (Berg et al., 2002; Cruz et al., 2013). Bile is essential for the digestion of triglycerides into smaller lipid globules and prepares these lipids for further digestion by pancreatic enzymes in the small intestine (Berg et al., 2002). Hepatocytes secrete bile into bile ductiles for transport throughout the biliary tree (Figure 1.2). A summary of cholesterol and bile synthesis is presented below.

Cholesterol Structure & Synthesis

Cholesterol ($C_{27}H_{45}OH$ or cholest-5-en-3β-ol) is a steroid with the characteristic tetracyclic hydrocarbon ring system of the steroid group (Brown, 2000, 483-490; Figure 1.3). It is also termed a sterol as it is an unsaponifiable steroid alcohol containing a hydroxyl group at C-3 on one end, and an aliphatic side chain of 8 hydrocarbons at C-17 on the other. The ring system has a double bond at C-5 and C-6, with two methyl groups at C-10 and C-13 (Myant, 1981, 1-51; Brown, 2000, 483-

490). The hydroxyl group, methyl groups (positioned above the plane of rings), the side chain at C-17, and the hydrogen atom at C-8 are in the β-configuration (Myant, 1981, 32; Brown, 2000, 483-490). The hydrogen atoms at C-9 and C-14 are at α configuration, with an *R* configuration at C-20 (Myant, 1981, 30-32). This stereochemistry confers the ring system a rigid planar structure, and flexibility in the side chain, allowing for the structural integrity that can be seen in cell membranes (Brown, 2000, 483-490).

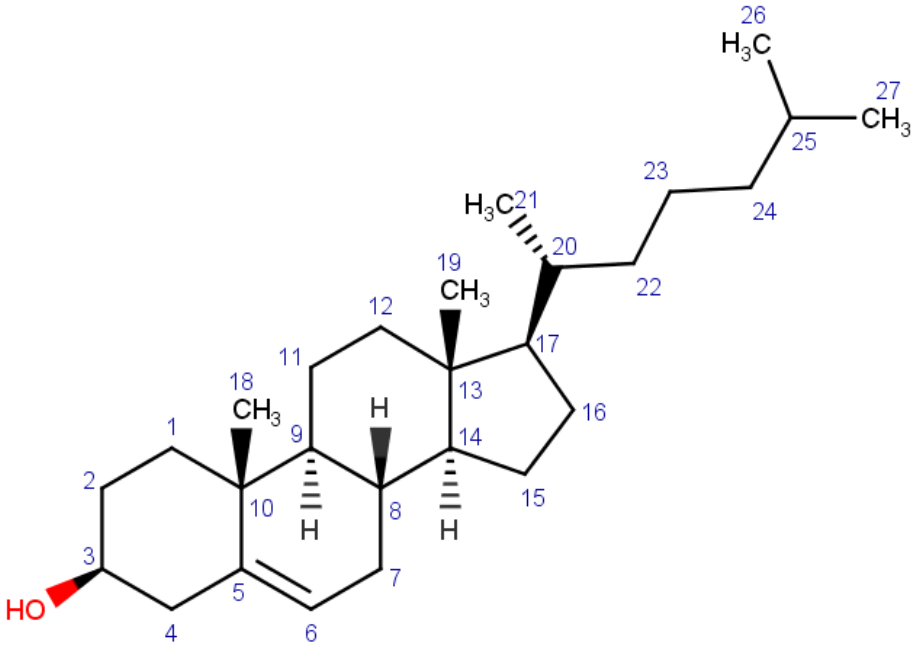

Figure 1.3 Molecular structure of cholesterol denoting α (dashed line) and β (bold line) stereochemistry (modified from Hardinger, 2017).

The ring system and side chain are non-polar, with the polar hydroxyl group end giving it only a moderate amount of solubility in aqueous solutions (Brown, 2000, 483-490). This is the primary reason lipoproteins are required to transport cholesterol throughout the body as it is insoluble in blood plasma (Brown, 2000, 483-490).

As the primary precursor for essential hormonal and digestive steroids, cholesterol is known as the parent steroid, and as such, its biosynthetic pathway is both highly regulated and selective (Brown, 2000). It involves approximately 30 different reactions and over a dozen enzymatic processes obtained from a variety of subcellular regions (Alphonse et al., 2016). The pathway can be summarised in to 5 main stages: (1) mevolonate synthesis, (2) isopentenyl phosphate synthesis, (3)

squalene formation, (4) lanosterol synthesis and finally (5) cholest-5-en-3β-ol synthesis. Biosynthesis takes place in hepatic cells and begins with the catalyzation of a single acetyl-CoA and acetoacetyl-CoA molecule to form 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) (Waterman, 2006; Alphonse et al., 2016). HMG-CoA is then reduced by HMG-CoA reductase via NADPH (utilised as a co-factor for all reduction reactions in this pathway) to form mevalonate (Waterman, 2006; Alphonse et al., 2016). This reaction is considered the principle regulatory pathway for cholesterol synthesis and is subsequently targeted by lipid lowering drugs, such as statins (Alphonse et al., 2016). Mevalonate is first phosphorylated by a number of kinases and then decarboxylated to form the isoprenoid, isopentenyl pyrophosphate (IPP). IPP undergoes a number of condensation reactions that are catalyzed by squalene synthase, forming squalene (Waterman, 2006; Alphonse et al., 2016). The cyclization of squalene leads to lanosterol synthesis, with a further series of enzymatic reactions leading to the synthesis of cholest-5-en-3β-ol (Waterman, 2006).

Bile Acid Structure & Synthesis

Cholesterol is converted into bile acids via two major synthesis pathways that begin in liver hepatocytes. Although the process is complex and involves at least 17 enzymes, the overall aim is the synthesis of powerful surfactants by the hydroxylation of the cholesterol ring system and the carboxylation of its side chain (Figure 1.4; Chiang, 2009; Eggert et al., 2014). The classical pathway is initiated by the oxysterol enzyme 7α-hydroxylase, and alongside the sterols 12α-hydroxylase and 27-hydroxylase, produce the primary bile acid cholic acid (3α, 7α, 12α-trihydroxy-cholanoic acid). The second (acidic) pathway, limits the steroid ring hydroxylation to two hydroxyl groups (making the compound more acidic) and is initiated by the oxysterol 27-hydroxylase; producing the second primary bile acid chenodeoxycholic acid (3α, 7α-dihydroxy-cholanoic acid) (Figure 1.4; Chiang, 2009; Kwong et al., 2015).

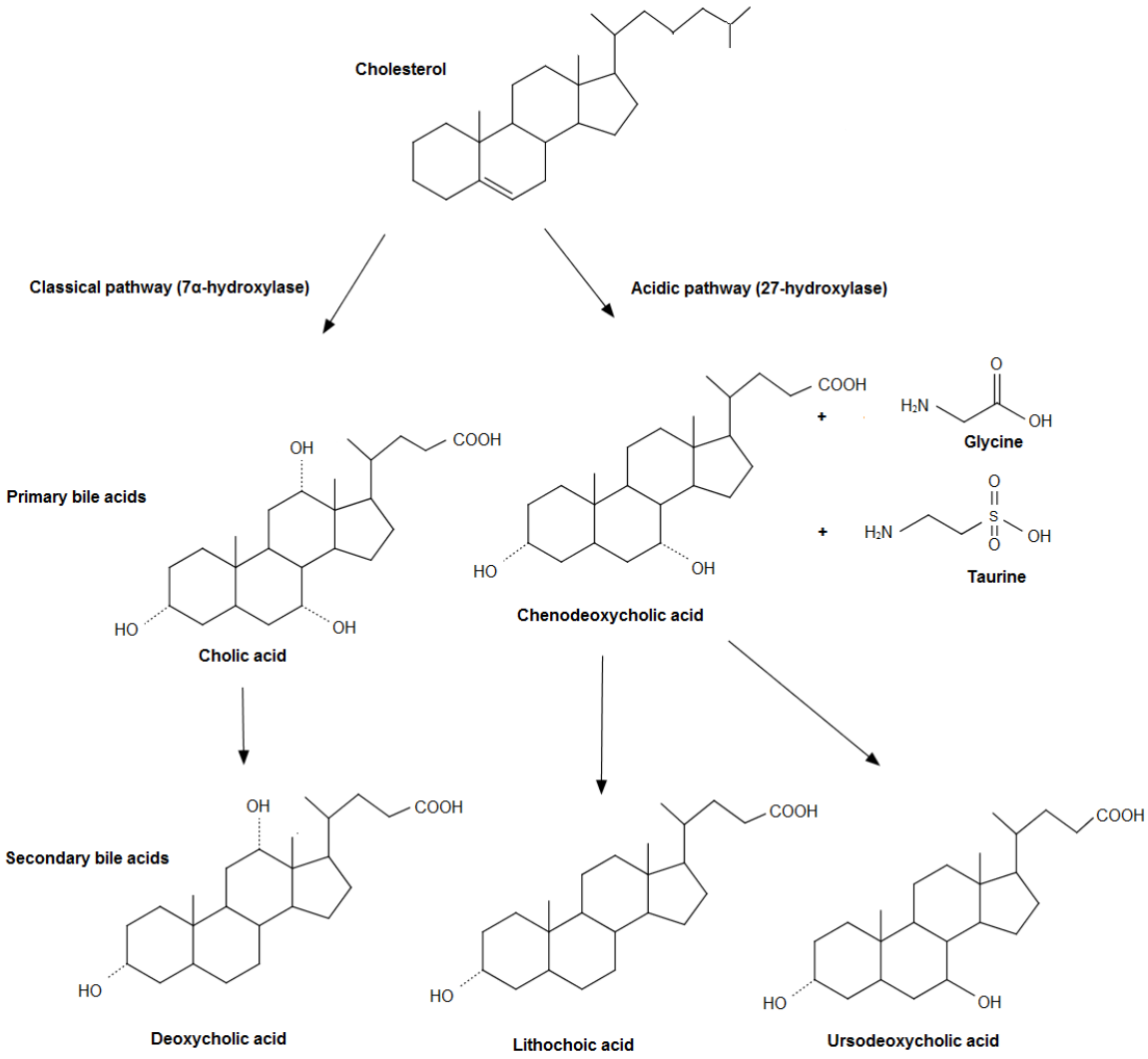

Figure 1.4 The structure and major biosynthetic pathways of the primary and secondary bile acids (modified from Fehrer, 2017).

The terminal step before release into bile canaliculi is the conjugation of bile acids into glychocholic and taurocholic acid (also termed bile salts). This addition of amide groups to the carboxyl group equally decreases toxicity and increases the solubility of the primary bile acids (Chiang, 2009). The metabolism by anaerobic bacteria in the intestines further removes hydroxyl groups from the primary bile acids, and produces the secondary bile acids deoxycholic (3α, 12-dihydroxy) and lithocholic acid (3α-monohydroxy); with small amounts of ursodeoxycholic acid (Figure 1.4; Chiang, 2009; Kwong et al., 2015; Eggert et al., 2014). The average adult liver synthesises approximately 500 mg of cholesterol into bile acids per day (Russell,

2003). Dependent on whether cholesterol levels are high or low, a strict regulatory system ensures lipid homeostasis by decreasing or increasing production of bile acids respectively (Russell, 2003). Bile acids are then stored in the gallbladder and secreted into the duodenum to aid in the digestion, absorption and transportation of lipids and nutrients (Chiang, 2009).

The Gallbladder

The gallbladder is a small (approx. 7-10 cm in length) pear shaped organ that sits under the liver and is joined to it via the cystic duct (Figure 1.5; Springhouse, 2002). The main role of the gallbladder is to concentrate and store bile, a lipid emulsification and digestive agent, until it is needed in the duodenum as the stomach lacks the ability to digest large lipid molecules, such as triglycerides (Springhouse, 2002; Betts et al., 2017).

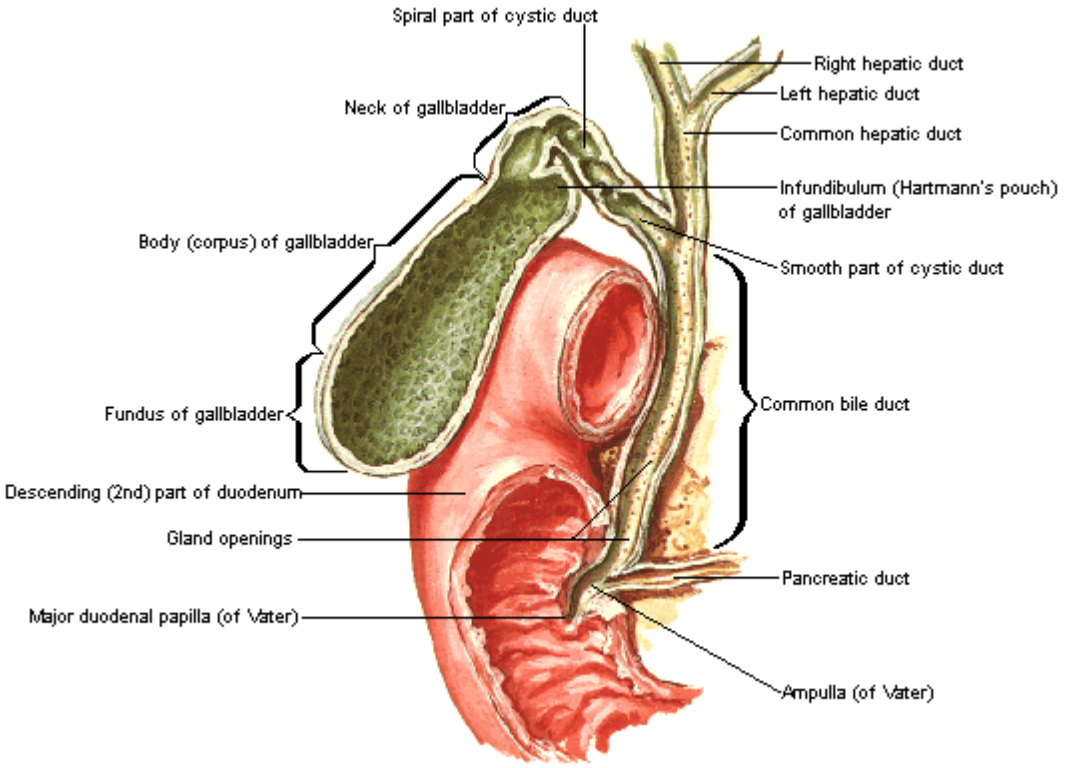

Figure 1.5 The major anatomical features of the human gallbladder and associated biliary ducts (modified from CSSA, 2018).

Bile, an alkaline (pH 7.6-8.6) brownish green liquid, is composed of potassium and sodium salts, bile acids (chenodeoxycholic acid, cholic acid), water, phospholipid lecithin, bile pigments bilirubin and biliverdin, electrolyte ions and cholesterol (Springhouse, 2002; Betts et al., 2017).

Bile is concentrated 10-fold in the gallbladder and can contain heavy metals and drugs, including antibiotics, which the liver secretes into bile as part of its detoxification processes (Springhouse, 2002; Tortora & Derrickson, 2009, p. 921-949). The gallbladder anatomy includes the fundus (broadest section), the corpus (central section), the infundibulum or Hartmann's pouch (above central section) and the neck (tapered section) (Figure 1.5; Betts et al., 2017). Its exterior is composed of visceral peritoneum – a layer of protective cells, while its interior is a layer of mucosa that lacks a submucosa (Springhouse, 2002; Tortora & Derrickson, 2009, p. 921-949). The smooth muscle of the mucosa contracts via peristalsis to eject bile into the cystic duct when hormones and chemical receptors signal deposition of chyme or fats into the duodenum. The liver secretes bile continuously at a rate of approximately 800-1000 mL per day with secretion increased during meals. During periods of fasting, the sphincter of Oddi remains closed, and bile flows into the gallbladder to be stored until the next round of digestion occurs (Springhouse, 2002; Betts et al., 2017).

The Pancreas

The pancreas is an elongated organ, approximately 12-15 cm long that lies beneath the liver and to the left of the duodenum of the small intestine (Figure 1.2). Its primary function is the secretion of insulin and glucagon for blood sugar regulation, and pancreatic juice for digestion (Springhouse, 2002; Betts et al., 2017). The major functional cells of the pancreas, termed acini, secrete approximately 1 litre of pancreatic juice into ducts that merge with the primary pancreatic duct (Springhouse, 2002; Tortora & Derrickson, 2009, p. 921-949). The pancreatic duct runs the length of the pancreas and connects to the common bile duct at the ampulla of Vater (Figure 1.5). Pancreatic juice is composed of water, salts, several enzymes, sodium bicarbonate and pancreatic lipase – the principle enzyme that digests triglycerides (Springhouse, 2002; Betts et al., 2017). The emulsification actions of bile reduce

large lipid molecules into smaller fat globules thereby increasing their surface area (Springhouse, 2002). This increased surface area allows pancreatic juice to further reduce these globules into fatty acids and glycerol for absorption by the small intestine for the body's use (Springhouse, 2002; Betts et al., 2017).

1.3 Pathophysiology, Diagnosis and Treatment

Gallstones are said to form when the ratio of cholesterol outweighs that of emulsification agents in bile (Figure 1.6). The optimum ratio to keep cholesterol from crystallising, and bile contents in micellar form, is said to be approximately 80% bile salts/acids, 15% phospholipid lecithin, and 5% cholesterol (Marked *P* in Figure 1.6). An imbalanced ratio is attributed to hypersecretion of cholesterol by the liver, diminished synthesis of bile salts and acids, and increased low density lipoprotein (LDL) cholesterol levels in the blood (Stinton & Shaffer, 2012; Blesso & Fernandez, 2018).

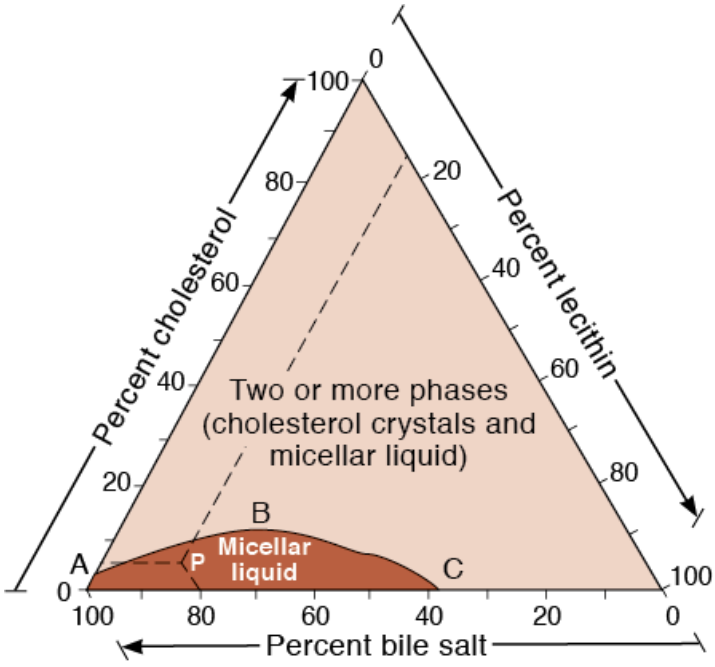

Figure 1.6 Solubility triangle depicting the major phases of cholesterol in a mixture containing Phospholipid (Lecithin) and Bile Salts. *P* indicates the ideal state in which cholesterol crystal precipitation is significantly limited (modified from Barrett et al., 2016).

Hypersecretion of cholesterol in the liver has been associated with genetics,

prescription drugs, and the female sex hormones oestrogen and progesterone (Stinton & Shaffer, 2012). Increased cholesterol in the blood (hypertriglyceridemia) has been linked to diets that are rich in triglycerides and LDL cholesterol, but low in beneficial high-density lipoprotein (HDL) cholesterol (Stinton & Shaffer, 2012; Blesso & Fernandez, 2018). These factors, in particular progestin hormones, have also been attributed to the impairment of gallbladder motility or emptying (stasis) (Lee et al., 2015). Gallbladder stasis can also occur with overuse (hypermotility) and the impairment of the sphincter of Oddi (Afamefuna & Allen, 2013). Stasis, and subsequent accumulation of concentrated cholesterol-rich bile, is also said to allow cholesterol crystals to aggregate over time to form macroscopic stones (Afamefuna & Allen, 2013). Gallstones are generally classified into four major types according to the ratio of cholesterol versus bilirubinate content in their matrix, and are designated cholesterol stones (≥70% cholesterol, ≤30% bilirubinate), brown pigmented stones (≤30% cholesterol, ≥70% bilirubinate), black pigmented stones (≥ 90% or purely bilirubinate), and mixed stones (between ≥30-70% cholesterol) (Bouchier, 1992; Qiao, 2013). Asymptomatic 'silent stones' account for the majority of presentations, with 1-4% of patients going on to experience symptomatic or acute Cholelithiasis (Lee et al., 2015). Although gallstone disease predominantly presents in the gallbladder, it is not restricted to it, and the term is used concurrently for a number of related conditions (Afamefuna & Allen, 2013). Biliary colic is an acute condition that can be re-occurring and presents as a sharp pain in the upper right quadrant of the abdomen due to the temporary blocking of the cystic duct by a gallstone (Lee et al., 2015). Vomiting, nausea and intolerance to fatty foods is said to follow the pain with most episodes managed by analgesics (Beckingham, 2001). If blockage of the cystic duct and pain persists, the result is inflammation of the gallbladder or Acute cholecystitis (Beckingham, 2001). Acute cholecystitis is accompanied by both upper abdominal pain and mild fever requiring hospitalisation (Beckingham, 2001). An associated condition is Mirizzi's syndrome, where the common bile duct is obstructed through compression due to a lodged stone in the neck or cystic duct of the gallbladder. The compression usually leads to cholangitis (inflammation of the biliary ducts) further abdominal pain, fever and jaundice (Lee et al., 2015). Porcelain gallbladder syndrome involves the calcification of the gallbladder wall and is considered to be a precursor for gallbladder cancer (Crawford, 2013). Gallbladder

polyps are also a concerning condition associated with gallstone disease. Gallbladder polyps are mostly a lesion of aggregated cholesterol crystals attached to the wall of the gallbladder that vary in size but can also become adenomatous polyps –a precursor for gallbladder cancer (Crawford et al., 2013). Choledocholithiasis is a condition in which gallstones migrate into or develop within the common bile duct. Acute cholangitis occurs when pathogenic bacteria, namely from the duodenum, infect the obstructed bile duct causing septicaemia; with urgent biliary decompression and antibiotics treatment required as a result (Beckingham, 2001). Gallstone disease can also affect the normal functioning of the Pancreas. Acute pancreatitis is a condition with a relatively high mortality rate (10%) and occurs in approximately 5% of patients with gallstone disease (Beckingham, 2001). It is caused by gallstones migrating to the ampulla of Vater in the duodenum, thereby obstructing the pancreatic duct, and causing a reflux of pancreatic fluid or bile back into the pancreatic duct (Beckingham, 2001). The resultant reflux of fluids, and build-up of pancreatic secretions, can cause inflammation and complete impairment of the organ (Lee et al., 2015; Beckingham, 2001). Symptoms are similar to associated gallstone disease conditions and include severe abdominal and back pain, nausea and vomiting requiring emergency hospitalisation (Choi & Silverman, 2018). These conditions may also cause biliary dyskinesia, the impaired functioning of the sphincter of Oddi, causing either hypersecretion, hyposecretion or compaction of bile and pancreatic fluids (Afamefuna & Allen, 2013; Choi & Silverman, 2018).

At present, gallstone disease treatment is centred on the management of the condition, as a universal non-invasive treatment regime which preserves the gallbladder and eliminates the underlying cause of stone formation is yet to be found (Demehri FR, Alam 2016). Initial screening is done via ultrasound, endoscopic ultrasound, computed tomography (CT) Scan, endoscopic retrograde cholangiopancreatography (ERCP), and magnetic resonance cholangiopancreatography (MRCP). Ultrasound is used to detect stones in the gallbladder, liver, pancreas and the biliary tree using soundwaves. It is considered highly effective, safe, and presently the first pass option when patients present with typical symptoms of abdominal pain, nausea and vomiting (Choi & Silverman, 2018). Endoscopic ultrasound is an endoscopic device, with an ultrasound probe attached to it, used to enter the intestines and obtain internal images of the

gallbladder, liver and pancreas. It is most effective when ultrasound imaging fails to detect stones in bile ducts, and in the screening of cancers in the biliary tree and pancreas (Choi & Silverman, 2018). CT scanning is less effective at detecting gallstones than ultrasound but has shown to be essential for resolving acute pancreatitis (Choi & Silverman, 2018). ERCP involves a thinner endoscope which can be inserted into the biliary tree and allows for the removal of gallstones therein, including the measurement of pressure within the sphincter of Oddi to diagnose biliary dyskinesia (Choi & Silverman, 2018). However, ERCP cannot remove gallstones for the gallbladder itself and requires a separate surgical procedure. MRCP is a non-invasive technique that uses magnetic resonance imaging to obtain images of the biliary tree with a similar resolution to ERCP. It is often conducted before therapy is performed by ERCP (Choi & Silverman, 2018).

Once gallstone disease is identified, a number of both invasive and non-invasive management options can be applied. Treatment is dependent on the severity of the condition and patient health. For patients with symptomatic presentations, the most common procedure is the removal of the gallbladder by key-hole or laparoscopic cholecystectomy. Laparoscopic cholecystectomy supersedes open cholecystectomy (exposing the upper right quadrant of the abdomen), and was first introduced in 1987, seeing continual refinement over the last 20 years (Crawford, 2013; Beckingham, 2001). It involves making three small incisions in the abdomen and the use of laparoscopes, fibre-optic instruments with a camera similar to endoscopes, to provide live images whilst small surgical tools are used to remove both gallstones and the gallbladder (Swanstrom & Soper, 2013, pp. 341-348). It is preferred over open cholecystectomy due to its low mortality rate (0.1% compared to 0.5% for open), reduced hospital stay (day procedure with an overnight stay), less pain, less scarring, and faster recovery time (within 10 days) (Beckingham, 2001). Although the success rate of the operation is uniformly high, especially in relation to open cholecystectomy, it is not without short comings. There is the possibility of post-surgery complications, such as bile leak, bile duct injury, Sphincter of Oddi dysfunction, dyspepsia, diarrhoea, bile reflux gastritis, and stone recurrence in the biliary tree (Beckingham, 2001; Crawford, 2013). Some reports have also reported an increased risk of colonic cancer in post-surgery patients (Zhang et al., 2016).

In recent years, research has shown that the gallbladder is vital for bile flow regulation, prompting attempts to develop a method to keep the organ intact and manage stone recurrence as it arises (Zhang et al., 2016). A relatively new technique termed Endoscopic-laparoscopic cholecystolithotomy (ELC) is leading efforts to achieve this outcome. It involves scooping gallstones out of the gallbladder, rather than the removal of the organ, and has shown to lower post-surgical digestive symptoms, recovery time, and stone recurrence compared to that of laparoscopic cholecystectomy (Zhang et al., 2016). Although increasing in popularity in Asia, it has yet to be adopted in western populations due to its relative infancy (Zhang et al., 2016). Types of non-invasive treatments include oral dissolution therapy (chenodeoxycholic, ursodeoxycholic acid), and shockwave lithotripsy (fragmenting stones with high-energy sound waves) (Beckingham et al., 2001). These treatments, however, are only suitable for less than 10% of patients who have mild symptoms, fewer than 4 stones of less than 20 mm diameter, and a normal functioning gallbladder. Success rates of the treatments also vary from patient to patient and can become too costly for patients who require lifelong treatment for recurrent stones (Beckingham et al., 2001).

1.4 Risk factors

Gender

The prevalence of gallstones is greater in females of reproductive age compared to those of males, with these rates equalising with increasing age (Stinton & Shaffer, 2012). The higher rate in females has been linked to the female sex hormones oestrogen and progesterone (Ko, 2006; Stinton & Shaffer, 2012). These hormones have been shown to influence cholesterol hypersecretion, reduce bile salts, and interfere with gallbladder motility and emptying (Ko, 2006; Stinton & Shaffer, 2012). These factors are exacerbated during pregnancy, as oestrogen and progesterone are significantly elevated, leading to an increase in biliary sludge (cholesterol, calcium bilirubinate, mucin gel) in the gallbladder (Stinton & Shaffer,

2012). This sludge is shown to occur in 5-30% of women during pregnancy, while approximately 5% go on to have definitive gallstones (Stinton & Shaffer, 2012). Oral contraceptives, hormonal imbalance, and hormone replacement therapy are also recognised risk factors in women (Ko, 2006; Stinton & Shaffer, 2012). In both genders, exogenous oestrogen sources have been shown to increase the risk of Cholelithiasis (Greer et al., 2018). These include certain prescribed medications (e.g. statins, synthetic steroids) and alcohol, which can have the effect of lowering testosterone and promoting its conversion into oestrogen (Schooling, 2013; Purohit, 2000).

Age

Increasing age is an established risk factor in gallstone disease, regardless of gender and ethnicity, becoming 4-10 times more likely in individuals over the age of 40 (Lee et al., 2015; Stinton & Shaffer, 2012). Although data is limited, and requires further verification, recent reports have also identified an increased risk of gallstone disease in children (up to the age of 10) with obesity, prematurity, and haemolytic disease (Greer et al., 2018). Early puberty is also considered alongside these factors for teenagers (Greer et al., 2018). Increased reports of the disease in children may also be due to the more frequent use of ultrasound when diagnosing other conditions; highlighting the need for further work in this area for a more accurate picture of Cholelithiasis prevalence in children (Greer et al., 2018).

Obesity, Weight loss, and Diet

Obesity, or a body mass index (BMI) greater than 25.0 kg/m^2, is considered a well-established risk factor for gallstone disease (Stinton & Shaffer, 2012). It is said to affect the cholesterol synthesis regulating enzyme 3-hydroxyl-3methyl-glutaryl co-enzyme A (HMG-CoA) in the liver, leading to hypersecretion of cholesterol into bile (Stinton & Shaffer, 2012). The increase of obesity in both developed and developing nations has also been linked to the increase of gallstone prevalence in recent decades (Stinton & Shaffer, 2012). Rapid weight loss at a rate of >1.5kg per week, in particular after obesity related surgery, is associated with risk of gallstone formation.

A low caloric diet or a history of dieting (whether moderate or extreme) can also be a disease precursor (Stinton & Shaffer, 2012). There is a general consensus that a diet high in cholesterol, carbohydrates, fatty acids, and legumes may increase chances of developing gallstones, whilst unsaturated fats, coffee, fibre, Vitamin C, calcium and moderate alcohol can decrease the risk (Stinton & Shaffer, 2012). However, recent research has shown diet to be a difficult area to analyse due the complexity of the disease and inconsistency of results, requiring further investigation (Stinton & Shaffer, 2012). In regards to exercise, increased activity of approximately 30 mins a day is considered a protective measure, with reduced activity increasing the risk of stone formation (Shaffer, 2006).

Ethnicity

The highest incident rates of gallstone disease have been reported in North American Indian populations (64.1% of women, 29.5% of men), and the aboriginal populations of South America (49.4% of women, 12.6% of men) (Stinton & Shaffer, 2012). High incident rates are also reported for female Mexican Americans (27%) with Mexican American men reported to have the equivalent rate of White American males (8.6%) (Everhart et al., 1999). Occurrence in White American women is reported to be 16.6%, whilst prevalence in Black Americans is said to be at 13.9% for women, and 5.3% for men. In Europe, occurrence rates are intermediate at 10-15% (females at the higher percentage range), with increased rates reported in Northern Europeans (Norway, Sweden, Denmark, Poland) at 11-30%. The occurrence rate in India is reported at 10-22%, with lower rates reported in Asia (Thailand, China, Taiwan, Japan) at 4-12%. An increase from 5% to 10% in Japan is associated with the introduction of a Western Diet and 4-10 times higher as both genders go beyond the age of 40 (Stinton & Shaffer, 2012).

From what data is available in the Pacific, the rates are relatively high (29%) with the Moari populations of Easter Island. The lowest rate of gallstone disease is reported for Sub-Saharan Black Africans at <5% (Stinton & Shaffer, 2012). In Australia, the trend is equivalent to western European nations at 10-15% for those under the age of 50, with an increase to 25-30% reported for both genders over the age of 50 (Australian Commission on Safety and Quality in Health Care, 2017). Recent studies

have reported an overall trend of increasing prevalence of gallstone disease in western populations (Stinton & Shaffer, 2012). There is also a trend in the types of gallstones per nation. Western populations such as the U.S, U.K, Canada, and Australia present with a predominance of (approx. 80%) cholesterol stones, while Asian populations predominantly present with pigmented type stones (Lee et al., 2015). In recent decades, however, there has been a rise in cholesterol type stones in Asian populations attributed to a transition to a more westernised diet (Lee et al., 2015).

Genetics and family history

There is yet to be a specific gene or group of genes identified that is a definitive indicator for gallstone disease. Instead, a number of variant genes associated with cholesterol synthesis and secretion have been identified that may predispose individuals to the disease. These are the apolipoproteins E and B (APOE, APOB), the cholesterol ester transporting protein (CETP), cholecystokinin receptor A (CCKAR), LDL receptor (LDLR), hepatic secretion genes ABCG8 19H and ABCB4, and cholesterol 7 α-hydroxylase (Stinton & Shaffer, 2012). Familial history studies indicate close to 5 times the risk of developing gallstones in relatives of patients with the condition. Twins are at higher risk with a rate of 6-12% if one of them presents with the disease. However, an overall pattern of inheritance is yet to be identified and does not account for all cases. Associated genes and familial history only account for approximately 11-30% of total presentations, indicating the complex nature of the disease which most likely involves a host of both environmental and genetic factors (Shaffer, 2006; Stinton & Shaffer, 2012).

Related conditions

Conditions that are associated with and put individuals at risk to gallstone disease are sickle cell disease, haemolytic anaemia, liver cirrhosis, Crohn's disease, cystic fibrosis, and metabolic syndrome (Stinton & Shaffer, 2012). Sickle cell disease and haemolytic anaemia relate to the irregular or abnormal production and increased breakdown of red blood cells in the body. Increased bilirubin secretion is one of the symptoms of these conditions, predisposing patients to the development of black

pigmented stones (Stinton & Shaffer, 2012). Pigmented stones are also associated with liver cirrhosis (damaged liver cells), cystic fibrosis (mucus trapped in lung air passages), and Chrohn's disease (inflammatory bowel disease), with each shown to lead to excessive bilirubin secretion (Stinton & Shaffer, 2012). The metabolic syndrome is a clustering of conditions that collectively have shown to not only increase risk of type 2 diabetes, stroke and heart disease, but also gallstone disease (Shaffer, 2006; Chew et al., 2006). It includes abdominal obesity, high blood sugar, high levels of LDL cholesterol, elevated blood pressure and insulin resistance (Chew et al., 2006).

1.5 Previous research

Although the central theory of gallstone nucleation is that phase separation of cholesterol crystals from supersaturated bile is key, and that a number of physiological risk factors influence this, a definitive model of growth for all gallstone types remains to be found (Stewart et al., 2002; Van Epercum, 2011). Indeed, even the most modest attempts at ascertaining conclusive compositional classifications of the four major stone types is met with confounding and often contradictory results when the vast number of these studies are compared (Sutor & Wooley, 1968; Bills & Lewis, 1975; Carey & Small, 1978; Carey, 1993; Strasbourg, 1998; Stewart et al., 2002 and references therein). For example, cholesterol stones were invariably seen to be composed of a calcium carbonate nidus formed by the active binding of Ca^{2+} cations to anionic bile agents, such as bilirubinate, phosphate and carbonate (Rege, 2002). Although this view persists today, it was shown as early as 1975 that the crystalline nature, orientation, distribution and method of formation of the calcium carbonate in cholesterol stones varied from stone to stone (Bills & Lewis, 1975; van den Berg, 2000). Some cholesterol stones were shown to have centres made entirely of monohydrate cholesterol crystals, with minimal amounts of calcium carbonate distributed radially in the stones' rings, whilst some stones were shown to have no calcium carbonate within their matrix at all (Bills & Lewis, 1975; van den Berg, 2000). Further, the precipitation of the three polymorphs of calcium carbonate - calcite, aragonite, and more rarely vaterite, were also shown to vary from stone to stone, with each assumed to precipitate separately via unique biliary and chemical

pathways (Bills & Lewis, 1975; Lee & Chen, 2009). However, reports of an unusual stone exhibiting all three polymorphs, an octahedral symmetrical structure, and no discernible nucleation or nidus point have also been seen (Sutor & Wooley, 1968). This incongruity is also evident in studies which focused solely on pigmented stones. In 1982, a consensus conference was held to codify stone types by colour, with most of these codifications and the understanding behind their appearance now considered obsolete (Stewart et al., 2002). Although these misconceptions persist today, it was assumed that black pigmented stones were predominant in Western populations, small (~ 3mm), sterile, and found almost exclusively in the gallbladder. Further, that they were composed primarily of Ca-bilirubinate, with some stones also exhibiting Ca-carbonate and Ca-phosphate (Stewart et al., 2002). Black stones were also associated with patients who had cirrhosis, and other haemolytic diseases, including alcoholism (Stewart et al., 2002). Conversely, brown pigmented stones were said to be predominant in Asian populations, of varying size, associated with infection, and invariably found in the common bile duct. Brown pigmented stone compositions were described as containing a combination of Ca-bilirubinate and Ca-palmitate, with lesser amounts of cholesterol (Stewart et al., 2002). It wasn't until the work of Maki (1966) and later Stewart et al. (1987; 2000; 2002) that these ideas were questioned, and the predominantly physico-chemical hypothesis of pigmented stone formation began to shift towards that of a biogenic model. Maki proposed that, regardless of the chemical make-up of bile constituents, the formation mechanism behind pigmented stones was in fact bacterially mediated (Maki, 1966). Bacterially produced ß-glucuronidase was seen to deconjugate bile acids, leading to the precipitation of calcium bilirubinate crystals, with these crystals further conjugated by an anionic glycoprotein (i.e. sodium alginate) leading to the agglomeration of the observed macroscopic stones (Maki, 1966). This was an intriguing idea at the time, as even in physico-chemical models, biliary 'sludge', which included mucin gel alongside common bile components, was considered a common pre-condition of the gallbladder before crystallisation events (Carey, 1993; Ko et al., 1999). However, Maki's findings were only applicable to bacteria that could produce ß-glucuronidase, such as *E.coli*, and did not account for the other bacterial species that were identified within pigmented stones (Stewart et al., 2000). The work by Stewart et al. (1987), furthered Maki's work by proposing that bacterial biofilms were more critical to

stone formation than ß-glucuronidase. The biofilm product glycocalyx, an anionic protein, was proposed as the central agglomerating factor (cementing Ca-palmitate and Ca-bilirubinate) in pigmented stone formation, and could explain the predominance of biofilm forming bacteria identified in a number of studies (Stewart et al., 1987, 2000, 2002; Cetta, 1991). Importantly, with the use of scanning electron microscopy, Fourier-transform Infrared spectroscopy (FTIR), and culture studies, Stewart et al. (1987; 2002) determined that most pigmented stones obtained from western patients, regardless of composition, morphology or colour, contained bacterial microcolonies that could produce biofilms. The identified bacteria were *Klebsiella*, *Enterococcus*, *Enterobacter*, *E. coli* and *Psuedomonas aeruginosa*, with *P. aeruginosa* the greatest biofilm producer of the group after *Citrobacter freundii* (Stewart et al., 2000). Similar studies corroborated these findings, but as per Maki and Stewart et al., they restricted bacterial mediation to pigmented stones alone, or those with pigmented centres, with little data obtained for cholesterol stones (Cetta,1991). This was largely due to the limitation of culture-based studies, in which only 1% of bacteria in complex environmental samples can be brought into culture providing a limited view of the total species present. It was assumed cholesterol stones were sterile, or contained only negligible concentrations of bacteria (Cetta, 1991). However, in recent decades, with the advent of new technology, such as NextGen sequencing, cholesterol stones can now be studied with instruments of greater sensitivity. Indeed, one recent 16S rRNA high-throughput profiling investigation of the microbial composition of 27 cholesterol gallstones, identified a predominance of *Enterobacteriaceae*, *Ruminococcoceae,* and to a lesser extent, *Bacteroidales, Lactococcus, Enterococcus and Clostridiales* within the analysed gallstones (Wu et al., 2013).

1.6 Methodology & Techniques

The following provides an overview of the approaches and techniques utilised in this thesis.

1.6.1 16S rRNA high-throughput profiling

The full body of knowledge concerning microorganisms in the past century was obtained by pure culture techniques, in which organisms were distinguished by their morphological or phenotypic characteristics (Clarridge, 2004). Although these techniques revealed important metabolic pathways and biochemical processes for isolated microorganisms, they provided a biased view of relative abundance and composition when examining complex environmental samples (Mardanov et al., 2017). Only up to 1% of bacteria from a complex environmental sample can be propagated in culture, for some microorganisms are unculturable, and the correct laboratory conditions cannot be simultaneously achieved for all species present (Amann, 1995). The discovery of DNA as a carrier of hereditary information encoded in an organism's genome gave rise to molecular biology and the genomic revolution (Clarridge, 2004; Mardanov et al., 2017). The pioneering work by Woese (1987) showed that phylogenetic relationships between bacterial species could be determined by comparing a conserved or stable part of genetic code universal to all prokaryotes; namely the approximately 1,550 base pair (bp) long 16S ribosomal RNA gene (Figure 1.7, Clarridge, 2004). The 16S rRNA gene was shown by Woese to act as a 'molecular clock' and thereby particularly consistent in evolutionary terms, making it a prime target for genomic analyses (Ramazzotti et al., 2017). This gene is part of the prokaryotic cellular ribosome consisting of a large subunit (50S), which includes the rRNA molecules 5S and 23S, and the small subunit (30S) which contains the 16S rRNA molecule (Figure 1.7).

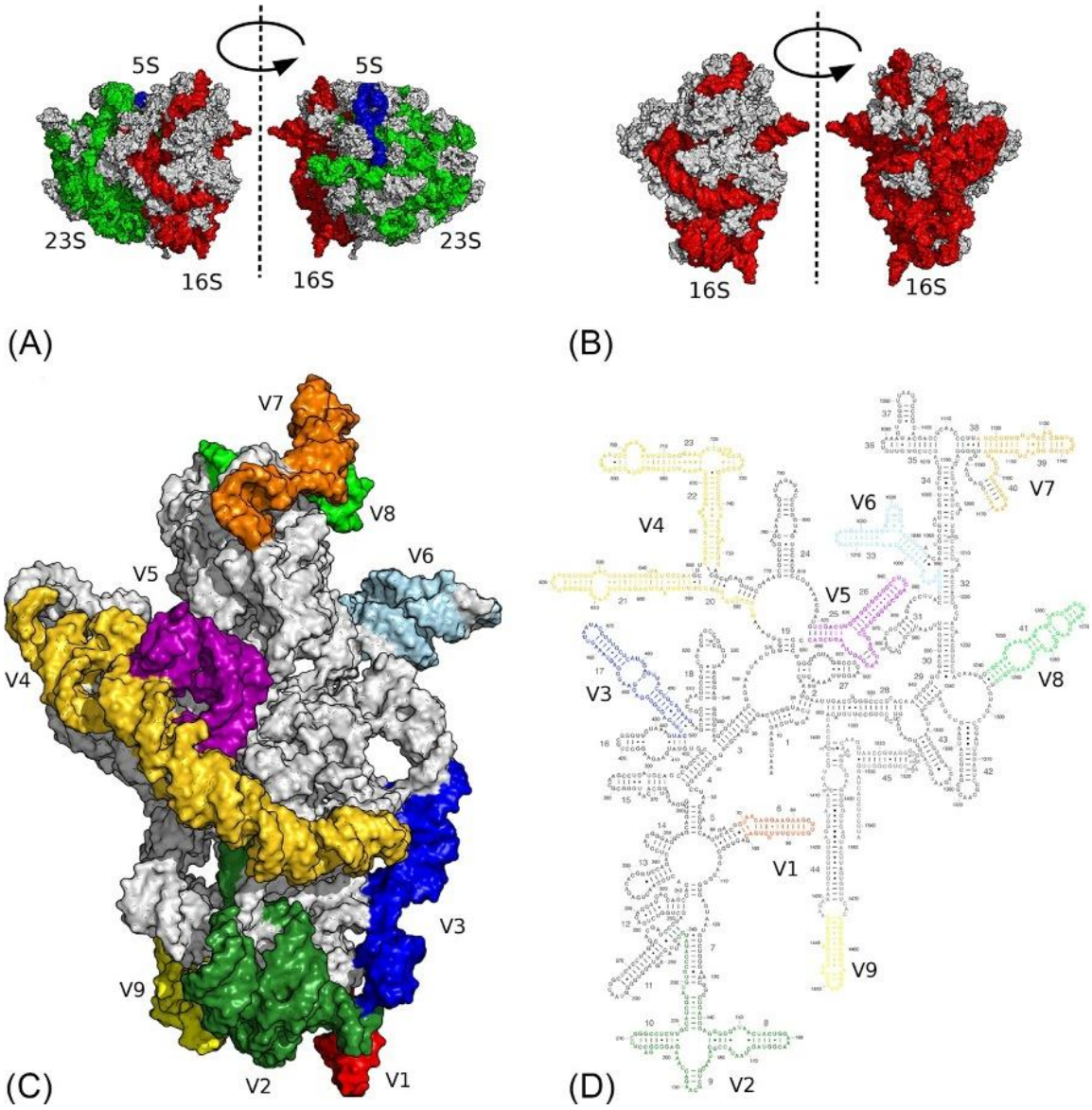

Figure 1.7 (A) The two sides of the prokaryotic ribosome, with the 5S rRNA (blue), 23S rRNA (green) molecules from the large subunit (50S), and the 16S rRNA (red) of the small subunit (30S) shown in 3D. (B) Depicts the two sides of the small subunit (30S) which contains both proteins (white) and the 16S rRNA molecule (red). (C) Depicts the location of the nine variable regions within the 16S rRNA gene. (D) A classical representation of the nine variable regions identified in the 16S rRNA gene of *E.coli.* The regions are colour coded as per image (C). (modified from Ramazzotti & Bacci, 2017).

