Ad meliora:

Space microbial research for the benefit of humanity

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May this research result in the betterment of humankind.

Ad benevolentiam

May the Hakka find a home at the next frontier.

Abstract

When humans go to space, they take their microbes with them. Microbiomes benefit many aspects of human health, but little is known about how spaceflight affects the physiology of our microbial commensals. The functional composition of the human vaginal microbiomes (i.e. eubiosis vs. dysbiosis) is well established and *Gardnerella vaginalis* is known to drive dysbiosis, being implicated in the development of bacterial vaginosis (BV). In fact, 10% of the medical issues arising during spaceflight are genitourinary, a decent portion from female astronauts, despite being underrepresented in space missions. Because *G. vaginalis* is a key species driving microbial dysbiosis, this thesis aimed to uncover the biological pathways of *G. vaginalis* affected by space conditions, particularly the lack of gravity (i.e. microgravity), and the potential implications it holds for human health in space. Untargeted Metabolomics by Gas Chromatography Mass Spectrometry was conducted to assess the changes in bacterial metabolites when *G. vaginalis* was subjected to simulated microgravity. In the process, a mix of supervised and unsupervised multivariate analysis methods were explored. Our result suggests that microbial metabolic changes induced by microgravity may alter the redox status which, in turn, may favour the growth of different species of bacteria in a reduced anaerobic environment. Additionally, we found eight compounds whose relative concentrations were reduced in comparison to gravity control after cross-validation. These include hydroxybenzoic acid and the amino acids aspartate, glutamate and (hydroxy)lysine with known specific roles on cellular responses. The former, for instance, is known to have estrogenic activity and antimicrobial properties and to affect bioavailability of metronidazole, the first line of drug to treat BV. These findings demonstrate that simulated microgravity has the potential of altering microbial metabolic responses that may lead to disturbances of the microbiomes and alterations on host physiology. The space environment has been viewed as an environment for accelerated disease modelling. Understanding metabolic and physiological responses to space will help humanity on Earth and to establish safe and long-term space exploration programs.

Index of Figures

List of Abbreviations

2D	2 dimensional
3D	3 dimensional
AGXX	Antimicrobial technology against germs and viruses
AMDIS	Automated Mass Spectral Deconvolution and Identification System
ATCC	American Type Culture Collection
BER	Balanced Error Rate
BRIC	Bacterial Research in Canisters
BV	Bacterial Vaginosis
CDC	cholesterol-dependent cytolysin
CE-MS	Capillary electrophoresis mass spectrometry
CTRL	Control
CV	Cross Validation
DAP	diaminopimelate
DIABLO	Data Integration Analysis for Biomarker discovery using Latent cOmponents
DNA	Deoxyribonucleic acid
GC	Gas Chromatography
GC-MS	Gas Chromatography Mass Spectrometry
GCCA	Generalised canonical correlation analysis
GCR	Galactic cosmic rays
GLDS	GeneLab Data Systems
GOX	Graphene Oxide
GSH	Glutathione
HA	Hydroxybenzoic acid
HARV	High Aspect Ratio Vessels

HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HBT	Human Blood bilayer-Tween
HIV	Human immunodeficiency viruses
ICA	Independent Component Analysis
IPCA	Independent Principal Component Analysis
IS	Internal Standards
ISS	International Space Station
LC	Liquid Chromatography
LC-MS	Liquid Chromatography Mass Spectrometry
LEO	Low Earth Orbit
LSMMG	Low-Shear Modelled Microgravity
LV	Latent Variable
MRSA	Methicillin-resistant Staphylococcus aureus
MPS	Microphysiological systems
NAD	Nicotinamide adenine dinucleotide
NASA	National Aeronautics and Space Administration
NIST	National Institute of Standards and Technology
NMR	Nuclear magnetic resonance
NYC	New York City
O/OREOS	Organism/Organic Exposure to Orbital Stresses
OD	Optical Density
PCA	Principal Component Analysis
PLS	Projection to Latent System
PLS-DA	Partial Least Square-Discriminant Analysis
PSD	Peptone-starch-dextrose
QC	Quality Control
QS	Quorum sensing
RCCS	Rotary Cell Culture System
RF	Random Forest

RNA	Ribonucleic acid
RPM	Random Positioning Machine
RWV	Rotating Wall Vessel
SAM	S-adenosylmethionine
SERRF	Systematic error removal using random forest
sg, sG	Simulated Gravity
SLS	Space Launch System
sPLS-DA	Sparse Partial Least Square-Discriminant Analysis
SPE	Solar particle events
STS	Space Transportation System
Sug, s-μg, sμg	Simulated microgravity
uG, μG	Microgravity
VLY	Vaginolysin

Chapter 1 - Introduction

1.1 The bacterium *Gardnerella vaginalis* and the human vaginal microbiome

1.1.1 Classification

First recognised, isolated from urine and cervical swabs from men and women, and published in the United States Arms Forces Medical Journal (Leopold, 1953), *Gardnerella vaginalis* has gone through many different classifications, and was widely believed to be the cause of Bacterial Vaginosis (BV) which results in vaginal discharge, higher than normal vaginal pH, and a 'fishy odor' (Morrill et al., 2020). An isolation on blood agar, and a Gram-negative stain, the perceived requirement of hemin and NAD for growth, paved the way to the acceptance of the *Haemophilus* species denotation, and the subsequent assignment of *Haemophilus vaginalis* (Catlin, 1992; Gardner & Dukes, 1955). During the media development for *Gardnerella*, it had come to the attention of Dunkelberg and McVeigh (1969) that hemin and NAD were not requirements for growth. This demonstrated that the *Haemophilus* denotation was no longer appropriate. These non-requirements in addition to the fact that *G. vaginalis* sometimes showed capability of crystal violet retention indicating a Gram-positive stain, and its resemblance to coryneform resulted in some adoption of the name *Corynebacterium vaginale* (Wilbur E. Dunkelberg et al., 1970; Zinnemann & Turner, 1963). Later in the century, analysis from series of biochemical tests, electron microscopy, and DNA-DNA hybridisation revealed that "*Haemophilus vaginalis*" did not fit well into either the established Gram-negative and Gram-positive genera (Greenwood & Pickett, 1980; Piot et al., 1980). This resulted in the need for a new genus, which was proposed to be *Gardnerella* (Greenwood & Pickett, 1980; Piot et al., 1980).

Vaginal microbiome studies utilising cpn60 barcode sequences often distinguish *Gardnerella* into four subgroups (Hilbert et al., 2017; Jayaprakash et al., 2012; Khan et al., 2019). In the mid-1980s, on the basis of activities from hippurate hydrolysis, β-galactosidase, and lipase, eight biotypes were identified; this was extended to 17 biotypes when arabinose, xylose, and galactose were included (Benito et al., 1986; Piot et al., 1984). An investigation by Vaneechoutte et al., (2019) on whole genome sequences from 81 *Gardnerella* full genome sequences resulted in a classification of 3 additional named species and 9 unnamed species, giving 13 species in total. Recent research from Putonti et al (2021) suggests a 14th species. The strain used in this thesis, ATCC 14018, has retained the identification *Gardnerella vaginalis*. It should be pointed out that the number of species and strains can still shift and change over time, I will refer to the species as *Gardnerella spp* or *Gardnerella vaginalis* as appropriate.

