

**AMINO ACIDS UTILISATION
BY *CLOSTRIDIUM DIFFICILE*
STRAINS**

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Abstract

The carbon and energy metabolism of the human pathogen *Clostridium difficile* is poorly understood. Amino acid metabolism by the Stickland reactions has previously been described as a primary source of energy in a number of *Clostridium* species, especially when grown in a medium containing only amino acids. Deeper insights may be gained by metabolic analyses using liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) platforms, but such experiments are best performed in well-defined growth media. A number of *C. difficile* strains have been successfully cultivated on defined media but these media provide an excess of nutrients, particularly in terms of amino acid provision, resulting in undesirable background growth in the absence of glucose. To overcome these challenges, three variants of a defined medium were developed that contain the essential nutrients that support the growth of this bacterium, in the presence of a carbon and energy source such as glucose together with an LC-MS/MS method that will simultaneously measure all twenty amino acids.

Since the focus in this study was amino acid utilisation, a comprehensive and most effective technique that will provide as much information as possible for understanding the metabolic requirements of eight *Clostridium difficile* strains (CD630 Δ erm, DH196, R20291, EK15, EK28, R12801, L26, O17 Serotype F) and two transposon mutants (CRG-2979, CRG-3887) was required. Due to high water solubility and the range of ionic characteristics of amino acids, an aqueous normal phase chromatographic method was considered to simultaneously separate a mixture of amino acids without derivatisation. Aqueous normal phase chromatographic method represents an important new technology with a capability of the silica-hydrate-base stationary phases, offering a distinct advantage for practical application with a high degree of reproducibility and long-term stability of polar and non-polar compounds.

The developed methods were subsequently adapted to study amino acid utilisation in the presence or absence of a fermentable carbohydrate and/or selenium. OD determinations were performed by measuring absorbance at 600

nm (or OD₆₀₀) using a Biomate 3 spectrophotometer. The concentration of individual amino acids remaining in the spent *C. difficile* culture media was measured after 24 h and/or 48 h using the developed LC-MS/MS method in order to determine which amino acids were being utilised by these organisms and in which order. This analysis should give further insight on the importance of amino acids for the survival of this bacterium in the gut, which may possibly lead to discovery of novel fermentable products and metabolic pathways and/or eventually aid in the successful control of the disease.

Data obtained for CD630 Δ erm, DH196, R20291, EK15, EK28, R12801, L26, O17 Serotype F strains showed that they all grew on the fully defined medium, with different growth profiles in terms of lag phase, growth rate, and maximum OD reached. The LC-MS/MS data generated suggest that cysteine, glutamine, isoleucine, leucine, serine, threonine and tyrosine are preferentially utilised, both in the presence and absence of glucose. Several other amino acids, including asparagine, glycine, phenylalanine, proline and valine were also utilised but to a lesser extent. Notable in this study was the lack of glutamate utilisation, except by strain L26, and the excretion of alanine after its initial uptake by most of the tested stains. The excretion of alanine may be due to the use of pyruvate as an amino acceptor during the degradation of preferentially fermented amino acids, whereas, glutamate is not a substrate for most *C. difficile* strains. Thus, LC-MS/MS profiling confirmed that these organisms derive most of their carbon and energy from the fermentation of a selected range of amino acids.

Given the importance of selenium-dependent Stickland reactions to the growth of this bacterium, further studies were undertaken to evaluate the metabolism of amino acids by two different *C. difficile* strains (630 Δ erm, R20291) and two transposon mutants CRG-2979 (defective in *hadB*, encoding one of the two subunits of hydroxyisocaproyl-CoA dehydratase required for reductive degradation of leucine) and CRG-3887 (defective in *selA* encoding selenocysteine synthase) in the presence or absence of glucose/selenium. LC-MS/MS data reveal that amino acid utilisation was affected by the presence of selenite, notably proline utilisation, which could be explained by the presence

of the enzyme proline reductase and the lack of glycine consumption, known to be selenium-dependent. The non-utilisation of glycine could be explained by the presence of proline which represses the formation of the necessary enzyme systems required for glycine degradation. Data generated for CRG-2979 reveals that this mutant could only thrive in the presence of selenium when glucose is present, possibly due to the presence of proline replacing leucine as the major Stickland acceptor. The results of the transposon mutant CRG-3887, were much of a surprise too, because this mutant was predicted to be deficient in proline and glycine breakdown which is also reliant on selenoenzymes. This suggests the presence of selenium independent proline fermentation pathway although this hypothesis is not supported by the existing literature.

Experimental data provided further evidence about the ability of this bacterium to obtain its carbon and energy in the absence of a fermentable carbohydrate; by Stickland reactions and that the presence or absence of certain amino acids could repress the utilisation or biosynthesis of other amino acids.

Declaration

I hereby declare that the content of this PhD thesis is the presentation of my original research work and efforts, and has not been submitted anywhere else for any other qualification. Where contributions of others are involved, every effort has been made to indicate clearly the extent of their contribution, with due reference to the literature and acknowledgement of collaborative research. The work was done under the supervisions of Professor David Barrett, Centre for Analytical Bioscience, Division of Molecular and Cellular Science, School of Pharmacy, University of Nottingham, UK and Dr Klaus Winzer, School of Life Sciences, Centre for Biomolecular Sciences, University of Nottingham, UK.

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Abbreviations

AA	Amino acid
ACN	Acetonitrile
Ala	Alanine
AMP	Adenosine monophosphate
ANOVA	Analysis of Variance
ANP	Aqueous normal phase
ANPC	Aqueous normal phase chromatography
APCI-MS	Atmospheric pressure chemical ionization coupled to mass spectrometry
Arg	Arginine
Asn	Asparagine
Asp	Aspartic acid
ATP	Adenosine triphosphate
BHI	Brain heart infusion
CaCl ₂ ·2H ₂ O	Calcium chloride dihydrate
cAMP	Cyclic adenosine monophosphate
CBS	Centre for Biomolecular Sciences
CDI	<i>Clostridium difficile</i> Infection
Cdt	<i>Clostridium difficile</i> transferase
CE	Capillary electrophoresis
CoCl ₂ ·6H ₂ O	Cobalt(II) chloride hexahydrate
CRG	Clostridia Research Group
Cys	Cysteine
dH ₂ O	deionised water
DNA	Deoxyribonucleic acid
ES	Electrospray
ES+	Positive ion electrospray
ESI	Electrospray ionisation
ESI-MS	Electrospray ionisation mass spectrometry
ESI-TOFMS	Electrospray ionization time-of-flight mass spectrometry
eV	Variable energy ionisation
FeSO ₄ ·7H ₂ O	Iron(II) sulfate heptahydrate

GC	Gas chromatography
GC-MS	Gas chromatography- mass spectrometry
Gln	Glutamine
Glu	Glutamic acid
Gly	Glycine
HCOOH	Formic acid
HILIC	Hydrophilic interaction liquid chromatography
HPLC	High performance liquid chromatography
HPLC-MS	High performance liquid chromatography-mass spectrometry
HPLC-MS/MS	High performance liquid chromatography coupled with tandem mass spectrometry
HPLC-UV	High performance liquid chromatography-ultra violet detection
His	Histidine
Ile	Isoleucine
KH ₂ PO ₄	Potassium dihydrogen phosphate
LC	Liquid Chromatography
LCESI-MS	Liquid chromatography coupled to electrospray-ionization mass spectrometry
LC-MS	Liquid chromatography – mass spectrometry
LC-MS/MS	Liquid chromatography-tandem mass spectrometry
Leu	Leucine
LOD	Limit of detection
LLOQ	Lower limit of quantification
Lys	Lysine
MCDM	Modified <i>Clostridium difficile</i> medium
Met	Methionine
MgCl ₂ ·6H ₂ O	Magnesium chloride hexahydrate
MnCl ₂ ·4H ₂ O	Manganese(II) chloride tetrahydrate
MRM	Multiple reaction monitoring
mRNA	Messenger ribonucleic acid
MS	Mass Spectrometry
MS/MS	Tandem mass spectrometry

<i>m/z</i>	Mass-to-charge ratio
Na ₂ HPO ₄	Sodium hydrogen phosphate
NaCl	Sodium chloride
NADH	Nicotinamide adenine dinucleotide hydrate
Na ₂ SeO ₄ ·10H ₂ O	Sodium selenate decahydrate
NaHCO ₃	Sodium hydrogen carbonate
NAP1	North America Pulsed-field gel electrophoresis type 1
(NH ₄) ₂ SO ₄	Ammonium sulphate
NMR	Nuclear magnetic resonance
NPLC	Normal-phase liquid chromatography
NPLC-MS	Normal-phase liquid chromatography mass spectrometry
NPLC-MS/MS	Normal-phase liquid chromatography-tandem mass spectrometry
OD	Optical density
OD ₆₀₀	Optical density, measured at a wavelength of 600 nm
PCR	Polymerase chain reaction
pH	Hydrogen ion concentration
Phe	Phenylalanine
pM	picomolar
Pro	Proline
RF	Radio frequency
RNA	Ribonucleic acid
RP	Reversed phase
RP-HPLC-MS	Reversed phase high performance liquid chromatography-mass spectrometry
RSD	Relative standard deviation
RT	Retention time
Sec	Selenocysteine
SECIS	Selenocysteine insertion sequence
<i>SelA</i>	Selenocysteine synthase
<i>SelD</i>	Selenophosphate synthase
SEM	Standard error of mean
Ser	Serine
TcdA	<i>Clostridium difficile</i> toxin A

TcdB	<i>Clostridium difficile</i> toxin B
Thr	Threonine
TIC	Total ion chromatography
TLC	Thin layer chromatography
tRNA	Transfer ribonucleic acid
Trp	Tryptophan
Tyr	Tyrosine
UGA	Uracil-guanine-adenine
UPLC	Ultra-Performance Liquid Chromatography
Val	Valine

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CHAPTER 1.

GENERAL INTRODUCTION

1. GENERAL INTRODUCTION

1.1 Review on Clostridia

Clostridia are anaerobic, spore-forming, rod-shaped, motile, Gram-positive bacteria of the genus *Clostridium*. They are widely distributed in nature, including the intestinal tract of animals and humans. A vast majority of these bacteria are not necessarily harmful in way, but have economic and medical benefits. For example, *Clostridium pasteurianum* is capable of converting nitrogen to ammonia by nitrogen fixation [1, 2], *Clostridium acetobutylicum* is able to produce organic solvents such as acetone and butanol [3, 4] while those that belong to the species *Clostridium sporogenes* and *Clostridium novyi* are associated with the treatment of cancer as previously reported [5, 6]. On the other hand, a sizeable number of the *Clostridium* species do not have beneficial properties because of their ability to cause disease to animals and humans via the production of toxins [7, 8]. Some of the important diseases caused by *Clostridium* include tetanus caused by *C. tetani*, gas gangrene by *C. perfringens*, *C. novyi*, *C. septicum*, *C. sordellii* and *C. histolyticum*, botulism caused by *C. botulinum*, *C. perfringens* type A-induced food poisoning and *C. perfringens* type C necrotising jejunitis (also known as pig-bel), pseudomembranous colitis and antibiotic-associated diarrhoea caused by *C. difficile* [7, 9, 10].

1.1.1 *Clostridium difficile* infection

The bacterium was first described in 1935 as part of the intestinal flora in healthy new-born infants [11, 12] but later on in 1978, it was shown to be the causative agent of antibiotic-associated diarrhoea [13], also referred to as *Clostridium difficile* infection (CDI). A few individuals naturally harbour these bacteria in their gut and as such they show no symptoms and would naturally not require any treatment. Infection would only occur when the normal flora has been destroyed, thereby making it possible for these competing bacteria to colonize and possibly overgrow the gut leading to a build-up of toxins and their effects [14-17]. It can be transmitted from one individual to another

through the faecal oral route [18, 19]. Symptoms of the disease range from mild diarrhoea sometimes accompanied with fever, abdominal pain, malaise, anorexia, leukocytosis, cramping to severe pseudomembranous colitis [14-16, 18, 20, 21]. In some patients, symptoms may not include diarrhea, but may be associated with ileus or toxic megacolon and failure to treat would result in a condition known as colonic perforation, or sepsis or death of the patient [12].

1.1.2 The hypervirulent strains

The problem of increased CDI prevalence has been attributed to the emergence of *C. difficile* strains belonging to restriction endonuclease analysis group BI, North American pulsed-field gel electrophoresis type 1 (NAP1) and PCR-ribotype 027 (BI/NAP1/027) [7, 22-24]. Clinical isolates obtained from CDI patients in recent times have become highly represented by these strains with a clear indication that BI/NAP1/027 type is now the most commonly isolated *C. difficile* type in most countries [7, 24-30]. This strain is linked with the emergence of community-associated infections, increased resistance to most commonly used antimicrobial agents, increased incidence of nosocomial infections and mortality [17, 24, 28, 31-33]. The ability to produce higher levels of toxins *via* an unknown mechanism has become more worrisome [7, 12, 14, 15, 34-36].

1.1.3 Pathogenicity

The pathogenesis of *C. difficile* begins with interruption to the existing normal microflora of the colon occasioned by indiscriminate use of broad spectrum antibiotics, followed by colonization [17, 19, 37, 38]. Upon colonization of the colon, the two toxins (TcdA and TcdB) released find their way through the receptor-mediated endocytosis to deactivate the GTPases and this disengages the cytoskeleton, causing damage to the intestinal epithelial cell as well as increasing permeability of tight junctions as previously reported [14, 39]. Furthermore, the release of these toxins leads to the production of pro-

inflammatory mediators, cytokines and activating the enteric nervous system, giving way for neutrophil chemotaxis and fluid secretion [39].

1.1.4 Factors that influence *C. difficile* infection

1.1.4.1 Virulence factors

The virulence potential of the infecting strains and the level at which the virulence is allowed to be expressed may determine the outcome of infection [8]. As previously reported, some of the several established and potential virulence factors of this bacterium relate to its ability to adhere to the intestinal mucosa, expression of fimbriae, production of a protective polysaccharide capsule, production of tissue degradative enzymes and more importantly the production of toxins [8]. Aside from toxins which have clearly been identified as the key virulence factors, the role of other virulence factors seems to be more speculative, considering the previous data obtained from our laboratory [40]. Most of the disease-causing strains of *C. difficile* are reported to produce either toxins TcdA or TcdB or both [21, 35, 40-42]. Toxins TcdA and TcdB have since been identified as the largest amongst the bacterial toxins, with approximate molecular masses of about 308 and 270 kDa respectively [14, 21, 43, 44]. Early reports indicated that both toxins are potent cytotoxins, but for most cell lines TcdB is much more cytotoxic than TcdA [8]. Where both toxins are produced, one increases the effectiveness of the other to upset the cytoskeleton of the intestinal epithelial cells [12, 15, 21]. As previously reported, toxin TcdA functions by loosening the tight junctions around the epithelial cells that line the colon and allows the invasion of the cell by the second toxin TcdB [21, 34]. Incidentally, toxin TcdB is a thousand times more toxic than toxin TcdA [21, 35]. Some *C. difficile* strains are reported to produce an adenosine diphosphate ribosyltransferase binary toxin (Cdt) in addition to the TcdA and TcdB toxins [45-47]. Its role in pathogenesis is not fully understood but it has been reported to act as an additional factor for *C. difficile* virulence [45, 48]. A number of the clinical isolates are said to produce toxins TcdA and TcdB, but not Cdt [17, 49]. Cdt is supposedly formed by two separate, unlinked polypeptide subunits, CdtA and CdtB [50]. CdtA is reported

to enter the cell following mediation by CdtB, which binds to a plasma membrane receptor [51].

1.1.4.2 Age of the host

People most vulnerable to CDI are the elderly [19]. Age related changes in the gut flora of the elderly have been reported to cause a reduction in the amount of disruption of colonisation resistance required for the bacterium to colonise [52-54]. In particular, toxin A is said to cause a greater chemotactic response of leukocytes, in elderly than in the young individual [55]. In young infants and new-borns, *C. difficile* infection is not very pronounced, even though they oftentimes harbour the bacteria in their gut [56]. This is made possible because the young infants have a developing and incomplete intestinal flora that is unable to exclude the bacteria [8]. Previous reports also indicated that the young infant lacks the receptors for the toxins to bind in their intestines, or even where they exist they seem not to be recognised by these toxins because they are in an immature form [57].

1.1.4.3 Disruption of colonisation resistance

The indiscriminate use of broad-spectrum antibiotics has been reported by several authors as one major factor influencing the outcome of CDI [16, 19, 38, 58]. Most of the broad-spectrum antibiotics such as clindamycin, penicillins, cephalosporins, and quinolones are capable of disrupting the host defences provided by indigenous microflora in the colon allowing *C. difficile* to colonise the gut and cause infection [14, 38, 43]. On the other hand, gastrointestinal tract surgery, use of medications that decrease intestinal motility and cancer chemotherapy treatment can also cause a disruption of colonisation resistance and predispose patients to *C. difficile* infection as previously reported [59-61].

1.2 Microbial Metabolism

All the biochemical reactions occurring in an organism to generate and use energy in order to synthesise cellular materials is known as metabolism [62]. Metabolism is central to microbial life and as previously reported, it arises from interactions of molecules in the cell environment, fuelling all cellular activities with building blocks and energy [63]. Chemical diversity of substrate oxidations/dissimilation reactions and uptake/utilisation of the organic or inorganic compounds necessary for growth and maintenance of a cellular steady state are some areas of focus in the study of microbial metabolism [62]. Overall, the scope covers the range in which an organism is able to live, function, and reproduce in a specified culture condition with the attendant chemical changes occurring during its transformation [62]. As previously described, an integrated enzyme system catalyse these respective energy-yielding and energy-requiring reactions within the living microbial cell, leading to the replication of the cell by itself. Bacteria for example, are described as highly specialised energy transformers, where high-energy compounds such as adenosine diphosphate (ADP) and adenosine triphosphate (ATP) or compounds containing the thioester bond (acetyl $\sim$ SCoA or succinyl $\sim$ SCoA) are conserved following the generation of chemical energy by substrate oxidations [62]. ADP and ATP here represent adenosine monophosphate (AMP) plus one and two high-energy phosphates (AMP $\sim$ P and AMP $\sim$ P $\sim$ P, respectively), storing energy as high-energy phosphate bonds. New complex organic compounds needed by the cell can be synthesised from compounds used as energy sources in the presence of proper enzyme systems. The formation and use of such high-energy compounds as previously reported will require all living cells to maintain steady-state biochemical reactions [62].

It is worthy of note that across divergent species the important metabolic functions are highly similar, even though there may be differences in lifestyle and habitats where these organisms live (Figure 1-1). All organisms, regardless of species must as a requirement hunt for food, and coordinate their chemical processes, monomer synthesis and macromolecule polymerization for biomass

synthesis and proliferation [62, 63]. Previous reports have recognised the use of ATP or the high-energy phosphate bond ($\sim P$) by bacteria, mammals and plant cells as their primary chemical energy source [62]. For growth and energy transformation requirement, bacterial cells have been reported to utilise the B-complex vitamins as functional coenzymes for many oxidation-reduction reactions. *Thiobacillus thiooxidans*, for instance will grow in an acid environment, particularly in a medium containing inorganic salts and elemental sulfur and utilizing carbon dioxide as carbon source [64, 65]. They are also able to synthesise large amounts of riboflavine, pantothenic acid, thiamine, biotin, pyridoxine and nicotinic acid. This concept of biochemical agreement has informed the use of bacteria as model systems for related biochemical reactions studies [62].

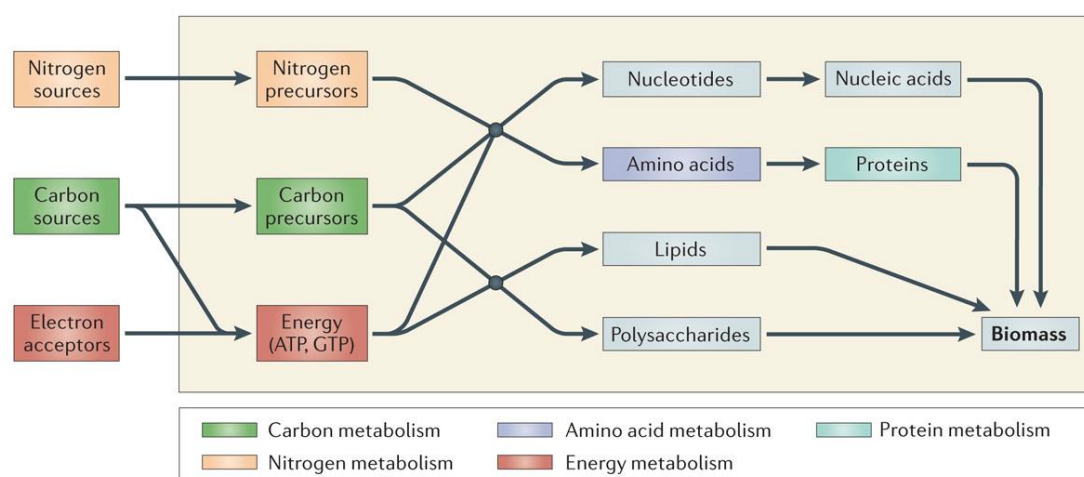


Figure 1-1: Coarse-grained view of different sectors that compose large parts of metabolism in many bacteria.

Microorganisms need to carry out a range of metabolic tasks to ensure a supply of metabolic fluxes through the sectors and thus sustain cell maintenance and growth. All organisms must regulate the uptake of nutrients and coordinate carbon, energy and nitrogen metabolism to balance monomer synthesis and macromolecule polymerization. Reproduced from Chubukov *et al.* [63].

1.2.1 Heterotrophic metabolism

Heterotrophic metabolism is a form of reaction in living cells generating usable energy through the transfer of electrons from one molecule to another. The process of heterotrophic metabolism, brings about the synthesis of ATP as the chemical energy source and permits the generation of precursor molecules needed by the organism for biosynthetic or assimilatory reactions as previously reported [62]. Serving as precursor molecules are the Krebs cycle intermediate compounds for the energy-requiring biosynthesis of complex organic compounds in bacteria. Previous reports indicate that preformed organic compounds are used aerobically or anaerobically by all heterotrophs as growth substrates to generate reducing equivalents such as reduced nicotinamide adenine dinucleotide, which end up as sources of chemical energy.

A vast majority of the heterotrophs are pathogenic and the most commonly studied are bacteria. They obtain their energy from oxidation of organic compounds such as glucose, lipids, and protein. Some heterotrophs produce enzymes and proteins that enables them to degrade polysaccharides to their constituent sugar units, accumulate sugar inside the cell using an appropriate transport system and convert sugar into one of the central intermediates of metabolism [66]. Some of the pathways used for carbohydrate metabolism include Embden-Meyerhof Parnas pathway, pentose phosphate pathway and the Entner-Doudoroff pathway [66, 67].

1.2.1.1 Fermentation

Fermentation is one of the examples of heterotrophic metabolism through which glucose metabolism produces energy for the cell. It uses an organic compound, such as a sugar or amino acid as a terminal electron acceptor [62]. It should be noted that the substrate being metabolised can determine the amount and type of fermentation end products excreted and the level of NADH generated for recycling [68]. Though these reactions may include a few steps, they ultimately lead to the direct formation of ATP [66]. The process of anaerobic dissimilation of glucose, eventually will lead to the formation of

simple organic end products. However, due to the incomplete biological oxidation process, simple organic end products are formed to serve as hydrogen and final electron acceptors. There follows the secretion of these organic end products (alcohol or acid) into the medium as waste metabolites. Though the oxidation process is supposedly incomplete, energy produced from organic substrate compounds is often times sufficient for microbial growth. Fermentation has previously been described as a vital process associated with the growth of specific microorganisms by Pasteur in the late 1850s and the principal organic end product such as lactic acid, ethanol, acetic acid, or butyric acid could be predicted from the type of fermentation process [67]. His butyric acid fermentation studies paved the way for the discovery of anaerobic microorganisms [67]. The glycolytic pathway is the result of glucose dissimilation in most microbial fermentations reactions. The most commonly generated organic compound is pyruvate or compounds derived enzymatically from pyruvate, which include acetaldehyde, α -acetolactate, acetyl $\sim$ SCoA, or lactyl $\sim$ ScoA. A number of organisms that ferment glucose produce carbon dioxide and hydrogen with diverse end products like acetate, succinate, lactate and formate. *Enterobacter aerogenes*, *Aeromonas*, *Serratia*, *Erwinia*, and *Bacillus* species are also known to produce ethanol, acetylmethylcarbinol, 2,3-butylene glycol, carbon dioxide and hydrogen. A vast majority of the clostridia, for example, *Clostridium saccharobutyricum*, *C. thermosaccharolyticum* and *Butyribacterium* species will ferment glucose to produce butyrate, acetate, carbon dioxide and hydrogen, while *C. acetobutylicum* and *C. butyricum* will produce butanol, acetone, isopropanol, formate, and ethanol [67].

1.2.1.2 Respiration

In respiration, the electron transfer reaction is the primary mode of generating energy. Electrons from molecules of sugar are transferred to an inorganic molecule instead of another organic molecule. Up to 38 molecules of ATP per molecule of glucose can be produced from the breakdown of sugar to carbon dioxide and water. Using the electron transport chain (an enzyme system and

cofactors found in the cell membrane) electrons are conveyed to oxygen and arranged in such a manner that electrons passing down the chain are aligned with the movement of protons across the cell membrane. The positively charged hydrogen ions are activated by the electron transport to move to the outside. Previous reports have described this process as a common way which organisms generate energy and store it. Also, due to the complete oxidation of the energy source during respiration, bacteria are far more able to produce more energy per sugar molecules. Unlike fermentation, all of the available energy is extracted as shown in the high level of ATP production for respiring bacteria. Using available nutrients, organism that use respiration are able to produce a greater quantity of cell materials with less toxic end products.

Respiration can occur under both aerobic and anaerobic conditions. Aerobic respiration, which is the most familiar respiratory process, uses oxygen as the final electron acceptor. Though the solubility of oxygen in water is limited, there seems to be a direct link between available oxygen and how far aerobic bacteria can grow or survive. Similarly, in anaerobic respiration, an inorganic molecule, such as nitrate, nitrite, sulphate or carbon dioxide is used as the final electron acceptor, although with a lesser energy yield available to the cells than in aerobic respiration. Nonetheless, the ability to use inorganic molecules as the final electron acceptor is of great importance to the environment. A few areas of such importance to the environment include, the conversion of nitrate to nitrite and dinitrogen gas as denitrification being performed by *Pseudomonas stutzeri*, and the reduction of carbon dioxide to acetate and methane being performed by *Acetobacterium woodii* and methanogenic archaea respectively.

1.2.2 Autotrophic metabolism

In autotrophic metabolism, carbon dioxide serves as a sole source of carbon for the synthesis of all cell constituents, while nitrogen is sourced from ammonia, nitrate or nitrogen compounds [62, 69]. Organic compounds are synthesised from carbon dioxide through the reductive pentose phosphate cycle, the

reductive tricarboxylic acid cycle, and/or the acetyl-CoA pathways [69-71]. The reductive pentose phosphate cycle, operational in a number of organisms including bacteria, is considered the most widely distributed pathway. Here a store of chemical energy can be created, whilst carbon dioxide is being reduced to glucose from which organic cellular matter is synthesized. The important reaction here is the combination of ribulose 1,5-bisphosphate with carbon dioxide, to produce two molecules of 3-phosphoglycerate, a precursor to glucose. Carbon fixation is maintained following the cell's ability to carry out photosynthetic or aerobic respiratory metabolism. Most autotrophs can make their food and as such are sometimes called producers. The process of making their own food could be by photosynthesis, in which case sunlight, carbon dioxide and water is used or by chemosynthesis using carbon dioxide, water and chemicals such as ammonia [72]. From a nutritional point of view, the autotrophs display a unique mode of metabolism, which may be defined by the electron donor they use [66]. For example, *Thiobacillus ferrooxidans*, *T. denitrificans* both sulfur-compound-oxidizing bacteria, occasionally exhibit a strictly autotrophic mode of metabolism, oxidizing thiosulfate and elemental sulphur to sulphate. Ferrous ions are oxidized by *Acidithiobacillus ferrooxidans* to ferric ions, ammonia is oxidized by *Nitrosomonas europaea* to nitrite, and in turn nitrite is oxidized by *Nitrobacter winogradskyi* to nitrate. *Oligotropha carboxidovorans*, on the other hand oxidizes carbon monoxide to carbon dioxide, while hydrogen gas is oxidized by *Alcaligenes eutrophus*. [66]

1.2.3 Phototrophic metabolism

Phototrophic metabolism is a mode of metabolism performed by some prokaryotes such as bacteria and cyanobacteria. Light energy from the Sun is trapped to release electrons with higher energy levels using pigments called chlorophylls. Early studies indicated that during day light hours phototrophic organisms accumulate polysaccharides in their cells especially when photosynthetic carbon dioxide fixation becomes a dominant metabolic process. On the other hand, much of the starch reserves are hydrolysed to sugars by amylase activity during the evening [68, 73, 74] and to pyruvate, through the

activity of glycolysis [68]. The electrons released are passed through a transport chain that generates energy to form a proton gradient that initiates the synthesis of adenosine triphosphate [66, 67]. Previous reports indicate that these electrons eventually return to the chlorophyll and the whole process fulfills the energy needs of the cell [66]. During biosynthetic processes, a source of electrons is needed to replenish electrons that have been utilised.

1.3 Microbial Amino Acid Metabolism in the Gastrointestinal Tract

1.3.1 Amino acid fermenting microbes

The intestinal microbiota is reported to contain the largest part of the human microbiome, with a highly diverse community of mostly anaerobic microorganisms [75-80]. Previous reports have recognised their very useful roles in preventing growth of pathogens, producing hormones and vitamins, regulating the development of the gut, regulating epithelial proliferation in the intestine, contributing to host energy metabolism including the fermentation of amino acids [75, 78, 81-87]. Early studies have shown the contribution of gut bacteria in amino acid homeostasis by comparing an altered distribution of free amino acids along the gastrointestinal tract between germ free mice and conventional mice [75, 88]. Peptides and amino acids can be hydrolysed from alimentary and endogenous proteins along the gastrointestinal tract by host and bacteria-derived proteases and peptidases. As previously reported both gut bacteria and the host can thereafter utilise the released amino acids and peptides [75, 89, 90]. If there are low levels of fermentable carbohydrate in the colon, protein breakdown and amino acid fermentation are increased and there are long intestinal transit times, with possible undesirable effects for the host [91, 92]. The vast majority of the microbial community are bacteria, with the foremost ones belonging to the phyla *Proteobacteria* (include a wide variety of pathogens, such as *Escherichia*, *Salmonella*, *Vibrio*, *Helicobacter*), genus *Clostridium* and *Bacillus-Lactobacillus-Streptococcus* [75, 93]. Clostridia and proteobacteria are the most dominant species involved in amino acid fermentation in the human intestine [93-95] and may play a role in protein digestion and amino acid absorption [75, 93].

1.3.2 Microbial amino acid catabolism and utilisation

Previous studies on amino acid metabolism in the gut have described the disappearance of amino acids to be the result of direct assimilation by bacteria, deamination, decarboxylation or their binding to the bacteria and host epithelial cells [92, 93]. In essence, bacterial cells can either directly utilise amino acids as protein building blocks or catabolise them through different pathways. This has previously been demonstrated in milk-fed piglets, showing that lysine incorporated into the mucosal proteins was quantitatively less than lysine catabolism in the intestinal mucosa [75, 96]. This has also been alluded to recently for other amino acids like methionine and phenylalanine, partly being utilised by the small intestinal microbiota [75, 93]. The amounts of methionine were reported to represent about half of the dietary methionine in the piglets, implying that part of the dietary methionine is consumed in the gastrointestinal tract. A related *in vitro* study also showed the catabolism of single amino acids by both monocultures and mixed bacterial cultures derived from the pig small intestine [97, 98], which is consistent with the ability of some bacteria to harbour highly active peptidases [99]. Considerable amounts of amino acids appear not to be assimilated in the human small intestine following the breakdown of protein [100]. The microbiotas in the colon is reported to subsequently use or transport these amino acids from the lumen into the portal blood stream while the host on the other hand produces mucins (glycoproteins) that contribute to the available amino acids within the colon as previously reported [75, 101]. There are considerably more bacteria in the more distal part of the human intestine than in the proximal part of the intestine where the concentrations of proteins, peptides, and amino acids are relatively high [75]. This could be explained by the fact that along the gastrointestinal tract there are huge differences in microbiota abundance and composition leading to the specificity of bacterial amino acid metabolism in the gut [75, 102].

In the large intestine, rather than being absorbed by the colonic mucosa, amino acids are instead being significantly metabolised by the large intestinal

microbiota [93]. Previous reports have linked this high rate of bacterial protein fermentation to the reduced amount of available carbohydrate coupled with the high pH level in the large intestine [75, 90]. The most metabolised amino acid substrates are, arginine, glycine, isoleucine, leucine lysine and valine [94], leading to the production of a complex mixture of metabolic end products such as ammonia, acetate, propionate, butyrate, valerate, isobutyrate, isovalerate and biogenic amines. As previously reported, these bacterial metabolites seem to play a role in the epithelial physiology by prompting signalling pathways in epithelial cells, regulating the host mucosal immune system [103-105] and regulating the degree of bacterial gene expression for the production of enzymes associated with amino acid metabolism [75, 106]. The biogenic amines are produced by decarboxylation of amino acids via the action of the resident microbiota [75]. For example, the decarboxylation of lysine and arginine produces cadaverine and agmatine respectively with significant physiological effects *in vivo* [75, 107]. A recent experiment with rats revealed the influence of agmatine on multiple physiological and metabolic functions by increasing tissue cAMP levels [108]. The elevated tissue cAMP levels cause a reduction in diet-induced weight gain, which has a replicating effect of caloric restriction with respect to metabolic reprogramming as previously reported [75, 108].

1.3.3 Microbial amino acids biosynthesis

Aside from serving as basic building blocks of proteins, amino acids also play vital roles in the synthesis of many important cellular molecules such as vitamins and nucleotides and can be grouped into six biosynthetic families (Figure 1-2) as previously reported [67]. The entire basic set of 20 amino acids can be synthesised by most microorganisms, through a variety of pathways that are critical for their survival [93]. Even though the pathways for the biosynthesis of amino acids may be different, they seem to share common features such as their carbon skeletons from intermediates of glycolysis, the pentose phosphate pathway, or the citric acid cycle [67].

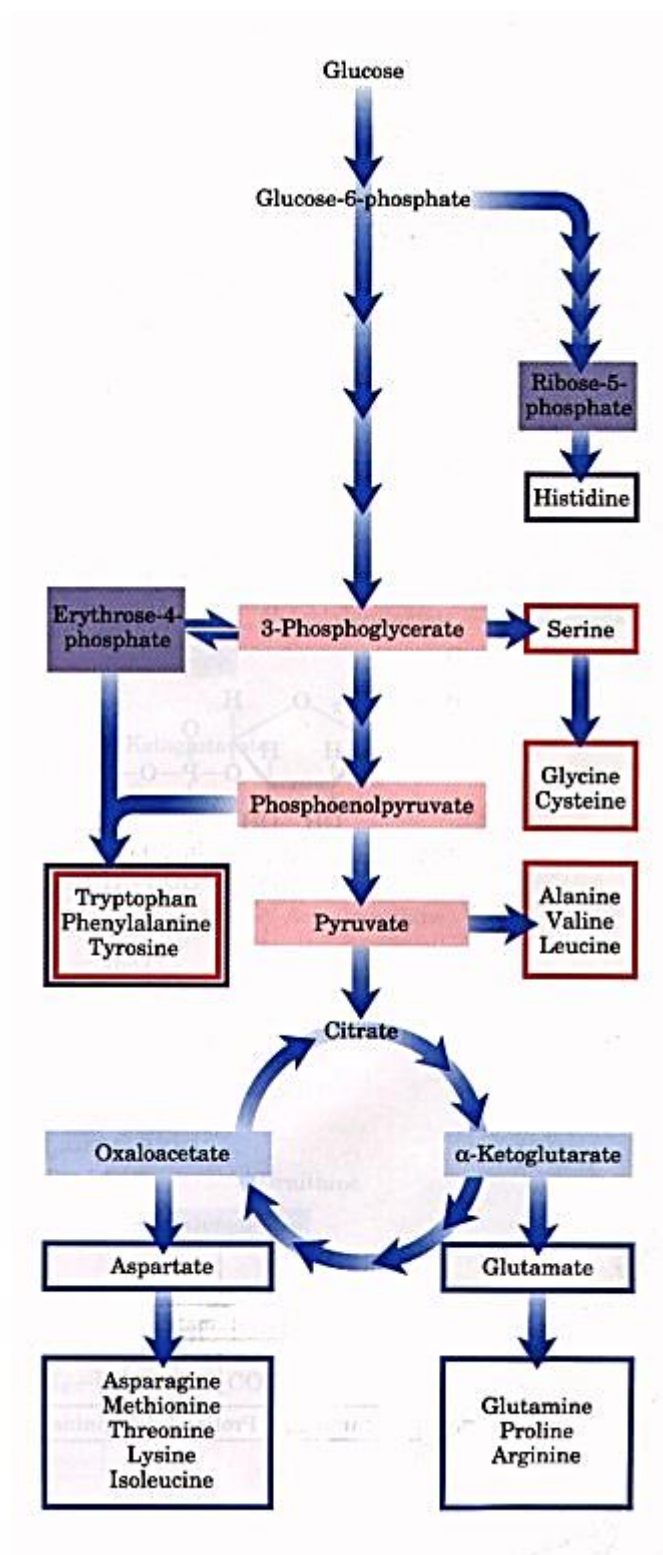


Figure 1-2: Overview of amino acid biosynthesis.

Precursors from glycolysis (red), the citric acid cycle (blue), and the pentose phosphate pathway (purple) are shaded, and the amino acids derived from them are boxed in the corresponding colours. Reproduced without modification [109].

In addition to the usage of amino acids, microorganisms, including bacteria also contribute in the production and *de novo* biosynthesis of amino acids [75, 93]. In a previous study, ruminal bacteria - *Streptococcus bovis*, *Selenomonas ruminantium*, and *Prevotella bryantii* were shown to have contributed in *de novo* synthesis of amino acids in the presence of physiological concentrations of peptides [75, 110]. Other studies have shown the absorption and incorporation of an essential amino acid, lysine (microbially derived) into host proteins [75, 111-113]. Another experiment comparing conventionalised and germ-free rats following the incorporation of ^{15}N from $^{15}\text{NH}_4\text{Cl}$ into body lysine showed that all ^{15}N -lysine detected was from microbial origin [114]. About 75% of the microbial ^{15}N -labeled lysine was later found to have been absorbed by the small intestine [115]. Further experiment incorporating ^{15}N from dietary $^{15}\text{NH}_4\text{Cl}$ and ^{14}C from dietary ^{14}C -polyglucose into amino acids in body tissues enabled the assessment of the synthesis of essential amino acids by gut bacteria and their absorption using a pig model [116]. ^{15}N and ^{14}C cannot be incorporated into lysine or essential amino acids respectively by pigs, therefore the detected incorporation into body protein in the previous experiment is an indication of a microbial origin [75]. An important contribution of microbial synthesis of amino acids was made by microbially derived lysine and threonine to the free plasma lysine and threonine pool in adult humans on nitrogen adequate diets [111, 117]. In related studies, amino acids in plasma were shown to have originated from microbially degraded urea in human infants [113], while the intestinal microbiota is enriched with genes involved in biosynthesis of essential amino acids using precursors derived from the human plasma [115].

1.3.4 Growth of *C. difficile* Stickland amino acid pairs

Amino acid degradation can occur through several pathways [118]. However, clostridia have been reported to ferment amino acids using specific pathways that are unique to them [119]. This unique feature also referred to as the

Stickland reaction involves the oxidation of an amino acid coupled to the reduction of another [118-121].

Figure 1-3 presents a generalized view of Stickland reaction from specific amino acids acting as donors or acceptors or both [120]. The oxidised amino acid is acted upon by amino acid hydrogenase and goes through a deamination process to lose two electrons and two protons [118, 122, 123] resulting in the formation of a keto acid. The keto acid formed then loses a carbon atom to carbon dioxide leading to ATP synthesis by substrate-level phosphorylation in the absence of a respiratory system [118, 120, 123]. Thereafter, electrons and protons are transferred from a coupled reaction via a reductive branch to another amino acid catalyzed by a reductase to support a reductive deamination [124-127]. *Clostridium sticklandii* is the most extensively studied of all *Clostridium* species, with special ability to utilise threonine, arginine, lysine and serine as reductants, glycine and proline as oxidants in the Stickland reaction. Other amino acid pair ornithine/proline or ornithine/lysine have also been described in the Stickland reaction [119, 128].