It was further shown that the bacterial 16S rRNA gene contained nine variable regions (V1-V9), with polymorphisms in these regions being species specific, allowing for diversity, abundance, and taxonomic differentiation in a given sample (Gray et al., 1984). The V4 region, in particular, was shown to be semi-conserved allowing for an even more reliable target for analyses (Lebret et al., 2016;

Ramazzotti & Bacci, 2017). The subsequent development of universal primers to target the 16S rRNA gene region, Polymerase Chain Reaction (PCR), and sequencing techniques, such as Sanger and 454 pyrosequencing enabled the culture-independent discovery of at least 215 novel bacterial species (Woo et al., 2008). Further, it provided greater in-situ insight into bacterial community interactions within a broad variety of sites, such as the human body and the greater environment (Woo et al., 2008). To date, GenBank - the world's largest nucleotide database, holds approximately 20 million sequences from a wide variety of genes including 90,000 partial and complete bacterial and archaeal 16S rRNA genes obtained from both cultivated and named taxa as well as from uncultivated environmental origin (Clarridge, 2004). The advent of Next-Generation high-throughput sequencing technologies, such as the Illumina MiSeq 2500 platform, have shown that the same information and accuracy (to at least the genus level) that can be gained from sequencing the full 16S rRNA gene, can also be achieved by targeting the 250-300 bp V4 region alone (Ramazzotti & Bacci, 2017). The increase in number of sequences per run with the high-throughput Illumina platforms, and at lower error rates, is on the order of 1,000-fold and has brought cost-effective and high-speed genomic analysis to labs world-wide (Caporaso et al., 2010; Lebret et al., 2016). With the rapid advance of computer technology and dedicated software applications, sample sequences can now be matched with high accuracy against databases like GenBank within hours; with approximately 99% accuracy achievable for known organisms (Clarridge, 2004).

1.6.2 Shotgun Metagenomics profiling

Metagenomics analysis utilising the Illumina HiSeq 2500 platform has taken genomic studies to the next level. Unlike the 16S rRNA profiling technique, which can only be used to identify bacterial and archaeal species, shotgun metagenomics profiling provides a broad overview of structural (e.g. rRNA) as well as functional genes that are present in an environmental sample (Mardanov et al., 2017). It enables the study of not only taxonomic composition and relative abundance of taxa, but also the genetic diversity, ecological role and metabolic pathways of microorganisms

living in niche ecosystems (or microbiomes), such as terrestrial or benthic systems, including the human body (Reisenfeld et al., 2004; Mardanov et al., 2017).

This non-targeted approach allows a greater part of organismal genomes to be identified, such as genes coding for specific proteins termed open reading frames (ORFs), enabling the functional potential of microorganisms (i.e. biofilm formation genes, or nitrate reduction genes) to be revealed (Madigan et al., 2012). The process of building metagenome libraries begins with the extraction, purification and enzymatic shearing of DNA into random fragments (Quail et al., 2009; Figure 1.8). Shearing can also be done through a nebulizer, which mechanically fragments the DNA as it passes through a small spray nozzle (Madigan et al., 2012). This random fragmentation process is the reason why the technique is also termed shotgun metagenomics, emphasising its unique non-targeted random approach (Madigan et al., 2012). Multiplex Oligos or adapters are then attached via PCR amplification to the fragments to enable Indexing (barcoding) to track multiple samples in a single run, and to produce higher yield libraries for sequencing (New England Biolabs, 2019). The Illumina platform clones the random fragments through solid-surface PCR resulting in large clusters of identical DNA fragments (Thomas et al., 2012). Depending on the concentration of DNA per sample, cloning maximises sequencing accuracy, particularly for organisms of low-abundance (Madigan et al., 2012). The fragments can be sequenced from either end via a ‘sequencing-by-synthesis’ process in which up to 300 bp (150 bp overlapping paired-end reads) of continuous information (contiguous sequences) can be obtained (Thomas et al., 2012). The high-throughput advantage of the Illumina HiSeq platform refers to its ability to sequence hundreds of millions of clustered fragments in a massively parallel process in which up to 16 channels can be utilised for enzymatic reactions per run (Illumina, 2016).

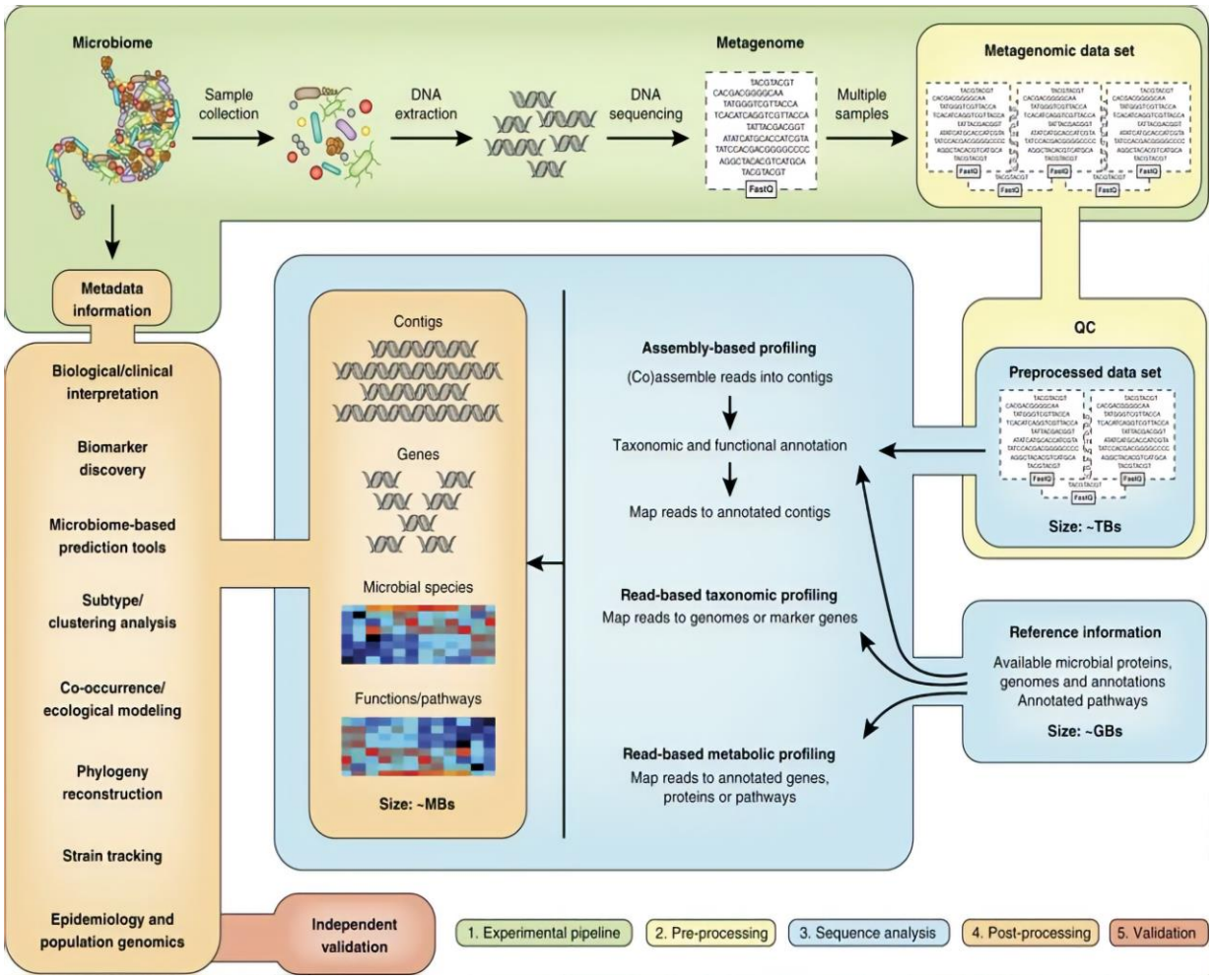

Figure 1.8 An overview of the shotgun metagenomics sequencing and analysis process (modified from Quince at al., 2017).

The subsequent steps involve state-of-the-art computing technology and bioinformatics software in which the sequences are screened for overlaps and are put into the correct order producing assembled genomes (Madigan et al., 2012). Assembled genomes can then be annotated or matched against known metagenomes in databases such as the National Center for Biotechnology Information (NCBI) nucleotide database (NCBI, 2019). The NCBI protein database or the KEGG, eggnog, COG and SEED subsystem databases can also be utilised for functional ORF annotation (Thomas et al., 2012). Although the taxonomic resolution is not as high as targeted approaches, shotgun metagenomics allows for an unprecedented view into the complex relationships between archaeal, viral, bacterial and protistal microorganisms and their functional potential (Ramazzotti & Bacci, 2017). To date, this technique has been used in combination with targeted approaches and has

advanced our understanding of nearly every ecological system within the biosphere (Mardanov et al., 2017).

1.6.3 Stable Isotope and Compound Specific Isotope Analysis (CSIA)

Stable isotope analysis refers to the study of a number of important non-radioactive (non-decaying) elements that form the bulk of biological systems, namely carbon, hydrogen, nitrogen, oxygen and sulfur (Michener & Lathja, 2007, 1-5; Newton, 2016). A variance in each element's neutron count creates an isotope of that element, with all isotopes of an element participating in the same chemical reactions, though they may do so at different rates (Newton, 2016). One of the most important and prevalent element on Earth is carbon-12 (^{12}C), which has an atomic mass of 12 (six protons and six neutrons) (NOAA, 2019). Due to naturally occurring physico-chemical processes, this element may gain or lose neutrons, developing isotopes of carbon, such as the carbon-13 isotope (^{13}C) (6 protons, 7 neutrons) (NOAA, 2019; Newman, 2016). The ratio between ^{12}C and ^{13}C in a given environment or food source may show ^{13}C enrichment or depletion (denoted as its $\delta^{13}C$ value) indicating a distinct carbon fixation pathway (i.e. the C3 or C4 pathway in plants) or carbon source (Kelly et al., 2005; Figure 1.9).

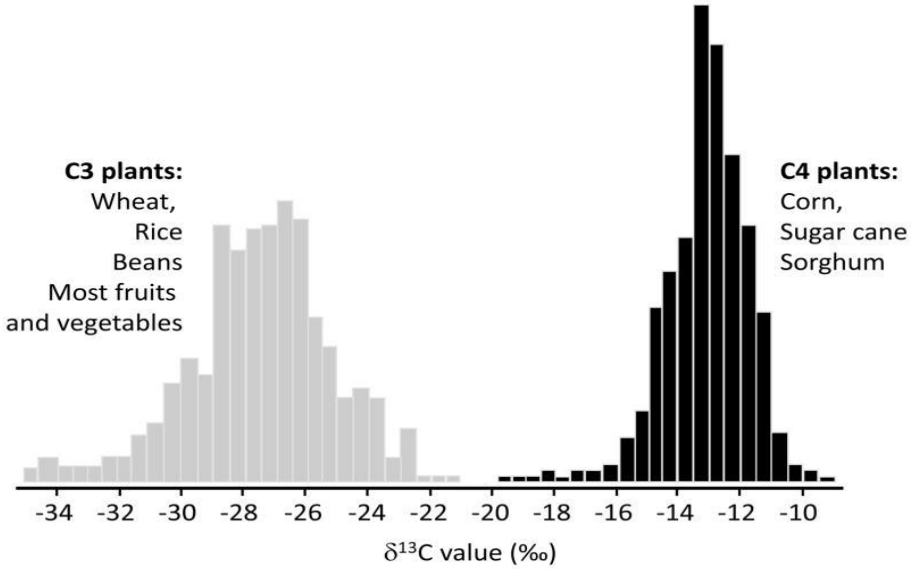

Figure 1.9 The $\delta^{13}C$ values associated with the most commonly consumed plants (modified from O'Brien, 2015).

Subsequent carbon isotope studies have shown this value to persist through the food chain allowing for trace origin and trophic structure analysis (Michener & Lathja, 2007, 240). The stable isotope ratios of oxygen ($^{18}O/^{16}O$) and hydrogen ($^{2}H/^{1}H$) have also been shown to vary geographically due to the differing water evaporation and precipitation cycles of continental regions, with this reflected in regional plants and in the tissues of the animals that consume them (O'Brien & Wooller, 2007; O'Brien, 2015). Subsequently, O and H isotope analysis has been utilised in geographic fingerprinting for migration and dietary studies, including the reconstruction of past climates (O'Brien & Wooller, 2007; O'Brien, 2015). Important information has also been obtained from the analysis of N, S and P studies (Figure 1.10).

Isotope ratio	Fractionation	Information
$^{2}H/^{1}H$	Evaporation, condensation, precipitation	Geographical
$^{13}C/^{12}C$	C3 and C4 plants	Diet (geographical proxy)
$^{15}N/^{14}N$	Trophic level, marine and terrestrial plants, agricultural practice	Diet (geographical proxy)
$^{18}O/^{16}O$	Evaporation, condensation, precipitation	Geographical
$^{34}S/^{32}S$	Bacterial	Geographical (marine)
$^{87}Sr/^{86}Sr$	Age of the rock and Rb/Sr ratio	Underlying geology, geographical

Figure 1.10 Shown are the most commonly studied isotope ratios of a number of environmental elements, how they may be fractionated by natural processes, and the information that can be gained by each (modified from Kelly et al., 2005).

The relative Isotopic abundance of a sample is measured via an isotope ratio mass spectrometer (IRMS) coupled to a gas chromatograph (GC-IRMS) (Kelly et al., 2005). The final ratio value of a given sample is calculated against a known international reference standard, namely Vienna standard mean ocean water for O and H isotopes (VSMOW), and Vienna Peedee Belemnite (VPBD) for C isotopes (Slater et al., 2001). The delta value, denoted in parts per thousand (‰ or per mil), is obtained by calculating the relative isotopic ratio difference between the sample and international standard value using the following equation:

$$\delta_{\text{sample}}(‰) = \left(\frac{R_{\text{sample}} - R_{\text{standard}}}{R_{\text{standard}}} \right) \times 1000$$

Where R_{sample} = [heavy]/[light] element in the sample, and R_{standard} = [heavy]/[light] element in the standard (modified from Urey 1948, McKinney et al., 1950).

Although Stable isotope analysis has revealed important insights in a number of disciplines, it has largely been conducted on the bulked samples which offer limited information (Evershed et al., 1999). The advent of coupling a GC to a combustion furnace alongside an IRMS has enabled source analysis of individual compounds (Matthews & Hayes, 1978; Hayes, 1983, Hayes et al., 1990). The combustion furnace allows for a known elemental signature provided by a reference gas (i.e. CO_2 for carbon) to be measured against carbon signatures in compounds that are separated via the GC (Grice, 2001; Lichtfouse, 2000). Termed compound specific isotope analysis (CSIA), this technique has allowed the determination of trace compounds in even the most complex chemical mixtures revealing both source and biosynthetic pathway patterns of individual compounds within the same sample (Hayes et al., 1990; Lichtfouse, 2000; Grice, 2001). It was shown by early developers of the technique, namely organic geochemists, that individual organic compounds in a given sample inherit specific isotopic signals (i.e. $^{13}C/^{12}C$) resulting from their origin and biosynthetic transformation pathways (Hayes et al., 1990; Grice et al., 1997, 2001). It enabled earth scientists to trace carbon flow through ancient environments by obtaining molecular 'biomarker' information from extinct systems, and thereby reconstruct past climates, depositional conditions, and CO_2 concentration changes through time (Hayes et al., 1990; Collister et al., 1992; Grice et al., 1997, 2001). It has subsequently become a power tool in a number of disciplines, such as forensic anthropology and archaeology. Stott & Evershed (1996) were able to reconstruct palaeodietary information by analysing the $\delta^{13}C$ signatures within cholesterol preserved in excavated teeth and femur bones at a coastal site in the U.K dating from the Saxon (ca. 500-1066) period to the 18th century. It was shown that the community analysed had a preference for marine foods for the last ~1500 years,

indicated by $\delta^{13}C$ values of cholesterol at roughly –22‰ over terrestrial foods –26‰ (Stott & Evershed, 1996).

To date, CSIA has become an important tool not only in environmental, archaeological, and forensic applications, but has also been applied to a limited number of biomedical studies, such as determining the source and biosynthetic fractionation pathway of glucose in patients with diabetes (Lichtfouse, 2000).

1.6.4 Field Emission and Focused Ion Beam – Scanning Electron Microscopy

Scanning Electron Microscopy (SEM) is a technique in which a thin probe of high energy electrons is focused via magnetic lenses and scanned across a given sample along parallel lines (Bogner et al., 2007). The impact of the electrons upon the sample's surface results in the generation of multiple signals that are retrieved by the instrument's detector and used to generate images or analyse the sample's chemical properties (Bogner et al., 2007). The generated signals range in energy level from the approximately 40 keV electrons backscattered from the probe itself, lesser eV secondary electrons, and X-rays typically used for chemical analysis (Bogner et al., 2007). Although the SEM instrument revolutionised microscopy techniques from its inception in 1965, lens imperfections and microscope column brightness limited its ability to obtain the high-resolution images commonly seen today (Joy, 1991; Bogner et al., 2007). The 1980s saw improvements in lens design and the development of the field emission (FE-SEM) and Schottky electron guns, allowing for both greater resolution and brightness to be achieved at even the nanoscopic scale (Joy, 1991). Recent advances in SEM technology include the development of the focused ion beam (FIB-SEM) technique, which uses energetic metal ions to mill or cut through a sample so that its interior structure can be imaged by SEM (Chen et al., 2013). The combination of FE-SEM, X-ray spectroscopy, and now FIB in a single instrument, such as the Lyra and Myra TESCAN instruments, has enabled powerful low-cost sample characterisation for a broad variety of scientific fields (Chen et al., 2013; Jiruše et al., 2014).

1.7 Aims of this thesis

The primary aim of this thesis is to contribute to the understanding of the role bacteria play in gallstone pathogenesis. Although bacteria have been identified in a number of stones, questions still remain as to how they survive and outcompete competitors in what is an extremely hostile gallbladder environment in which toxic agents are concentrated 10-fold. The role and presence, if any, of archaea, protists, fungi, or viral components in gallstone pathogenesis is also unknown. Further, although 16S rRNA profiling enabled the detection of bacteria on cholesterol stones, and culture studies have identified a number of genera in pigmented stones, there has yet to be a consensus on the key genera responsible for bacterial biofilm production across all major stone types using this technique. A secondary aim of this thesis is to ascertain any possible physico-chemical processes that may also lead to or influence gallstone formation, such as the source of dietary cholesterol in all major stone types. There is relatively little data on this aspect of gallstone disease, and it is still unknown whether cholesterol signatures vary from patient to patient, or whether a patients' native stones retain similar profiles. Lastly, an investigation of the morphology of large solitary stones, an often overlooked and understudied stone-type, will be provided in order to elucidate possible formation processes that may be obscured in smaller stones.

In order to achieve the first aim, state of the art shotgun metagenomics profiling will be employed. This technique is yet to be conducted on human gallstones, and allows for the resolution of putative bacterial gene activity in complex environments, including that of archaea, protists, fungi and viral components. This technique will be seconded by 16S rRNA profiling, a more targeted technique, to resolve bacterial signatures and their concentrations in all major stone types. To investigate the secondary aims of this thesis, dietary source fingerprinting will be attempted by utilising gas-chromatography mass-spectroscopy (GCMS) and isotope ratio mass spectroscopy (GCirMS) also known as compound specific isotope analysis (CSIA). Previous investigations conducted by Haigh and Lee (2001), in which a GCMS method was developed to better characterise the unique chemistry of gallstones, revealed novel oxysterols within a number of samples. Due to this research area

being relatively under studied, our study will attempt to further the work of Haigh and Lee and examine the possible presence of saturated and aromatic compounds alongside the more polar sterols that are within the most likely cholesterol rich stones. Although techniques such as liquid chromatography-mass spectroscopy (LCMS) are more amenable to the study of polar and higher-molecular weight compounds such as choelsterol, this pilot study will aim to continue the line of inquiry and methodology developed by Haigh & Lee by screening the stones for lower molecular weight compounds first before developing a new method for LCMS. We further this approach by applying CSIA to human gallstones for the first time to discover metabolic pathway and/or dietary source information for cholesterol and any other compounds identified within the gallstones. CSIA has routinely been applied to energy, environmental and paleoenvironmental research, but rarely applied to medical studies. The morphological study on large solitary stones will be accomplished by employing scanning electron microscopy (SEM) and X-ray diffraction techniques to aid in visualising the stone matrices. It is hoped that this may reveal clues as to the formation processes of the stone matrices and the possible visualisation of microbes that may be imbedded within.

In **Chapter 2**, we conducted shotgun metagenomics profiling on human gallstones from patients with dissimilar backgrounds: the pigmented stones of a 76-year old male patient, and the cholesterol stones of a 30-year old female patient. This study aimed to reveal the mechanisms bacteria and other organisms may employ to survive, persist and outcompete in the extreme environment of the human gallbladder and to identify key players in these processes. Further, whether patient background or certain risk factors influenced the organisms present. The study is enhancedby the stable carbon and hydrogen isotopic analyses of cholesterol in each of the patients' stones in order to establish whether homogeneity exist in either native or patient-patient settings.

In **Chapter 3**, the findings of the first study are extended to a cohort of 16 patients of varying backgrounds and stone types. A more targeted approach is applied here using 16S rRNA profiling and is further complimented by stable isotopic carbon and hydrogen analyses of cholesterol in each of the stones. This was done with the aim of confirming whether the observations in the first study can be consistently seen across a broader and more varied sample set.

In **Chapter 4**, large and solitary stones are explored in greater detail using SEM, XRD, and stable isotopic carbon and hydrogen analyses of cholesterol in each of the stones to ascertain formation mechanisms that may be elusive in the majority of gallstones where sizes range from 0.5-1.5 cm in diameter.

Lastly, In **Chapter 5**, a summary of findings from the three studies will be provided, including suggestions for future work in this area that may aid research efforts in ultimately revealing the mechanisms behind gallstone pathogenesis.

References

Alphonse, P.A.S., & Jones P.J.H. (2016). Revisiting Human Cholesterol Synthesis and Absorption: The Reciprocity Paradigm and its Key Regulators. *Lipids*, *51*, 519–536.

Amann, R.I., Ludwig, W., & Schleifer, K.H. (1995). Phylogenetic identification and in situ detection of individual microbial cells without cultivation. *Microbiology Reviews*, *59*, 143–69.

Australian Institute of Health and Welfare. (2015). *Admitted patient care 2013-14: Australian hospital statistics* (Chapter 6: What procedures were performed?) Retrieved from https://www.aihw.gov.au/reports/hospitals/ahs-2013-14-admitted-patient-care/contents/

Australian Institute of Health and Welfare. (2017). *Hospital admitted patient expenditure 2004–05 to 2012: Australian health expenditure—demographics and diseases: 13.* (Health and welfare expenditure series Number 59) Retrieved from https://www.aihw.gov.au/reports/health-welfare-expenditure/australian-health-expenditure-demographics-disease/contents/

Australian Commission on Safety and Quality in Health Care. (2017). *The second Australian atlas of healthcare variation*. Sydney: Australian Commission on Safety and Quality in Health Care.

Barrett, K.E., Barman, S.M., Botano, S., & Brooks, H.L (Eds.). (2016). *Ganong's Review of Medical Physiology* (25th edition). New York: McGraw-Hill.

Beckingham, I.J. (2001). Gallstone disease. *BMJ*, *322(7278)*, 91-94.

Berg, J.M., Tymoczko, J.L., & Stryer, L. (Eds.). (2002). Chapter 26. The Biosynthesis of Membrane Lipids and Steroids. In *Biochemistry* (5th ed., sec. 26.4). Retrieved from https://www.ncbi.nlm.nih.gov/books/NBK22339/

Betts, J.G., Young, K.A., Wise, J.A., Johnson, E., Poe, B., Kruse, D.H…DeSaix, P. (2017). *Anatomoy and Physiology*. Rice University: Houston. Retrieved from https://openstax.org/details/books/anatomy-and-physiology

Bills, P.M., & Lewis, D. (1975). A structural study of gallstones. *Gut*, *16*(8), 630-637.

Blesso, C.N., & Fernandez, M.L. (2018). Dietary Cholesterol, Serum Lipids, and Heart Disease: Are Eggs Working for or Against You? *Nutrients*, *10(4)*, 426.

Bouchier, I.A. (1992). The formation of gallstones. *The Keio Journal of Medicine*. *41(1)*, 1–5.

Brown, W.H. (Ed.) (2000). *Introduction to Organic Chemistry* (2nd Edition) Florida: Harcourt Brace & Company.

Campbell, J.L., Reinmann, A.B., & Templer, P.H. (2014). Soil freezing effects on sources of nitrogen and carbon leached during snowmelt. *Soil Science Society of America Journal*, *78(1)*, 297–308.

Caporaso, J. G., Lauber, C. L., Walters, W. A., Berg-Lyons, D., Lozupone, C. A., Turnbaugh, P. J., Fierer, N., … Knight, R. (2010). Global patterns of 16S rRNA diversity at a depth of millions of sequences per sample. *Proceedings of the National Academy of Sciences of the United States of America*, *108 Suppl 1*(Suppl 1), 4516-22.

Carey, M.C., & Small, D.M. (1978). The physical chemistry of cholesterol solubility in bile. Relationship to gallstone formation and dissolution in man. *The Journal of Clinical Investigation*, *61*(4), 998–1026.

Carey, M.C. (1993). Pathogenesis of Gallstones. *The American Journal of Surgery*, *165(4)*, 410-419.

Cetta, F. (1991). The role of bacteria in pigment gallstone disease. *Annals of Surgery*, *213*(4), 315-326.

Chen, H., Grassian, V.H., Saraf, L.V., & Laskin, A. (2013). Chemical imaging analysis of environmental particles using the focused ion beam/scanning electron microscopy technique: microanalysis insights into atmospheric chemistry of fly ash. *Analyst*, *138(2)*, 451-460.

Chew, G.T., Gan, S.K., &Watts, G.F. (2006). Revisiting the metabolic syndrome.

The Medical Journal of Australia, *185(8)*, 445-449.

Chiang, J.Y.L. (2009). Bile acids: regulation of synthesis. *Journal of Lipid Research, 50(10)*, 1955–1966.

Choi, Y., & Silverman, W.B. (2018). American College of Gastroenterology: Biliary tract disorders, gallbladder disorders, and gallstone pancreatitis.. Retrieved from http://patients.gi.org/topics/biliary-tract-disorders-gallbladder-disorders-and-gallstone-pancreatitis.

Clarridge, J.E. (2004). Impact of 16S rRNA Gene Sequence Analysis for Identification of Bacteria on Clinical Microbiology and Infectious Diseases. *Clinical Microbiology Reviews*, *17(4)*, 840–862.

Crawford, M. (2013). Biliary pain: work-up and management in general practice. *The Right Upper Quadrant*, *42(7)*, 458-461.

Cruz,P.M., Mo, H., McConathy, W.J., Sabnis, N., & Lacko, A.G. (2013). The role of cholesterol metabolism and cholesterol transport in carcinogenesis: a review of scientific findings, relevant to future cancer therapeutics. *Frontiers in Pharmacology*, *4*, 119.

Collister, J.W., Summons, R.E., Lichtfouse E., and Hayes, J.M., (1992) An isotopic biogeochemical study of the Green River oil shale. *Organic Geochemistry*, *19*, 265-276.

Colorado Springs Surgical Associates. (2018). Retrieved from https://www.coloradosurgical.com/images/Documents/lap-chole-instructions.pdf

Demehri, F.R., & Alam, H.B. (2016). Evidence-based management of common gallstone-related emergencies. *Journal of Intensive Care Medicine*, *31(1)*, 3-13.

Eggert, T., Bakonyi, D., Hummel, W. (2014). Enzymatic routes for the synthesis of ursodeoxycholic acid. *Journal of Biotechnology*, *191(10)*, 11-21.

Everhart, J.E., Khare, M., Hill, M., & Maurer, K.R. (1999). Prevalence and Ethnic Differences in Gallbladder Disease in the United States. *Gastroenterology*, *117(3)*, 632–639.

Evershed, R., Dudd, S., Charters, S., Mottram, H., Stott, A., Raven, A., . . . Bada, J. (1999). Lipids as Carriers of Anthropogenic Signals from Prehistory [and

Discussion]. *Philosophical Transactions: Biological Sciences, 354*(1379), 19-31.

Feher, J. (2017). *Quantitative Human Physiology: An Introduction* (2nd ed.) Amsterdam: Elsevier.

Feingold, K.R., Grunfeld, C. (2000). Structure of Lipoproteins. In De Groot, L.J., Chrousos, G., Dungan, K., et al., (Ed.), *Introduction to Lipids and Lipoproteins.* South Dartmouth (MA): MDText.com, Inc. Retrieved from https://www.ncbi.nlm.nih.gov/books/NBK305896/#lipid_intro.toc-structure-of-lipoproteins.

Fu, P., Zhang, S., Dai, K., Zheng, K.,…Zhang, C. (1984) Gallstone classified based on sectional structure and chemical composition. *Chinese Journal of Surgery, 22*(5), 258–260.

Goad, J. & Akihisa, T. (1997). *Analysis of Sterols*. United Kingdom: Chapman and Hall.

Gray, M.W., Sankoff, D., & Cedergren, R.J. (1984). On the evolutionary descent of organisms and organelles: a global phylogeny based on a highly conserved structural core in small subunit ribosomal RNA. *Nucleic Acids Research, 12(14)*, 5837–52.

Greer, D., Heywood, S., Croaker, D., & Gananadha, S. (2018). Is 14 the new 40: trends in gallstone disease and cholecystectomy in Australian children. *Pediatric Surgery International*, *34(8)*, 845–849.

Grice, K., Schaeffer, P., Schwark, L., and Maxwell, J.R., (1997) Changes in palaeoenvironmental conditions during deposition of the Permian Kupferschiefer (Lower Rhine Basin, N.W. Germany) inferred from molecular and isotopic compositions of biomarker components. *Organic Geochemistry, 26*, 677-690.

Grice, K., 2001. $\delta^{13}C$ as an indicator of paleoenvironments: A molecular approach, in: Unkovich, M., Pate, J., McNeill, A., Gibbs, J. (Eds.), Application of stable isotope techniques to study biological processes and functioning ecosystems. Kluwer Academic Publishers, pp. 247-281.

Hardinger, S.A. (2017). Molecular structure of cholesterol. Illustrated Glossary of Organic Chemistry. Retrieved from http://www.chem.ucla.edu/~harding/IGOC/C/cholesterol.html.

Hayes, J.M., (1983) *In*: Meinschein W.G. (*ed*.) Organic Geochemistry of Contemporaneous and Ancient Sediments, Great Lakes Section, Society of Economic Palaeontologists and Mineralogists, Bloomington, Indiana, pp. 5-31.

Hayes, J. M., Freeman, K.H., Popp, B.N., and Hoham, C.H., (1990). Compound-specific isotopic analyses: A novel tool for reconstruction of ancient biogeochemical processes. *Organic Geochemistry*, *16*, 1115-1128.

Illumina, Inc. (2016). Illumina Sequencing by Synthesis. Retrieved from https://www.illumina.com/company/videohub/fCd6B5HRaZ8.html?langsel=/us/

Jiruše, J., Havelka, M., &Lopour, F. (2014). Novel field emission SEM column with beam deceleration technology. *Ultramicroscopy*, *146*, 27-32.

Joy, D.C., 1991. The theory and practice of high-resolution scanning electronmicroscopy. *Ultramicroscopy*, *37*, 216–233.

Kelly, S., Heaton, K., & Hoogewerff, J. (2005). Tracing the geographical origin of food: The application of multi-element and multi-isotope analysis. *Trends in Food Science & Technology*, *16*, 555–567.

Ko, C.W., Sekijima, J.H., & Lee, S.P. (1999). Biliary sludge. *Annals of Internal Medicine*, *130(4)*, 301-311.

Ko, C.W. (2006). Risk Factors for Gallstone-Related Hospitalization DuringPregnancy and the Postpartum. *American Journal of Gastroenterology*, *101(10)*, 2263–2268.

Kwong, E., Li, Y,. Hylemon, P.B., & Zhou, H. (2015). Bile acids and sphingosine-1-phosphate receptor 2 in hepatic lipid metabolism. *Acta Pharmaceutica SinicaB*, *5(2)*, 151–157.

Lebret, K., Schroeder, J., Balestreri, C., Highfield, A., Cummings, D., Smyth, T., & Schroeder, D. (2016). Choice of molecular barcode will affect species prevalence but not bacterial community composition. *Marine Genomics*, *29*, 39-43.

Lee, J.Y., Keane, M.G., & Pereira, S. (2015). Diagnosis and treatment of gallstone disease. *Practitioner*, *259(1783)*, 15-19.

Lee, T., & Chen, J.G. (2009). Biomimetic Gallstone Formation: Crystallization of Calcium Carbonate by the Evolving Taurocholate−Lecithin−Cholesterol Complex Lipid System. *Crystal Growth & Design*, *9*(8), 3737–3748.

Lichtfouse, E. (2000). Compound-specific isotope analysis. Application to archaelogy, biomedical sciences, biosynthesis, environment, extraterrestrial chemistry, food science, forensic science, humic substances, microbiology, organic geochemistry, soil science and sport. *Rapid Communications in Mass Spectrometry*, *14*, 1337–1344.

Maki, T. (1966). Pathogenesis of calcium bilirubinate gallstone: Role of E. coli, beta glucuronidase and coagulation by inorganic ions, polyelectrolytes, and agitation. *Annals of Surgery*, *164*(1), 90-100.

Matthews, D.E., and Hayes, J.M., (1978) Isotope-ratio monitoring gas chromatography- mass spectrometry. *Analytical Chemistry*, *50*, 1465-1473.

Mardanov, A.V., Kadnikov, V.V., & Ravin, N.V. (2017). Metagenomics: A Paradigm Shift in Microbiology in Nagarajan, M. (Ed.). *Metagenomics: perspectives, methods, and applications*. Retrieved from http://ebookcentral.proquest.com.

McKinney C. R., McCrea J. M., Epstein S., Allen H. A. and Urey H. C. (1950) Improvements in mass spectrometers for the measurement of small differences in isotope abundance ratios. *Rev. Sci. Instrum. 21*, 724-730.

Michener, R. & Lajtha, K. (2007). *Stable Isotopes in Ecology and Environmental Science* (2nd ed) Blackwell Publishing, Singapore.

Myant, N.B. (1981). *The Biology of Cholesterol and Related Steroids*. London: William Heinemann Medical Books Ltd.

National Center for Biotechnology Information (NCBI). (2019). Genomes. Retrieved from https://www.ncbi.nlm.nih.gov/home/genomes/

National Oceanic & Atmospheric Administration (NOAA). (2019). Stable and radiocarbon isotopes of carbon dioxide. Retrieved from https://www.esrl.noaa.gov/gmd/outreach/isotopes/chemistry.html

New England BioLabs. (2019). NEBNext Multiplex Oligos for Illumina. Retrieved from https://www.nebiolabs.com.au/tools-and-resources.

Newton, J. (2016). Stable isotopes as tools in ecological research. In: eLS. John Wiley & Sons, Ltd: Chichester.

O'Brien D.M. & Wooller, M.J. (2007). Tracking human travel using stable oxygen and hydrogen isotope analyses of hair and urine. *Rapid communications in mass spectrometry*, *21*, 2422-2430.

O'Brien D.M. (2015). Stable isotope ratios as biomarkers of diet for health research. *Annual Review of Nutrition*, 35, 565–594.

Organisation for Economic Co-operation and Development. (2016). *Health care utilisation: surgical procedures (shortlist)*. Retrieved from https://stats.oecd.org/index.aspx?queryid=30167

Purohit, V. (2000). Can alcohol promote aromatization of androgens to estrogens? A review. *Alcohol, 22(3)*, 123-7.

Qiao, T., Ma, R-h., Luo, X-b., Yang, L-q., Luo, Z-l., & Zheng P-m. (2013). The Systematic Classification of Gallbladder Stones. *PLoS ONE, 8(10)*, e74887.

Quail, M. A., Swerdlow, H., & Turner, D. J. (2009). Improved protocols for the illumina genome analyzer sequencing system. *Current protocols in human genetics*, *Chapter 18*, Unit 18.2.

Ramazzotti, M., & Bacci, G. (2017). 16S rRNA-Based Taxonomy Profiling in the Metagenomics Era in Nagarajan, M. (Ed.). *Metagenomics: perspectives, methods, and applications*. Retrieved from http://ebookcentral.proquest.com.

Rege, R.V. (2002). The role of biliary calcium in gallstone pathogenesis. *Frontiers in Bioscience*, *7*, 315-325.

Russell, D.W. (2003). The enzymes, regulation, and genetics of bile acid synthesis. *Annual Review of Biochemistry*. *72*, 137-174.

Schooling, C.M., Au Yeung, S.L., Freeman, G., Cowling, B.J. (2013). The effect of statins on testosterone in men and women, a systematic review and meta-analysis of randomized controlled trials. *BMC Medicine*, *11(57)*, 2-9.

Sears, J. (1992). Anatomy and Physiology of the liver. *Baillière's Clinical Anaesthesiology*, *6(4)*, 697-727.

Slater, C., Preston, T., & Weaver, L.T. (2001). Stable isotopes and the international system of units. *Rapid Communications in Mass Spectrometry*, *15(15)*, 1270-1273.

Springhouse. (Ed.). (2002). *Lippincott Professional Guides: Anatomy & Physiology* (2nd Edition). Philadelphia: Lippincott Williams & Wilkins.

Stevenson, A. (Ed). (2010). *Oxford Dictionary of English* (3rd edition), United Kingdom: Oxford University Press.

Stewart L., Smith A.L., Pellegrini, C.A., Motson, R.W. & Way, L.W. (1987). Pigment gallstones form as a composite of bacterial micro-colonies and pigment solids. *Annals of Surgery*, *206*(3), 242-250.

Stewart, L., Ponce, R., Oesterk, A.L., Griffiss, J.M., & Way. L.W. (2000). Pigment gallstone pathogenesis: Slime production by biliary bacteria is more important than beta-glucuronidase production. *Journal of Gastrointestinal Surgery*. *4*(5), 547-543.

Stewart, L., Oesterle, A.L., Erdan, I, Griffiss, J.M. & Way, L.W. (2002). The pathogenesis of pigment gallstones in western societies: the central role of bacteria. *Journal of Gastrointestinal Surgery*, *6*(6), 891–904.

Stinton, L.M., & Shaffer, E.A. (2012). Epidemiology of Gallbladder Disease: Cholelithiasis and Cancer. ***Gut and Liver***, *6(2)*, 172-187.

Stott, A.W., & Evershed, R.P. (1996). delta ^{13}C analysis of cholesterol preserved in archaeological bones and teeth. *Analytical Chemistry*, *68(24)*, 4402-8.

Strasberg, S.M. (1998). The pathogenesis of cholesterol gallstones—a review. *Journal of Gastrointestinal Surgery*, 2(2), 109-125.

Sutor, D.J., & Wooley, S.E. (1968) Gallstone of Unusual Composition: Calcite, Aragonite, and Vaterite. *Science*, *159*(3819), 1113-111.

Swanstrom, L. L., & Soper, N. J. (2013). *Mastery of endoscopic and laparoscopic surgery*. U.S.A: Wolters Kluwer Health.

Tortora, G.J., & Derrickson, B. (Eds.). (2009). *Principles of Anatomy and Physiology* (12th edition). U.S.A: Wiley & Sons.

Urey H. C. (1948) Oxygen isotopes in nature and in the laboratory. *Science*, *108*, 489-496.

van den Berg, A.A., van Buul, J.D., Ostrow, D.J., & Groen, A. K. (2000) Measurement of cholesterol gallstone growth in vitro. *Journal of Lipid Research*, *41*(2),189–194.

van Erpecum, K.J. (2011). Pathogenesis of cholesterol and pigment gallstones: An update. *Clinics and Research in Hepatology and Gastroenterology*, *35*(4), 281-287.

Waterham, H.R. (2006) Defects of cholesterol biosynthesis. *FEBS Letters*, *580(23)*, 5442-5449.

Woese, C. R. (1987). Bacterial evolution. *Microbiology Reviews*, *51*, 221–271.

Woo, P.C., Lau, S.K., Teng, J.L., Tse, H., & Yuen, K.Y. (2008). Then and now: use of 16S rDNA gene sequencing for bacterial identification and discovery of novel bacteria in clinical microbiology laboratories. *Clinical Microbiology and Infection*, *14(10)*, 908-34.

Wu, T., Zhang, Z., Liu, B., Hou, D., Liang Y., Zhang, J., & Shi, P. (2013). Gut microbiota dysbiosis and bacterial community assembly associated with cholesterol gallstones in large-scale study. *BMC Genomics*, *14*(669), 1-11.

Zhang, Y., Peng, J., Li, X., & Liao, M. (2016). Endoscopic-Laparoscopic Cholecystolithotomy in Treatment of Cholecystolithiasis Compared With Traditional Laparoscopic Cholecystectomy. *Surgical laparoscopy, endoscopy & percutaneous techniques*, *26(5)*, 377-380.

Chapter 2:

Metagenomics of pigmented and cholesterol gallstones: the putative role of bacteria

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Scientific Reports, 8:11218, 1-13. (2018).

Abstract

There is growing evidence for bacteria playing a role in the pathogenesis and formation of pigmented gallstones from humans. These studies mainly involved cultivation of gallstone-associated bacteria and 16S rRNA profiling, providing an indirect link between processes involved in gallstone formation by the bacteria *in-situ*. Here, we provide functional metagenomic evidence of a range of genes involved in bile stress response, biofilm formation, and anaerobic energy metabolism by Gram-negative *Klebsiella* in pigmented gallstones from a 76-year-old male patient. *Klebsiella* was also present in one cholesterol-type stone in a 30-year-old female patient who had additional cholesterol gallstones characterised by Gram-positive bacteria. Pigmented stones further revealed a predominance of genes involved in carbohydrate metabolism, whilst cholesterol stones indicated a profile dominanted by protein metabolism possibly reflecting known chemical differences between Gram-negative and Gram-positive biofilm matrices. Archaeal genes were not detected. Complementary carbon and hydrogen isotopic analyses of cholesterol within the patients' stones revealed homogeneity, suggesting a common diet or cholesterol biosynthesis pathway that has little influence on microbial composition. This pilot study provides a framework to study microbial processes that play a potential role in gallstone formation across markedly different types of stones and patient backgrounds.

Introduction

The focus on bacteria and its role in gallstone pathogenesis and formation began most notably in 1966 by Maki (1966), and was furthered by Stewart et al. (1987; 2000). In those studies, bacteria were suggested to play a causal role in the pathogenesis of pigmented and the pigmented portion of mixed stones only, as cholesterol stones rarely exhibited bacterial signatures (Stewart et al, 1987). A conclusive definition of gallstone types and their bacterial compositions are yet to be determined due to the complex nature of gallstones. However, researchers do generally group the stone types into cholesterol (predominantly composed of cholesterol, that may have pigmented centres), pigmented (predominantly composed of the bilepigment bilirubin, derived from the breakdown of aged red blood cells by the liver giving its brown colour), mixed (a compositional mixture of the cholesterol and pigmented) and black (assumed to be pigmented but with a black 'volcanic glass' type appearance). For a detailed discussion on stone types and compositions see Stewart et al (2002). A further study restricted gallstone pathogenesis *via* bacterial action to only brown pigmented stones, ruling out cholesterol, mixed pigmented and black pigmented gallstones altogether (Cetta, 1991). Subsequently, bacterial studies were focused on pigmented stone formation with the dominant theory behind the mechanism of formation for this type of stone through the activity of bacterially produced ß-glucuronidase (Maki, 1966). ß-glucuronidase was observed to be the deconjugating factor that led to the precipitation of calcium bilirubinate crystals, with these crystals conjugated by an anionic glycoprotein (i.e. sodium alginate) leading to the agglomeration of the bilirubinate calcium crystals into macroscopic stones (Maki, 1966). However, Maki's findings related to *Escherichia coli* (a known producer of ß-glucuronidase), and did not explore non-ß-glucuronidase producing bacterial species that are often found in gallstone studies (Stewart et la., 2000). Biofilm formation as an alternative mechanism for the role of bacteria in pigmented stone formation was initially proposed by Stewart et al (1987). In that culture-based study, the biofilm product glycocalyx (an anionic glycoprotein), was suggested to be the central agglomerating factor, with ß-glucuronidase having a comparatively minor role. *Klebsiella*, *Enterococcus*, *Enterobacter*, *E. coli* and *Pseudomonas aeruginosa*, were reported to be the most prevalent cultured bacteria

across 61 stones (predominantly pigmented and mixed stones), with *P. aeruginosa* the greatest biofilm producer of the group after *Citrobacter freundii* (Stewart et al., 2002). A more recent study involving mice as a host identified *Salmonella, via* scanning electron microscopy (SEM) and culture analysis, as a primary producer of biofilms on the surface of cholesterol gallstones (Marshall et al., 1991). However, only up to 1% of bacteria in complex environmental samples can generally be brought into culture, which provides a biased view into the relative abundance of the total species present. A recent cultivation-independent investigation of the microbial composition of 27 cholesterol gallstones using high-throughput 16S rRNA profiling identified a predominance of *Enterobacteriaceae* and *Ruminococcoceae* and to a lesser extent, *Bacteroidales, Lactococcus, Enterococcus and Clostridiales* within the analysed gallstones (Wu et al., 2013). However, 16S rRNA profiling does not provide information on the role the identified bacteria play in the formation of gallstones and what mechanisms they possess to survive in the human gallbladder.