1.1.2 General biology

Gardnerella spp are Gram-positive coccobacilli, and characterised microscopically as Gram-negative (figure 1.1) to Gram-variable as they are most often stained as Gram-negative bacteria (Catlin, 1992; Greenwood & Pickett, 1980; Vaneechoutte et al., 2019). An analysis on the cell wall structure with electron microscopy showed the absence of lipopolysaccharides in the cell wall, and a peptidoglycan layer that lacked diaminopimelate (DAP) which meant that *Gardnerella* is very much a Gram-positive bacterium (Harper & Davis, 1982; O'Donnell et al., 1984; Reyn et al., 1966; Sadhu et al., 1989). DAP is frequently found in Gram-negative cell walls, but rarely found in a Gram-positive bacterial species (Harper & Davis, 1982).

Beveridge et al. (1988) suggested that the Gram reaction is dependent on the amount of peptidoglycan on the cell wall. Species and strains that have been identified as Gram-variable can appear as Gram-positive during exponential growth phase but shifts to Gram-negative as

a slowdown in growth also results in a conservative peptidoglycan layer that can no longer retain crystal violet (Beveridge et al., 1988; Sadhu et al., 1989). It is accepted that the thin cell wall is responsible for the variability of Gram-staining for *Gardnerella spp* (Harper & Davis, 1982; Sadhu et al., 1989).

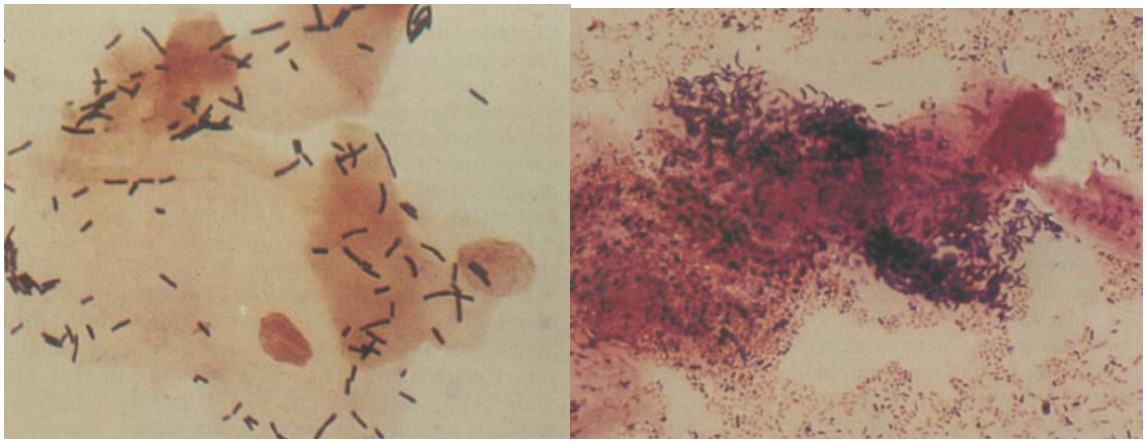

Figure 1.1 On the left is a Gram-stained vaginal smear from women with normal vaginal flora (left) showing lactobacillus morphotypes, where the image on the right has no lactobacilli with lots of gram-negative rods and curved rods (not limited to *Gardnerella spp*) (Nugent et al., 1991).

Mattarelli et al., (2014) showed that *Gardnerella* has a substantial 16S rRNA gene sequence similarity (93.1%) to *Bifidobacterium bifidum*, a Gram-positive bacterium. *Gardnerella*'s phylogeny also frequently branches next to or within other bifidobacterium species (Lugli et al., 2017; G. Zhang et al., 2016). Fructose 6-phosphate phosphoketolase, an activity that was believed to be exclusive to the bifidobacteriaceae family, is also seen in *Gardnerella* (Mattarelli et al., 2014). Vaginolysin (VLY), a toxin from the cholesterol-dependent cytolysin (CDC) family (Gelber et al., 2008) is produced by most strains of *Gardnerella,* and CDCs are common in Gram-positive pathogens (J. Castro et al., 2015; Gelber et al., 2008; Hotze et al., 2013). Although the microbiota from both BV-positive and healthy women have VLY, the former tend to have higher levels of it (Nowak et al., 2018).

The PSD medium for *Gardnerella spp* was first developed by Dunkelberg and McVeigh (1969). Consisting of six bases (adenine sulfate, cytosine, guanine·HCl, thymine, uracil and xanthine),

glucose, maltose, five vitamins, and a vitamin-free casein (W. E. Dunkelberg & McVeigh, 1969). In this thesis, the NYC III media was used. The original NYC media, first developed for *Neisseria gonorrhoeae*, makes use of yeast dialysate as additional nutrients (Faur et al., 1973) have shown to be useful in growing other vaginal-related bacteria such as *Atopobium vaginae*, *Lactobacillus iners*, *Peptostreptococcus anaerobius*, and *Prevotella bivia* (Rosca, Castro, & Cerca, 2020). Though it has not been compared with the PSD medium, the NYC III media is considered the gold standard and showed significant planktonic growth for *Gardnerella spp* and other bacterial vaginosis-associated anaerobes (Marrs et al., 2012; Rosca, Castro, & Cerca, 2020). NYC III media consist of proteose peptone, glucose, HEPES, NaCl, Yeast extract, and is supplemented with inactivated horse serum (Rosca et al., 2020).

There are a lot of discrepancies in test results for *Gardnerella spp*. In the event of identification from metabolic reactions, the following is used: *Gardnerella spp* is catalase-negative, beta-hemolysis on Human Blood bilayer-Tween 80 agar (HBT agar), positive for Hippurate hydrolysis, Starch hydrolysis, ***α***-Glucosidase, negative for ***β***-Glucosidase, generates acid from Maltose, but not from Mannitol, Inhibition zone with 50 µg Metronidazole (Piot et al., 1982).

When Dunkelberg and McVeigh (1969) developed the PSD medium, they saw that the *Gardnerella* 594^{T} strain required adenine whereas strain 317 did not. The study by Vaneechoutte et al., (2019) pointed out that only the strains of *G. vaginalis* produced β-galactosidase, which was not identified in the other *Gardnerella spp*, and there will be metabolic differences between the *Gardnerella spp*. Cornejo et al., (2018) carried out a comparison between *Gardnerella* and *Bifidobacterium* and saw that the *Bifidobacterium* genome had 785 protein families that were absent in *Gardnerella. Gardnerella* did not possess many metabolic capabilities that were present in *Bifidobacterium spp*. It was found that *Gardnerella* had unique protein families, many of which were annotated as toxin-related, and transport proteins, so it can be seen that *Gardnerella* uses these to acquire nutrients that it could not obtain itself (Cornejo et al., 2018). *Gardnerella* has also been observed as the

provider of amino acids to *Prevotella bivia* where in response *P. bivia* produces ammonia which is in turn used by *Gardnerella spp* (Pybus & Onderdonk, 1997). In terms of metabolism, this shows *Gardnerella spp* as highly resourceful, extractive, but can also be cooperative. *Gardnerella* will kill other bacteria with their toxin-antitoxin system to obtain certain nutrients (Cornejo et al., 2018), but cooperate with other bacteria, such as *P. bivia* that it can exchange nutrients with (Pybus & Onderdonk, 1997).

1.1.3 The vaginal microbiome, bacterial vaginosis, and biofilms

A variety of definitions used for the microbiome. However, many of the definitions only capture certain essences of the microbiome (G. Berg et al., 2020). Berg et al., (2020) suggests a revival of an 1988 definition originally conceived for the plant's rhizosphere (Whipps and Karen, 1988).

The revived Whipps and Karen (1988) definition as stated by Berg et al., (2020): “The microbiome is a microbial community with defined characteristics where it occupies a defined habitat with distinct physio-chemical properties. This includes not only the microorganisms, but their activities, which forms a specific ecological niche. The microbiome is an interactive micro-system and can change in scale over time, and can have a macro-ecosystem integration with hosts, which have an impact on their health and functions.”