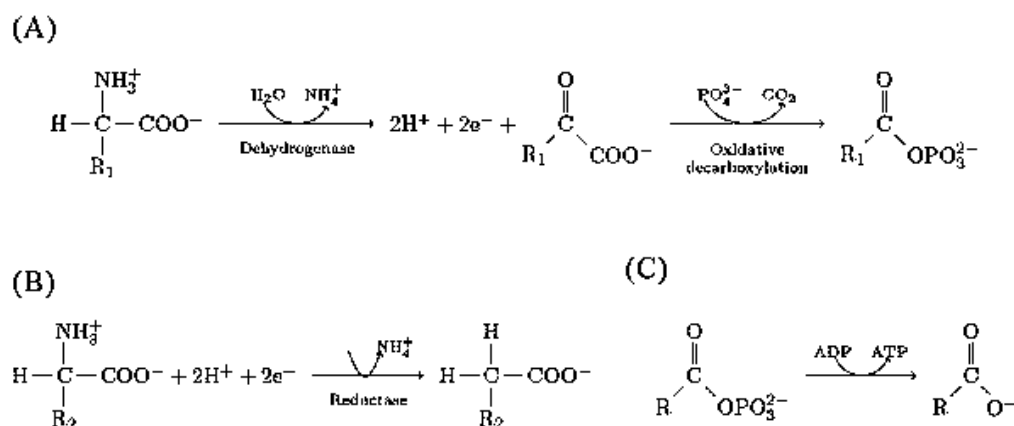


Figure 1-3: The Stickland reaction.

The two steps of amino acid fermentation in *Clostridium*. (A) Oxidative deamination of the amino acid with the higher oxidation state yields an α -keto acid, ammonium, and two protons. For example, if R1 is a methyl group (-CH₃), then the donor amino acid is an alanine, and the product of the oxidative deamination would be pyruvate, a central compounds of the intermediary metabolism. (B) Inorganic phosphate attaches at the carboxyl terminus. The second proton takes the place of the amino radical. The product of this reaction is an acyl-phosphate. For example when R2 is a hydrogen (thus the acceptor amino acid is a glycine) this product corresponds to acetyl-phosphate. The two phosphate compounds from A and B are substrates for the synthesis of ATP from ADP. Naturally, in *Clostridium* spp. all these reactions occur with the aid of hydrogenases (for the electron donors) and reductases (for the electron acceptors). ATP synthesis is catalysed by kinases. Reproduced from de Vlader [129].

1.4 Growth and Nutritional Requirements of *C. difficile*

Generally, the ability of a particular microorganism to grow depends on the physical and chemical conditions present as well as the presence of all essential nutrients. With this in mind, great attention must be paid to observe these conditions when growing, isolating or characterising the different types of organisms. In a controlled environment such as the laboratory, all essential

nutrients required for maintenance, energy generation and cellular biosynthesis must be present in the required amount in the media. As previously reported, a large variety of culture media are now being employed for growth, isolation, maintenance and identification of microorganisms [130]. Such media must meet a number of these conditions;

- Optimum temperature –Most pathogenic bacteria are reported to thrive well at 37°C and this is possibly why the human body plays the perfect host for *C. difficile* [131, 132]; below minimum or above maximum range growth will be affected.
- Relative acidity or alkalinity – must be taken into consideration in the changing milieu of bacterial waste products that accumulate during growth.
- Moisture – necessary to facilitate diffusion into the cell, glycolysis and slowing down the formation of spores.
- Carbon and energy sources – a requirement for all organisms and for *C. difficile* in particular, which cannot make its own food, but relies on the provision of organic substances for growth [133]. If faced with a choice of nutrient sources, a sizeable number of bacterial species including *C. difficile* will adapt their physiology to the preferred substrate in order to survive [134-136], but then, when the condition becomes unfavourable, the organism is able to enter a dormant state and form a highly resistant spore. When conditions become favourable again, the bacterial spore is able to return to its vegetative state [135].
- The right amount of essential macro and micro elements.
- Growth factors – such as vitamins, certain amino acids, purines and pyrimidines, which may be needed because of a blocked or missing metabolic pathway in the cell. They are also required, to fulfil specific roles in biosynthesis, even though this may be provided by very small amounts.
- Gaseous requirements – strict anaerobes like *C. difficile*, do not use oxygen for growth and metabolism, rather they live by anaerobic fermentation [133].

1.5 Analytical Methodologies for Identification and Quantification of Microbial Metabolites

The elastic nature of many bacterial species has enabled them to produce or utilise primary and/or secondary metabolites that provide selective advantage over other species in their natural habitat [137]. Such metabolism can also be induced in the laboratory by establishing a defined culture condition(s) that are specific to the bacterial population. A vast majority of microorganisms are able to modify their metabolism to make the most of the available nutrients [138-141]. They are able to do so by altering gene expression (specialized regulon) in response to the availability or deprivation of amino acids, carbon and energy sources, availability or depletion of nitrogen and phosphorus, changes in pH and temperature [137, 140, 142]. Such response has been associated with the level at which both intracellular and extracellular pathogens are able to cause a disease [140, 143].

1.5.1 Bacterial metabolome

The bacterial metabolome is comprised of a large number of diverse metabolites that are produced or consumed as a result of biological activity and invariably could convey critical information about metabolic changes over the lifetime of an organism [144-150]. The diverse metabolites are the low molecular weight chemical products of enzyme-catalysed reactions, which are highly important for the central biological processes. They are reported to function as a direct link between the different pathways that operate within a living cell [151] whose major role is to provide energy and building blocks for bacterial cell growth and division. Therefore, approaches that accelerate measurement of metabolite levels directly from cells and tissues are important and should increase our understanding of the diverse roles of metabolites and potentially lead to discovery of novel metabolites and metabolic pathways as previously reported [152]. For *C. difficile*, this is very important, especially when biological functions are to be quantified with respect to the observed pathogenicity associated with the infection. An identification of the pathways

and metabolites that serve as signals for repression of toxin production and metabolic switching would possibly provide therapeutic avenues to interfere with toxin expression in *C. difficile*.

1.5.2 Analytical technique

Mass spectrometers are increasingly being used for profiling of intracellular and extracellular metabolites associated with microbial activity [148, 153-156]. This analytical technique has been applied in the identification and quantification of very complex, biological mixtures and compounds ionized directly from liquid into gaseous phase [157-159]. As previously reported, different metabolites (or molecules) can be identified or quantified based on molecular mass or weight [160, 161]. The molecular mass of the metabolite of interest as well as its physical and chemical properties can be determined respectively by elemental composition, arrangement and types of atoms, using ionization techniques as previously reported [157, 161]. Considering that different metabolites are sometimes present in complex mixtures (such as the defined media used in this study) and the fact that the same mass value may be shared by more than one metabolite (especially the metabolites of interest), a separation technique described as secondary separation system may be required [157, 162]. Separation techniques such as gas chromatography-mass spectrometry (GC-MS) and liquid chromatography-mass spectrometry (LC-MS) are the most commonly used [146, 151, 163-173]. The core of this technique is liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS), which has become an increasingly relevant tool to investigate metabolic changes in microorganisms [139, 152, 174, 175]. These techniques are ideally suited for measurement of large numbers of metabolites from microbial extracts due to their selectivity, sensitivity, reproducibility and high resolution in a relatively short time [151, 159, 176-182]. A more detailed description of the liquid chromatography-mass spectrometry technique is provided in chapter two.

1.5.2.1 Analytical approaches

Two distinct categories or approaches are currently available for LC-MS analysis and include, the qualitative approach, which can identify large numbers of metabolites from a single analysis using high resolution mass spectrometry [159, 179, 183, 184]. The second approach is quantitative, using triple quadrupole mass spectrometry to measure known metabolites of interest with high sensitivity and high resolution in the multiple reaction monitoring (MRM) mode [180, 185, 186]. This study focused on the quantitative analysis of spent *Clostridium difficile* media using MRM mode to measure amino acids that have been utilised by *Clostridium difficile* strains and if so, the order in which they have been utilised.

1.5.3 Data analysis

This stage of analysis has been described as being very fundamental in generating good quality data [187]. Raw data generally need to be converted into an appropriate format for data analysis. This is because, raw data obtained in the mass spectrometer typically represent a series of full scan mass spectra acquired at a given time point, consisting of mass-to-charge (m/z), time, and intensity triplets that describe the strength of the ion beam for every detected ion mass. Processing and interpreting these data correctly is very challenging due to a number of factors including diversity [187]. However, the availability of modern software packages containing automated algorithms for integrating peaks has made it possible to convert data into a standard and uniform format, which could also be exported into Word/Excel processing packages or spreadsheets as previously reported [187-190].

1.6 Clinical Importance of the Study

The rate and severity of CDI has been reported to be on the increase even as it is now a major cause of nosocomial diarrhoea worldwide [11, 191, 192]. This is in spite of several interventions put in place towards the prevention and

eradication of the *C. difficile* infection. Some of the attributing factors as earlier mentioned include the indiscriminate use of broad-spectrum antibiotics, the organism's ability to produce spores, and the emergence of hyper virulent antibiotic-resistant strains with ability to produce greater quantities of toxins [7, 20, 22-24, 193-195]. This is of great concern, hence the urgent need to develop novel therapies to tackle *C. difficile* infection. Most of the findings have concentrated on understanding disease pathogenesis, diagnosis and therapeutics [196]. *C. difficile* toxin production appears to be greatly influenced by nutritional factors present in the medium [197-200]. However, not much is known about the precise role of nutritional and metabolic factors in the control of this menace. The survival of most pathogenic organisms has been associated with their adaption to the environment [137, 142] and for *C. difficile*, this is very important, with respect to the observed pathogenicity. Therefore, a thorough understanding of the mechanisms underlying their metabolism and survival is important and may eventually aid in the successful diagnosis of the disease using the mass spectrometry platforms as previously reported [153, 159, 165, 179, 201-203].

1.7 General Aims and Objectives

This study seeks to understand the importance of amino acids for the survival of *C. difficile* in the gut through the functional application of mass spectrometry-based metabolomics platform. The specific objectives were;

- To establish an LC-MS/MS platform capable of quantitative determination of a large number of metabolites in clostridial pathogens.
- To establish defined culture conditions that allow reproducible growth.
- To measure the extracellular metabolome using the established LC-MS/MS method in order to determine possible changes and if so, the order in which the changes occurred.

CHAPTER 2.

DEVELOPMENT OF LIQUID CHROMATOGRAPHY – TANDEM MASS SPECTROMETRY METHODOLOGY FOR THE STUDY OF AMINO ACIDS UTILIZATION IN CLOSTRIDIAL PATHOGENS

2 DEVELOPMENT OF LIQUID CHROMATOGRAPHY – TANDEM MASS SPECTROMETRY METHODOLOGY FOR THE STUDY OF AMINO ACIDS UTILIZATION IN CLOSTRIDIAL PATHOGENS

2.1 Introduction

2.1.1 LC-MS methods for amino acids profiling

The choice of analytical platform is largely dependent on the type of analyte, with gas chromatography- mass spectrometry (GC-MS) most suitable for volatile and semi-volatile analytes, capillary electrophoresis (CE) most suitable for charged analytes and liquid chromatography-mass spectrometry (LC-MS) most suitable for polar and non-volatile analytes. Other platforms have been developed, including separation by high performance liquid chromatography (HPLC), nuclear magnetic resonance spectroscopy (NMR), thin layer chromatography (TLC) [146, 151, 163-173]. The MS platforms in particular, are frequently being used to provide sensitive and reproducible detection of hundreds to thousands of metabolites in known and unknown samples. The analytical capacity of the MS is further extended by the retention ability of LC, which helps to overcome common problems encountered in direct MS analysis like ion suppression and isobaric complications [204-206].

Once metabolites have been extracted from samples, analysis consists of separation, identification and possibly quantitation, which requires a high throughput tool capable of identifying the structure of complex mixtures simultaneously [207]. For the detection of metabolites in complex biological samples, previous reports have shown that LC coupled to electrospray-ionization MS (LC-ESI-MS) is the most favoured. C-18 columns on LC-ESI-MS reversed-phase mode are considered more appropriate in the separation of semi-polar compounds such as flavonoids, alkaloids, phenolic acids, glycosylated steroids and other glycosylated species [158]. Other studies have shown that polar compounds such as amino acids, sugars, nucleotides, vitamins and carboxylic acids would best be separated using hydrophilic

interaction liquid chromatography (HILIC) [181, 208, 209]. The normal-phase liquid chromatography (NPLC) has also been reported to be an option for separating polar compounds method [181], but the use of non-polar organic mobile phases makes it more compatible with atmospheric pressure chemical ionization coupled with mass spectrometry (APCI-MS) instead of ESI-MS. Similarly, NPLC-APCI-MS could be applied for the analysis of non-polar lipids such as triacylglycerols, sterols and fatty-acid [210].

A number of literature reviews has shown preference in the use of liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) in amino acid analysis, due to its selectivity, sensitivity, reproducibility and high resolution in a relatively short time [151, 153, 159, 165, 176-180, 201-203]. Though the widespread use of LC-MS/MS for amino acid profiling is recent, there has been a continuing increase in its application in various fields of endeavours. In the biological sciences for instance, LC-MS/MS has been applied to the understanding of gene function [153, 154], while in clinical sciences it has become a powerful approach for clinical diagnosis. Currently it is attracting more scientific attention from multiple fields, ranging from genetics, pharmaceuticals, food science, environmental analysis to biochemistry [211-215] and advanced microbiological research. Table 2-1, gives a representative overview of LC-MS methods for amino acids profiling developed over the years. The ionisation method presented in the table varies with the type of sample that is being analysed with majority using Electrospray Ionisation (ESI). Also shown are some of the available mass analysers currently in use, some of which include quadrupoles, time-of-flight analysers and quadrupole time of flight. Quadrupole analysers, whether configured as single, triple or four quadrupole, are widely used in clinical biochemistry LC-MS applications due to the ease of scanning and the quality data gotten [216]. The quadrupole consists of four cylindrical rods, set parallel to each other, responsible for filtering sample ions, based on their mass-to-charge ratio (m/z). The transmission of a narrow band of m/z values along the axis of the rods is made possible via a constant and varying (radio frequency) voltages. Changing the voltages with time as previously described will enable scanning across a range of m/z values, resulting in a mass spectrum [216]. For pharmacokinetics,

environmental studies, drug metabolism and biological analyses, the triple quadrupole mass spectrometer has been reported to yield lower detection and quantitation limits as well as allowing for increased sensitivity [217].

Table 2-1: A representative overview of LC-MS methods for amino acids profiling developed over the years.

Analytical method	Analytical conditions	Aim	Sample	Mass spectrometer	References
LC-MS/MS	C-18 column (Perkin-Elmer, 220 mm x 4.6 mm x 5 mm). Mobile phase (A) water with 0.1% (v/v) formic acid and (B) methanol/water (50:50) 0.1% (v/v) formic acid	Understanding the interaction between free amino acids and physiological processes	Seed of <i>Amaranthus</i>	Triple quadrupole	[218]
LC-MS/MS	C-18 column (3.5 mm, 2.16 x 150 mm) Mobile phase - 0.01% formic acid in water-acetonitrile (90:10) at a flow rate of 0.3 ml/min	Diabetes risk assessment	Serum	Triple quadrupole	[219]
ESI-TOFMS	XDB-C18 column (2.1 x 50mm) with a 1.8 mM particle size (Agilent Technologies) Mobile phase (A). 0.5mM tridecafluoroheptanoic acid (TDFHA) in water, (B). 100% acetonitrile.	Quantification of free amino acids	Plasma	Time of flight	[220]
UPLC-qTOF-MS	Waters ACQUITY UPLC BEH amide column (1.7 µm, 2.1 mm x 100 mm) with a VanGuard HSS T3 (1.8 µm). Mobile phase: (A). water, 10 mM ammonium formate, + 0.15 % formic acid (B). Acetonitrile, 1 mM ammonium formate, + 0.15 % formic acid.	Dietary intervention study	Urine	Quadrupole time of flight	[221]
HPLC-MS/MS	C-18 (3.5 µm x 2.1 mm x 100 mm, Waters). Mobile phase: (A). 1 mM perfluoroheptanoic acid in water (B). 100% Acetonitrile	Assessment of metabolic phenotypes	Amino acids	Quadrupole	[222]
UPLC-ESI-MS/MS	C-18 (1.7 µm x 2.1 mm x 100 mm, Waters) Mobile phase: (A).1 mM perfluoroheptanoic acid in H ₂ O (B). 100% Acetonitrile	Detection and quantification of low abundance amino acids in complex samples	Blood, Plant leaf (<i>Arabidopsis thaliana</i>)	Quadrupole	[223]
LC-MS/MS	Primesep (5 µm x 2.1 mm x 100 mm). Mobile phase: (A). 0.3 mL/L trifluoroacetic acid in 0.3 L/L aqueous acetonitrile (B). 4.5 mL/L trifluoroacetic acid in 0.3 L/L aqueous acetonitrile	To certify analytes concentration	Amino acids	Triple quadrupole	[224]
HILIC-MS/MS	ZIC-HILIC column (3.5 µm x 4.6 mm x 150 mm). Mobile phase: (A). 98 % v/v water, 1 % v/v acetonitrile, 1 % v/v formic acid (B). 98 % v/v acetonitrile, 1 % v/v water, 1 % formic acid)	Analysis of underivatised amino acids	Amino acids	Triple quadrupole	[225]
HILIC-ESI-MS	ZIC-HILIC column (5 µm x 2.1 mm x 250 mm) Mobile phase: (A). 10 mM acetic acid in water (B). 100 % Acetonitrile	Accurate quantification of underivatised amino acids from hydrolysed peptide/protein	Protein hydrolysates	Single quadrupole	[226]

Analytical method	Analytical conditions	Aim	Sample	Mass spectrometer	References
HPLC-ESI-MS/MS	C-18 column (5 μ m x 2.1 mm x 50 mm). Mobile phase: (A). 0.1 % TDFHA in water (B). 0.1 % TDFHA in acetonitrile	Metabolic disorder	Dry blood, Plasma, Urine	Quadrupole	[227]
LC-MRM-MS	Zorbax SB-Aq column (4.6 mm x 250 mm). Mobile phase: (A). 0.1 % formic acid in water (B). 0.1 % formic acid in 90 % acetonitrile/9.9 % water	Measurement of hydroxyproline after acid hydrolysis	Animal tissue	Quadrupole	[228]
LC-ESI-MS/MS	C-18 column (2.1 mm i.d.x100 mm, 3.5 μ m; Waters)	Synthesis and application of a new derivatization reagent,	Amino acids	Triple quadrupole	[229]
(RP) LC-ESI-MS/MS	Mobile phase: Acetonitrile-water	Diagnosis and management of inherited disorders of amino acid metabolism	Plasma, Urine	Triple quadrupole	[230]
LC-MS/MS	Mobile phase: Acetonitrile-water	Screening for disorders in fatty acid, organic acid, & amino acid metabolism	Dried blood	Four quadrupole	[231]
NP-LC-MS/MS	CN normal phase column. Mobile phase: water/acetonitrile/trifluoroacetic acid, flow rate of 1 ml/min.	Diagnosis of metabolic disease	Plasma, Dried blood spot	Quadrupole	[232]
LC-ESI-MS/MS	C(18) capillary column	To search for chemical signatures of life in future planetary explorations	Terrestrial crust	Quadrupole	[233]
LC-MS/MS	Devalosil C30-UG-5 (5 μ m x 4.6 mm x 250mm). Mobile phase: (A). 0.2% acetic acid in water (B). 0.2% acetic acid in acetonitrile	Metabolic flux analysis	Fed-batch culture of <i>E. coli</i>	Quadrupole	[234]
LC-ESI-MS/MS	Teicoplanin-based chiral stationary phase	Simultaneous chiral separations of underivatized amino acids	Amino acids	Quadrupole	[235]
LC-MS	Purospher RP-18e or a Supelcosil ABZ+Plus column Mobile phase: Acetonitrile-water	Determination of 20 underivatized proteinic amino acids	Amino acids	Quadrupole	[236]
LC-ESI-MS/MS	Strong cation exchange (SCX) column (Luna 5 μ m SCX 100 Å) with Mobile Phase: (A) 30 mM ammonium acetate in water; (B)5% acetic acid in water	Determination of proteinogenic and ¹⁵ N-labeled amino acids.	Plant	Quadrupole	[237]
LC-MS/MS	Column 1-porous graphitic carbon column, 3 μ m Hypercarb, 4.6 mm ID x 50 mm); Column 2-fused-core (Advanced Materials Technology, 2.7 μ m Halo C18, 2.1 mm ID x 100 mm). Mobile phase: (A).1 mM TDFHA in water, (B). 1 mM TDFHA in 100% acetonitrile	Diagnosing and monitoring patients with inherited metabolic disorders	Plasma, urine, and cerebrospinal fluid	Quadrupole	[238]

Despite all these advances, including current applications in microbial research to understanding the metabolic as well as systems behaviours of important microorganisms [144, 155, 212, 239], not much has been done to determine importance of amino acids for the survival of *C. difficile* in the gut using the LC-MS/MS platform. In most bacterial cells, amino acids play a very crucial role in biological processes, such as protein synthesis and in primary and secondary metabolic pathways [163, 240, 241]. As previously reported, the synthetic, metabolic and signalling pathways of the individual cells within the organisms can be activated in response to the prevailing environmental conditions in order to benefit the whole organism [242]. Therefore, a thorough understanding of these metabolic changes is essential and would involve the identification and quantification of the compounds in the metabolome of bacterial cells in different states [138]. Such analysis forms the centre of modern efforts to define critical functions of biological systems and the subsequent identification of relevant metabolites, which in a way has continued to generate interest in practically every aspect of life [163, 164, 223, 240, 241, 243]. A proper analysis and interpretation of LC-MS results may reveal potential metabolite functions within the organism that sometimes are not detected by other approaches [144, 153, 207, 244]. Furthermore, it should enable the understanding of metabolic pathways driven by numerous biological mechanisms in a variety of contexts of pathological and physiological activities in response to diverse stimuli such as disease, drug action, gene modification, toxicity and environmental changes [153, 159].

2.1.2 Application of amino acid profiling in microorganisms

Amino acids, sugars, organic acids and a number of other molecules or metabolites are present and actively involved in the life processes of most living organisms. As previously described, they are building blocks for many other biological components (for example proteins, RNA, DNA and cell walls), central in intermediary metabolism, providing many necessities for life (for example ATP for energy release) and also playing an active role in regulation and signalling [174, 245]. They are also seen to play a very important role in connecting the different pathways that operate within a living cell [151] and their composition conveys the relationship of the metabolic changes over the lifetime of an organism [144-146] which is also a

reflection of enzymatic pathways and networks encoded within the genome [144]. Since microorganisms possess the capacity to modify their metabolic composition due to prevailing circumstance [138] it is therefore imperative to determine the probable changes that may be related to genetic differences, disease, antimicrobials or environmental influences via a MS platform as previously reported [174]. Available literature has described the use of C-18 columns in either reversed-phase or hydrophilic interaction liquid chromatography or normal phase chromatography methods for analysis of amino acids. In most cases, amino acids were either derivatised or underivatised, but using ion-pairing agents [218, 220, 229-232, 246-249]. The analytical conditions were similar as majority of them used methanol or acetonitrile with formic or acetic acid to control pH of the mobile phase. A range of these MS platforms will be described in detail.

2.1.2.1 Reversed Phase High-Performance Liquid Chromatography Mass Spectrometry (RP-HPLC-MS)

RP-HPLC-MS is capable of moderate to high throughput analysis with a reasonable dynamic range, and separating molecules on the basis of their hydrophobicity [250-252]. Hydrophobic binding of the solute molecule enhances separation from the mobile phase to the immobilized hydrophobic ligands attached to the stationary phase and is suitable for compounds of medium and low polarity [250, 251]. Firstly, the solute mixture is applied to the sorbent in the presence of aqueous buffers, and the addition of organic solvent to the mobile phase allows or brings about the elution of the solutes. Here solutes would elute in order of increasing molecular hydrophobicity if concentration of organic solvent is kept constant (isocratic method), or by increasing the amount of organic solvent over time (gradient elution) [250]. C-18 is the most commonly employed column in RP-HPLC-MS analysis and is composed of a non-polar stationary phase, typically silica, chemically modified by a derivatised silane bearing an n-alkyl hydrophobic ligand [250, 253]. The more polar compounds such as amino acids and some lipids are very difficult to retain and often require the use of graphitized carbon columns, pre-column derivatization or ion-pairing agents [222, 223, 229, 254-256]. Previous report has shown the use of derivatization on reversed-phase liquid chromatography for the analysis of 19

naturally occurring amino acids, detected in fingerprints of 20 donors [257] and the profiling of free amino acid in grain amaranth [218].

2.1.2.2 Hydrophilic Interaction Liquid Chromatography (HILIC)

The HILIC method, was first introduced for the separation of compounds such as peptides, nucleic acids, and amino acids by Alpert [208], later for drug research by Strege [258] and provides an alternative to effectively separating small polar compounds on polar stationary phases [259]. HILIC uses a polar stationary phase analogous to normal phase chromatography and aqueous/polar organic solvents [260]. The aqueous rich sub layer formed adsorbs to the polar surface of the stationary phase into which analytes partition. Up till now, the retention mechanism in this method are still being debated, but is thought to be a combination of hydrophilic partitioning interaction, and secondary electrostatic and hydrogen bonding mechanisms [261]. In contrast to the order in which analytes elute from a reversed phase column, separation of analytes occurs based on analyte polarity and degree of solvation [208, 258, 261]. The mobile phase employed is highly organic in nature, typically acetonitrile [262] though other solvents such as methanol, dichloromethane, isopropanol and ethanol have equally been used [263-266]. The type of column used is dependent on the application (non-bonded silica or polar-bonded silica) and includes aminopropylamide-, cyanopropyl-, poly (succinimide)-, diol-, sulfoalkylbetane silica phases. Previous reports have shown their successful applications in microbial studies, especially for separations of polar compounds in complex matrices, small organic acids, carbohydrates [262, 267] peptides [268, 269], polar pharmaceuticals [265, 270], basic drugs, and many other neutral and charged substances [259].

2.1.2.3 Aqueous Normal Phase Chromatography (ANPC)

This describes the combination of effects occurring as a result of the interactions that take place because of the aqueous nature of the mobile phase and those that are due to a decreasing retention as the mobile phase becomes more non-polar [271]. The term was introduced to account for the behaviour observed with silica-hydrate stationary phases in displaying a dual mechanism and is often used synonymously and interchangeably with hydrophilic interaction chromatography as it relates to the

retention of polar compounds [271]. The retention behaviour in ANPC is comparable to that found in normal phase chromatography due to the addition of water as part of the binary solvent in the mobile phase [272]. Very interesting aspects of the separation performance, is the possibility for both polar and non-polar compounds to have reasonable retention over [271, 272]. Most commonly used columns are made up of silica-hydrate materials that are especially advantageous for analysis of biological samples, including amino acids due to their excellent retention capabilities for hydrophilic and hydrophobic compounds [273]. Depending on the analytes of interest, the mobile phase can be either organic solvent-rich or water-rich in composition [273]. Previous studies on endogenous metabolites in biological matrices have shown the use of ANPC in either the normal-phase or reversed-phase modes, with a special feature of retaining both polar and nonpolar compounds simultaneously [254, 271, 273-276].

2.1.3 Liquid chromatography – tandem mass spectrometry

Tandem mass spectrometric detection (usually referred to as MS/MS) may be applied for identification, quantification and mass analysis of a very wide range of compounds ranging from small molecules to peptides and proteins [181, 182]. Detection and quantification can be achieved using electrospray ionization (ESI) in multiple reaction monitoring (MRM) mode to monitor transitions of the protonated molecules to their specific product ions [222]. It uses two or more mass analysers to analyse individual compounds in a mixture.

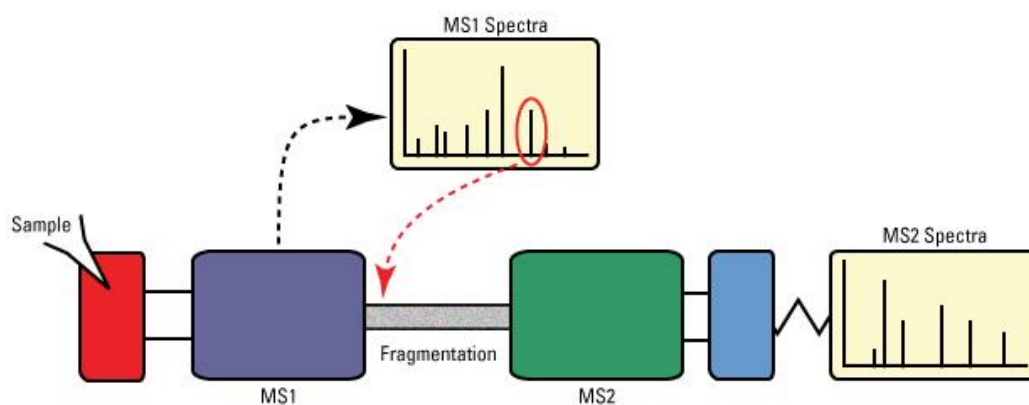


Figure 2-1: Diagram of tandem mass spectrometry (MS/MS).

A sample is injected into the mass spectrometer, ionized, accelerated and analysed by mass spectrometry (MS1). Ions from the MS1 spectra are then selectively fragmented and analysed by a second stage of mass spectrometry (MS2) to generate the spectra for the ion fragments. Adapted without modification [277].

As shown in the Figure 2-1, the sample is ionized in the source (electrospray ionisation, ESI or an atmospheric pressure chemical ionisation source, APCI) and the selected ion undergoes fragmentation as it passes through the first mass filtering device (MS1) into the collision chamber and into the second mass filtering device (MS2) and then the detector (also known as electron multiplier). Here, the product ions may be detected and in so doing, the identity and structure of analytes from unknown samples can be confirmed [278]. LC-MS/MS could be performed on instruments such as ion trap, time of flight, Fourier transform, but previous reports indicate that the triple quadrupole is the most frequently used mass spectrometer for MS/MS probably due to cost and ease of use. Besides from the selective (or multiple) reaction monitoring, previous report shows that LC-MS/MS could be used within the limits of an instrument's sensitivity and mass resolution, to perform other different kinds of experiments namely full scan, product ion scan, precursor ion scan, neutral loss scan [278] and presented in Figure 2-2 - Figure 2-6.

2.1.3.1 Full Scan

Here the user is able to see all ions that are present in a sample. As shown in Figure 2-2, a full scan is performed in part or across the entire mass range of the first and second filters (Q1 & 3), with no collision gas in Q2.

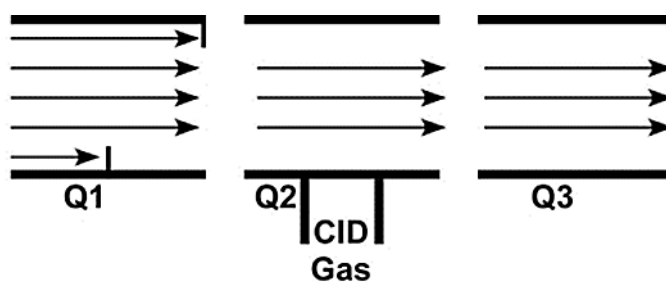


Figure 2-2: Scanning Q1 and Q3 with no collision gas in Q2.

2.1.3.2 Product Ion Scan

Here one specific ion (m/Q) is selected and then Q2 is filled with low pressure gas for collision-induced dissociation of the selected ion to be scan in part or across the entire mass range of Q3 as shown in Figure 2-3. In this case, the user is able to see all fragment and/or productions of the selected precursor ion.

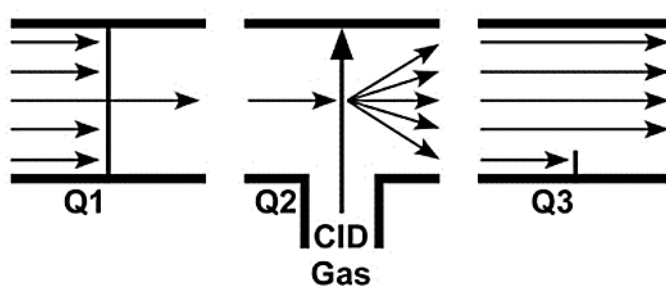


Figure 2-3: Q1 is fixed and scanning Q3, collision gas present in Q2.

2.1.3.3 Precursor Ion Scan

The precursor ion scan is performed in part or across the entire mass range of Q1, while Q2 is filled with collision gas to enable the fragmentation of all ions in the scan range, which is then followed by the selection of one specific m/Q in Q3 (Figure 2-4). This scan enables the user to determine which m/Q precursor ion(s) might have given rise to the selected product ion.

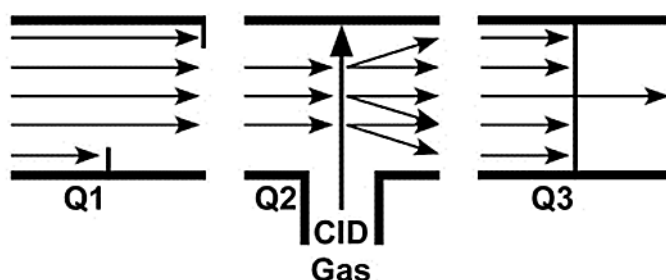


Figure 2-4: Scanning performed in part or across Q1 and Q3 while collision gas in Q2 is fixed.

2.1.3.4 Neutral Loss Scan

Neutral loss scan enables all precursors that have lost a selected common chemical group to be easily identified. As shown in Figure 2-5 below, scan is performed in part or across the entire mass range of Q1, with collision gas in Q2 to enable the fragmentation of all ions in the scan range, which is followed by the scanning of Q3, but in this case across a predetermined range.

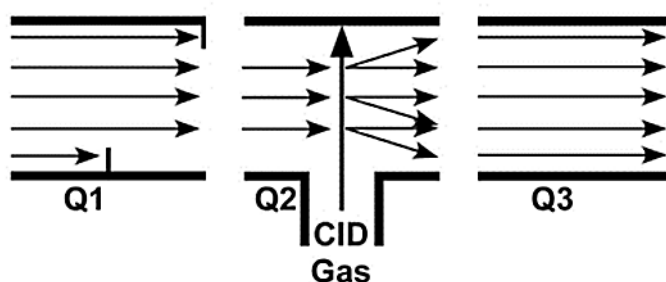


Figure 2-5: Scanning performed in part or across Q1 and Q3 while Q2 is filled with collision gas.

2.1.3.5 Selective (or Multiple) Reaction Monitoring (SRM or MRM)

The SRM or MRM are widely used for mass spectrometric quantitation. SRM or MRM scan allows fragmented ions to be monitored and quantified in the mist of very complicated matrix [279]. Scan is performed by selecting a specific m/Q in Q1, while filling Q2 with collision gas in order to fragment the selected m/Q . The fragmented ion, which should be specific, is then selected in Q3 (Figure 2-6).

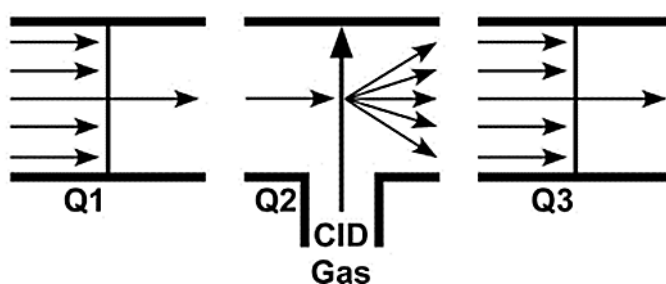


Figure 2-6: Q1 and Q3 are fixed while Q2 is filled with collision gas.

2.1.4 Analytical strategy

2.1.4.1 Extraction

Previous report has described the extraction process as the most difficult and time consuming step towards analysing different metabolites of different molecular type [151, 280, 281]. Here, analytes from complex matrices will have to be brought into a format that is compatible with the analytical procedure to be employed, whilst eliminating matrix components, which usually cause interference in chromatography. A proper sample preparation procedure should separate unwanted components (such as phospholipids, salts, proteins, nucleic acids, sugars, etc.) from sample matrix, provide the target analyte(s) in solution and at a concentration appropriate for detection or measurement in very low concentration [187, 189, 190]. Some of the sample preparation methods stated in the literature include; liquid-liquid extraction, solid-phase extraction, direct injection, supercritical fluid extraction, ultracentrifugation, microwave-assisted extraction, protein precipitation and accelerated solvent extraction.

For the analysis of microbial cells, both the intra and extracellular metabolites would need to be measured. Depending on type of sample and the metabolites of greatest interest, intracellular metabolites could be extracted using organic solvent, such as ethanol or methanol, methanol-water mixtures, acetonitrile:methanol:water mixtures [213, 281-288]. The extracellular metabolites on the other hand, would require only centrifugation to separate cells from the culture media [280]. Often times the analysis of free amino acids in biological samples will require protein precipitation (with acid or organic solvent) and ultrafiltration prior to analysis. In addition, there may be a need for the addition of internal standard and sample clean up in order to decrease assay variability [187, 189, 190]. Thereafter, a homogenous / representative sample is taken and maintained at the appropriate condition which ensures that samples do not degrade before analysis [187, 189, 190].

2.1.4.2 Quenching

Another very crucial step in the analysis of microbial cell extract is quenching. This is due to a number of factors including high exchange rate, size of metabolites of

interest and their sensitivity to variation in the cell environment [177, 282, 283, 289, 290]. In this regard, an ideal quenching method should involve the immediate stoppage of all enzymatic activities without necessarily changing the cell environment. This step as previously reported must be fast, efficient and reproducible in order to measure cellular metabolites and allow direct comparisons in a large sample set [177]. Methods that have previously been adapted for quenching in microbial cells include; quenching with cold methanol, maintaining the sample temperature below -20°C, centrifugation prior to extraction [290-296] and quenching of the cells through fast heating [292].

2.1.4.3 Chromatographic methods

Interfering compounds from analytes can be separated via an improved chromatography. For instance, a reduced analysis time, improved quality of the separation and the detection limit could be achieved by adopting the gradient method. Here, the composition of the mobile phase is varied during separation bringing about an increase in eluent strength. Also, the retention, selectivity and sensitivity of the separation can be affected if the analytes are ionizable [182]. For high throughput analysis, previous report has suggested pretreatment of samples to reduce matrix effect caused by fast gradient LC, which reduces chromatographic separation between analytes and endogenous compounds. Other options of improving chromatography are derivatisation, ion-pairing agents or the use of a stationary phase with a different selectivity, such as HILIC, which combines the use of bare silica or polar bonded stationary phases and mobile phases with a high content of organic solvents, for the analysis of polar compounds in biological samples [246, 247, 297]. Speed, resolution and sensitivity of the method may also be improved upon by using columns with small diameter particles and high pressures in ultra-performance liquid chromatography technology [182].

2.1.4.4 Quantitative LC-MS/MS

The selective quantification of specific analytes of interest in very complex mixtures can be enhanced by the high specificity and sensitivity of Multiple Reaction Monitoring (MRM), which allows for identification and quantification. For the development of a specific LC-MS/MS method, this will require a careful

examination of the physical and chemical characteristics of the analytes of interest, and optimisation of experimental conditions where the analytes are measured [298]. Such knowledge whether coming from literature review or practical experience would be useful for the possible estimation of the requirements for each experiment and the specific precautions to be taken throughout the entire method development, validation and analysis stage. For the bioanalysis of unstable small molecules like amino acids, their instability may not be readily determined, except necessary stability assessment has been conducted using quality control samples against freshly prepared calibration standards [298]. As previously reported, sample collection, processing, storage, extraction and analysis may affect analytes stability at some points during bioanalytical process, hence the need for strategies that will incorporate specific guidance such as addition of enzyme inhibitors or antioxidants [298].

2.1.4.5 Method validation

This is a way of ensuring confidence in bioanalytical data through the use of specific laboratory investigations to assess the performance of a method and that it is reliable and suitable for the intended analytical applications [299]. For a bioanalytical method, validation should include demonstration of selectivity; accuracy, precision, and recovery; the calibration curve; sensitivity; reproducibility; and stability of analyte in spiked samples [299]. Depending on the type of sample, a full, partial or cross validation may be necessary in order to meet certain criteria for it to be applied in a number of studies.

Calibration Curve and Quality Control - This involves the preparation of calibration solution [300], containing known concentration of sample. The composition of the calibration samples should normally be very similar to that of the actual sample being analysed, whilst the quality control samples are included as part of the analysis or injected throughout an extended analysis time, to monitor assay precision and bias, and to check instrument stability and response [187, 189, 190]. Calibration solution along with the quality control samples should be injected in six replicates into the LC-MS-MS system as previously reported [176, 301]. A linear calibration model is accepted if precision and reproducibility for each concentration is within $\leq 15\%$ of the calculated regression equation [176, 300, 301].