In this study, we analysed functional metagenomes (Orsi et al., 2018) to investigate the diversity and metabolic potential of microbial communities in pigmented *vs.* cholesterol stones, and whether they possess genes involved in the formation of biofilms or other processes including bile resistance that could lead to gallstone formation. The sequencing of functional metagenomes was furthermore used to provide parallel information on the functional and/or taxonomic diversity of all domains of life not limited to bacteria (e.g. fungi, archaea, and viruses) that may be present and underexplored in gallstones (Haigh & Lee, 2001; Miettinen et al., 1996). To the best of our knowledge, the only other related shotgun metagenomics study was perfomed on human bile samples (Shen et al., 2015). However, it has been shown that bacterial biofilms may persist on the surface of gallstones, even when the patient's bile is culture negative (Swidinski & Lee, 2001).

We complement the shotgun metagenomics analysis with compound specific isotopic analysis (CSIA) of cholesterol in the gallstones - namely carbon ($\delta^{13}C$ values) and hydrogen (δD values) isotopes to ascertain possible dietary or exogenous environmental factors that may be associated with or divergent from the bacteria identified in this study.

Results and Discussion

Gallstones were collected from the gallbladders of a 76-year old male patient (PM1) with pigmented type gallstones (n=4) and one 30-year old female patient (CF4) with cholesterol type gallstones (n=4) (Table 2.1). Both patients were diagnosed with gallstone disease (cholelithiasis) by ultrasound imaging and computed tomography scanning at the Fiona Stanley and St John of God Hospital, Murdoch, WA, Australia. Neither patient had been given a course of antibiotics for treatment. Pigmented gallstones contained 57.2 ± 36.6 ng per stone genomic DNA while the DNA content in the cholesterol gallstones was 16.5 ± 10.9 ng per stone. This DNA served as template for the construction of the metagenomics libraries discussed below.

Table 2.1 Patient Information.

Sample ID	Age	Sex	BMI	Antibiotics	Type	Size	No.	Site
PM1	76	M	24	Nil	Pigmented	Small 0.5-1 cm	4	FSH
CF4	30	F	40.5	Nil	Cholesterol	Small 0.5-1 cm	4	SJOG

Metagenomics

Metagenomic analysis revealed distinct differences between the microbial diversity of the two patients. The following section focuses on the taxonomic diversity and gene functions potentially associated with gallstone pathogenesis and formation.

Principle component analysis (PCoA; Figure 2.1) on open reading frames (ORFs) revealed that the first component explains 83.7% of the variance in microbial diversity in the analysed gallstones. The second component explains 9.8% of the variation, with both components combined accounting for 93.5% of the variation between the two patients. Only 5.5% of the variation remains unexplained. The microbial composition between the four stones found in patient PM1 showed a high level of similarity at 80%. A 60% similarity in microbial diversity was observed between stones 1, 3 and 4 of patient CF4. Stone 2, however, only shared 40% similarity to the group (Figure 2.1).

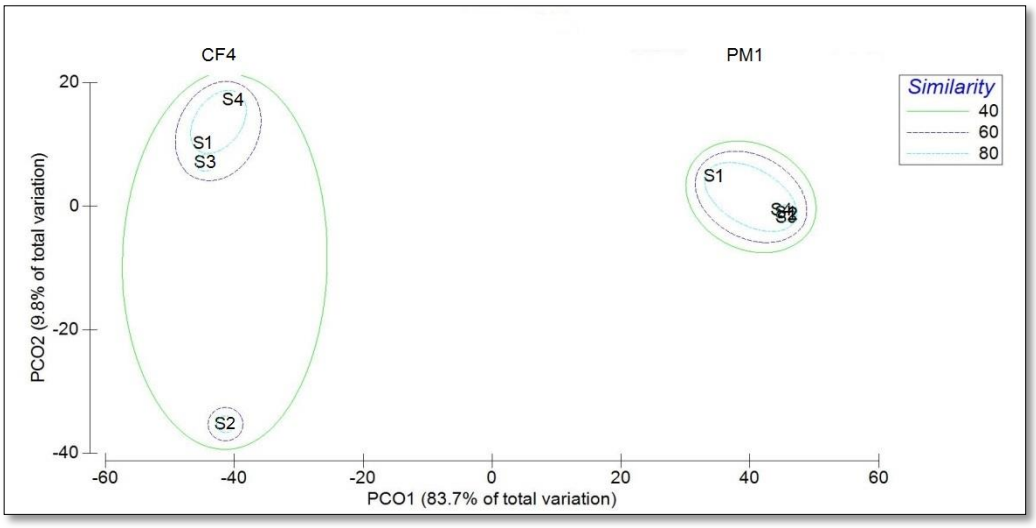


Figure 2.1 Principle coordinate analysis (PCoA) ordination of Bray-Curtis similarity (square root transformed) between total bacterial genera in the four gallstones of patients PM1 and CF4. Shown is the first two principle coordinate axes, which combined explain 93.5% of the variation between the patients. Coloured ellipses signify the percentage of similarity between the patients' native stones.

Taxonomic affiliation

Taxonomic analysis of the ORFs of 4 replicate pigmented gallstones of patient PM1 revealed a predominance of bacteria (97.65%) while no archaeal ORFs were recovered (Figure 2.2). Eukaryotes comprised 1.98% of the ORFs with the majority (80.49%) of human origin. A small proportion of the ORFs were of viral origin (0.3%) and misidentified reads (0.07%).

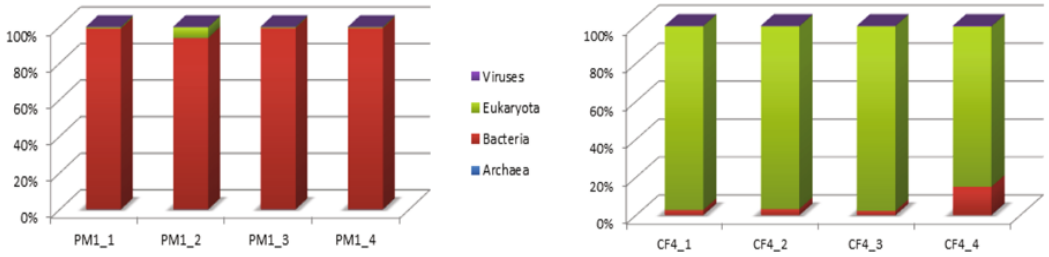


Figure 2.2 Relative abundance of bacteria, archaea, eukaryotes and viruses recovered from metagenomes in patients PM1 (left) and CF4 (right).

Taxonomic analysis of bacterial ORFs comprising >1% of the total community revealed that Gram-negative *Klebsiella* was the most abundant genus in PM1 (69-79 %), followed by *Enterococcus* (3.7-13.6%), *Escherichia* (2.9-8.0%), *Salmonella*

(1.7-2.2%), and *Enterobacter* (1.1-1.3%). *Meiothermus* was abundant in stone 1 (17.4%), but was not detected in the other stones of PM1 (Figure 3.3).

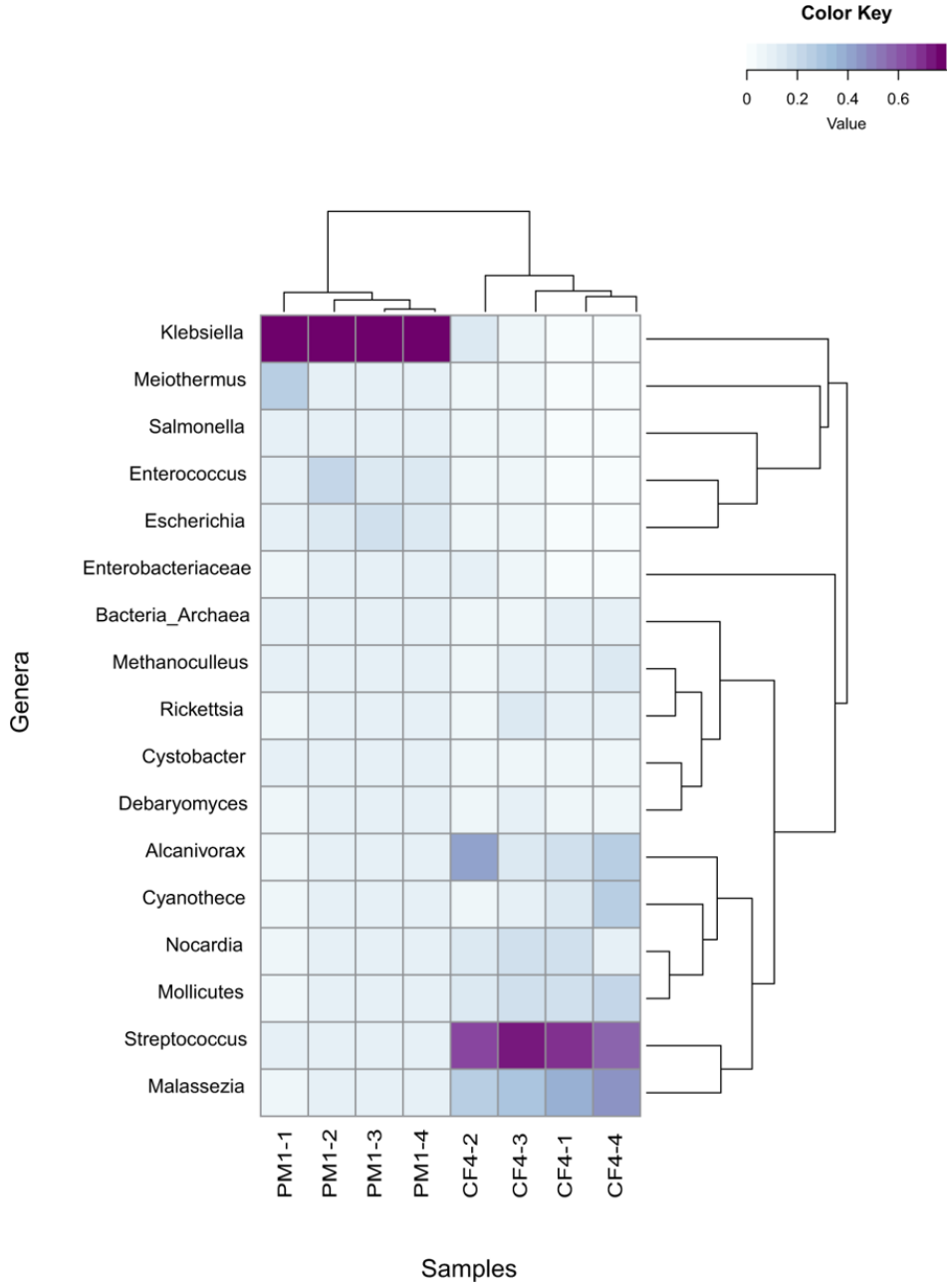


Figure 2.3 Heatmap with the major bacterial genera identified in the gallstones (n=4) of patients PM1 (pigmented) and CF4 (cholesterol). The color key shows the relative abundance of the genera in the gallstones. The dendrograms illustrate the relationship between samples showing that the distribution of genera is relatively similar between replicate stones, but greatly differ between the two patients.

Metagenomic profiling of 4 replicate cholesterol gallstones of patient CF4 revealed a predominance of eukaryotes (96.05%) with the majority (98.5%) stemming from the patient or comprising human cells, with others (1.46%). Bacteria comprised only 3.6% of the ORFs. In agreement with the metagenomes of PM1 stones, a small

portion of the ORFs were of viral origin (0.3%) and misidentified reads (0.05%), while no archaeal ORFs were recovered (Figure 2.2).

Taxonomic analysis of bacterial ORFs comprising >1% of the total bacterial community revealed that Gram-positive *Streptococcus* was the most abundant genus within all 4 stones in CF4 (38.4-54.7%) (Figure 2.3), followed by *Alcanivorax* (6.4-27.8%), *Mollicutes* (6.3-15.1%), *Nocardia* (7.0-13.0%), *Methanoculleus* (5.5-8.1%), *Ricketssia* (4.9-6.9%), and *Cystobacter* (2.7-4.1%). Stone 2 only showed 40% similarity with the other three stones as it did not contain *Nocardia*, *Methanoculleus*, *Ricketssia*, and *Cystobacter*. It was, however, the only stone to exhibit *Klebsiella* (6.8%) and other *Enterobacteriaceae* (4.5%). *Geobacillus* was found only in stones 3 and 4 (1.1-1.2%).

As mentioned earlier, a consensus is yet to be drawn on what type of bacterial communities are common to pigmented and cholesterol gallstones (Wu et al., 2013). This is in part due to the complex and unique interplay between a patient's health history, microbiome, environment, and a predominance of culture studies in which total community resolution is limited (Wu et al., 2013). Nevertheless, in our study, patient PM1's pigmented stone bacterial community do approximately align with a previous culture analysis in which *Klebsiella*, *Enterococcus*, *Enterobacter*, *E. coli* and *Pseudomonas aeruginosa* were found to be the most prevalent genera across 61 pigmented and mixed gallstones (Stewart et al., 2000).

Less is known about microbial compositions in cholesterol gallstones, due to their propensity to have low amounts of bacterial DNA. However, this assertion may be due to the limitation of culture studies, as a recent investigation of the microbial composition of 27 cholesterol gallstones using high-throughput 16S rRNA profiling identified *Enterobacteriaceae* to be abundant (Wu et al., 2013), whereas the less abundant genera differed from those present in patient CF4 in our study.

Quantitative PCR revealed a two orders of magnitude higher bacterial 16S rRNA copy number (~ $9.9 \times 10^6 \pm 4.6 \times 10^6$) in pigmented gallstones of PM1 compared to the cholesterol-type gallstones of CF4 ($3.1 \times 10^4 \pm 2.0 \times 10^4$ copies per stone). The results of this cultivation-independent molecular quantification approach are consistent with previous culture studies that report a greater microbial biomass in pigmented *vs.* cholesterol stones (Maki, 1966; Stewart et al., 1987, 2000, 2002; Wu et al., 2013). We provide a conservative estimate of bacterial cell numbers (1.2×10^6

$\pm$ 5.8 x $10^{5)}$ bacterial cells per stone in PM1 and 7.7 x 10^3 $\pm$ 5.0 x 10^3 cells per stone in CF4) based on known number of ribosomal operons in the most abundant genera found in PM1 (*Klebsiella* with 8 rRNA operons) and CF4 (*Streptococcus* with 4 rRNA operons) (Klappenbach et al., 2000; Iwase et al., 2016).

Functional metagenomics profiling

Genes involved in Bile Stress response

Microorganisms have been known to survive and thrive in a range of extreme environments, including the human body where variations in pH, nutrient limitations, low oxygen levels, an established diverse bacterial microbiome, and host immunity responses provide long-term obstacles for survival (Begley et al., 2005; Wu et al., 2011; Sistrunk et al., 2016).

The human gallbladder environment poses its own unique challenges. The liver secretes approximately 800-1000 mL of bile into the gallbladder per day (Tortora & Derrickson, 2014). Bile acts as a detergent or emulsification agent for the digestion and absorption of lipids and contains sodium and potassium salts, bile acids (namely chenodeoxycholic acid, cholic acid), cholesterol, phospholipids, and bile pigments such as bilirubin (Tortora & Derrickson, 2014). This environment is made further hostile to bacteria as bile is concentrated 5-10 fold in the gallbladder, with commonly used antibiotic drugs, and heavy metals being secreted into bile as per the liver's detoxification and enterohepatic cycling processes (Begley et al., 2005; Tortora & Derrickson, 2014).

The strategies bacteria employ to resist toxic agents such as bile and antibiotics are often complex and involve a variety of methods that include and are not limited to efflux pumps (which pump the toxic agent out of the cell), reduction of cell permeability, enzymatic modification or destruction of toxic agents either within or without the cell wall barrier, and the modification of the toxic agent's target either *via* genetic mutation, enzymatically, or by presenting an alternate target (Walsh, 2000; Lebeaux et al., 2014). Bile, as a detergent, and consisting of a variety of toxic agents has been shown to cause membrane perturbations, DNA damage and oxidative stress in bacteria (Lebeaux et al., 2014). This is shown to be consistent with our current study where multidrug export efflux pumps, DNA and cell wall repair

proteins were identified as important in bile resistance. Of the seventeen taxa in both patients combined that comprised at least 1% of the total sequenced gene pool, we identified six genera that harbour genes involved in bile stress survival, which are linked to the production of biofilms that may be associated with pigmented as well as cholesterol-type gallstone formation (Table 2.2, 2.3 and 2.4) as discussed in detail below.

Table 2.2 Loci disrupted in bile-sensitive mutants and the functions of gene products.

Genes disrupted in bile-sensitive mutants	Function of gene products/putative function	Reference(s)
Gram-negative bacteria		
Klebsiella		
marA	Multiple antibiotic resistance protein - Regulatory genes	Shen.,2015;Wu.,2011;Walsh, 2000
marB	Multiple antibiotic resistance protein - Regulatory genes	Shen., 2015;Wu ., 2011;Walsh, 2000
marC	Multiple antibiotic resistance protein Regulatory genes	Shen., 2015;Wu., 2011;Walsh, 2000
marR	Regulatory genes	Shen.,2015;Wu.;2011;Walsh, 2000
TolC precursor	Efflux pump function	Wu., 2011; Walsh 2000.
dam	DNA adenine methylase	Prouty., 2004.
emrE	Efflux pump –drug resistance	Leverrier., 2004
emrB	Efflux pump – drug resistance	Leverrier., 2004
mdtABCD	Efflux pump –multi drug resistance	Leverrier., 2004;Sulavik., 1997
cmeA	Efflux pump	Leverrier., 2004;Prouty., 2004
cmeB	Efflux pump	Leverrier., 2004; Prouty., 2004
sbcC	Exonuclease	Thanassi., 1997
MutS	DNA mismatch repair	Thanassi., 1997
nifJ homolog	Pyruvate flavodoxin oxidoreductase	Thanassi., 1997
yvaG	3-oxoacyl-acyl-carrier protein reductase	Thanassi., 1997
dgt homolog	Deoxyguanosinetriphosphate triphosphohydrolase	Thanassi., 1997
Escherichia		
yvaG	3-oxoacyl-acyl-carrier protein reductase	Thanassi., 1997
Shigella		
PhoQ	Regulatory genes	Dal Santo et al., 2012
Gram-positive bacteria		
Enterococcus		
emrB	Efflux pump – drug resistance	Leverrier., 2004
sbcC homolog	Exonuclease	Thanassi., 1997
MutS	DNA mismatch repair	Thanassi., 1997
nifJ homolog	Pyruvate-flavodoxin oxidoreductase	Thanassi., 1997
yvaG	3-oxoacyl-acyl-carrier protein reductase	Thanassi., 1997

dgt homolog	Deoxyguanosinetriphosphate triphosphohydrolase	Thanassi., 1997

Table 2.3 Promoters, proteins or open reading frames (ORFs) induced by bile and their functions.

ORFs/promotors/proteins induced by bile	Function of gene products/putative function	Reference(s)
Gram-negative bacteria		
Klebsiella		
DnaK	Molecular chaperone	Sistrunk., 2016; Thanassi., 1997
GroEL/GroES	Heat shock protein- Molecular chaperone	Sistrunk., 2016; Thanassi., 1997
Ohr	Organic hydroperoxide resistance	Shen., 2015; Sistrunk., 2016; Thanassi., 1997
Hsp20	Heat shock protein, molecular chaperone	Sistrunk., 2016
ClpB	ATP-binding chain of an ATP-dependent protease	Sistrunk., 2016
RecR homolog	Recombinase (DNA repair)	Shen., 2015; Smart., 2013
sodA	Manganese superoxide dismutase	Sistrunk., 2016; Lin., 2003
BCCP	Biotin-containing carboxyl carrier protein of acetyl-CoA carboxylase	Sistrunk., 2016; Perez., 2009
CysK homolog	Putative cysteine synthase	Sistrunk., 2016; Smart., 2013
ORF002	NADPH dependent aldo or keto-oxidoreductase	Heithoff., 2001; Le Breton., 2002
MutB	Putative Methylmalonyl-CoA mutase	Shen., 2015; Smart., 2013
AspA	Putative Aspartate ammonia-lyase	Sistrunk., 2016;
G6PD	Glucose-6-phosphate 1-dehydrogenase	Rince., 2001; Hu., 2010
ATPG	ATP synthase gamma chain	Sistrunk., 2016; Perez., 2009
HemH homolog *dmsABC*	Putative Ferrochelatase, protoheme ferro-lyase Anaerobic dimethyl sulfoxide reductase chain A, B & C	Sistrunk., 2016; Smart., 2013; Chowdhury., 1996;Lee.,1998 ;Fink., 2007
Escherichia		
ORF002	NADPH dependent aldo or keto-oxidoreductase	Rince., 2001; Hu., 2010
Shigella		
ORF001	NADPH dependent aldo or keto-oxidoreductase	Rince., 2001; Hu., 2010
ORF002	NADPH dependent aldo or keto-oxidoreductase	Rince., 2001; Hu., 2010
Serratia		
ClpB	ATP Binding Chain on ATP-dependent protease	Sistrunk., 2016;
Gram-positive bacteria ***Enterococcus***		
DnaK	Molecular chaperone	Sistrunk., 2016; Thanassi., 1997
GroEL/GroES	Heat shock protein- Molecular chaperone	Sistrunk., 2016; Thanassi., 1997
Ohr	Organic hydroperoxide resistance	Shen., 2015,26
Gsp	General stress protein	Shen., 2015, Sistrunk.,2016; Thanassi., 1997
clpB	ATP Binding Chain on ATP-dependent protease	Sistrunk., 2016

sodA	Manganese superoxide dismutase	Sistrunk., 2016,Lin., 2003
BCCP	Biotin-containing carboxyl carrier protein of acetyl-CoA carboxylase	Sistrunk., 2016; Perez., 2009
CysK homolog	Putative cysteine synthase	Sistrunk., 2016; Smart., 2013
OppD homolog	Oligopeptide transport ATP-binding protein	Sistrunk., 2016; Arese., 2012
G6PD	Glucose-6-phosphate 1-dehydrogenase	Rince., 2001; Hu., 2010
ATPG	ATP synthase gamma chain	Sistrunk., 2016; Perez., 2009
Bacillus		
OppD homolog	Oligopeptide transport ATP-binding protein	Sistrunk., 2016; Arese., 2012

Table 2.4 Genes associated with biofilm production and their functions/putative functions

EPS related genes	Function of gene products/putative function	Reference(s)
Gram-negative bacteria		
Klebsiella		
CsgD	Transcriptional regulator	Swidinski., 2001
Gsp	General stress protein 18	Gralnick., 2006
fim Type I	Type 1 fimbriae fimA,B,D,E,L,F,G	Swidinski., 2001;Chowdhury., 1996
fim Type IV	Type IV fimbrial assembly, ATPase PilB	Swidinski., 2001;Chowdhury., 1996
wza	Polysaccharide export lipoprotein	Swidinski., 2001;Chowdhury., 1996
wzc	Tyrosine-protein kinase	Swidinski., 2001;Chowdhury., 1996
Ribose ABC Transport System	Ribose ABC transport system, ATP-binding protein RbsA	Chowdhury., 1996
Ribose ABC Transport System	Ribose ABC transport system, permease protein RbsC	Chowdhury., 1996
Autoinducer 2(AI-2)	ABC transport system, fused AI2 transporter subunits and ATP-binding component	Chowdhury., 1996
CP4-57- integrase	putative CP4-57-type integrase	Chowdhury., 1996
Polyphosphate kinase	Polyphosphate kinase- Biofilm development	Rashid, 2000
sugE	Quaternary ammonium compound-resistance protein	Swidinski., 2001
ClpX	ATP-dependent Clp protease ATP-binding subunit	Swidinski., 2001
RapA	RNA polymerase associated protein reg. yhcQ, YeeZ	Wu., 2011
LuxR	Transcriptional regulator	Swidinski., 2001
CspD	Cold shock protein CspD	Swidinski., 2001
Escherichia		
wzb	Low molecular weight protein-tyrosine-phosphatase	Chowdhury., 1996
wzc	Tyrosine-protein kinase	Chowdhury., 1996
Gram-positive bacteria		
Enterococcus		
RbsA	Ribose ABC transport system, ATP-binding protein	Chowdhury., 1996
galE	UDP-glucose 4-epimerase	Fink., 2007
sugE	Quaternary ammonium compound-resistance protein SugE	Swidinski., 2001
ClpX	ATP-dependent Clp protease ATP-binding subunit	Swidinski., 2001
LuxR	Transcriptional regulator	Swidinski., 2001
CspD	Cold shock protein CspD	Swidinski., 2001

Gram-negative Bacteria

Klebsiella (bile-sensitive genes)

The *marABC* and *marR* operons (for all genes identified see Table 2.2) are regulatory genes that control multiple antibiotic drug resistance (Sulavik et al., 1997) and have been shown to be activated in the presence of the bile salt deoxycholate, with the level of gene expression dependent on the salts' concentration (Begley et al., 2005; Prouty et al., 2004). The *Tol* protein and derivatives are important in many Gram-negative bacterial transport systems and act as an outer membrane pore function or efflux pump (Bina et al., 2001). Mutations in *Tol* genes destabilise the membrane allowing for greater bile salt entry, thereby affecting bile resistance (Bina et al., 2001). The *TolC* efflux pump (Table 2.2), in particular, has been shown to be upregulated in biofilms and is associated with the removal of toxic compounds and antibiotics (Lebeaux et al., 2014). The *emrEB, mdtABCD, cmeAB* genes similarly correspond to efflux pump action and are essential for bile resistance (Bina et al., 2001). The *emrEB* multidrug efflux pump systems have been shown to actively efflux the bile salt chenodeoxycholic acid (Thanassi et al., 1997). Furthermore, over-expression of *mdtABCD,* a multidrug resistance efflux pump cluster, leads to increased deoxycholate resistance (Baranova et al., 2002). The *cmeAB* has been shown to function as a multidrug efflux pump in *C. Jejuni* by effectively mediating resistance to bile salts (Lin et al., 2003). The *Dam* (DNA adenine methylase) enzyme has been associated with repairing damage to *Salmonella* DNA after bile acid exposure (Heithoff et al., 2001). Similarly, the *sbcC* and *MutS* (DNA repair), *yvaG* (rebuilding the cell membrane after stress), *nifJ* (oxidative response) and *dgt* (dGTP hydrolysis) homologs were associated with DNA and cell wall repair in response to bile stress in *Enterococcus faecelis* (Le Breton et al., 2002).

Klebsiella (ORF's, promotors, proteins induced by bile)

Experiments with bile salt treatments to *Enterococcus faecelis* (Le Breton et al., 2002; Begley et al., 2005) identified an increased production of a number of stress proteins (*Gsp*). Three of these stress proteins were identified in this study as the *DnaK* and *GroEL/GroES* molecular chaperones (Le Breton et al., 2002) and the organic hydroperoxide resistance protein *Ohr* (Begley et al., 2005; Rince´ et al., 2001) within *Klebsiella* (Table 2.3). Further experiments with *Propinobacterium*

freudenraichii revealed genes involved in a variety of stress responses (heat, acid, bile salts) termed *GSPs* (General stress proteins), with bile salts in particular associated with oxidative stress responses (Leverrier et al., 2004). A number of these were annotated to *Klebsiella* in this study and include the molecular chaperones *Hsp20* (heat stress), *DnaK, GoEL, AspA and ClpB* that are associated with acid stresses (Leverrier et al., 2004). *SodA,* an oxidative damage remediation gene, was also identified in this study and has been shown to be involved in stress responses within *Lactobacillus lactis* (oxygen stress), *Bacillus subtilis* (heat, salt and ethanol stresses), and *B. cereus* (heat, salt and ethanol stress) (Leverrier et al., 2004; Hu et al., 2010). Further oxidative damage reduction and remediation proteins identified for *Klebsiella* were *ORF002*, *G6PD*, and the *CysK*, *HemH* homologs. The NADPH dependent aldo or keto-oxidoreductase *ORF002* protein is an important part of the glutathione cellular defense system that is involved in the reduction of oxidative stress caused by reactive oxygen species (ROS) associated with bile (Hu et al., 2010; Smart et al., 2013). The *G6PD* (Glucose-6-phosphate 1-dehydrogenase) protein has been shown to be activated in the presence of ROS, that arise due to stresses such as high levels of salt, and considered vital for cellular redox balance (Dal Santo et al., 2012; Arese et al., 2012). Similarly, the *CysK* (cysteine synthase) and *HemH* (ferrochetalse) homologs have also been shown to be overexpressed when exposed to bile-salt stresses (Leverrier et al., 2004; Lu et al., 1990). Other acid stress proteins identified were *BCCP* (a biotin containing carboxyl carrier protein) and *ATPG* (ATP synthase gamma chain) (Leverrier et al., 2004; Oliveira et al., 2017). The DNA damage repair proteins *MutB* (Methylmalonyl-CoA mutase) and *RecR* (Recombinase) were also identified (Begley et al., 2005; Lu et al., 1990).

Genes encoding the dissimilatory dimethylsulfide reductase A, B and C (dmsABC, Table 2.3) were retrieved in *Klebsiella,* indicating its capacity for anaerobic metabolism and use of dimethylsulfoxide (DMSO) as a terminal electron acceptor. DMSO respiration is energetically favourable under anaerobic conditions in bacteria that contain this metabolic potential (Gralnick et al., 2006). Further, the dmsABC operons are controlled by the oxidative regulator *fnr*, important for oxidative stress response and anaerobic metabolism in pathogenic bacteria (Chowdhury et al., 1996; Lee et al., 1998; Fink et al., 2007). This ability by *Klebsiella* may explain its successful survival and growth in the anoxic conditions present in the human

gallbladder and its dominance in the present study. Other gram-negative and gram-postive bacteria identified in this study may be out competed by *Klebsiella* or utilise fermentation for energy metabolism instead, a less efficient form of energy conservation than DMSO (Poole et al., 2011).

Escherichia (bile-sensitive genes)

Similar to *Klebsiella*, *Escherichia* exhibited the oxido reductase gene *yvaG* or membrane composition and repair protein in our study (Table 2.2).

Escherichia (ORF's, promotors, proteins induced by bile)

Like *Klebsiella*, *Escherichia* exhibited the NADPH dependent aldo or keto-oxidoreductase *ORF002* protein involved in the reduction of oxidative stress caused by ROS associated with bile (Table 2.3).

Shigella (bile-sensitive genes)

Shigella exhibited the *PhoQ* regulatory protein (Table 2.2). *PhoQ* is closely associated with the *PhoP* regulon. The combined *PhoP-PhoQ* proteins have been associated with various bacteria and their ability to sense and resist bile stress (van Velkinburgh et al., 1999). Bacterial mutants missing *PhoP-PhoQ* were killed at significantly lower concentrations of bile than those with these proteins, and those with *PhoP* alone surving a >60% concentration of bile in lab conditions (van Velkinburgh et al., 1999).

Shigella (ORF's, promotors, proteins induced by bile)

Similar to *Klebsiella* and *Escherichia*, *Shigella* exhibited the NADPH dependent aldo or keto-oxidoreductase *ORF001* and *ORF002* proteins involved in bile oxidative stress reduction (Table 2.3).

Serratia (ORF's, promotors, proteins induced by bile)

Bile sensitive genes for *Serratia* were not identified. However, *Serratia* exhibited the *ClpB* molecular chaperone involved in acid stress reponses (see *Klebsiella* above).

Gram-positive bacteria

Enterococcus (bile sensitive genes)

Similarly to *Klebsiella*, the important proteins for bile resistance in *Enterococcus* were the multidrug export efflux pump system *emrB*, and those associated with DNA and cell wall repair; *sbsC homolog*, *MutS*, *yvaG*, *nifJ* and *dgt* homologs.

Enterococcus (ORF's, promotors, proteins induced by bile)

As per *Klebsiella*, we see the following same bile stress, oxidative stress and DNA repair genes associated with *Enteroccoccus*: *Dnak*, *GroEL/GroES*, *Ohr*, *Gsp*, *clpB*, *sodA*, *BCCP*, *G6PD*, *ATPG* and the *CysK homolog*. The *OppD homolog* (ABC transporter – ATP binding protein), involved in the efflux of bile and antibiotic resistance during biofilm formation, was also present (Leverrier, et al., 2004; Ito et al., 2009).

Bacillus (ORF's, promotors, proteins induced by bile)

Bile sensitive genes for *Bacillus* were not identified in the metagenomes. However, Bacillus exhibited the *OppD homolog* as described above for *Enterococcus*.

Genes involved in biofilm production

In addition to mediating toxic substances *via* the resistance strategies described above, microorganisms can group together, attach to either living and non-living surfaces, and form what is called a biofilm (Donlan, 2002). Biofilms comprise a variety of microorganisms enclosed in an extracellular polymeric substance (EPS) or matrix made up of mostly polysaccharides and other environmental specific materials (Donlan, 2002). Biofilm formation has considerable advantages and has shown to protect bacterial communities from UV light, heavy metals, acidity, hydration or salinity changes and host immune responses, including large doses of antimicrobial drugs that would be lethal to the same community if in a planktonic state (Lebeaux et al., 2014; Hentzer et al., 2003). There are three main stages involved in biofilm formation: initial adherence to a surface, development of a community structure and ecosystem, and eventual detachment (Donlan, 2002). Each stage is a complex process regulated by a variety of genes that are often environmental, bacterial strain or stressor specific (Lebeaux et al., 2014). Common

mechanisms include the development of curli fimbriae (adherence or attachment mechanisms), and quorum-sensing or cross community communication to coordinate biofilm attachment, development, detachment, and resistance (Hentzer et al., 2003; Landini, 2009). The main resistance mechanisms afforded by the EPS matrix include drug indifference, in which the EPS works as a barrier between the drug and the targeted microbial cell membrane, the allowance of antibiotics to slowly diffused through the EPS, so that time is given for resistance mutations to develop, efflux pumps, and the secretion of periplasmic glutans that keep toxic substances away from intracellular targets (Lebeaux et al., 2014). Certain organisms may provide the base biofilm, whilst others live either competitively or symbiotically within it, with environmental and community composition in a state of constant change (Hentzer et al., 2003). As previously reported, biofilms with *Salmonella* were identified through SEM and culture analysis, on the surface of cholesterol gallstones (Marshall et al., 2014). Our study also suggested the presence of a biofilm on the surface of both pigmented and cholesterol type gallstones since stringent UV sterilisation initially resulted in a reduction of the yield of extracted genomic DNA by over 90%. Indeed the presence of a microbial biofilm on the surface of the patients' gallstones was confirmed by taxonomic and functional metagenomics analysis. The following genera were identified in the gallstone metagenomes that harbor genes, which are putatively associated to biofilm production.

Klebsiella

The *CsgD* gene is a transcriptional activator involved in the regulation of curli fimbriae biosynthesis (Landini, 2009) (Table 2.4). Curli fimbriae have been identified to be significant EPS components within *Enterobacteriaceae* and involved in bacterial adherence to abiotic substances and cell adhesion during symbiotic or infectious processes (Landini, 2009). *Klebsiella*, an enterobacterium, has exhibited the *CsgD* gene, alongside the Type 1 and Type IV fimbriae in this study. The Type 1 and Type IV fimbriae are part of the gene cluster *fim*, containing all the components required for fimbrial assembly, and associated with capsule and pilin processes - significant factors in colonisation and biofilm production (Wu et al., 2011). Type 1 and Type IV fimbriae have also been shown to facilitate biofilm assembly on both abiotic and host-derived extracellular matrix protein surfaces (Landini, 2009). The

wza and *wzc* genes encode for surface molecules involved in capsule assembly and are considered to be important in the early stages of biofilm formation by *Klebsiella pneumoniae* (Landini, 2009; Wu et al., 2011). A study isolating the genes involved in biofilm formation of the *K. pneumoniae* strain causing Pyogenic Liver Abscess found *SugE* an important gene that affects biofilm production by modulating capsular polysaccharide production and biofilm mucoviscosity (Wu et al., 2011). The *ClpX* and *LuxR* regulatory genes, part of the sugar-specific phosphotransferase systems, and the cold shock protein *cspD* were also implicated in biofilm production (Wu et al., 2011).

Recently, various strains of *Klebsiella* were tested to determine those with the highest biofilm production and the genes associated with this process (Landini, 2009). The strain identified with the highest output of biofilm implicated the *RbsA* and *RbsC* genes alongside the quorum sensing molecule Autoinducer 2 (AI-2*)* and the prophage CP4-57 integrase as putitavively involved in the process (Landini, 2009). These important genes associated with *Klebsiella* were also identified in our study (Table 2.4) The *RapA* gene identified has been shown to play a role in regulating the *yhcQ* gene that encodes a putative multidrug efflux pump and *yeeZ*, a gene associated with biofilm production (Lebeaux et al., 2014). Polyphosphate kinase has been linked with biofilm development, quorum sensing and virulence in *P. aeruginosa* and was also annotated to *Klebsiella* in our study (Rashid et al., 2000; Bandeira et al., 2017).

Escherichia

The surface molecule encoding *wzb* and *wzc* genes, important for capsule assembly and early stage biofilm formation in *K. pneumoniae* (Begley et al., 2005; Landini, 2009), were also associated with *Escherichia* in our study (Table 2.4).

Enterococcus

Similar to genes involved in biofilm formation (the *RbsA, SugE, ClpX, LuxR, CspD*) discussed for *Klebsiella* above were associated with *Enteroccoccus* in our study. *Enterococcus* also exhibited the *galE* gene shown to influence lipopolysaccharide structure, colonisation and biofilm formation (Nesper et al., 2001).

Other relevant cellular processes

Consistent with a more prominent presence of number of species and genes encoding for processes associated with resistance to oxidative stress from bile and biofilm production, patient PM1 also exhibitied an overall higher abundance of genes involved in stress response, cell wall and capsule production, cluster-based subsystem activity as well as carbohydrate metabolism compared to patient CF4 (Figure 2.4). A potentially enhanced carbohydrate metabolism in PM1 may be attributed to low levels of nutrients in the gallbladder resulting in microbes to metabolise excess biofilm (namely polysaccharides), as reported previously (Bhatt et al., 2013). In contrast, Gram-positive bacteria, mainly associated with gallstones of patient CF4, reveal a higher relative abundance of genes involved in protein metabolism. One hypothesis is that these bacteria are involved in the decomposition of dead human cells associated with the gallstones of this patient (Figure 2.4) as inferred from the high relative abundance of human genes compared to PM1. Another explanation for the observed difference in a carbohydrate *vs.* protein dominated microbial metabolism between the gallstone types is that biofilms of Gram-negative and Gram-positive bacteria differ chemically from each other. EPS produced by Gram-negative bacteria, which predominate in PM1, exhibit anionic properties (attributed to uronic acids) that enable calcium and magnesium ions to bind with polymer strands providing a more tightly bound biofilm architecture[45]. Gram-positive bacteria (mainly in CF4) have been shown to exhibit a more cationic EPS charge, and be composed of teichoic acid mixed with small quantities of protein (Donlan et al., 2002; Hussain et al., 1993), which may explain a higher relative abundance of genes involved in protein metabolism.

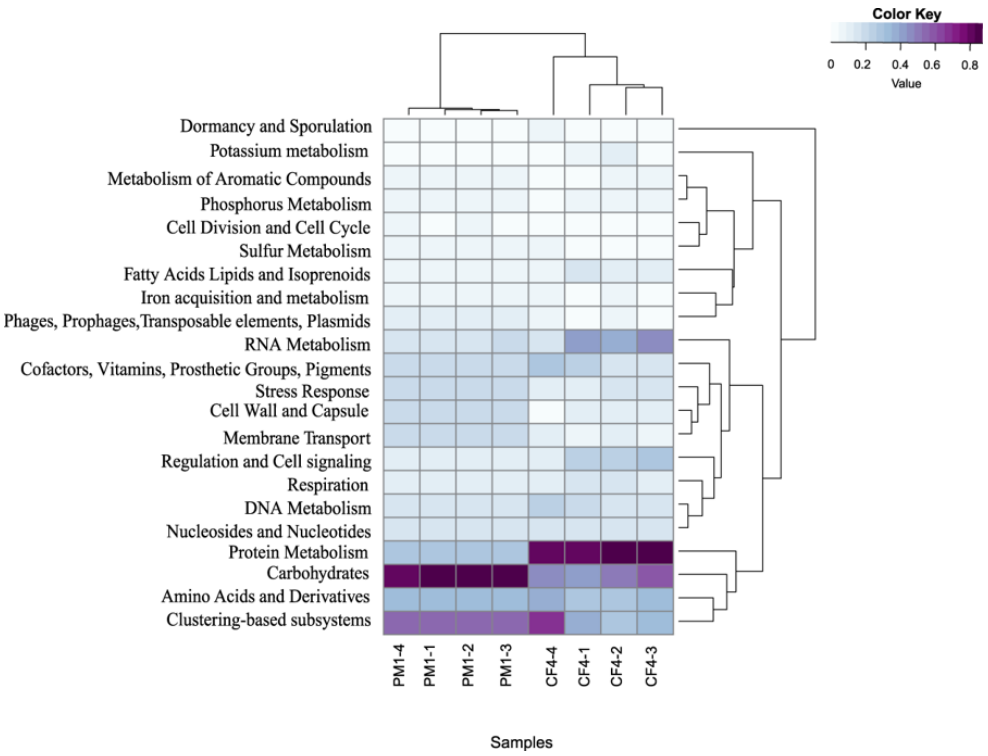


Figure 2.4: Heatmap with the major functional gene categories (acquired from the Subsystems, SEED Database) identified in the gallstones (n=4) of patients PM1 (pigmented) and CF4 (cholesterol). The color key shows the relative abundance of the gene categories in the gallstones. The dendrograms illustrate the relationship between samples showing that the distribution of genes is relatively similar between replicate stones, but greatly differ between the two patients. For example, bacterial genes involved in the carbohydrate vs. protein metabolism were more abundant in gallstones from PM1 vs. CF4.

Cholesterol analysis

We conducted compound specific isotopic analysis (CSIA) of the patients' carbon ($\delta^{13}C$) and hydrogen (δD) isotopes of cholestrol to ascertain possible dietary or exogenous environmental factors that may be associated with or divergent from the bacteria identified in this study. Individual compounds in a complex mix (i.e. a gallstone made from patient-specific cholesterol/bile mixtures) can have distinct differences in their carbon, hydrogen, oxygen, nitrogen or sulfur isotopic signatures (Mancini et al., 2008). The marked difference between the isotopic weight of a $\delta^{13}C$ value of an identified compound in two gallstones, for example, can indicate that a different source or mechanism was utilised during the synthesis of the compound (Mancini et al., 2008). We investigated whether or not the patients' native stones were homogenous and if homogeneity existed between the patients themselves.

The only molecular component identified by GC-MS within the stones of each patient was the cholest-5-en-3β-ol compound with trace amounts of 5α-cholest-7-en-3β-ol (see Appendix 1). Subsequently, CSIA was conducted on the non-derivatised cholest-5-en-3β-ol compound of each of the patients' stones (Table 2.5).

Table 2.5. Compound Specific $\delta^{13}C$ and δD values for the patients PM1 and CF4. The isotopic value is followed by the standard deviation (in brackets) obtained by the number of repeat injections (3).

$\delta^{13}C$ (‰VPBD)				**δ^{D}(‰VSMOW)**			
PM1-1	−24.7(0.1)3	CF4-1	−23.1(0.1)3	PM1-1	−231(0)3	CF4-1	−252(0)3
PM1-2	−25.9(0.1)3	CF4-2	−23.5(0.1)3	PM1-2	−222(1)3	CF4-2	−253(0)3
PM1-3	−24.8(0.4)3	CF4-3	−23.3(0)3	PM1-3	−218(3)3	CF4-3	−254(0)3
PM1-4	−24.9(0.1)3	CF4-4	−23.7(0)3	PM1-4	−221(1)3	CF4-4	−254(1)3

The cholest-5-en-3β-ol $\delta^{13}C$ values obtained for patient PM1 showed a range between –24.7 and –25.9 ‰, and a range between –23.1 and –23.7 ‰ for patient CF4, resulting in an approximately 1 ‰ difference across the four stones analysed for each patient. The cholest-5-en-3β-ol δD showed a value range of –218 and –231 ‰ for patient PM1, resulting in an approximately 13 ‰ difference across the four stones analysed for this patient. The cholest-5-en-3β-ol δD showed a value range of –252 and –254 ‰ for patient CF4, resulting in a negligible 2 ‰ difference across the four stones analysed for this patient.