The status of a microbiome should be viewed as a spectrum from eubiosis (healthy state) to dysbiosis (unbalanced and potentially pathogenic). For the vaginal microbiota, eubiosis is where microbes from the *Lactobacillus* genus are commonly seen, and will prevent the growth of adversarial microorganisms (Ceccarani et al., 2019; Ravel et al., 2011). Copious amounts of lactic acid is produced by *Lactobacillus* which causes acidification of the vaginal pH to around 3.5 and precludes colonisation of other microorganisms (Aldunate et al., 2015).

Contradictions exist for *Lactobacillus iners*. This may be an exception to eubiosis since this species can release toxins such as inerlysin, which is found among BV women, and better coexists with other BV-related species (Pleckaityte, 2019; Vaneechoutte, 2017).

Bacterial vaginosis (BV) is a common dysbiotic condition, and has association with pregnancy complications, infertility, and sexually transmitted infections (Arif, 2018; Atashili et al., 2008; Brotman et al., 2010; Brusselaers et al., 2019; Lokken et al., 2017; Rosca, Castro, Sousa, et al., 2020). *G. vaginalis* has been documented to metabolise tenofovir, an anti-HIV drug, and for decreasing the bioavailability, and its effectiveness (Klatt et al., 2017). In BV, there is a great diversity of bacterial species, excluding most lactobacilli. In this condition, the vaginal pH raises above 4.5, and a range of short chain fatty acids is released to the vaginal environment (Aldunate et al., 2015). It is still important to point out that while *Gardnerella spp* is commonly seen in large numbers during BV, it is frequently present in healthy women as well (Shipitsyna et al., 2019). *Gardnerella spp* is far more abundant and ubiquitous in women with BV than women with protective vaginal lactobacilli (Fredricks et al., 2007b; Shipitsyna et al., 2013). A dysbiotic vaginal microbiota can be a result of hormonal changes, douching, and/or sexual activities that lead to a great diversity of microorganisms which eventually become together a BV pathotype (Hawes et al., 1996); characterised by the presence of biofilms (Swidsinski et al., 2008).

Biofilms consist of a mono- or multispecies bacterial community enclosed into a self-synthesised extracellular matrix and are attached to surfaces or as aggregates adhering to porous spaces (Costerton et al., 1995; Patterson et al., 2010). Biofilms often provide an effective method for microorganisms to decrease the effects of environmental stressors (Jung et al., 2017). They are found in industrial and natural environments, and can become a health hazard once it becomes a prolonged interaction with humans and their systems (Costerton et al., 1995; Stoodley et al., 2002). Biofilms help microorganisms evade host defense mechanisms, it prevents the full penetration of antibiotics, tolerance to pressure gradients,

alternative spreading and accumulation of nutrients, which allows the development of antibiotic resistance, and these properties contribute to recurrent disease and infections (Parra et al., 2021; R. Singh et al., 2010; Stoodley et al., 2002; Swidsinski et al., 2008; Thurlow et al., 2011).

Biofilm is the strongest feature of BV, and this is largely absent in healthy females (Swidsinski et al., 2008). *Gardnerella* biofilms can be sexually transmitted between females and males (Swidsinski et al., 2010). *Gardnerella spp* is considered the initial key coloniser of the vaginal epithelium during BV being the microorganism that initiates the formation of this biofilm (Alves et al., 2014; Schwebke et al., 2014). Compared to other BV-associated bacteria, *Gardnerella spp* has better adherence to the vaginal epithelium, a higher level of cytotoxicity, and has a greater ability to form biofilms than a range of vaginal bacterial species as demonstrated by Patterson et al., (2010). In BV, *Gardnerella vaginalis* and other virulent strains of *Gardnerella spp*. are often the ones to initiate biofilm formation which other pathogenic bacteria use to evade stressors (Muzny et al., 2019). *Gardnerella spp* biofilms have been documented to survive current standard treatments to BV (Muzny et al., 2019; Swidsinski et al., 2008, 2011).

1.2 Spaceflight and microorganisms

1.2.1 Introduction

Space has induced wonders throughout humanity's history. Seven decades after the launch of the first dog Laika, humankind has established 2 decades of permanent human presence in low Earth orbit (LEO) (Afshinnekoo et al., 2020). During this time, a greater understanding of the universe and how space affects Earth organisms have been uncovered. Spaceflight

biological research has created a wealth of knowledge and new technologies for benefits in terrestrial applications, including those in the biological and medical fields. Scientific discovery in space has often required the advancement of technology, with examples including artificial limbs, fluorescent chromatid paints, bioelectric life support systems and others (NASA Technology Transfer Program, 2019). In his autobiography, Scott Kelly describes rashes, severe leg swelling, nausea, and pain in the days returning to Earth after spending a year in space, and explains that “no one in the hospital will have seen the symptoms of having been in space for a year” (Kelly, 2017). With the commercialisation of space access, private spaceflight passengers that differ from medically fit astronauts come into the equation (Ehrenfried & von Ehrenfried, 2020; Kluge et al., 2013; Lambright & Henry Lambright, 2018; Weinzierl, 2018).