Limit of detection and quantification (LOD/LLOQ) - The LOD and LLOQ can be evaluated by analysing up to five replicates of spiked samples prepared at appropriate concentration. As previously reported the LOD can be assessed by a 3:1 or higher signal to noise ratio for the target ions, and the LLOQ by measuring the lowest concentration level for each of the amino acids six times [300, 302].

Analysis - To demonstrate selectivity, speed or sensitivity, it will be necessary to start by injecting a known solution, with a sample containing internal standard and comparing its response to previous data before the actual commencement of analysis [187, 189, 190]. Carryover can be assessed by injecting blank samples during the start of the run, in between the run and after the highest calibration standards as previously reported [300, 302].

Performance Monitoring and Practical Consideration - Since many factors could affect the performance of an LC-MS system [216], some practical measures may be taken to check the sensitivity and integrity of the instrument. They include injecting known concentration of the internal standard in triplicate and visually checking plot of the response, peak shape and retention time to detect decreases in sensitivity.

2.1.5 Justification of the study

Although, a variety of LC-MS method have been developed to separate, detect and quantify amino acids in bacterial samples [222, 223, 240] they would often times require some form of pre or post column derivatization procedures or analysis without derivatisation but using ion-pairing agents in order to reduce polarity, and make the analytes amenable to retention and separation [164, 222, 240]. There is not yet an underivatized LC-MS/MS method (without ion-pairing agents) available for amino acid profiling in *Clostridium difficile*. This could possibly be due to high water solubility, range of ionic characteristics, non-availability of a universal and selective chromophore that would enable the simultaneous separation of amino acids in a large sample set [222, 303], coupled with specific growth requirements of this bacterium [197]. As a result, there is limited literature available on the use of LC-MS/MS for the analysis of amino acids in this bacterium.

Previous literature reports have shown that the use of ion-pairing agents, pre or post column derivatization have some kind of drawback such as complete evaporation of solvent before derivatization, which may interfere with the quality of data. This could be due to instability of derivatives and/or the inconsistency in yields of the derivatised amino acids, longer analysis time, low analyte recovery and increased impurities in samples [254, 304]. In most cases, a manual derivatisation may be required and this could result in a more complicated analytical system [164, 305-307]. Additionally, ion-pairing may only be achieved using mobile phase components that are sometimes not compatible with detection by mass spectrometry [254]. This limitation has reduced the widespread use of this method for biological samples, due to the presence of heterogeneous matrices [308].

Although the HILIC method has been shown to offer an alternative choice to the retention and separation of polar compounds, including amino acids, the method is not without its drawbacks. There is the likelihood of not providing effective separation of samples containing a mixture of both hydrophilic and hydrophobic analytes as previously reported [271, 309], high levels of salts or buffer requirements [272]. Since the chromatographic behavior here is typically the opposite of that obtained with the reversed phase method, there is also the challenge of deciding which types of solute need to be retained [271].

Based on previous report, there is no doubt that the quantification of underderivatised amino acids would be more challenging. Nevertheless, detection methods not requiring the use of ion-pairing agents or derivatisation of amino acids are most desirable, not only for simplicity or convenience or accuracy, but to avoid the challenges that are associated with the use of ion-pairing agents or derivatisation procedures [240]. The aqueous normal phase chromatography represents an important new technology to achieving this feat. The capabilities of the silica-hydride-base stationary phases in ANPC, have been reported to offer a distinct advantage for practical application with a high degree of reproducibility and long-term stability [272, 310]. These advantages have been demonstrated through a number of studies using the right gradient, mobile phase and modifier [271, 272, 311]. This methodology should therefore increase our understanding of the dynamics in *C. difficile* metabolism and pathways as previously reported [175, 179].

2.1.6 Aims and objectives

The focus in this chapter was to establish an LC-MS/MS amino acid profiling platform that is fast and reliable and apply this platform to analyse spent microbiological media in order to provide clear resolution of the analytes and demonstrate its suitability for future application. This would be achieved by;

- Optimising LC gradient conditions using amino acids standards
- Exploring the retention capabilities of a reversed phase column and/or an aqueous normal phase chromatography to simultaneously separate amino acids mixtures using compatible mobile phase.
- Adapting the established LC-MS method to analyse a spent *C. difficile* medium.
- Optimizing mass spectrometer for MRM analysis using amino acids standards.

2.2 Materials and Methods

2.2.1 Reagents and materials

All twenty amino acids (L-alanine, L-arginine, L-asparagine, L-aspartic acid, L-glutamine, L-glutamic acid, glycine, L-histidine, L-isoleucine, L-leucine, L-lysine, L-methionine, L-phenylalanine, L-proline, L-serine, L-threonine, L-tryptophan, L-tyrosine, L-valine, L-cysteine), norleucine, acetonitrile containing 0.1 % formic acid, water containing 0.1 % formic acid were sourced from Sigma-Aldrich (Gillingham, UK). All chemicals and reagents listed above were of analytical grades and used with minimum handling to avoid possible contamination.

2.2.2 Columns

2.2.2.1 C-18

Silica Type: Luna 5 μ C18 (2) - 3 μ m, 100Å; dimensions, 2 x 150 mm; sourced from Phenomenex, Macclesfield, Cheshire, UK

2.2.2.2 Cogent Diamond Hydride

Silica Type: Cogent Diamond Hydride - 4 μ m, 100Å; dimensions, 2.1 x 150 mm; sourced from MicroSolv Technology Corporation, Eatontown, USA. Both columns were equilibrated prior to use with mobile phase according to manufacturer's recommendation.

2.2.3 Sample preparation

2.2.3.1 Amino acid standards, internal standards, calibration solution and quality control samples

100 mM solutions for each amino acid and their mixtures were prepared by dissolving in LC-MS chomasolv water containing 0.1 % formic acid and filtered using 0.45 μ m membrane filters. Aspartic acid, glutamic acid, tryptophan and tyrosine were the exceptions for which lower concentrations were made as they are not very soluble. Thereafter 1 ml amounts of the individual amino acid and their

mixtures were transferred separately into sterile 2 ml vials, sealed, labelled and maintained at – 20 °C and brought to room temperature before use.

10 mM concentration of the internal standard was prepared by dissolving norleucine in acetonitrile:water (50:50, v/v), stored at – 20°C and brought to room temperature before use. A working solution containing 5 µM norleucine (internal standard) was prepared with acetonitrile:water (50:50, v/v). Thereafter, the working solution was used to make the calibration solutions by diluting the amino acids stock solutions at concentration levels of 1 µM, 2 µM, 4 µM, 6 µM, 8 µM, 10 µM. The quality control samples were set to fall in the low (1 µM), middle (6 µM) and high regions (10 µM) of the standard solution.

2.2.3.2 Preliminary sample for microbiological experiment

An initial experiment to test the LC-MS/MS method was performed with an overnight culture of *C. difficile* 630 wild type, inoculated 1:100 in to sterile Brain Heart Infusion, yeast extract and L-cysteine (BHIS) media supplemented with *C. difficile* selective supplement (cycloserine and cefoxitine), in triplicate [197]. After 2 hours of anaerobic growth at 37 °C, one set of cultures were moved to 5 % oxygen conditions in the hypox cabinet set at 37 °C. Samples were taken after 48 hours; cells were spun down by centrifugation at 13,200 rpm for one minute. The resulting supernatants were then filter sterilised through 0.2 µm filters and stored at -20 °C until ready for use.

Four volumes of cold methanol amounting to 800µl were added to 200 µl volumes of each the filtered sterilized samples in eppendorf tubes and the mix was vortexed for 30 seconds. The samples were spun down at 14000 x g at 4 °C for 10 minutes using the Harrier 18/80 centrifuge. The supernatants were transferred into another set of eppendorf tubes without disturbing the precipitate, then diluted with 200 µl cold methanol and speed vacuum dried at 0 °C in the Jouan centrifugal evaporator. The samples were reconstituted to 200 µl volumes with 90 % water / 10 % acetonitrile mixture. Thereafter, 50 µl of each sample were dispensed into 2 ml vials ready for LC/MS analysis.

2.2.4 Liquid chromatography

2.2.4.1 Preliminary method development on C-18 and Cogent Diamond Hydride columns

Initial LC analysis was performed on two different columns listed in sub-section 2.2.2. The procedure was a modification of an established method reported in the literature to determine the most appropriate condition for this experiment [272, 312, 313]. The initial optimisation was performed using a high performance benchtop LC-MS mass spectrometer - the Exactive powered by Orbitrap™ technology, equipped with electrospray ionisation source operating simultaneously in the negative and positive ionisation modes. The instrument's data are controlled and processed by Thermo Scientific XCalibur software. Following several repeats of the process, a final optimized mobile phase consisting of [A] 100 % water with 0.1 % v/v formic acid, and [B] 100 % acetonitrile with 0.1 % v/v formic acid was obtained. The optimised ionization source settings were as follows; mass range of m/z between 50-1,000, spray voltage at 3.00 kV, skimmer voltage of 20 V and capillary voltage set at 27.50 V. The heater temperature and capillary temperatures were set at 300 °C and 350 °C respectively. The condition and performance parameters of the columns used are presented below:

Using a reversed phase (RP), C18 column an isocratic elution was performed at different time lag of 30, 20, 10, 7 and 5 minutes respectively. The percentage of the organic solvent (B) varied with each run starting from 5 % and changing to 100% or vice versa. A flow rate of 0.25 ml/min, was initially chosen and then changing to 0.3 ml/min with a constant injection volume of 5 μ l. Similarly, the composition of the mobile phase was varied (gradient elution) at an initial flow rate of 0.25 ml/min, changing to 0.3 ml/min with injection volume of 5 μ l. Several and continuous repetitions of the run conditions were performed, after 30, 15, 10, 5 minutes and followed by further testing and adjustments to obtain the final gradient conditions shown in Table 2-2.

Table 2-2: Final gradient condition on silica type – Luna 5 μ C18 (2) - 3 μ m, 100 Å; dimensions, 2 x 150 mm.

Time (min)	A (%)	B (%)	Flow Rate (mL/min)
0	100	0	0.3
5	80	20	0.3
10	0	100	0.3
13	0	100	0.3
14	100	0	0.3
16	100	0	0.3

A = 100 % water containing 0.1 % formic acid; B = 100 % acetonitrile containing 0.1 % formic acid.

Aqueous normal phase (ANP) using cogent diamond hydride column - an isocratic run from 0% to 100% [B] were performed with the run time spanning between 3 & 30 minutes at a flow rate of 0.4 and then to 0.6 ml/min with an injection volume of 5 μ l. As shown in Table 2-3, the gradient conditions were obtained after several testing and adjustment of the run conditions.

Table 2-3: Final gradient conditions on silica type - cogent diamond hydride, 4 μ m, 100Å; dimensions, 2.1 x 150 mm.

Time (min)	A (%)	B (%)	Flow Rate (mL/min)
0	0	100	0.6
2	0	100	0.6
3	10	90	0.6
6	10	90	0.6
7	25	75	0.6
13	25	75	0.6
15	60	40	0.6
17	60	40	0.6
21	0	100	0.6
22	0	100	0.6

A = 100 % water containing 0.1 % formic acid; B = 100 % acetonitrile containing 0.1 % formic acid.

2.2.5 Method application for the analysis of *C. difficile* spent medium

To further explore the retention capabilities of the columns as well as the method, a complex *Clostridium difficile* medium was analysed on LC-MS system. The extracted culture medium was injected in 5µl amounts onto the LC-MS system and eluted using the same conditions stated in subsection 2.2.4. In this analysis, all metabolites were measured, not just amino acids, and a direct comparison was made between C18 and cogent diamond hydride columns.

2.2.6 Targeted profiling by LC-MS/MS

In this experiment, the option was to use the cogent diamond hydride column only (in view of its advantage over the C18 as shown in the result) and to improve upon the method to separate polar compounds also as previously discussed. Using the same LC conditions described in subsection 2.2.4, the experiment was carried out on a Micromass Quattro Ultima, high performance benchtop triple quadrupole mass spectrometer designed for routine LC-MS/MS operation. Quattro Ultima is coupled to a HPLC system with or without an autosampler, an infusion pump, a syringe pump, mass detector, column oven, ion source using MassLynx version 4.1 chromatographic data system. As previously described, targeted profiling is intended to identify and quantify specific compounds of interest [314, 315], which was accomplished using a Multiple Reaction Monitoring (MRM) scan to identify all target analytes. Since MRM scans are extremely sensitive and selective, this allows fragmented ions to be monitored and quantified in the midst of very complicated matrix [279]. Analysis was performed using cogent diamond hydride with the gradient conditions and mobile phase previously described in subsection 2.2.4.

2.2.6.1 Final chromatographic optimisation

Having achieved good separation on the Exactive (subsection 2.2.4) it was necessary to further optimize the chromatographic parameter in order to assess the sensitivity of the instrument described in subsection 2.2.6, and its ability to achieve the desired limit of detection, high resolution and efficiency. Series of standards were injected at

different times (intra-batch) and days (inter-batch) to ensure validity of the method. As previously reported, optimization was carried out using the standards prepared for the standard curve [316]. Separation was realised using gradient elution previously described in subsection 2.2.4 at a flow rate of 0.6 mL/min with an injection volume of 5 μ l.

2.2.6.2 Optimisation of the mass spectrometer for MRM analysis

After the optimisation of the LC, described in the previous section, optimisation of the mass spectrometer settings for MRM analysis was also required. Previous reports have shown that assay performance and optimal conditions are most times dependent on the type of instrument used as well as the analyte itself [216]. Tuning was therefore performed in order to optimise the response for the individual amino acids (ions), whose values could vary with the type of instrument. As previously reported, the tuning parameters which could affect signal quality are source temperature, nebulization gas, desolvation gas, collision gas, ion path potentials, collision energy, mobile phase flow rate, ionization voltages, which were adjusted to correspond with analyte concentration and response [317]. Here a constant stream of each analyte (prepared in the same mobile phase used for the LC separation) was introduced into the ion source using a syringe infusion pump as previously reported [187, 317]. During the continuous infusion of the analytes, the ion parameters and collision energy for each of the analyte were optimized. The best ionization mode was determined with the best informative fragment being chosen [187, 216] and presented in Table 2-4.

2.2.6.3 Mass spectrometry conditions

The focus here was to adjust those parameters which could affect ion transfer, fragmentation in the collision cell and mass analyser as well as ionization and desolvation as previously reported [187]. The optimised MRM-MS conditions for the target profiling experiment are as presented below; polarity - ES⁺, capillary (kV) - 3.55, cone (V) - 35, RF lens 1- 16.9, aperture (V) - 0.4, RF lens 2 - 0.2, source temperature (°C) - 125, desolvation temperature (°C) - 350, cone gas flow (L/Hr) - 189, desolvation gas flow (L/Hr) - 627, LM 1 resolution - 12.5, HM 1 resolution -

12.9, ion energy 1 - 1.1, entrance – 3, collision – 20, Exit – 12, LM 2 resolution - 12.5, HM 2 resolution - 12.5, ion energy 2 - 1.7, multiplier (V) - 650, collision cell pressure (mbar) - 2.26e-3, flow (µl/min) – 600, stop time (minutes) - 22.0, min pressure (bar) – 0, max pressure (bar) – 400, oven temperature left(°C) - 40.0, oven temperature right(°C) - 40.0. Instrument was operated with a positive ion electrospray, with simultaneous acquisition of full scan data using the parameters fine-tuned by MassLynx.

Table 2-4: MRM transitions, optimized source cone voltages and collision cell voltages for LC–MS/MS analysis of twenty amino acids used.

Compounds	Parent ions (m/z)	Daughter ions (m/z)	Cone Voltage (V)	Collision Energy (eV)	*T _R (min)	Quantification ion pair (m/z)
Alanine	90.15	44	35	10	9.94	90.15>44
Arginine	175	70.18	35	25	16.03	175>70.18
Asparagine	133.1	74	35	15	10.09	133.1>74
Aspartic acid	134.13	74.12	35	14	9.34	134.13>74.12
Cysteine	122.13	76	35	12	9.37	122.12>76
Glutamine	147.08	130	35	10	10.26	147.08>130
Glutamic acid	148	84	35	15	9.49	148>84
Glycine	76.05	30.3	35	10	10.03	76.05>30.3
Histidine	156	110.18	35	12	15.88	156>110.18
Isoleucine	132	86.01	35	10	9.46	132>86.01
Leucine	132	86.14	35	10	9.46	132>86.14
Lysine	147.15	84.16	35	15	16.17	147.15>84.16
Methionine	150	104.04	35	12	9.40	150>104.04
Phenylalanine	166	120	35	15	9.26	166.0>120
Proline	116	70.09	35	12	10.14	116>70.09
Serine	106.11	88	35	12	9.83	106.11>88
Threonine	120.15	74	35	12	9.77	120.15>74
Tryptophan	205.1	188	35	10	9.23	205.1>188
Tyrosine	182.25	136.2	35	12	9.29	182.25>136.2
Valine	118.19	72.2	35	12	9.69	118.19>72.2

*T_R = Retention Time

2.2.7 Method validation

2.2.7.1 Calibration curve

Here linearity was tested by assessing signal responses of target analytes as previously described [318]. Linearity was shown in the range of 1 μM – 10 μM using linear calibration function fit curve and weighting method with offset $1/X^2$ [300, 319]. The ratio of peak area of amino acids standards to that of internal standard was plotted against the applied concentrations of the amino acids. Calibration solution along with the quality control samples (presented in subsection 2.2.3.1), were injected in six replicates into the LC-MS-MS system as previously reported [176, 301]. A linear calibration model was accepted if precision and reproducibility for each concentration were within $\leq 15\%$ of the calculated regression equation [176, 300, 301].

2.2.7.2 Limit of detection and quantification (LOD/LLOQ)

The LOD and LLOQ samples were prepared by diluting the amino acids stock solutions presented in subsection 2.2.3 above to concentration levels of 1 pM, 5 pM, 10 pM, 1 nM, 2 nM, 4 nM, 6 nM, 8 nM, 1 μM , 2 μM , 4 μM , 6 μM , 8 μM , and 10 μM . To assess the LOD, a 3:1 or higher signal to noise ratio was set for the target ions, while the LLOQ was assessed by measuring the lowest concentration level for each of the amino acids six times as previously reported [300, 302]. Acceptable precision of the LOD and LLOQ was set at $\leq 20\%$ [176] for the point at which the instrument deviates from acceptable performance.

2.2.7.3 Precision

A one way analysis of variance was used to evaluate the precision of the method and this was achieved by calculating the percentage deviation of the quality control samples injected in six replicates. The closeness of the data obtained for individual amino acid measured to the multiple samples were determined and calculated as relative standard deviation (RSD). Previous report indicates that it should normally not exceed $> 20\%$ at LLOQ or $> 15\%$ at higher concentration [320, 321]. Quality control samples were used to evaluate inter-batch and intra-day precision and also

provided the bases for rejection or acceptance of the results. The criterion for accepting the result is that at least 67 % of quality control samples should be within RSD of 15 %.

2.2.7.4 Carryover

This was determined by injecting blank samples during the start of the run, in between the runs after the highest calibration standards as previously reported [300, 302].

2.2.7.5 Quality control

The following practical measures were taken to check the sensitivity and integrity of the instrument;

- injecting known concentration of the internal standard in triplicate
- including quality control samples as part of the analysis
- injecting quality control samples throughout an extended analysis time, to monitor assay precision and bias, and check instrument stability and response
- visually checking plot of the response, peak shape and retention time to detect decreases in sensitivity.

2.2.8 Data analysis

Raw data acquired from the instruments were exported onto the group database (kingfisher) and pre-processed to convert data into a standard and uniform format using MassLynx V4.1. The processed data were thereafter exported onto excel spreadsheets as previously reported [187-190] to generate calibration curves from which the interpolated concentrations of the individual amino acids were determined and thereafter exported onto the Graph Pad Prism 6 for further processing.

2.3 Results and Discussion

2.3.1 Liquid chromatography

The retention capability of the C18 column in the reversed-phase mode and the cogent diamond hydride column in aqueous normal phase were explored for the separation of the mixture of amino acids in the first instance. As noted earlier, we aimed to achieve good resolution, sensitivity and symmetrical peak shape with a possible short run time. Analysis commenced under isocratic conditions and later to gradient elution with several mobile phases being created and experimented upon. The initial LC detection was also performed as described in subsection 2.2.4. This involved altering the proportions and time of mobile phase B as well as instrument parameters until the best retention and well resolved peaks were obtained using acetonitrile (ACN) with 0.1% formic acid (HCOOH) as the organic solvent and water (H₂O) with 0.1% formic acid as the aqueous solvent. An overview of the final RP on C-18 column conditions is presented in Figure 2-7. Here, retention of the amino acids decreased as the amount of organic solvent (acetonitrile) increased. Only the hydrophobic amino acids isoleucine, leucine, phenylalanine, tryptophan and tyrosine were retained on the column and there was little or no separation of the majority of the amino acids (Table 2-5). This is in agreement with previous literature reports indicating that amino acids must be derivatised to enable good separation by reversed phase C-18 columns [247, 322, 323]. The choice of column was informed by prior knowledge on the type of analyte which are oftentimes very useful in setting up the initial experimental conditions as previously reported [263, 324]. Also, our initial decision on the suitability of the mobile phase in particular, was made based on the previous reports showing acetonitrile as the most effective solvent for the separation of polar/semi-polar compounds [208, 263, 267, 324].

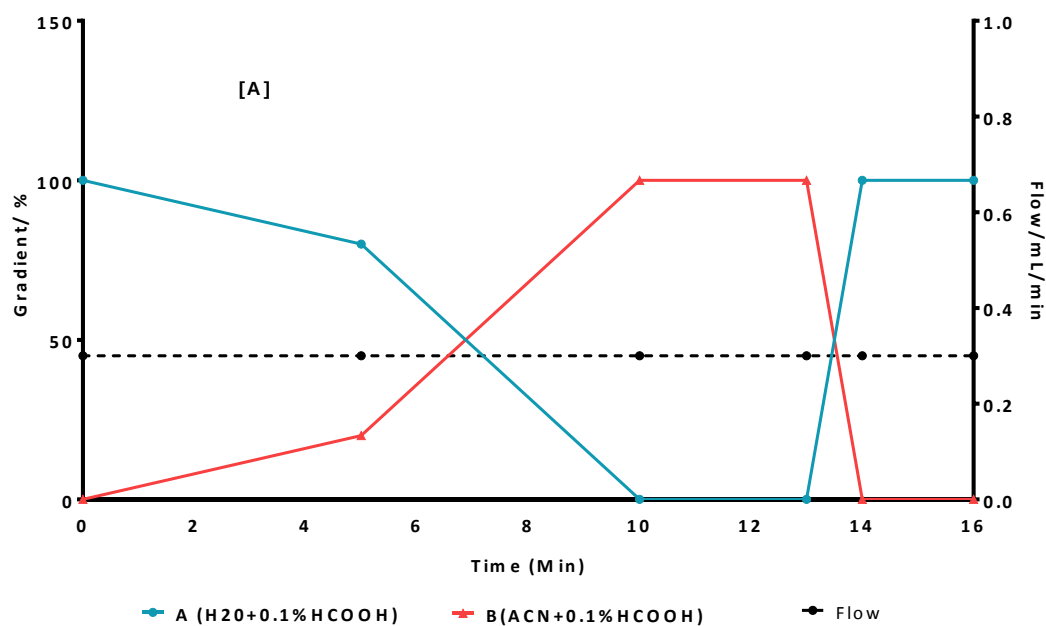


Figure 2-7: Final LC gradient and flow rate on RP C-18 column.

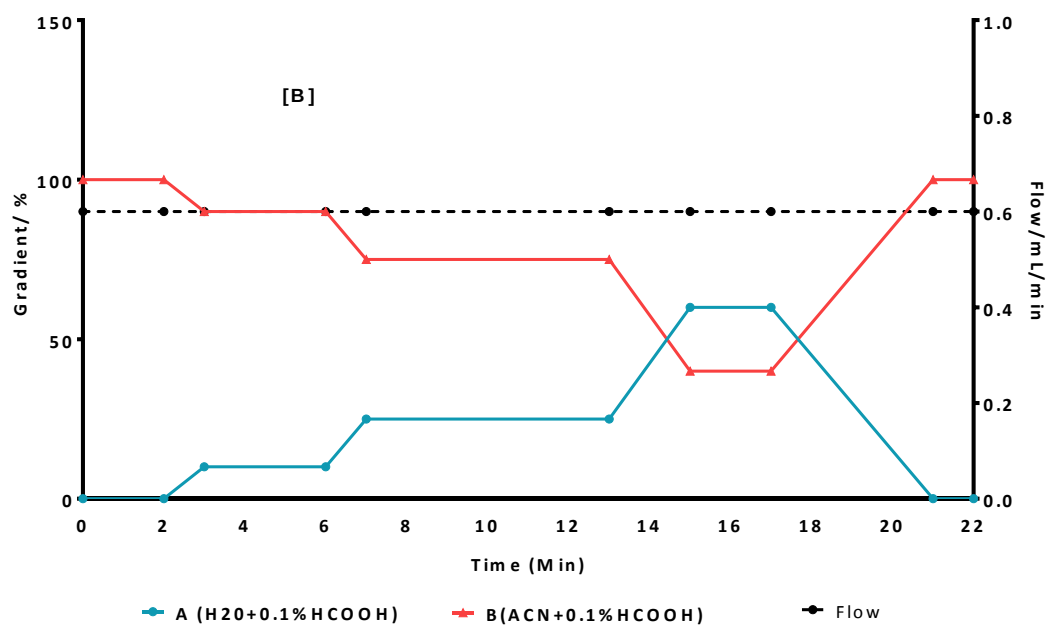


Figure 2-8: Final LC gradient and flow rate on ANP Diamond hydride column.

An overview of the LC gradient using the cogent diamond hydride column in aqueous normal phase mode is presented in Figure 2-8. Retention here decreases (data not shown) as the concentration of organic solvent (acetonitrile) decreases, which is typical of an aqueous mode separation, affirming what has previously been reported [247, 325]. The result of the simultaneous separation of all twenty amino acids is presented in Figure 2-9.

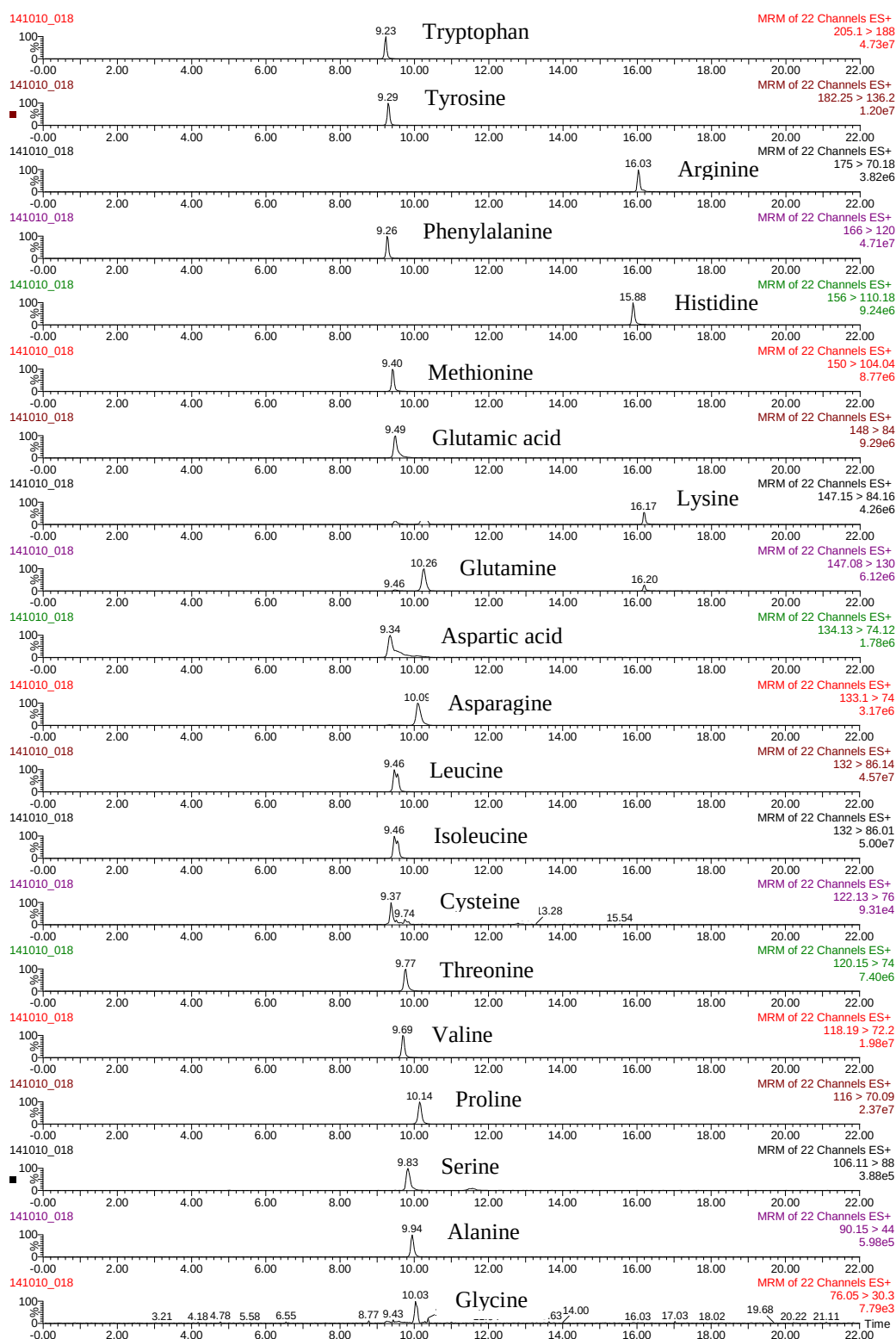


Figure 2-9: Total Ion Chromatogram of twenty amino acids at 10 μM using Cogent Diamond Hydride column.

Ala, RT 9.94 min; Arg, RT 16.03 min; Asn, RT 10.09 min; Asp, RT 9.34 min; Cys, RT 9.37 min; Gln, RT 10.26 min; Glu, RT 9.49 min; Gly, RT 10.03 min; His, RT 15.88 min; Ile, RT 9.46 min; Leu, RT 9.46 min; Lys, RT 16.17 min; Met, RT 9.40 min; Phe, RT 9.26 min; Pro, RT 10.14 min; Ser, RT 9.83 min; Thr, RT 9.77 min; Trp, RT 9.23 min; Tyr, RT 9.29 min; Val, RT 9.69 min.

A brief comparison of the two columns used, showed that it was not possible to simultaneously separate the amino acid mixture on the C-18 column, as only a few of them were selectively detected (Table 2-5). Like many other polar compounds, they are reported to have poor retention and elute near the void volume in the C-18 reversed-phase method and this has continued to pose a great challenge as reported by several authors [323]. As previously discussed, most of the approaches to overcoming this challenge have been to either derivatise, use ion pairing agent or normal phase or hydrophilic interaction chromatography methods [218, 220, 229-232, 246-249]. As shown also in Table 2-5, all twenty amino acids were detected on the cogent diamond hydride column, affirming its ability to separate both polar and non-polar compounds with reasonable retention over a range of organic solvent concentration in the mobile phase as previously reported [271, 272]. From this result therefore, the use of the C-18 column was discontinued due to poor retention of the amino acids.

Table 2-5: Comparison of the retention capability between C-18 and Cogent Diamond Hydride columns.

Amino Acids	C-18 Column	Cogent Diamond Hydride column
Alanine	ND	+
Arginine	ND	+
Asparagine	ND	+
Aspartic acid	ND	+
Cysteine	ND	+
Glutamine	ND	+
Glutamic acid	ND	+
Glycine	ND	+
Histidine	ND	+
Isoleucine	+	+
Leucine	+	+
Lysine	ND	+
Methionine	ND	+
Phenylalanine	+	+
Proline	ND	+
Serine	ND	+
Threonine	ND	+
Tryptophan	+	+
Tyrosine	+	+
Valine	ND	+

+ = Detected, ND = Not Detected

2.3.2 Targeted profiling by LC-MS/MS using the Cogent Diamond Hydride column

2.3.2.1 Chromatographic optimisation

For a satisfactory quantitation of amino acids in their native form, previous reports have stated the importance of completely resolving chromatographic peaks in order to correspond to their isomers, isobars and/or isotopomers [204, 206, 236, 326, 327]. This is in spite of the specificity which the tandem mass spectrometer provides, especially when operated in the MRM mode [204, 206, 236, 326]. Without derivatization or use of ion-pairing agents, the method developed enabled the quantification of all twenty amino acids within a total analysing time of 22 min using the Cogent Diamond Hydride column. Representative results of the twenty amino acids mix with retention time for each of the amino acid extracted from the chromatogram is presented in Figure 2-9. Though there seems to be an overlap amongst the amino acids shown in Figure 2-9 and

Figure 2-10, a majority of the literature reviewed showed very similar fragmentation patterns of the target ions, despite having different chemical structures [204-206]. They are reported to have the same MRM mass transitions due to their isomeric chemical structure and identical molar mass [204, 328]. In previous work, quantification was carried out by chromatographic baseline separation or selection of additional fragment ions for the isomers/isobars. As shown in this work, the isobaric and/or isomeric sets, namely, leucine/isoleucine, glutamine/lysine, glutamine/glutamic acid and asparagine/aspartic acid were very clearly discriminated in our chromatographic method with all the amino acids showing their respective MRM transition (Table 2-4 and Figure 2-9). The results were reproducible, with not more than 5 seconds average variation in the retention times recorded between the different batches analysed (data not shown). This method provided an excellent chromatographic resolution for the clear identification of peaks all the 20 amino acids without derivatization or use of ion-pairing agents as compared with previous methods [204-206].

2.3.2.2 Optimisation of mass spectrometer for MRM analysis

Strong and stable signals of all twenty amino acids were individually observed during optimization of the mass spectrometric parameter. These were in the form of their $[M + H]^+$ molecular ions with mass to charge ratios as presented in Table 2-4. Following on from the previously described method, each of the parent ions was subjected to collision-induced dissociation in order to determine the resulting daughter ions [329]. A considerable response and/or signals were formed giving values for collision energy of between 10 and 25 eV, while cone voltages were however maintained at 35 V. Consequently, the ion transitions with the corresponding ion pairs, and collision energy presented in Table 2-4 were selected for MRM of the amino acids.

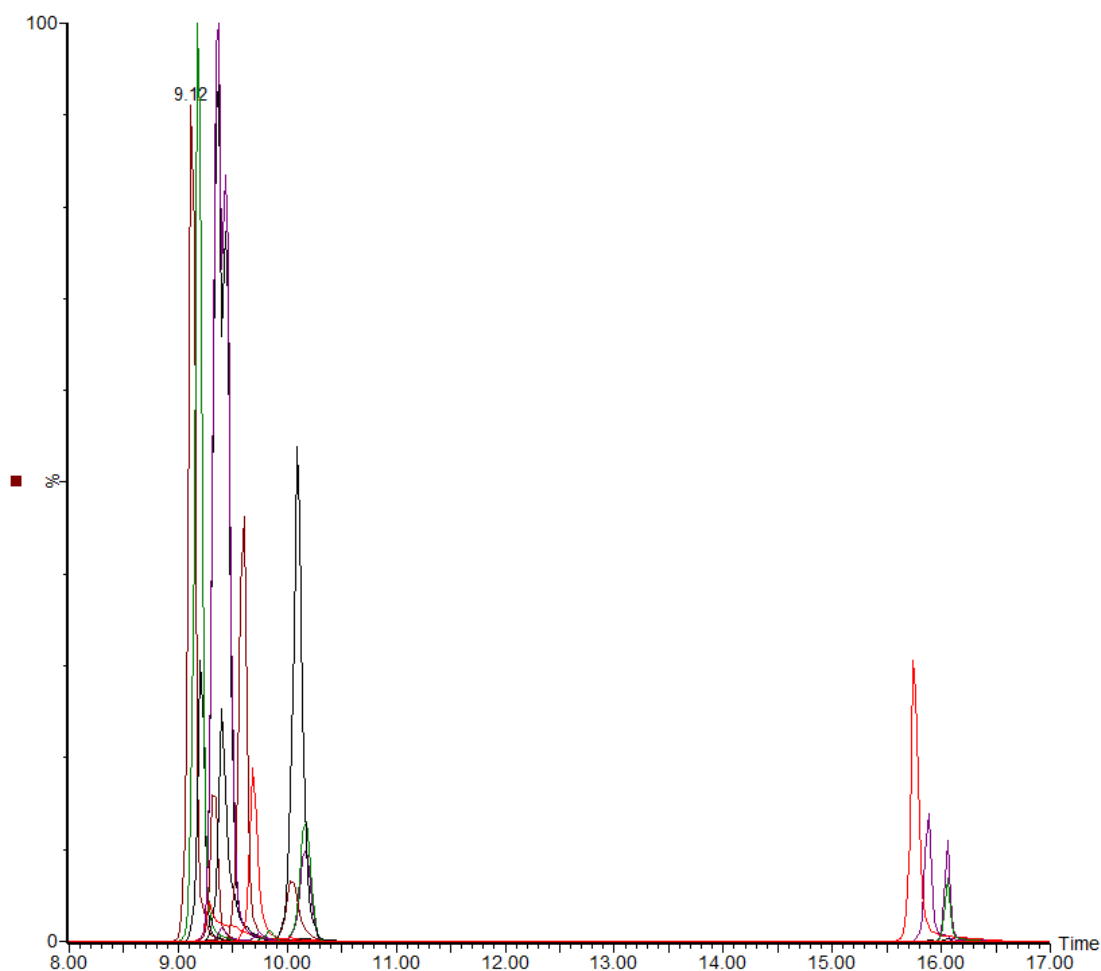


Figure 2-10: Chromatogram showing separation of the twenty amino acids mix by LC-MS/MS in positive ESI mode using the Cogent Diamond Hydride column (4 μm x 2.1 mm x 150 mm).

The x-axis represents retention time (mins) and the y-axis represents relative abundance (%). Mobile phase: [A] 100 % water with 0.1 % v/v formic acid, and [B] 100 % acetonitrile with 0.1 % v/v formic acid. Total run time was 22 min at a constant flow rate of 0.6 mL/min with an injection volume of 5 μl .

2.3.3 Method validation

The performance of the LC-MS/MS method for the analysis of twenty amino acids was evaluated by measuring linearity (regression coefficient and dynamic range), sensitivity (detection limits) and precision (relative standard deviation) as previously reported [204, 299].

2.3.3.1 Calibration curve

Linearity was shown in the concentration range of 1 μM – 10 μM with the correlation coefficients (R^2) of all twenty amino acids, except lysine above 0.99 (Table 2-6). As noted previously in subsection 2.3.2.1, not more than 5 seconds average variation in the retention times was recorded between the different batches analysed, indicating good linearity of the curve. A representative plot of the calibration curve showing linearity of the method as well as regression equations (y) are presented in Figure 2-11 and Table 2-6 respectively.

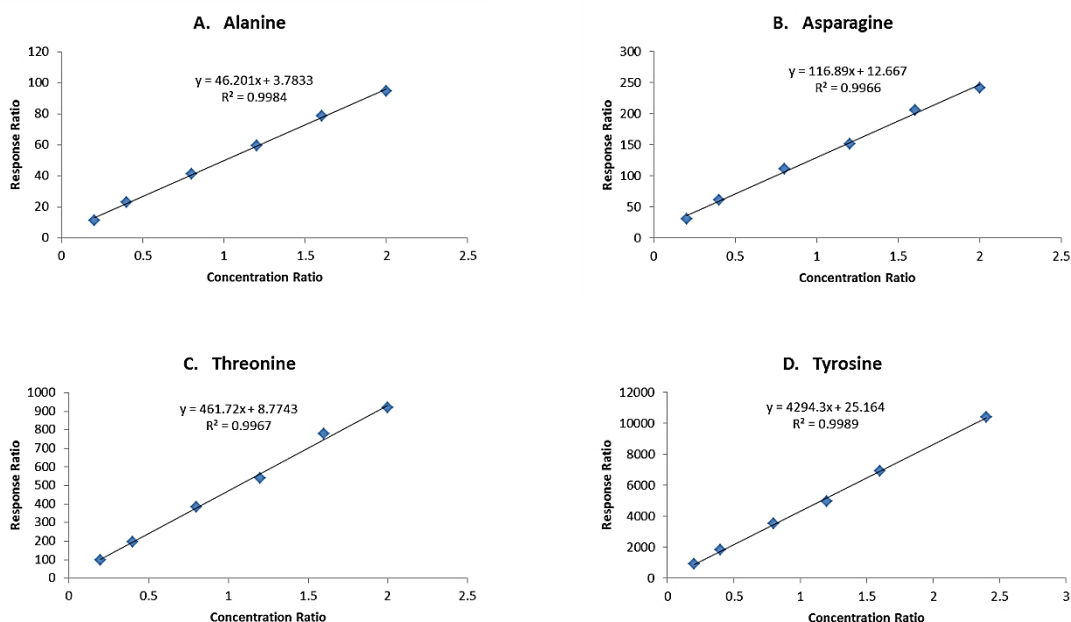


Figure 2-11: Representative plot of the calibration curve showing linearity.