Within each patient both the $\delta^{13}C$ and δD values for cholest-5-en-3β-ol were not significantly different amongst the 4 stones analysed, supporting the possibility of a common source for cholest-5-en-3β-ol. Between the two patients the $\delta^{13}C$ and δD values differed only by minor amounts also supporting a common source for cholest-5-en-3β-ol.

Conclusions

This pilot study explored taxonomic and functional metagenomics and sterol homogeneity within two patients of diverse backgrounds to elucidate a possible universal factor at play in gallstone pathogenesis and formation. For the first time, functional genes were identified that were associated with bile stress response and biofilm development as possible microbial processes leading to the formation of both pigmented and cholesterol-type gallstones. In the analysed pigmented stones, genes involved in biofilm formation were mainly recovered from clinically pathenogenic *Klebsiella* and *Enterococcus* while bile resistance genes were present also in *Escherichia*, *Shigella*, *Serratia* and *Bacillus*. *Klebsiella* was also present in one of the chlolesterol gallstones, while the remaining analysed cholesterol stones showed a predominance of Gram-positive bacteria that were not identified within the pigmented stones. *Klebsiella* was also the only genus to exhibit DMSO respiration, giving it a distinct advantage in the anoxic environment of the human gallbladder. This, in conjuction with being the genus to exhibit the highest number of genes involved in bile stress response, and biofilm formation, may place *Klebsiella* as a major player in gallstone pathogenesis and formation. Further, pigmented stones, predominated by Gram-negative bacteria, revealed a high proportion of genes involved in carbohydrate metabolism, whilst cholesterol stones indicated a profile dominated by protein metabolism. A possible explanation for the observed difference in a carbohydrate vs. protein dominated microbial metabolism between the gallstone types is that biofilms of Gram-negative and Gram-positive bacteria differ chemically from each other resulting with the latter having a higher protein content in the EPS matrix. Fungal and archaeal genes were not detected in both types of stones. Complementary carbon and hydrogen isotopic analyses of cholesterol within the patients' stones revealed homogeneity, suggesting a common diet or cholesterol synthesis pathway that has only a minor influence on microbial composition.

This pilot study provides a framework to study microbial processes that play a potential role in gallstone formation across markedly different types of stones and patient backgrounds. In addition, future studies could also involve metatranscriptomic profiling to ultimately reveal which bacteria are actively

expressing genes involved in processes such as bile stress response and biofilm formation that could contribute to the pathogenesis of gallstones.

Methods

Sample collection

Samples were collected whilst the patients were undergoing laproscopic cholecystectomy and were immediately rinsed in sterile saline solution (9 g L^{-1} NaCl) and placed in sterile glass containers. The samples were immediately stored at −80 °C until further processing. All patients provided written informed consent upon enrolment to the study. The study was designed with the aid of the National Health and Medical Research Council (NHMRC), according to the guidelines stipulated in the National Statement on Ethical Conduct in Human Research 2007 (NHRMC, 2007a) and the Australian Code for the Responsible Conduct of Research 2007 (NHMRC, 2007b). The study, which includes all associated experiments and methods, was approved by and met the ethical guidelines of the South Metropolitan Health Service Human Research Ethics Committee (HREC Reference: 15-136), the Fiona Stanley Human Research Ethics Committee (Ref: 2015-136), the St John of God Health Care Human Research Ethics Committee (Ref: 1021), and the Curtin University Human Research Ethics Committee (Ref: HR229/2015).

DNA extraction

Genomic DNA of 4 gallstones from each patient were obtained from extractions following the procedure described by Haigh and Lee (2001). Inside a HEPA-filtered laminar flow bench, individual gallstones (~100 mg each) were pulverized using a heat-sterilized mortar (500 ºC, 8h). 700 uL of 1% SDS solution was added to each pulverized gallstone and incubated under rotation at room temperature for 12h). Lithium chloride was added (final concentration of 1.5 M) following cell lysis through homogenisation in a FastPrep 96 Instrument (MP Biomedicals LLC, NSW, Australia) (1600 rpm, 60 sec). After centrifugation (5 min, 10,000 rcf) 1 vol of Phenol-Chloroform-isoamylalcohol 25:24:1 (PCI) pH 8 was added to the supernatant, vortexed for 1 min., and centrifuged for 5 min at 10,000 rcf. The PCI extraction was repeated once and 0.4 vol of molecular grade 80 vol% ethanol was added to the aqueous phase. The sample was then transferred to a Spin™ Filter and

DNA was eluted from the filter using Solution C6 following the guidelines of the PowerSoil DNA Isolation Kit (Mo Bio Laboratories Inc, CA, U.S.A). PCR-inhibiting impurities were completely removed using the OneStep PCR Inhibitor Removal Kit (Zymo Research, U.S.A). The DNA concentration was quantified fluorometrically (NanoDrop 3300 Fluorospectrometer; Thermo Fisher Scientific, MA, U.S.A) using the Quant-iT PicoGReen dsDNA Assay kit (Life Technologies, VIC, Australia).

Quantitative PCR

To quantify the amount of bacterial 16S rRNA gene copies, an aliquote of the extracted and purified genomic DNA was subjected to quantitative polymerase chain reaction (qPCR) using general primers (Caporaso et al., 2012) targeting the V4 region of bacterial 16S rRNA. All reactions were performed using SYBR Premix Ex Taq (TLi RNase H Plus) (Takara Bio Inc) in a Realplex quantitative PCR cycler (Eppendorf) and involved initial denaturing (1 min at 95°C), followed by 32 cycles including denaturing (5 s at 95 °C), primer annealing (30 sec at 60 °C), primer extension and imaging of newly formed fluorescent (SYBR®green I labelled) double-stranded DNA (72 °C for 60 sec). Between 10^1 and 10^6 copies (10-fold dilution series) of bacterial 16S rRNA were added to reaction mixtures and served as standards during qPCR to calibrate the copy numbers of bacterial 16S rRNA in the gallstone samples.

Metagenomic library preparation and sequencing

Metagenomic libraries were prepared using the NEBNext Ultra II DNA Library Prep Kit for Illumina (New England BioLabs Inc.) according to manufacturer's instructions. The amplification involved 13-15 cycles. The resulting libraries were concentrated to a volume of 20 uL using Amicon Ultra centrifugal filer units Ultra-0.5 MWCO 30KDa. Gel electrophoresis (2 wt%, 50 min, 120V) was performed with 10 uL of the concentrated libraries and gel fragments (200-500 bp) were excised and gel purified with the Monarch DNA Gel Extraction Kit (New England BioLabs Inc). The final volume after gel purification for each barcoded library (n=8) was 20 uL and were sent to the Australian Genomic Research Facility (AGRF) in Perth, Western Australia for final quality checking and sequencing. At AGRF, the Illumina HiSeq2500 platform was used to generate 2 × 100-bp pair-end sequencing reads. The

HiSeq Control Software (HCS) v2.2.68 and Real Time Analysis (RTA) v1.18.66.3 software performed real-time image analysis and base calling on the HiSeq instrument computer. The AGRF Illumina bcl2fastq 2.19.0.316 pipeline was used to generate the sequence data.

Processing of sequence data and bioinformatics

Approximately 280 million paired-end sequence reads (see Supplementary Table S1 online) were imported into CLC Genomics Workbench 8.0 (CLC Bio) and trimmed of ambiguous reads to a quality limit of 0.5. Contigs were assembled using the CLC Genomics Workbench paired-end Illumina (*de novo*) read assembler with automatic bubble and word size, length fraction of 0.5, similarity fraction of 0.95, and a minimum contig size cut-off of 300 nucleotides. Contigs were assembled without scaffolding to reduce the formation of chimaeric assemblies. The CLC Genomics Workbench read mapping option was used to map reads onto contigs. ORFs within the contigs were detected using FragGeneScan (Orsi et al., 2018). Taxonomic assignments of contigs were performed using the NCBI BLASTp software suite against the SEED database of predicted proteins from cultivated microbial genomes with assigned taxonomy. The basis for taxonomic assignment of the ORFs was amino acid similarity of >60% over an alignment length of >50 amino acids to predicted proteins present in the database with an assigned taxonomy (Orsi et al., 2018).

A matrix showing the relative abundance (average coverage) of annotated ORFs deriving from specific taxa per sample was produced using a python script publicly available online (bitbucket.org/wrf) and was subsequently used for downstream analysis. Heatmaps were performed in R (http://www.r-project.org/) using the vegan (http://vegan.r-forge.r-project.org/) and the Bioconductor Heatplus (https://bioconductor.org/biocLite.R) package. The data was normalized, with the Hellinger function used to produce the taxonomy overview heatmap to show species that may have been obscured by the dominant reads (Figure 2.3). The overview of Subsystems, Level 1, cellular processing category annotations were obtained from the SEED database *via* MG-RAST (Project ID: mgp81110-81111; metagenomics.anl.gov). The Primer-E software package (http://www.primer-e.com/) was used to generate principle coordinate analysis (PcoA) plots using the Bray-Curtis distance metric.

Gas Chromatography Isotope Ratio Mass Spectrometry

Four gallstones from each patient were individually crushed in heat-sterilized (500 ºC, 8h) mortars. The ground powder was then extracted *via* sonication (1 h) with dichloromethane (DCM) and methanol (9:1). The extracts were then fractionated by small-scale column liquid chromatography (Bastow et al., 2007). Approximately 2 mg of the total extract was placed on top of a small column (5 x 0.5 cm i.d.) of activated silica gel (160 ºC, 8 h). The first hydrocarbon fraction was eluted with *n*-hexane (2 mL), the second hydrocarbon fraction with DCM in *n*-hexane (1:4, 2 mL), and the more polar fraction with an equal mixture of DCM and methanol (1:1, 2 mL). The fractions were analysed by gas chromatography-mass spectrometry (GC-MS).

The polar fractions (containing cholest-5-en-3β-ol) were each analysed by compound specific isotope analyses to obtain $\delta^{13}C$ and δD values of cholest-5-en-3β-ol. The polar fractions were run as un-derivatised free sterols. Although this method allows for timely and convenient analysis, if samples containg exchangeable hydrogens are inadequately prepared, there is the potential that these hydroxyl groups can exchange with hydrogen from atmospheric water vapour, effecting δD values. All precautions were taken to limit atmospheric exposure in this study. The samples were removed from the patient and immediately placed into sterile containers that were in-turn placed into a liquid nitrogen thermos within the aseptic operational theatre. Once transported the the main analysis site, the samples were kept in a -20 freezer as lower temperatures reduce the rate of exchange. Further, as cholesterol has only one exchangeable hydrogen atom and 45 non-exchangeable hyrdogens, the overall effect on the δD value is considered here to be low (Schimmelmann et al., 2006).

The instrument used was a Thermo Delta V Advantage isotope ratio monitoring mass spectrometer (irMS), coupled to a Thermo Trace GC Ultra via a GC Isolink and Conflo IV. The column used was an Agilent DB-5MS Ultra-Inert, 60 m long, 0.25 mm (i.d.), with 0.25 µm film thickness. An aliquot of 1 µL of each fraction was injected into the split/splitless injector in splitless mode, held at 280 °C. The GC oven was increased from 40 to 325 °C at 10 °C/min, then held at 325 °C for 10 min. The carrier gas used was helium held at a constant flow of 1.5 mL/min.

For the carbon isotope analysis, GC column outflow was passed through the GC Isolink combustion reactor (copper oxide / nickel oxide, 1000 °C) to combust

hydrocarbons to CO_2. For hydrogen isotope analysis, the outflow passed through the high-temperature conversion reactor (graphite-lined, 1420 °C) and was thermally converted to H_2. The CO_2 / H_2 passed through the Conflo IV interface to the irMS, which measured m/z 44, 45 and 46 (for CO_2) or m/z 2 and 3 (for H_2). The $\delta^{13}C$ and δD values were calculated from the measured masses by Thermo Isodat software, and calibrated to the VPDB (for CO_2) and VSMOW (for H_2) scales by comparison with a mixture of *n*-alkane standards of known isotopic composition.

References

Arese, P., Gallo, V., Pantaleo, A., & Turrini, F. (2012). Life and death of Glucose-6-Phosphate Dehydrogenase (G6PD) deficient erythrocytes – Role of redox stress and Band 3 modifications. *Transfusion Medicine and Hemotherapy, 39,* 328-334.

Bandeira, M., Borges, V., Gomes, J.P., Duarte, A. & Jordao, L. (2017). Insights on *Klebsiella pneumoniae* biofilms assembled on different surfaces using phenotypic and genotypic approaches. *Microorganisms, 5,* 2-16.

Baranova, N. & Nikaido, H. (2002). The BaeSR two-compo-nent system activates transcription of the yegMNOB (mdtABCD) transporter gene cluster in *Escherichia col*i and increases its resistance to novobiocin and deoxycholate. *Journal of Bacteriol*ogy, *184,* 4168–4176.

Bastow, T.P., van Aarssen, B.G.K. & Lang, D. (2007). Rapid small-scale separation of saturate, aromatic and polar components in petroleum. *Organic Geochemistry, 38,* 1235-1250.

Begley, M., Gahan, C.G. & Hill, C. (2005). The interaction between bacteria and bile. *FEMS Microbiology Reviews, 29,* 625–651.

Bhatt, V.D., Dande, S.S., Patil, N.V. & Chaitanya, G. J. (2013). Molecular analysis of the bacterial microbiome in the forestomach fluid from the dromedary camel (*Camelus dromedarius*). *Molecular Biology Reports, 40,* 3363-3371.

Bina, J.E. & Mekalanos, J.J. (2001). Vibrio cholerae tolC is required for bile resistance and colonization. *Infection and Immunity, 69,* 4681–4685.

Caporaso, J.G., Lauber, C.L., Walters, W.A., Berg-Lyons, D., Huntley, J., Fierer, N.,…Knight, R. (2012). Ultra-high-throughput microbial community analysis on the Illumina HiSeq and MiSeq platforms. *The ISME Journal,6,* 1621–1624.

Cetta, F. (1991). The role of bacteria in pigment gallstone disease. *Annals of Surg*ery, *213,* 315-26.

Chowdhury, R. Gautam, K.S. & Jyotirmoy, D. (1996). Stress response in pathogenic bacteria. *Journal of Bioscience*, *21,* 149–160.

Dal Santo, S., Stampfl, H., Krasensky, J., Kempa, S., Gibon, Y., Elena Petutschnig, E.,…Jonak, C. (2012). Stress-induced GSK3 regulates the redox stress response by phosphorylating glucose-6-phosphate dehydrogenase in *Arabidopsis*. *Plant Cell*, *24,* 3380-92.

Donlan, R.M. (2002). Biofilms: Microbial Life on Surfaces. *Emerging Infectious Diseases*, *8*, 881-890.

Fink, R. C., Evans, M. R., Porwollik, S., Vazquez-Torres, A., Jones-Carson, J., Troxell, B., Libby, S. J., McClelland, M., … Hassan, H. M. (2007). FNR is a global regulator of virulence and anaerobic metabolism in Salmonella enterica serovar Typhimurium (ATCC 14028s). *Journal of bacteriology*, *189*(6), 2262-73.

Gralnick, J.A., Vali, H., Lies, D.P & Newman, D.K. (2006). Extracellular respiration of dimethyl sulfoxide by *Shewanella oneidensis* strain MR-1. *PNAS*, *103,* 4669-4674.

Haigh, W.G., & Lee, S.P. (2001). Identification of oxysterols in human bile and pigment gallstones. *Gastroenterology, 121,* 118–123.

Heithoff, D.M., Enioutina, E.Y., Daynes, R.A., Sinsheimer, R.L., Low, D.A. & Mahan, M.J. (2001). *Salmonella* DNA adenine methylase mutants confer cross protective immunity. *Infection and Immununity, 69,* 6725–6730.

Hentzer, M. & Givskov, M. J. Clin. (2003). Pharmacological inhibition of quorum sensing for the treatment of chronic bacterial infections. *Journal of Clinical Investigation*, *112,* 1300–1307.

Hu, Y., Lu, P., Zhang, Y., Li, L. and Chen, S. (2010). Characterization of an aspartate-dependent acid survival system in *Yersinia pseudotuberculosis*. *FEBS Letters*, *584*, 2311–2314.

Hussain M, Wilcox MH, White PJ. (1993). The slime of coagulase-negative-staphylococci: biochemistry and relation to adherence. *FEMS Microbiology Reviews*, *104*, 191–208.

Ito, A., Taniuchi, A., May, T., Kawata, K. & Okabe, S. (2009). Increased antibiotic resistance of *Escherichia coli* in mature biofilms. *Applied and environmental microbiology*, *75*, 4093–4100.

Iwase, T., Ogura, Y., Hayashi, T. & Mizunoe, Y. (2016). Complete genome sequence of *Klebsiella pneumoniae* YH43. *Genome Announcements*, *4*, 00242-16.

Klappenbach, J. A., Dunbar, J. M., & Schmidt, T. M. (2000). rRNA operon copy number reflects ecological strategies of Bacteria. *Applied and Environmental Microbiology*, *66*, 1328–1333.

Landini, P. (2009). Cross-talk mechanisms in biofilm formation and responses to environmental and physiological stress in *Escherichia coli*. *Research in Microbiology*, *160*, 259-66.

Le Breton, Y., Maze, A., Hartke, A., Lemarinier, S., Auffray, Y., & Rince´, A. (2002). Isolation and characterization of bile salts sensitive mutants of *Enterococcus faecalis*. *Current Microbiol*ogy, *45*, 434–439.

Lebeaux, D., Ghigo, J.M. & Beloin, C. (2014). Biofilm-Related Infections: Bridging the gap between clinical management and fundamental aspects of recalcitrance toward antibiotics. *Microbiology and Molecular Biology Reviews*, *78*, 510–543.

Lee, H. S., Lee, Y. S., Kim, H. S., Choi, J. Y., Hassan, H. M. & Chung, M. H. (1998). Mechanism of regulation of 8-hydroxyguanine endonuclease by oxidative stress: roles of *fnr*, *arcA*, and *fur*. *Free Radical Biology and Medicine, 24*, 1193-1201.

Leverrier, P., Vissers, J.P.C., Rouault, A., Boyaval, P. & Jan, G. (2004). Mass spectrometry proteomic analysis of stress adaptation reveals both common and distinct response pathways in *Propionibacterium freudenreichii*. *Archives of Microbiology*, *181*, 215–230.

Lin, J., Sahin, O., Overbye Michel, L., & Zhang, Q. (2003). Critical role of multidrug efflux pump CmeABC in bile resistance and in vivo colonization of *Campylobacter jejuni*. *Infection and Immunity*,*71*, 4250–4259.

Lu, A.L., Cuipa, M.J., Ip, M.S. & Shanabruch, W.G. (1990). Specific A/G-to-CG mismatch repair in *Salmonella typhimurium* LT2 requires the mutB gene product. *Journal of Bacteriology*, *172*, 1232–1240.

Maki, T. (1996). Pathogenesis of calcium bilirubinate gallstone: Role of *E. coli*, beta glucuronidase and coagulation by inorganic ions, polyelectrolytes, and agitation. *Annals of Surgery*, *164*(1), 90-100.

Mancini, S.A., Lacrampe-Couloume, G. & Lollar, B.S. (2008) Source differentiation for benzene and chlorobenzene groundwater contamination: A field application of stable carbon and hydrogen isotope analyses. *Environmental Forensics*, *9*, 177-186.

Marshall, J.M., Fletchtner, A.D., La Perle, K.M & Gunn, J.S. (2014). Visualization of extracellular matrix components within sectioned *Salmonella* biofilms on the surface of human gallstones. *PLoS One*, *9*, 1-7.

Miettinen, T.E., Kesaniemi Y.A., Gylling, H., Jarvinen, H., Silvennoinen, E., & Miettinen, T.A. (1996). Noncholesterol sterols in bile and stones of patients with cholesterol and pigment stones. *Hepatology*, *23*, 274 –280.

National Health and Medical Research Council (NHMRC). (2007a). Australian Code for the Responsible Conduct of Research. Retrieved from https://www.nhmrc.gov.au/guidelines-publications/r39

National Health and Medical Research Council (NHMRC). (2007b). National Statement on Ethical Conduct in Human Research. Retrieved from https://www.nhmrc.gov.au/book/national-statement-ethical-conduct-human-research.).

Nesper, J. Lauriano, C.M., Klose, K.E., Kapfjammer, D., Kraiss, A. & Reidl, J. (2001). Characterization of *Vibrio cholerae* 01 El tor galU and galE mutants: influence on lipopolysaccharide structure colonization, and biofilm formation. *Infection and Immun*ity, *69*, 435–445.

Oliveira L.C. Saraiva, T.L.D., Silva, W.M., Pereira, U.P., Campos, B.C.J., Benevides, L.J.,…Soares, S.C. (2017). Analyses of the probiotic property and stress resistance-related genes of *Lactococcus lactis* subsp. *lactis* NCDO 2118 through comparative genomics and in vitro assays. *PLoS One*, *12*, e0175116.

Orsi, W. D., Richards, T. A., & Francis, W. R. (2018). Predicted microbial secretomes and their target substrates in marine sediment. *Nature Microbiology*, *3*, 32–37.

Perez, M. J. & Briz, O. (2009). Bile-acid-induced cell injury and protection. *World Journal of Gastroenterology, 15*, 1677–1689.

Poole, R.K. (Ed.) (2011). *Advances in Microbial Physiology: Volume 58*. Elsevier: San Diego.

Prouty, A.M., Brodsky, I.E., Falkow, S. & Gunn, J.S. (2004). Bile-salt-mediated induction of antimicrobial and bile resistance In *Salmonella typhimurium*. *Microbiology*, *150*, 775–783.

Rashid, M.H., Rumbaugh, K., Passador, L., Davies, D.G., Hamood, A.N., Iglewski, B.H., Kornberg, A. (2000). Polyphosphate kinase is essential for biofilm development, quorum sensing, and virulence of *Pseudomonas aeruginosa*. *Proceedings of the National Academy of Sciences USA*, *97*, 9636-41.

Rince´, A., Giard, J.C., Pichereau, V., Flahaut, S. and Auffray, Y. (2001). Identification and characterization of gsp65, an organic hydroperoxide resistance (ohr) gene encoding a general stressprotein in *Enterococcus faecalis*. *Journal of Bacteriology*, *183*, 1482–1489.

Schimmelmann, A., Sessions, A.L. & Mastalerz, M. (2006). Hydrogen Isotopic (D/H) Composition of Organic Matter During Diagenesis and Thermal Maturation *Annu. Rev. Earth Planet. Sci*., *34*, 501–33.

Shen, H., Ye, F., Xie, L., Yang, J., Li, Z., Xu, P.,…Zhang, X. (2015). Metagenomic sequencing of bile from gallstone patients to identify different microbial community patterns and novel biliary bacteria. *Scientific Reports, 5*, 17450.

Sistrunk, J.R., Nickerson, K.P., Chanin, R.B., Rasko, D.A., Faherty, C.S. (2016). Survival of the Fittest: How bacterial pathogens utilize bile to enhance infection. *Clinical Microbiology Reviews*, *29*, 819-836.

Smart R.C., & Hodgson, E. (2013) *Molecular and Biochemical Toxicology*, New Jersey: John Wiley & Sons.

Stewart L., Smith, A.L., Pellegrini, C.A., Motson, R.W. & Way, L.W. (1987). Pigment gallstones form as a composite of bacterial micro-colonies and pigment solids. *Annals of Surgery*, *206*, 242-250.

Stewart, L., Ponce, R., Oesterk, A.L., Griffiss, J.M., & Way. L.W. (2000). Pigment gallstone pathogenesis: Slime production by biliary bacteria is more important

than beta-glucuronidase production. *Journal* of *Gastrointestinal Surgery*, *4*, 547.

Stewart, L., Oesterle, A.L., Erdan, I, Griffiss, J.M. & Way, L.W. (2002). The pathogenesis of pigment gallstones in western societies: the central role of bacteria. *Journal of Gastrointestinal Surgery*, *6*, 891–904.

Sulavik, M.C., Dazer, M. & Miller, P.F. (1997). The *Salmonella typhimurium* mar locus: molecular and genetic analyses and assessment of its role in virulence. *Journal of Bacteriol*ogy, *179*, 1857–1866.

Swidinski, A., & Lee, S.P. (2001). The role of bacteria in gallstone pathogenesis. *Frontiers in Bioscience, 6,* e93-103.

Thanassi, D.G., Cheng, L.W. & Nikaido, H. (1997). Active efflux of bile salts by *Escherichia coli*. *Journal of Bacteriology*, *179*, 2512–2518.

Tortora, G. J., & Derrickson, B.H. (2014). *Principles of anatomy & physiology* (12th Edition). U.K: John Wiley & Sons.

van Velkinburgh, J.C. & Gunn, J.S. (1999). PhoP-PhoQ-regulated loci are required for enhanced bile resistance in *Salmonella* spp. *Infection and Immunity, 67,* 1614-22.

Walsh, C. (2000). Molecular mechanisms that confer antibacterial drug resistance, *Nature*, *406*, 775-781.

Wu, M., Lin, T. Hsieh, P., Yang, H. & Wang, J. (2011). Isolation of genes involved in biofilm formation of a *Klebsiella pneumonia* strain causing Pyogenic Liver Abscess. *PLoS One*, *6,* 1-11.

Wu, T., Zhang, Z., Liu, B., Hou, D. Liang, Y., Zhang, J., & Shi, P. (2013). Gut microbiota dysbiosis and bacterial community assembly associated with cholesterol gallstones in large-scale study. *BMC Genomics*, *14*, 1-11.

Acknowledgements

The Fiona Stanley and St John of God, Murdoch hospitals are thanked for facilitating patient consent, sample collection and ethics approval for the study. The authors would like to acknowledge WA-OIGC, TIGeR, Curtin University, and the contribution of an Australian Government Research Training Program Scholarship in

supporting this research. K.G. thanks the Australian Research Council for infrastructure support (LE110100119).

Author contributions

S.H.K, M.J.L.C & K.G designed the experiments. S.H.K, M.J.L.C, W.D.O & K.G carried out the analyses and data interpretation. S.H.K and M.J.L.C wrote the main manuscript text with contributions from K.G, W.D.O and M.B.

Competing interests

The authors declare no competing interests.

Data availability

Data are available as raw sequence reads from the NCBI Short
Read Archive (SRA) under accession number SRP136827 and as assembled contigs in MG RAST (metagenomics.anl.gov) under accession numbers 4754155.3, 4754325.3, 4754326.3, 4754607.3, 4754608.3, 4754609.3, 4754610.3, 4754611. Fasta files containing the expressed ORFs with signal peptides are available from the authors upon request.

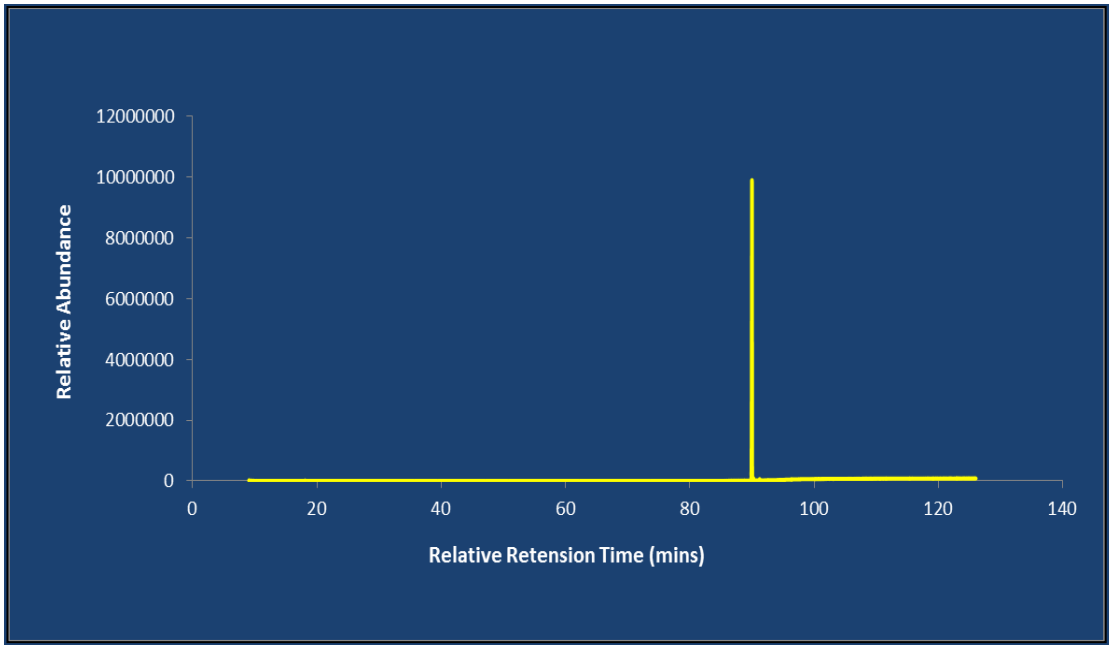
Appendix 1

Table A1: Sequence Data Summary

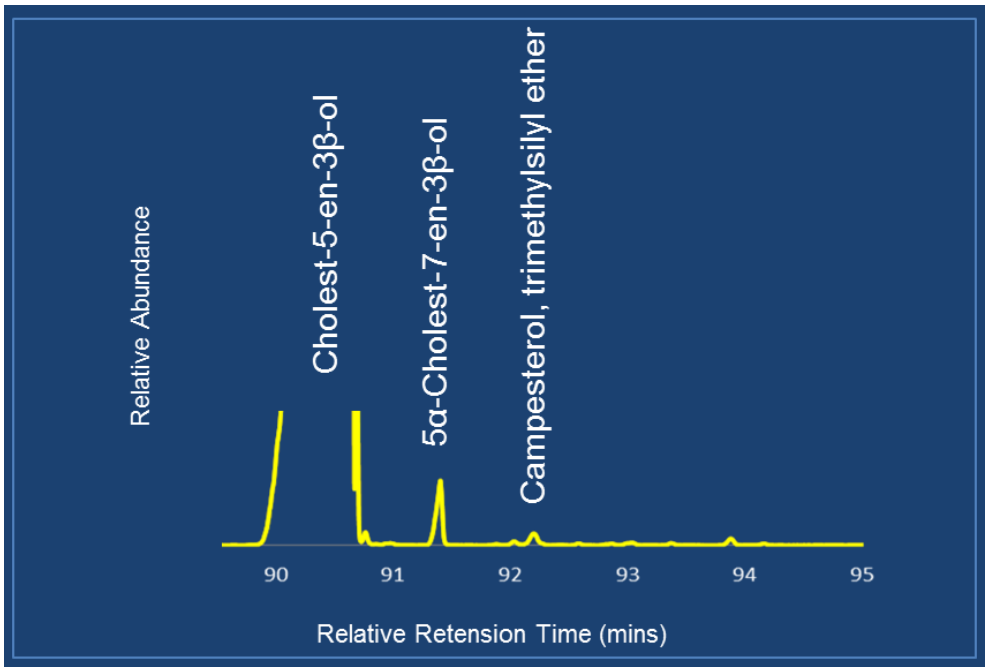
	Pigmented Stones (N=4)	Cholesterol Stones (N=4)	Total (N=8)
Total Paired-End Sequence Reads	262,254,326	19,347,392	281,601,718
Paired-End Sequence Reads per sample	$65{,}563{,}582 \pm 26.5\ 10^6$	$4{,}836{,}848 \pm 1.16\ 10^6$	$70{,}400{,}430 \pm 30.0\ 10^6$
Total ORFs	22,393,091	392,392	22,785,483
ORFs per sample	$5{,}598{,}273 \pm 2.75\ 10^6$	$98{,}098 \pm 41.1\ 10^3$	$5{,}696{,}371 \pm 2.75\ 10^6$

Figure A1:

Example of predominant GCMS Results for all gallstone polar fractions:



Sample PM1 with single cholest-5-en-3b-ol peak.



Sample PM1 magnified

Chapter 3:

Biofilm-producing bacteria identified in all major human gallstone types

S.H. Kose, K. Grice, M. Ballal, A. Suthananthan, C. Wuchter, and M.J.L. Coolen. *PLOS One*, (*under preperation)*. (2018).

Abstract

Recently reported functional metagenomic profiling of microbial communities associated with pigmented and cholesterol-type gallstones from a 76-year-old male and a 30-year-old female patient revealed that bile-resistance, biofilm formation, and an efficient energy metabolism are important traits of gallstone-associated bacteria and that these processes may play an important role in gallstone disease. The greatest number of genes involved in these processes was associated with *Klebsiella* and *Enterococcus* in pigmented and cholesterol stones, respectively. Knowing the key players in these processes we undertook high-throughput amplicon sequencing of the taxonomic marker gene 16S rRNA to provide a comprehensive overview of the microbial diversity in all major gallstone types (black, pigmented, cholesterol, and solitary) from a larger cohort of patients (n=16) with diverse backgrounds.

In this study, *Enterobacteriaceae* species were found to predominate in the majority of the gallstones analysed. This further suggests that gallbladder infection by *Enterobacteriaceae*, most notably biofilm-forming and bile resistant *Klebsiella*, is not limited to pigmented stones alone and may be an intrinsic factor in gallstone pathogenesis.

Introduction

Gallstone disease (GSD) continues to be an increasing burden to health care systems worldwide and is now being linked to various gastrointestinal cancers (Stinton & Shaffer, 2012). In 2013 alone, Australia was reported to have one of the highest rates of laparoscopic cholecystectomy among the Organisation for Economic Co-operation and Development (OECD) nations (AIHW, 2016). Traditional risk factors for gallstone disease include ethnicity, genetics, age, female gender, metabolic syndrome, diabetes mellitus, obesity and a sedentary lifestyle (Stinton & Shaffer, 2012; AIHW, 2016). In recent decades, however, these risk factors are being re-examined as an increasing number of patients, contrary to known risk factors, present with GSD (Stinton & Shaffer, 2012). GSD is now seen as an increasingly complex disease with no tangible cure (besides invasive surgery) in sight; prompting a continual re-examination of established research (Stinton & Shaffer, 2012). For example, traditionally and informally categorised into four main types (brown and black pigmented, cholesterol, mixed and solitary), investigators now suggest as many as eight distinct gallstone types based on variations in chemistry (Qiao et al., 2013). Further, what was considered a solely physico-chemical phenomenon relating to hormonal imbalance, gallbladder motility, and oversaturation of cholesterol or bilirubinate in bile (Stinton & Shaffer, 2012; Shaffer, 2005), a growing body of evidence has begun to implicate the role of bacteria in gallstone pathogenesis [Maki, 1966; Swidinksi & Lee, 2001; Marshall et al., 2014; Shen et al., 2015; Stewart et al., 1987, 2000, 2002; Cetta, 1991). With most of these studies placing a particular emphasis on the brown pigmented-type variety in which bacteria are often found to be in larger concentrations (Maki, 1966; Stewart et al., 1987, 2000, 2002; Cetta, 1991). Bacterial investigations have now begun to focus on bacterially produced biofilm or extra polymeric substance (EPS) matrices, with the implication that biofilm provides the glycoproteins necessary to initiate the binding process that forms the gallstone nidus (Marshall et al., 2014; Stewart et al., 2000, 2002). In a recent pilot study, we explored the role of bacteria in gallstone pathogenesis by undertaking functional metagenomics profiling of two patients; a 76-year old male patient with brown pigmented-type gallstones and a 30-year old female patient with cholesterol-type stones. Functional metagenomics profiling allows for the

identification of all domains of life in a given sample, and includes protists, fungi, and archaea, which are generally overlooked in human microbiome investigations (Laforest-Lapointe & Arrieta, 2018). Our analysis revealed only trace levels of sequences from archaeal, protistal or fungal origin, confirming the majority of DNA extracted from the analysed gallstones was bacterial in origin (Kose et al., 2018). We identified 53 putative genes related to bile resistance, stress response, and biofilm formation revealing the survival strategies bacteria may be utilising to withstand and shield them from what is the extreme environment of the human gallbladder (Kose et al., 2018). These genes were predominantly associated with the pathogenic genera *Klebsiella* and *Enteroccocus*. Further, dimethylsulphoxide (DMSO) genes associated to *Klebsiella* were identified, revealing a possible respiration strategy, which enables this genus to outcompete other bacteria and may explain its dominance in the study. This was followed by an analysis of the stable C and H isotopic compositions of the stones' cholesterol, which revealed that dietary or environmental factors may not play a significant role in selecting the types of bacterial species and their potential activities in the gallstones.

Here, we follow up our pilot study with a larger group of 16 patients representing various ages, BMIs, gender and gallstone types (black, pigmented, cholesterol, solitary/mixed) to ascertain any correlation between these factors and the bacterial taxonomy identified in the gallstones. While the functional metagenomics survey revealed the potential microbial processes and those involved, we used a more targeted approach, high throughput 16S rRNA amplicon sequencing, to lower the detection limit for less dominant taxa and to provide a more comprehensive overview of the bacterial communities present within the gallstones. Our goal was to verify if the same microbes involved in biofilm formation and that possess the genetic machinery to withstand the hostile gallbladder environment occurred in all major gallstone types. Quantitative PCR was used to estimate the amount of microbial abundance in various gallstones. In addition, we used compound specific isotopic analysis (C and H) of the cholesterol present in each of the patients' gallstones to ascertain if diet or a particular source of cholesterol influenced which bacteria were present.

Materials and methods

Sample collection

Patients diagnosed with GSD were recruited to participate in the study in consultation with their medical advisor and provided written informed consent upon enrolment. The study was designed with the aid of the National Health and Medical Research Council (NHMRC), according to the guidelines stipulated in the National Statement on Ethical Conduct in Human Research 2007 (NHMRC, 2007a), and the Australian Code for the Responsible Conduct of Research 2007 (NHMRC, 2007b). The study was approved for a maximum number of 16 patients and met the ethical guidelines of the South Metropolitan Health Service Human Research Ethics Committee (HREC Reference: 15-136), the Fiona Stanley Human Research Ethics Committee (Ref: 2015-136), the St John of God Health Care Human Research Ethics Committee (Ref: 1021), and the Curtin University Human Research Ethics Committee (Ref: HR229/2015). Samples were collected during surgery and were immediately rinsed in sterile saline solution (9 g L^{-1} NaCl) and placed in sterile glass containers. The samples were immediately stored at −80 °C until further processing. See Table 3.1 for patient and sample information.

Patient Information

Table 3.1. Patient and sample Information.

Sample ID	Age	Sex	BMI	Overweight	Antibiotics	Type	Size
BM2	50	M	25.9	Y	Nil	Black Pigmented	Small 0-1 cm
BF3	52	F	30.0	Y	Nil	Black Pigmented	Small 0-1 cm
BF4	56	F	28.2	Y	Nil	Black Pigmented	Small 0-1 cm
PM1	76	M	24.0	N	Nil	Pigmented	Small 0-1 cm
PF3	28	F	20.4	N	Nil	Pigmented	Small 0-1 cm
CM1	63	M	29.3	Y	Nil	Cholesterol	Small 0-1 cm
CM2	88	M	28.7	Y	Nil	Cholesterol	Small 0-1 cm
CM3	36	M	26.1	Y	Nil	Cholesterol	Small 0-1 cm
CF4	30	F	40.5	Y	Nil	Cholesterol	Small 0-1 cm
CF5	32	F	30.6	Y	Nil	Cholesterol	Small 0-1 cm
CF7	44	F	28.9	Y	Nil	Cholesterol	Medium 1-2 cm
SF1	27	F	32	Y	Nil	Solitary	Medium 1-2 cm
SF2	71	F	31	Y	Nil	Solitary	Medium 1-2 cm
SM1	38	M	25.8	Y	Nil	Solitary	Medium 1-2 cm
SM2	65	M	24	N	Nil	Solitary	Medium 1.2 cm
SM4	69	M	30.5	Y	Nil	Solitary	Large 2-3 cm

DNA extraction

Inside a HEPA-filtered laminar flow bench, individual gallstones (~100 mg each) were pulverized using a heat-sterilized mortar (500 ºC, 8h). DNA was then extracted from the pulverized gallstone after Haigh and Lee (2001) with modifications as described in detail in Kose et al., (2018). PCR-inhibiting impurities were completely removed using the OneStep PCR Inhibitor Removal Kit (Zymo Research, U.S.A). The DNA concentration was quantified fluorometrically (NanoDrop 3300 Fluorospectrometer; Thermo Fisher Scientific, MA, U.S.A) using the Quant-iT PicoGreen dsDNA Assay kit (Life Technologies, VIC, Australia).

rRNA gene amplification, quantification, and bioinformatic analysis

An aliquot of the extracted DNA was subjected to quantitative polymerase chain reaction (qPCR) using the bacterial forward primer P5_U519f (5′-CAG CMG CCG

CGG TAA-3′) and reverse primer U806r (5′-GGA CTA CHV GGG TWT CTA AT-3′) as per Caporaso et al. (2012) targeting the V4 region of prokaryotic 16S rRNA. The reverse primer was barcoded, with equimolar amounts of the barcoded amplicons pooled, concentrated and gel purified. All reactions were performed using SYBR Premix Ex Taq (TLi RNase H Plus) (Takara Bio Inc) in a Realplex quantitative PCR cycler (Eppendorf) and involved initial denaturing (1 min at 95°C), followed by 32 cycles including denaturing (5 sec at 95 °C), primer annealing (30 sec at 60 °C), primer extension and imaging of newly formed fluorescent (SYBR®Green I labelled) double-stranded DNA (72 °C for 60 sec). Between 10^1 and 10^6 copies (10-fold dilution series) of bacterial 16S rRNA were added to reaction mixtures and served as standards during qPCR to calibrate the copy numbers of bacterial 16S rRNA in the gallstone samples.

The 16S rRNA barcoded gene libraries were sequenced on the Illumina MiSeq sequencing platform using the facilities of the Australian Genome Research Facility, Harry Perkins Institute for Medical Research, Perth, Australia. The sequenced bacterial libraries resulted in the generation of 2 x 300bp (paired-end) reads for each sample with approximately 2.2 million reads across the 16 samples. The resultant DNA sequences were processed in QIIME 2 following the standard protocol for analysing paired-end reads (Caporaso et al., 2010). Initial demultiplexing of the dataset was followed by correction of Illumina-sequenced amplicon errors using the q2-dada2 and dada2-denoise-single plugins (Callahan et al., 2016). Both forward and reverse read pairs were trimmed and filtered to between 15 bp – 289 bp with all base pairs with quality (Phred) scores below 25 having been removed. Additional phiX reads and chimeric sequences within the dataset were removed. The final sequence count after quality control was 1,149,854 reads across the sample set.

The reads were assigned taxonomy *via* the pre-trained q2-feature-classifier: a Naive Bayes classifier trained on the Greengenes database (13_8 99% OTUs, trimmed to 250 bp) (Callahan et al., 2016). Reads comprising >1% of the total community were verified to 99% sequence identity to the nearest cultured study through BLASTn searches against the NCBI nucleotide (nr) database (Madden, 2003). The resulting data was used to construct a genus-level OTU table for downstream biostatistical analyses.

Heatmap images were generated in R (http://www.r-project.org/) using the vegan (http://vegan.r-forge.r-project.org/) and bioconductor Heatplus (https://bioconductor.org/biocLite.R) packages. The data was normalized, with the Hellinger function used to show species that may have been obscured by the dominant reads. The Primer-E software package (http://www.primer-e.com/)was used to perform permutational multivariate analysis of variance (PERMANOVA) and the Monte-Carlo permutation procedure with up to 999 permutations.

Gas Chromatography Isotope Ratio Mass Spectrometry

The 16 samples were individually crushed in heat-sterilized (500 ºC, 8h) mortars. The ground powder was then extracted *via* sonication (1 h) with dichloromethane (DCM) and methanol (9:1). The extracts were then fractionated by small-scale column liquid chromatography (Bastow et al., 2007). Approximately 2 mg of the total extract was placed on top of a small column (5 x 0.5 cm i.d.) of activated silica gel (160 ºC, 8 h). The first hydrocarbon fraction was eluted with *n*-hexane (2 mL), the second hydrocarbon fraction with DCM in *n*-hexane (1:4, 2 mL), and the more polar fraction with an equal mixture of DCM and methanol (1:1, 2 mL).

The polar fractions were each analysed by the Thermo Trace GC Ultra with only cholest-5-en-3β-ol identified in each of the samples. Subsequently compound specific isotope analyses was undertaken to obtain $\delta^{13}C$ and δD values of cholest-5-en-3β-ol in each of the samples. The polar fractions were run as un-derivatised free sterols. Although this method allows for timely and convenient analysis, if samples containg exchangeable hydrogens are inadequately prepared, there is the potential that these hydroxyl groups can exchange with hydrogen from atmospheric water vapour, effecting δD values. All precautions were taken to limit atmospheric exposure in this study. The samples were removed from the patient and immediately placed into sterile containers that were in-turn placed into a liquid nitrogen thermos within the aseptic operational theatre. Once transported the the main analysis site, the samples were kept in a -20 freezer as lower temperatures reduce the rate of exchange. Further, as cholesterol has only one exchangeable hydrogen atom and 45 non-exchangeable hyrdogens, the overall effect on the δD value is considered here to be low (Schimmelmann et al., 2006).