1.2.2 Physical stressors during spaceflight.

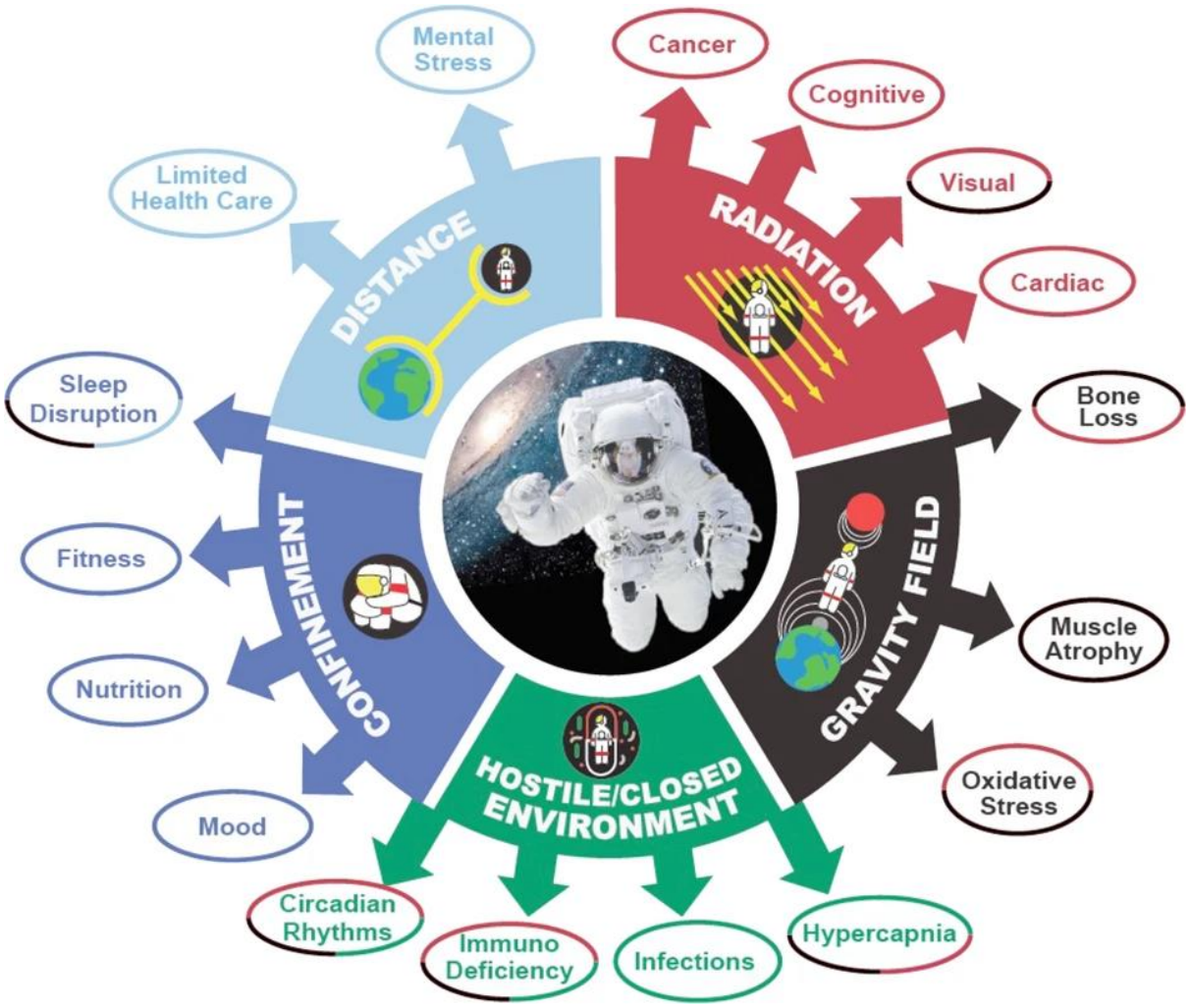

Figure 1.2 Physical and biological factors affecting human space exploration and their associated stressors including isolation, long confinement in closed environments, microgravity, and ionizing radiation. Figure adapted from NASA Genelab (R. Barker et al., 2021).

Space is an unnatural environment for all life on Earth, and spacecraft subject humans to considerable health hazards can pose performance issues to astronauts. These challenges can include changes to gravitational forces, access to healthcare, space radiation, and social issues such as sleep deprivation and isolation (Afshinnekoo et al., 2020). In addition, spacecraft and humans are carrying microorganisms to space. For microorganisms, the two main health hazards of spaceflight are space radiation and microgravity. Space radiation can arise from solar wind particles (figure 1.2), the heavy ions of galactic cosmic rays (GCR), which originate from outside the solar system, and high-energy protons made during solar particle

events (SPEs) (Chancellor et al., 2014). During interaction with the spacecraft material, these particles produce a secondary intravehicular radiation hazard, which still deliver significant radiation dosage (Chancellor et al., 2014). Apart from the crewed missions of Apollo, all other crewed missions have taken place in LEO where they are protected from most radiation due to the Earth's magnetosphere (Hassler et al., 2014). More relevant to this thesis is the role of microgravity as a space health hazard. Gravity has defined how all life on Earth evolved and has adapted. The effects of gravity are mostly removed in space, a condition known as microgravity. Microgravity has been shown to cause fluid redistribution, induce cellular and molecular changes, it affects the genome and proteome, which creates risk for the development of pathologies (Demontis et al., 2017). The effects of microgravity and its underlying mechanisms on microorganisms are less obvious and will require extended investigations.

1.2.3 On-ground simulation of microgravity.

There are obvious technical limitations to study the effects of microgravity to any living system in a reproducible experimental set-up. Additionally, there are also strong limitations to conduct experiments in a real spaceflight situation such as astronaut time and access to equipment (Matin and Lynch, 2005), and the cost of launching payloads. Some costs can be reduced when utilising environments such as parabolic flights, drop towers, sounding rockets (Huang et al., 2018). CubeSats, or small satellites, have also been used to reduce the cost, noise and error from human interaction (Zea et al., 2021). The drop tower, parabolic flight offer seconds of microgravity, sounding rockets provide a couple minutes of microgravity, while recoverable satellites offer a few months of microgravity (Huang et al., 2018). But even these require a substantial amount of infrastructure, costs, and the inflexibility in the amount of time often means limitations in the type of experiments that can be carried out.

Ground-based simulation models are often carried out instead, which results are then used to build an argument why certain experiments should be sent up to space. A range of models for simulating microgravity have been developed. Examples include the Random Positioning Machine (RPM), Hindlimb unloading, rotating wall vessels (RWV), clinostats (Bonnefoy et al., 2021; Globus & Morey-Holton, 2016; Grimm et al., 2018). A study that tested the differences between constant rotation (clinostats, rotating wall vessels) to the RPM demonstrated that a highly sensitive organism will show detectable mechanical stress when using the RPM (Hauslage et al., 2017). It may be tempting to attach a three-dimensional (3D) aspect of the RPM as a better representation of microgravity simulation, but this would not count if it ends up producing unnecessary mechanical stress.

The RWVs is the instrument used to simulate microgravity in this thesis. RWVs, sometimes referred as High Aspect Ratio Vessel (HARV)(Figure 1.3)(Wolf et al., 1992).Originally developed by the NASA Biotechnology Group at the Johnson Space Center in Houston Texas, the RWV minimises turbulence, shear, and keeps cells in suspension (T. G. Hammond & Hammond, 2001; Schwarz et al., 1992). The effect termed for the RWV is the Low-Shear Modelled Microgravity (LSMMG), it consists of a rotating cylindrical culture vessel with a hollow cylinder filled with a liquid medium (T. G. Hammond & Hammond, 2001; Nickerson et al., 2004). As the inner and outer parts of the vessel rotates with the same angular velocity, the laminar flow fluid velocity is minimised radially which results the actual laminar flow to be minimised and eliminates the shear stress (Goodwin et al., 1993). The culture is gently mixed which can reduce sedimentation, or as Wolf (1991) points out that the resulting sedimentations can result in a secondary flow pattern. This is quite important as sedimentation of particles is absent during spaceflight. The reduction of air bubbles in the vessel can also reduce turbulence. The RWV's main advantage is the suspension of cells without turbulence and shear, providing the required nutrients, and the exchange of gas if required (T. G. Hammond & Hammond, 2001). The rotation of the RWV induces centrifugal and Coriolis forces, which

are dependent on terminal velocity and the two forces are minimised via the reduction of terminal velocity (T. G. Hammond & Hammond, 2001; Tsao et al., 1994). It is important to point out that the development of the RWV was meant to replicate the expected culture conditions during experimentation in space, but it does not simulate microgravity *per se* (Goodwin et al., 1993; Wolf, 1991). The use of the term 'simulating microgravity' in the literature is used for simplicity and attachment.

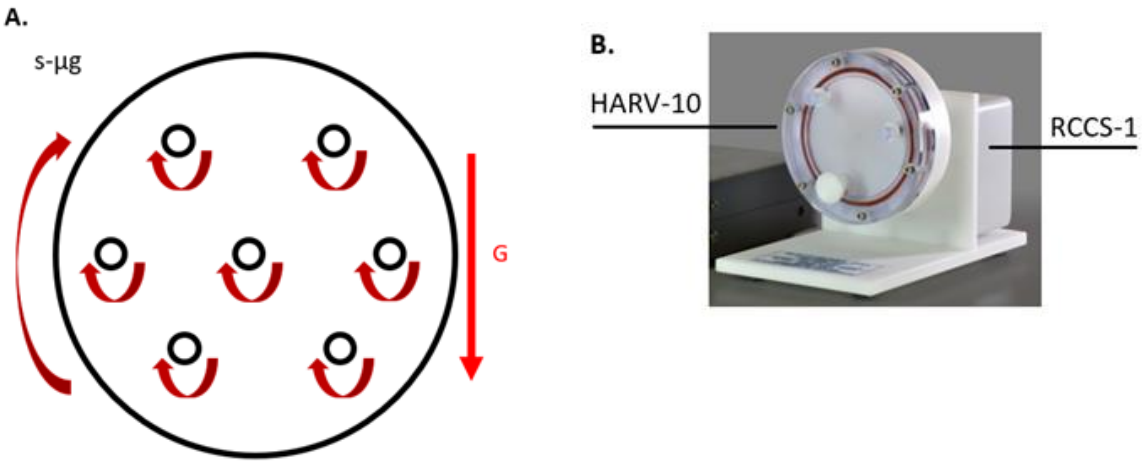

Figure 1.3 The vessel HARV-10 (B) is rotated by RCCS-1 (Lynch et al., 2006). Figure 1.3A is an interpretation of the cellular movements within the HARV vessel.