A) Alanine, B) Asparagine C) Threonine D) Tyrosine. Response ratio on the y-axis represents peak area divided by internal standard area, while concentration ratio on the x-axis is the concentration of the amino acid standard divided by internal standard area.

Table 2-6: Summary of calibration equation and correlation coefficients (R^2)

Compounds	Calibration	Correlation coefficient (R^2)
Alanine	$y = 46.201x + 3.7833$	0.9984
Arginine	$y = 69.496x - 4.9455$	0.9939
Asparagine	$y = 116.88x + 12.666$	0.9966
Aspartic acid	$y = 71.022x + 3.7203$	0.9969
Cysteine	$y = 18.748x - 2.9468$	0.9940
Glutamine	$y = 62.108x + 17.957$	0.9967
Glutamic acid	$y = 64.661x + 4.3247$	0.9959
Glycine	$y = 8.1513x + 1.0992$	0.9900
Histidine	$y = 48.619x - 1.106$	0.9963
Isoleucine	$y = 1.4653x + 1.1178$	0.9914
Leucine	$y = 1.7319x + 1.0282$	0.9943
Lysine	$y = 43.312x - 2.3057$	0.9898
Methionine	$y = 592.14x - 11.832$	0.9948
Phenylalanine	$y = 472.7x + 0.4972$	0.9915
Proline	$y = 468.54x + 9.8605$	0.9939
Serine	$y = 91.8x + 13.196$	0.9914
Threonine	$y = 461.72x + 8.7743$	0.9967
Tryptophan	$y = 676.91x - 0.8472$	0.9911
Tyrosine	$y = 4288.2x + 25.128$	0.9989
Valine	$y = 295.34x + 20.6$	0.9937

2.3.3.2 Limit of detection (LOD) and quantification (LLOQ)

As previously described, the LOD and LLOQ were assessed in accordance with baseline noise method and the values obtained ranged from 1 pM to 10 μ M (Table 2-7), with the coefficient variation ≤ 15 % (except for glycine), indicating the sensitivity of the method with high reproducibility and excellent chromatography properties.

2.3.3.3 Precision

The result of precision of the method is presented in Table 2-7. As shown in Table 2-7, precision was well within ± 20 % for each amino acid at low and high concentration levels of analytes, indicating high reproducibility and acceptability of the method. Previous report on using 6-aminoquinolyl-n-hydroxysuccinimidyl carbamate derivatisation for amino acids analysis [204] are comparable to those obtained in our study, even though our amino acid samples were not derivatised.

Table 2-7: Precision for low and high concentration levels of analytes

Compounds	Low Concentration levels	Precision (% RSD, n=6)	High Concentration levels	Precision (% RSD, n=6)
Alanine	1 μ M	14.6	10 μ M	5.52
Arginine	1 pM	7.52	10 μ M	15.0
Asparagine	10 pM	2.46	10 μ M	3.72
Aspartic acid	1 pM	5.31	10 μ M	9.51
Cysteine	8 nM	4.86	10 μ M	2.39
Glutamine	1 μ M	11.4	10 μ M	12.3
Glutamic acid	1 pM	0.54	10 μ M	4.41
Glycine	1 μ M	31.4	10 μ M	7.68
Histidine	1 pM	2.23	10 μ M	3.28
Isoleucine	1 pM	1.20	10 μ M	5.95
Leucine	1 pM	1.59	10 μ M	5.17
Lysine	1 pM	6.63	10 μ M	5.14
Methionine	1 nM	1.85	10 μ M	3.08
Phenylalanine	1 pM	0.75	10 μ M	5.42
Proline	1 pM	5.62	10 μ M	9.92
Serine	1 pM	1.93	10 μ M	2.69
Threonine	10 pM	9.39	10 μ M	4.75
Tryptophan	1 pM	2.16	10 μ M	2.46
Tyrosine	1 pM	7.16	10 μ M	2.21
Valine	1 μ M	5.33	10 μ M	7.21

2.4 Conclusions

Method development is an important step in this chapter because of its direct effect on the quality of the raw data as well as the need to separate and identify compounds of interest present in complex mixtures as previously discussed. The whole process follows a series of steps that are often times revisited. As a first step in this process, the retention characteristics of the analytes of interest were determined using a simple gradient and a high retention column, which allowed for selection of the most probable conditions to be adopted. As mentioned earlier on it involves several repetitions, adjustment of the procedures with the view to producing the best retention, resolution of the analyte of interest and then further testing and adjustments in order to obtain a final method.

In line with the goals of this study, a reliable LC-MS/MS platform was developed, capable of quantitative determination of a large number of metabolites using a cogent diamond hydride column and aqueous normal phase chromatography. The advantage of the method in comparison to other available methods is the ability to simultaneously separate a mixture of amino acids without any derivatisation step or use of ion-pairing agent. More so, its ability to clearly discriminate between the isobaric and/or isomeric sets, namely, leucine/isoleucine, glutamine/lysine, glutamine/glutamic acid and asparagine/aspartic acid. The established method was validated and found to be simple, rapid, sensitive and reproducible with an excellent stability of the retention time of calibration standards and samples injected over time without any particular column care. It was successfully applied in the analysis of a complex *C. difficile* spent medium under aerobic and anaerobic conditions. Consequently, the method will be adapted to study amino acid uptake in *C. difficile*, which will be discussed in the subsequent chapters. Hopefully, the outcome of such study should increase our understanding of the dynamics in the bacterial metabolism and possibly provide more information on the importance of amino acids for survival in the gut.

CHAPTER 3.

DEVELOPMENT OF *CLOSTRIDIUM* *DIFFICILE* MEDIA FOR AMINO ACID UPTAKE STUDY

3 DEVELOPMENT OF *CLOSTRIDIUM DIFFICILE* MEDIA FOR AMINO ACID UTILISATION STUDY

3.1 Introduction

Clostridium difficile like all bacteria requires energy and nutrients to build proteins, generate cell structures such as membranes, cell walls and flagella, and drive biochemical processes. Since the organism is heterotrophic [133, 330], it requires sources of organic carbon in addition to nitrogen, phosphorous, iron and a large number of other minerals. Several studies have shown the ability of this bacterium to ferment carbohydrates such as glucose to obtain its carbon and energy and, in the absence of a fermentable carbohydrate, by activating specific pathways and/or enzymes to degrade amino acids (AA) by fermentation processes in order to create ATP [126, 331]. The latter are thought to proceed via a special mechanism known as ‘Stickland reaction’ [126, 332, 333], which involves coupled oxidations and reductions among amino acid pairs [331]. Presently, the carbon and energy metabolism of this bacterium has not been fully understood in spite of its medical importance. Deeper insights may be gained by comprehensive carbon source utilisation studies utilising mass spectrometry based approaches, but such experiments are best performed in well-defined growth media.

3.1.1 Culture media for the propagation of *C. difficile*

Appropriate nutritional and biophysical environments are essential requirements for the propagation of *C. difficile* for any purpose. In the laboratory, the nutritional environment is made available as a culture medium, which can be constituted depending on the need and use of the organism in question. Culture media may be in liquid, semi-solid or solid forms and used for the cultivation, isolation, identification or maintenance of pure cultures of the organism as previously reported [334-336]. *C. difficile* culture media can be classified into the categories of defined minimal or complex media in order to determine the roles of nutritional and metabolic factors in the control of virulence associated traits.

- a) Complete defined medium is composed of pure biochemicals off the shelf, and the exact chemical composition is known. If it provides only the exact nutrients needed by the organism in question, then it is considered to be a minimal

medium. Such media have been reported to be useful in the study of nutritional requirements of microorganisms, for enrichment cultures, elicitation and accumulation of toxins, determination of growth requirements, auxotrophic mutants' characterisation and a wide variety of physiological studies [337-341].

- b) Complex (undefined) media (as opposed to the defined media) usually contain complex materials of biological origin whose exact chemical composition is not determined. The biological materials include, but are not limited to extracts from beef, yeasts, or plants, or digest of proteins. Partially digested protein products called peptones, provides the energy, carbon, nitrogen, and sulfur needed in the media, while organic growth factors and vitamins are provided by yeast or meat extracts. These media may be used for cultivation of bacterial pathogens, fastidious bacteria or organisms that require a large number of growth factors, known or unknown [198, 334].

3.1.2 Study of bacterial growth

The study of bacterial growth seems quite complex due a number factors including the physical and biochemical requirements, the bacterium itself and the possible competition between the bacteria being studied. Nevertheless, the capacity to grow and multiply is the most important criterion for assessing viability [342-344]. A non-viable cell on the other hand, should not be able to increase in size and number even if cultured for a long period of time in a growth supporting medium [342, 344]. As previously described, bacterial growth requires a coordinated synthesis of a range of complex macro and micromolecules, an energy source and essential intermediary metabolites, which must be present in utilizable forms [342]. However, due to a constantly changing physical and chemical environment, most microorganisms can readily modify their metabolism to make the most of the available nutrients [138, 139]. They are able to do so by altering gene expression, which may lead to changes in their structural, and transport proteins, enzymes and possibly the production of toxins. This is of great importance, especially when biological functions are to be quantified as changes in the culture medium may be a direct response to changes in the bacterial metabolism as previously reported [139, 174, 345, 346].

For a bacterial growth study, viable cells are obtained and inoculated into sterile broth and incubated at appropriate temperatures (mostly at 37°C for *C. difficile*) and

times (between 16 and 48 h) [347]. The inoculated cells are expected to utilise the components of the media and increase in size and mass. An increase in cell mass and size will eventually result in cell division and the ensuing increase in cell numbers may be measured using a spectrophotometer [347, 348]. The spectrophotometer measures the amount of light transmitted through the bacterial suspension to a photoelectric cell and the value obtained is recorded as optical density (OD) [349]. OD measurements are based on light scattering, and this depends not only on the number of cells, but also their size, shape and surface structure. If measurements are made at intervals after inoculation, the log of OD measured can be plotted in relation to incubation time and the resulting curve is known as growth curve [350]. The typical curve (for batch cultures under non-limiting conditions) shows the following phases of the growth cycle: lag, exponential, stationary phases and the phase of decline (Figure 3-1), all of which would be useful in determining the dynamics of cell growth [347, 350, 351].

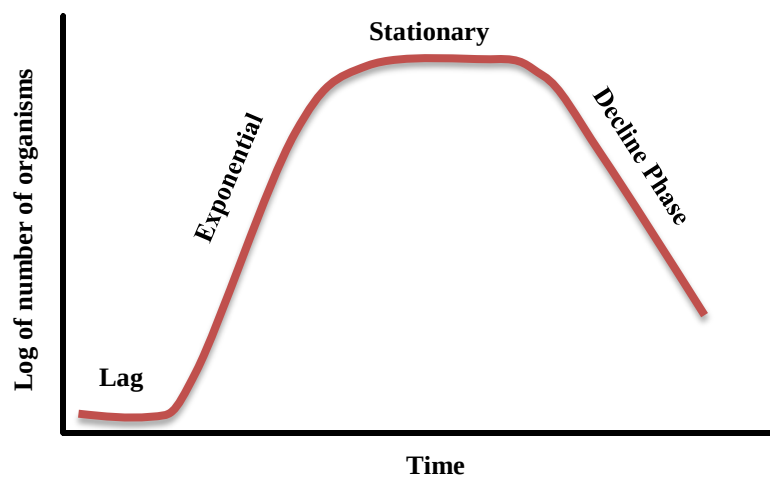


Figure 3-1: A typical bacterial growth curve under selected condition. Adapted with modifications [347, 350, 352].

3.1.3 Justification of the study

Changes in environmental conditions have been reported to play a role in the expression of virulence in most pathogenic bacteria [337]. For *C. difficile*, toxin production appears to be greatly influenced by nutritional factors present in the medium [42, 197, 198, 332]. However, not much is known about the precise role of nutritional and metabolic factors in the control of virulence associated traits.

This could possibly be due to the non-availability of a fully defined medium that would support the growth of a majority of the *Clostridium difficile* strains. The available media which have been used for the cultivation of VPI 10463, KZ 1626, KZ 1630, KZ 1647 and KZ 1748 strains [197] did not include any recently isolated and relevant clinical isolates. Also, the semi-defined *Clostridium difficile* media, developed by Cartman and currently used for the cultivation of the 630 Δ erm strain [353] allow a high level of undesirable background growth in the absence of fermentable carbohydrate. This would interfere with the analysis of alternative carbon and energy sources and the media as described are therefore unsuitable for the determination of *C. difficile*'s nutritional requirements. The development of a defined minimal medium is important in order to ensure reproducibility of studies investigating metabolic changes in this bacterium. Knowledge coming from such experiments should increase our understanding of the diverse roles of metabolites and potentially lead to discovery of novel metabolites and metabolic pathways as previously reported [152].

The starting point for the development of an improved minimal medium was an existing semi-defined *C. difficile* medium containing casein hydrolysate (casamino acids) as the sole source of essential amino acids. In such a minimal medium, we would expect to see very little background growth in the absence of sugars, making it suitable for the analysis of amino acids/Stickland pairs that can be utilised and the fermentation products which are formed. Secondly, a richer but still fully defined medium in which, ideally, all *C. difficile* strains can grow would be desirable. This could be used for experiments where all or selected amino acids are present at increased concentrations to observe the order in which they are being utilised as carbon and energy sources.

3.1.4 Aim

For this study, we were particularly interested in establishing defined culture conditions that, ideally, would be based on mineral salt solutions only containing defined carbon, energy sources and essential amino acids, allowing very little or no background growth in the absence of a carbon and energy source.

3.2 Materials and Methods

3.2.1 Bacterial strains

The *Clostridium difficile* 630 Δ erm strain used in this study was obtained from the Clostridia Research Group (CRG) culture collection at the Centre for Biomolecular Sciences (CBS), University of Nottingham.

3.2.2 Chemicals and reagents

Chemicals and reagents used in this study were Casamino acids, salts (potassium dihydrogen phosphate, sodium hydrogen phosphate, sodium chloride, sodium hydrogen carbonate, trace salts (calcium chloride dihydrate, magnesium chloride hexahydrate, manganese(II) chloride tetrahydrate, ammonium sulphate, cobalt (II) chloride hexahydrate, iron (ferrous sulfate), glucose (D-glucose), vitamins (calcium-D-pantothenate, pyridoxine-hydrochloride, D-biotin), amino acids (already listed in subsection 2.2.1), sourced from Sigma-Aldrich (Gillingham, UK). All chemicals and reagents listed above were of analytical grades and used with minimum handling to avoid possible contamination.

3.2.3 Growth media

3.2.3.1 Modified *Clostridium difficile* medium (MCDM)

The MCDM was based on the recipe described by Steve Cartman [44, 353] with modification in weight (g) per 100 ml of sterile anaerobic dH₂O. This medium was made by mixing appropriate volume(s) of each set of stock solutions (Table 3-1) together with sterile anaerobic distilled water. All stock solutions were either filtered sterilized or autoclaved and maintained in the cold room for a short time in between experiments or kept at -20°C until ready for use.

Table 3-1: Stock solutions for preparation of the modified *C. difficile* medium and its derivatives in weight (g) per 100 ml of dH₂O.
Based on *Clostridium difficile* minimal medium [353] with modifications.

Composition	Weight (g)
*Amino acid solution A [(5X) (filter-sterilised)]	
Casamino acids	5.00
L-Tryptophan	0.25
L-Cysteine	0.25
* Casamino acid solution [(5X) (filter-sterilised)]	
Casamino acid hydrolysate	5.00
*Salt solution 1 [(10X) (autoclaved)]	
KH ₂ PO ₄	0.90
Na ₂ HPO ₄	5.00
NaCl	0.90
NaHCO ₃	5.00
*Salt solution 2 [(100X) (autoclaved)]	
CaCl ₂ •2H ₂ O	0.26
*Salt solution 3 [(100X) (autoclaved)]	
MgCl ₂ • 6H ₂ O	0.20
MnCl ₂ • 4H ₂ O	0.10
(NH ₄) ₂ SO ₄	0.40
CoCl ₂ • 6H ₂ O	0.01
*Glucose solution [(20X) (filter-sterilised)]	
D-Glucose	20.0
*Trace element solution [(SL9) (100X) (filter-sterilised)]	
FeSO ₄ • 7H ₂ O	0.04
*Vitamin solution [(100X) (filter-sterilised)]	
Calcium-D-pantothenate	0.01
Pyridoxine-HCl	0.01
D-Biotin	0.01

Table 3-2: Composition of the Standard medium.
Adapted with modification [353].

Composition	Volume used (in 100ml)
Amino acid solution A (5X)	20 ml
Salt solution 1 (10X)	10 ml
Salt solution 2 (100X)	1 ml
Salt solution 3 (100X)	1 ml
Trace element solution (SL9)	0.2 ml
Glucose solution	5 ml
Vitamin solution (100X)	1 ml
Sterile anaerobic H ₂ O	61.8 ml

The MCDM (which was also the control medium), presented in Table 3-2 was made by mixing appropriate volume(s) of each stock solution (Table 3-1) to generate 100 ml amount. This medium was further modified when required and as described in the Results, by modifying the concentration of amino acids provided and adding additional supplements such as selenate.

3.2.3.2 Brain heart infusion supplemented with L-cysteine (BHIC)

Brain heart infusion (BHI) is a general purpose medium used for the isolation, cultivation, and maintenance of a variety of fastidious and nonfastidious microorganisms [335, 354]. BHI supplemented with L-cysteine (0.1 %; Sigma, United Kingdom) is widely used to obtain good growth and high toxin production of *C. difficile* [197]. BHIC was prepared as described by Mc Gann *et al.* [355].

3.2.4 Inoculum preparation

Starter cultures were grown by streaking *C. difficile* 630 Δ erm from frozen brain heart infusion broth-glycerol stock cultures onto a freshly prepared BHIC agar medium and incubating at 37 °C in an anaerobic workstation (Don Whitley, Yorkshire, United Kingdom) for 24 h as previously described [15]. Three colonies from the BHIC agar plates were inoculated in triplicate into Eppendorf tubes containing 1 ml of modified *C. difficile* medium and grown overnight for 24 h. The overnight culture was then inoculated at a 1 in 100 dilution into 5 ml or 50 ml of the MCDM and/or defined media.

3.2.5 Culture of bacteria

Viable cells of *C. difficile* 630 Δ erm strains as described in sub-section 3.2.4 were inoculated into the MCDM and/or defined media in triplicate and incubated in the anaerobic cabinet at 37 °C. They were incubated from 24 to 48 h maximum to ensure that the final OD of slower growing cultures would be accurately monitored.

3.2.5.1 Sample processing

Two sets of samples were collected at different time points as follows; every 2 h (0, 2, 4, 6, 8, 10, 12, 14, 16); also after 24, 26, 28, 30, 32 and 48 h. The first set was for the determination of optical density performed at 600 nm using a Biomate 3 spectrophotometer. Cultures were diluted for a more accurate measurement if OD₆₀₀ readings exceeded 0.2. The second sets of samples were spun down by centrifugation at 5400 rpm for ten minutes. The resulting supernatants were filtered sterilised into another set of eppendorf tubes using 0.2 µm filters. The supernatants were reconstituted with 50 % water / 50 % acetonitrile mixture and injected into the LC-MS/MS or stored at -20 °C until ready for use as previously reported [133, 356-358].

3.2.6 Data processing/reporting

All experiments reported herein were performed in triplicate and at least three times to ensure reproducibility. Mean OD₆₀₀ values were used to generate graphs (bar chart) in relation to time or the analytes tested using Graphpad Prism 6. Growth curve was obtained by plotting log of OD measured against incubation time. Data were analysed by Analysis of Variance (ANOVA) using Graphpad Prism 6 and data sets obtained are indicated in the respective chart. The standard error of the mean (SEM) was estimated as standard deviation of the error in the sample mean with respect to the true mean [359-362]

3.3 Results and Discussion

Previous studies have shown the importance of using a defined minimal medium for the determination of the nutritional requirements, examination of microbial physiology, toxins accumulation in microbial cells and other traits [337-339]. Here, we attempt to develop a medium that is simple and contains the essential nutrients that support good growth of *C. difficile* 630 Δ *erm* strain at very low concentrations. This medium will be useful for physiological studies to determine (i) amino acid requirements (for protein synthesis), (ii) extent and order of amino acid utilisation (as carbon and energy sources) and (iii) also for future studies on virulence and pathogenesis as previously reported [139, 181, 345].

3.3.1 Growth of *C. difficile* in modified *C. difficile* medium

C. difficile 630 Δ *erm* was grown in MCDM in the presence and absence of glucose to enable the determination of a suitable time point for obtaining final (maximal) optical density readings. These readings were required to determine the suitability of a given medium variant or condition to support *C. difficile* growth. This was achieved by measuring OD₆₀₀ of an actively growing culture (at a wavelength of 600 nm) every 2 hours (0, 2, 4, 6, 8, 10, 12, 14, 16 h); also after 24, 26, 28, 30, 32 and 48 hours. The result presented in Figure 3-2 shows that *C. difficile* 630 Δ *erm* reached maximum growth in the standard medium after approximately 24 h of incubation with an OD₆₀₀ value of 0.60. There is substantial, undesirable background growth in the absence of glucose. It indicates that components present in the casamino acid preparation act as excellent carbon and energy sources. Optimisation of this medium would aim at replacing the casamino acids with defined amino acid mixtures and reducing the amount to reduce background growth in absence of the primary carbon source glucose.

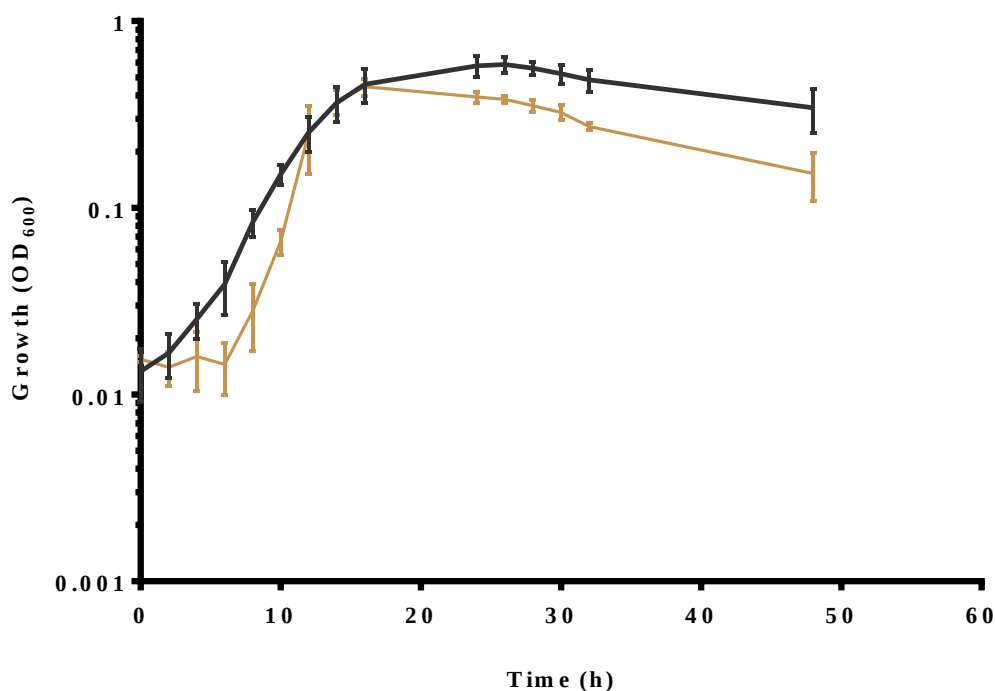


Figure 3-2: Growth of *C. difficile* in MCDM in the presence and absence of glucose. Error bars represent the mean $\pm$ s.e.m. of triplicate experiments, — with glucose, — without glucose.

3.3.2 Effect of reduced casamino acids in the presence and absence of fermentable carbohydrates

Casamino acids act as a convenient amino acids source and are therefore commonly used in studies of the growth requirements of microorganisms [363]. However, when provided in excess they can also promote undesirably high levels of background growth in the absence of the main carbon and energy source. As a first attempt at medium optimisation, we investigated the effect of reducing casamino acid content of the standard medium in the presence of cysteine as a reducing agent. This experiment aimed at obtaining information on the minimal amount of casamino acids required for good growth. The different percentages of casamino acid solution used were (in relation to the original medium) 50%, 25%, 12.5%, 6.25%, 0%, respectively, with the final volume adjusted with water accordingly. As shown in Figure 3-3, the mean OD₆₀₀ value recorded in the presence and absence of glucose, respectively, for each condition was as follows: 0.61, 0.39 (100%); 0.45, 0.20 (50%); 0.33, 0.13 (25%); 0.16, 0.10 (12.5%); 0.12, 0.09 (6.25%); 0.006, 0.002 (0%). Results

obtained were not surprising, as we expected to see a progressive decrease in growth yields, both in the presence and absence of glucose as casamino acids were reduced, confirming the importance of casamino acids in the medium as a source of amino acids for protein synthesis [363] but also their utilisation as alternative carbon and energy sources. The results also show that whilst growth is severely restricted at low casamino acid concentrations, the background growth in the absence of glucose is still considerable, making this medium unsuitable for detailed uptake studies.

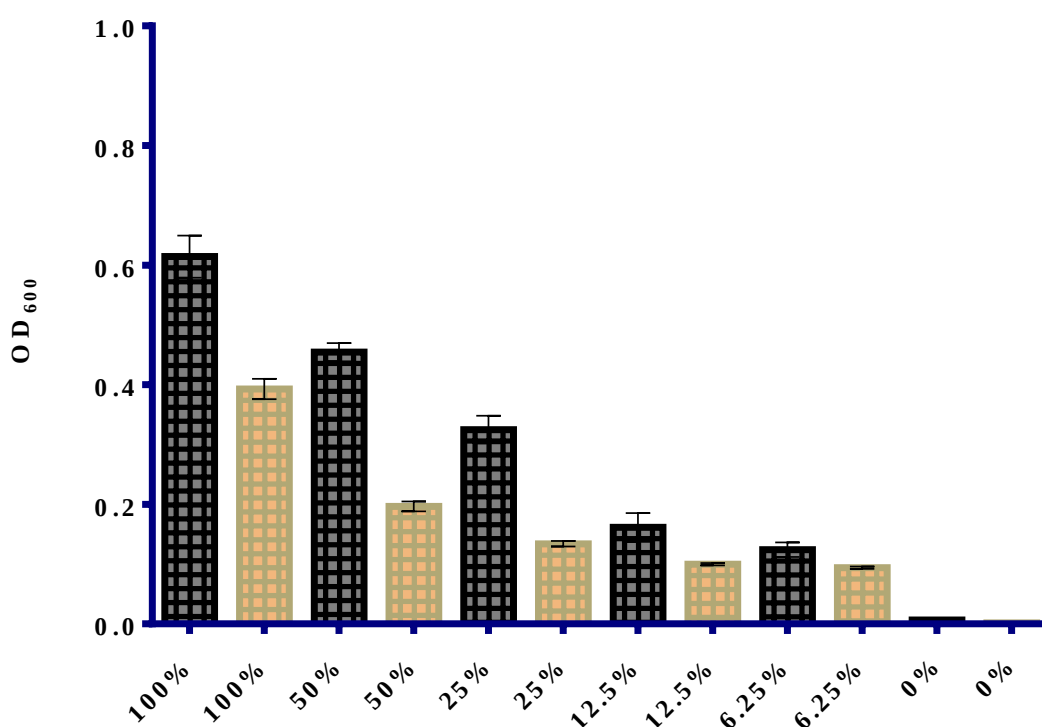


Figure 3-3: Growth of *C. difficile* in MCDM medium variants containing reduced amounts of casamino acids after 24 h of cultivation, in the presence and absence of glucose.

Error bars represent the mean $\pm$ s.e.m. of triplicate experiments, $P < 0.0001$, ■■■ MCDM with glucose, ■■■ MCDM without glucose.

3.3.3 Effect of replacing casamino acids with proteinogenic amino acid mix at concentrations of 0.2 mM and 1 mM in the presence and absence of fermentable carbohydrates.

In a preliminary experiment growth was observed in the absence of casamino acids when the Roswell Park Memorial Institute (RPMI) 1640 medium (used for the culture of human normal and neoplastic leukocytes) was added together with the six essential amino acids although it remained considerably lower than in standard medium (MCDM). Based on this outcome further experiments were conducted with all amino acids (except isoleucine) supplied at concentrations of 0.2 mM and 1 mM in the presence of fermentable carbohydrate. Isoleucine is considered to be essential for *C. difficile* and was therefore set up as a negative control experiment. Initially, all amino acids were provided at a final concentration of 0.2 mM, apart from tyrosine (0.25 mM) and cysteine which also served as a reducing agent and was therefore provided at 4.2 mM [335]. Based on the results obtained for other species [364-366], we anticipated that under these conditions all requirements in terms of protein synthesis would be completely satisfied, whilst the provided amounts would still be too low to support notable background growth in the absence of an alternative carbon and energy source. However, mean OD₆₀₀ values obtained in the presence of glucose after 24 and 48 h incubation were 0.24 and 0.21 and in the absence of glucose 0.11 and 0.08 respectively (Figure 3-4). Thus, growth was considerably reduced even in the presence of glucose, meaning that for at least some amino acids the concentration required to achieve good growth was not provided for.

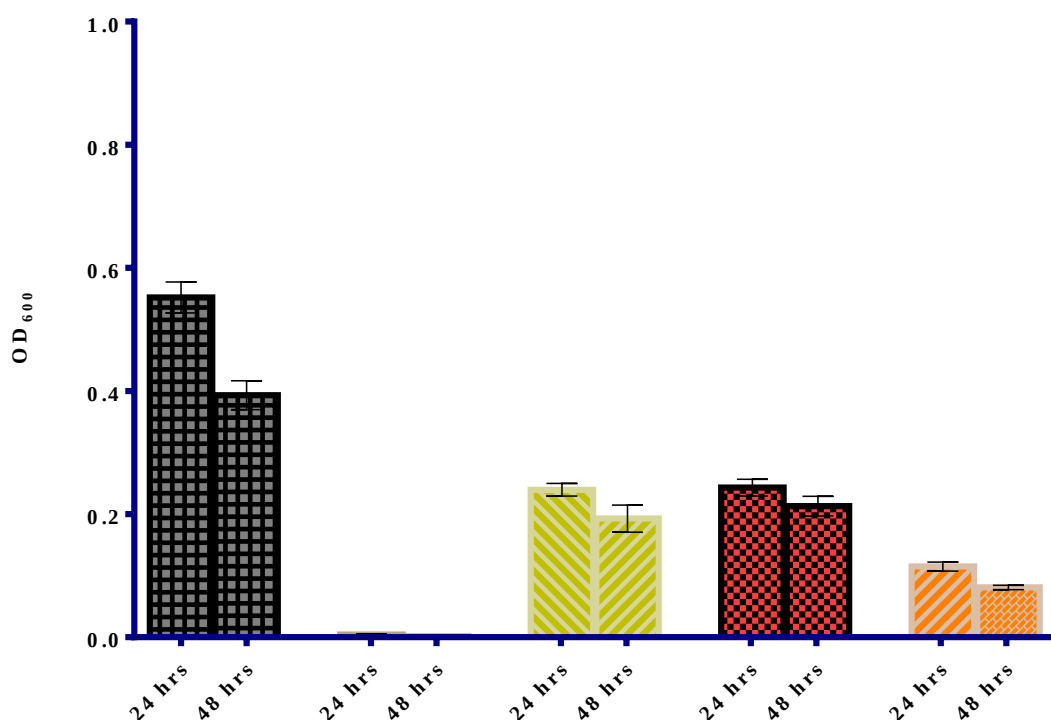


Figure 3-4: Growth of *C. difficile* 630 Δ erm in the presence of all proteinogenic amino acids at 0.2 mM concentration after 24 and 48 h of incubation.

Error bars represent the mean $\pm$ s.e.m. of triplicate experiments, $P < 0.0001$, ■■ MCDM medium with glucose (positive control), ■ MCDM medium without casamino acids, but with glucose (negative control), ■ amino acid (AA) mix (0.2 mM) except tyrosine (0.25 mM) and cysteine (4.2 mM) without isoleucine & glucose, ■ AA mix (0.2 mM) except tyrosine (0.25 mM) and cysteine (4.2 mM), with glucose, ■ AA mix (0.2 mM) except tyrosine (0.25 mM) and cysteine (4.2 mM) without glucose.

Building on the results presented in Figure 3-4, we attempted to improve growth in the fully defined medium by providing all twenty proteinogenic amino acids at 1 mM, with the exception of tyrosine and cysteine, which were provided at 0.25 mM and 4.2 mM respectively. This was considerably higher than what would be expected to be required for protein synthesis alone [110]. Results obtained were highly satisfactory with a mean OD₆₀₀ value of approximately 0.94 after three repeats in the presence of glucose after 24 hours incubation, demonstrating that the medium satisfied the nutritional needs of this bacterium. This medium is subsequently being referred to as 'DMaa1mM'. However, after 48 h of incubation, the mean OD₆₀₀ was reduced to a value of 0.64. Similarly, in the absence of glucose, a comparatively poor growth was observed with an average OD₆₀₀ value of 0.16 and 0.12 respectively after 24 and 48 h of incubation (Figure 3-5). Interestingly, a negative control experiment in which the amino acid isoleucine had been omitted (in the absence of glucose) showed considerable growth with an OD₆₀₀ of around 0.5 after 48 h. A possible explanation could be that the provided amino acids contained low amounts of contamination in the form of isoleucine or one of its biosynthetic precursors. Alternatively, it could be that this amino acid was provided by the small amount (1:100) of unwashed pre-culture which was added to inoculate. However, the concentration would have been below 10 μ M, and thus far below the concentration shown to be required for growth. This possibility was excluded in further studies, by LC-MS-MS based analysis of the developed defined medium.

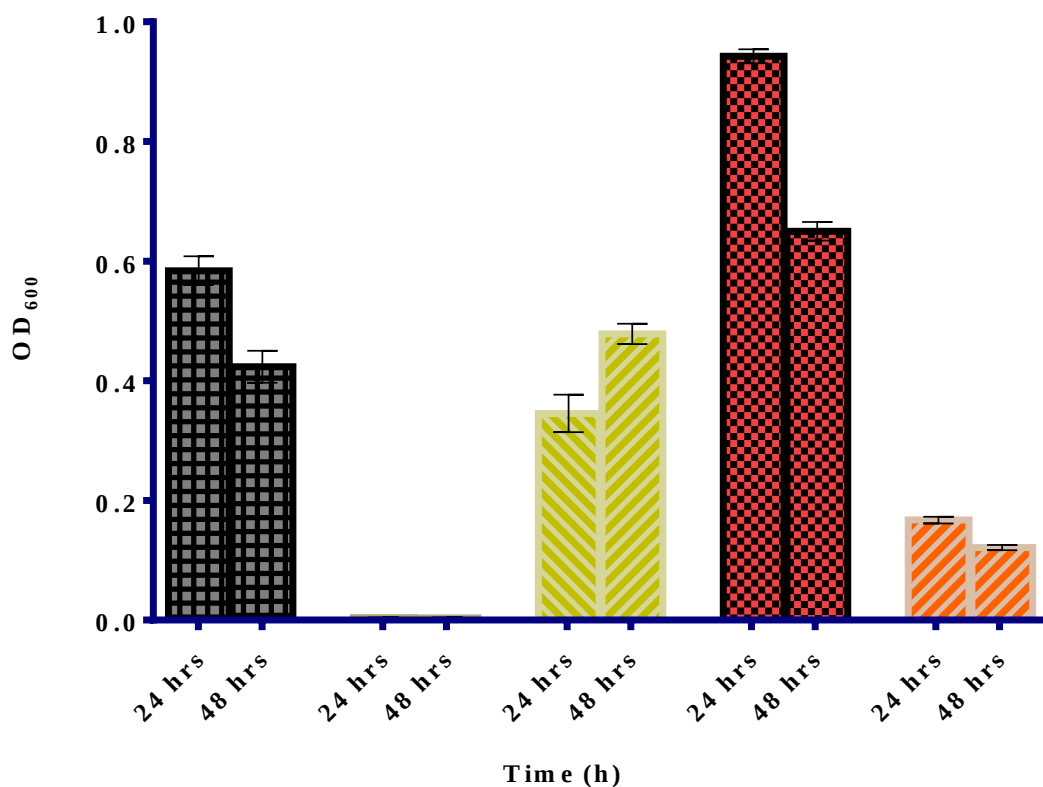


Figure 3-5: Growth of *C. difficile* 630 Δ erm in the presence of all proteinogenic amino acids at 1 mM concentration after 24 and 48 h of incubation.

Error bars represent the mean $\pm$ s.e.m. of triplicate experiments, $P < 0.0001$ ■■■ MCDM medium with glucose (positive control), ■■■ MCDM medium without casamino acid with glucose (negative control), ■■■ AA mix (1 mM) except tyrosine (0.25 mM) & cysteine (4.2 mM) without isoleucine & glucose, ■■■ AA mix (1 mM) except tyrosine (0.25 mM), cysteine (4.2 mM) with glucose, ■■■ AA mix (1 mM) except tyrosine (0.25 mM), cysteine (4.2 mM) without glucose.

3.3.4 Identification of amino acids essential for *C. difficile* growth in the presence and absence of glucose

To further optimise DMaa1mM medium, we tried to identify the minimal amino acid requirements for good growth by omitting individual amino acids as previously described [197] from the otherwise complete medium. Results obtained in the presence of glucose showed that growth was not much affected when alanine, arginine, asparagine, glycine, histidine, lysine, phenylalanine, proline, serine, threonine, aspartic acid, glutamic acid, tryptophan, tyrosine were absent. Growth was considerably reduced in the absence of glutamine, methionine, and almost abolished in the absence of cysteine, leucine, valine and isoleucine (in the first 24 h of incubation) Figure 3-6. This suggested that glutamine and methionine are growth promoting amino acids, whereas, cysteine, leucine, valine and possibly isoleucine are likely to be essential amino and cannot be synthesised by the *C. difficile* 630 Δ erm strain. Considering that isoleucine showed considerable growth with an OD600 of around 0.5 after 48 h. it therefore means that *C. difficile* 630 Δ erm growth was partially restored after an increased period of incubation, presumably because the strain was not completely auxotrophic for isoleucine and would probably have activated a pathway for the de novo synthesis of this amino acid. This is in contrast to previous findings which suggested that the organism is auxotrophic for all branched chain amino acids [197].

In a related study by Karasawa *et al.* [197] a defined medium containing the minimal amino acids and vitamins required for growth of *C. difficile* strains was developed. Their data showed that cysteine, isoleucine, leucine, proline, tryptophan and valine were essential for bacterial growth, whereas arginine, glycine, histidine, methionine and threonine were found to be growth-enhancing amino acids. Another study by Karlsson *et al.* [44] describing the impact of various growth conditions on the expression of toxins and other proteins by *C. difficile* VPI 10463 associated high toxin expression in post-exponential peptone yeast extract cultures with the reduced levels of certain amino acids. Although the essential and growth promoting amino acids identified in this study were not identical to those stated in the previous studies [44, 197, 333], the results seem to support the fact that certain amino acids greatly influence growth of *C. difficile*.