The instrument used was a Thermo Delta V Advantage isotope ratio monitoring mass spectrometer (irMS), coupled to the Thermo Trace GC Ultra via a GC Isolink and Conflo IV. The column used was an Agilent DB-5MS Ultra-Inert, 60 m long, 0.25 mm (i.d.), with 0.25 μm film thickness. An aliquot of 1 μL of each fraction was injected into the split/splitless injector in splitless mode, held at 280 °C. The GC oven was increased from 40 to 325 °C at 10 °C/min, and then held at 325 °C for 10 min. The carrier gas used was helium held at a constant flow of 1.5 mL/min.

The stable carbon isotope analysis utilised a GC column outflow which was passed through a GC Isolink combustion reactor (copper oxide / nickel oxide, 1000 °C) to combust hydrocarbons to CO_2 and H_2O. The H_2O was removed *via* a Nafion water trap. For hydrogen isotope analysis, the outflow passed through the high-temperature conversion reactor (graphite-lined, 1420 °C) and was thermally converted to H_2. The CO_2 / H_2 passed through the Conflo IV interface to the irMS, which measured *m/z* 44, 45 and 46 (for CO_2) or *m/z* 2 and 3 (for H_2 and DH). The H_3^+ correction factor was measured daily during δD analyses and was always less than 13 ppm. The $\delta^{13}C$ and δD values were calculated from the measured masses by Thermo Isodat software and calibrated to the VPDB (for CO_2) and VSMOW (for H_2 or D/H) scales by comparison with a mixture of *n*-alkane standards of known isotopic composition. The resulting data was interpreted *via* the Isodat Workspace v3.0, Gas Isotope Ratio MS Software, developed by Thermo Scientific.

Results

Bacterial diversity per gallstone type:

Black pigmented stones

For simplicity, we only describe bacteria identified at the genus level that comprised >1% of the total reads in each gallstone and in the order as listed in Table 3.1. Male patient BM2 contained a high relative abundance of *Klebsiella* (43.8%) and *Escherichia* (49.0%), while *Rhodopirellula* (3.0%) and *Alloiococcus* (2.0%) represented the remainder of the identified genera. In the black pigmented stone of female patient BF3, *Streptococcus* was the only dominant genus comprising 99% of the total population (Figure 3.1). *Escherichia* (45.9%) and *Klebsiella* (34.5%) were found in female patient BF4, followed by *Verrucombimicrobium* (5.2%), *Acinetobactor* (5.0%), *Tepidomonas* (3.6%), *Alicyclobacillus* (3.5%) and *Pseudomonas* (2.2%).

Brown pigmented stones

The brown pigmented gallstone of male patient PM1 also showed a predominance of *Klebsiella* (92.6%) and *Enterococcus* (6.8%) with the remainder of genera representing <1% of the reads. The female patient PF3 showed a higher diversity, but was also predominated by *Klebsiella* (78.9%) followed by *Escherichia* (12.9%), *Enhydrobacter* (2.6%), *Comamonadaceae* (1.8%) and *Streptococcus* (1.5%) (Figure 3.1).

Cholesterol stones

In the male patients who presented with cholesterol gallstones, *Enterobacter* comprised 97.1% of the bacterial community of patient CM1, with the remaining 2.9% comprising genera that are less than 0.5% of total reads. Patient CM2 revealed a predominance of *Escherichia* (55.4%), *Klebsiella* (36%), and *Streptococcus* (2.4%) as well as *Bilophila* (2.7%), *Tepidimonas* (1.8%), and *Rhodococcus* (1.7%). Patient CM3 showed a predominance of *Pseudomonas* (87.7%) and *Klebsiella* (12.3%).

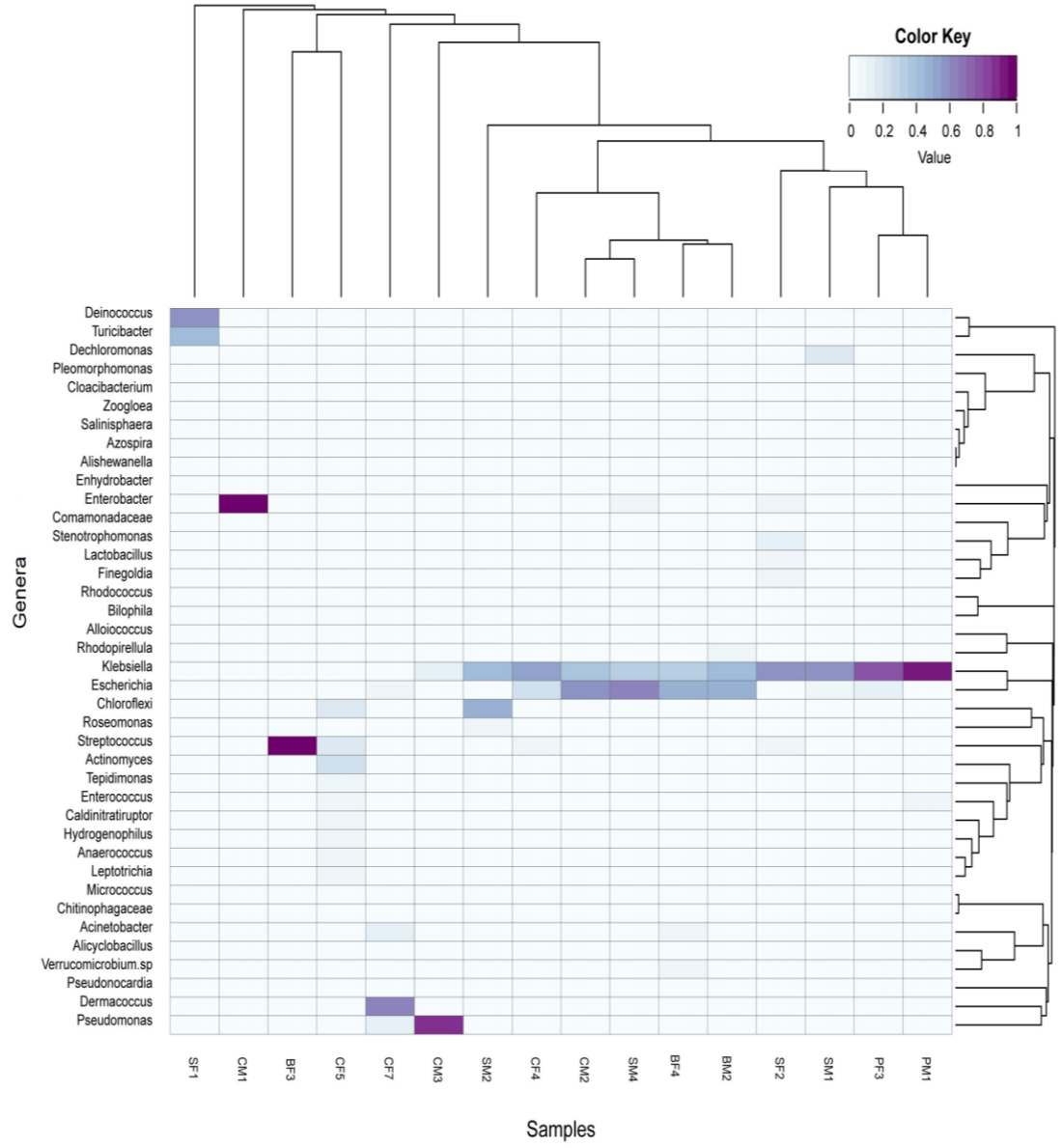


Figure 3.1 Heatmap with the predominant bacterial genera identified in the gallstones of 16 patients within the study. The colour key shows the relative abundance of the genera that are >1% in the gallstones starting with yellow at 1% and progressing to darker shades with increasing abundance. The data was normalized, with the Hellinger function used to reduce the weight of dominant taxa and to increase visibility of the less abundant taxa. The dendrograms illustrate the relationship between samples.

In the female patients who presented with cholesterol gallstones, *Klebsiella* (51%) was a predominant genus in the analysed stones from patient CF4, followed by *Escherichia* (24%), *Streptococcus* (5.3%), *Chloroflexi* (5.2%), *Tepidimonas* (4.3%), *Chitinophagaceae* (3.1%), *Micrococcus* (3.0%), *Enhydrobacter* (1.2%) and *Acinetobacter* (1%). *Actinomyces* (22.4%), *Chloroflexi* (15.7%) and *Streptococcus*

(15.9%) predominated the more diverse cholesterol stone of patient CF5, followed by *Tepidimonas* (8.9%), *Anaerococcus* (8.8%), *Hydrogenophilus* (8.2%), *Enterococcus* (7.4%), *Leptotrichia* (7.3%) and *Caldinitratiruptor (5.4%)*.
The cholesterol stone of patient CF7 was predominated with *Dermacoccus* (64.6%), *Acinetobacter* (12.2%), *Pseudomonas* (11.4%) and to a lesser extent *Pseudonocardia* (3.6%) and *Tepidimonas* (1.0%) (Figure 3.1).

Solitary stones

In the male patients who presented with solitary gallstones, patient SM1, showed a relatively high diversity with a predominance of *Klebsiella* (56.3%), followed by *Dechloromonas* (17.4%), *Escherichia* (9.8%), *Pleomorphomonas* (3.3%), *Cloacibacterium* (2.1%), *Tepidimonas* (1.9%), *Salinisphaera* (1.8%), *Azospira* (1.7%), *Alishewanella* (1.7%), *Comamonadaceae* (1.6%), and *Zoogloea* (1.5%). Patient SM2 exhibited a predominance of *Chloroflexi* (46.7%) followed closely by *Klebsiella* (44.6%), and to a lesser extent *Roseomonas* (8.7%). The solitary stone of patient SM4 exhibited a predominance of *Escherichia* (60.9%) and *Klebsiella* (31.7%), followed by *Enterobacter* (7.4%) (Figure 3.1).

In the female patients who presented with solitary gallstones, patient SF1 revealed a unique microbial community that was predominated by only two genera: *Deinococcus* (56.7%) and *Turicibacter* (43%). *Klebsiella* was found to be the most abundant genus in the solitary stone of patient SF2 (55.1%), followed by *Stenotrophomonas* (13.8%), *Enterobacter* (8.4%), *Lactobacillus* (8.3%), *Finegoldia* (5.1%), and *Comamonadaceae* (3.7%) (Figure 3.1).

The majority of identified genera in the sample set were Gram-negative (see S1 Fig.), with all genera identified having the potential to produce biofilm or associated with biofilm producing communities (Table 3.1). Previous research has shown Gram-negative bacteria to have the ability to withstand a variety of toxic agents, including bile acids (Merritt et al., 2009). This ability is largely attributed to the second cell wall membrane, conferring a natural defence in these organisms, and investigations showing up-regulated DNA and cell wall repair genes by enteric Gram-negative bacteria in response to bile stress (Merritt et al., 2009; Gunn, 2000). In what limited

data is available for Gram-positive bacteria, recent reports have also identified similar bile resistance genes for the genera *Enterococcus* and *Streptoccocus* (Kose et al., 2018, Begley et al, 2005). We indicate this predicted ability alongside the biofilm producing bacteria identified in the study (Table 3.1).

Table 3.2. Overview of identified Gram-negative and Gram-positive bacteria associated with biofilm production and predicted bile resistance.

Genus	Gram-stain	Biofilm production/ association	Bile stress resistance/ association	Reference
Acinetobacter	Negative	Y	Y	Merritt.,2009;Gunn,2000; Gurung.,2013
Azospira	Negative	Y	Y	Merritt.,2009;Gunn,2000; Xiao., 2015
Cloacibacterium	Negative	Y	Y	Merritt.,2009;Gunn,2000, Nouha.,2016
Deinococcus	Negative	Y	Y	Merritt.,2009;Gunn,2000, Shukla., 2017;Makarova.,2001
Enterobacter	Negative	Y	Y	Stewart., 2000; Kose., 2018 Merritt.,2009;Gunn,2000
Escherichia	Negative	Y	Y	Stewart., 2000; Kose., 2018 Merritt.,2009;Gunn,2000
Klebsiella	Negative	Y	Y	Stewart., 2000; Kose., 2018 Merritt.,2009;Gunn,2000
Pseudomonas	Negative	Y	Y	Stewart.,2000;Merritt.,2009;Gunn, 2000; El-Masry., 1995
Roseomonas	Negative	Y	Y	Merritt.,2009;Gunn,200 Michael., 2016; Diesendorf.,2017)
Salinisphaera	Negative	Y	Y	Merritt.,2009;Gunn,2000; Azúa-Bustos.,2009
Stenotrophomonas	Negative	Y	Y	Merritt.,2009;Gunn,2000; Donelli,2015
Tepidimonas	Negative	Y	Y	Merritt.,2009;Gunn,2000; Zumsteg, 2017.
Verrucomicrobium	Negative	Y	Y	Merritt.,2009;Gunn,2000; Springs, 2016
Zoogloea	Negative	Y	Y	Merritt.,2009;Gunn,2000; Farkas., 2014
Alishwenella	Negative	Y	Y	Merritt.,2009;Gunn,2000; Charles, 2015
Bilophila	Negative	Y	Y	Merritt.,2009;Gunn,2000; Donelli, 2015
Caldinitratiruptor	Negative	Y	Y	Merritt.,2009;Gunn,2000; Fang., 2017; Fardeau., 2010
Chitinophagaceae	Negative	Y	Y	Merritt.,2009;Gunn,2000; Luo., 2017
Chloroflexi	Negative	Y	Y	Merritt.,2009;Gunn,2000; Luo ., 2017; Schmitt., 2011
Comamonadaceae	Negative	Y	Y	Merritt.,2009;Gunn,2000; Che.,2017
Dechloromonas	Negative	Y	Y	Merritt.,2009;Gunn,2000
Enhydrobacter	Negative	Y	Y	Merritt.,2009;Gunn,2000; Lyautey.,2005
Hydrogenophilus	Negative	Y	Y	Merritt.,2009;Gunn,2000; Salmassi.,2006
Leptotrichia	Negative	Y	Y	Merritt.,2009;Gunn,2000; Eriksson.,2017
Pleomorphomonas	Negative	Y	Y	Merritt.,2009;Gunn,2000; Yamamuro., 2012
Rhodopirellula	Negative	Y	Y	Merritt.,2009;Gunn,2000; Lage., 2012
Turicibacter	Positive	Y		Bengelsdorf., 2015
Actinomyces	Positive	Y		Arai., 2015
Alicyclobacillus	Positive	Y		Dos Anjos., 2013
Enterococcus	Positive	Y	Y	Stewart.,2000;Kose.,2018;Begley., 2005
Streptococcus	Positive	Y	Y	Stewart.,2000;Kose.,201 Begley., 2005
Finegoldia	Positive	Y		Donelli., 2015
Alloiococcus	Positive	Y		Chan., 2017
Anaerococcus	Positive	Y		Dowd., 2008
Dermacoccus	Positive	Y		Soto-Giron.,2016
Lactobacillus	Positive	Y		Salas-Jara.,2016
Micrococcus	Positive	Y		El-Masry., 1995
Pseudonocardia	Positive	Y		Holmes., 2016
Rhodococcus	Positive	Y		Al-Akhrass., 2012

Shown are the analysed gallstones that comprise at least 1% of the total Illumina reads. The cited references indicate relevant studies where members of these genera have been reported to be involved in or associated with biofilms and bile resistance.

Taxonomic similarity analysis: Patient Age, BMI and Gender

Permutational analysis of variance (PERMANOVA) on total reads revealed that microbial communities were not significantly different in gallstones from patients that differed in gender (*p=0.133*, n=8 male, n=8 female), or patient age <50 years (n=7) and >50 years (n=9), *p*=0.238). Overweight patients (n=13 or 84%) indicated by a BMI >25.0 predominated the study; however, no difference was observed in the microbial communities identified between these patients and those with a BMI less than 25.0 (n=3). The greatest similarity between the gallstones is that they shared a dominance of biofilm producing and bile resistant pathogenic bacteria, with *Enterobacteriaceae* species, such as *Klebsiella* and *Escherichia*, over-represented in the sample set (*p*=0.006).

Microbial abundance

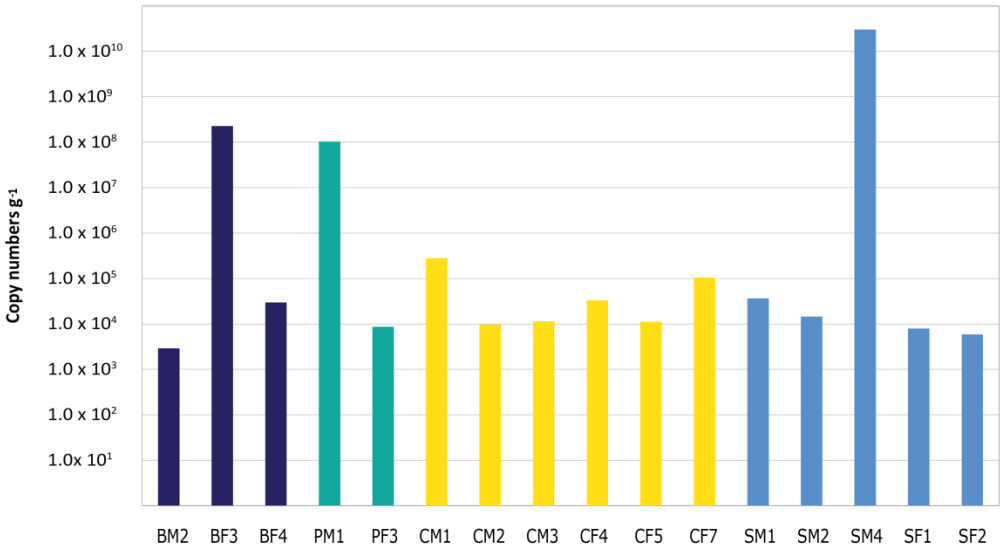


Figure 3.2 Bacterial 16S copy numbers per gram gallstone material in the different gallstone types across 16 patients. Colour coding describes the gallstone types, Dark Blue = black pigmented, Teal Green = brown pigmented, Yellow = cholesterol, Blue = solitary stones.

Quantitative PCR revealed seven orders of magnitude variation in the total number of bacterial 16S rRNA gene copies per gram of gallstone material (Figure 3.2). The large solitary stone from male patient SM4 revealed the highest bacterial 16S rRNA content (3×10^{10} copies g^{-1} gallstone). SM1 and SM2 represented male patients with large solitary stones with orders of magnitude lower bacterial 16S copies than SM4 (3.7×10^{4} and 1.5×10^{4} copies g^{-1} respectively). Comparably low bacterial 16S copy numbers were quantified in the large solitary stones of female patients SF1 at 7.9×10^{3} copies g^{-1} and SF2 at 5.9×10^{3} copies g^{-1} (Figure 3.2).

The pigmented stone of male patient PM1 (1.0×10^{8} copies g^{-1}) and the black stone of female patient BF3 (2.3×10^{8} copies g^{-1}) showed the next highest abundance of bacteria. In contrast, the female patient with pigmented gallstones (PF3) had only 8.7×10^{3} copies g^{-1} and the other analysed black stones contained only 2.9×10^{2} (BM2) and 3.0×10^{4} (BF4) bacterial copies g^{-1}.

The lowest variation in bacterial 16S copy numbers was found in the pure cholesterol stones. The bacterial 16S content in the cholesterol stones from the male patients (CM1, CM2, CM3) ranged between 9.7×10^{3} and 2.8×10^{5} copies g^{-1}. This was comparable with the female patients (CF4, CF5, CF7) whose stone bacterial content ranged between 1.1×10^{4} and 1×10^{5} copies g^{-1}.

Based on known rRNA operons of the main bacteria present in the stones (Klappenbach et al., 2000) we provide a conservative cell number estimate range between 3.63×10^{2} and 4.33×10^{9} bacterial cells g^{-1} stone.

Cholesterol analysis

Compound specific isotope analysis (CSIA) allows for the isotopic analyses of compounds in a complex mixture by linking a GC to an IRMS via a reaction furnace where combustion or pyrolysis takes place. The marked difference between the $\delta^{13}C$ value of an identified compound in two gallstones could indicate that a different source or mechanism was utilised during the synthesis of the compound of interest (Peng et al., 2014). We conducted CSIA of the patients' carbon ($\delta^{13}C$ values) and hydrogen (δD values) isotopes of cholesterol to ascertain possible dietary or exogenous environmental factors that may be associated with the bacteria identified in this study.

The only component identified within the stones of each patient was the cholest-5-en-3β-ol with trace amounts of 5α-cholest-7-en-3β-ol. This identification is based on our initial GCMS screening results, with the instrument prepared as per Haigh & Lee (2001) who were able to resolve two oxysterols amongst others in their study. We were unable to reproduce these results in the present study, suggesting either gallstone variability or the need for a technique more amenable to the analysis of polar and higher molecular weight compounds, such as liquid chromatography-mass spectrometry (LCMS) (Peng et al., 2014). Subsequently, CSIA was conducted on the non-derivatised cholest-5-en-3β-ol compound identified in each of the patients' stones (Figure 3.3).

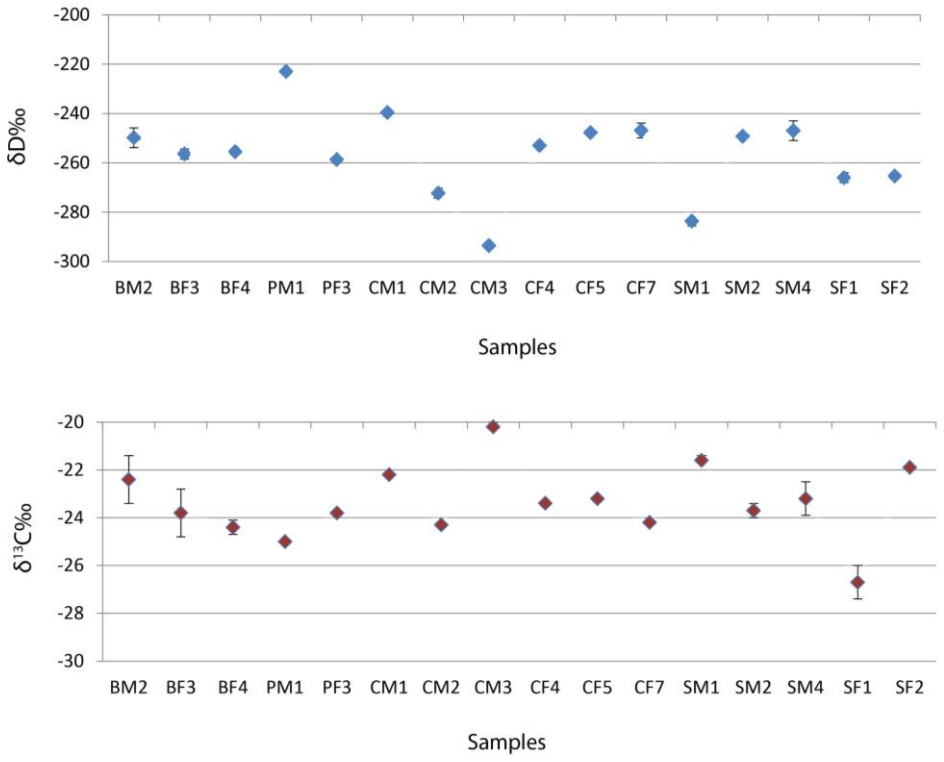


Figure 3.3 Compound specific $\delta^{13}C$ (Vienna Pee Dee Belemnite reference standard) and δD (Standard mean ocean water) results for the 16 patients. The error bars correspond to replicate measurements (N=3).

The cholest-5-en-3β-ol $\delta^{13}C$ values ranged between –20.2‰ to –26.7‰ (with an average of –23.3‰) across the 16 stones, whilst the δD values ranged between –

223‰ to –293‰ (with an average of –256‰) across the 16 stones. The $\delta^{13}C$ values are consistent with the majority of C3 vegetation consumed in Australian diets, such as wheat, rice, potatoes, soy and common fruits and vegetables (excluding corn) in which $\delta^{13}C$ values have been shown to range between –20‰ to –34‰ (Peng et al., 2014). Heavier $\delta^{13}C$ values that range between –10‰ to –20‰ would indicate a diet rich in C4 plants such as, corn, sugar cane and sorghum grass as the common source of the cholest-5-en-3β-ol identified in the patients' stones. Stable hydrogen isotope values of organic samples have been used as a type of environmental/geographic fingerprint, and have been applied extensively for animal and human migration studies (Mancini et al., 2008). This approach has only recently begun to be applied to human health and dietary studies and requires further verification and continued study (Mancini et al., 2008). Nevertheless, a recent study has identified dairy products in the U.S as having the uppermost (lightest) δD values, with butter the highest at –228‰, followed by a variety of cheeses that range between –145‰ to –198.9‰. This is consistent with the results obtained in this study, in which the average δD value is –256‰; suggesting a diet that may be rich in dairy products for all 16 patients. However, as dietary and geographical studies using δD isotopes are limited in number, additional investigations with larger samples sizes are required to distinguish whether these signals are indeed related to dairy rather than geographical/regional effects.

Discussion

In this study, a predominance of pathogenic biofilm-producing and bile resistant bacteria was identified in the gallstones of 16 patients diagnosed with gallstone disease. The majority of patients (n=11) revealed gallstones predominated by bacteria from the *Enterobacteriaceae* family, most notably the pathogenic genera *Klebsiella*, *E. coli*, and *Enterobacter*. The remaining five patients exhibited known prolific biofilm producers, such as the genera *Pseudomonas* (CM3, CF7), *Streptococcus* (BF3), *Actinomyces* (CF5), and *Deinococcus* (SF1).

Of the patients examined, only three were found to have a BMI less than 25.0. Nevertheless, these patients (PM1, PF3, and SM2) remained aligned with the others by presenting with a predominance of *Klebsiella* and *E. coli* in their gallstones. The trend continued with both genders and age groups (<50 and >50) exhibiting a predominance of *Enterobacteriaceae* with no significant differences in the microbial make-up between the groups. These findings suggest that the presence of these bacteria do not necessarily correlate with the traditional gallstone risk factors of female gender, advanced age or a BMI greater than 25.0 (Stinton & Shaffer, 2012; AIHW, 2013).

The results of the present study align with and progress previous studies in which *Enterobacteriaceae* were found to be predominant in the gallstones analysed. These were mostly culture studies which concentrated on pigmented stones alone due to low bacterial reads on other types (Maki, 1996; Stewart et al., 2000; Stewart et al., 2002), and a study focusing solely on cholesterol stones using culture-independent 16S rRNA profiling (Wu et al., 2013). Although we were limited in the number of pigmented stones (n=2) analysed due to ethical constraints, with favour given to stone types least studied, these samples nevertheless correlate with the majority of previous pigmented stone studies (Maki, 1966; Stewart et al., 2000, 2002). These previous findings, alongside the current finding of a predominance of *Enterobacteriaceae* across all major gallstone types, suggest that gallstone formation may indeed be concomitant with bacterial processes directly relating to bile resistance and biofilm formation (Stewart et al., 2000, 2002; Kose et al., 2018). Of interest is that all other described less abundant genera are known to contain species frequently reported from microbial biofilms in a variety of environmental settings

(Table 3.1). This further suggests that any communal growth or survival in, what is the extreme environment of the human gallbladder, may pre-necessitate the ability to form or to be associated with biofilms. Our findings further suggest that a gallstone type cannot be attributed to a particular bacterial community; as described in previous studies where *Enterobacteriaceae* were only associated in the formation of pigmented stones (Maki, 1966; Stewart et al., 2000, 2002). As with concretionary structures that are the result of bacterial processes in nature, in which the local chemical, biological and physical environment determine the shape, size, colour and composition of the concretions (Bosak et al., 2013), a patient's unique gallbladder environment (i.e., differences in bilirubinate versus cholesterol ratio) may be the reason behind compositional differences seen in the major gallstone types. The common link between the two systems being, the production of biofilms by bacteria that enable binding and trapping of detrital matter or precipitation of minerals determined directly by the local environment (Bosak et al., 2013).

Although Gram-positive genera were identified in this study, of particular interest is the predominance of Gram-negative bacteria identified across both current and previous studies and their presence in the human gallbladder. The *Enterobacteriaceae* are Gram-negative, facultative anaerobic bacilli and are known to frequent the gastrointestinal tract (CDC, 2012). They are often implicated in a number of infections ranging from catheter related applications and urinary tract infection (UTI) to pneumonia and meningitis (Qureshi, 2014). Gram-negative bacteria are conferred a natural defence against toxic agents by way of a second cell wall, which may be the reason for their predominance in the human gallbladder, as opposed to Gram-positive genera.

In recent decades, the misuse of broad-spectrum antibiotics alongside global connectivity has facilitated the emergence of multi-drug resistant Gram-negative bacteria (MDRGN), which include members of the *Enterobacteriaceae* (Bassetti et al., 2016). MDRGNs are now increasingly being considered a global threat in the fight against bacterial antibiotic resistance (Bassetti et al., 2016; Vasoo et al., 2015). Of particular concern to clinicians are *E. coli* and the *Klebsiella* species *K. pneumoniae* and *K. oxytoca* (Rawat & Nair, 2010; Bassetti et al., 2016; Vasoo et al., 2015). These bacteria have recently developed the ability to produce extended-spectrum β-lactamases (ESBLs) which have now been shown to provide broad

spectrum resistance to virtually all β-lactams, a class of antibiotics that act on the bacterial cell wall, and provide co-resistance to multiple other antibiotic classes (Rawat & Nair, 2010; Vasoo et al., 2015). Often the last defence against ESBL producing Gram-negative strains is the antibiotic class of carbapenems (CDC, 2012; Rawat & Nair, 2010; Vasoo et al., 2015). However, the recent discovery of carbapenemase producing *K. pneumoniae* (KPC) has significantly limited options for infection control and as a consequence increased global morbidity and mortality rates (CDC, 2012; Qureshi, 2014; Nordmann et al., 2009).

As shown in our previous functional metagenome survey (Kose et al., 2018), we found a predominance of genes associated with multiple-drug resistance, bile stress resistance and biofilm formation in gallstone associated *Klebsiella*. The predominance of this genus across multiple gallstone studies may indicate that they are associated with ESBL and KPC producing strains. If this is the case, it is not surprising then, to find *Klebsiella* and *E. coli* thriving in the extreme environment of the human gallbladder, where toxic agents such as heavy metals, multiple-drugs (both prescribed and environmental) alongside bile acids are often concentrated ten-fold (Kose et al., 2018). Although KPC producing strains have been difficult to detect in the past with conventional methods (Nordmann et al., 2009), techniques such as metatranscriptomics, in which specific gene expression can be determined, may point the way forward to resolving the presence and role of these strains in the human gallbladder.

An interesting question to ask is whether the pressure and stressors within the human gallbladder act as a hosting ground for pathogenic bacteria and trigger multiple-drug resistance genes to be expressed, or whether these bacteria are already equipped to withstand these stressors pre-entry to the gallbladder. The implications of the former may be concerning, as continued exposure to gallbladder stressors by bacteria other than *Klebsiella* and *E. coli* may see adaptation and resistance to multiple-drug classes in those genera also. The implications of the latter could also be concerning, as the predominance of *Klebsiella* and *E. coli* in such an environment may mean the human gallbladder may provide the ideal site in which these pathogens may develop even greater resistance before infecting other sites within the human body.

Our cholesterol analysis may hold the clue to answering this question. Although the $\delta^{13}C$ values were consistent with an average vegetation diet across the 16 patients,

the δD values were quite elevated and might be consistent with a diet rich in dairy products. This may be of key significance as there have been numerous studies showing *Klebsiella pneumoniae*, *E.coli* and other Gram-negative bacteria as common food borne pathogens found in Milk and the dairy farm environment; even after hygienic production measures and pasteurisation (Oliver et al., 2005; Munoz et al., 2006; Leedom, 2006). Indeed, the consistent use of antibiotics in the dairy industry, which can lead to increased bacterial resistance, may be a contributing factor (Oliver et al., 2011). Further verification of environmental and dietary isotopic values to ascertain any involvement of dairy products, a larger patient cohort size including stone type replicates, and continued research into the gut microbiome may ultimately reveal the source of these pathogens.

Conclusions

Gallstone disease is a complex condition that requires a multidisciplinary and multifaceted approach if future investigations are to be successful. Although physico-chemical factors may be at play in gallstone lithiasis, the findings of the current and previous studies suggest the role of bacteria, in particular, those of the Gram-negative *Enterobacteriaceae* family, may be significant and should be considered alongside other interdisciplinary work. The analysis of a larger cohort of patients and stone type replicates will further aid research in this area. Future work, utilising state-of-the-art ‘omics technologies, and continued study into the unique interplay between the gallbladder environment, diet and pathogenic bacteria, will further the effort in resolving the role of bacteria in gallstone disease.

Acknowledgements

The Fiona Stanley and St John of God, Murdoch hospitals are thanked for facilitating patient consent, sample collection and ethics approval for the study. The authors would like to acknowledge TIGeR, WA-OIGC, Curtin University, and the contribution of an Australian Government Research Training Program Scholarship in supporting this research. We thank the ARC infrastructure grant (LE110100119) for the CSIA facility.

Data availability

Data are available as raw sequence reads from the NCBI Short Read Archive (SRA) under accession number SRP148965.

References

Al-Akhrass F, Al-Wohoush I, Chaftari AM, Reitzel R, Jiang Y, Ghannoum M., Raad, I. (2012). *Rhodococcus* bacteremia in cancer patients is mostly catheter related and associated with biofilm formation. *PLoS One, 7,* 1-6.

Arai, T., Ochiai, K., Senpuku, H. (2015). *Actinomyces naeslundii* GroEL-dependent initial attachment and biofilm formation in a flow cell system. *Journal of Microbiology Methods, 109,* 160-6.

Australian Institute of Health and Welfare (AIHW). (2016). Admitted patient care 2014-15: Australian hospital statistics. Canberra: (Cat. No. HSE 172; Health Services Series No. 68.)

Azúa-Bustos, A., González-Silva, C., Mancilla, R.A., Salas, L, Palma, R.E., Wynne, J.J.,…Vicuna, R. (2009). Ancient photosynthetic eukaryote biofilms in an atacama desert coastal cave. *Microbial Ecology, 58,* 497.

Bassetti, M., Pecori, D., & Peghin, M. (2016) Multidrug-resistant Gram-negative bacteria-resistant infections: epidemiology, clinical issues and therapeutic options. *Italian Journal of Medicine,10,* 364-375.

Bastow, T.P., van Aarssen, B.G.K., & Lang, D. (2007). Rapid small-scale separation of saturate, aromatic and polar components in petroleum. *Organic Geochemistry, 38,* 1235-1250.

Begley, M., Gahan, C.G. & Hill, C. (2005). The interaction between bacteria and bile. *FEMS Microbiology Reviews, 29,* 625–651.

Bengelsdorf, F.R., Gabris, C., Michel, L., Zak, M., Kazda, M. (2015). Syntrophic microbial communities on straw as biofilm carrier increase the methane yield of a biowaste-digesting biogas reactor. *AIMS Bioengineering, 2,* 264-276.

Bosak, T., Knoll, A.H., Petroff, A.P. (2013). The meaning of Stromatolites. *Annual Review of Earth and Planetary Sciences, 41,* 21–44.

Centers for disease control and prevention (CDC). (2018). *Klebsiella pneumoniae* in healthcare settings. Retrieved from http://www.cdc.gov/HAI/organisms/klebsiella/klebsiella.html.

Chan, C.L., Richter, K., Wormald, P.J., Psaltis, A.J., & Vreugde, S. (2017). *Alloiococcus otitidis* forms multispecies biofilm with *Haemophilus influenzae*: effects on antibiotic susceptibility and growth in adverse conditions. *Frontiers in cellular and infection microbiology*, *7*, 344.

Callahan, B.J., McMurdie, P.J., Rosen, M.J., Han, A.W., Johnson, A.J., & Holmes SP. (2016). DADA2: High-resolution sample inference from Illumina amplicon data. *Nature Methods, 13,* 581–583.

Caporaso, J.G., Lauber, C.L., Walters, W.A., Berg-Lyons, D., Huntley, J., Fierer, N., Knight, R. (2012). Ultra-high-throughput microbial community analysis on the Illumina HiSeq and MiSeq platforms. *The ISME Journal,6,* 1621–1624.

Caporaso, J.G., Kuczynski, J., Stombaugh, J., Bittinger, K., Bushman, F.D., Knight, R. (2010). QIIME allows analysis of high-throughput community sequencing data. *Nature Methods, 7,* 335-336.

Cetta, F. (1991). The role of bacteria in pigment gallstone disease. *Annals of Surg*ery, *213,* 315-26.

Charles, C.J., Rout, S.P., Garratt, E.J., Patel, K., Laws, A.P., & Humphreys, P.N. (2015). The enrichment of an alkaliphilic biofilm consortia capable of the anaerobic degradation of isosaccharinic acid from cellulosic materials incubated within an anthropogenic, hyperalkaline environment. *FEMS microbiology ecology, 91,* 1-11.

Che J, Li, Z., Xie, J., Wang, G., Yu, E., Xia, Y.,…Zhang, K. (2017). Microbial succession in biofilms growing on artificial substratum in subtropical freshwater aquaculture ponds. *FEMS Microbiology Letters, 364,* 1-7.

Diesendorf, N., Köhler, S., Geißdörfer, W., Grobecker-Karl, T., Karl, M., & Burkovski, A. (2017). Characterisation of *Roseomonas* mucosa isolated from the root canal of an infected tooth. *BMC Research Notes,10,* 212.

Donelli, G. (Ed). (2015). Biofilm-based healthcare-associated Infections: Vol 1. Switzerland: Springer International Publishing.

dos Anjos, M.M., Ruiz, S.P., Nakamura, C.V., de Abreu Filho, B.A. (2013). Resistance of *Alicyclobacillus acidoterrestris* spores and biofilm to industrial sanitizers. *Journal of Food Protection, 76,* 1408-13.

Dowd, S.E., Wolcott, R.D., Sun, Y., McKeehan, T., Smith, E., Rhoads, D. (2008). Polymicrobial nature of chronic diabetic foot ulcer biofilm infections determined using bacterial tag encoded FLX amplicon pyrosequencing (bTEFAP). *PLoS One, 3,* e3326.

El-Masry, M.H., Hassouna, M.S., El-Rakshy, N., & Mousa, I.E. (1995). Bacterial populations in the biofilm and non-biofilm components of a sand filter used in water treatment. *FEMS Microbiology Letters, 131,* 263-269.

Eriksson, L.L., Holgerson, P., & Johansson, I. (2017). Saliva and tooth biofilm bacterial microbiota in adolescents in a low caries community. *Scientific Reports,7,* 1-12.

Fang, H., Chen, Y., Huang, L., & He, G. (2017). Analysis of biofilm bacterial communities under different shear stresses using size-fractionated sediment. *Scientific Reports, 7,* 1-4.

Fardeau ML, Barsotti V, Cayol JL, Guasco S, Michotey V, Joseph M.,…Ollivier, B. (2010). *Caldinitratiruptor microaerophilus,* gen. nov., sp. nov. isolated from a French hot spring (Chaudes-Aigues, Massif Central): a novel cultivated facultative microaerophilic anaerobic thermophile pertaining to the Symbiobacterium branch within the Firmicutes. *Extremophiles, 14,* 241-7.

Farkas, M., Táncsics, A., Kriszt, B., Benedek, T., Tóth, E., Kéki, Z., Veres, P.G., & Szoboszlay, S. (2014). *Zoogloea oleivorans* sp. nov., a floc-forming, petroleum hydrocarbon-degrading bacterium isolated from biofilm. *International Journal of Systems and Evolutionary Microbiology, 64,* 274-279.

Gunn, J.S. (2000). Mechanisms of bacterial resistance and response to bile. *Microbes and Infection, 2,* 907.

Gurung, J., Khyriem, A.B., Banik, A., Lyngdoh, W.V., Choudhury, B., & Bhattacharyya, P. (2013). Association of biofilm production with multidrug resistance among clinical isolates of *Acinetobacter baumannii* and *Pseudomonas aeruginosa* from intensive care unit. *Indian Journal of Critical Care Medicine, 17,* 214–218.

Haigh, W.G., & Lee, S.P. (2001). Identification of oxysterols in human bile and pigment gallstones. *Gastroenterology, 121,* 118–123.

Holmes NA, Innocent TM, Heine D, Al Bassam M, Worsley SF, Trottmann F, Hutchings., M.I. (2016). Genome analysis of two *Pseudonocardia* phylotypes associated with *Acromyrmex* leafcutter ants reveals their biosynthetic potential. *Frontiers in Microbiology, 7,* 2073.

Klappenbach, J.A., Dunbar, J.M., & Schmidt, T.M. (2000). rRNA Operon copy number reflects ecological strategies of bacteria. *Applied and environmental microbiology, 66,* 1328–1333.

Kose, S.H., Grice, K., Orsi, W.D., Ballal, M., & Coolen, M.J.L. (2018). Metagenomics of pigmented and cholesterol gallstones: the putative role of bacteria. *Scientific Reports, 8,* 1-13.

Laforest-Lapointe, I. & Arrieta, M. (2018). Microbial Eukaryotes: a missing link in gut microbiome studies. *mSystems, 3,* 1-5.

Lage, O.M., & Bondoso, J. (2012). Bringing *Planctomycetes* into pure culture. *Frontiers in Microbiology, 3,* 405.

Leedom, J.M. (2006). Milk of Nonhuman Origin and Infectious Diseases in Humans. *Clinical Infectious Diseases, 43,* 610–615.

Luo, J., Lv, P., Zhang, J., Fane, A.G., McDougald, D., & Rice, S.A. (2017). Succession of biofilm communities responsible for biofouling of membrane bioreactors (MBRs). *PLoS One, 12,* e0179855.

Lyautey, E., Jackson, C.R., Cayrou, J., Rols, J.L., & Garabétian, F. (2005). Bacterial community succession in natural river biofilm assemblages. *Microbial Ecology, 50,* 589-601.

Madden, T. (2002). The BLAST Sequence Analysis Tool. In McEntyre J, Ostell J, (Eds). *The NCBI Handbook*. Available from: http://www.ncbi.nlm.nih.gov/books/NBK21097/

Makarova, K.S., Aravind, L., Wolf, Y.I., Tatusov, R.L., Minton, K.W., Koonin, E.V., & Daly, M.J. (2001). Genome of the Extremely Radiation-Resistant Bacterium *Deinococcus radiodurans* viewed from the perspective of comparative genomics. *Molecular Biology Reviews, 65,* 44-79.

Maki, T. (1996). Pathogenesis of calcium bilirubinate gallstone: role of *E. coli,* beta

glucuronidase and coagulation by inorganic ions, polyelectrolytes, and agitation. *Annals of Surgery,164*, 90-100.

Mancini, S.A., Lacrampe-Couloume, G., Lollar, B.S. (2008). Source differentiation for benzene and chlorobenzene groundwater contamination: A field application of stable carbon and hydrogen isotope analyses. *Environmental Forensics, 9*, 177-186.

Marshall, J.M., Fletchtner, A.D., La Perle, K.M., Gunn, J.S. (2014). Visualization of extracellular matrix components within sectioned *Salmonella* biofilms on the surface of human gallstones. *PLoS One, 9,* 1-7.

Merritt, M.E. & Donaldson, J.R. (2009). Effect of bile salts on the DNA and membrane integrity of enteric bacteria. *Journal of Medical Microbiology, 58,* 1533–1541.

Michael, V., Frank, O., Bartling, P., Scheuner, C., Göker, M., Brinkmann, H., & Petersen, J. (2016). Biofilm plasmids with a rhamnose operon are widely distributed determinants of the 'swim-or-stick' lifestyle in roseobacters. *The ISME Journal*, *10,* 2498-513.

Moen, B., Røssvoll, E., Måge, I., Møretrø, T., & Langsrud, S. (2016). Microbiota formed on attached stainless steel coupons correlates with the natural biofilm of the sink surface in domestic kitchens. *Canadian Journal of Microbiology, 62*, 148-60.

Munoz, M.A., Ahlström, C., Rauch, B.J., & Zadoks, R.N. (2006). Fecal Shedding of *Klebsiella pneumoniae* by Dairy Cows. *Journal of Dairy Science, 89*, 3425-3430.

National Health and Medical Research Council (NHMRC). (2007a). Australian Code for the Responsible Conduct of Research. Retrieved from https://www.nhmrc.gov.au/guidelines-publications/r39

National Health and Medical Research Council (NHMRC). (2007b). National Statement on Ethical Conduct in Human Research. Retrieved from https://www.nhmrc.gov.au/book/national-statement-ethical-conduct-human-research.).

Nordmann, P., Cuzon, G., & Naas, T. (2009). The real threat of *Klebsiella pneumoniae* carbapenemase-producing bacteria. *Lancet Infectious Diseases, 9,* 228-36.