Inside the bioreactor (Fig 1.3B), the cells are held in a circular motion and in suspension via the use of slow rotation (Fig.1.3A). This suspension replicates culture conditions in spaceflight.

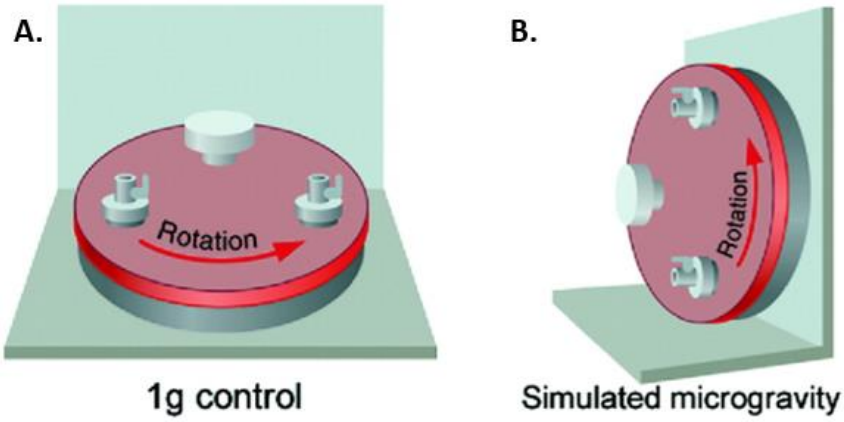

Figure 1.4: A rotation (A) in the vertical axis serves as the control for simulating normal gravity or 1g control, and a rotation in the horizontal axis is used to keep cells in suspension (B) (Lynch et al., 2006).

1.2.4 Microbial responses to microgravity.

Ease of handling, rapid life cycle, and their ability to impact humans and their resources mean that microorganisms remain ideal for studying responses to changes in the environment (Senatore et al., 2018). Subjecting microorganisms to the effects of spaceflight has shown to have effects on cell physiology, growth rate, metabolism, morphology, genetic transfers, gene expression, host interaction, viral reactivations (Horneck et al., 2010; Nickerson et al., 2004; Taylor, 2015). Changes in the skin microbiome is speculated to contribute to the experience of frequent skin rashes by astronauts (Voorhies et al., 2019). Between 1995 to 1998, crew-members aboard the Mir space station were documented to have microbial infections such as dental infections, conjunctivitis, and respiratory infections (Guéguinou et al., 2009; Institute of Medicine et al., 2001).

Due to the threat microbes have on the health of astronauts, considerable focus has been on cellular growth and secondary metabolism. This is because the growth of pathogens represent

a threat to astronauts' health and immune systems (Huang et al., 2018). Secondary metabolism has been the focus as these metabolites can be toxic to the host or have medicinal properties (Benoit et al., 2006; Rosado et al., 2010). Experiments have shown that bacteria can potentially change their secondary metabolism in response to simulated microgravity. *Escherichia coli* ZK650 was shown to produce microcin B17, an antibacterial polypeptide it usually produces, inhibited. Antibiotic production of β-lactam was inhibited in *Streptomyces clavuligerus*, similarly to rapamycin in the case of *Streptomyces hygroscopicus* (Demain & Fang, 2001; Fang et al., 1997). It should be also pointed out that Fang et al., (1997) showed that *Bacillus brevis*, on the other hand, had no changes in gramicidin S (GS) antibiotics production while Luo et al., (1998) showed an increase in the production of nikkomycins antibiotics from *Streptomyces ansochromogenus.* These published reports exemplify that, when it comes to this common space stressor (i.e. simulated microgravity), there is no a definite or universal response from microorganisms.

Microbial response to the spaceflight environment seems to be dependent on secondary metabolites and pathways, cell motility, and the environment around the microbial cells (Huang et al., 2018; Todd & Klaus, 1996). Cellular response to gravity is believed to be attained by three pathways. A "direct" effect, a "indirect" effect, and third being an interaction and integration of the two pathways (Todd & Klaus, 1996). The direct effect is based on organelles or molecules that has, or also function as a gravireceptor, and the indirect effect is cellular adaptive response to a different environment, such as nutrient distribution and metabolic transport by fluid dynamics (Huang et al., 2018; Todd & Klaus, 1996).

1.2.5 Biofilms in microgravity

The effects that space exploration might have on microbial physiology has been seen on space shuttles and stations, particularly because of biofilms that these microorganisms produce (Figure 1.6 and 1.7) (W. Kim et al., 2013; Zea et al., 2018). Abroad the Salyut 6, 7, and the Mir space station, pipes, the water recycling system, thermal control systems, oxygen electrolysis block, have shown microbial contamination (Klintworth et al., 1999). The responsible microorganisms to the erosion of a navigation window on the Mir space station were determined to be the actions of *Aspergillus sp*, *Penicillium rubens* (previously known as *P. chrysogenum*), and *Bacillus polymira* (Klintworth et al., 1999; Mauclaire & Egli, 2010) (Klintworth et al., 1999; Mauclair and Egli., 2010). On the International Space Station (ISS), biofilms were documented on viewing windows, rubber seals, and a variety of hardware surfaces (Gu, 2007). This renders long-term uses and explorations of spacecraft as unsustainable (Klintworth et al., 1999). *Pseudomonas aeruginosa* (figure 1.5) was reported to have biofilm formation during the shuttle flight on STS-95, and it was a decade later where *P. aeruginosa* was proven to have an increased biofilm formation (W. Kim et al., 2013; McLean et al., 2001). Currently, a range of pathogenic bacteria in spaceflight have shown to have greater formation of biofilms, increased virulence and is suggested to have a competitive advantage over their ground counterparts (Rosenzweig et al., 2010; Schiwon et al., 2013; Taylor, 2015). Increased aggregation from *Salmonella typhimurium* was shown during spaceflight, and aggregations are precursor to biofilms (Wilson, Ott, zu Bentrup, et al., 2007). *Klebsiella pneumoniae* showed an upregulation in biofilm associated genes coupled with increased formation of biofilms (H. Wang et al., 2016). *Micrococcus luteus*, a normal flora on the skin of mammals, also found in air, dust, water, and the soil, was shown to have higher biofilm biomass when subjected to simulated microgravity (Mauclaire & Egli, 2010). When biofilms of *E. coli* was formed under simulated microgravity, it was reported to be thicker than the ones produced under normal gravity, and with increased resistance to chemicals such as penicillin G, ethanol, chloramphenicol, and salt (Lynch et al., 2006)). This demonstrates that spaceflight can result in the increase of biofilms when compared to ground controls.