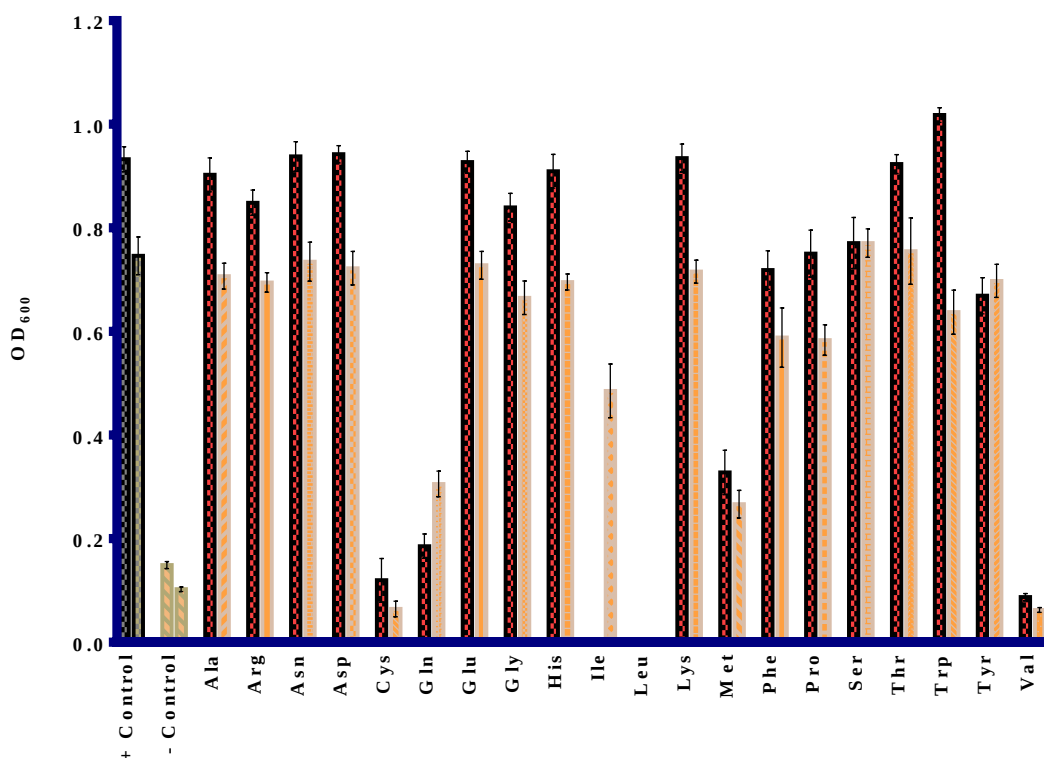


Figure 3-6: Determination of essential and growth promoting amino acids.

Error bars represent the mean $\pm$ s.e.m. of triplicate experiments, $P < 0.0001$,  all amino acids (1 mM) except tyrosine (0.25 mM) & cysteine (4.2 mM) in the presence of glucose (positive control),  all amino acids (1 mM) except tyrosine (0.25 mM) and cysteine (4.2 mM) in the absence of glucose (negative control),  all amino acids present as specified for the controls, apart from the ones indicated under the respective bars, 24 h of incubation in the presence of glucose,  all amino acids present as specified for the controls, apart from the ones indicated under the respective bars, 48 h of incubation in the presence of glucose.

3.3.5 Growth in the absence of non-essential amino acids.

Following the determination of amino acid requirements in the previous experiment, we carried out another experiment to see whether provision of growth-promoting essential amino acids alone (Figure 3-6) would be sufficient to produce substantial growth. The aim was to develop our medium further into a minimal medium only containing nutrients essential for good growth of this bacterium with little background growth in the absence of sugar. However, results presented in Figure 3-7 show that this aim could not be achieved. Surprisingly, whilst growth in standard medium was as expected, growth in the presence of the above amino acids was almost non-existent. Neither the essential amino acids (cysteine, leucine, valine and isoleucine) alone, nor the essential ones in combination with growth promoting amino acids (glutamine and methionine), produced any tangible growth (Figure 3-7). This means that although some amino acids may have appeared to be non-essential, they would still be needed in combination with the essential ones to support growth [44, 197].

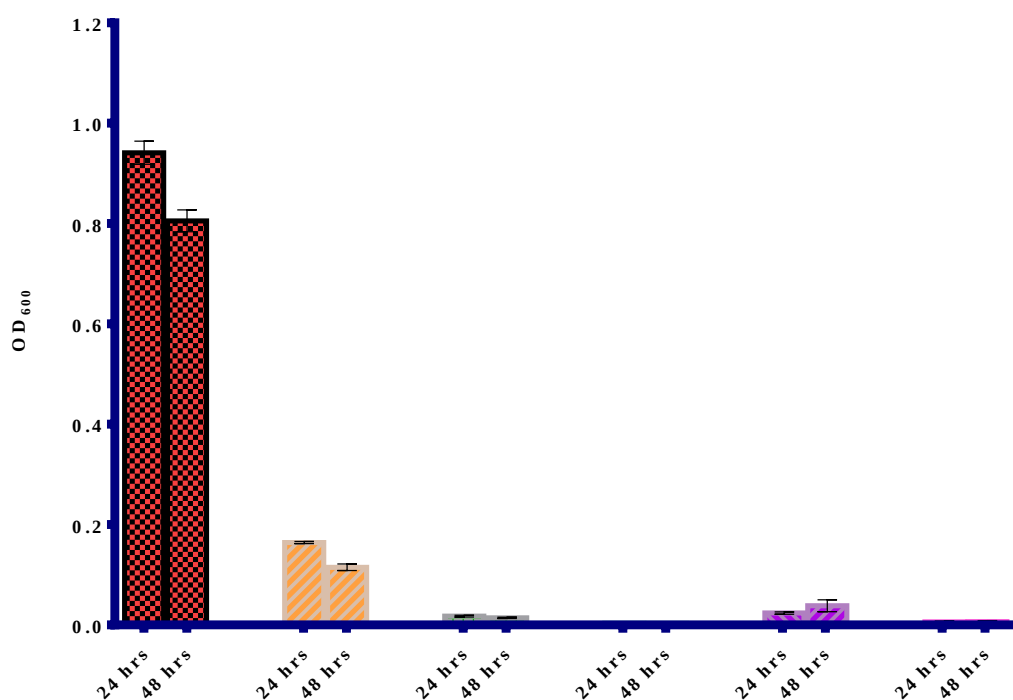


Figure 3-7: Growth of *C. difficile* 630 Δ erm in the presence of essential and growth-promoting amino acids after 24 h and 48 h with and without glucose.

Error bars represent the mean $\pm$ s.e.m. of triplicate experiments, $P < 0.0001$, ■ all amino acids (1 mM) except tyrosine (0.25 mM) & cysteine (4.2 mM) in the presence of glucose (positive control), ■ all amino acids (1 mM) except tyrosine (0.25 mM) & cysteine (4.2 mM) in the absence of glucose (negative control), ■ cysteine, leucine, valine and isoleucine only with glucose, ■ cysteine, leucine, valine and isoleucine only without glucose, ■ cysteine, leucine, valine, isoleucine, glutamine, methionine with glucose, ■ cysteine, leucine, valine, isoleucine, glutamine, methionine without glucose.

3.3.6 Growth in the presence of essential and selected non-essential amino acids

Since the above combination of all essential and growth promoting amino acids (Figure 3-7) failed to produce growth in the presence of fermentable carbohydrate, the growth promoting effect of those amino acids previously classified as non-essential was further examined. This was necessary to determine which of the non-essential amino acids were required to restore maximum growth. The objective was to keep the medium as simple as possible, with little background growth in the absence of sugar as it may also be used to analyse utilisation of alternative carbon sources such as amino acids/Stickland pairs and their fermentation products.

However, due to the very large number of possible combinations, only a small number of amino acids were tested, a choice based mainly on the complexity of their *de novo* biosynthetic pathways. As shown in Figure 3-8, good growth was achieved by addition of tyrosine and phenylalanine together with cysteine, glutamine, isoleucine, leucine, methionine and valine in the medium. The mean OD₆₀₀ value obtained was 0.82 after 48 h of incubation in the presence of glucose and 0.09 in the absence of glucose. However, growth was slower compared to the control which was grown in the presence of all proteinogenic amino acids and reached comparable ODs already after 24 h of incubation. This result suggests that tyrosine and phenylalanine are required to support the growth of *C. difficile* 630 Δ *erm* strain under these conditions. However, it is not yet clear why the inclusion of further amino acids such as proline and serine reduced growth to ODs below 0.31 and 0.40 after 24 and 48 h of incubation, respectively. As previously described, this medium is now being referred to as DMMaa1mM. Previous studies have identified cysteine, proline, tryptophan, isoleucine, leucine and valine as essential amino acids for a number of *C. difficile* strains [197], although the *C. difficile* 630 Δ *erm* was not included in these analyses. In this study, we found that only cysteine, leucine and valine were required under all conditions tested. However, under certain conditions, phenylalanine and tyrosine were identified as growth enhancing amino acids. Our results further revealed that proline and tryptophan in contrast to other *C. difficile* strains [197] were not essential for the *C. difficile* 630 Δ *erm* strain.

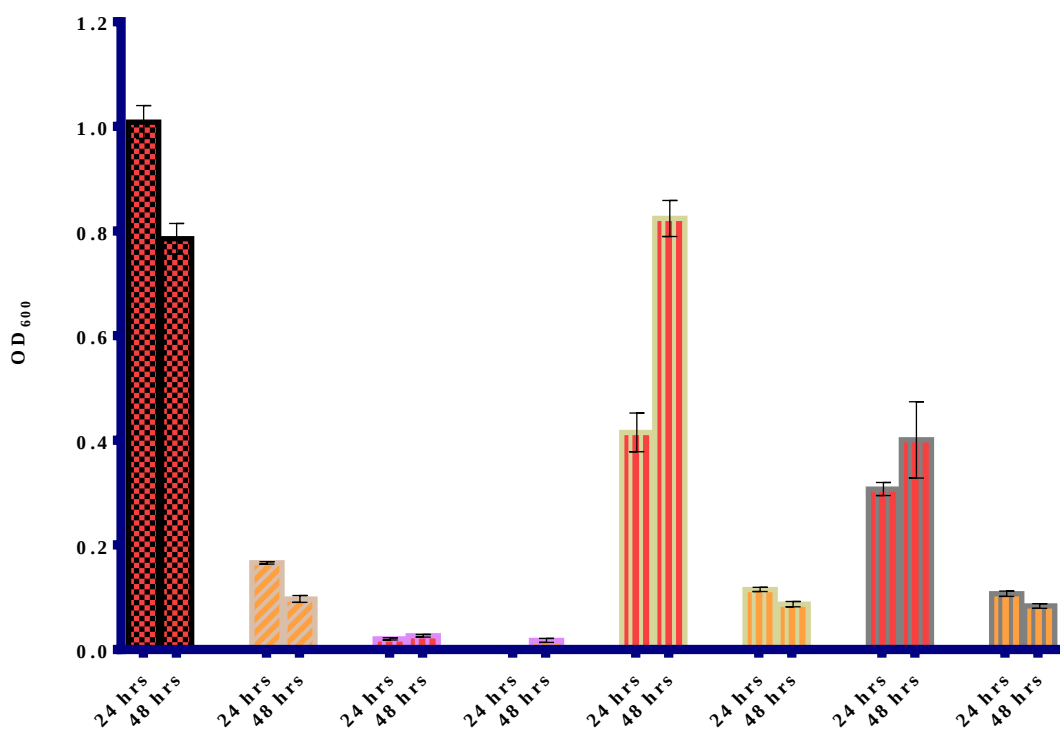


Figure 3-8: Growth in the presence of different combinations of essential (cysteine, isoleucine, leucine, valine), growth-promoting (glutamine, methionine) and non-essential amino acids (tyrosine, phenylalanine, proline, serine) after 24 h and 48 h of incubation.

Error bars represent the mean $\pm$ s.e.m. of triplicate experiments, $P < 0.0001$,  all AA (1 mM) except tyrosine (0.25 mM) and cysteine (4.2 mM) with glucose (positive control),  all AA (1 mM) except tyrosine (0.25 mM) and cysteine (4.2 mM) without glucose (negative control),  cysteine, glutamine, isoleucine, leucine, methionine, valine, tyrosine with glucose,  cysteine, glutamine, isoleucine, leucine, methionine, valine, tyrosine without glucose,  cysteine, glutamine, isoleucine, leucine, methionine, valine, tyrosine, phenylalanine with glucose,  cysteine, glutamine, isoleucine, leucine, methionine, valine, tyrosine, phenylalanine without glucose,  cysteine, glutamine, isoleucine, leucine, methionine, valine, tyrosine, phenylalanine, proline, serine with glucose,  cysteine, glutamine, isoleucine, leucine, methionine, valine, tyrosine, phenylalanine, proline, serine without glucose.

3.3.7 Effect of providing all twenty proteinogenic amino acids mix at higher concentrations (from 2.5 mM up to 20 mM).

Having established a medium that provided excellent growth in the presence of glucose and very little in its absence, an attempt was made to develop another medium derivative, which would allow for the study of utilisation of complex amino acid mixtures as carbon and energy sources. In such a medium all amino acids should be present at sufficiently high concentrations so that excellent growth is observed in the absence of glucose or other sugars. Here, it was necessary to establish whether the medium developed in subsection 3.3.3 could be improved by increasing growth in the presence of glucose even further whilst maintaining very low background growth in its absence. In addition, establishing whether any inhibitory effects were associated with higher amino acid concentrations. Consequently, the concentration of all amino acids except tyrosine was therefore increased to 2.5 mM, 5 mM, 7.5 mM, 10 mM, 15 mM and 20 mM, respectively. As shown in Figure 3-9, the average OD₆₀₀ values obtained after 24 h of incubation in the presence of glucose were 0.80, 0.88, 0.65, 0.31, 0.03, and 0.01, for 2.5 mM, 5 mM, 7.5 mM, 10 mM, 15 mM and 20 mM and in the absence of glucose 0.18, 0.29, 0.31, 0.33, 0.11, and 0.02, respectively. Similarly, after 48 h incubation the following results were obtained; in the presence of glucose 0.70, 0.47, 0.46, 0.34, 0.14 and 0.02 for 2.5 mM, 5 mM, 7.5 mM, 10 mM, 15 mM and 20 mM and in the absence of glucose 0.11, 0.16, 0.19, 0.17, 0.23, 0.21 respectively.

The results shown in Figure 3-9 revealed that growth in the presence of glucose could not be further improved by increasing the amino acid concentration. Growth remained high when amino acids were supplied at concentrations of 2.5 and 5 mM, but declined at higher concentrations, confirming the inhibitory effects associated with such concentrations as previously reported for other species [367]. However, in the absence of glucose, a steady increase in growth was observed as the concentration of amino acids increased to 5 mM, 7.5 mM and 10 mM. Above 10 mM, inhibitory effects were observed. The increase in growth was not unexpected and suggested the activation of pathways for the breakdown of certain amino acids by this bacterium in the absence of glucose. Thus, the observations in this experiment are in agreement with previous experiments showing the usage of certain amino acids as alternative carbon and energy sources by some species of

Clostridium, including *C. difficile* [119, 358]. Further analysis of growth in the presence of 10 mM amino acid mix will be conducted in Chapter 4 to establish which amino acids are being utilised and in which order, in an approach similar to that described recently for *Clostridium sticklandii* by Fonknechten *et al.* [119].

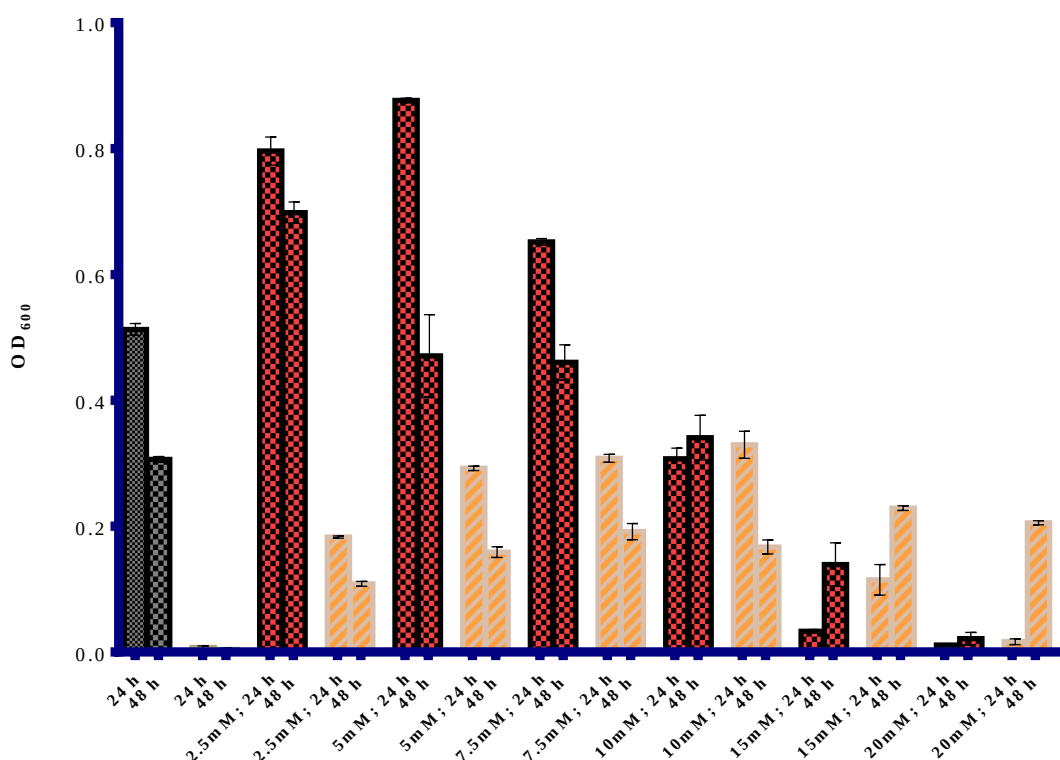


Figure 3-9: Growth of *C. difficile* 630Δerm in the presence of all proteinogenic amino acids (except tyrosine) at concentrations of 2.5 to 20 mM.

Growth was monitored after 24 and 48 h of incubation, in the presence and absence of glucose, respectively. Error bars represent the mean ± s.e.m. of triplicate experiments, $P < 0.0001$. ■ standard medium with glucose (positive control), ■ standard medium without casamino acids with glucose (negative control), ■ AA mix provided as indicated in the respective bars, except tyrosine (0.25 mM) in the presence of glucose, ■ AA mix provided as indicated in the respective bars, except tyrosine (0.25 mM) in the absence of glucose.

3.4 Conclusions

Three variants of a defined medium have been developed for *C. difficile* 630 Δ erm that allow good growth in the presence of a carbon and energy source such as glucose. These media were designed to serve different purposes in the physiological and metabolic analysis of this and other toxinogenic strains.

Defined medium DMaa1mM, contains all proteinogenic amino acids at a concentration of 1 mM, except tyrosine (0.25 mM, due to its low solubility) and cysteine (4.2 mM, due to its additional role as reducing agent). This medium supports very good growth in the presence of a dedicated carbon and energy source and very little in its absence. Thus, this medium can be used to investigate utilisation of alternative carbon sources such as defined Stickland amino acid pairs and other growth factors.

Defined medium DMaa10mM, contains all proteinogenic amino acids at a concentration of 10 mM, except tyrosine (0.25 mM). This medium allows good growth in the presence and absence of carbohydrate based carbon and energy sources due to its comparatively high concentration in amino acids, some of which can be broken down and utilised by the organism. This medium variant will be useful in identifying which amino acids (and potentially other added carbon sources) will be preferentially utilised, co-metabolised, and in which order.

Defined medium DMMaa1mM can be considered a defined minimal medium as it only contains essential and growth promoting (under these conditions) amino acids, i.e. cysteine, glutamine, isoleucine, leucine, methionine, valine, tyrosine, and phenylalanine. Similar to medium DMaa10mM, this medium can be used to investigate utilisation of alternative carbon sources such Stickland amino acids and other growth factors. However, as it promotes slower growth and, in the absence of carbohydrate, similar growth yields as medium DMaa10mM, the latter would be the preferred choice for such studies.

In contrast to other strains [197] *C. difficile* 630 Δ erm was found to have an absolute requirement for cysteine and the branched chain amino acids, leucine, valine, while phenylalanine and tyrosine were identified as growth enhancing amino acids. Supply

of only the essential and other identified growth promoting amino acids was insufficient to support growth. The reasons for this are unclear.

The defined media developed herein represent a significant step forward in determining the roles of nutritional and metabolic factors in the control of virulence in this bacterium using a mass spectrometry platform. Medium DMaa10mM will further be tested on other representative strains of the *C. difficile* pathogens, which will be discussed in the next chapter.

CHAPTER 4.

THE STUDY OF AMINO ACID UTILISATION BY EIGHT *CLOSTRIDIUM DIFFICILE* STRAINS

4 THE STUDY OF AMINO ACID UTILISATION BY EIGHT *CLOSTRIDIUM DIFFICILE* STRAINS

4.1 Introduction

Previous studies have shown the important role played by amino acids (AA) in nutrition, physiology and as critical regulatory factors of metabolic pathways [97, 368-370]. They are predictably one of the major nutrients in human and animal diets as well and this makes them an important substrate for protein biosynthesis in many gut-associated microorganisms [371]. In the absence of an exogenous source, the synthesis of these amino acids is vital for survival of most organisms. In addition to being an important product of protein degradation, they also play key roles in the synthesis of nucleotides and vitamins. Amino acids readily support the growth of some bacteria as well as their hosts and can serve as an alternate source of carbon and energy, especially when a crucial metabolic process has been compromised [119, 371, 372]. Previous report has described amino acids as an important source of nutrients for most potential pathogens [373]. Majority of these organisms are reported to possess the ability to modify their metabolic requirements, in response to environmental conditions to maximally make the most of the available nutrients including amino acids [138, 139, 373]. Such organisms can readily differentiate into non-replicating or less metabolically demanding cells. Other organisms that can't alter their metabolic requirement are able to avoid amino acid starvation in a way by manipulating host amino acid uptake and production systems in order to make protein or meet their nutrient requirements [373].

4.1.1 Colonic microbiota

The utilization of amino acids is widely distributed amongst bacterial strains that are resident in the human digestive tract. Among them are: *Fusobacterium* spp, *Peptostreptococcus* spp, *Veillonella* spp, *Megasphaera elsdenii*, *Acidaminococcus fermentans*, *Selenomonas ruminantium* and many others [371]. Previous studies have shown that microbes in the gastrointestinal tract may play an important role in host protein and amino acid metabolism. As previously reported, the gastrointestinal tract of humans is highly populated by a complex community of bacteria, estimated to be around 10^{14} cells [81, 374]. One important role they play is to provide resistance to colonization and infection by pathogenic organisms. They are also known to be

actively involved in the metabolism of dietary compounds, and at the same time interacting with intestinal epithelial cells of the host [371, 374].

About 20 to 60 g of complex carbohydrates have been estimated to enter the colon every day. These complex carbohydrate compounds are fermented each time by the colonic microbiota into short chain fatty acids, with their attendant benefit in the gut [374]. The colonic microbiota are also reported to be involved in regulating amino acids metabolism, lipid metabolism, protein digestion in many other host and bacterial metabolic reactions [374]. Exogenous proteins are hydrolysed by host enzymes into amino acids and smaller peptides, which are further utilized by the colonic microbiota to produce ammonia. However, *Clostridium* species are reported to be the most common amino acid fermenting bacteria in the gut [374]. They have been shown to ferment amino acids, using specific pathways [119] and enzymes with exceptional activities such as B12-dependent aminomutases, selenium-containing oxidoreductase and 2-hydroxyacyl-CoA dehydratases [119, 121, 375, 376]. Of particular interest is their ability to ferment amino acids via the Stickland reaction, as first described in 1934 [119, 377]. The term ‘Stickland reaction’ here refers to the ability to oxidize an amino acid coupled to the reduction of another and in so doing, conserve energy by ATP formation via substrate-level phosphorylation [119]. The fermentation of amino acids by the gut microbiota could generate products beneficial to the host such as acetate, butyrate, propionate, valerate, isobutyrate, isovalerate, as well as harmful products like phenols, indoles, ammonia, amines, thiols and hydrogen sulphide [374].

4.1.2 Justification of the study

The work presented focuses on LC-MS/MS as a tool for the analysis of amino acids in spent *Clostridium difficile* medium. Even though some quantitative studies of uptake and metabolism of peptides and amino acids have been performed using different bacteria, not much seems to have been done with *Clostridium* species using LC-MS/MS techniques. Mass spectrometry techniques in the biological sciences have currently gone beyond the conventional chemical categorization and metabolic phenotype analysis to understanding gene function [153, 154]. It has become a very useful tool for clinical diagnostics and is currently attracting more scientific attention from multiple biological fields [159, 212-215, 378], including advanced

microbiological research [139, 152, 174, 175] to understand the metabolic changes as well as systems behaviours of important microorganisms [155].

As previously mentioned, the carbon and energy metabolism of *C. difficile* seems not to have been fully understood in spite of its medical importance. Amino acid metabolism by the Stickland reactions has previously been described as a primary source of energy in a number of *Clostridium* species, especially when grown in a medium containing only amino acids [332], given the ability of these bacteria to obtain its carbon and energy in the absence of a fermentable carbohydrate, by activating specific pathways and/or enzymes to degrade amino acids by fermentation processes in order to create ATP [118, 126, 331]. One amino acid can act as an electron donor, while the second amino acid will act as an electron acceptor [118, 126, 332, 333]. Most of the *C. difficile* strains tested have shown absolute requirement for isoleucine, leucine, proline and valine, whereas arginine, glycine, histidine and threonine were classified as growth enhancing amino acids [197]. For *C. difficile*, toxin production (a key factor associated with the infection) appears to be greatly influenced by nutritional factors present in the medium [42, 197, 198, 332]. Therefore, in order to gain deeper insights to the carbon and energy metabolism of this organism, comprehensive carbon source utilisation studies utilising the mass spectrometry based approaches was performed. As previously reported such studies are best performed using a well-defined growth medium [44, 197, 199, 379]. Although a number of *C. difficile* strains, have been successfully cultivated on defined media, these media provide excess of nutrients, particularly in terms of amino acid provision, resulting in undesirable background growth in the absence of glucose. To overcome these challenges, a defined medium was developed that allows good growth of this bacterium, in the presence of a carbon and energy source such as glucose (already discussed in chapter 3) together with an LC-MS/MS method, that will simultaneously measure all twenty amino acids (details also presented in chapter 2). The application of both methods (presented in chapters two and three) will provide further insight into the metabolic activities of this bacterium, increase understanding of the importance of amino acids for the survival of *C. difficile* in the gut and possibly lead to discovery of novel metabolites and metabolic pathways as previously reported [152, 175, 179].

4.1.2.1 Strain selection

As discussed in chapter 3, the amino acid requirements of *C. difficile* 630 Δ erm differed from those of other strains tested [197, 380]. Thus, to obtain a more general picture of amino acid utilisation in this species, a number of different clinical isolates was selected for the work described here. The selection of a limited number of representative strains is a somewhat challenging task. However, the choices in this study were made on the basis of their wide distribution, outbreaks caused and their association with increases in the incidence, severity of the disease and mortality worldwide [7, 24-29, 381, 382]. These strains have been reported to be resistant to commonly used antimicrobial agents such as fluoroquinolones, and to be associated with increased incidence of nosocomial infections and their ability to produce more potent toxins via an unknown mechanism [7, 12, 14, 15, 17, 24, 28, 31-36]. The acquisition of resistance has been attributed to the continuous use of antimicrobial agents resulting in their continuous spread and persistence [24]. In order to obtain metabolic information that may aid the development of novel therapies to tackle *C. difficile* infection, we tested the performance and suitability of our fully defined media [337-339] for the cultivation of eight important *C. difficile* strains, from which we would quantify the amount of amino acids utilised.

4.1.3 Aims and objectives

The focus in this chapter was to apply the methodology developed in chapters two and three to study amino acid utilisation by eight *Clostridium difficile* strains. The specific objectives includes, measuring the concentration of amino acids left in spent *Clostridium difficile* culture media as well as determining the order in which different amino acids are being utilised.

4.2 Materials and Methods

4.2.1 Chemicals

Details of all the chemicals used are as previously described in chapters two (subsection 2.2.1) and three (subsection 3.2.2).

4.2.2 Bacterial culture and growth conditions

The *Clostridium difficile* strains used in this study and their origin are presented in Table 4-1 below.

Table 4-1: Origin of *C. difficile* strains used

Strain name	PCR Ribotype	Origin	References
CD630 Δ erm	PCR ribotype 012	Strain obtained from Peter Mullany lab, UCL (London)	[383]
DH196	PCR ribotype 027	SW England, Torbay isolate (Val Hall)	[384]
R20291	PCR ribotype 027	R20291: Jon Brazier (Cardiff), Stoke Mandeville outbreak	Nottingham strain
EK15	PCR ribotype 014	Strain obtained from E. J. (Ed) Kuijper, Netherlands.	[29, 385]
EK28	PCR ribotype 078	Strain obtained from E. J (Ed) Kuijper, Netherlands	[29, 386]
R12801	PCR ribotype 106	SW England, Torbay isolate (Val Hall)	[387]
L26	PCR ribotype 001	Strain obtained from E. J. (Ed) Kuijper, Netherlands isolate	[29]
17serogroupF	PCR ribotype 017	Jon Brazier/Ed Kuijper	[388, 389]

4.2.3 Inoculum preparation

All eight *Clostridium difficile* strains were prepared as previously described in chapter three (subsection 3.2.4).

4.2.4 Growth experiments for amino acid utilisation

The defined medium (DMaa10mM) was prepared as previously described in chapter three (subsection 3.3.7 and Table 4-2) and inoculated with the eight *Clostridium difficile* strains in eight separate flasks at a 1 in 100 dilution. An uninoculated flask containing the defined medium was used as blank to compare the different samples. The inoculated and uninoculated flasks were maintained in the anaerobic cabinet at 37°C. Two sets of samples were collected every 2 hours (0, 2, 4, 6, 8, 10, 12, 14, 16); also after 24, 26, 28, 30, 32 and 48 hours. The first set of samples was for the determination of the optical density (at a wavelength of 600 nm), and a logarithmic plot made using Graph Pad Prism 6. The second set of samples was prepared by centrifugation at 5400 rpm for ten minutes. The resulting supernatants were then filter sterilised through 0.2 µm filters and stored at -20°C until ready for use.

4.2.5 Amino acid analysis

The supernatant obtained in subsection 4.2.4 was diluted 1:1000 using acetonitrile:water mixture (50:50, v/v), so that any unused amino acid would be present at a concentration of 10 µM. Thereafter, 100 µl of each sample (thoroughly mixed) was dispensed into a 2 ml vial ready for LC/MS analysis. The MS detection was performed using Quattro (described in subsection 2.2.6) equipped with electrospray ionization source operating simultaneously in the negative and positive ionization modes. The LC conditions adapted for this experiment are presented in chapter 2 (subsections 2.2.4 and 2.2.6).

4.2.6 Quantification of amino acids/data analysis

The raw data were acquired from the research group instrument's database (Kingfisher) and pre-processed to convert data into a standard and uniform format using MassLynx V4.1. The processed data were thereafter exported into Excel to generate a calibration curve from which the individual amino acids concentrations were determined. The interpolated concentrations were exported into the Graph Pad Prism 6 to plot a graph of concentration against time. Data were analysed by Analysis of Variance (ANOVA) using Graphpad Prism 6 and data sets obtained are indicated in the respective chart.

Table 4-2: Composition of the modified medium.

Adapted with modification [353].

Composition	Concentrations of the fully defined media/ volume used (in 100ml)	
	1 mM	10 mM
Amino acid Solution A*, (100 mM)	1 ml	10 ml
Amino acid Solution B*, (50 mM)	2 ml	20 ml
Amino acid Solution C*, (200 mM)	0.5 ml	5 ml
Amino acid Solution D*, (1 M)	0.1 ml	1 ml
Amino acid Solution E*, (2.5 mM)	1 ml	10 ml
Salt solution 1 (10X) (see section 3.2.3)	10 ml	10 ml
Salt solution 2 (100X) (subsection 3.2.3)	1 ml	1 ml
Salt solution 3 (100X) (subsection 3.2.3)	1 ml	1 ml
Trace element solution (SL9)	0.2 ml	0.2 ml
Glucose solution (20 %)	5 ml	5 ml
Vitamin solution (100X)	1 ml	1 ml
Selenium solution (1 mM)	0.1 ml	0.1 ml

A* = A mix of alanine, arginine, asparagine, glutamine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, proline, serine, threonine, valine; B* = A mix of aspartic acid, glutamic acid, tryptophan; C* = Cysteine; D* = Glycine; E* = Tyrosine. This medium was made by mixing appropriate volumes of each solution together with sterile anaerobic distilled water to adjust the final volume to 100 ml. Prepared media were maintained in the cold room for a few days or kept in the -20°C freezer until use.

4.3 Results and Discussion

4.3.1 Growth performance of *C. difficile* strains in fully defined DMaa10mM medium

In order to demonstrate the suitability of the developed DMaa10mM medium for amino acids utilisation studies, we tested the performance of eight different *C. difficile* strains in this fully defined medium which contains all proteinogenic amino acids at a concentration of 10 mM, except tyrosine (0.25 mM) as previously described in chapter three. Growth curves were determined for each of the *C. difficile* strains as described under subsections 3.2.3 and 4.2.4. All eight strains grew differently in the presence and absence of glucose and the results are presented in Figure 4-1. In the presence of glucose, most strains showed lag phases below 5 h, except L26 (6 h) and 017 (14 h). Overall, growth curves were similar for strains CD630 Δ erm, EK15, EK28, L26, R12801 DH196 and R20291, although there were significant differences in the OD's reached after 24 h (stationary phase), with the latter two strains reaching considerably lower levels. EK28 was particularly fast growing and reached stationary phase before all other strains tested. However, ODs for this strain started to decline again after 24 h and reached a value of approximately 0.1 after 48 h. Strain 017 serotype F was particularly slow growing and reached stationary phase only after 30 h with the lowest maximal OD of all the strains tested (below 0.2), whereas strain L26 reached the highest OD (0.71).

In the absence of glucose, lag phase below 5 h was observed for CD630 Δ erm, EK28, R12801 and R20291, whereas DH196, EK15, L26, and 017 serotype F showed recovery times of 6, 8, 10 and 16 h, respectively. Interestingly, following inoculation ODs for all strains initially started to decline under these conditions. The maximum ODs achieved were also lower for most of the tested strains. An exception was strain 017 which appeared to grow equally well if not slightly better in the absence of glucose.

The adaptation period (lag phase) for all *C. difficile* strains except 017 serotype F strain was between 0 and 10 h. The 017 serotype F strain lag phase lasted up to 14 h in the presence and absence of glucose. Previous experiments have shown that it is a period where most bacterial cells begin to exploit their new environment,

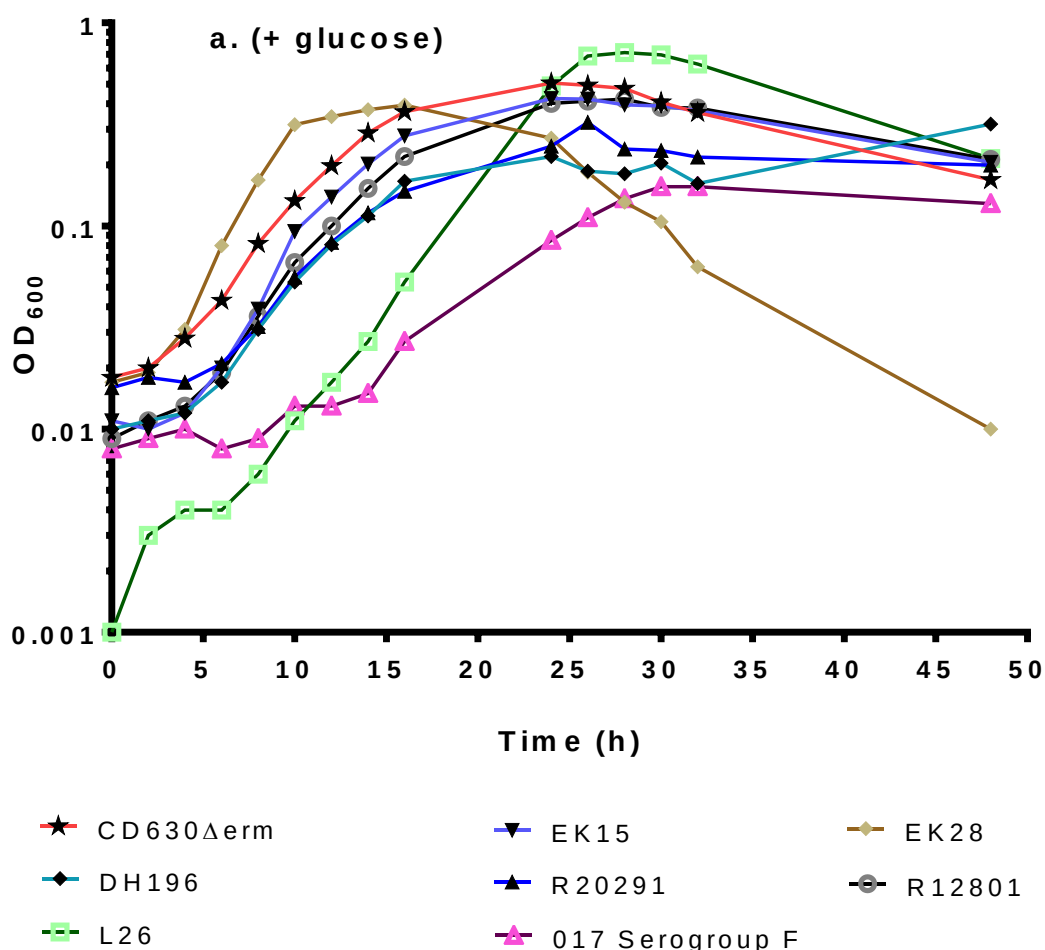
synthesizing cellular components necessary for growth and the repair of damaged macromolecules [350]. How long this period will last, is dependent on a number factors such as the type of organism, variation in environmental parameters, and the period the starter culture has undergone in starved conditions before the new settings [390]. Once the organism is fully adapted, growth rate and the number of bacteria increase rapidly up to a maximum such as the one recorded by some of the strains tested (Figure 4-1, Table 4-3). This would largely be attributed to increased metabolic activities, DNA replication by binary fission occurring at a constant rate and possibly the exploitation of the culture medium at a maximal rate [390, 391].

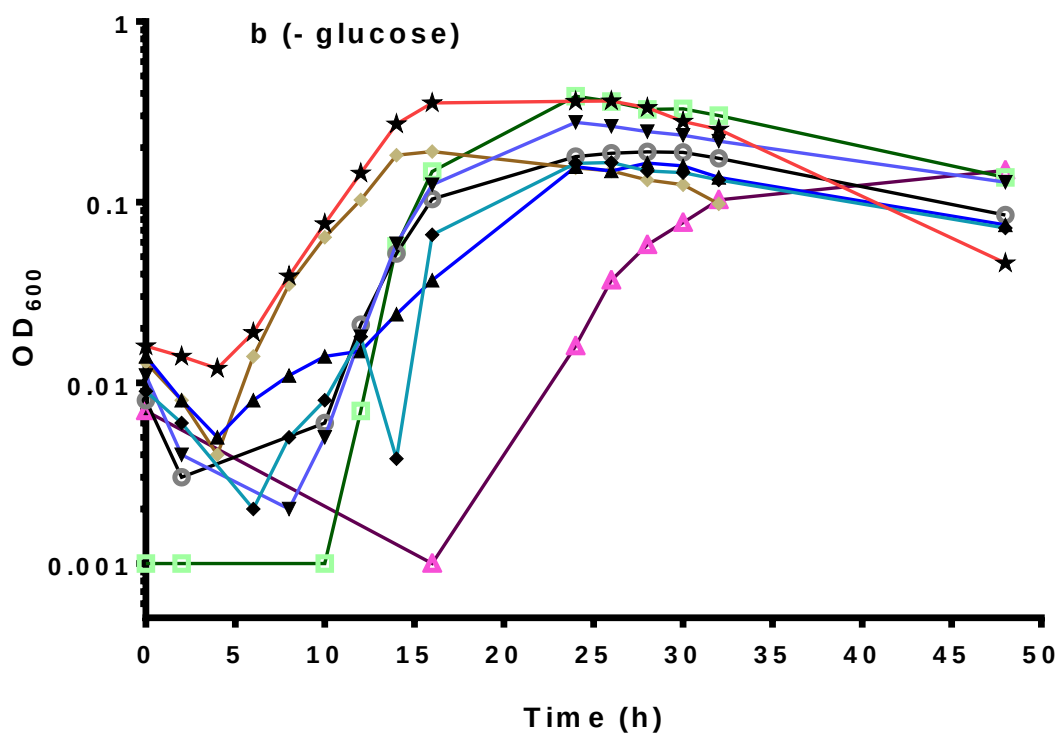
Table 4-3: Growth rates during exponential growth in the presence and absence of glucose.