Nouha, K., Kumar, R.S., & Tyagi, R.D. (2016). Heavy metals removal from wastewater using extracellular polymeric substances produced by *Cloacibacterium normanense* in wastewater sludge supplemented with crude glycerol and study of extracellular polymeric substances extraction by different methods. *Bioresource Technology, 212,* 120-129.

O'Brien, D.M. (2015). Stable Isotope Ratios as Biomarkers of Diet for Health Research *Annual Review of Nutrition, 35,* 565–594.

O'Brien, D.M., Wooller, M.J. (2007). Tracking human travel using stable oxygen and hydrogen isotope analyses of hair and urine. *RCM, 21,* 2422-2430.

Oliver, S.P., Jayarao, B.M., & Almeida, R.A. (2005). Foodborne Pathogens in Milk and the Dairy Farm Environment: Food Safety and Public Health Implications. *Foodborne Pathogens and Disease, 2,* 115-129.

Oliver, S.P., Murinda, S.E., & Jayarao, B.M. (2011). Impact of antibiotic use in adult dairy cows on antimicrobial resistance of veterinary and human pathogens: A comprehensive review. *Foodborne Pathogens and Disease, 8,* 337-355.

Paterson, D.L. (2006). Resistance in gram-negative bacteria: *Enterobacteriaceae*. *American Journal of Medicine, 119,* 62-70.

Peng, C., Tian, J., Lv, M., Huang, Y., Tian, Y., & Zhang, Z. (2014). Development and validation of a sensitive LC-MS-MS method for the simultaneous determination of multicomponent contents in artificial Calculus Bovis. *Journal of chromatographic Science, 52,* 128-36.

Qiao, T., Ma, R.H., Luo, X.B., Yang, L.Q., Luo, Z.L., & Zheng, P.M. (2013) The systematic classification of gallbladder stones. *PLoS One, 8,10,* 1-11.

Qureshi, S. (2014). *Klebsiella* Infections. Medscape. Retrieved from http://emedicine.medscape.com/article/219907-overview.

Rawat, D., & Nair, D. (2010). Extended-spectrum β-lactamases in Gram negative bacteria. *Journal of global Infectious disease*, 3, 263–274.

Salas-Jara, M.J., Ilabaca, A., Vega, M., & García, A. (2016). Biofilm forming *Lactobacillus*: new challenges for the development of probiotics. *Microorganisms, 4,* 35.

Salmassi, T.M., Walker, J.J., Newman, D.K., Leadbetter, J.R., Pace, N.R., & Hering, J.G. (2006). Community and cultivation analysis of arsenite oxidizing biofilms at Hot Creek. Environ Microbiol. 2006; 8: 50-9.

Shaffer, E.A. (2005) Epidemiology and risk factors for gallstone disease: has the paradigm changed in the 21st century? *Current Gastroenterology Reports, 7,* 132-140.

Shen, H., Ye, F., Xie, L., Yang, J., Li, Z., Xu, P.,…Zhang, X. (2015). Metagenomic sequencing of bile from gallstone patients to identify different microbial community patterns and novel biliary bacteria. *Scientific Reports, 5,* 17450.

Schmitt, S., Deines, P., Behnam, F., Wagner, M., & Taylor, M.W. (2011). *Chloroflexi* bacteria are more diverse, abundant, and similar in high than in low microbial abundance sponges. *FEMS microbiology ecology, 78,* 497–510.

Shukla, S.K., & Toleti, S.R. (2017). The first recorded incidence of *Deinococcus radiodurans* R1 biofilm formation and its implications in heavy metals bioremediation. *bioRxiv, 234781,* 1-27.

Soto-Giron, M.J., Rodriguez, L.M., Luo, C., Elk, M., Ryu, H., & Hoelle, J. (2016). Biofilms on hospital shower hoses: characterization and implications for nosocomial infections. *Applied Environmental Microbiology, 82,* 2872-2883.

Spring, S., Bunk, B., Spröer, C., Schumann, P., Rohde, M., Tindall, B.J., & Klenk, H.P. (2016). Characterization of the first cultured representative of *Verrucomicrobia* subdivision 5 indicates the proposal of a novel phylum. *The ISME Journal, 10,* 2801–2816.

Stewart L., Smith, A.L., Pellegrini, C.A., Motson, R.W. & Way, L.W. (1987). Pigment gallstones form as a composite of bacterial micro-colonies and pigment solids. *Annals of Surgery, 206,* 242-250.

Stewart, L., Ponce, R., Oesterk, A.L., Griffiss, J.M., & Way. L.W. (2000). Pigment gallstone pathogenesis: Slime production by biliary bacteria is more important than beta-glucuronidase production. *Journal* of *Gastrointestinal Surgery, 4,* 547.

Stewart, L., Oesterle, A.L., Erdan, I, Griffiss, J.M. & Way, L.W. (2002). The pathogenesis of pigment gallstones in western societies: the central role of bacteria. *Journal of Gastrointestinal Surgery, 6,* 891–904.

Stinton L.M., Shaffer, E.A. (2012). Epidemiology of gallbladder disease: cholelithiasis and cancer. *Gut and Liver, 6,* 172-187.

Swidinski, A. & Lee, S.P. (2001). The role of bacteria in gallstone pathogenesis. *Frontiers of Bioscience, 6,* 93-103.

Vasoo, S., Barreto, J.N., & Tosh, P.K. (2015). Emerging issues in gram-negative bacterial resistance: an update for the practicing clinician. Mayo clinical procedures, 90, 395-403.

Wu, T., Zhang, Z., Liu, B., Hou, D. Liang, Y., Zhang, J., & Shi, P. (2013). Gut microbiota dysbiosis and bacterial community assembly associated with cholesterol gallstones in large-scale study. *BMC Genomics*, *14*, 1-11.

Xiao, Y., Zheng, Y., Wu, S., Zhang, E.H., Chen, Z., Liang, P.,…Zhao, F. (2015). Pyrosequencing reveals a core community of anodic bacterial biofilms in bioelectrochemical systems from China. *Frontiers in Microbiology, 16,* 1410.

Yamamuro, A., Kouzuma, A., Abe, T., & Watanabe, K. (2014) Metagenomic analyses reveal the involvement of syntrophic consortia in methanol/electricity conversion in microbial fuel cells. *PLoS ONE, 9,* e98425.

Zumsteg, A., Urwyler, S.K., & Glaubitz, J. (2017). Characterizing bacterial communities in paper production—troublemakers revealed. *Microbiology Open, 6,* 1-6.

Appendix 2

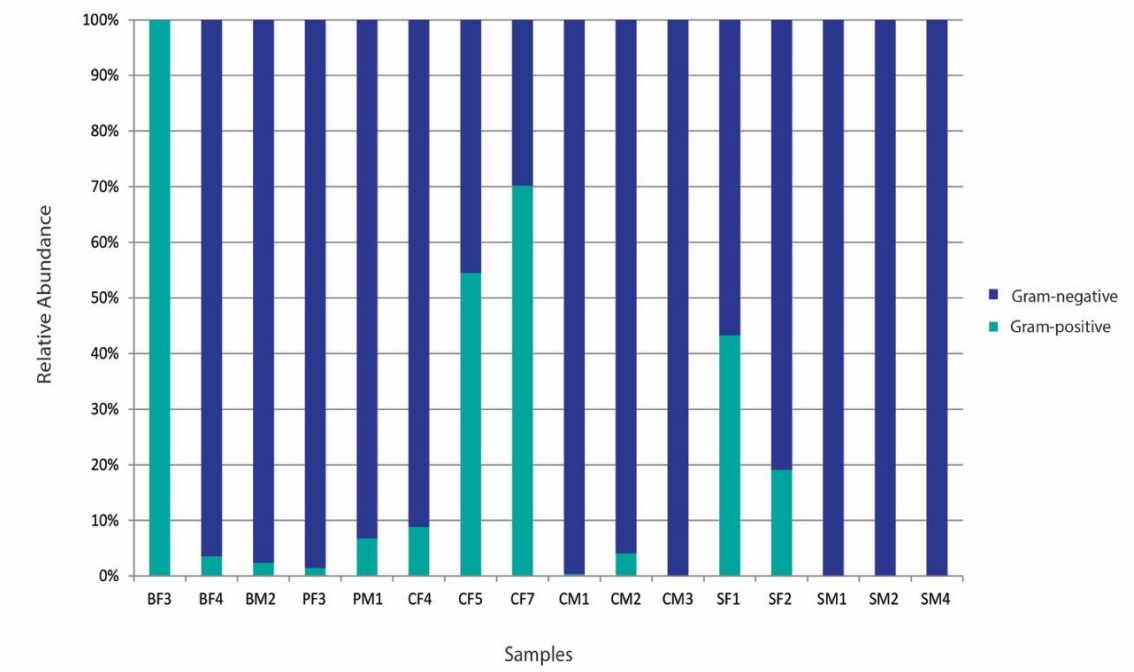


Figure A2. The relative abundance percentage of Gram negative and Gram positive bacteria in the gallstones of 16 patients within the study based on the total reads acquired by MiSeq 16S High-throughput sequencing.

Chapter 4:

Characterisation of large and solitary human gallstones: A case study for a natural biomineralisation model.

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(*under preparation).*

Abstract

Gallstone disease is a complex condition which accounts for the majority of elective surgery requests in OECD nations and poses a considerable morbidity and financial cost to health systems worldwide. Previous studies have linked gallstone formation to a variety of causes which include genetics, medical history, physico-chemical imbalance and most recently, bacterial processes. A definitive model is yet to be made that can account for the composition, morphology and growth mechanisms behind all gallstone disease reports and findings. This is mostly due to the increasingly observed complexity of the disease and a history of incongruent data. The majority of studies have concentrated on multiple small sized (0.5-1 cm) cholesterol and pigmented stones as they are considered to be the prevailing presentation types. Few studies, however, have investigated large (2-4 cm), solitary or unusual stones in considerable detail. Further, a number of researchers have begun to explore the possible connection between gallstone lithification and biomineralisation events observed in nature which have been shown to exhibit similar morphologies to gallstones. In this study, we explore five large gallstones in greater detail using state-of-the-art SEM microscopic techniques to elucidate possible formation mechanisms that may be obscured in smaller stones. The observed stone morphology is compared to known biomineralisation models in nature, and a proposed theoretical model for gallstone formation is provided. We complement this with compound specific isotopic analysis of cholesterol in the rings of a particularly large solitary stone to ascertain possible environmental factors that may be at play in stone formation.

Introduction

The prevailing hypothesis of gallstone pathogenesis is that a number of physiological conditions influence an imbalance in the ratio between cholesterol, phospholipids, and bile acids in the gallbladder, altering the normal micellar phase of bile to that which facilitates both cholesterol and calcium bilirubinate crystal precipitation (Stewart et al., 2002; Van Epercum, 2011). In 1982, a conference was held to codify the most commonly observed gallstones into four broad types determined mostly by colour and appearance (Stewart et al., 2002). These were white or yellow cholesterol

stones (≥70% cholesterol, ≤30% ca-bilirubinate), brown pigmented stones (≤30% cholesterol, ≥70% ca-bilirubinate), black pigmented stones (≥ 90% or purely ca-bilirubinate), and mixed stones (between ≥30-70% cholesterol) (Bouchier, 1992; Qiao, 2013). Though these four broad categories continue to be used for descriptive convenience, a number of studies have suggested as many as eight stone types (Fu et al., 1984; Ha & Park, 2018). Nevertheless, the direct mechanism as to how crystallised precipitates aggregate to form the larger observed stones has yet to be determined. Initial attempts emphasised a physico-chemical model, in which cholesterol was seen to accrete around a calcium carbonate nidus formed by the active binding of Ca^{+2} cations to the anionic bile constituents bilirubinate, phosphate and carbonate (Rege, 2002). In other studies it has been shown that the nidus can precipitate into one of three calcium carbonate polymorphs; calcite, aragonite, or the rarer vaterite, with each forming via a unique biochemical pathway (Bills & Lewis, 1975; Lee & Chen, 2009). Brown or black pigmented stones were shown to have a higher concentration of bilirubin (a break down product of red blood cells) giving them their characteristic colour, with their formation attributed to increased precipitation of ca-bilirubinate rather than calcium carbonate (Lee & Chen, 2009; Liu & Hu, 2002; Ha & Park, 2018). An alternative theory involved the mediation of bacterial biofilms providing the glue that bound precipitates together, with this model restricted to brown pigmented stones alone as other stone types were thought to be sterile (Stewart et al., 2001, 2002). Although these formation models persist today, a number of studies, alongside the advent of new technology, have rendered them obsolete. For example, as early as 1968, a particularly unusual stone was found to consist of all three calcium carbonate polymorphs, a symmetrical octahedral structure, and no discernible nidus point (Sutor & Wooley, 1968). Subsequent reports revealed gallstones with nidus points composed entirely of monohydrate cholesterol crystals, with calcium carbonate only within the stones' radial ring structure, or no calcium carbonate in the stone matrix at all (Bills & Lewis, 1975; van den Berg, 2000). In recent years, improved microbial techniques, such as NextGen sequencing, revealed biofilm forming bacteria in not only pigmented stones, but all major stone types (Kose et al., 2018; 2019 (under review). Of particular note is the sparsity of investigations focusing on large or solitary stones (2.0-2.9 cm), which have been

suggested to predispose patients to double the risk of gallbladder cancer, with this risk increasing 10-fold for stones > 3 cm (Diehl, 1983; Heuman, 2017).

In the present study, we take an alternative approach and provide an interdisciplinary investigation into the morphology of five large gallstones (2-4 cm in diameter) in greater detail using light microscopy, state-of-the-art SEM, and energy-dispersive X-ray spectroscopy. To our knowledge few studies have investigated large or solitary stones in sufficient depth. This may be due to their rarity or the complex matrices they present with. A greater examination of the morphology of these stones may reveal the processes behind the aggregation and growth of all stone types and those that may be obscured in smaller stones (<1 cm) (Diehl, 1983; Heuman, 2017). We complement this study with cholesterol analysis of individual rings within a particularly large gallstone (approx. 3 cm in length), using compound specific Isotope analysis (CSIA) (Mancini et al., 2008). This technique allows for the measurement of the carbon ($\delta^{13}C$ values) and hydrogen (δD values) isotopes of individual compounds (i.e. cholesterol) to ascertain whether environmental or dietary changes occurred as the stone ring system developed over time. Our results show that the formation mechanism behind a number of stones may be indicative of those observed in microbial concretions within natural ecosystems. We provide a novel formation and growth model for all gallstone types based on these natural concretions and offer greater insight into the bacterial hypothesis of gallstone pathogenesis.

Materials and methods

Sample collection

The study was designed with the aid of the National Health and Medical Research Council (NHMRC), according to the guidelines stipulated in the National Statement on Ethical Conduct in Human Research 2007 (NHMRC, 2007a), and the Australian Code for the Responsible Conduct of Research 2007 (NHRMC, 2007b). The study was approved for a maximum number of 16 patients, of which only five presented with the large or solitary gallstone type. The study met the ethical guidelines of the South Metropolitan Health Service Human Research Ethics Committee (HREC Reference: 15-136), the Fiona Stanley Human Research Ethics Committee (Ref: 2015-136), the St John of God Health Care Human Research Ethics Committee (Ref: 1021), and the Curtin University Human Research Ethics Committee (Ref: HR229/2015). Samples were collected during surgery and were immediately rinsed in sterile saline solution (9 g L^{-1} NaCl) and placed in sterile glass containers. The samples were immediately stored at −80 °C until further processing. Patients presenting with gallstone disease were recruited to participate in the study in consultation with their medical advisor and provided written informed consent upon enrolment. See Table 4.1 for patient and sample information.

Patient Information

Table 4.1 Patient and sample Information.

Sample ID	Age	Sex	BMI	Overweight	Antibiotics	Type	Size
LM_1	36	M	26.1	Y	Nil	Large/Multiple	1-2 cm
LM_2	36	M	26.1	Y	Nil	Large/Multiple	1-2 cm
LS1	27	F	32	Y	Nil	Large/Solitary	1-2 cm
LS2	71	F	31	Y	Nil	Large/Solitary	1-2 cm
LS3	69	M	30.5	Y	Nil	Large/Solitary	2-3 cm

Light Microscopy

Images of stones at 0.5 (mm) x magnification were captured with a 1996 Nikon SMZ800 stereomicroscope located at the John de Laeter Centre of Curtin University, Australia. All images were captured at stereo setting 1, and analysed by the ToupView 3.3 Imaging software.

FE-SEM

The Tescan Mira 3 field emission – scanning electron microscope (FE-SEM), located at the John de Laeter Centre of Curtin University, Australia, was utilised to obtain intact surface images of a number of gallstone samples. The SEM was operated between 10-20 kV, using a low-pressure vacuum setting (LVSTD) for low magnification images, and secondary (SE) or back scattered electron detectors (BSE) utilised for image magnifications greater than 100 µm. Images were complemented by spot and area chemical analysis via the instrument's in-built energy-dispersive X-ray spectrometer (EDS). The Oxford Instruments AZtec imaging software v. 3.3 SP1 was used for subsequent image and chemical analysis.

FIB-SEM

Images of the samples were acquired from fracture surfaces from gallstones. The sample fragments were mounted onto an aluminium stub and coated with a thin layer of platinum. Imaging was performed using a Tescan Lyra 3 focused ion beam - scanning electron microscope (FIB-SEM) located in the John de Laeter Centre of Curtin University, Australia. The SEM was operated at acceleration voltage of 10 kV and images were captured using secondary electron (SE) and back scattered electron detectors (BSE). In some cases, the images were complemented by spot and area chemical analysis collected using the instrument's EDS spectrometer. The Tescan Lyra 3 FIB–SEM was used to produce site-specific cross-sections in order to image the sample below the surface. A platinum strip was deposited over the region of interest and the FIB was used to sputter away a trench approximately 10 µm wide and 8 µm deep. The cross-sectional surface was polished with a 1 nA ion beam current. The Oxford Instruments AZtec imaging software v. 3.3 SP1 was used for subsequent image and chemical analysis.

Gas Chromatography Isotope Ratio Mass Spectrometry (CSIA)

Isotope and cholesterol analysis were conducted on sample LS3 using Gas Chromatography Mass spectrometry and Gas Chromatography Isotope Ratio Mass spectrometry. A sub sample was taken from the outer ring, inner-most ring, and centre core section of the sample, with each sub sample crushed in individual heat-sterilized (500 ºC, 8h) mortars. The ground powder was then extracted *via* sonication (1 h) with dichloromethane (DCM) and methanol (9:1). The extracts were then fractionated by small-scale column liquid chromatography (Bastow et al., 2001). Approximately 2 mg of the total extract from each sub sample was placed on top of a small column (5 x 0.5 cm i.d.) of activated silica gel (160 ºC, 8 h). The first hydrocarbon fraction was eluted with *n*-hexane (2 mL), the second hydrocarbon fraction with DCM in *n*-hexane (1:4, 2 mL), and the more polar fraction with an equal mixture of DCM and methanol (1:1, 2 mL). The fractions were analysed by gas chromatography-mass spectrometry (Hewlett-Packard/Agilent HP6890 Series GC with HP5973 Mass Selective Detector), with the instrument prepared as per Haigh & Lee (Haigh & Lee, 2001).

The polar fractions of each sub sample were analysed by compound specific isotope analyses to obtain $\delta^{13}C$ and δD values of cholest-5-en-3β-ol. The polar fractions were run as un-derivatised free sterols. Although this method allows for timely and convenient analysis, if samples containg exchangeable hydrogens are inadequately prepared, there is the potential that these hydroxyl groups can exchange with hydrogen from atmospheric water vapour, effecting δD values. All precautions were taken to limit atmospheric exposure in this study. The samples were removed from the patient and immediately placed into sterile containers that were in-turn placed into a liquid nitrogen thermos within the aseptic operational theatre. Once transported the the main analysis site, the samples were kept in a -20 freezer as lower temperatures reduce the rate of exchange. Further, as cholesterol has only one exchangeable hydrogen atom and 45 non-exchangeable hyrdogens, the overall effect on the δD value is considered here to be low (Schimmelmann et al., 2006).

The instrument used was a Thermo Delta V Advantage isotope ratio monitoring mass spectrometer (irMS), coupled to a Thermo Trace GC Ultra via a GC Isolink and Conflo IV. The column used was an Agilent DB-5MS Ultra-Inert, 60 m long, 0.25 mm (i.d.), with 0.25 μm film thickness. An aliquot of 1 μL of each fraction was injected into the split/splitless injector in splitless mode, held at 280 °C. The GC oven was increased from 40 to 325 °C at 10 °C/min, then held at 325 °C for 10 min. The carrier gas used was helium held at a constant flow of 1.5 mL/min. The stable carbon isotope analysis utilised a GC column outflow which was passed through a GC Isolink combustion reactor (copper oxide / nickel oxide, 1000 °C) to combust hydrocarbons to CO_2 and H_2O. The H_2O was removed *via* a Nafion water trap. For hydrogen isotope analysis, the outflow passed through the high-temperature conversion reactor (graphite-lined, 1420 °C) and was thermally converted to H_2. The CO_2 / H_2 passed through the Conflo IV interface to the irMS, which measured *m/z* 44, 45 and 46 (for CO_2) or *m/z* 2 and 3 (for H_2). The H_3^+ correction factor was measured daily during δD analyses and was always less than 13 ppm. The $\delta^{13}C$ and δD values were calculated from the measured masses by Thermo Isodat software and calibrated to the VPDB (for CO_2) and VSMOW (for H_2 or D/H) scales by comparison with a mixture of *n*-alkane standards of known isotopic composition. The resulting data was interpreted *via* the Isodat Workspace v3.0, Gas Isotope Ratio MS Software, developed by Thermo Scientific.

Results

Light Microscopy analysis

Overview images of samples LM_1, LM_2, LS1, and LS2 (Figure 4.1) revealed nodular, elongated or roughly spherical exterior morphology. Samples LM_1, LM_2 and LS1 exhibited medium to dark brown colouring on their exterior surface, while sample LS2 showed a pale-yellow exterior alongside spots of white or pale green. Conversely, compared with the brown of their exterior and rim, the interior overview images of samples LM_1, and LM_2 exhibited a distinctly bright crystalline yellow

colouring throughout the stone matrix, with central cores that revert back to a lighter shade of brown particulate matter.

Sample LS1 retained its brown colouring throughout its internal matrix, but a closer look into its centre reveals a rich yellow crystalline core mixed with brown and dark green particulate matter. Sample LS2 exhibits a rich yellow crystalline internal rim, with a brown particulate, non-crystalline internal matrix. There was also no discernible crystalline core in this sample, with the brown particulate matter uniform across the stone's matrix. A closer look into the morphology of each of the stones' external surfaces revealed aggregated or clustered grape-like morphology, regardless of the internal or external colour, particulates, or arrangement within the stones' matrices.

The largest stone in the sample set, sample LS3, exhibited an elliptical shape, alongside a smooth dark brown external surface morphology (Figure 4.3). Cross-section images of the samples interior revealed an alternating brown, dark brown, ring system of particulate matter. The central area of the sample exhibited a distinct concentrated white to pale yellow crystalline core (Figure 4.3). Due to its large size and unique morphology, this sample is presented separately and examined in greater detail in the following section.

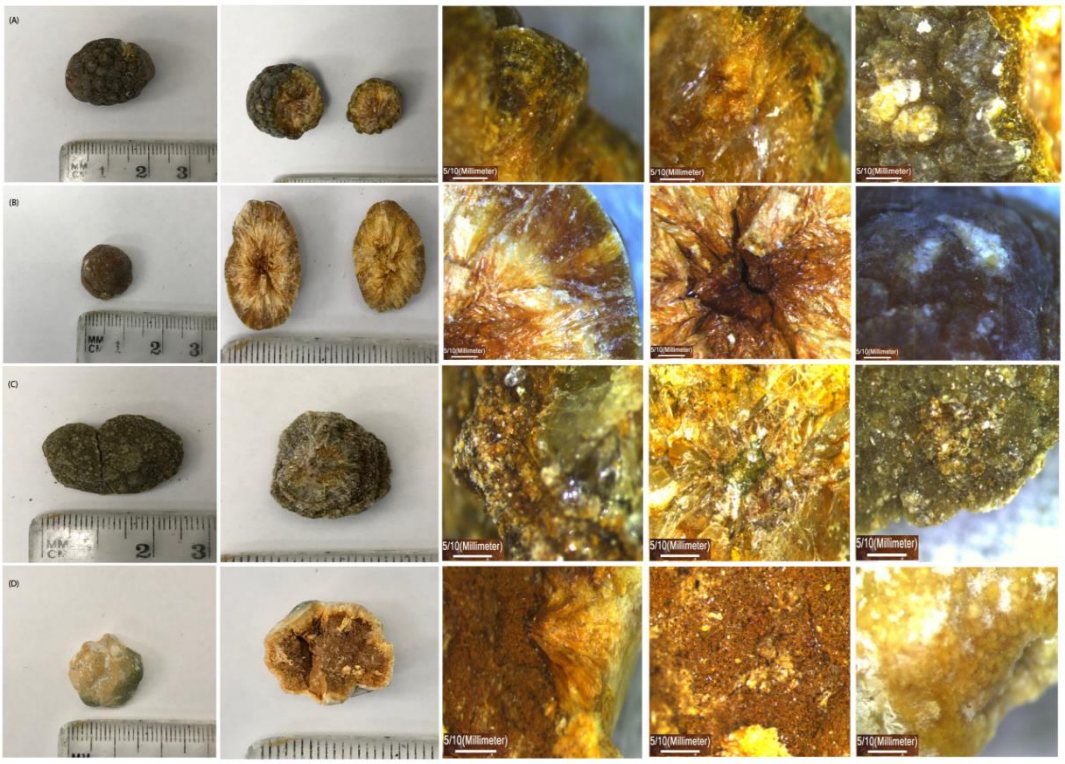


Figure 4.1 Overview images of samples LM_1 (A), LM_2 (B), LS1 (C), and LS2 (D). The last three panels show the Light Microscope images of the edge, centre and outside morphology of each sample. An aggregated, grape-like morphology can be observed in all four samples.

SEM Imaging

A closer examination of the external grape-like surface morphology of sample LM1_1 revealed an agglomeration of thin micro plate-like structures (see full page Figure 4.2). Examination of the stone's interior revealed a similar stacking of micro-plates (Figure 4.2). Spot chemical analysis of both the exterior and interior revealed a predominance of carbon with no other elements detected (see Appendix 3 for spectra). The observed micro plates are indicative of the planar structure of cholesterol and align with our previous analysis of the gallstones of 16 patients in which the primary chemical signal recovered for each was cholest-5-en-3β-ol (Kose et al., 2018). It is assumed hereafter that the following observed micro plate-like structures are cholesterol micro-plates.

Sample LM1_2 also exhibited a predominantly cholesterol micro-plate interior matrix. Higher magnification of its core section revealed not only cholesterol micro-plates, but microspheres within, and imbedded into, one of the observed plates

(Figure 4.2). Spot chemical analysis of the internal edge and core area also showed a predominance of carbon with no other elemental signals detected. Both the internal outer rim and central core section of sample LS1 similarly exhibited cholesterol micro-plate morphology (Figure 4.2). Spot chemical analysis of the internal rim and central core of LS1 revealed a predominance of carbon, with a possible trace of calcium and oxygen detected on the rim surface (Appendix 3). FIB analysis was conducted in the central core area of LS1 to examine whether the uniform plate morphology persisted with depth. No structural changes were observed however this could be due to the susceptibility of cholesterol to melt as the ion beam cut into the stone's surface (Figure 4.2). A similar result was found for sample LS2. The brown particulate matter which runs throughout its interior revealed the same cholesterol micro-plate structure seen with the other stones. FIB analysis of LS2's core similarly revealed the cholesterol structure to persist with depth (Figure 4.2). Spot chemical analysis of LS2 revealed a predominance of carbon and possible traces of phosphorous, calcium and sodium in the internal rim surface of the stone (Appendix 3). The central core area exhibited a predominance of carbon with no other elemental traces detected.

SEM analysis of the largest stone (LS3) revealed a unique micro-morphology. A sub-sample comprised of part ring section and part of the centre core was examined in greater detail. The brown, friable ring section revealed a porous micro morphology (Figure 4.3). The centre core area revealed the characteristic cholesterol micro-plate morphology exhibited by the previous four stones. Spot chemical analysis of the outer-most ring and inner-most section of the ring system (Figure 4.3; Appendix 3), showed a predominance of carbon with trace amounts of oxygen and calcium (see Appendix 3). The central core region revealed only a carbonate dominated matrix. A higher magnification image of this area revealed what appears to be a mucilaginous substance on one of the plate surfaces that resembles biofilm. This material was not observed in the other examined plate-like surfaces and may not be immediately visible.

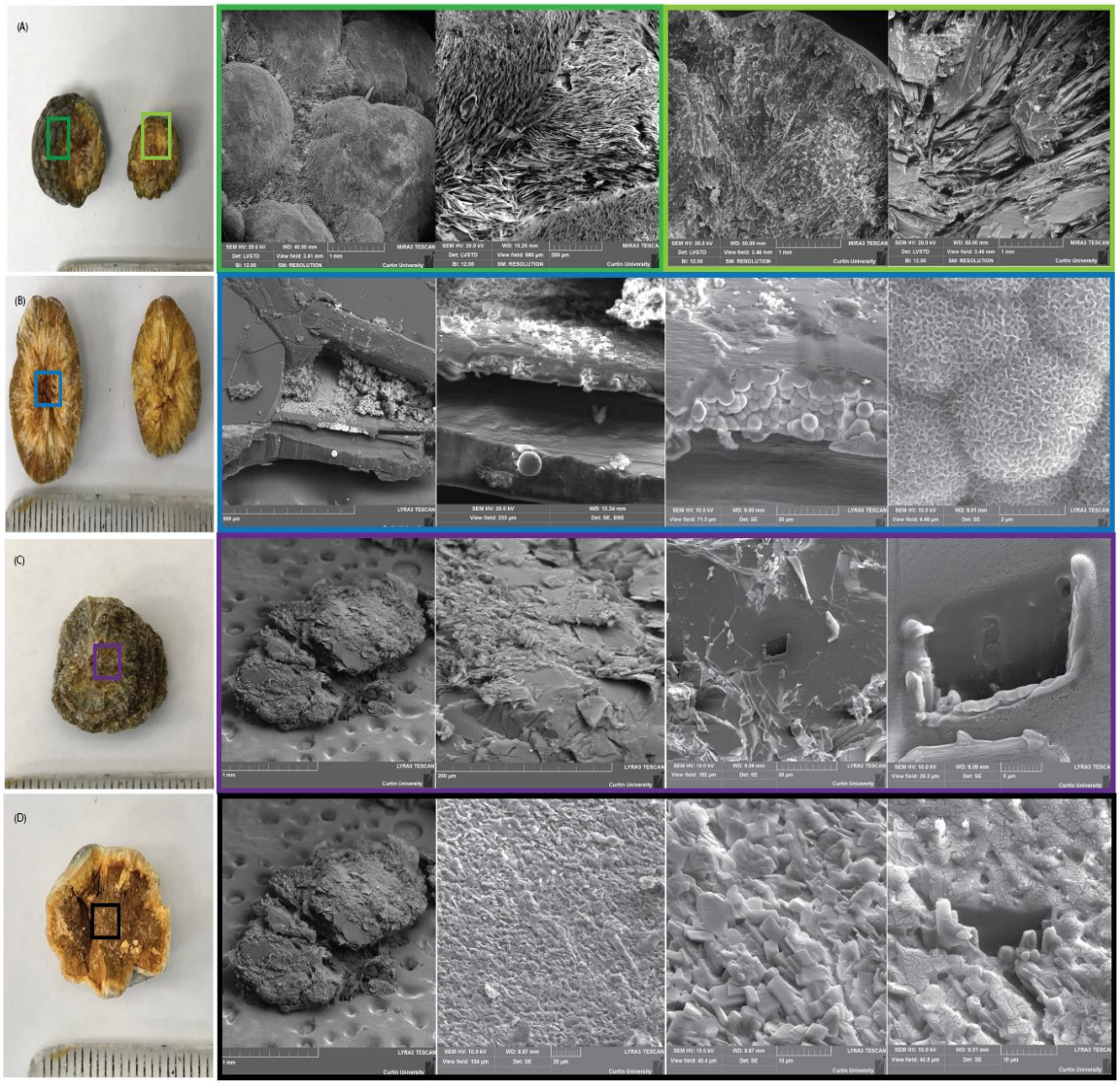


Figure 4.2 Overview and SEM images of samples LM_1 (A), LM_2 (B), LS1 (C), and LS2 (D).The first two SEM images for sample (A) show the stone's outer region morphology, with the last two images indicated the stone's centre region. The centre region of samples (B), (C), and (D) are shown in increasing magnification. Sample LM_2 (B) showed an aggregated grape-like microsphere structure in its centre was LM_2 (B). FIBSEM analysis was conducted on LS1 (C), and LS2 (D), revealing the tightly packed cholesterol micro-plate structure persisting at depth in these stones.

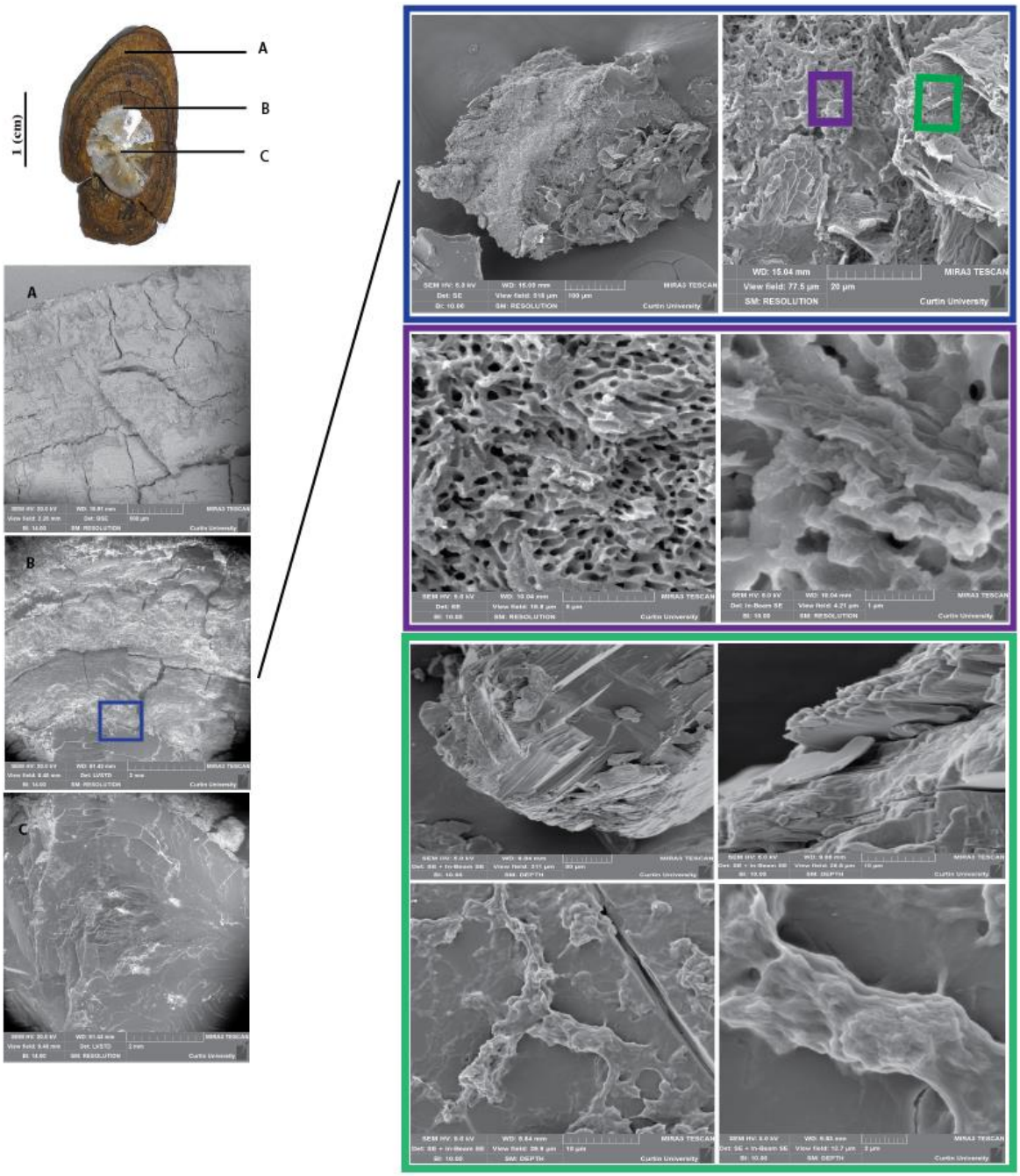


Figure 4.3 FESEM images of sample LS3. The outer ring (A), inner-most ring and partial centre (B) and centre core crystallised area (C) are indicated. Image B is shown magnified in greater detail revealing a friable porous area within the pigmented section (purple) and plate like morphology in the centre section (green). The plate like morphology in the centre section is magnified further to reveal what is possibly traces of bacterial biofilm.

Cholesterol analysis

Cholesterol analysis was performed on samples LM1_1, LM_2, LS1, LS2 in a previous study and revealed a predominance of cholest-5-en-3β-ol with trace amounts of 5α-cholest-7-en-3β-ol in each of the stones (Kose et al., 2019 under review). The $\delta^{13}C$ values for the studied stones averaged –23.3‰, with the δD values averaging –256‰. The same cholest-5-en-3β-ol compound predominated the interior outer-ring (A), inner most-ring (B) and central core (C) section of sample LS3 (Figure 4.3, Table 4.2). As per the previous study, compound specific isotope analysis was performed on the non-derivatised cholest-5-en-3β-ol compound identified in each section of LS3. The $\delta^{13}C$ values were consistent with the previous study, and revealed possible homogeneity with an average of –23.1‰ with a 1 per mil difference between the three sections examined (Table 4.2). The δD values were consistent between the outer and inner-most ring section with a 2 per mil difference between the two (Table 4.2). The difference between the ring sections and the central core revealed a 6-7 per mil difference, with the average between the three –247‰.. The δD average of the sections within sample LS3 show an 8 per mil difference with the previous study.

Table 4.2 Compound specific $\delta^{13}C$ and δD values for sample LS3. The values are followed by the standard deviation (in brackets) and the number of repeat injections (3).

$\delta^{13}C$(‰VPBD)		δ^{D}(‰VSMOW)	
A	−22.1(0.1)3	A	−244 (1)3
B	−23.4(0.1)3	B	−246 (0)3
C	−23.9(0.1)3	C	−252 (1)3

Discussion

The light microscope examination of the four large stones at lower magnification revealed grape-like clusters in each of the stones' external morphology. Greater magnification of both the internal outer rim and the central core section of these stones showed a predominance of what are most likely aggregated cholesterol micro-plates. Calcium carbonate nidus points were not identified in any of the stones examined, with the central core morphology, particularly in samples LM_1, LS1 and LS3, attributed here to cholesterol monohydrate crystallisation. Further, EDS spectroscopy of the stones revealed a predominance of carbonate material throughout the matrices of the stones, particularly in the centre, with only some stones exhibiting trace levels of calcium or oxygen in their outer ring sections. The CSIA of cholesterol in each of the stones was consistent with our previous studies, revealing isotope values associated with C3 vegetation and those rich in fats typically associated with dairy products (Kose et al., 2018; Kose et al., 2019 under review). The first three samples (LM1_1, LM1_2, LS1) and the largest sample (LS3) would generally be categorised as pigmented or mixed stones, with sample LS2 considered a cholesterol stone. However, both the internal and external matrices of these stones, regardless of their colour, shape, or source of environmental cholesterol, suggest a uniform formation process. Of particular note, is the grape-like clusters or microspheres seen on the surface morphology of the first four analysed stones, and the core section of sample LM1_2. This type of morphology, including accretion of particulate matter around a concentrated core, has been studied in-depth within biomineralised stones in natural settings (Catuneanu et al., 2011; Batchelor et al., 2018; Mariotti et al., 2018). Commonly known as concretions or microbialites, these organosedimentary structures are said to form by the detrital trapping or binding of sediments as a result of the growth and metabolic activity of microbial communities (Burne & Moore, 1987). The most commonly studied microbialites are ooids and stromatolites. The morphology observed in this study is most resemblant of microbially mediated ooid formation models (Figure 4.4). Although the exact formation mechanism of ooid morphology is yet to be resolved, a number of broad formation models exist based on a near century of observation in this area (Batchelor et al., 2018). Those concerned with microbial influence are shown to accumulate via

the successive coating, cementing and binding of carbonate particulates by bacterial biofilms (Catuneanu et al., 2011; Mariotti et al., 2018; Figure 4.5). This particulate matter may be excreted or precipitated by the microbial communities in question, or be particulate matter in the immediate environment incidentally trapped by the accumulating biofilms (Burn & Moore, 1987; Catuneanu et al., 2011; Mariotti et al., 2018). Of note is the grape-like clusters that are shown to accumulate and in-turn be bound together in successive stages of ooid stone growth and development leading to pelloid nuclei and botryoidal macro-stone morphologies (Figure 4.4, Figure 4.5). These morphologies are strikingly similar to that observed in the gallstones in both current and previous studies where all key stages of ooid development are readily observable (Figure 4.4, Figure 4.5).

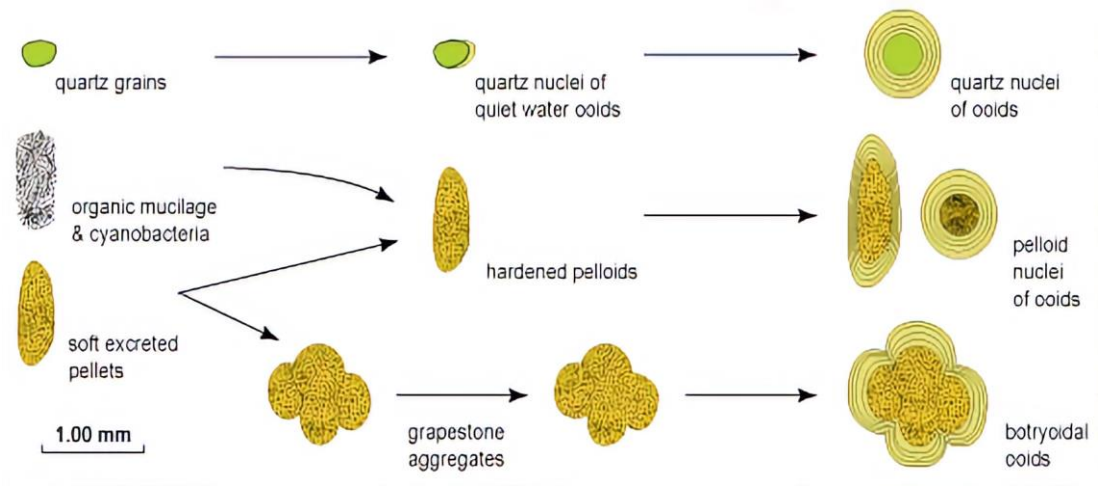


Figure 4.4 A popular biomineralisation model depicting the accumulation and aggregation of a variety of ooid morphologies. The images depict stone growth as turbidity increases (from left to right) in shallow lagoon systems. The formation mechanism shown in the centre and bottom images involve the mediation of bacterial biofilm as a key component of stone cementation, aggregation and growth (modified from Catuneanu et al., 2011).

The connection between gallstone morphology and natural organosedimentary structures is not new and was first suggested in 1968 with the analysis of a particularly large, solitary, and unusual gallstone (Sutor & Wooley, 1968; Figure 4.5). This gallstone had a botryoidal macro-morphology that has only been observed in ooid formation models that involve microbial mediation (Figure 4.4, Figure 4.5).

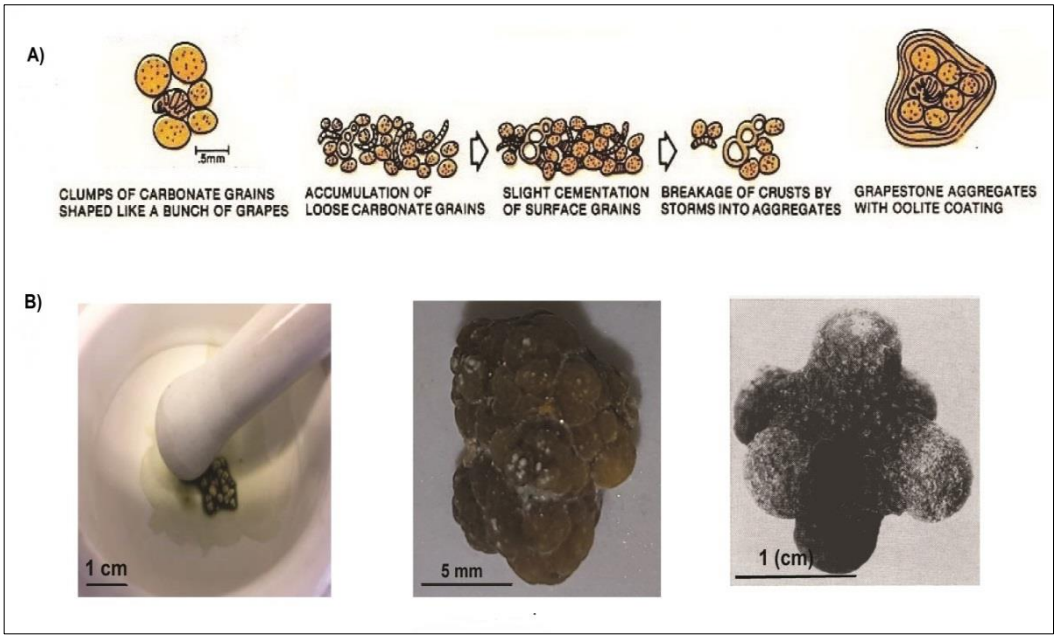


Figure 4.5 Examples of gallstones from previous studies (B) matching the environmental biomineralised models shown above (A). The last gallstone image is the botryoidal gallstone morphology first observed by Sutor & Wooley in 1968 (modified from Catuneanu et al., 2011; Kose et al., 2019 (under review); Suter & Wooley, 1968).