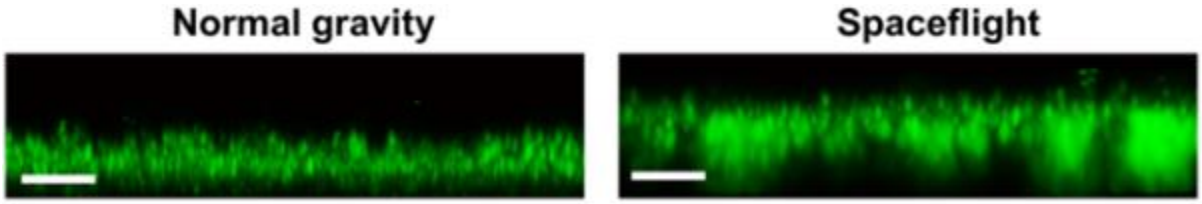

Figure 1.5: A comparison of 3 day old *Pseudomonas aeruginosa* biofilms formed in normal gravity and in spaceflight (W. Kim et al., 2013).

Figure 1.6 The inlet to the Water Processor Mostly Liquid Separator of the International Space Station, with biofilm formation at many of the channels. Image: NASA, (Zea et al., 2018).

Figure 1.7 The inlet of the Russian condensate processor, with formation of biofilms inside the condensate plumbing**.** Image: NASA, (Zea et al., 2018).

It is important to point out that, based on the literature, it seems that there is no common effect that applies to all microbes affected by microgravity. *Salmonella enteritidis* showed an increase in the growth, amikacin resistance, but genes associated with the formation of biofilm were downregulated (B. Zhang et al., 2019). Subjecting *Staphylococcus aureus* under the effects of simulated microgravity led to decreased virulence, but an increase in the formation of biofilms (S. L. Castro et al., 2011). After spaceflight, *Acinetobacter baumannii* showed a decrease in the ability to form biofilms (Zhao et al., 2019). This suggests that even biofilms

themselves do not respond to this stressor in the same manner. It is tempting to speculate that as all organisms on Earth have evolved in gravity, we cannot expect that they will all have the same response to microgravity at least in a short-term before evolution can act on selective traits. A study looking at the transcriptome of *Arabidopsis* by Paul et al., (2017) suggested that it is that some genes have responses to microgravity that are non-adaptive, have no real consequences on growth and can be sometimes inappropriately activated or misinterpreted. A study investigating the transcriptomic changes in the squid *Euprymna scolopes* and its beneficial microbial symbiont *Aliivibrio fischeri*, showed that *A. fischeri* were essentially able to turn off immune and oxidative stress response under simulated microgravity whereas this was not the case without the symbiont (Casaburi et al., 2017). This shows that some species can be quite synergistic with another species on an evolutionary level and is required for the stability of the host's health in spaceflight.

Bacterial infections during spaceflight would require the use of broad-spectrum antibiotics for treatment, and this can encourage the development of resistance (Barratt & Pool, 2008). Recent methods on creating an unsafe environment for pathogens include the use of surface coating with silver and ruthenium, an antimicrobial coating termed AGXX®, which has been shown to reduce the microbial load on the surfaces of the International Space Station, and be quite effective against human pathogens including *Enterococcus faecalis*, *Legionella*, MRSA, and *E. coli* (Clauss-Lendzian et al., 2018; Guridi et al., 2015; Sobisch et al., 2019; Vaishampayan et al., 2018). Another method being graphene oxide GOX which uses electrostatic attraction to immobilise bacteria (Wischer et al., 2020). Biofilms are secreted by microorganisms after they adhere to surfaces, persistent attachment will result in corrosion and bioleaching by the microorganisms as demonstrated in Figure 1.8 (Kanematsu & Barry, 2015; Parra et al., 2021; Vera et al., 2013; Wischer et al., 2020). A study on graphene coating has been shown effective in reducing the ability of biofilm induced corrosion and bioleaching for *Cupriavidus metallidurans* (Figure 1.8) (Parra et al., 2021). GOX has been shown to be effective against both Gram-positive (*S. aureus*) and Gram-negative bacteria (*E. coli*) (Ahmed

et al., 2020). While both AGXX® and GOX have been shown to be quite effective in reducing surface-based colonisation by airborne and pathogenic bacteria, these methods would not necessarily be feasible if an invasive procedure is required.

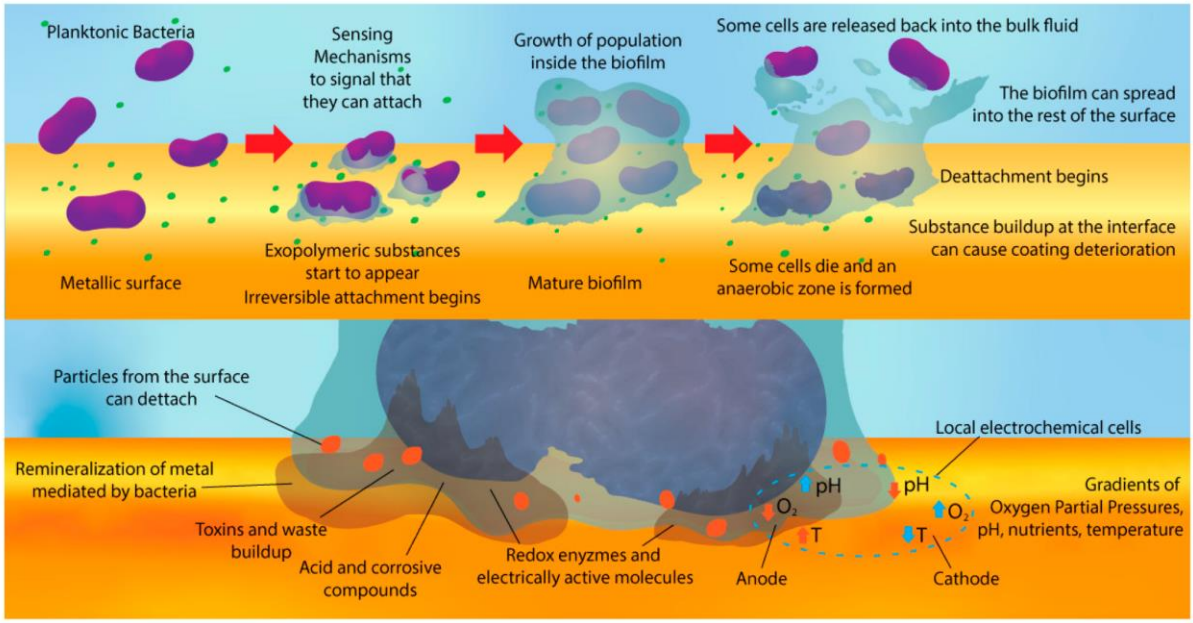

Figure 1.8 Diagram shows the stages of biofilm growth (top half), and the mechanisms that lead to metal corrosion. Figure adapted from Parra et al., (2021).

1.3 Research hypothesis and objectives

Studying how a spaceflight stressing factor (i.e. simulated microgravity) affects a model human-colonizing bacteria implicated on dysbiosis (i.e. *Gardnerella spp)*, will help us understand the potential health threats that astronauts might face during space exploration, paving a way for the design of mitigation strategies. The induction of certain bacterial behaviours via microgravity could also lead us to envisage potential applications.

In this thesis, we propose to investigate omics datasets with the **major aim** of understanding global biological responses to microgravity. But how would we analyse data from biological datasets? Omics datasets contain a lot of data, with many variables. The size and complexity of omics datasets mean that there is always more that can be learnt from a dataset. But getting the most out of it requires sophisticated algorithms to tackle in-depth analysis. My **first objective** (Chapter 2) was to develop a pipeline which could be used in processing and analysing a range of different spaceflight omics, and omics datasets in general.