<i>C. difficile</i> strain	Period of exponential growth (h)	
	With glucose	Without glucose
CD 630 Δ <i>erm</i>	2 – 16	6 – 16
DH 196	8 – 20	10 – 20
R20291	8 – 24	8 – 24
EK 15	4 – 16	8 – 16
EK 28	2 – 10	2 – 10
R12801	6 – 16	8 – 14
L 26	2 – 20	8 – 16
017 Serotype F	14 – 48	16 – 24

As shown in Figure 4-1, all the strains except 017 Serotype F grew well in our medium in the presence and absence of fermentable carbohydrate. The 017 Serotype F seems to grow slowly, having a long lag phase [392]. Overall, growth of all the strains was satisfactory despite the variation in the specific growth rates of the individual strains. Though there was growth in the absence of glucose, but a little more enhanced in the presence of glucose, which could be attributed to the high concentration of amino acids in the medium. Most *C. difficile* strains have been reported to ferment many carbohydrates, including glucose and to use it as a source of carbon and energy [374, 393]. A medium that supports the growth of a number of relevant *C. difficile* strains is essential in order to obtain general and reproducible results relating to the amino acid utilisation of this species. Experimental data

obtained were reproducible and therefore suitable in identifying which amino acids will be preferentially metabolized as previously reported [119, 337, 372]. By the use of DMaa10mM medium, we were able to demonstrate clearly the extent to which the eight different *C. difficile* strains tested can use certain amino acids in the presence and absence of glucose to derive the energy required for growth. With this medium, it is possible to quantify the amount of amino acid used by these strains, which is in agreement with previous studies showing the importance of using a defined medium for the determination of the nutritional changes in microbial cells and other traits [337-339].





CD630Δerm

EK15

EK28

DH196

R20291

R12801

L26

017 Serogroup F

Figure 4-1: Growth of eight *C. difficile* strains in DMaa10mM medium in (a) presence and (b) absence of glucose.

Data shown are from two independent cultures. Error bars are not shown for reasons of clarity. P value for growth in DMaa10mM medium in (a) presence <0.01 and (b) absence of glucose <0.01.

4.3.2 LC-MS/MS analysis of amino acid utilisation by *C. difficile* strains

The aim of this experiment was to measure the concentration of individual amino acids in spent *C. difficile* culture media to determine which amino acids were being utilised by the organism and in which order. *C. difficile* strains were grown for 48 h in DMaa10mM as shown in Figure 4-1 and samples taken as indicated for the OD readings. The concentration of individual amino acids in these samples was determined by LC-MS/MS as described in subsection 2.2.4 and the results obtained are presented in Table 4-4.

Based on the concentrations remaining in the medium after 48 h of incubation, the amino acids were divided into four groups as shown in Table 4-4. Firstly, the highly utilized amino acids, with more than 60 % disappearing from the medium (highlighted in green), secondly the moderately utilized amino acids, with 30 to 59 % disappearing from the medium (highlighted in blue), thirdly the poorly utilized amino acids, with less than 30 % disappearing from the medium (highlighted in yellow) and lastly not utilized amino acids with less than 10 % disappearing from the medium (highlighted in pink).

Strains L26 and 017 displayed a markedly different amino acid utilisation pattern when compared to the other strains. L26 is utilising several amino acids not metabolised by the other seven strains or utilising them to a larger extent, whereas L26 is utilising several amino acids to a lesser extent compared to the other strains tested (cysteine, glutamine, isoleucine, leucine and valine).

For most strains and amino acids, utilisation was comparable in the presence and absence of glucose. Exceptions showing considerable differences were for instance valine for strain 630 Δ erm, DH196, R20291, EK15, and EK28, methionine for strain EK28, arginine, glutamate, histidine, proline, and tryptophan for L26, and leucine, isoleucine and threonine for strain 017, which were all used to a larger extent in the absence of glucose.

There appeared to be no net consumption of alanine, although further analyses revealed (see below) that this amino acid was first partially consumed and then actively produced by most of the strains. There was also generally little or no

consumption of arginine, aspartate, glutamate, histidine, lysine, methionine and tryptophan, except by strain L26.

Amino acids that were utilised to a large extent by most strains were cysteine, glutamine, isoleucine, leucine, serine, threonine and tyrosine.

None of the strains, apart from L26, used notable amounts of glutamate, arginine, histidine, and lysine. And only two strains utilised aspartate (L26, 017), methionine (L26, EK28) and tryptophan (L26, 630 Δ *erm*). The lack of glutamate utilisation is surprising given that it is an important nitrogen (amino group) donor [394-397] and that the enzyme glutamate dehydrogenase, required for the first step of glutamate breakdown, is secreted by almost all *C. difficile* strains and is in fact a key marker for its presence in faecal samples [398-400].

Table 4-4: Percentage utilization of amino acids by eight different *C. difficile* strains after 48 h incubation in DMaa10mM in the presence and absence of glucose.

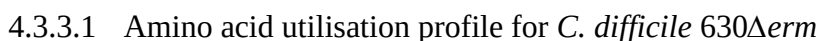
Amino acids	CD 630 Δ erm		DH 196		R20291		EK 15		EK 28		R12801		L 26		017 SerotypeF	
	+ve glucose	-ve glucose	+ve glucose	-ve glucose	+ve glucose	-ve glucose	+ve glucose	-ve glucose	+ve glucose	-ve glucose	+ve glucose	-ve glucose	+ve glucose	-ve glucose	+ve glucose	-ve glucose
Ala*	≤1	≤1	≤1	≤1	≤1	≤1	≤1	≤1	≤1	≤1	≤1	≤1	≤1	≤1	≤1	≤1
Arg	5	5	5	5	5	5	5	5	≤1	≤1	≤1	≤1	35	55	≤1	≤1
Asn	5	5	17	20	20	15	30	35	15	20	14	15	50	65	≤1	≤1
Asp	≤5	≤5	≤1	≤1	5	5	5	5	≤1	≤1	≤1	≤1	35	42	24	23
Cys	88	90	65	70	70	62	60	85	85	95	90	85	95	95	≤5	≤5
Gln	73	70	30	35	85	80	82	85	55	65	75	76	85	88	≤1	≤1
Glu	≤5	≤5	≤1	≤1	5	5	5	5	≤1	≤1	≤1	≤1	25	50	≤1	≤1
Gly	10	10	15	15	5	5	30	30	≤1	≤1	≤1	≤1	10	10	54	52
His	≤5	≤5	≤1	≤1	≤1	≤1	≤1	≤1	≤1	≤1	≤1	≤1	40	60	≤1	≤1
Ile	72	95	70	90	90	100	70	90	70	100	90	95	100	100	25	62
Leu	75	95	70	85	88	100	75	90	75	100	85	92	100	100	21	59
Lys	5	5	≤1	≤1	5	5	5	5	≤1	≤1	≤1	≤1	35	48	≤1	≤1
Met	≤5	≤5	≤1	≤1	≤1	≤1	5	5	18	31	≤1	≤1	65	75	≤1	≤1
Phe	10	10	≤1	≤1	5	5	26	20	26	21	≤1	≤1	60	70	≤1	≤1
Pro	40	32	5	5	37	26	30	35	10	15	52	55	41	65	≤1	≤1
Ser	95	100	85	70	80	85	100	85	100	100	100	85	100	98	60	65
Thr	100	100	55	60	75	75	90	85	65	100	93	85	90	98	21	45
Trp	10	12	≤1	≤1	5	5	5	5	≤1	≤1	≤1	≤1	35	60	≤1	≤1
Tyr	22	25	70	99	18	22	75	80	55	65	75	80	100	100	37	35
Val	30	57	21	45	40	60	30	50	1	70	28	25	60	77	≤5	≤5

Pink = <10 %; Yellow = ≥10-29 %; Blue = ≥30-59 %; Green = ≥60 %; *Excreted into the medium

The results suggest these organisms derive most of their carbon and energy from the fermentation of glucose and a selected range of amino acids as highlighted above. Fonknechten *et al.* [119] have previously shown that *Clostridium sticklandii* could metabolise certain amino acids in a particular order, when cultured in a defined medium with all amino acids present at the same concentration. Interestingly, *Clostridium sticklandii* has been reported to share a similar energy metabolism with *Clostridium difficile* and could serve as an excellent reference organism [119]. In a related studies with *Streptomyces lividans* D’Huys *et al.* [392] also demonstrated the preferred manner in which certain amino acids were utilised by this bacterium, when cultured on a minimal medium supplemented with casamino acids [392]. Most recently, Neumann-Schaal *et al.* [120] demonstrated that *C. difficile* 630 Δ erm in particular, could degrade certain amino acids in a preferential way, depending on the composition of the medium, suggesting that these organisms are optimally adapted to the conditions observed in the intestine.

4.3.3 Amino acids utilisation at different phases of growth

After establishing the general trends for amino acid utilisation by *C. difficile* in the subsection 4.3.2, we tried to determine the changes in amino acid content in DMaa10mM culture medium over time and in relation to the growth phase. This was done by superimposing the interpolated concentrations obtained from the LC-MS analysis on the growth curves previously obtained (Figure 4-1). As shown in Figure 4-1 to 4.9 considerable changes were observed in the concentrations of most amino acids measured, although, during the lag phase of growth, the level of amino acids, remained largely unchanged most likely due to the fact that the cells were just beginning to adjust to the new environment. However, during the exponential phase, experimental data showed that many of the amino acids were decreasing in concentration presumably due to their uptake and metabolic breakdown, although this assumption can only be confirmed by measuring both the intra and extracellular metabolome or by employing isotope labelled compounds. The sequences of changes recorded during the exponential phase of growth by the different *C. difficile* strains are shown in detail in the subsections below:



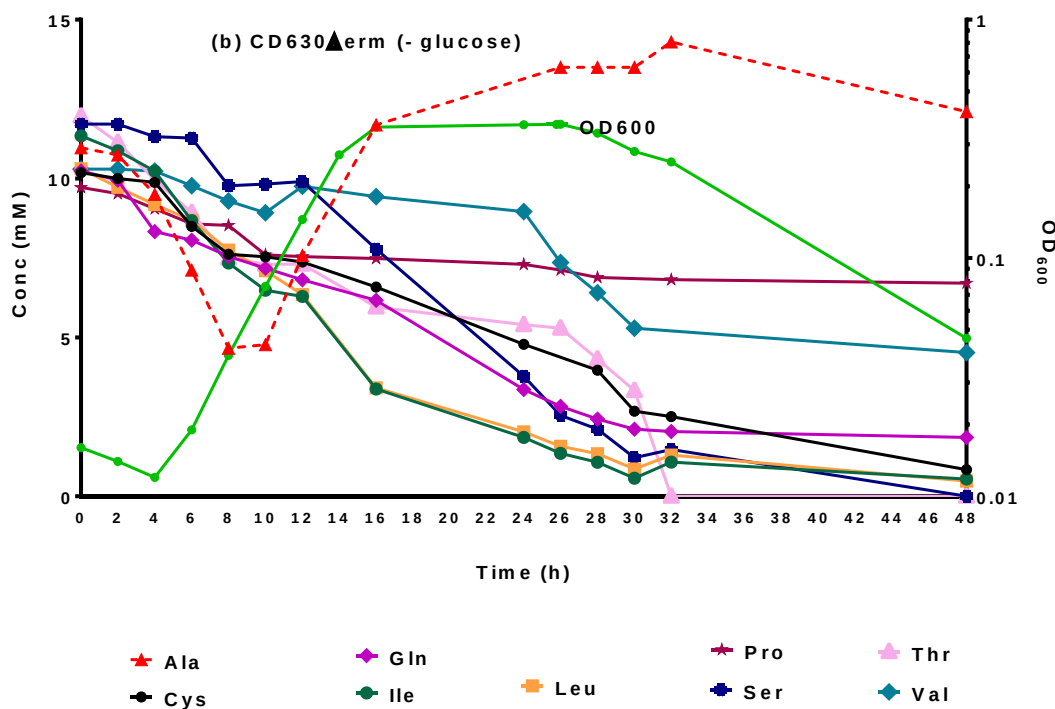
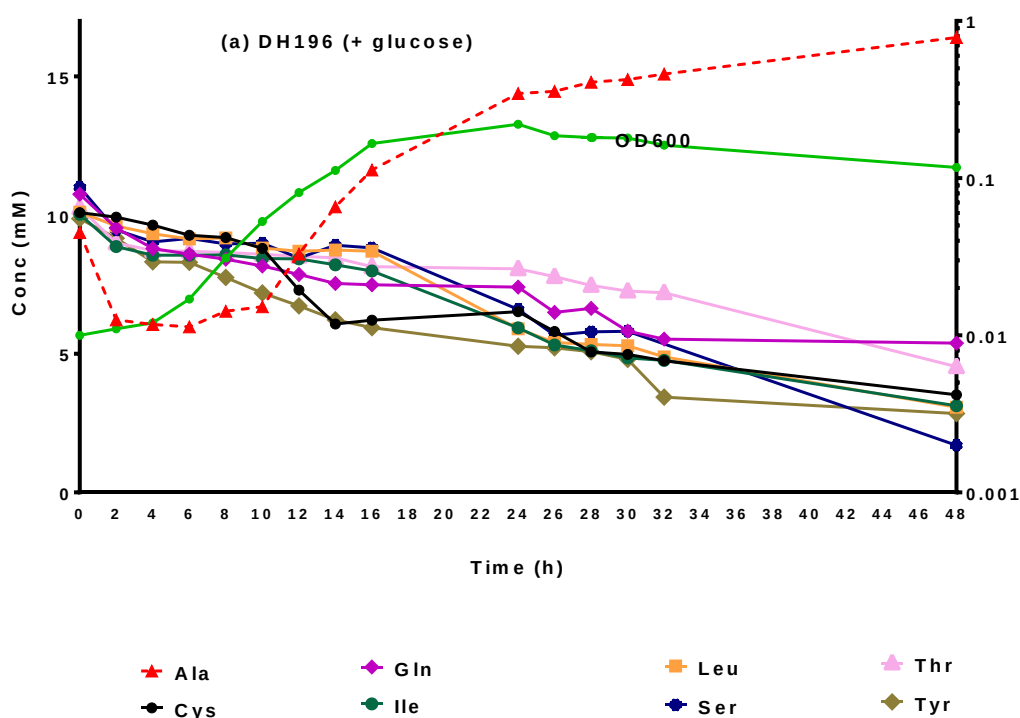


Figure 4-2: Amino acid utilisation profile for *C. difficile* 630 Δ erm grown in DMaa10mM for 48 h in the presence (a) and absence (b) of glucose.

Shown are profiles for amino acids for which an overall consumption of more than 30 % was observed under at least one of the indicated conditions. Data shown are from three independent cultures. Growth (OD₆₀₀) is indicated in green. Error bars not shown for clarity. P value for growth in DMaa10mM medium in (a) presence <0.0001 and (b) absence of glucose <0.0001.

4.3.3.2 Amino acid utilisation profile for *C. difficile* DH 196

When the DH196 strain was grown in DMaa10mM medium, most of the consumed amino acids steadily declined in their concentration (Figure 4-3). The alanine concentration declined rapidly to about 6 mM within the first 2 h (lag phase) but increased again during the late exponential and stationary phases. Some of the amino acids, e.g. cysteine, isoleucine, leucine, serine, and threonine initially showed little or no consumption in the presence of glucose. In the absence of glucose, tyrosine declined more rapidly and is completely consumed after 24 h, whereas isoleucine and leucine also declined more rapidly but only after cultures have reached the transition to stationary phase.



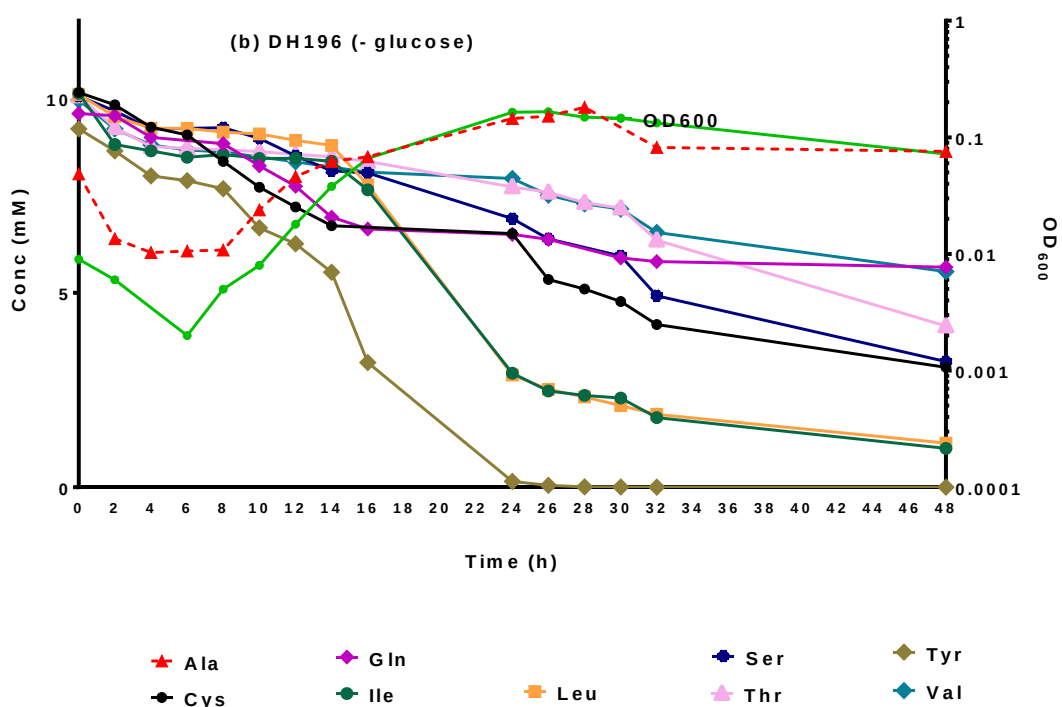
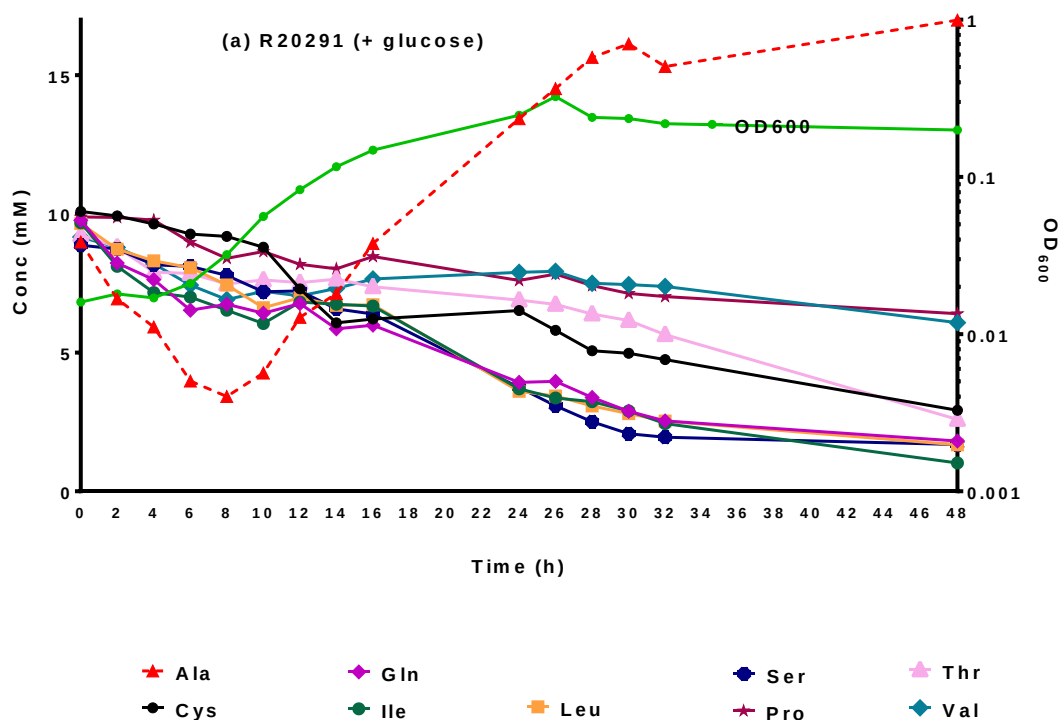


Figure 4-3: Amino acid utilisation profile for *C. difficile* DH196 grown in DMaa10mM for 48 h in the presence (a) and absence (b) of glucose. Shown are profiles for amino acids for which an overall consumption of more than 30 % was observed under at least one of the indicated conditions. Data shown are from three independent cultures. Growth (OD₆₀₀) is indicated in green. Error bars not shown for clarity. P value for growth in DMaa10mM medium in (a) presence <0.0001 and (b) absence of glucose <0.0001.

4.3.3.3 Amino acid utilisation profile for *C. difficile* R20291

As shown in Figure 4-4 following inoculation there was a rapid uptake of glutamine, isoleucine, leucine, valine, serine and threonine in the presence and absence of glucose, for the first 6 h until cells started to grow (Figure 4-4a). As seen for all other strains alanine was also utilised during the first 6 h and from then on secreted. Proline utilisation in the presence of glucose was only observed following the mid exponential growth phase. Glutamine and cysteine both steadily declined during growth both in the presence and absence of glucose. Leucine, isoleucine, serine and glutamine were metabolised both in the presence and absence of glucose.



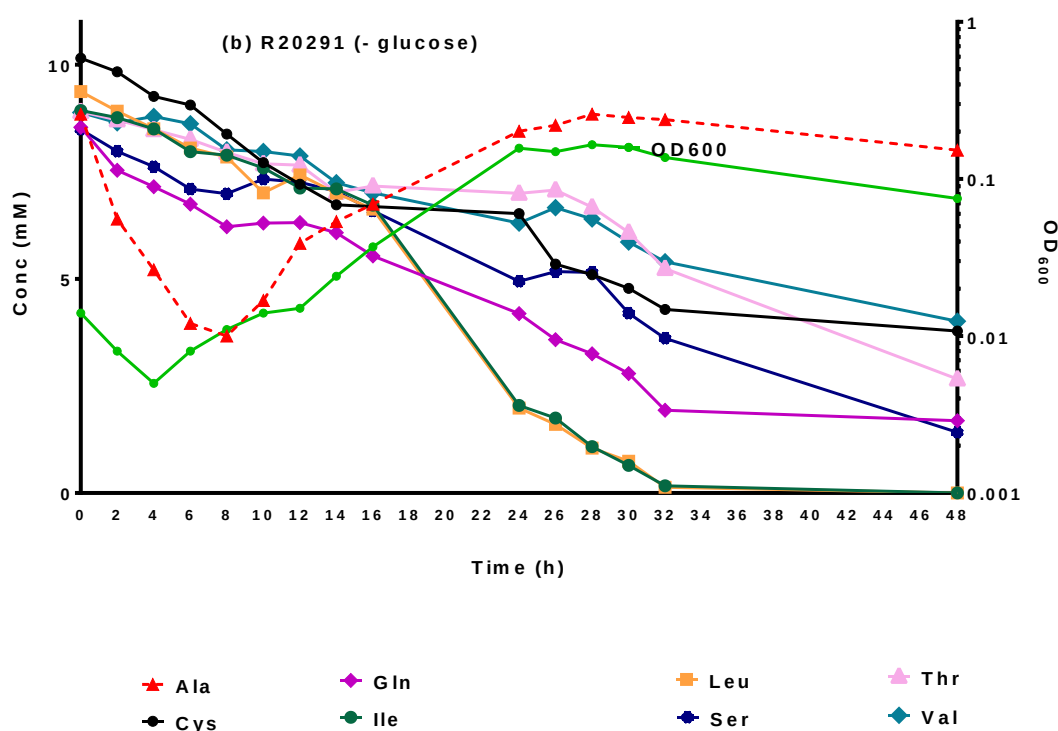
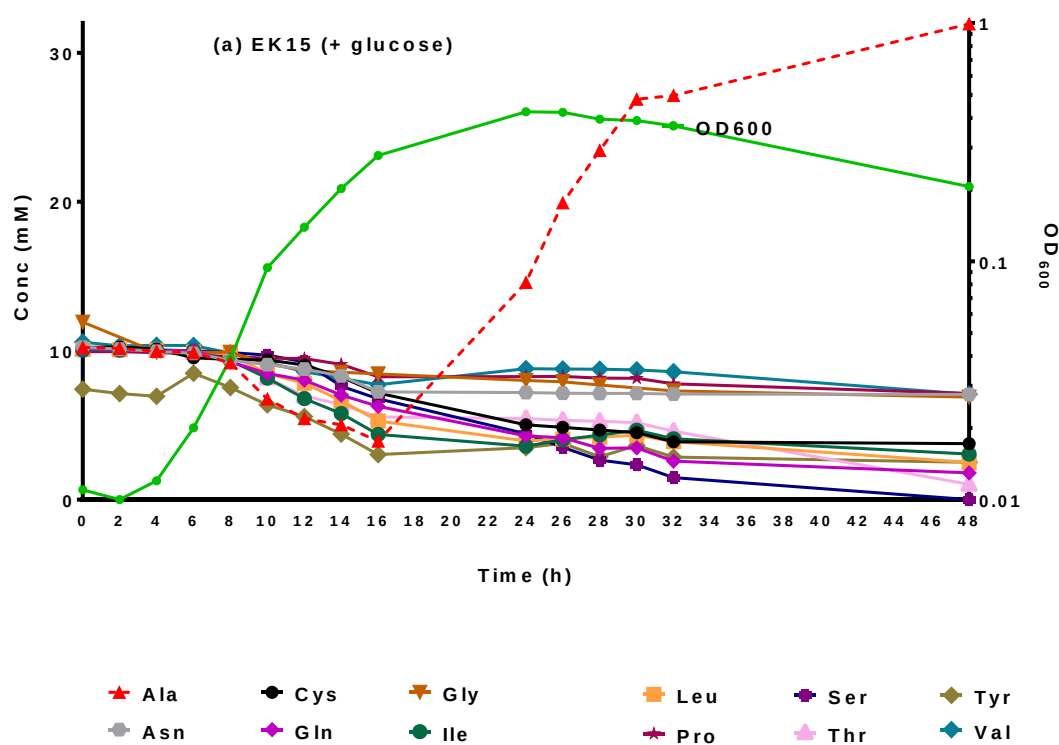


Figure 4-4: Amino acid utilisation profile for *C. difficile* R20291 grown in DMaa10mM for 48 h in the presence (a) and absence (b) of glucose.

Shown are profiles for amino acids for which an overall consumption of more than 30 % was observed under at least one of the indicated conditions. Data shown are from three independent cultures. Growth (OD₆₀₀) is indicated in green. Error bars not shown for clarity. P value for growth in DMaa10mM medium in (a) presence <0.0001 and (b) absence of glucose <0.0001.

4.3.3.4 Amino acid utilisation profile for *C. difficile* EK15

For the EK15 strain, in the presence of glucose utilisation of twelve amino acids was only observed following the mid exponential growth phase (cysteine, glutamine, asparagine, proline isoleucine, leucine, serine, valine, threonine, glycine, alanine and tyrosine (Figure 4-5). As seen for other strains, alanine was initially degraded both in the presence and absence of glucose but concentrations in the medium increased again from early stationary phase onwards. This increase was considerable and resulted in a final concentration of above 30 mM in the presence of glucose. Glycine, which was also taken up following the mid-exponential phase, was also excreted following entry into stationary phase but only with a moderate increase in concentration.



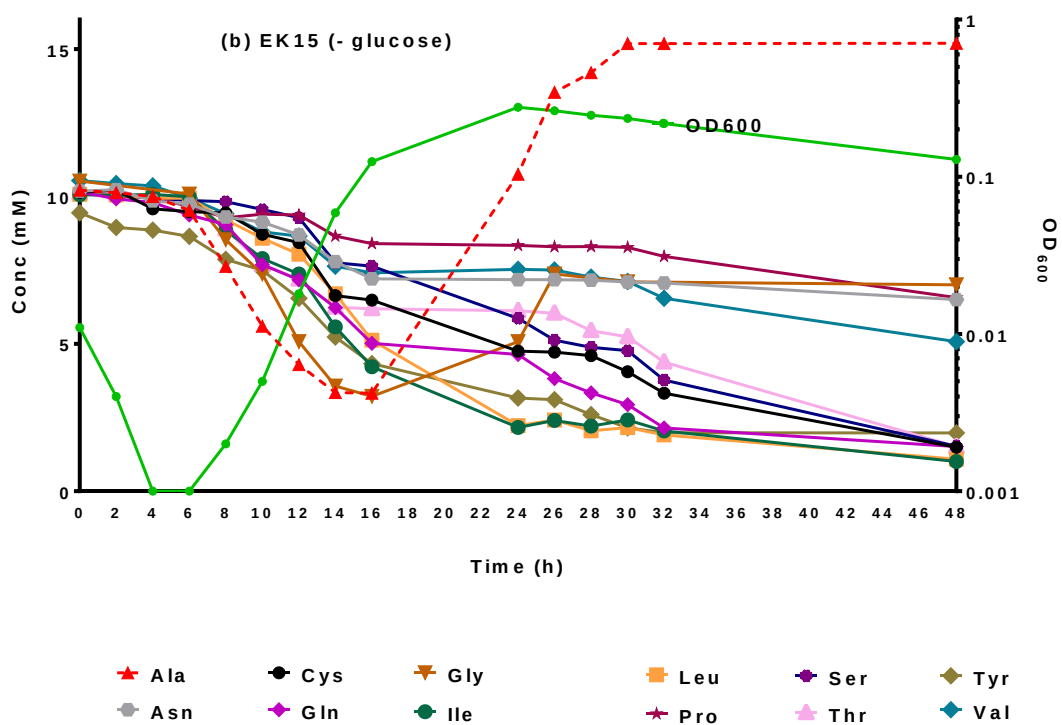
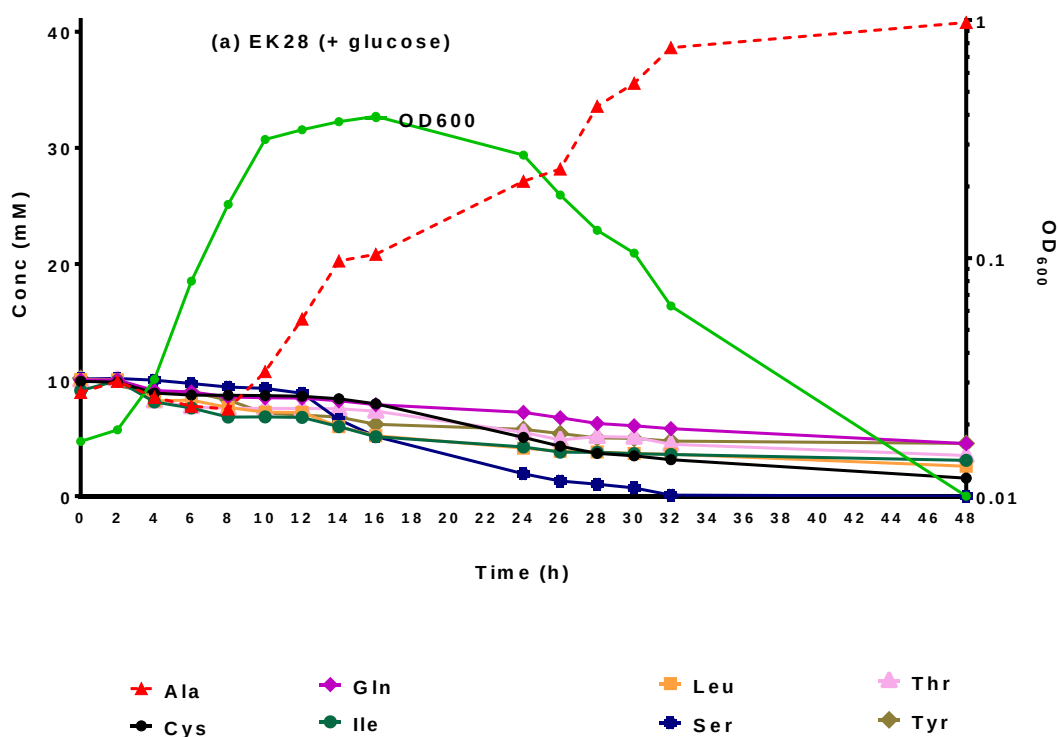


Figure 4-5: Amino acid utilisation profile for *C. difficile* EK15 grown in DMaa10mM for 48 h in the presence (a) and absence (b) of glucose.

Shown are profiles for amino acids for which an overall consumption of more than 30 % was observed under at least one of the indicated conditions. Data shown are from three independent cultures. Growth (OD₆₀₀) is indicated in green. Error bars not shown for clarity. P value for growth in DMaa10mM medium in (a) presence <0.0001 and (b) absence of glucose <0.0001.

4.3.3.5 Amino acid utilisation profile for *C. difficile* EK28

For this strain, a steady uptake and utilisation of cysteine, isoleucine, leucine, serine, threonine, glutamine and tyrosine, was observed in the presence of glucose from the early exponential phase onwards (Figure 4-6). Serine was completely consumed after 32 h and cysteine and threonine after 48 h. Alanine was consumed to a small extent before being excreted to a very high final concentration (40 mM) during late exponential and stationary phase. In the absence of glucose, utilisation of some amino acids was already observed during the lag phase, e.g. leucine, isoleucine, glutamine, threonine and tyrosine. Among these, leucine, isoleucine, threonine but also serine and cysteine were removed to a large extent after only 30 h of incubation. Tyrosine was also utilised to a considerable degree under these conditions, whereas alanine was only metabolised during the lag phase and excreted during exponential and stationary phase. Glutamine was moderately taken up in the presence and absence of glucose, while tyrosine uptake occurred in the presence and absence of glucose. A moderate uptake of methionine and valine occurred in the absence of glucose only.



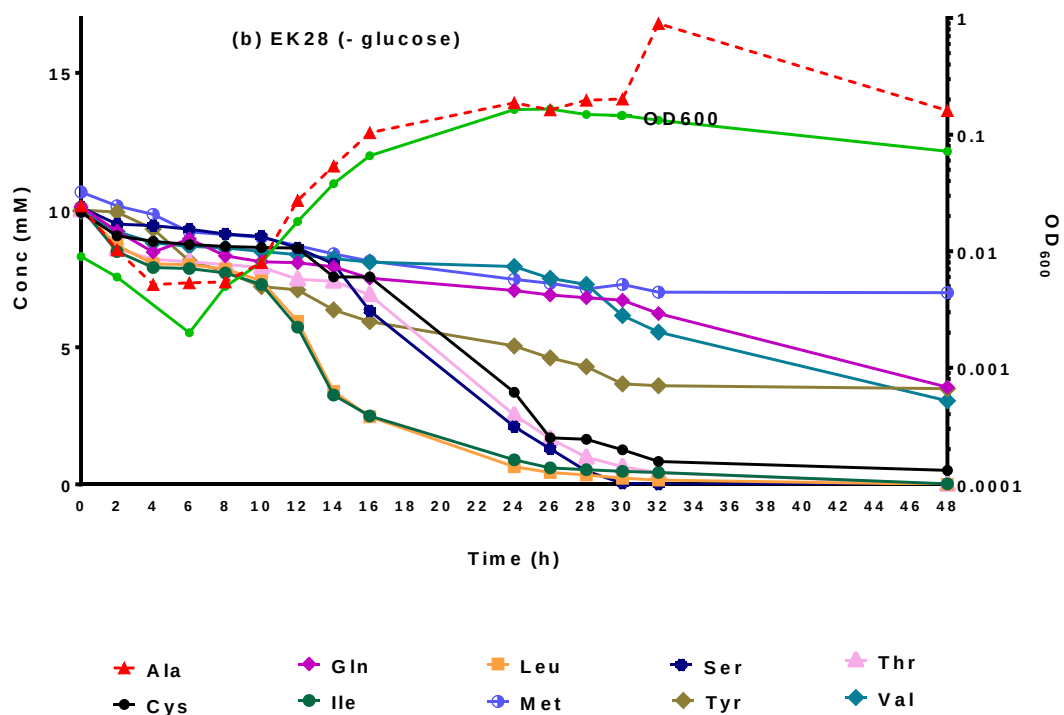


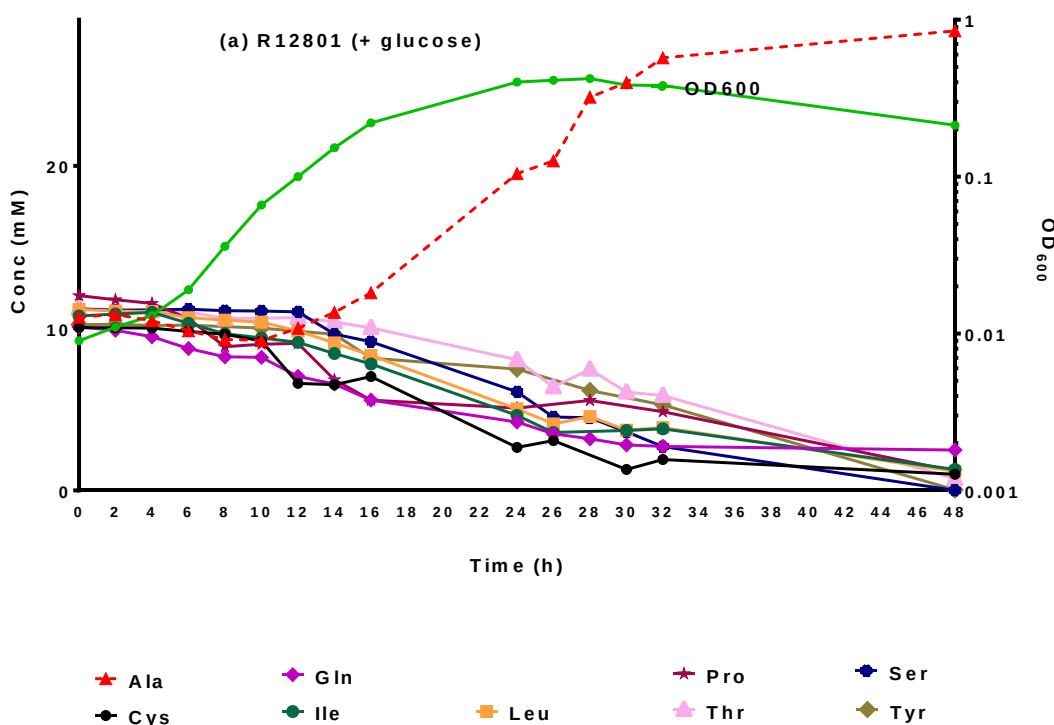
Figure 4-6: Amino acid utilisation profile for *C. difficile* EK28 grown in DMaa10mM for 48 h in the presence (a) and absence (b) of glucose.

Shown are profiles for amino acids for which an overall consumption of more than 30 % was observed under at least one of the indicated conditions. Data shown are from three independent cultures. Growth (OD₆₀₀) is indicated in green. Error bars not shown for clarity. P value for growth in DMaa10mM medium in (a) presence <0.0001 and (b) absence of glucose <0.0001.

4.3.3.6 Amino acid utilisation profile for *C. difficile* R12801

In the presence of glucose strain R12801 showed a short lag phase and little or no consumption of amino acids (alanine, cysteine, leucine, isoleucine, proline, serine, threonine and tyrosine) before the culture reached the mid-exponential growth phase (Figure 4-7). An exception was glutamine which the strain started to consume during early exponential phase. There was little consumption of alanine before the mid-exponential phase but a considerable amount of this amino acid was produced after that point and accumulated to a concentration of about 28 mM. Interestingly, in the presence of glucose all of the other aforementioned amino acids were almost completely utilised at the end of the experiment after 48 h.

In the absence of glucose, growth was decreased but interestingly utilisation of most amino acids did also not commence to a noticeable degree before the culture had reached the exponential phase, with the exception of glutamine, which started after 4 h. (Figure 4-7) Similarly, alanine accumulated to high concentrations (28 mM) and all of the other aforementioned amino acids were almost completely utilised at the end of the experiment after 48 h.