The work of Fu et al. (1984), furthered this connection by examining a number of gallstones and suggested a re-classification of stones into 8 distinct types based on their similar morphology with known organosedimentary structures observed in natural ecosystems. These were radial, radial annual (tree) ring-like, rock strata-like stromatolite, cast amorphous, sand bed-like stromatolite, silt-like, black, and complex stones (Fu et al., 1984; Ha & Park, 2018). The results of this study suggest a more simplified view. Namely, that cholesterol hypersecretion leads to increased precipitation of cholesterol crystals/micro-plates, which, together with incidental particulates in biliary sludge, are bound together by successive coatings of microbial biofilm. Through turbidity and abrasion, the bound particulates form microspheres (grape-like clusters), with this process repeating until the observed macro-stones are formed (Figure 4.6). The colour, shape and exterior morphology of the stones may only look different due to each patients' unique biliary environment (i.e. greater levels of bilirubinate or cholesterol), or physical activity (including gallbladder activity) causing abrasion, compression or break-up of successive stones into a number of shapes and sizes. The tightly packed core area of the stones may be caused by successive layers, or other stones, compressing the entire matrix of the

stone inducing melting, re-crystallisation, and concentration of cholesterol crystals in the centre (Figure 4.6).

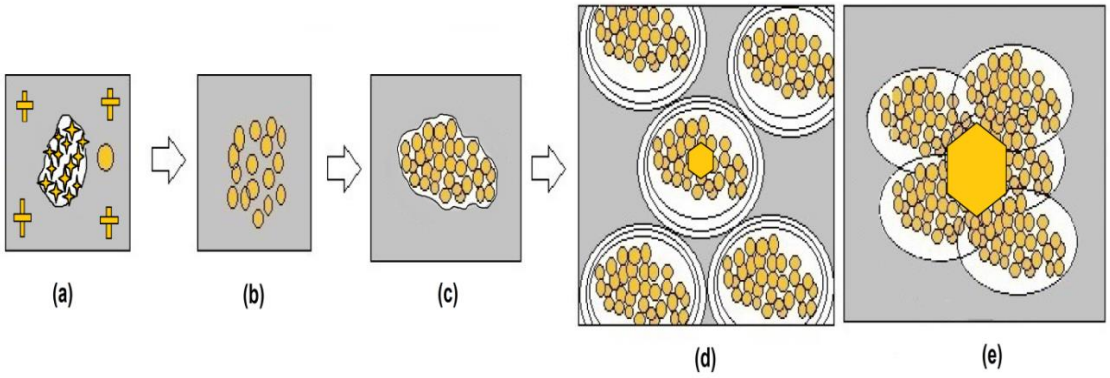


Figure 4.6 Panel (a), (b), and (c) depict cholesterol microcrystals or plates accumulating (yellow) in biliary sludge (light grey) and aggregating into larger individual or solitary gallstones via the coating and cementation of bacterial biofilms (white). Panel (d) depicts cholesterol clusters melting and recrystallizing in the stone centre due to compression from other stones, the gallbladder wall, or as the stone grows consecutive rings over time. The rounding out or smoothing of the stone exterior is similarly shown to occur with either abrasion from other stones or the gallbladder wall. Rings are formed around the clusters as detrital matter attaches to consecutive layers of biofilm coating. Panel (e) depicts an alternative model in which a number of clusters form into a larger grape-like or botryoidal stone morphology. Time, turbidity, abrasion, gallbladder stasis, and bile constituents ultimately influence the colour, ring structures and morphology seen in each type of stone.

As stones in both natural and gallbladder settings are seen to take a number of years to develop, an individual's bile chemistry may change over time, causing some layers of a gallstone to have different particulate matter trapped within it (i.e more bilirubinate than cholesterol). This may explain the change in colour of the ring system of some gallstones as seen with sample LS3, supported by what is possibly biofilm on its core cholesterol micro-plate surface (Figure 4.3). The 6-7 per mil difference between the central core and ring system of sample LS3 could be attributed to the concentrated cholesterol (lighter isotopic value) in the central core of the stone as opposed to the outerring in which the brown colour suggests heme products with a heavier isotopic value. Indeed, a lighter isotopic value suggesting a higher fat concentration in the core and a heavier value at the outer rings would support the theory of compression as per the above model. However, further studies

investigating the isotopic differences in the core and outer rings of larger stones will be required in order to establish if such a trend exists within all gallstones.

Our previous studies support a formation model in which bacterial mediation and biofilm is central to stone pathogenesis and growth. In those studies, we identified a predominance of prolific biofilm producing Gram-negative bacteria across the gallstones of 16 diverse patients, and provided insight as to how biofilm formation and bile resistance was key to bacterial survival in what is the extreme environment of the human gallbladder (Kose et al., 2018; 2019 under review). The current study suggests that biliary sludge and stone development is due to bile imbalance in which cholesterol is able to precipitate into crystals, and aggregate via successive biofilm coatings produced by prolific biofilm forming and bile resistant pathogenic bacteria. This formation model provides a framework for future work in this area, initiating greater focus on the importance of bacterial processes, and how they may influence and manipulate environmental and human systems in similar ways.

Conclusions

This study highlights the need for greater interdisciplinary research into the area of biomineralisation processes in humans, and how these may intersect and be aided by a near century of detailed investigations into biomineralised fabrics in nature. That these disparate systems produce similar stone morphologies, with bacteria the only connection between them, should not be taken lightly. Further work in this area, in particular ex-vivo stone growth in laboratory settings, may ultimately reveal the detailed mechanisms behind gallstone pathogenesis and growth.

Acknowledgements

The Fiona Stanley and St John of God, Murdoch hospitals are thanked for facilitating patient consent, sample collection and ethics approval for the study. The Microscopy and Microanalysis facility, John de Laeter Centre, Curtin University, is acknowledged for providing access to the Light Microscope, FESEM and FIBSEM

instruments. The authors would like to acknowledge Ms. Elaine Miller for assistance with the FESEM and Light Microscope, and Dr. William Rickard for assistance with the FIBSEM. The authors would also like to acknowledge TIGeR, WA-OIGC, Curtin University, and the contribution of an Australian Government Research Training Program Scholarship in supporting this research. We thank the ARC infrastructure grant (LE110100119) for the CSIA facility.

References

Bastow, T.P., van Aarssen, B.G.K., & Lang, D. (2007). Rapid small-scale separation of saturate, aromatic and polar components in petroleum. *Organic Geochemistry, 38(8),* 1235-1250.

Batchelor, M.T., Burn, R.V., Bruce, I.H., Li, F. & Paul, J. (2018). A biofilm and organomineralisation model for the growth and limiting size of ooids. *Scientific Reports, 8(559),* 1-9.

Bouchier, I.A. (1992). The formation of gallstones. *The Keio Journal of Medicine*. 41(1), 1-5.

Burne, R.V., Moore, L.S. (1987). Microbialites: organosedimentary deposits of benthic microbial communities**.** *PALAIOS, 2(3),* 241-254.

Catuneanu, O., Galloway, W.E., Kendall, G.S.C., Miall, A.D., Posamentier, H.W., Strasser, A., et al. (2011). Sequence Stratigraphy: Methodology and Nomenclature. *Newsletters on Stratigraphy, 44(3),* 173–245.

Cao, C., Jiang, J., Sun, H., Huang, Y., Tao, F., Lian, B. (2016). Carbonate mineral formation under the Influence of limestone-colonizing *Actinobacteria*: Morphology and polymorphism. *Frontiers in Microbiology, 23(7),* 366.

Diehl, A.K. (1983). Gallstone size and the risk of gallbladder cancer. *JAMA, 250(17),* 2323-2326.

Fu, P., Zhang, S., Dai, K., Zheng, K., Zhang, C., et al. (1984) Gallstone classified based on sectional structure and chemical composition. Chinese Journal of Surgery, 22(5), 258–260.

Ha, B.J., & Park, S. (2018). Classification of gallstones using Fourier-transform infrared spectroscopy and photography. *Biomaterials Research, 22(18),* 1-10.

Haigh, W.G., Lee, S.P. (2001). Identification of oxysterols in human bile and

pigment gallstones. *Gastroenterology, (121)*, 118–123.

Heuman, D.M. (2017). Gallstones (Cholelithiasis) Treatment & Management. Retrieved from https://emedicine.medscape.com/article/175667-treatment#d8.

Mancini, S.A., Lacrampe-Couloume, G. & Lollar, B.S. (2008). Source differentiation for benzene and chlorobenzene groundwater contamination: A field application of stable carbon and hydrogen isotope analyses. *Environmental Forensics, 9*, 177-186.

National Health and Medical Research Council (NHMRC). (2007a). Australian Code for the Responsible Conduct of Research. Retrieved from https://www.nhmrc.gov.au/guidelines- publications/r39.

National Health and Medical Research Council (NHMRC). (2007b). National Statement on Ethical Conduct in Human Research. Retrieved from https://www.nhmrc.gov.au/book/national-statement-ethical-conduct-human-research

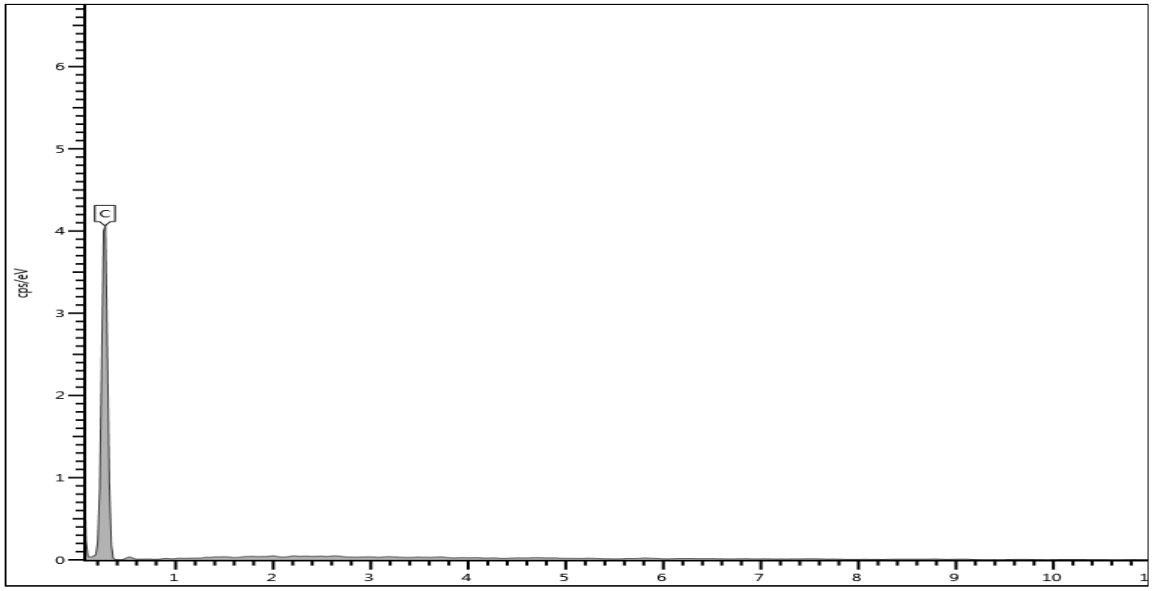
Qiao, T., Ma, R-h., Luo, X-b., Yang, L-q., Luo, Z-l., & Zheng P-m. (2013). The Systematic Classification of Gallbladder Stones. *PLoS ONE, 8(10)*, e74887.

Sutor, D.J. & Wooley, S.E. (1968). Gallstone of Unusual Composition: Calcite, Aragonite, and Vaterite. *Science, 159(3819)*, 1113-1114.

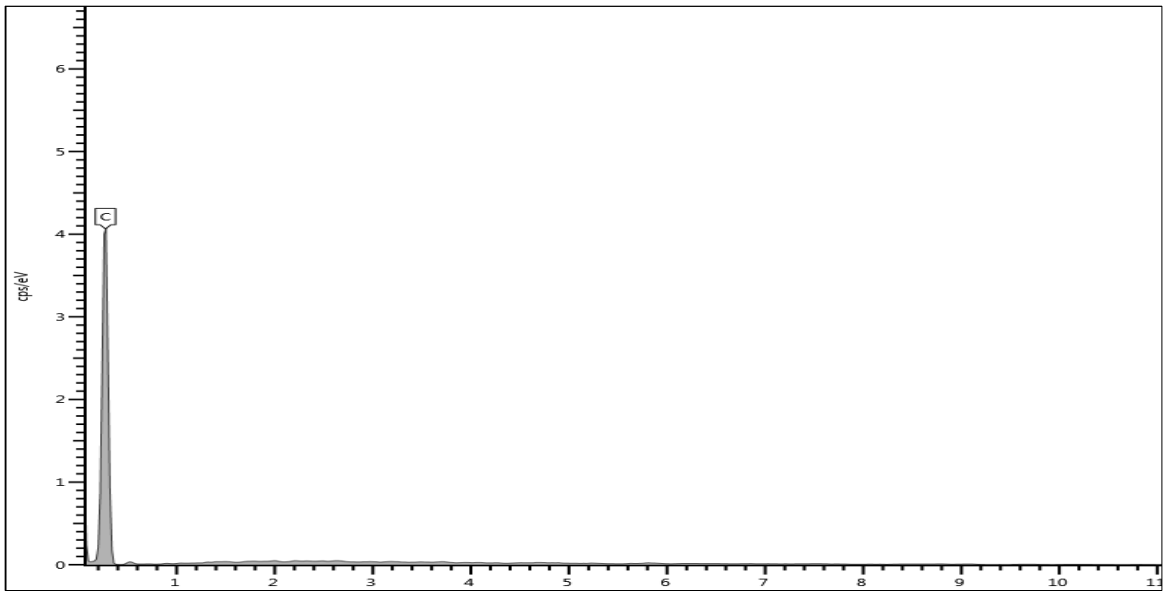
Whiting M.J., Watts J.M. (1986). Chemical composition of common bile duct stones. *British Journal of Surgery, 73(3)*, 229-32.

Appendix 3

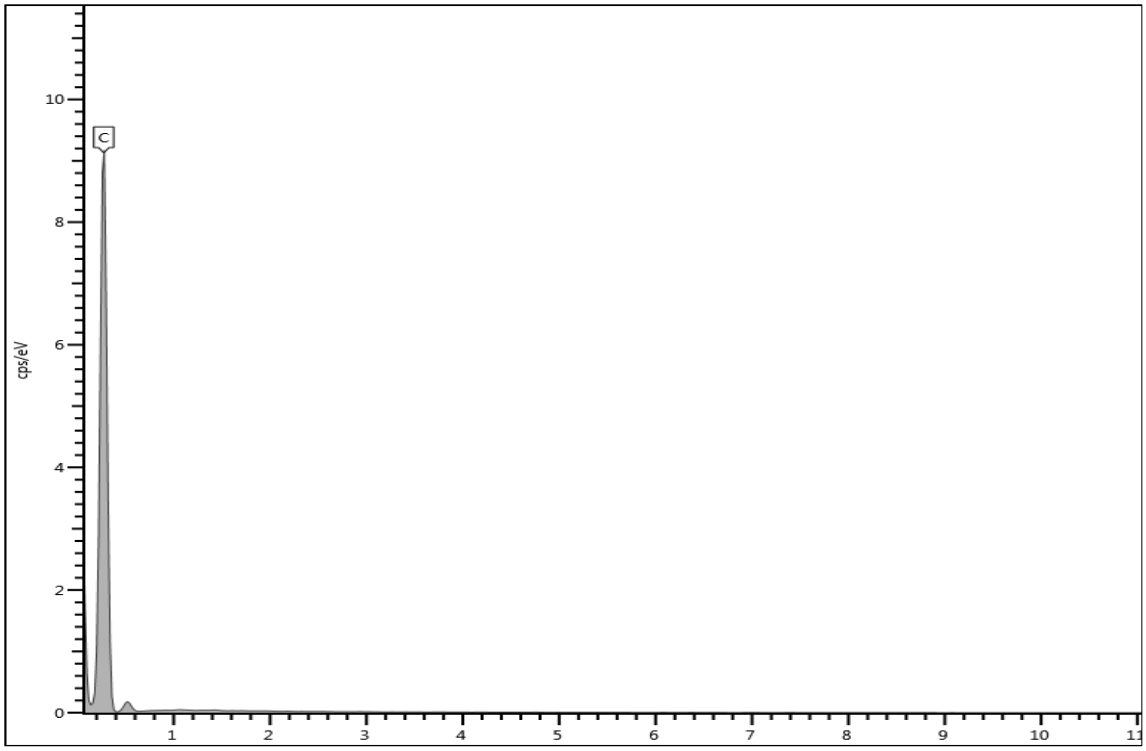
Figure A3. EDS spectra for each of the studied samples.



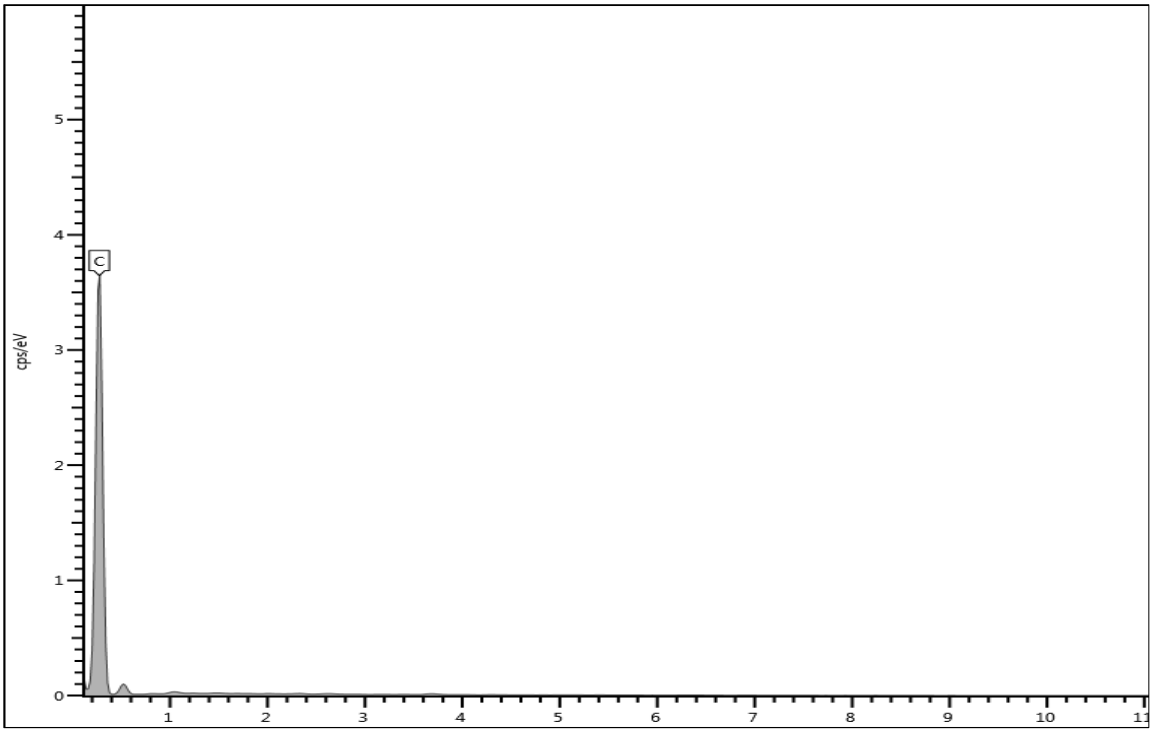
LM1_1 Core



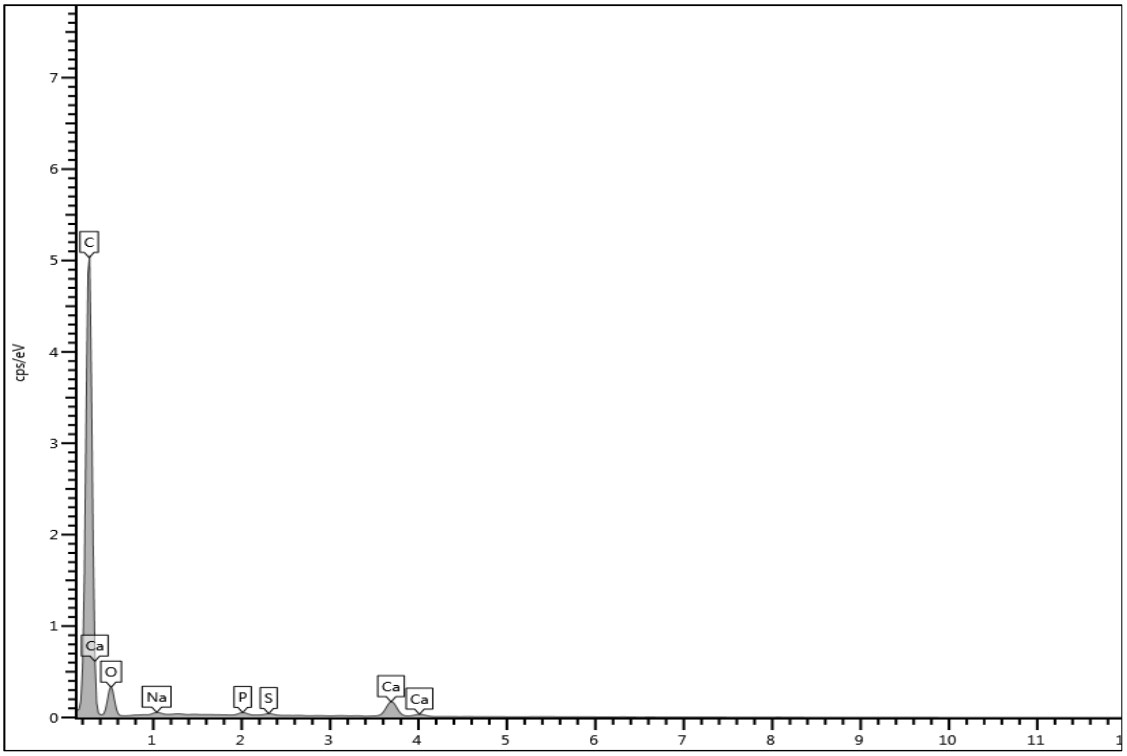
LM1_1 Exterior surface



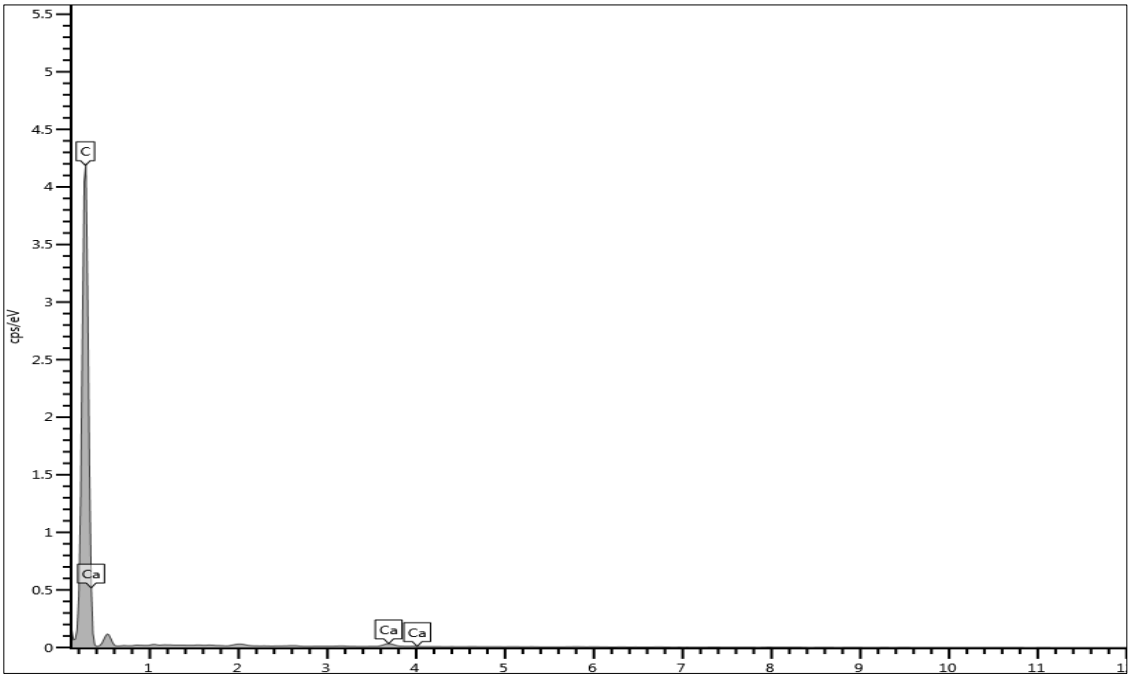
LM1_2 Interior rim



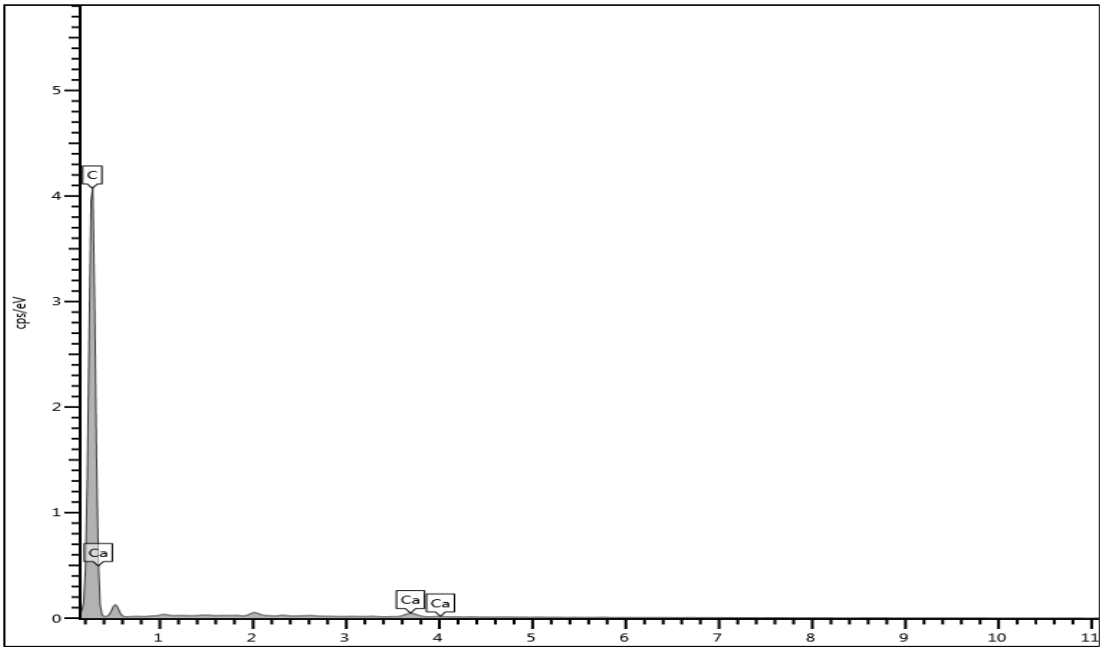
LM1_2 Core



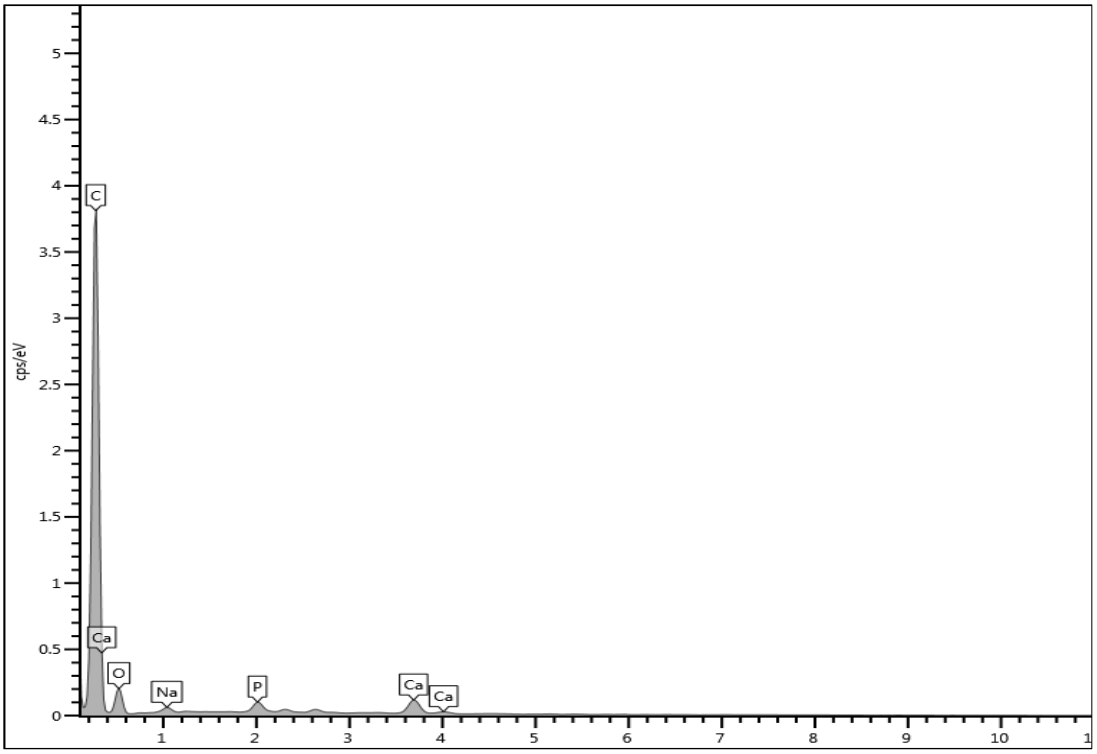
LS1 Interior rim



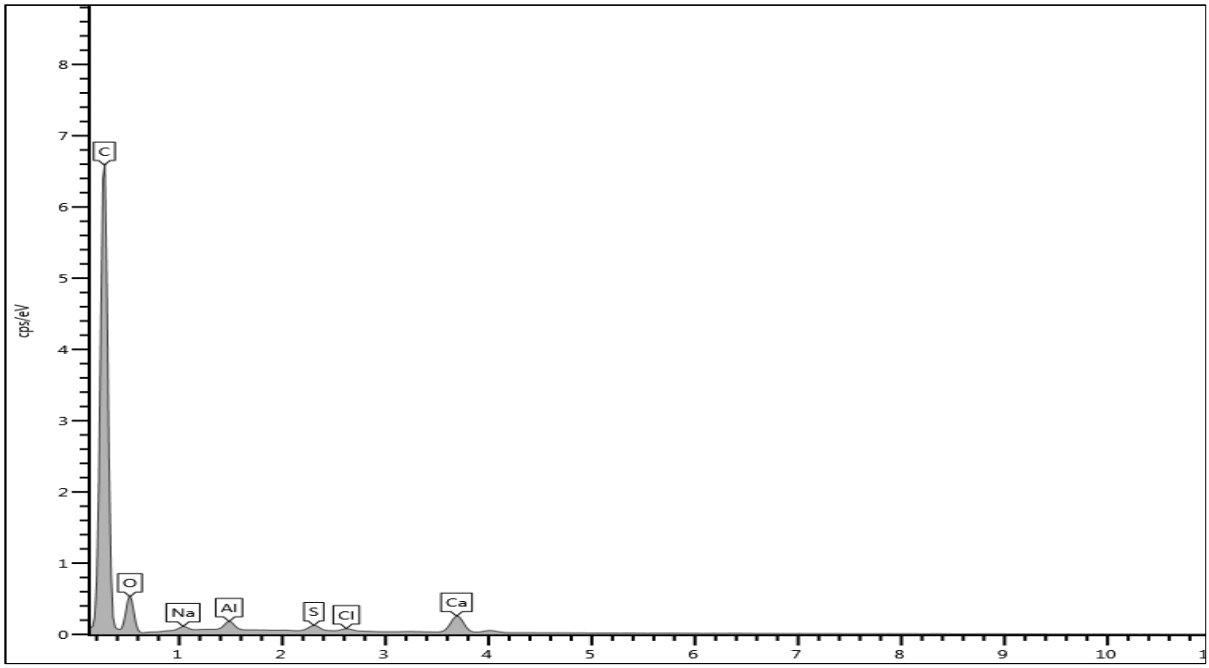
LS1 Core



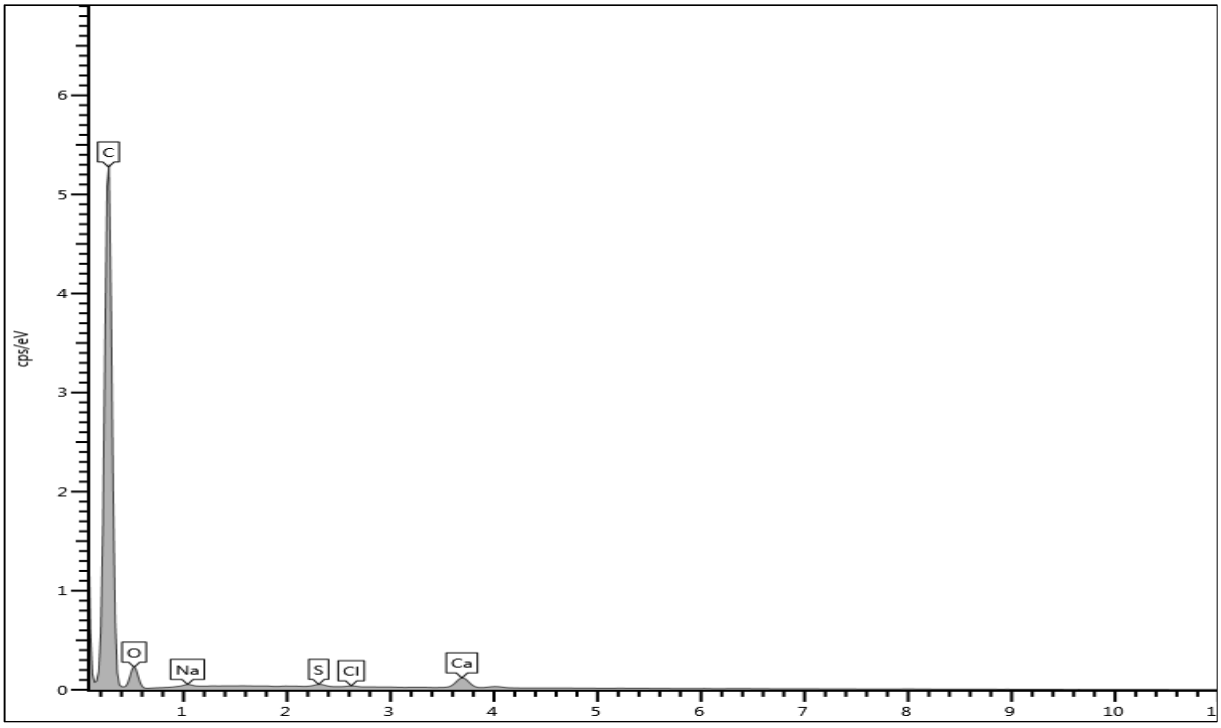
LS2 Interior rim



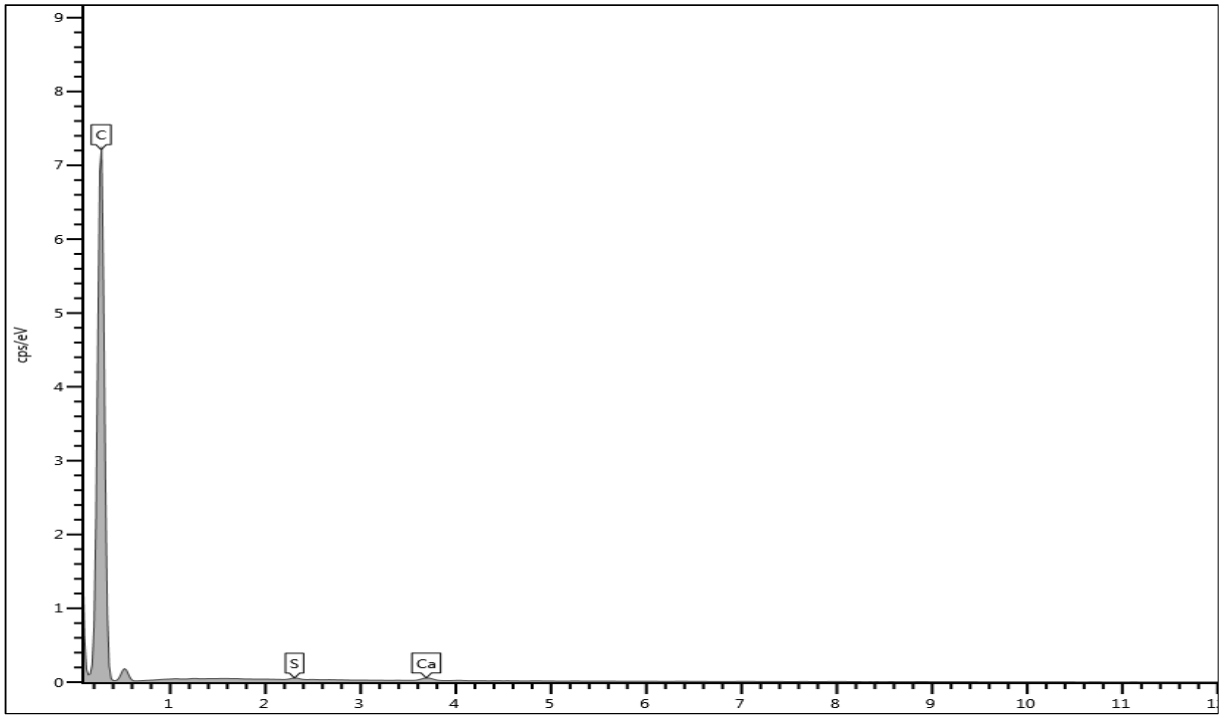
LS2 Core



LS3 outer-most-ring



LS3 inner-most ring



LS3 Core

Chapter 5:

Conclusions and Future Outlook

This thesis investigates the gallstones of 16 patients of diverse backgrounds and provides novel insights into the bacterial and physico-chemical processes that may lead to gallstone disease pathogenesis. It provides a more targeted framework for future research into this area, whilst simultaneously broadening its scope by applying hitherto untried interdisciplinary techniques to medical settings.

Microbial hypothesis

Previous studies investigating the involvement of bacteria in gallstone pathogenesis were largely conducted using culture-based techniques, and resulted in a biased view in which only pigmented stone formation was associated with bacterial biofilms. This research applied Shotgun Metagenomics profiling to human gallstones for the first time to test first and foremost if any universal factors are at play in gallstone pathogenesis. Putative genes relating to bile stress response and biofilm formation were identified in bacteria isolated from both pigmented and cholesterol gallstones from a 76-year old male patient and a 30-year old female patient. The predominance of these genes was associated with the genus *Klebsiella*. DMSO related respiration genes were also identified and annotated to *Klebsiella*, suggesting the reason why this genus may be out-competing other genera in the study. Metagenomics allows for the detection of all domains of life in an environmental sample, and as such, this study determined that bacteria alone predominate the gallstones of both patients with no fungal, viral, archaeal, or protistal signatures identified. In chapter 3, this research is followed up by the characterisation of the bacterial communities within the gallstones of 16 patients using 16S rRNA profiling. This more targeted technique allowed the detection of bacteria within the entire sample set, implicating bacterial

mediation in all stone types. It identified Gram-negative bacteria in greater than 90% of patients, with *Klebsiella* found to be predominant in 10 of the 16 patients investigated. Of the patients with a predominance of *Klebsiella*, 5 of them also had a high abundance of *Escherichia*. The remaining 6 patients had a predominance of strong biofilm producing and multiple stressor resistant bacteria such as, *Enterobacter*, *Streptococcus*, *Pseudomonas*, *Dermacoccus*, *Deinococcus* and *Turicibacter*. These results suggest Gram-negative, highly resilient bacteria may be a key factor in gallstone disease pathogenesis across all stone-types and furthers the microbial hypothesis of gallstone formation. Future work utilising Metatranscriptomics and a larger patient cohort would enable researchers to further advance this area. Metatranscriptomics allows for both bacterial community analysis and gene function activity (whether the gene was switched on or off at the time of sampling) to be determined. Further work investigating the source of the mostly pathogenic bacteria identified in this study would also be useful.

Physico-chemical hypothesis

Previous physico-chemical studies have focused on gallstone nidus characterization and resulted in conflicting theories of stone formation. This research conducted compound specific isotope analysis (CSIA) of cholesterol on human gallstones for the first time to further characterise and trace possible dietary sources that may lead to gallstone pathogenesis. Initial chemical characterization of the gallstones resulted in a predominance of cholest-5-en-3β-ol and only trace amounts of campesterol, regardless of stone-type or patient background. Cholest-5-en-3β-ol is the highly regulated end product of the cholesterol synthesis pathway that begins in liver hepatocytes. It is an essential precursor to steroid hormones, bile acid synthesis, and vital to cell membrane integrity. Although it is not surprising that this compound is the major chemical constituent identified in the stones it is, however, surprising that it was the only compound detected across all 16 patients. The GCMS instrument and methodology for this study was modelled after research conducted be Haigh and Lee (2001), in which novel oxysterols were identified. The reason why this study was not able to resolve these sterols could be a question of extraction methods, instrument capability, or a unique condition or gallstone matrix sampled by Haigh & Lee that

may not be reproducible and is patient dependent. Subsequently, the hydrogen (δD values) and carbon ($\delta^{13}C$ values) isotopes within the cholest-5-en-3β-ol identified in each gallstone was analysed in order to investigate biosynthetic pathway and environmental source information. The isotopic values were homogenous between all 16 patients, with δD values corresponding to possibly dairy product (butter, cheese) values identified in previous dietary studies, and the $\delta^{13}C$ values attributed to C3 plants common to the diet. These results may indicate a common dietary source for all 16 patients. However, this cannot be stated with certainty. As this research area is in its infancy (particularly with hydrogen isotopes), more work is required, especially in large scale studies comprising multiple geographic regions in order to develop an isotopic reference library that will aid in the distinguishment of regional effects and diet and whether either of these have an important role in gallstone pathogenesis. Another important objective to be addressed is whether animal fats or protein play a role in the cholesterol identified in gallstone studies. Nitrogen ($\delta^{15}N$) isotopes have been used as a biomarker for investigating the source/trophic level of protein in a sample. For example, previous CSIA studies have targeted the $\delta^{15}N$ signatures within the amino acids, glutamic acid and phenylalanine, which have been shown to increase with trophic level and remain stable during trophic transfer within animals respectively. Although this study was limited by time constraints and instrument availability, future work utilising $\delta^{15}N$ isotopes would provide further insights into diet and its association with gallstone disease.

This research investigated five large (>2cm) gallstones in closer detail using a variety of SEM techniques in order to reveal formation processes that may have been obscured in the majority of investigations in which smaller stones (0.5-1cm) predominate. Grape-like clusters were observed on the external morphology of four out of five stones. In every stone examined, cholesterol micro-plate like structures made up both the external and internal fabric of the stones. Energy-dispersive X-ray spectroscopy of both the core and external areas of the stones revealed carbon as the primary element in the stones. In one of the stones, grape-like clusters were shown to be embedded into the observed cholesterol micro-plates. The only morphology that is observed in natural settings that matches the morphology observed in the five stones is that of ooids. Although

unresolved, ooid formation models suggest a step by step process in which carbonate particles accumulate and are cemented together via bacterial biofilms to form grape-like clusters. These clusters can form a number of structures, such as pelloid and botryoidal stone morphologies. Their colour, size, chemical composition, and shape are determined by the environment in which they form. This study was able to show physical evidence of stone development from each step of the ooid formation process. This research suggests gallstone formation may follow a similar unified model in which cholesterol hypersecretion associated with a number of risk factors and bacterial biofilms, act together to form particle accumulation and growth. It proposes that there are no particular stone-types, but that shape, size, colour, and chemical composition (i.e bilirubinate vs cholesterol ratio) is dependent on an individual's gallbladder environment.

The homogenous results observed in this study in both the microbial and chemical analyses suggest that the traditional risk factors of female gender and advanced age may need to be re-examined. These risk factors may be based on biased sampling due to the higher frequency in which these two groups are screened for a number of conditions (pregnancy, check-ups) in which asymptomatic gallstones are detected as a by-product of the screening process. Although pregnancy can predispose an individual to cholesterol hypersecretion, the fact that not all post-natal women go on to develop stones should be considered further. The combined results of this research suggest cholesterol hypersecretion, host immunity, and microbial community may be more important than traditional risk factors in which only hypersecretion is reported as the primary cause of disease.