Following from this, my **second objective** (Chapter 3) was to move beyond open access datasets to analyse my own metabolomics experiment on the effects of simulated microgravity on *Gardnerella sp*.

In my final chapter, I discuss the technical challenges and pitfalls during this research and summarize the main findings. What worked on open access omics data, for instance? What extra considerations were to be required when conducting our own omics experiment? How potential metabolic changes are matched to phenotypic changes? What are the future prospects of this and related research and how may they contribute to advancement of space science and society?

Chapter 2 - Analysis of spaceflight biological data

2.1 Omics

High-throughput technologies are being used to obtain a wide view of biological molecules that make up an organism (Aizat et al., 2018). These technologies are often termed ‘Omics’. Omics leads to the generation of a large amount of biological data, and these technologies form the backbone of Systems Biology. Systems Biology was initiated to understand the cell as a whole (Aizat et al., 2018). Omics datasets often contain a number of samples with hundreds to thousands of features. These features can include metabolites (metabolomics), proteins (proteomics), mRNA (transcriptomics), and genes (genomics). Prior to Omics, biological experiments tended to be hypothesis-driven, whereas omics tend to be hypothesis-generating (Horgan & Kenny, 2011). Omics are well-used in biomarker discoveries (Kell, 2006; Montaner et al., 2020), and it also be used for uncovering spaceflight-related insights.

The omics workflow and biological molecules can be seen in Figure 2.1. Genomics is the study of an organism's genome, and is used to unveil abnormalities such as chromosomal insertions and deletions, genomic hybridisation, down to single nucleotide polymorphisms (Horgan & Kenny, 2011). The transcriptome is the amount of detected mRNA or expressed genes of an organism or cell at the time of measuring. The proteome is the expressed proteins of an organism, tissue, or cell, and can be used to better understand protein pathways and networks (Petricoin et al., 2002; Theodorescu & Mischak, 2007). Metabolomics is the expressed metabolites of a system at a given time, and the systems can include cells, tissue, and organisms, media and other liquids (Goodacre et al., 2004). The linearity of the central dogma is being tested in favour of multi-directionality (Franklin & Vondriska, 2011).

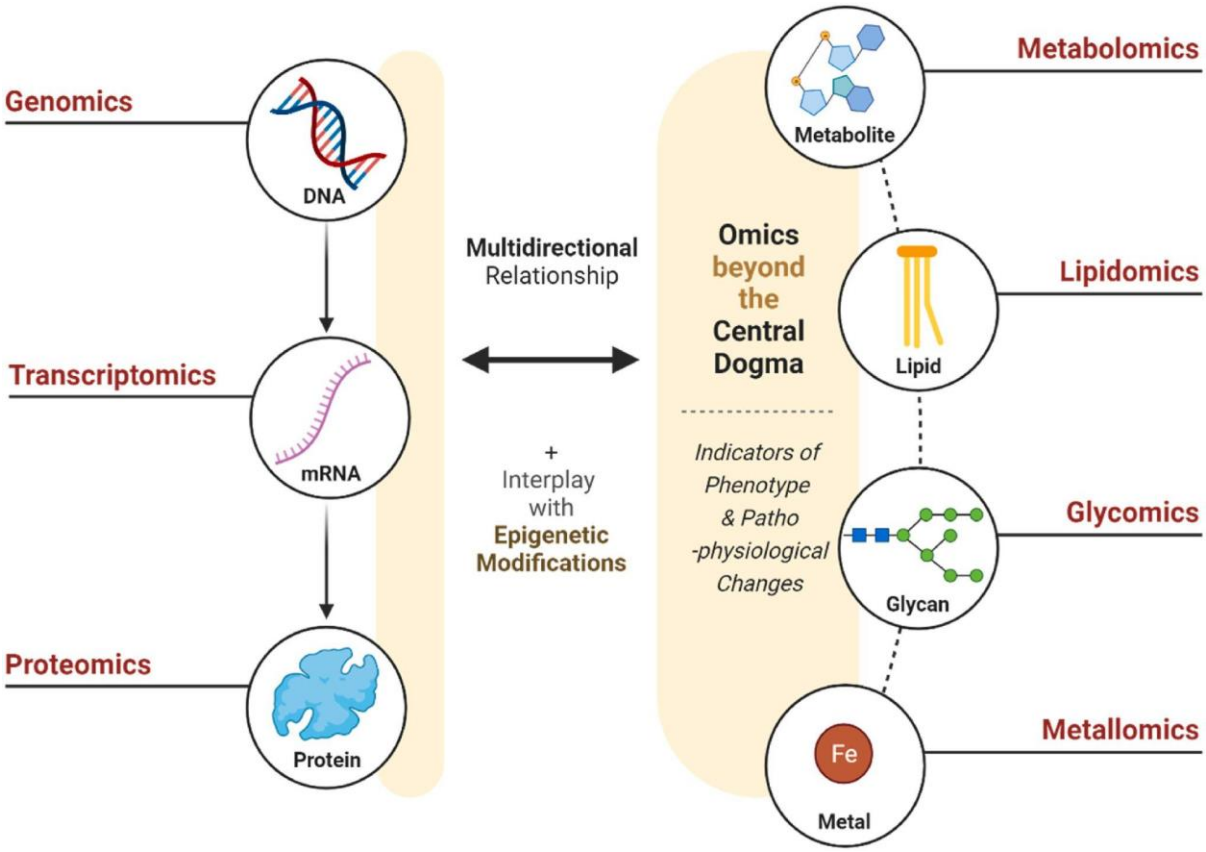

Figure 2.1 Holistic view of Omics relationships. Reproduced from Lim et al., (2021).

Small changes in the transcriptome and proteome can have a large impact on the metabolome (Kell, 2006). In both theory (Mendes, 1996) and in practice (Mendes, 1996; Raamsdonk et al., 2001; Urbanczyk-Wochniak et al., 2003).

2.2 Pipeline for Omics Analysis

Omics are large datasets, and this presents opportunities for new multivariate methods. The main aim of this chapter is to explore a few multivariate methods, and potentially explain when a particular multivariate method is useful.

2.2.1 Data transformation in Metabolomics

In omics research, several steps are required before a dataset is suitable for analysis. Chapter 3 of this thesis will focus on metabolomics. As seen in Figure 2.2, raw data is first obtained, and biological samples are extracted and prepared for analysis. This is followed by data pre-processing to form clean data (Shurubor et al., 2005; Werf et al., 2005), which is commonly observed as normalised peaks of the intracellular metabolite concentrations (R. A. van den Berg et al., 2006). These datasets go through pre-treatment and treatment before analysis. The main aim of these processes is to reduce the influence of noise from measurements. Chapter 2 will focus on the analyses while Chapter 3 will explore the normalisation and data transformation methods as seen in Figure 2.3. Normalisation is considered to be part of the data pre-treatment process.