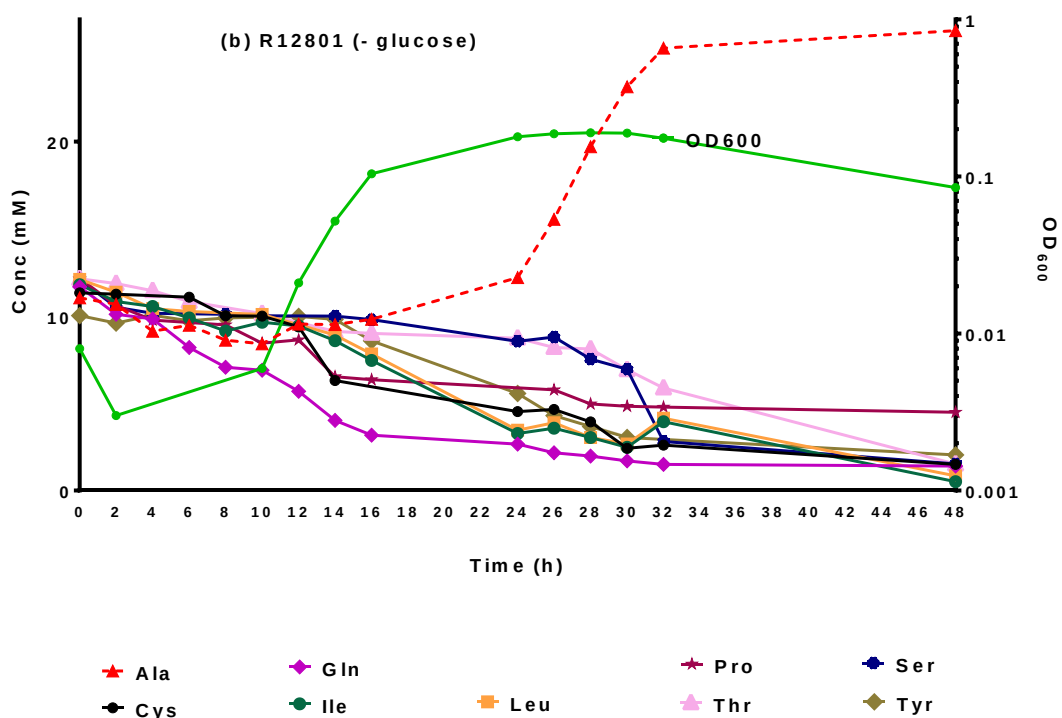
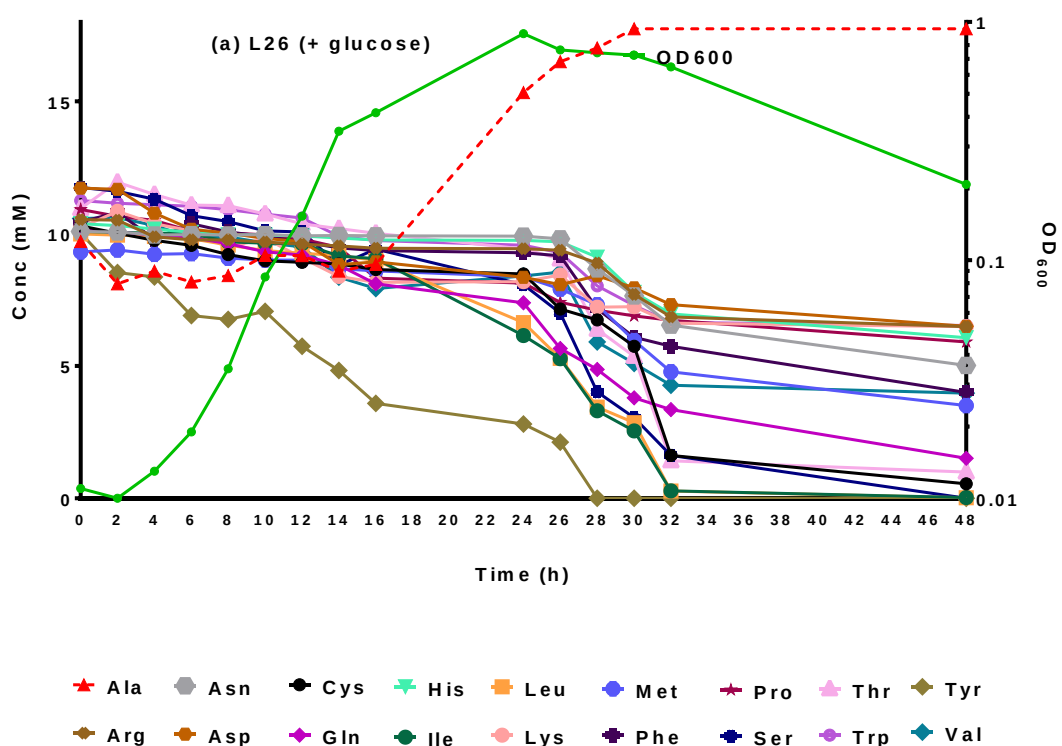


Figure 4-7: Amino acid utilisation profile for *C. difficile* R12801 grown in DMaa10mM for 48 h in the presence (a) and absence (b) of glucose.

Shown are profiles for amino acids for which an overall consumption of more than 30 % was observed under at least one of the indicated conditions. Data shown are from three independent cultures. Growth (OD₆₀₀) is indicated in green. Error bars not shown for clarity. P value for growth in DMaa10mM medium in (a) presence <0.0001 and (b) absence of glucose <0.0001.

4.3.3.7 Amino acid utilisation profile for *C. difficile* L 26

Strain L26 grew very well in DMaa10mM medium both in the presence and absence of glucose and, as already shown in Table 4-4, its amino acid utilisation profile differed considerably from that of the other strains in that a larger number of amino acids was being metabolised. Further differences were noted when amino acid consumption was analysed over time (Figure 4-8). For instance, in the presence of glucose there was very little or no consumption of amino acids before the end of the exponential growth phase, with the exception of tyrosine whose concentration steadily decreased after inoculation until it was used up after 28 h of growth. Following entry into stationary phase, alanine was excreted under these conditions and the extracellular concentration increased to approximately 17.5 mM, whilst isoleucine and leucine breakdown was induced resulting in their complete consumption after 32 h of growth. Interestingly, after 24 h of growth, i.e. in stationary phase, utilisation of the previously not (or little) utilised amino acids was induced, and consumption of tyrosine, leucine and isoleucine appeared to increase even further. Only two of the twenty amino acids added remained unchanged in the presence of glucose or changed comparatively little (below 25 %) and these were glycine and glutamate, respectively.



In the absence of glucose, only glycine showed limited use below 10 % (data not shown). However, tyrosine, leucine and isoleucine reached very low concentrations after 24 h of growth and serine, threonine, valine, cysteine and lysine were also drastically reduced. In contrast to all other strains tested, alanine could be excreted but balanced by uptake in the absence of glucose.

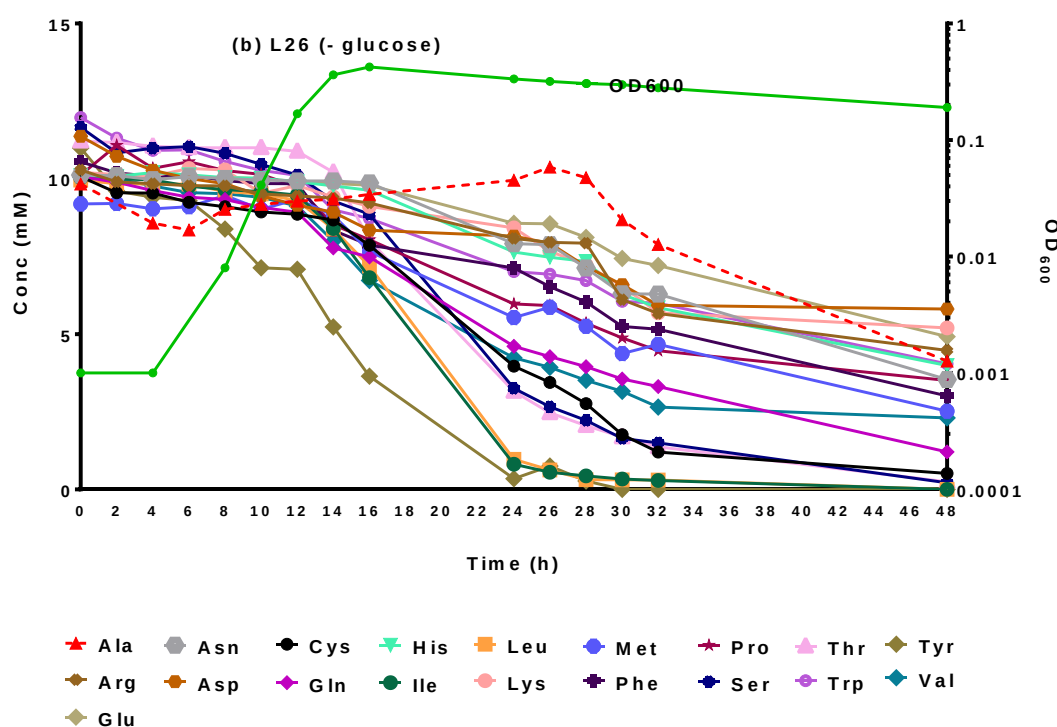


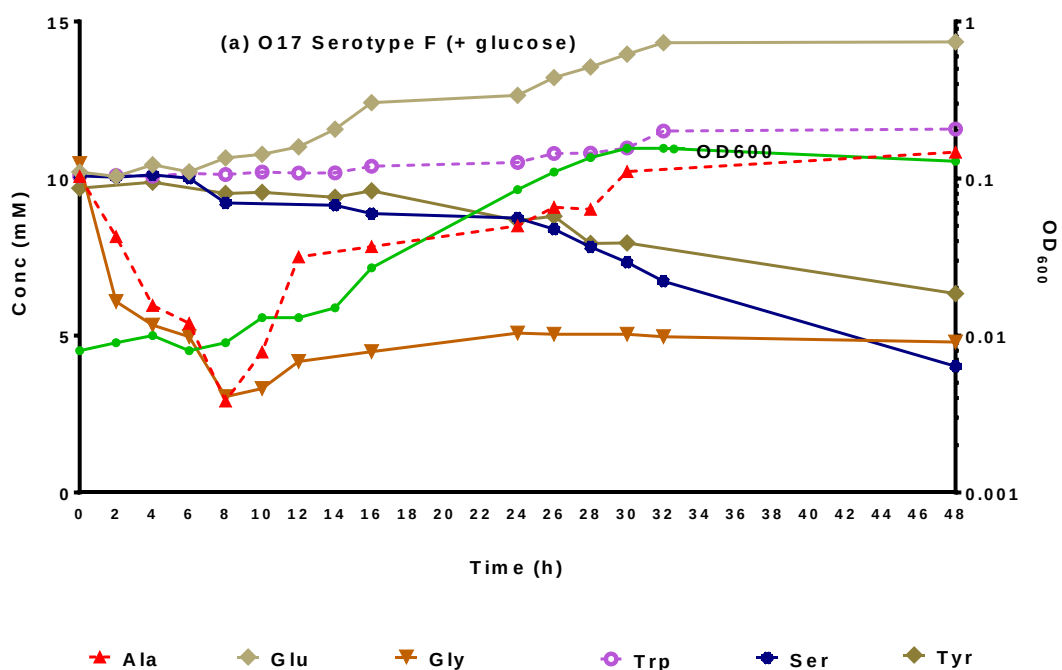
Figure 4-8: Amino acid utilisation profile for *C. difficile* L26 grown in DMaa10mM for 48 h in the presence (a) and absence (b) of glucose.

Shown are profiles for amino acids for which an overall consumption of more than 30 % was observed under at least one of the indicated conditions. Data shown are from three independent cultures. Growth (OD₆₀₀) is indicated in green. Error bars not shown for clarity. P value for growth in DMaa10mM medium in (a) presence <0.0001 and (b) absence of glucose <0.0001

4.3.3.8 Amino acid utilisation profile for *C. difficile* 017 Serotype F

Strain 017 reached low final ODs in DMaa10mM and exhibited long lag phases both in the presence and absence of glucose (Figure 4-9). Amino acid utilisation also differed in many ways from that observed for most other strains. For instance, apart from L26, 017 was the only strain to consume a small amount of aspartate (less than 30 %), both in the presence and absence of glucose. It was also the only strain that utilised considerable amounts of glycine under these conditions and excreted tryptophan and glutamate. It was also the only strain not to use valine at least under one of the conditions tested. However, like all other strains alanine was first utilised and later on excreted, albeit to levels lower than those observed for other strains.

Interestingly, utilisation of alanine and glycine took place during the first 8 h of the lag phase, whereas other amino acids such as tyrosine and serine were consumed to a small degree during the exponential phase and to a large extent after entry into stationary phase, both in the presence and absence of glucose. In contrast to the other strains, notably leucine, isoleucine utilisation only started at the beginning of stationary phase in the absence of glucose, while serine utilisation occurred both in the presence and absence of glucose.



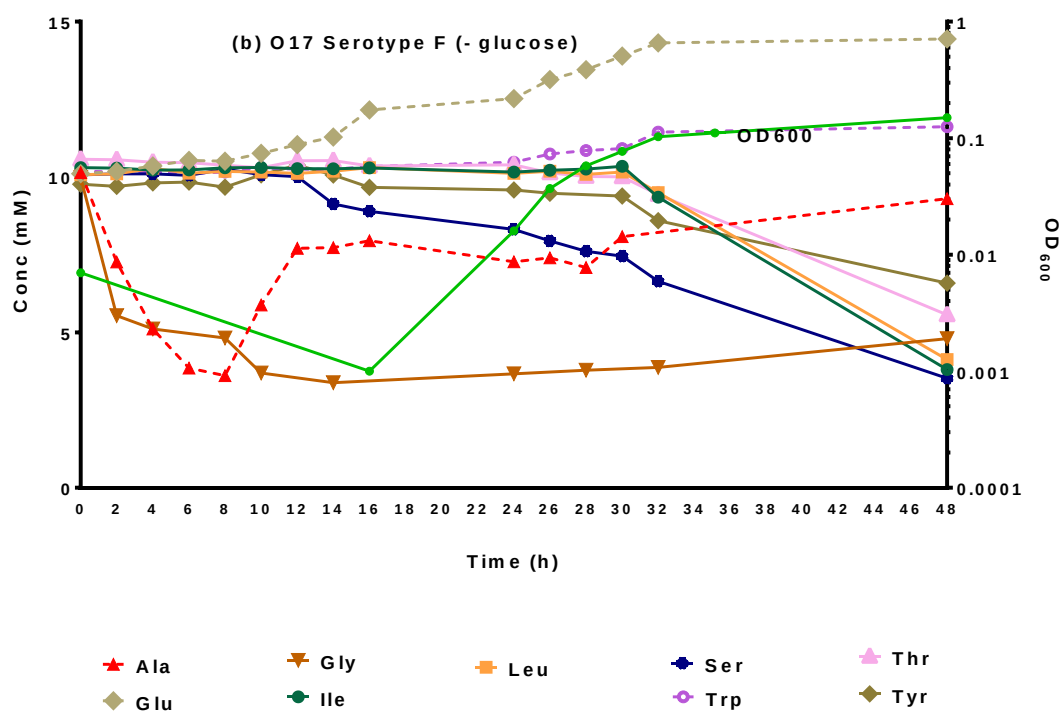


Figure 4-9: Amino acid utilisation profile for *C. difficile* 017 grown in DMaa10mM for 48 h in the presence (a) and absence (b) of glucose.

Shown are profiles for amino acids for which an overall consumption of more than 30 % was observed under at least one of the indicated conditions. Data shown are from three independent cultures. Growth (OD₆₀₀) is indicated in green. Error bars not shown for clarity. P value for growth in DMaa10mM medium in (a) presence <0.0001 and (b) absence of glucose <0.0001

Having tested the growth performance of these strains in DMaa10mM (subsection 4.3.1), we investigated further to see which amino acids were preferably metabolized (subsection 4.3.2) and at which growth phase using Liquid Chromatography – Mass Spectrometry. Results obtained under the tested conditions suggest that growth of *C. difficile* is dependent on its ability to utilise certain amino acids in the presence and absence of a fermentable carbohydrate. Although the utilisation of amino acids by most strains tested was comparable in the presence and absence of glucose, they however differed in their preference. This may be explained by the differences in the bacterial strains as well as their nutritional requirements, which have previously been reported by Nuemann-Schaal *et al.* [120] and Wallace *et al.* [401]. From the results, lag, log and stationary phase of growth were clearly established in this medium with the level of amino acids staying approximately the same during the lag phase of growth for most strains. This may largely be attributed to the fact that the cells are just beginning to adapt to the growth conditions by synthesizing RNA and protein, and for this require considerably less external carbon and energy sources as when actively growing, or possibly modifying their intracellular amino acids pools, which for many strains takes time as previously reported [350, 392]. Once the cells are adjusted and have started growing exponentially, there is an increased demand for nutrients due to the expression of the appropriate enzymes for growth. Nuemann-Schaal *et al.* [120] recently demonstrated under their test conditions, that the entry of these organisms into the stationary phase of growth was not necessarily caused by the lack of energy source, but by the lack of a carbon source usable for biomass production. These authors further established a clear difference in the utilisation of amino acids and formation of fermentation products by *C. difficile* 630 Δ erm similar to the findings in this study. For example, glutamate was not used despite its importance as a nitrogen (amino group) donor [394-397] and the fact that most *C. difficile* strains are able to produce the enzyme glutamate dehydrogenase, required for the first step of glutamate breakdown [398-400]. The increased demand for isoleucine and leucine as the preferred substrates by most *C. difficile* strains has been reported by a number of authors [120, 332, 402-404]. Alanine, like in previous reports [119, 120, 392], has been shown to be an exceptional amino acid in this study. As already mentioned, for most of the strains tested, this amino acid was initially utilised during the first 6 to 8 h and from then on secreted into the medium. The reasons for this are unclear but it could be due to the use of pyruvate as an amino acceptor during the uptake of and metabolism of preferred amino acids.

Previous studies by Fonknechten *et al.* [119] on a related strain, *C. sticklandii*, revealed that alanine was excreted into the medium during the exponential phase and subsequently disappeared in the stationary phase [119]. Under the growth conditions tested, these authors suggested that the excretion of alanine could be due to the breakdown of ornithine which was used to constitute the medium [119].

4.4 Conclusions

As illustrated in the current work, *C. difficile* 630 Δ erm, DH 196, R20291, EK 15, EK 28, R12801, L26, 017 Serotype F strains were successfully grown on DMaa10mM medium, containing all proteinogenic amino acids at a concentration of 10 mM, except tyrosine (0.25 mM) in the presence and absence of glucose. Although all strains grew on the fully defined medium, growth profiles were different in terms of lag phase, growth rate, and maximum OD reached. The LC-MS/MS analysis enabled the identification of amino acids that were preferentially utilised. Comparison of the amino acid LC-MS/MS profiles indicates that strains L26 and 017 differed remarkably in their utilisation pattern when compared to others. L26 is shown to have utilised several amino acids not metabolised by the other seven strains or utilised them to a larger extent, whereas 017 was the only strain to consume a moderate amount of aspartate, both in the presence and absence of glucose. 017 was also the only strain not to use valine, but used considerable amounts of glycine and excreted tryptophan and glutamate under the tested conditions. For most strains, data generated showed that cysteine, glutamine, isoleucine, leucine, serine, threonine and tyrosine were utilised to a large extent, whereas arginine, aspartate, glutamate, histidine, lysine, methionine and tryptophan were not or marginally consumed, except by strain L26. Particularly surprising, is the lack of glutamate utilisation which is an important nitrogen (amino group) donor [394-397] and the fact that enzyme glutamate dehydrogenase, required for the first step of glutamate breakdown, is secreted by most of the *C. difficile* strains [398-400]. This result seems to agree with previous studies suggesting that glutamate is not a substrate for most *Clostridium* species [119, 120, 128, 405].

As shown in previous studies [119, 120, 392], alanine is an exceptional amino acid in this study. Alanine was initially degraded in the presence and absence of glucose by most of the tested stains, but concentrations in the medium increased afterward following entry into the exponential or stationary phases of growth. The reason for this was not definitively established but may be due the use of pyruvate as an amino acceptor during the degradation of preferentially fermented amino acids.

Overall, the data obtained confirm that this organism can derive carbon and energy from the fermentation of amino acids in agreement with previous reports [126, 332, 406-408].

CHAPTER 5.

EFFECT OF SELENIUM ON THE METABOLISM OF AMINO ACIDS BY *CLOSTRIDIUM DIFFICILE*

5 EFFECT OF SELENIUM ON THE METABOLISM OF AMINO ACIDS BY C. DIFFICILE

5.1 Introduction

Selenium is one amongst several elements that play key roles in the proper functioning of a number of living organisms [409-413]. Early reports have shown that certain cell processes and enzymes have an absolute requirement for selenium, but also that this element can become toxic at elevated levels [413-415]. First described by the Swedish Chemist and Doctor Jöns Jakob Berzelius in 1817 as a chalcogen, it shares many physical properties with sulphur and, as a result, is readily taken up and metabolized via sulphur transporters and pathways [409, 416, 417]. Selenium is reported to be present in at least twenty five identified selenoproteins, as selenocysteine (Sec), a rare amino acid associated with redox reactions occurring in the active site of many enzymes and proteins [409, 418, 419]. In 1973, the first selenoproteins were discovered in which selenium is covalently bound, glycine reductase present in *Clostridium sticklandii*, formate dehydrogenase present in *Clostridium thermoacetum* [420, 421] and glutathione peroxidase present in humans [422]. Selenoproteins have been identified in many bacterial pathogens such as *Salmonella typhimurium*, *Escherichia coli*, *Campylobacter jejuni* and *Haemophilus influenzae* using their genome sequences [423]. Selenocysteine, also referred to as the 21st amino acid [424], is uniquely described amongst the proteinogenic amino acids and encoded by an opal stop codon, uracil-guanine-adenine (UGA) [413, 416, 425, 426]. In the biogenesis of selenocysteine proteins, four gene products are said to be directly involved namely selenocysteine synthase (SelA), a special translation factor that binds guanine nucleotides and specifically recognizes selenocystyl-tRNA^{Sec} (SelB), a Sec-specific tRNA (SelC) and the selenophosphate synthase (SelD) [413, 427, 428]. It is well established that UGA normally serves as a termination codon that dictates the cessation of protein synthesis, but when present in the correct context it can be recognised by the translation machinery as selenocysteine-encoding by several organisms [425].

Selenium requirements seem to stem largely from its role in selenoenzymes as well as its incorporation into selenocysteine [413]. Although not all organisms require selenium for growth, those that do need selenium are able to incorporate it into a catalytic form within proteins and enzymes as an important micronutrient [423].

More importantly for this study, selenocysteine containing enzymes are also known to play a role in *C. difficile* amino acid metabolism, notably the breakdown of glycine and proline using two selenoenzymes, glycine reductase and D-proline reductase [423, 429, 430].

5.1.1 Selenium metabolism in bacteria

Selenium metabolism can be found in all domains of life including Bacteria, Archaea, Eukarya and viruses [425, 431-433]. As previously reported, selenium can be incorporated into amino acids such as cysteine and subsequently into selenium containing proteins [434]. Selenium has been associated with a number of metabolic functions such as detoxification, assimilation, anaerobic respiration and methylation [434]. In bacteria, selenium is readily metabolized and processed in a non-specific or specific manner, if these organisms are challenged with low molecular weight selenium compounds [435]. The non-specific manner is attributed to the chemical similarity between sulphur and selenium, meaning that microorganisms may absorb selenium inside the cell interior by the use of transport systems [416, 435-437]. For instance, the presence of sulphate (VI) in the culture medium would influence the transport of selenate (VI). This means that the absent of sulphur in the medium, will increase the organism's ability to absorb selenium [435] as previously reported for selenate uptake via a sulphate transporter in *Escherichia coli* and yeast [438]. As depicted in Figure 5-1, in the presence of selenite in *E. coli* at a concentration higher than 1 μM , the sulphite reduction pathway is intruded by selenium and it is metabolised along the routes of sulphur metabolism [416, 437]. According to previous reports, selenium in the form of selenate is reduced to selenide via the assimilatory sulphate reduction system, having initially been taken up by the sulphate transport system [437]. However, when present in the form of selenite, Bock [437] speculated that the reduction of selenium occurs by chemical interaction with thiol compounds such as glutathione. Free selenocysteine is formed as the first organic selenium compound and converted to selenocystathionine and thereafter to selenomethionine. Similarly, selenocysteine presents as a substrate for cysteyl-tRNA ligase which forms selenocysteyl-tRNA^{cys}, incorporating in proteins at cysteine positions [437]. The relative catalytic efficiencies of cysteyl-tRNA synthetase and

cytathionine synthetase for the substrate cysteine may determine the incorporation of selenium non-specifically as selenocysteine or selenomethionine [437].

The specific manner of selenium metabolism on the other hand, is not performed by a majority of microorganisms. Previous report indicated that bacteria with this ability will require several specific *cis* and *trans* acting proteins to incorporate selenocysteine into proteins [439]. The UGA codon determines the positions at which selenocysteine inserts into the nascent polypeptides as well as the secondary and tertiary structure, designated selenocysteine insertion sequence (SECIS) that is within the messenger RNA. The *trans* element consists of a transfer ribonucleic acid (tRNA) species, tRNA^{Sec}, which is charged by the cellular seryl-tRNA synthetase and is associated with conversion of the seryl moiety into the selenocysteyl product, catalysed by the biosynthetic enzyme selenocysteine synthase [437]. Other elements for cotranslational incorporation at particular stop codons include a selenophosphate synthase and a selenocysteine-specific elongation factor [439, 440]. Selenocysteine-specific elongation factor, which binds selenocysteyl-tRNA^{Sec} is reported to display a sequence similarity to elongation factor Tu, with the capacity to discriminate between serylated and selenylated tRNA^{Sec} [439, 441, 442]. During translation, the SECIS element recruits Sec-tRNA^{Sec} complex in order to facilitate the recoding of the UGA stop codon as previously reported [439, 441, 443, 444]. The best understood system for the specific manner of selenium metabolism has been described in *E. coli* in which the translational mechanism has been reported by a number of authors [430, 445].

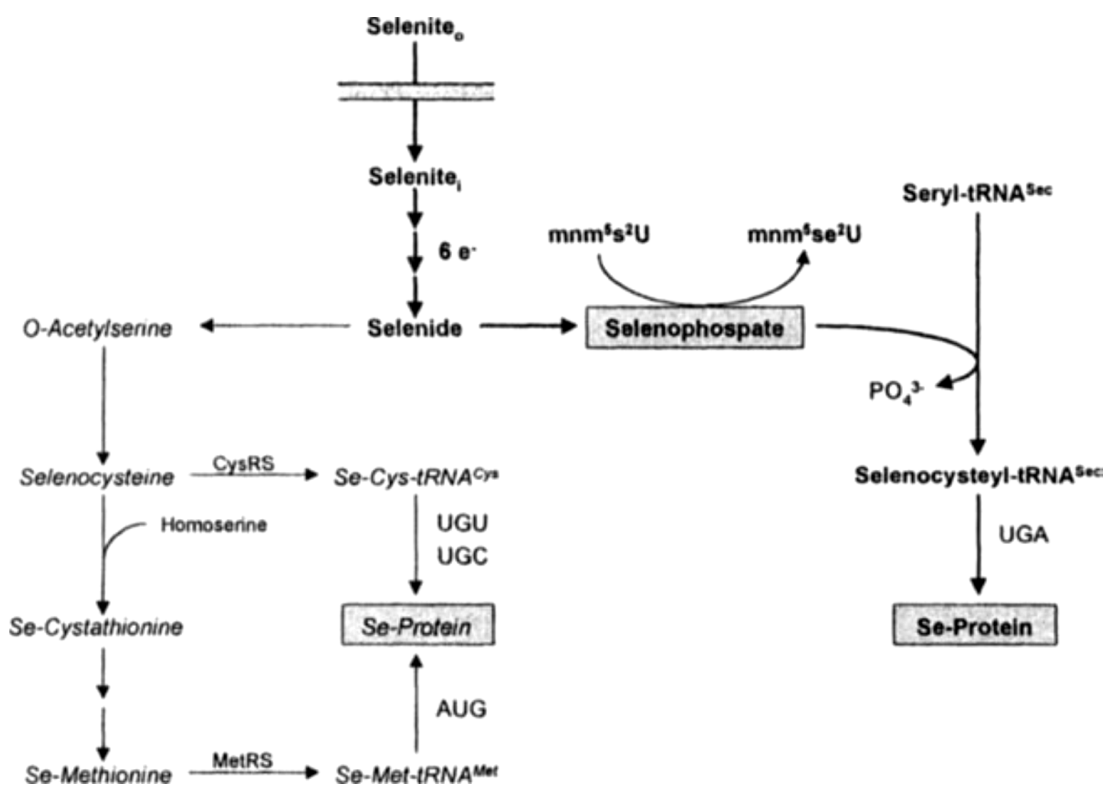


Figure 5-1: Specific and non-specific metabolism and incorporation of selenium into macromolecules.

The specific pathway is highlighted in bold. $\text{Mnm}^5\text{S}^2\text{U}$ is the abbreviation for 5-methylamino-methyl-2-thiouridine and $\text{mnm}^5\text{se}^2\text{U}$ for 5-methyl-aminomethyl-2-selenouridine. Reproduced without modifications [437].

5.1.2 Global and biochemical selenium cycles

Microorganisms are known to play a key role in the cycling of selenium compounds in nature [434]. As an essential element, selenium is assimilated from selenate or selenite by microbes and plants at the base of the food web and then by animals, and thereafter incorporated into organoselenides (i.e., selenoproteins) in living organisms. The environment on the other hand, receives back selenium via the decomposition of dead organisms [434]. Selenium can be released into the atmosphere, soil, and water in soluble forms (i.e., selenate and selenite) through mining operations, combustion of fossil fuels, agriculture, volcanic eruptions, and nuclear fuel cycle operations [434]. Figure 5-2, presents the complexity of the selenium cycle in nature, which is attributed to the wide range of its oxidation state from $-II$ to VI as well as its availability in different chemical and physical forms [434].

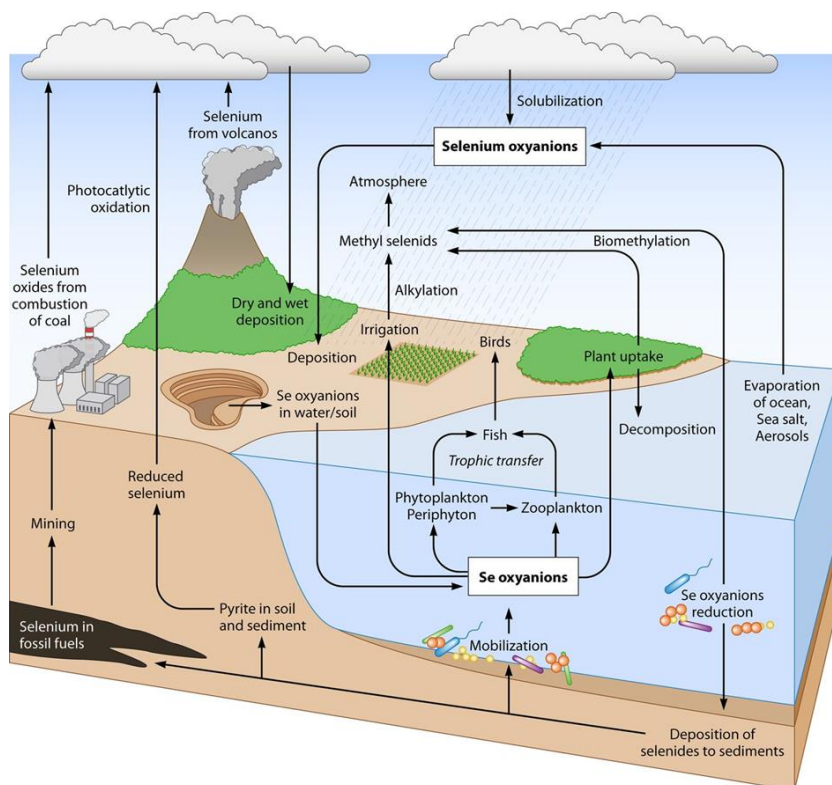


Figure 5-2: Global selenium cycle in nature. Reproduced from Nancharaiah and Lens [434].

As an essential trace element, selenium's biochemical cycle in nature is currently receiving increased attention, even as selenium-respiring bacteria are becoming widespread and metabolically active and consequently affecting the carbon, nitrogen and phosphorus cycles in nature. As already mentioned above, the transformation of selenium in nature is being mediated by both biological and chemical mechanisms. As shown in Figure 5-3, the role of microorganisms in performing the oxidation and reduction reactions is becoming increasingly evident [434], especially in the oxidation of elemental selenium to yield selenide (Se^{2-}) or selenite (SeO_3^{2-}) as the end products [434, 446-448]. Here, disproportionation occurred via the simultaneous reduction and oxidation to form two different products. Selenium reducing-bacteria are ubiquitous in natural settings and reported to have a key role to play in the biochemical selenium cycle as many have the ability to use selenate (SeO_4^{2-}) and/or selenite as a terminal electron acceptor in anaerobic respiration. The degradation of organic matter in anaerobic sediments for instance is coupled to the reduction of

selenium oxyanions. Under anaerobic conditions, selenium oxyanions can be reduced to elemental selenium by bacteria. In assimilatory selenate and selenite reduction, selenide is formed and incorporated into selenoproteins by some bacteria, while the dissimilatory reduction of selenate and selenite to elemental selenium seems to be a major sink for selenium oxyanions in anoxic sediments. For example, *Sulfurospirillum barnesii* and *Bacillus arseniciselenatis* have been reported to reduce Se(VI) or Se(IV) to insoluble elemental selenium, while reduction of elemental selenium by *Bacillus selenitireducens* leads to the formation of selenide. Selenide on the other hand is then oxidized aerobically, for instance by species of the genus *Thiobacillus* and *Thiothrix* (so called selenium-oxidizing bacteria). Figure 5-3 also depicts the alkylation reactions, which produce volatile dimethylselenide - $(\text{CH}_3)_2\text{Se}$ and $(\text{CH}_3)_2\text{Se}_2$, as well as dealkylation mediated by microorganisms in soil and water [434].

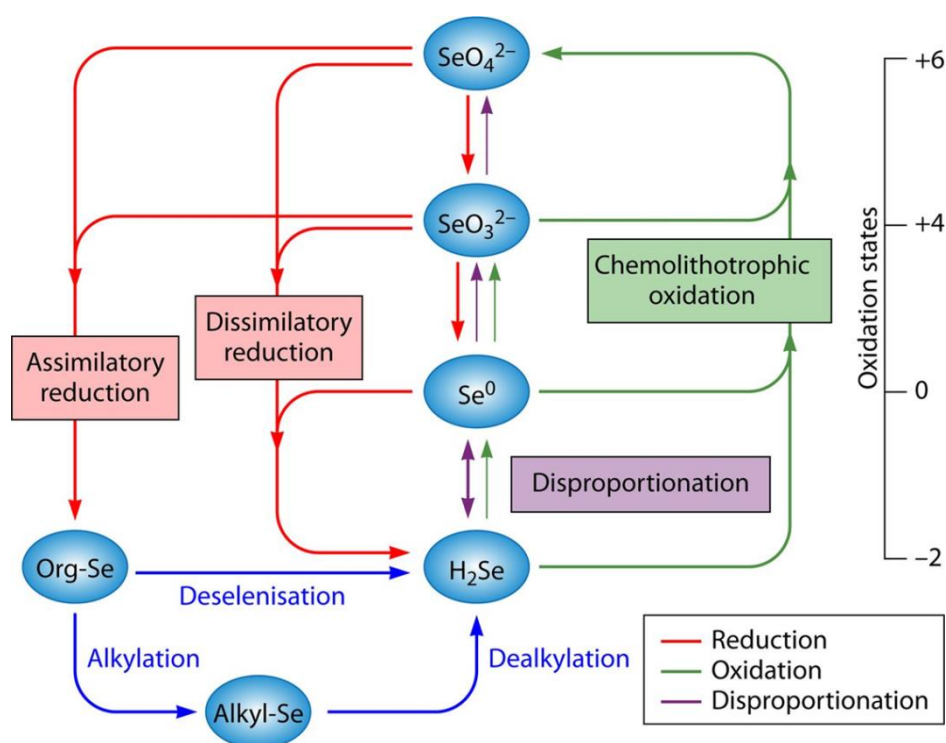


Figure 5-3: Selenium transformations in nature.

Geologic and anthropogenic sources release selenium as SeO_4^{2-} into the environment. In natural settings, Selenium-respiring bacteria are said to be ubiquitous and play vital roles in the biochemical selenium cycle. Selenium-respiring bacteria in anaerobic respiration, are reported to possess special ability to use selenate and/or selenite as a terminal electron acceptor. The reduction of selenium oxyanions (SeO_4^{2-} and SeO_3^{2-}) is coupled to the degradation of organic matter in anaerobic sediments. Under anaerobic conditions, SeO_4^{2-} and SeO_3^{2-} can be reduced to Se^0 by bacteria. Some bacteria reduce SeO_4^{2-} and can incorporate it into organic compounds. A major sink for Se oxyanions in anoxic sediments seems to be the reduction of SeO_4^{2-} to Se^0 . The production of insoluble elemental Se^0 can result in the reduction of Se (VI) or Se (IV) by selenium-respiring bacteria like *Sulfurospirillum barnesii* and *Bacillus arseniciselenatis*. Similarly, oxidation of elemental selenium, can occur in soils and sediments, albeit at lower rates. Selenide may be formed by a further reduction of elemental Se^0 by other microorganisms like *Bacillus selenitireducens*. This selenide is then oxidized aerobically by *Thiobacillus*, *Thiothrix* (selenium-oxidizing bacteria). Other important processes mediated by microorganisms in soil and water include, dealkylation, alkylation reactions, which produce volatile $(\text{CH}_3)_2\text{Se}$ and $(\text{CH}_3)_2\text{Se}_2$. Reproduced from Nancharaiah and Lens [434].

5.1.3 Justification of the study

Although selenium has been shown to be an essential component for the growth and metabolism of most living organisms, its effect on metabolism of amino acids, particularly by *Clostridium difficile*, is largely unstudied. Perhaps, this is due to the specific growth requirement of the majority of the *Clostridium difficile* strains, coupled with the thin line that exists between the amount of selenium that exerts toxic effects and that which has beneficial effects on an organism [409, 416, 434, 438, 449-451]. Experiments with two *C. difficile* strains (ATCC 9689, 630) showed that the presence of certain amino acids reduced or increased growth yield in the presence and absence selenium/glucose in the medium [126]. In particular, it has been shown that the addition of certain amino acids such as proline and glycine in the presence of selenium could enhance the growth of these bacteria due to the presence of an appropriate enzyme system, proline reductase and glycine reductase [120, 332, 452-454]. Proline and glycine amongst other amino acids have been identified as the most efficient electron acceptors in coupled Stickland reaction metabolism by *C. sporogenes* and *C. sticklandii* [332, 453-456]. As already mentioned, the reduction of these two amino acids is performed by two selenium-dependent reductases, D-proline reductase and glycine reductase. Previous reports indicate that proline reductase reductively cleaves D-proline to 5-aminovalerate, while glycine reductase on the other hand catalyses the reductive deamination of glycine to acetyl phosphate and ammonium [332, 452, 457]. D-proline reductase in particular, has been associated with the generation of proton motive force, which is presumably the major energy-yielding pathway for a majority of Stickland amino acids fermentative bacteria [452, 458]. Previous reports have revealed that selenoprotein expression in various organisms reaches a maximum value at low concentration of selenium in the medium as opposed to higher concentrations which result in lipid peroxidation of cell membranes [459]. Costilow [408] previously described the optimal growth of *C. sporogenes* in a basal medium, using L-alanine as the reductant and DL-proline as the oxidant. In this medium, glycine was substituted for proline, and at the end of incubation period no growth was observed beyond the background level observed without added oxidant. However, when sodium selenite was added at a concentration of 1 μ M, the total growth observed with glycine was equal to that observed with proline. In related studies by Kessi *et al.* [460] it was

shown that in the presence of selenite in a culture medium at a concentration of 1.5 mM, the phototrophic bacterium *Rhodospirillum rubrum* reached final cell densities that were only about 15% of the control final cell density. The cell densities were found to have remained nearly constant during the stationary phase for all tested concentrations. In the previous chapter, we observed amino acid utilisation in the absence of selenium, although small amounts are likely to have been introduced as contaminants together with the other nutrients added. Standard media used for *C. difficile* are usually not supplemented with defined amounts of selenate or selenite, although selenium may be introduced via certain ingredients such as yeast extracts. Thus the effect of selenium on growth, sporulation, toxin production and metabolism has not yet been carefully assessed. Here we wanted to see whether and how amino acid fermentation changed when selenium was provided. Understanding the role of selenium in amino acid fermentation may provide a viable pathway for antimicrobial development.

5.1.4 Aim and objectives

The objective of the undertaken study was to evaluate the metabolism of amino acids by *C. difficile* cultivated in control and experimental defined media enriched with selenium. This is to be achieved by;

- Applying the methodology developed in chapters two and three to study effect of selenium on the growth of *C. difficile* strains
- Measuring amino acid concentrations in spent *Clostridium difficile* culture media to determine possible changes and, if so, the order in which different amino acids are being utilised.

5.2 Materials and Methods

5.2.1 Bacterial strains

The *Clostridium difficile* strains used in this study and their origin are presented in Table 5-1 below.

Table 5-1: Origin of *C. difficile* strains used

Strain name	PCR Ribotype	Origin	References
CD630 Δ <i>erm</i> *	PCR ribotype 012	Strain obtained from Peter Mullany lab, UCL (London)	[383]
R20291*	PCR ribotype 027	R20291: Jon Brazier (Cardiff), Stoke Mandeville outbreak	Nottingham strain
CRG-2979**	PCR ribotype 027	Dr Daniella Heeg, University of Nottingham, unpublished	Nottingham strain
CRG-3887***	PCR ribotype 027	Dr Daniella Heeg, University of Nottingham, unpublished	Nottingham strain

CRG = Clostridium Research Group, Centre for Biomolecular Studies (CBS), University of Nottingham. *Used as reference strains; **mutant defective in *hadB*, encoding one of the two subunits of hydroxyisocaproyl-CoA dehydratase required for reductive degradation of leucine; ***mutant defective in *sela* encoding selenocysteine synthase.