Future laboratory work in which stones are grown *ex-vivo* using a number of bile substrates and bacterial species may ultimately reveal the mechanisms behind gallstone disease pathogenesis.

Bibliography

A

Al-Akhrass F, Al-Wohoush I, Chaftari AM, Reitzel R, Jiang Y, Ghannoum M.,…Raad, I. (2012). *Rhodococcus* bacteremia in cancer patients is mostly catheter related and associated with biofilm formation. *PLoS One*, *7*, 1-6.

Alphonse, P.A.S., & Jones P.J.H. (2016). Revisiting Human Cholesterol Synthesis and Absorption: The Reciprocity Paradigm and its Key Regulators. *Lipids*, *51*, 519–536.

Amann, R.I., Ludwig, W., & Schleifer, K.H. (1995). Phylogenetic identification and in situ detection of individual microbial cells without cultivation. *Microbiology Reviews*, *59*, 143–69.

Arai, T., Ochiai, K., Senpuku, H. (2015). *Actinomyces naeslundii* GroEL-dependent initial attachment and biofilm formation in a flow cell system. *Journal of Microbiology Methods*, *109*, 160-6.

Arese, P., Gallo, V., Pantaleo, A., & Turrini, F. (2012). Life and death of Glucose-6-Phosphate Dehydrogenase (G6PD) deficient erythrocytes – Role of redox stress and Band 3 modifications. *Transfusion Medicine and Hemotherapy*, *39*, 328-334.

Australian Institute of Health and Welfare. (2015). *Admitted patient care 2013-14: Australian hospital statistics* (Chapter 6: What procedures were performed?) Retrieved from https://www.aihw.gov.au/reports/hospitals/ahs-2013-14-admitted-patient-care/contents/

Australian Institute of Health and Welfare (AIHW). (2016). Admitted patient care 2014-15: Australian hospital statistics. Canberra: (Cat. No. HSE 172; Health Services Series No. 68.)

Australian Institute of Health and Welfare. (2017). *Hospital admitted patient expenditure 2004–05 to 2012: Australian health expenditure—demographics and diseases: 13.* (Health and welfare expenditure series Number 59) Retrieved from https://www.aihw.gov.au/reports/health-welfare-expenditure/australian-health-expenditure-demographics-disease/contents/

Australian Commission on Safety and Quality in Health Care. (2017). *The second Australian atlas of healthcare variation*. Sydney: Australian Commission on Safety and Quality in Health Care.

Azúa-Bustos, A., González-Silva, C., Mancilla, R.A., Salas, L, Palma, R.E., Wynne, J.J.,…Vicuna, R. (2009). Ancient photosynthetic eukaryote biofilms in an atacama desert coastal cave. *Microbial Ecology, 58,* 497.

B

Bandeira, M., Borges, V., Gomes, J.P., Duarte, A. & Jordao, L. (2017). Insights on *Klebsiella pneumoniae* biofilms assembled on different surfaces using phenotypic and genotypic approaches. *Microorganisms, 5,* 2-16.

Baranova, N. & Nikaido, H. (2002). The BaeSR two-compo-nent system activates transcription of the yegMNOB (mdtABCD) transporter gene cluster in *Escherichia col*i and increases its resistance to novobiocin and deoxycholate. *Journal of Bacteriol*ogy, *184,* 4168–4176.

Barrett, K.E., Barman, S.M., Botano, S., & Brooks, H.L (Eds.). (2016). *Ganong's Review of Medical Physiology* (25th edition). New York: McGraw-Hill.

Bassetti, M., Pecori, D., & Peghin, M. (2016) Multidrug-resistant Gram-negative bacteria-resistant infections: epidemiology, clinical issues and therapeutic options. *Italian Journal of Medicine,10,* 364-375.

Bastow, T.P., van Aarssen, B.G.K. & Lang, D. (2007). Rapid small-scale separation of saturate, aromatic and polar components in petroleum. *Organic Geochemistry, 38,* 1235-1250.

Beckingham, I.J. (2001). Gallstone disease. *BMJ, 322(7278),* 91-94.

Begley, M., Gahan, C.G. & Hill, C. (2005). The interaction between bacteria and bile. *FEMS Microbiology Reviews, 29,* 625–651.

Betts, J.G., Young, K.A., Wise, J.A., Johnson, E., Poe, B., Kruse, D.H…DeSaix, P. (2017). *Anatomoy and Physiology.* Rice University: Houston. Retrieved from https://openstax.org/details/books/anatomy-and-physiology

Bengelsdorf, F.R., Gabris, C., Michel, L., Zak, M., Kazda, M. (2015). Syntrophic microbial communities on straw as biofilm carrier increase the methane yield of a biowaste-digesting biogas reactor. *AIMS Bioengineering, 2,* 264-276.

Berg, J.M., Tymoczko, J.L., & Stryer, L. (Eds.). (2002).Chapter 26. The Biosynthesis of Membrane Lipids and Steroids. In *Biochemistry* (5th ed., sec. 26.4). Retrieved from https://www.ncbi.nlm.nih.gov/books/NBK22339/

Bhatt, V.D., Dande, S.S., Patil, N.V. & Chaitanya, G. J. (2013). Molecular analysis of the bacterial microbiome in the forestomach fluid from the dromedary camel (*Camelus dromedarius*). *Molecular Biology Reports*, *40,* 3363-3371.

Bills, P.M., & Lewis, D. (1975). A structural study of gallstones. *Gut*, *16*(8), 630-637.

Bina, J.E. & Mekalanos, J.J. (2001). Vibrio cholerae tolC is required for bile resistance and colonization. *Infection and Immunity, 69,* 4681–4685.

Blesso, C.N., & Fernandez, M.L. (2018). Dietary Cholesterol, Serum Lipids, and Heart Disease: Are Eggs Working for or Against You? *Nutrients, 10(4),* 426.

Bouchier, I.A. (1992). The formation of gallstones. *The Keio Journal of Medicine*. *41(1),* 1–5.

Bosak, T., Knoll, A.H., Petroff, A.P. (2013). The meaning of Stromatolites. *Annual Review of Earth and Planetary Sciences, 41,* 21–44.

Brown, W.H. (Ed.) (2000). *Introduction to Organic Chemistry* (2nd Edition) Florida: Harcourt Brace & Company.

C

Callahan, B.J., McMurdie, P.J., Rosen, M.J., Han, A.W., Johnson, A.J., & Holmes SP. (2016). DADA2: High-resolution sample inference from Illumina amplicon data. *Nature Methods, 13,* 581–583.

Campbell, J.L., Reinmann, A.B., & Templer, P.H. (2014). Soil freezing effects on sources of nitrogen and carbon leached during snowmelt. *Soil Science Society of America Journal*, *78(1)*, 297–308.

Caporaso, J. G., Lauber, C. L., Walters, W. A., Berg-Lyons, D., Lozupone, C. A., Turnbaugh, P. J., Fierer, N., … Knight, R. (2010). Global patterns of 16S rRNA diversity at a depth of millions of sequences per sample. *Proceedings of the National Academy of Sciences of the United States of America*, *108 Suppl 1*(Suppl 1), 4516-22.

Caporaso, J.G., Lauber, C.L., Walters, W.A., Berg-Lyons, D., Huntley, J., Fierer, N.,…Knight, R. (2012). Ultra-high-throughput microbial community analysis on the Illumina HiSeq and MiSeq platforms. *The ISME Journal*,*6*, 1621–1624.

Carey, M.C., & Small, D.M. (1978). The physical chemistry of cholesterol solubility in bile. Relationship to gallstone formation and dissolution in man. *The Journal of Clinical Investigation*, *61*(4), 998–1026.

Carey, M.C. (1993). Pathogenesis of Gallstones. *The American Journal of Surgery*, *165*(4), 410-419.

Centers for disease control and prevention (CDC). (2018). *Klebsiella pneumoniae* in healthcare settings. Retrieved from http://www.cdc.gov/HAI/organisms/klebsiella/klebsiella.html.

Cetta, F. (1991). The role of bacteria in pigment gallstone disease. *Annals of Surgery*, *213*(4), 315-326.

Chan, C.L., Richter, K., Wormald, P.J., Psaltis, A.J., & Vreugde, S. (2017). *Alloiococcus otitidis* forms multispecies biofilm with *Haemophilus influenzae*: effects on antibiotic susceptibility and growth in adverse conditions. *Frontiers in cellular and infection microbiology*, *7*, 344.

Charles, C.J., Rout, S.P., Garratt, E.J., Patel, K., Laws, A.P., & Humphreys, P.N. (2015). The enrichment of an alkaliphilic biofilm consortia capable of the anaerobic degradation of isosaccharinic acid from cellulosic materials incubated within an anthropogenic, hyperalkaline environment. *FEMS microbiology ecology*, *91*, 1-11.

Che J, Li, Z., Xie, J., Wang, G., Yu, E., Xia, Y.,…Zhang, K. (2017). Microbial succession in biofilms growing on artificial substratum in subtropical freshwater aquaculture ponds. *FEMS Microbiology Letters*, *364*, 1-7.

Chen, H., Grassian, V.H., Saraf, L.V., & Laskin, A. (2013). Chemical imaging analysis of environmental particles using the focused ion beam/scanning electron microscopy technique: microanalysis insights into atmospheric chemistry of fly ash. *Analyst*, *138(2)*, 451-460.

Chew, G.T., Gan, S.K., &Watts, G.F. (2006). Revisiting the metabolic syndrome. *The Medical Journal of Australia*, *185(8)*, 445-449.

Chiang, J.Y.L. (2009). Bile acids: regulation of synthesis. *Journal of Lipid Research*, *50(10)*, 1955–1966.

Choi, Y., & Silverman, W.B. (2018). American College of Gastroenterology: Biliary tract disorders, gallbladder disorders, and gallstone pancreatitis.. Retrieved

from http://patients.gi.org/topics/biliary-tract-disorders-gallbladder-disorders-and-gallstone-pancreatitis.

Chowdhury, R. Gautam, K.S. & Jyotirmoy, D. (1996). Stress response in pathogenic bacteria. *Journal of Bioscience*, *21*, 149–160.

Clarridge, J.E. (2004). Impact of 16S rRNA Gene Sequence Analysis for Identification of Bacteria on Clinical Microbiology and Infectious Diseases. *Clinical Microbiology Reviews, 17(4),* 840–862.

Crawford, M. (2013). Biliary pain: work-up and management in general practice. *The Right Upper Quadrant*, *42(7)*, 458-461.

Cruz, P.M., Mo, H., McConathy, W.J., Sabnis, N., & Lacko, A.G. (2013). The role of cholesterol metabolism and cholesterol transport in carcinogenesis: a review of scientific findings, relevant to future cancer therapeutics. *Frontiers in Pharmacology*, *4*, 119.

Collister, J.W., Summons, R.E., Lichtfouse E., and Hayes, J.M., (1992) An isotopic biogeochemical study of the Green River oil shale. *Organic Geochemistry*, *19*, 265-276.

Colorado Springs Surgical Associates. (2018). Retrieved from https://www.coloradosurgical.com/images/Documents/lap-chole-instructions.pdf.

D

Dal Santo, S., Stampfl, H., Krasensky, J., Kempa, S., Gibon, Y., Elena Petutschnig, E.,…Jonak, C. (2012). Stress-induced GSK3 regulates the redox stress response by phosphorylating glucose-6-phosphate dehydrogenase in *Arabidopsis*. *Plant Cell*, *24*, 3380-92.

Demehri, F.R., & Alam, H.B. (2016). Evidence-based management of common gallstone-related emergencies. *Journal of Intensive Care Medicine*, *31(1)*, 3-13.

Diesendorf, N., Köhler, S., Geißdörfer, W., Grobecker-Karl, T., Karl, M., & Burkovski, A. (2017). Characterisation of *Roseomonas* mucosa isolated from the root canal of an infected tooth. *BMC Research Notes,10,* 212.

Donelli, G. (Ed). (2015). Biofilm-based healthcare-associated Infections: Vol 1. Switzerland: Springer International Publishing.

Donlan, R.M. (2002). Biofilms: Microbial Life on Surfaces. *Emerging Infectious Diseases*, *8*, 881-890.

dos Anjos, M.M., Ruiz, S.P., Nakamura, C.V., de Abreu Filho, B.A. (2013). Resistance of *Alicyclobacillus acidoterrestris* spores and biofilm to industrial sanitizers. *Journal of Food Protection, 76,* 1408-13.

Dowd, S.E., Wolcott, R.D., Sun, Y., McKeehan, T., Smith, E., Rhoads, D. (2008). Polymicrobial nature of chronic diabetic foot ulcer biofilm infections determined using bacterial tag encoded FLX amplicon pyrosequencing (bTEFAP). *PLoS One, 3,* e3326.

E

Eggert, T., Bakonyi, D., Hummel, W. (2014). Enzymatic routes for the synthesis of ursodeoxycholic acid. *Journal of Biotechnology, 191(10),* 11-21.

El-Masry, M.H., Hassouna, M.S., El-Rakshy, N., & Mousa, I.E. (1995). Bacterial populations in the biofilm and non-biofilm components of a sand filter used in water treatment. *FEMS Microbiology Letters, 131,* 263-269.

Eriksson, L.L., Holgerson, P., & Johansson, I. (2017). Saliva and tooth biofilm bacterial microbiota in adolescents in a low caries community. *Scientific Reports,7,* 1-12.

Everhart, J.E., Khare, M., Hill, M., & Maurer, K.R. (1999).

Prevalence and Ethnic Differences in Gallbladder Disease in the United States. *Gastroenterology, 117(3),* 632–639.

Evershed, R., Dudd, S., Charters, S., Mottram, H., Stott, A., Raven, A., . . . Bada, J. (1999). Lipids as Carriers of Anthropogenic Signals from Prehistory [and Discussion]. *Philosophical Transactions: Biological Sciences, 354*(1379), 19-31.

F

Fang, H., Chen, Y., Huang, L., & He, G. (2017). Analysis of biofilm bacterial communities under different shear stresses using size-fractionated sediment. *Scientific Reports, 7,* 1-4.

Fardeau ML, Barsotti V, Cayol JL, Guasco S, Michotey V, Joseph M.,…Ollivier, B. (2010). *Caldinitratiruptor microaerophilus*, gen. nov., sp. nov. isolated from a French hot spring (Chaudes-Aigues, Massif Central): a novel cultivated facultative microaerophilic anaerobic thermophile pertaining to the

Symbiobacterium branch within the Firmicutes. *Extremophiles, 14*, 241-7.

Farkas, M., Táncsics, A., Kriszt, B., Benedek, T., Tóth, E., Kéki, Z., Veres, P.G., & Szoboszlay, S. (2014). *Zoogloea oleivorans* sp. nov., a floc-forming, petroleum hydrocarbon-degrading bacterium isolated from biofilm. *International Journal of Systems and Evolutionary Microbiology, 64*, 274-279.

Feher, J. (2017). *Quantitative Human Physiology: An Introduction* (2nd ed.) Amsterdam: Elsevier.

Feingold, K.R., Grunfeld, C. (2000). Structure of Lipoproteins. In De Groot, L.J., Chrousos, G., Dungan, K., et al., (Ed.), *Introduction to Lipids and Lipoproteins.* South Dartmouth (MA): MDText.com, Inc. Retrieved from https://www.ncbi.nlm.nih.gov/books/NBK305896/#lipid_intro.toc-structure-of-lipoproteins.

Fink, R. C., Evans, M. R., Porwollik, S., Vazquez-Torres, A., Jones-Carson, J., Troxell, B., Libby, S. J., McClelland, M., … Hassan, H. M. (2007). FNR is a global regulator of virulence and anaerobic metabolism in Salmonella enterica serovar Typhimurium (ATCC 14028s). *Journal of bacteriology*, *189*(6), 2262-73.

Fu, P., Zhang, S., Dai, K., Zheng, K.,…Zhang, C. (1984) Gallstone classified based on sectional structure and chemical composition. *Chinese Journal of Surgery*, *22*(5), 258–260.

G

Goad, J. & Akihisa, T. (1997). *Analysis of Sterols*. United Kingdom: Chapman and Hall.

Gralnick, J.A., Vali, H., Lies, D.P & Newman, D.K. (2006). Extracellular respiration of dimethyl sulfoxide by *Shewanella oneidensis* strain MR-1. *PNAS, 103*, 4669-4674.

Gray, M.W., Sankoff, D., & Cedergren, R.J. (1984). On the evolutionary descent of organisms and organelles: a global phylogeny based on a highly conserved structural core in small subunit ribosomal RNA. *Nucleic Acids Research, 12(14)*, 5837–52.

Greer, D., Heywood, S., Croaker, D., & Gananadha, S. (2018). Is 14 the new 40: trends in gallstone disease and cholecystectomy in Australian children. *Pediatric Surgery International*, *34(8)*, 845–849.

Grice, K., Schaeffer, P., Schwark, L., and Maxwell, J.R., (1997) Changes in palaeoenvironmental conditions during deposition of the Permian Kupferschiefer (Lower Rhine Basin, N.W. Germany) inferred from molecular and isotopic compositions of biomarker components. *Organic Geochemistry, 26*, 677-690.

Grice, K., 2001. δ13C as an indicator of paleoenvironments: A molecular approach, in: Unkovich, M., Pate, J., McNeill, A., Gibbs, J. (Eds.), Application of stable isotope techniques to study biological processes and functioning ecosystems. Kluwer Academic Publishers, pp. 247-281.

Gunn, J.S. (2000). Mechanisms of bacterial resistance and response to bile. *Microbes and Infection*, *2*, 907.

Gurung, J., Khyriem, A.B., Banik, A., Lyngdoh, W.V., Choudhury, B., & Bhattacharyya, P. (2013). Association of biofilm production with multidrug resistance among clinical isolates of *Acinetobacter baumannii* and *Pseudomonas aeruginosa* from intensive care unit. *Indian Journal of Critical Care Medicine, 17*, 214–218.

H

Haigh, W.G., & Lee, S.P. (2001). Identification of oxysterols in human bile and pigment gallstones. *Gastroenterology*, *121*, 118–123.

Hardinger, S.A. (2017). Molecular structure of cholesterol. Illustrated Glossary of Organic Chemistry. Retrieved from http://www.chem.ucla.edu/~harding/IGOC/C/cholesterol.html.

Hayes, J.M., (1983) *In*: Meinschein W.G. (*ed*.) Organic Geochemistry of Contemporaneous and Ancient Sediments, Great Lakes Section, Society of Economic Palaeontologists and Mineralogists, Bloomington, Indiana, pp. 5-31.

Hayes, J. M., Freeman, K.H., Popp, B.N., and Hoham, C.H., (1990). Compound-specific isotopic analyses: A novel tool for reconstruction of ancient biogeochemical processes. *Organic Geochemistry*, *16*, 1115-1128.

Heithoff, D.M., Enioutina, E.Y., Daynes, R.A., Sinsheimer, R.L., Low, D.A. & Mahan, M.J. (2001). *Salmonella* DNA adenine methylase mutants confer cross protective immunity. *Infection and Immununity, 69*, 6725–6730.

Hentzer, M. & Givskov, M. J. Clin. (2003). Pharmacological inhibition of quorum sensing for the treatment of chronic bacterial infections. *Journal of Clinical Investigation*, *112*, 1300–1307.

Holmes NA, Innocent TM, Heine D, Al Bassam M, Worsley SF, Trottmann F,…Hutchings., M.I. (2016). Genome analysis of two *Pseudonocardia* phylotypes associated with *Acromyrmex* leafcutter ants reveals their biosynthetic potential. *Frontiers in Microbiology*, *7*, 2073.

Hu, Y., Lu, P., Zhang, Y., Li, L. and Chen, S. (2010). Characterization of an aspartate-dependent acid survival system in *Yersinia pseudotuberculosis*. *FEBS Letters*, *584*, 2311–2314.

Hussain M, Wilcox MH, White PJ. (1993). The slime of coagulase-negative-staphylococci: biochemistry and relation to adherence. *FEMS Microbiology Reviews*, *104*, 191–208.

I

Illumina, Inc. (2016). Illumina Sequencing by Synthesis. Retrieved from https://www.illumina.com/company/videohub/fCd6B5HRaZ8.html?langsel=/us/

Ito, A., Taniuchi, A., May, T., Kawata, K. & Okabe, S. (2009). Increased antibiotic resistance of *Escherichia coli* in mature biofilms. *Applied and environmental microbiology*, *75*, 4093–4100.

Iwase, T., Ogura, Y., Hayashi, T. & Mizunoe, Y. (2016). Complete genome sequence of *Klebsiella pneumoniae* YH43. *Genome Announcements*, *4*, 00242-16.

J

Jiruše, J., Havelka, M., &Lopour, F. (2014). Novel field emission SEM column with beam deceleration technology. *Ultramicroscopy*, *146*, 27-32.

Joy, D.C., 1991. The theory and practice of high-resolution scanning electronmicroscopy. *Ultramicroscopy*, *37*, 216–233.

K

Kelly, S., Heaton, K., & Hoogewerff, J. (2005). Tracing the geographical origin of

food: The application of multi-element and multi-isotope analysis. *Trends in Food Science & Technology*, *16*, 555–567.

Klappenbach, J.A., Dunbar, J.M., & Schmidt, T.M. (2000). rRNA Operon copy number reflects ecological strategies of bacteria. *Applied and environmental microbiology, 66,* 1328–1333.

Ko, C.W., Sekijima, J.H., & Lee, S.P. (1999). Biliary sludge. *Annals of Internal Medicine, 130(4),* 301-311.

Ko, C.W. (2006). Risk Factors for Gallstone-Related Hospitalization During Pregnancy and the Postpartum. *American Journal of Gastroenterology, 101(10),* 2263–2268.

Kose, S.H., Grice, K., Orsi, W.D., Ballal, M., & Coolen, M.J.L. (2018). Metagenomics of pigmented and cholesterol gallstones: the putative role of bacteria. *Scientific Reports, 8*, 1-13.

Kwong, E., Li, Y,. Hylemon, P.B., & Zhou, H. (2015). Bile acids and sphingosine-1-phosphate receptor 2 in hepatic lipid metabolism. *Acta Pharmaceutica SinicaB, 5(2),* 151–157.

L

Laforest-Lapointe, I. & Arrieta, M. (2018). Microbial Eukaryotes: a missing link in gut microbiome studies. *mSystems, 3*, 1-5.

Lage, O.M., & Bondoso, J. (2012). Bringing *Planctomycetes* into pure culture. *Frontiers in Microbiology, 3,* 405.

Landini, P. (2009). Cross-talk mechanisms in biofilm formation and responses to environmental and physiological stress in *Escherichia coli. Research in Microbiology, 160,* 259-66.

Lebret, K., Schroeder, J., Balestreri, C., Highfield, A., Cummings, D., Smyth, T., & Schroeder, D. (2016). Choice of molecular barcode will affect species prevalence but not bacterial community composition. *Marine Genomics, 29,* 39-43.

Le Breton, Y., Maze, A., Hartke, A., Lemarinier, S., Auffray, Y., & Rince´, A. (2002). Isolation and characterization of bile salts sensitive mutants of *Enterococcus faecalis*. *Current Microbiol*ogy, *45,* 434–439.

Lebeaux, D., Ghigo, J.M. & Beloin, C. (2014). Biofilm-Related Infections: Bridging the gap between clinical management and fundamental aspects of recalcitrance toward antibiotics. *Microbiology and Molecular Biology Reviews, 78,* 510–543.

Lee, H. S., Lee, Y. S., Kim, H. S., Choi, J. Y., Hassan, H. M. & Chung, M. H. (1998). Mechanism of regulation of 8-hydroxyguanine endonuclease by oxidative stress: roles of *fnr*, *arcA,* and *fur*. *Free Radical Biology and Medicine, 24,* 1193-1201.

Lee, J.Y., Keane, M.G., & Pereira, S. (2015). Diagnosis and treatment of gallstone disease. *Practitioner, 259(1783),* 15-19.

Lee, T., & Chen, J.G. (2009). Biomimetic Gallstone Formation: Crystallization of Calcium Carbonate by the Evolving Taurocholate−Lecithin−Cholesterol Complex Lipid System. *Crystal Growth & Design*, *9*(8), 3737–3748.

Leedom, J.M. (2006). Milk of Nonhuman Origin and Infectious Diseases in Humans. *Clinical Infectious Diseases, 43,* 610–615.

Leverrier, P., Vissers, J.P.C., Rouault, A., Boyaval, P. & Jan, G. (2004). Mass spectrometry proteomic analysis of stress adaptation reveals both common and distinct response pathways in *Propionibacterium freudenreichii*. *Archives of Microbiology*, *181,* 215–230.

Lichtfouse, E. (2000). Compound-specific isotope analysis. Application to archaelogy, biomedical sciences, biosynthesis, environment, extraterrestrial chemistry, food science, forensic science, humic substances, microbiology, organic geochemistry, soil science and sport. *Rapid Communications in Mass Spectrometry*, *14*, 1337–1344.

Lin, J., Sahin, O., Overbye Michel, L., & Zhang, Q. (2003). Critical role of multidrug efflux pump CmeABC in bile resistance and in vivo colonization of *Campylobacter jejuni*. *Infection and Immunity*,*71*, 4250–4259.

Lu, A.L., Cuipa, M.J., Ip, M.S. & Shanabruch, W.G. (1990). Specific A/G-to-CG mismatch repair in *Salmonella typhimurium* LT2 requires the mutB gene product. *Journal of Bacteriology, 172,* 1232–1240.

Luo, J., Lv, P., Zhang, J., Fane, A.G., McDougald, D., & Rice, S.A. (2017). Succession of biofilm communities responsible for biofouling of membrane bio-reactors (MBRs). *PLoS One, 12*, e0179855.

Lyautey, E., Jackson, C.R., Cayrou, J., Rols, J.L., & Garabétian, F. (2005). Bacterial community succession in natural river biofilm assemblages. *Microbial Ecology, 50,* 589-601.

M

Madden, T. (2002). The BLAST Sequence Analysis Tool. In McEntyre J, Ostell J, (Eds). *The NCBI Handbook*. Available from: http://www.ncbi.nlm.nih.gov/books/NBK21097/

Makarova, K.S., Aravind, L., Wolf, Y.I., Tatusov, R.L., Minton, K.W., Koonin, E.V., & Daly, M.J. (2001). Genome of the Extremely Radiation-Resistant Bacterium *Deinococcus radiodurans* viewed from the perspective of comparative genomics. *Molecular Biology Reviews, 65,* 44-79.

Maki, T. (1966). Pathogenesis of calcium bilirubinate gallstone: Role of E. coli, beta glucuronidase and coagulation by inorganic ions, polyelectrolytes, and agitation. *Annals of Surgery, 164*(1), 90-100.

Mancini, S.A., Lacrampe-Couloume, G., Lollar, B.S. (2008). Source differentiation for benzene and chlorobenzene groundwater contamination: A field application of stable carbon and hydrogen isotope analyses. *Environmental Forensics, 9,* 177-186.

Marshall, J.M., Fletchtner, A.D., La Perle, K.M., Gunn, J.S. (2014). Visualization of extracellular matrix components within sectioned *Salmonella* biofilms on the surface of human gallstones. *PLoS One, 9,* 1-7.

Mardanov, A.V., Kadnikov, V.V., & Ravin, N.V. (2017). Metagenomics: A Paradigm Shift in Microbiology in Nagarajan, M. (Ed.). *Metagenomics: perspectives, methods, and applications*. Retrieved from http://ebookcentral.proquest.com.

Matthews, D.E., and Hayes, J.M., (1978) Isotope-ratio monitoring gas chromatography- mass spectrometry. *Analytical Chemistry, 50,* 1465-1473.

McKinney C. R., McCrea J. M., Epstein S., Allen H. A. and Urey H. C. (1950) Improvements in mass spectrometers for the measurement of small differences in isotope abundance ratios. *Rev. Sci. Instrum. 21*, 724-730.

Merritt, M.E. & Donaldson, J.R. (2009). Effect of bile salts on the DNA and membrane integrity of enteric bacteria. *Journal of Medical Microbiology, 58,*

1533–1541.

Michael, V., Frank, O., Bartling, P., Scheuner, C., Göker, M., Brinkmann, H., & Petersen, J. (2016). Biofilm plasmids with a rhamnose operon are widely distributed determinants of the 'swim-or-stick' lifestyle in roseobacters. *The ISME Journal*, *10,* 2498-513.

Michener, R. & Lajtha, K. (2007). *Stable Isotopes in Ecology and Environmental Science* (2nd ed) Blackwell Publishing, Singapore.

Miettinen, T.E., Kesaniemi Y.A., Gylling, H., Jarvinen, H., Silvennoinen, E., & Miettinen, T.A. (1996). Noncholesterol sterols in bile and stones of patients with cholesterol and pigment stones. *Hepatology*, *23*, 274 –280.

Moen, B., Røssvoll, E., Måge, I., Møretrø, T., & Langsrud, S. (2016). Microbiota formed on attached stainless steel coupons correlates with the natural biofilm of the sink surface in domestic kitchens. *Canadian Journal of Microbiology, 62*, 148-60.

Munoz, M.A., Ahlström, C., Rauch, B.J., & Zadoks, R.N. (2006). Fecal Shedding of *Klebsiella pneumoniae* by Dairy Cows. *Journal of Dairy Science, 89*, 3425-3430.

Myant, N.B. (1981). *The Biology of Cholesterol and Related Steroids*. London: William Heinemann Medical Books Ltd.

N

National Center for Biotechnology Information (NCBI). (2019). Genomes. Retrieved from https://www.ncbi.nlm.nih.gov/home/genomes/

National Health and Medical Research Council (NHMRC). (2007a). Australian Code for the Responsible Conduct of Research. Retrieved from https://www.nhmrc.gov.au/guidelines-publications/r39

National Health and Medical Research Council (NHMRC). (2007b). National Statement on Ethical Conduct in Human Research. Retrieved from https://www.nhmrc.gov.au/book/national-statement-ethical-conduct-human-research.).

National Oceanic & Atmospheric Administration (NOAA). (2019). Stable and radiocarbon Isotopes of carbon dioxide. Retrieved from https://www.esrl.noaa.gov/gmd/outreach/isotopes/chemistry.html

Nesper, J. Lauriano, C.M., Klose, K.E., Kapfjammer, D., Kraiss, A. & Reidl, J. (2001). Characterization of *Vibrio cholerae* 01 El tor galU and galE mutants: influence on lipopolysaccharide structure colonization, and biofilm formation. *Infection and Immun*ity, *69*, 435–445.

New England BioLabs. (2019). NEBNext Multiplex Oligos for Illumina. Retrieved from https://www.nebiolabs.com.au/tools-and-resources.

Newton, J. (2016). Stable Isotopes as Tools in Ecological Research. In: eLS. John Wiley & Sons, Ltd: Chichester.

Nordmann, P., Cuzon, G., & Naas, T. (2009). The real threat of *Klebsiella pneumoniae* carbapenemase-producing bacteria. *Lancet Infectious Diseases, 9,* 228-36.

Nouha, K., Kumar, R.S., & Tyagi, R.D. (2016). Heavy metals removal from wastewater using extracellular polymeric substances produced by *Cloacibacterium normanense* in wastewater sludge supplemented with crude glycerol and study of extracellular polymeric substances extraction by different methods. *Bioresource Technology, 212,* 120-129.

O

O'Brien D.M. & Wooller, M.J. (2007). Tracking human travel using stable oxygen and hydrogen isotope analyses of hair and urine. *Rapid communications in mass spectrometry, 21,* 2422-2430.

O'Brien D.M. (2015). Stable Isotope Ratios as Biomarkers of Diet for Health Research. *Annual Review of Nutrition,* 35, 565–594.

Oliveira L.C. Saraiva, T.L.D., Silva, W.M., Pereira, U.P., Campos, B.C.J., Benevides, L.J.,…Soares, S.C. (2017). Analyses of the probiotic property and stress resistance-related genes of *Lactococcus lactis* subsp. *lactis* NCDO 2118 through comparative genomics and in vitro assays. *PLoS One, 12,* e0175116.

Oliver, S.P., Jayarao, B.M., & Almeida, R.A. (2005). Foodborne Pathogens in Milk and the Dairy Farm Environment: Food Safety and Public Health Implications. *Foodborne Pathogens and Disease, 2,* 115-129.

Oliver, S.P., Murinda, S.E., & Jayarao, B.M. (2011). Impact of antibiotic use in adult dairy cows on antimicrobial resistance of veterinary and human pathogens: A comprehensive review. *Foodborne Pathogens and Disease, 8,* 337-355.

Organisation for Economic Co-operation and Development. (2016). *Health care utilisation: surgical procedures (shortlist)*. Retrieved from https://stats.oecd.org/index.aspx?queryid=30167

Orsi, W. D., Richards, T. A., & Francis, W. R. (2018). Predicted microbial secretomes and their target substrates in marine sediment. *Nature Microbiology, 3*, 32–37.

P

Paterson, D.L. (2006). Resistance in gram-negative bacteria: *Enterobacteriaceae*. *American Journal of Medicine, 119,* 62-70.

Peng, C., Tian, J., Lv, M., Huang, Y., Tian, Y., & Zhang, Z. (2014). Development and validation of a sensitive LC-MS-MS method for the simultaneous determination of multicomponent contents in artificial Calculus Bovis. *Journal of chromatographic Science, 52,* 128-36.

Perez, M. J. & Briz, O. (2009). Bile-acid-induced cell injury and protection. *World Journal of Gastroenterology, 15*, 1677–1689.

Poole, R.K. (Ed.) (2011). *Advances in Microbial Physiology: Volume 58*. Elsevier: San Diego.

Prouty, A.M., Brodsky, I.E., Falkow, S. & Gunn, J.S. (2004). Bile-salt-mediated induction of antimicrobial and bile resistance In *Salmonella typhimurium*. *Microbiology, 150,* 775–783.

Purohit, V. (2000). Can alcohol promote aromatization of androgens to estrogens? A review. *Alcohol, 22(3)*, 123-7.

Q

Qiao, T., Ma, R-h., Luo, X-b., Yang, L-q., Luo, Z-l., & Zheng P-m. (2013). The Systematic Classification of Gallbladder Stones. *PLoS ONE, 8(10)*, e74887.

Quail, M. A., Swerdlow, H., & Turner, D. J. (2009). Improved protocols for the illumina genome analyzer sequencing system. *Current protocols in human genetics, Chapter 18*, Unit 18.2.

Quince, C., Walker, A.W., Simpson, J.T., Loman, N.J. & Segata, N. (2017). Shotgun metagenomics, from sampling to analysis. *Nature Biotechnology, 35*, 833–844.

Qureshi, S. (2014). *Klebsiella* Infections. Medscape. Retrieved from http://emedicine.medscape.com/article/219907-overview.

R

Ramazzotti, M., & Bacci, G. (2017). 16S rRNA-Based Taxonomy Profiling in the Metagenomics Era in Nagarajan, M. (Ed.). *Metagenomics: perspectives, methods, and applications*. Retrieved from http://ebookcentral.proquest.com.

Rashid, M.H., Rumbaugh, K., Passador, L., Davies, D.G., Hamood, A.N., Iglewski, B.H., Kornberg, A. (2000). Polyphosphate kinase is essential for biofilm development, quorum sensing, and virulence of *Pseudomonas aeruginosa*. *Proceedings of the National Academy of Sciences USA, 97,* 9636-41.

Rawat, D., & Nair, D. (2010). Extended-spectrum β-lactamases in Gram negative bacteria. *Journal of global Infectious disease*, 3, 263–274.

Rege, R.V. (2002). The role of biliary calcium in gallstone pathogenesis. *Frontiers in Bioscience*, *7*, 315-325.

Rince´, A., Giard, J.C., Pichereau, V., Flahaut, S. and Auffray, Y. (2001). Identification and characterization of gsp65, an organic hydroperoxide resistance (ohr) gene encoding a general stressprotein in *Enterococcus faecalis*. *Journal of Bacteriology, 183*, 1482–1489.

Russell, D.W. (2003). The enzymes, regulation, and genetics of bile acid synthesis. *Annual Review of Biochemistry*. *72*, 137-174.

S

Salas-Jara, M.J., Ilabaca, A., Vega, M., & García, A. (2016). Biofilm forming *Lactobacillus*: new challenges for the development of probiotics. *Microorganisms, 4,* 35.

Salmassi, T.M., Walker, J.J., Newman, D.K., Leadbetter, J.R., Pace, N.R., & Hering, J.G. (2006). Community and cultivation analysis of arsenite oxidizing biofilms at Hot Creek. Environ Microbiol. 2006; 8: 50-9.

Schooling, C.M., Au Yeung, S.L., Freeman, G., Cowling, B.J. (2013). The effect of statins on testosterone in men and women, a systematic review and meta-analysis of randomized controlled trials. *BMC Medicine, 11(57),* 2-9.

Sears, J. (1992). Anatomy and Physiology of the liver. *Baillière's Clinical Anaesthesiology, 6(4),* 697-727.

Shaffer, E.A. (2005) Epidemiology and risk factors for gallstone disease: has the paradigm changed in the 21st century? *Current Gastroenterology Reports, 7,* 132-140.

Shen, H., Ye, F., Xie, L., Yang, J., Li, Z., Xu, P.,…Zhang, X. (2015). Metagenomic sequencing of bile from gallstone patients to identify different microbial community patterns and novel biliary bacteria. *Scientific Reports, 5,* 17450.

Schimmelmann, A., Sessions, A.L. & Mastalerz, M. (2006). Hydrogen Isotopic (D/H) Composition of Organic Matter During Diagenesis and Thermal Maturation *Annu. Rev. Earth Planet. Sci., 34,* 501–33.

Schmitt, S., Deines, P., Behnam, F., Wagner, M., & Taylor, M.W. (2011). *Chloroflexi* bacteria are more diverse, abundant, and similar in high than in low microbial abundance sponges. *FEMS microbiology ecology, 78,* 497–510.

Shukla, S.K., & Toleti, S.R. (2017). The first recorded incidence of *Deinococcus radiodurans* R1 biofilm formation and its implications in heavy metals bioremediation. *bioRxiv, 234781,* 1-27.

Sistrunk, J.R., Nickerson, K.P., Chanin, R.B., Rasko, D.A., Faherty, C.S. (2016). Survival of the Fittest: How bacterial pathogens utilize bile to enhance infection. *Clinical Microbiology Reviews, 29,* 819-836.

Slater, C., Preston, T., & Weaver, L.T. (2001). Stable isotopes and the international system of units. *Rapid Communications in Mass Spectrometry, 15(15),* 1270-1273.

Smart R.C., & Hodgson, E. (2013) *Molecular and Biochemical Toxicology*, New Jersey: John Wiley & Sons.

Soto-Giron, M.J., Rodriguez, L.M., Luo, C., Elk, M., Ryu, H., & Hoelle, J. (2016). Biofilms on hospital shower hoses: characterization and implications for nosocomial infections. *Applied Environmental Microbiology, 82,* 2872-2883.

Spring, S., Bunk, B., Spröer, C., Schumann, P., Rohde, M., Tindall, B.J., & Klenk, H.P. (2016). Characterization of the first cultured representative of *Verrucomicrobia* subdivision 5 indicates the proposal of a novel phylum. *The ISME Journal, 10,* 2801–2816.

Springhouse. (Ed.). (2002). *Lippincott Professional Guides: Anatomy & Physiology* (2nd Edition). Philadelphia: Lippincott Williams & Wilkins.

Stevenson, A. (Ed). (2010). *Oxford Dictionary of English* (3rd edition), United Kingdom: Oxford University Press.

Stewart L., Smith A.L., Pellegrini, C.A., Motson, R.W. & Way, L.W. (1987). Pigment gallstones form as a composite of bacterial micro-colonies and pigment solids. *Annals of Surgery, 206*(3), 242-250.

Stewart, L., Ponce, R., Oesterk, A.L., Griffiss, J.M., & Way. L.W. (2000). Pigment gallstone pathogenesis: Slime production by biliary bacteria is more important than beta-glucuronidase production. *Journal of Gastrointestinal Surgery*. *4*(5), 547-543.

Stewart, L., Oesterle, A.L., Erdan, I, Griffiss, J.M. & Way, L.W. (2002). The pathogenesis of pigment gallstones in western societies: the central role of bacteria. *Journal of Gastrointestinal Surgery*, *6*(6), 891–904.

Stinton, L.M., & Shaffer, E.A. (2012). Epidemiology of Gallbladder Disease: Cholelithiasis and Cancer. ***Gut and Liver***, *6(2)*, 172-187.

Stott, A.W., & Evershed, R.P. (1996). delta ^{13}C analysis of cholesterol preserved in archaeological bones and teeth. *Analytical Chemistry*, *68(24)*, 4402-8.

Strasberg, S.M. (1998). The pathogenesis of cholesterol gallstones—a review. *Journal of Gastrointestinal Surgery*, 2(2), 109-125.

Sutor, D.J., & Wooley, S.E. (1968) Gallstone of Unusual Composition: Calcite, Aragonite, and Vaterite. *Science, 159*(3819), 1113-111.

Sulavik, M.C., Dazer, M. & Miller, P.F. (1997). The *Salmonella typhimurium* mar locus: molecular and genetic analyses and assessment of its role in virulence. *Journal of Bacteriol*ogy, *179*, 1857–1866.

Swanstrom, L. L., & Soper, N. J. (2013). *Mastery of endoscopic and laparoscopic surgery*. U.S.A: Wolters Kluwer Health.

Swidinski, A. & Lee, S.P. (2001). The role of bacteria in gallstone pathogenesis. *Frontiers of Bioscience, 6***,** 93-103.

T

Thanassi, D.G., Cheng, L.W. & Nikaido, H. (1997). Active efflux of bile salts by *Escherichia coli*. *Journal of Bacteriology*, *179*, 2512–2518.

Tortora, G.J., & Derrickson, B. (Eds.). (2009). *Principles of Anatomy and Physiology* (12th edition). U.S.A: Wiley & Sons.

U

Urey H. C. (1948) Oxygen isotopes in nature and in the laboratory. *Science*, *108*, 489-496.

V

Vasoo, S., Barreto, J.N., & Tosh, P.K. (2015). Emerging issues in gram-negative bacterial resistance: an update for the practicing clinician. Mayo clinical procedures, 90, 395-403.

van den Berg, A.A., van Buul, J.D., Ostrow, D.J., & Groen, A. K. (2000) Measurement of cholesterol gallstone growth in vitro. *Journal of Lipid Research. 41*(2),189–194.

van Erpecum, K.J. (2011). Pathogenesis of cholesterol and pigment gallstones: An update. *Clinics and Research in Hepatology and Gastroenterology*, *35*(4), 281-287.

van Velkinburgh, J.C. & Gunn, J.S. (1999). PhoP-PhoQ-regulated loci are required for enhanced bile resistance in *Salmonella* spp. *Infection and Immunity, 67,* 1614-22.

W

Walsh, C. (2000). Molecular mechanisms that confer antibacterial drug resistance, *Nature*, *406*, 775-781.

Waterham, H.R. (2006) Defects of cholesterol biosynthesis. *FEBS Letters*, *580(23)*, 5442-5449.

Woese, C. R. (1987). Bacterial evolution. *Microbiology Reviews, 51,* 221–271.

Woo, P.C., Lau, S.K., Teng, J.L., Tse, H., & Yuen, K.Y. (2008). Then and now: use of 16S rDNA gene sequencing for bacterial identification and discovery of novel bacteria in clinical microbiology laboratories. *Clinical Microbiology and Infection*, *14(10)*, 908-34.

Wu, M., Lin, T. Hsieh, P., Yang, H. & Wang, J. (2011). Isolation of genes involved in biofilm formation of a *Klebsiella pneumonia* strain causing Pyogenic Liver Abscess. *PLoS One*, *6,* 1-11.

Wu, T., Zhang, Z., Liu, B., Hou, D., Liang Y., Zhang, J., & Shi, P. (2013). Gut microbiota dysbiosis and bacterial community assembly associated with cholesterol gallstones in large-scale study. *BMC Genomics*, *14*(669), 1-11.

X

Xiao, Y., Zheng, Y., Wu, S., Zhang, E.H., Chen, Z., Liang, P.,…Zhao, F. (2015). Pyrosequencing reveals a core community of anodic bacterial biofilms in bioelectrochemical systems from China. *Frontiers in Microbiology, 16,* 1410.

Y

Yamamuro, A., Kouzuma, A., Abe, T., & Watanabe, K. (2014) Metagenomic analyses reveal the involvement of syntrophic consortia in methanol/electricity conversion in microbial fuel cells. *PLoS ONE, 9,* e98425.

Z

Zhang, Y., Peng, J., Li, X., & Liao, M. (2016). Endoscopic-Laparoscopic Cholecystolithotomy in Treatment of Cholecystolithiasis Compared With Traditional Laparoscopic Cholecystectomy. *Surgical laparoscopy, endoscopy & percutaneous techniques, 26(5),* 377-380.

Zumsteg, A., Urwyler, S.K., & Glaubitz, J. (2017). Characterizing bacterial communities in paper production—troublemakers revealed. *Microbiology Open, 6,* 1-6.

Appendix 4

Co-Author Letter of Acknowledgement:

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