5.2.2 Reagents and materials

Sodium selenate decahydrate ($\text{Na}_2\text{SeO}_4 \cdot 10\text{H}_2\text{O}$) was of high purity (99.999%) and obtained from Sigma-Aldrich (Gillingham, UK). It was used with minimum handling to avoid possible contamination. Details of all other chemicals used are as previously described in Chapters 2 (subsection 2.2.1) and 3 (subsection 3.2.2).

5.2.3 Sample preparations

Amino acid standards, internal standards, calibration solution and quality control samples used in this experiment were prepared as previously described in chapter two (subsection 2.2.3).

5.2.4 Cultivation of bacterial strains

5.2.4.1 Composition of the growth medium

Cultivation of the bacterial strains was performed on the fully defined media previously described in chapter three (subsection 3.2.3) with modifications. The modifications were the addition of sodium selenate to a final concentration of 1 μ M to DMaa1mM and DMaa10mM medium, respectively (see Table 4-2). For this a 1000 fold concentrated (1 mM) stock solution was prepared (in water), filter-sterilised, and stored at 4 °C.

5.2.4.2 Inoculum preparation

All *Clostridium difficile* strains used in this experiment were prepared as previously described in chapter three (subsection 3.2.4).

5.2.4.3 Growth experiment

The defined media were inoculated with *Clostridium difficile* 630 Δ erm, CRG-2979, CRG-3887, and R20291 strains in triplicates at a 1 in 100 dilution. An uninoculated flask containing defined media only was used as blank to compare with inoculated flasks. The inoculated and uninoculated flasks were maintained in the anaerobic cabinet at 37 °C for up to 48 hours. Determination of the optical density of an actively growing culture was performed by measuring absorbance at 600nm (or OD₆₀₀) using Biomate 3 spectrophotometer. Cultures were diluted for a more accurate measurement if OD readings were greater than 0.2.

5.2.5 Sample Processing for LC-MS analysis

Samples were collected every 2 hours (0, 2, 4, 6, 8, 10, 12, 14, 16); also after 24, 26, 28, 30, 32, 48 hours for CD 630 Δ erm strain, 0 and 24 hours for the CRG-2979, CRG-3887, R20291 strains and processed as previously described in chapter 4 (subsection 4.2.4). The obtained supernatants were diluted to an approximate concentration of 10 μ M using 50 % water : 50 % acetonitrile mixture. 100 μ l of each sample (thoroughly mixed) was dispensed into 2 ml vials ready for LC/MS analysis.

5.2.6 Liquid chromatography-mass spectrometry analysis

Liquid chromatographic analysis of *C. difficile* 630 Δ erm, CRG-2979, CRG-3887, R20291 spent medium was performed as previously described in chapter 2 (subsection 2.2.4) using a cogent diamond hydride column with mobile phase consisting of [A] 100 % water with 0.1 % v/v formic acid, and [B] 100 % acetonitrile with 0.1 % v/v formic acid. The LC-MS conditions are described in chapter 2 (subsections 2.2.4 and 2.2.6).

5.2.7 Data processing/reporting

To ensure reproducibility, all experiments were performed in triplicate and at least three times. Mean OD₆₀₀ values of triplicate tests performed were used to generate graphs or bar charts in relation to time using Graphpad Prism 6 as previously described in chapter three (subsection 3.2.6). LC-MS data acquired in this experiment were processed as previously described in chapter 4 (subsection 4.2.6). Data were analysed by Analysis of Variance using Graphpad Prism 6 and data sets obtained are indicated in the respective chart.

5.3 Results and Discussion

5.3.1 Growth performance of *C. difficile* 630 Δ erm in the presence of selenium

The effect of selenium addition on growth and amino acid utilisation was explored for the *C. difficile* 630 Δ erm. Growth media DMaa1mM and DMaa10mM, containing all amino acids at 1 mM and 10 mM concentration, respectively, except tyrosine (see subsection 0) were supplemented with sodium selenate to a final concentration of 1 μ M. An overview of the growth performance of *C. difficile* 630 Δ erm in these media is presented in Figure 5-4. Addition of selenate to DMaa1mM reduced growth considerably, both in the presence and absence of glucose, rather than enhancing it. In fact, when selenate was present, very similar and limited growth was observed with and without glucose addition. In contrast, the addition of selenate to DMaa10mM resulted in moderate OD decreases or no changes, depending on conditions. Although growth in the presence of glucose was somewhat reduced when selenate was added, no significant final OD changes were observed in its absence. Growth rates for DMaa10mM cultures containing selenate appeared to be somewhat increased, but otherwise the growth curves obtained for these cultures looked very similar to the ones obtained for DMaa10mM without glucose and without selenate (Figure 5-4 b). The reasons for the growth inhibitory effect of selenate in DMaa1mM medium remain unclear. If selenate had general toxic effects at this concentration, due to non-specific metabolism along the sulphur pathways [461], this should also have been observed in DMaa10mM medium. The fact that growth was not affected in the latter when glucose was absent, and that selenate addition even appeared to increase growth rates, suggests that the observed phenomenon has a regulatory basis. It is possible that under conditions of sufficient amino acid supply, glucose utilisation is reduced or suppressed even though this leads to a reduction in growth yield. This hypothesis can be tested in future experiments by analysing glucose consumption under the above conditions.

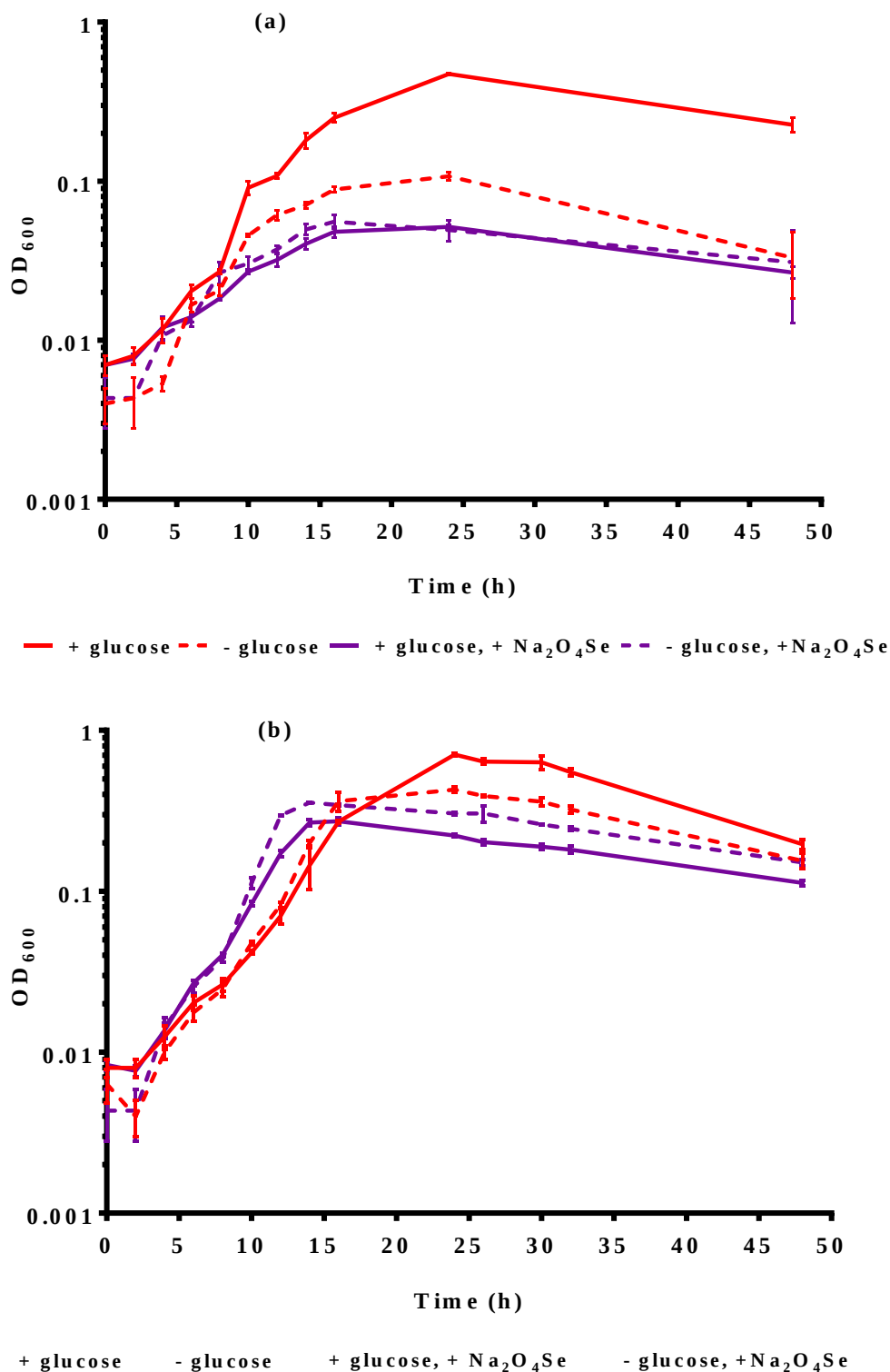


Figure 5-4: Growth of *C. difficile* 630 Δ erm in DMaa1mM (a) and DMaa10mM (b) media, with and without selenium and glucose.

Selenium was added in the form of sodium selenate at a concentration of 1 μ M. DMaa1mM (a) contained all amino acids at a concentration of 1 mM with the exception of tyrosine (0.25 mM), $P < 0.0001$; DMaa10mM (b) contained all amino acids at a concentration of 10 mM with the exception of tyrosine (0.25 mM), $P < 0.0001$. Data shown are from three independent cultures. Error bars indicate standard deviations.

5.3.2 LC-MS analysis of *C. difficile* 630 Δ *erm* spent medium

To measure the changes in amino acid content in the above culture media after 24 hours of incubation, LC-MS analysis was performed as previously described (section 2.2.4 and 2.2.6) and the results obtained are presented in Table 5-2. As shown, the amino acids were divided into four groups based on their consumption after 24 h: highly utilized amino acids, with more than 60 % disappearing from the medium (highlighted in green), moderately utilized amino acids, with 30 to 59 % disappearing from the medium (highlighted in blue), the poorly utilized amino acids, with less than 30 % disappearing from the medium (highlighted in yellow) and those not utilized or utilised to less than 10 % (highlighted in pink). Addition of selenate to DMaa10mM had several interesting effects on the utilisation of amino acids by *C. difficile* 630 Δ *erm* and these were independent of the presence of glucose. As shown before (chapter 4) strain 630 Δ *erm* highly or moderately consumed ten of the offered twenty amino acids. Among these, in the presence of selenate only two remained unchanged (leucine, isoleucine), five were consumed to a lesser degree (cysteine, glutamine, glycine, threonine, tyrosine, valine) and two showed increased utilisation (proline and serine). There was little or no consumption of alanine, arginine, asparagine, aspartic acid, glutamic acid, histidine, lysine, methionine, phenylalanine and tryptophan. The increased use of proline was expected as the key enzyme involved in fermentative proline breakdown, proline reductase, is selenium dependent [332, 452, 462]. Based on previous findings [120, 332, 452-454] an increase in the utilisation of glycine would also have been expected, as the key enzyme glycine reductase also contains selenium in the form of selenocysteine. However, the size of glycine (smallest possible amino acid) and possibly the concentration remaining in the spent medium, made it impossible to measure accurately enough in order to show differences. Thus, in conclusion, addition of selenate considerably altered the amino acids utilisation profile of strain 630 Δ *erm*.

Table 5-2: Percentage utilization of amino acids by *C. difficile* 630 Δ erm after 24 hour incubation in DMaa10mM medium in the presence and absence of glucose and selenium.

Amino acids	With glucose		Without glucose	
	- Na ₂ O ₄ Se	+ Na ₂ O ₄ Se	- Na ₂ O ₄ Se	+ Na ₂ O ₄ Se
Alanine*	≤1	≤1	≤	≤1
Arginine	5	5	5	5
Asparagine	4	3	3	3
Aspartic acid	≤5	≤5	≤5	≤5
Cysteine	58	42	60	40
Glutamine	60	28	55	32
Glutamic acid	≤5	≤5	≤5	≤5
Glycine	10	10	10	10
Histidine	≤5	≤5	≤5	≤5
Isoleucine	65	60	75	73
Leucine	68	62	81	78
Lysine	≤5	≤5	≤5	≤5
Methionine	≤5	≤5	≤5	≤5
Phenylalanine	5	5	5	5
Proline	38	48	35	52
Serine	63	100	70	100
Threonine	100	55	80	48
Tryptophan	5	5	5	5
Tyrosine	26	5	35	15
Valine	32	10	39	23

Pink = <10 %; **Yellow** = ≥10-29 %; **Blue** = ≥30-59 %; **Green** = ≥60 %; *Excreted into the medium; +Na₂SeO₄ = with sodium selenate, -Na₂SeO₄ = without sodium selenate.

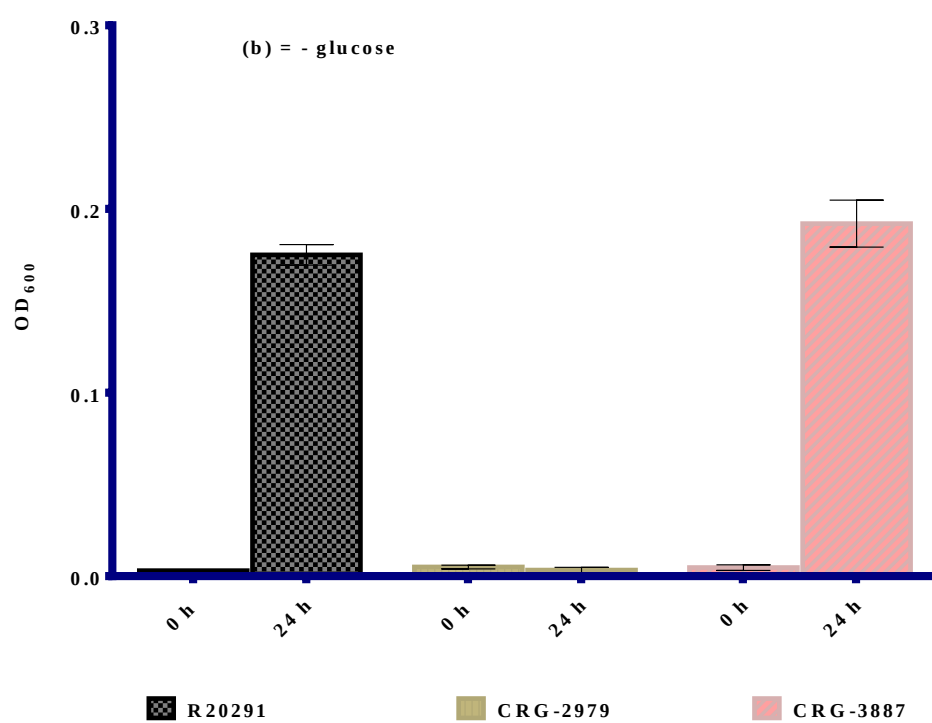
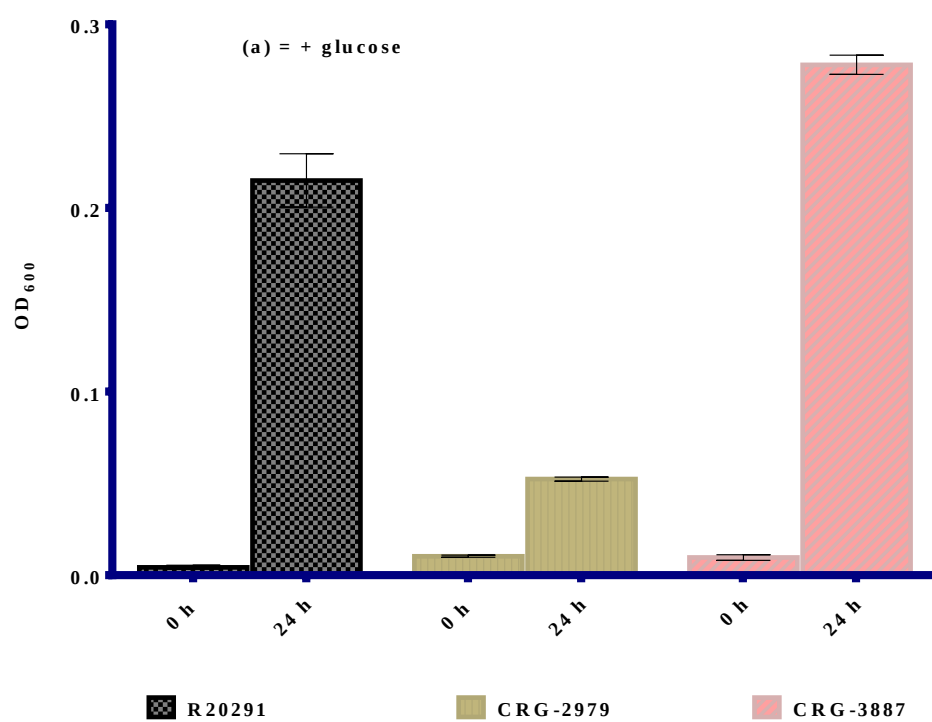
5.3.3 Growth performance of R20291 (parent strain), CRG-2979 (leucine fermentation mutant), CRG-3887 (selenocysteine synthase mutant) in the presence and absence of selenium.

To further examine the effect of selenium on growth and amino acid metabolism, we cultivated the *C. difficile* R20291 parent strain, CRG-2979 mutant (lacking the reductive branch of leucine fermentation) and CRG-3887 mutant (defective in selenocysteine synthase) in the fully defined DMaa10mM with and without selenium, in the presence and absence of glucose. Selenium was supplemented in the form of sodium selenate at a concentration of 1 μ M and the resultant changes monitored using a spectrophotometer. The results presented in Figure 5-5, shows that only mutant CRG-2979 was unable to grow in the absence of glucose. Although there was a little growth in the presence of glucose, but not as much as *C. difficile* R20291 parent strain and CRG-3887 mutant. In the presence of selenium, both mutants as well as the parent strain seems to thrive well in the presence and absence of glucose. Although the OD value recorded in the presence of selenium was lower than what was recorded in the absence of selenium.

This results seems to suggest that Stickland reactions are essential for growth, at least in the absence of glucose, proceeding via leucine in the absence of selenium or selenium-dependent reactions in the absence of leucine. Stickland reactions also appear to be required for optimal growth in the presence of glucose, as shown by the much reduced growth of CRG-2979 in the absence of selenium. The importance of leucine fermentation is in line with the observations made in chapter 4, which showed that this amino acid was highly utilised by almost all strains tested, both in the presence and absence of glucose (Table 4-3). Although mutants defective in the breakdown of other preferentially used amino acids were not analysed in this study, it appears that leucine acts a major Stickland acceptor in the absence of selenium [463-465]. Its role is not essential, however, when selenium is present, as this allows the bacterium to produce the selenocysteine-containing enzymes proline reductase and glycine reductase, which play key roles in the fermentation of proline and glycine, respectively [406, 466].

Jackson *et al.* [452] had previously demonstrated that Stickland reaction was essential for the growth of *C. difficile* in the presence of selenium. These authors showed that the addition of Stickland donor alanine had no effect on growth, but when D-proline and glycine (Stickland acceptors) were added, significant increase in growth was recorded. Further analysis with the addition of glycine to the medium, but lacking in selenium, showed that growth of the organism was inhibited. The observed inhibition was reported by Jackson *et al.* [452] to have been caused by the presence of glycine and subsequently resolved by the addition of a Stickland acceptor (D-proline) or by the Stickland donor (alanine) to the medium.

Again, is not clear why the addition of only 1 μ M sodium selenate had such drastic effects on the final OD, similarly to what was observed for strain 630 Δ *erm*.



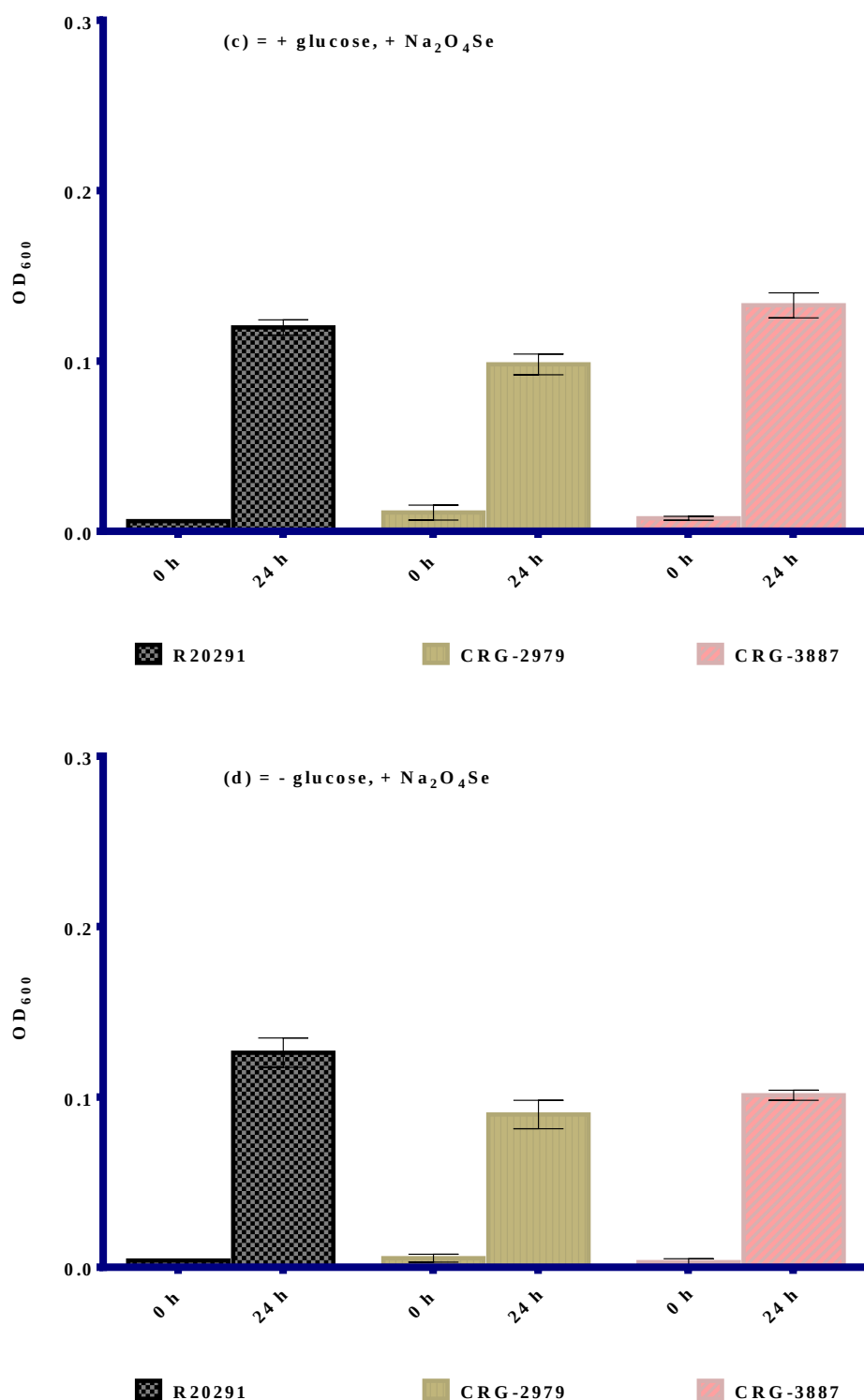


Figure 5-5: Effect of selenate on growth of *C. difficile* R20291 (parent strain), CRG-2979 (leucine fermentation mutant) and CRG-3887 (selenium selenocysteine synthase mutant).

Strains were incubated for 24 h in DMaa10mM medium under the following conditions: (a) in the presence of glucose, $P < 0.0001$, (b) in the absence of glucose, $P < 0.0001$, (c) in the presence of glucose and 1 μ M Na-selenate, $P < 0.0001$, (d) in the presence of 1 μ M Na-selenate and no glucose, $P < 0.0001$. Data shown are from three independent cultures with error bars indicating standard deviations.

5.3.4 LC-MS analysis of *C. difficile* R20291, CRG-2979, CRG-3887 spent medium

Having established the growth yields of R20291, CRG-2979, and CRG-3887 in DMaa10mM medium in the presence and absence of sodium selenate, it was necessary to determine the extent to which individual amino acids were being utilized under the different conditions. As described in chapter 4, LC-MS analysis was used to study the culture supernatants of the above strains with the results presented in Table 5-3. Based on the concentrations of amino acids remaining in the culture supernatant, the amino acids were divided into four groups as previously described in subsection 5.3.2.

Addition of selenate leads to complete or almost complete consumption of proline in parent strain and both mutants, regardless of whether glucose is present or not. Surprisingly, glycine was not consumed even when selenate was present; perhaps proline is preferentially used.

As expected leucine consumption is reduced in the leucine mutant under most conditions; the leucine that is consumed presumably serves as Stickland donor, i.e. is fermented via the oxidative branch. The exception is in the absence of glucose, with selenium being present. Under these conditions considerably higher amounts of leucine are consumed. Presumably there is an increased capacity for leucine oxidation because under these conditions proline serves as main electron acceptor.

There was little or no consumption of arginine (except the parent strain), aspartate (except by CRG-3887), glutamate, glycine, histidine, lysine and tryptophan (except by parent strain and CRG-2979 in the absence of glucose, CRG-3887 in the presence and absence of glucose), methionine (except CRG-2979 in the absence of glucose and CRG-3887 in the presence and absence of glucose). The moderately utilized amino acids were asparagine, phenylalanine (except the parent strain and CRG-2979 mutant in the absence of glucose and by CRG-3887 mutant in the presence and absence of glucose).

Alanine was first partially utilized and then actively produced by all the strains (except by CRG-2979 and CRG-3887 mutants in the absence of glucose) and CRG-2979 mutant in the absence of selenium.

Amino acids that were utilized to a large extent by all the strains were cysteine, glutamine and isoleucine regardless of whether glucose was present or not. There was a considerable difference in the utilisation of serine, threonine, tyrosine and valine for both mutants as well as the parent strain.

A related study by Fonknechten *et al.* [119] revealed a maximal utilization of arginine, asparagine, cysteine, glycine, histidine, lysine, proline, serine, threonine and valine by *C. sticklandii* from a defined medium with amino acids present at the same concentration. Under their growth conditions (without selenate), the aromatic amino acids were not utilized, while alanine, aspartate and glutamate were excreted into the medium. Isoleucine, leucine and alanine have previously been identified by a number of authors as the most efficient Stickland donors, with glycine, proline and hydroxyproline as the most efficient electron acceptors [126, 332, 454, 455]. This seems to be in agreement with the findings in this study, especially as it relates to the utilisation of cysteine, proline, serine, threonine and the non-utilisation of alanine. As previously reported, *C. difficile* can derive its carbon and energy from glucose [126, 467] or by Stickland reactions when grown with amino acids as the sole sources of carbon and nitrogen [126, 332, 406-408]. This further buttresses the fact that the availability of certain amino acids could play key roles in the growth yields of this bacterium as previously described for the related *C. sticklandii* [119].

Table 5-3: Percentage utilization of amino acids by *C. difficile* R20291 (parent strain), CRG-2979 (leucine mutant), CRG-3887 (selenocysteine mutants) in the presence and absence of glucose/selenate based on a 24 h incubation period.

Amino acids	R20291				CRG-2979				CRG-3887			
	+glucose	-glucose	+glucose, + Na ₂ SeO ₄	-glucose, + Na ₂ SeO ₄	+glucose	-glucose	+glucose, + Na ₂ SeO ₄	-glucose, + Na ₂ SeO ₄	+glucose	-glucose	+glucose, + Na ₂ SeO ₄	-glucose, + Na ₂ SeO ₄
Alanine*	≤1	≤1	≤1	≤1	≤1	2	≤1	5	≤1	≤1	≤1	5
Arginine	4	≤1	25	20	8	9	5	6	2	2	4	3
Asparagine	12	15	15	15	15	25	15	28	15	12	13	15
Aspartic acid	3	2	5	5	1	3	5	8	3	5	10	10
Cysteine	65	70	68	85	60	75	58	90	68	78	70	80
Glutamine	64	70	72	73	60	64	65	75	56	59	64	64
Glutamic acid	2	≤1	≤1	5	5	1	≤1	6	3	5	1	1
Glycine	≤5	≤5	≤5	≤5	≤5	≤5	≤5	≤5	≤5	≤5	≤5	≤5
Histidine	3	≤1	2	2	2	2	≤1	2	3	2	2	2
Isoleucine	66	85	74	86	76	71	70	73	61	90	76	73
Leucine	60	80	73	78	10	25	25	68	63	95	65	70
Lysine	3	≤1	≤1	≤1	2	1	≤1	2	3	1	1	2
Methionine	3	≤1	≤1	5	5	10	≤1	6	22	18	20	20
Phenylalanine	4	≤1	≤1	≤1	5	15	≤1	60	35	20	18	20
Proline	40	32	95	100	25	32	100	100	45	28	100	100
Serine	50	94	41	38	26	17	16	47	57	55	60	45
Threonine	64	65	46	53	15	20	16	60	31	33	38	30
Tryptophan	3	4	5	10	5	10	7	6	14	12	13	13
Tyrosine	12	20	45	50	20	39	36	47	67	69	35	31
Valine	30	36	32	33	11	15	11	37	35	36	31	36

*Excreted into the medium, +Na₂SeO₄ = with sodium selenate, -Na₂SeO₄ = without sodium selenate + = with glucose, - = without glucose, **Pink** = <10 %; **Yellow** = ≥10-29 %; **Blue** = ≥30-59 %; **Green** = ≥60 %.

5.4 Conclusions

Growth and utilisation of amino acids were tested for two different strains, 630 Δ *erm* and R20291, in the presence and absence of glucose and sodium selenate. Both conditions influenced the amino acid utilisation profile, although the effects of glucose were limited, at least for strain 630 Δ *erm*, and had comparatively little effect on the use of preferred amino acids such as isoleucine and leucine as compared to R20291.

Selenate also influenced the pattern of utilisation, the most notable effect being the increased consumption of proline. This was expected as proline breakdown is dependent on the selenoenzyme proline reductase [332]. Surprisingly, however, there was little effect on glycine consumption, which is also known to be selenium-dependent [332, 452]. A possible explanation could be that glycine reduction is only initiated when proline is not present in the culture media [452, 468]. Previous reports indicate that the presence of proline represses the formation of the necessary enzyme systems [468, 469].

Amino acid utilisation profiles for two transposon mutants were also generated. Mutant CRG-2979 is defective in *hadB*, encoding one of the two subunits of hydroxyisocaproyl-CoA dehydratase required for reductive degradation of leucine. Accordingly this mutant was unable to grow in the absence of glucose and could only thrive in the presence of selenium, presumably because proline replaced leucine as the major Stickland acceptor. Mutant CRG-3887, defective in *selA* encoding selenocysteine synthase was predicted to be deficient in proline and glycine reduction which is reliant on selenoenzymes. However, surprisingly, proline utilisation was not affected in this mutant suggesting the presence of an active site that play a part in proline metabolism [339, 470]. Therefore making glycine, a poor substitute for proline as an electron acceptor for growth in this mutant even in the presence of selenium as previously reported [339].

CHAPTER 6.

GENERAL DISCUSSION, CONCLUSIONS AND FUTURE WORK

6 General discussion

Given the medical importance of the superbug *C. difficile*, and the poor understanding of its carbon and energy metabolism, a comprehensive carbon source utilisation study utilising the most effective techniques is necessary in order to provide as much information as possible on its metabolic requirements and substrate preference. Of particular interest is the ability of this bacterium to obtain its carbon and energy in the absence of a fermentable carbohydrate, by activating specific pathways and/or enzymes to degrade amino acids by fermentation processes in order to create ATP [118, 126, 331].

In this study, several methodologies were developed to provide further insight into the importance of amino acids for the survival of this bacterium in the gut, which may possibly lead to discovery of novel metabolites and metabolic pathways and/or eventually aid in the successful control of the disease. Following the specific growth requirements of this bacterium [197], three variants of a defined medium that contain the essential nutrients that support good growth of *C. difficile* 630 Δ erm in the presence of a carbon and energy source such as glucose was developed. These media will be useful for physiological studies to determine amino acid requirements, determine the extent and order of amino acid utilisation (as carbon and energy sources) and for future studies on virulence and pathogenesis as previously reported [139, 181, 345]. Details on the defined media and other significant developments have been described in chapter 3.

Also, considering the high water solubility coupled with the range of ionic characteristics, and non-availability of a universal and selective chromophore that would enable the simultaneous separation of amino acids in a large sample set [222, 303] an LC-MS/MS platform was developed as previously described [139, 152, 174, 175]. This technique is ideally suited for measurement of large numbers of metabolites from microbial extracts due to its selectivity, sensitivity, reproducibility and high resolution in a relatively short time [151, 159, 176-182]. The developed method is an aqueous normal phase chromatography, representing an important new technology with a capability of the silica-hydride-base stationary phases, offering a distinct advantage for practical application with a high degree of reproducibility and long-term stability [272, 310]. Based on a previous report, a detection method not

requiring the use of ion-pairing agents or derivatisation of amino acids was chosen, not only for simplicity or convenience or accuracy, but to avoid the challenges that are associated with the use of ion-pairing agents or derivatisation procedures [240]. The focus here was the separation of amino acids, which presents an interesting separation challenge in metabolomics studies, obviously due to the range of polarities previously mentioned. The selection of the mobile phase in this study was made based on previous reports showing acetonitrile as the most effective solvent for the separation of polar/semi-polar compounds [208, 263, 267, 324]. After several repeats and adjustment of the procedures, the best retention was noted, which resolved all analytes of interest, and this was followed by further testing and adjustments in order to obtain a final method. The method developed enabled the simultaneous separation and quantification of all twenty amino acids within a total analysing time of 22 min. Summary of main conclusions in the LC-MS/MS method development are presented in chapter 2.

The performance of the LC-MS/MS method was evaluated by measuring linearity, sensitivity and precision [204, 299] after which it was applied in the initial analysis of a complex *C. difficile* 630 Δ erm spent medium. The developed method was subsequently adapted to study amino acid utilisation by other *C. difficile* strains in the presence or absence of a fermentable carbohydrate and selenium. Experimental data showed that all strains grew on the fully defined medium, with different growth profiles in terms of lag phase, growth rate, and maximum OD reached. The concentration of individual amino acids remaining in the spent *C. difficile* culture media was measured after 24 h and/or 48 h using the developed LC-MS/MS method in order to determine which amino acids were being utilised by these organisms and in which order. The utilisation of amino acids by most strains tested was comparable, although in the absence and presence of glucose there were preferences for individual amino acids. Notable in this study, was the pattern of utilisation exhibited by strains L26 and O17, differing remarkably from the other strains. For example, a considerable number of the amino acids not metabolised by the other seven strains were utilised to a larger extent by strain L26, while O17 was the only strain to consume a moderate amount of aspartate both in the presence and absence of glucose. O17 was also the only strain that metabolised a considerable amounts of glycine and excreted tryptophan and glutamate under the tested conditions. Details of

the amino acids utilisation pattern and the order in which they were metabolised have been presented already in chapter 4 of this thesis. These results suggest that growth of this bacterium is highly dependent on its ability to utilise certain amino acids in the presence or absence of a fermentable carbohydrate. The increased demand for isoleucine and leucine as the preferred substrates by most *C. difficile* strains has been reported by a number of authors [120, 332, 402-404]. A recent study by Neumann *et al.* [120] showed a clear difference in the utilisation of amino acid and formation of fermentation products by *C. difficile* 630 Δ erm similar to the findings in this study.

The most significant developments in this study were the lack of glutamate utilisation and the excretion of alanine by most of the tested stains. Theoretically, the use of glutamate was expected being an important source of nitrogen (amino group donor) [394-397] and the fact that the enzyme glutamate dehydrogenase, required for the first step of glutamate breakdown, is secreted by most of the *C. difficile* strains [398-400]. This result suggests that glutamate is not a substrate required for growth by all *C. difficile* strains and is therefore in agreement with previous studies [119, 120, 128, 405]. Alanine was shown to be an exceptional amino acid in this study, having initially been consumed by most strains in the presence and absence of glucose. However, following entry into the exponential or stationary phases of growth concentrations in the medium were seen to increase considerably and similar to what has been reported for other bacterial species [119, 120, 392]. The reason for this was not definitively established but may be due to the use of pyruvate as an amino acceptor during the degradation of preferentially fermented amino acids.

Further studies undertaken to evaluate the metabolism of amino acid by two different *C. difficile* strains (630 Δ erm, R20291), and two transposon mutants (CRG-2979, CRG-3887) in the presence and absence of glucose led to their cultivation in a control and experimental defined medium enriched with selenium in the form of selenate. Summary of the main conclusions are presented in chapter 5 of this thesis. The transposon mutant, CRG-2979 is defective in *hadB*, encoding one of the two subunits of hydroxyisocaproyl-CoA dehydratase required for reductive degradation of leucine [471], while the CRG-3887 mutant is defective in *sela* encoding selenocysteine synthase [428] (see Table 5-1). Selenium requirements stem largely

from its role in selenoenzymes as well as its incorporation into selenocysteine [413], but important for this study is its established role in *C. difficile* amino acid metabolism, especially in the breakdown of glycine and proline using two selenoenzymes, glycine reductase and D-proline reductase [423, 429, 430]. Previous studies by Jackson *et al.* [452] showed that Stickland reaction was essential for the growth of *C. difficile* in the presence of selenium.

As demonstrated in this study, the presence or absence of glucose had some effect on the profile of amino acid utilisation by 630 Δ *erm* and the R20291 strains. For strain 630 Δ *erm*, the effects of glucose were limited to some extent with comparatively small effects on the use of preferred amino acids like isoleucine and leucine. The pattern of utilisation was also affected by the presence of selenate, particularly in the usage of proline, which was not unexpected. Previous studies have shown that the presence of selenium allows this bacterium to produce the selenocysteine-containing enzyme proline reductase, which plays a key role in the fermentation of proline [406, 466]. A little more surprisingly, was the lack of glycine consumption, which is also known to be selenium-dependent [332, 452]. However, a review of previous literature reveals that the presence of proline seems to repress the formation of the necessary enzyme systems required for glycine degradation [468, 469]. The implication of this is that glycine reduction is only initiated when proline is not present in the culture medium and therefore in agreement with previous findings [452, 468].

For the transposon CRG-2979 mutant, the absence of glucose had a considerable effect on growth as compared to when glucose was present. This mutant could only thrive in the presence of selenium, probably due to the presence of proline replacing leucine as the major Stickland acceptor. The results for the transposon mutant CRG-3887, were also unexpected because this mutant was predicted to be deficient in proline and glycine breakdown which is reliant on selenoenzymes. Proline utilisation as demonstrated in this study, suggests the presence of selenium independent proline fermentation pathway although this hypothesis is not supported by the existing literature.

In conclusion, three variants of a defined medium for the cultivation of *C. difficile* strains in the presence of a carbon and energy source such as glucose and a reliable LC-MS/MS platform were developed and validated for the study of amino acids utilisation by *C. difficile* strains. The application of these methods provided useful information on the importance of amino acids for the survival of this bacterium in the gut. Experimental data provided further evidence about the ability of this bacterium to obtain its carbon and energy in the absence of a fermentable carbohydrate, by activating specific pathways and/or enzymes to degrade certain amino acids by fermentation processes in a preferential way. Interestingly, in this study were the lack of glutamate utilisation, except by strain L26 and the excretion of alanine after its initial degradation by most of the tested stains. The defined media developed herein represent a significant step forward in determining the roles of nutritional and metabolic factors in the control of virulence in this bacterium using a mass spectrometry platform. Overall, the evidence in this thesis forms the basis for further investigation of the previously reported link between amino acids metabolism and toxin production in *C. difficile*.

Future Work

Future experiments that could be performed following the outcome of this study include a more comprehensive analysis using the same conditions to measure the complete spectrum of intra and extracellular metabolites in order to determine possible changes and if so, the order in which different carbon and energy sources are being utilised. For instance, measuring the rate of glucose utilisation and the generation of excreted fermentation products may provide further insights into the fermentation metabolism of this organism and possibly identify important metabolic and regulatory networks. In this study, the influence of selenium on fermentation metabolism was examined for only two wild-type strains and two transposon mutants. However it would be desirable to expand the scope to include a larger number of strains, in particular clinical isolates, as well as stable, defined mutants defective in established or predicted fermentation pathway genes.

CHAPTER 7.

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7 REFERENCES

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