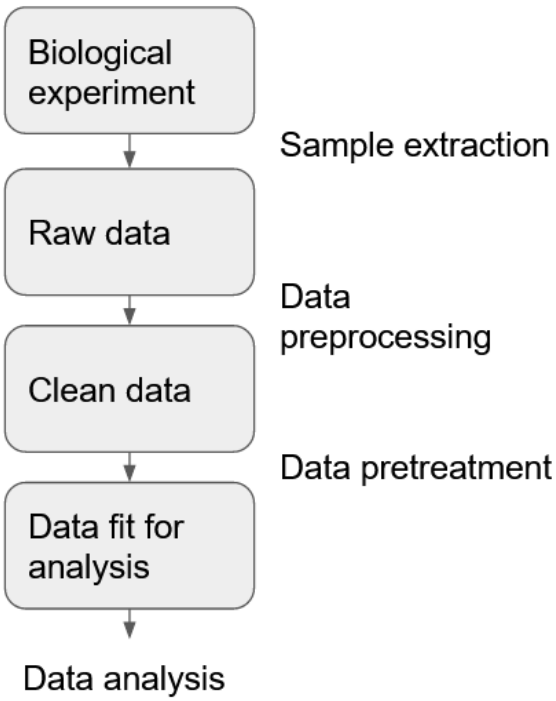

Figure 2.2 Steps required for analysis of metabolomics. Adapted from van den Berg et al., (2006).

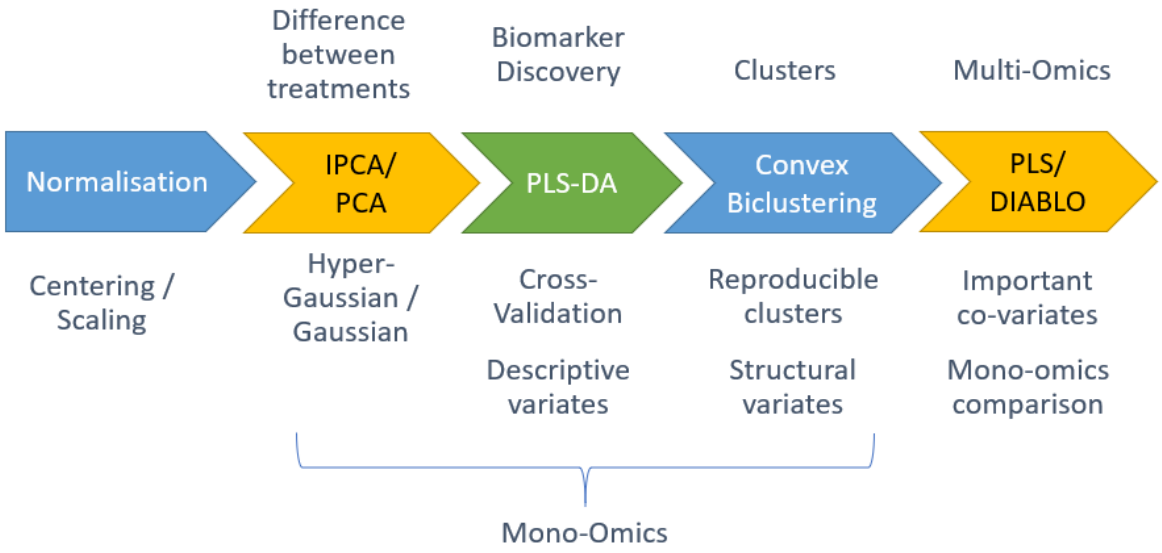

Figure 2.3 The proposed pipeline for analysing high-throughput biological datasets. IPCA stands for Independent Principal Component Analysis, and respectively for PCA. PLS-DA stands for Partial Least Square Discriminative Analysis. PLS stands for Projection to Latent System. DIABLO is an acronym of **D**ata **I**ntegration **A**nalysis for **B**iomarker discovery using **L**atent variable approaches for **O**mics studies. Mono-omics is used in this diagram to represent single omics.

In this thesis, I have developed a pipeline for analysing omics datasets using a series of machine learning and/or multivariate analysis techniques. The main goal of this chapter is not to investigate biological learnings, but to explore these different investigative methods on spaceflight biological datasets. When choosing between univariate and multivariate analyses, Nimon and Williams (2009) recommended multivariate methods when sphericity is largely violated ($\varepsilon < 0.7$) and when sample size is not small ($n > 30$). The sphericity assumption is more likely to be violated during small sample size and when measurement occasions occur in a noticeable manner (Haverkamp & Beauducel, 2017). One of the methods (Girden, 1992) identified for measuring sphericity is the examination of differing variances between paired occasions of measurement. A range of sources, such as measurement errors, sample preparation, and biological variation from the abundance of

metabolites (relative concentration) cause omics datasets to suffer from variability (Schwämmle & Jensen, 2018). Sample size is often small due to cost, and technological constraints that results in the measurement occasions causing the differing variances. This should make multivariate analyses the *de facto* standard for studying omics datasets. It should also be pointed out that while sometimes I discuss metabolomics exclusively as it is a big part of my analyses, statistical methods aren't exclusive to any omics or particular type of datasets. I will go through several multivariate algorithms and in what manner they become useful, and under circumstances when particular multivariate methods become useful.

In the analysis for Chapter 2, the publicly available datasets of analysis were retrieved from genelab.nasa.gov hosted by NASA Genelab ("NASA GeneLab", 2020). Three datasets were analysed, GLDS-138, GLDS-145, which features the effects of spaceflight on *Bacillus subtilis*, and *Staphylococcus aureus* respectively across transcriptomics, proteomics, and metabolomics, and GLDS-251, which was on the transcriptomic response of *Arabidopsis thaliana* to fractional gravity under blue light and while during spaceflight. Data processing for metabolomic datasets of GLDS-138 and GLDS-145 was carried out together; this is possible as GLDS-138 and GLDS-145 are extracted together. The transcriptomic datasets for GLDS-138, 145, and 251 were processed and normalised by Genelab (S.G. Gebre, personal communication, March 27, 2020). The data pre-processing step for GLDS-138 and GLDS 145 were carried out by NASA Genelab (S.G. Gebre, personal communication, April 1, 2020). The pre-processing steps for metabolomics are explored in Chapter 3, but for chapter 2, the main objective is developing a pipeline.

In the data pre-treatment steps of GLDS-138 and GLDS-145, all samples undergo a row-wise correction step; this was conducted as samples have technical and biological variation. There are many different methods for correction variation. Since no internal standards were present in the datasets, we correct the variation by dividing by the sum of all peak intensities. This was conducted as it normalises the data by the total number of detector hits in the mass

spectrometer (R. Bang, personal communication, February 28, 2020). For the baseline correction, we made use of the Tryptic Soy Agar medium to subtract from peak responses.

The next step of the pre-treatment process is scaling. There are many different methods for normalising metabolomics data (van den Berg et al., 2006). Data transformation is important as omics data often have orders of magnitude differences between metabolite concentrations, differences in fold changes, differences in fluctuations of metabolites, technical variation, and heteroscedasticity (R. A. van den Berg et al., 2006). Heteroscedasticity is important as variations in biological sampling and measurements assume that the data has equal standard deviations, are symmetrically distributed, and are centered around zero (R. A. van den Berg et al., 2006). This assumption is not true, heteroscedasticity is often present, and introduces additional data structure (H. R. Keller et al., 1992). Methods aimed at removing variations due to heteroscedasticity include the *Log Transformation (Purohit et al., 2004)*, the *Auto Scaling* (Hu & Xu, 2013), *Range Scaling* (Smilde et al., 2005), *Level Scaling* (R. A. van den Berg et al., 2006), and *Centering* (Bro & Smilde, 2003). Rather than settle on a popular scaling method, vanden Berg et al., (2006) suggests that the pre-treatment method depends on the dataset itself, and choosing the incorrect pre-treatment method can lead to an incorrect biological interpretation.

In Figure 2.4, the pre-normalised data appears to be almost the same as the data after log transformation, where there are only a few large peaks. Both level and centre scaling are not significantly different from the pre-normalised data. Autoscaling and range scaling led to many large peaks.

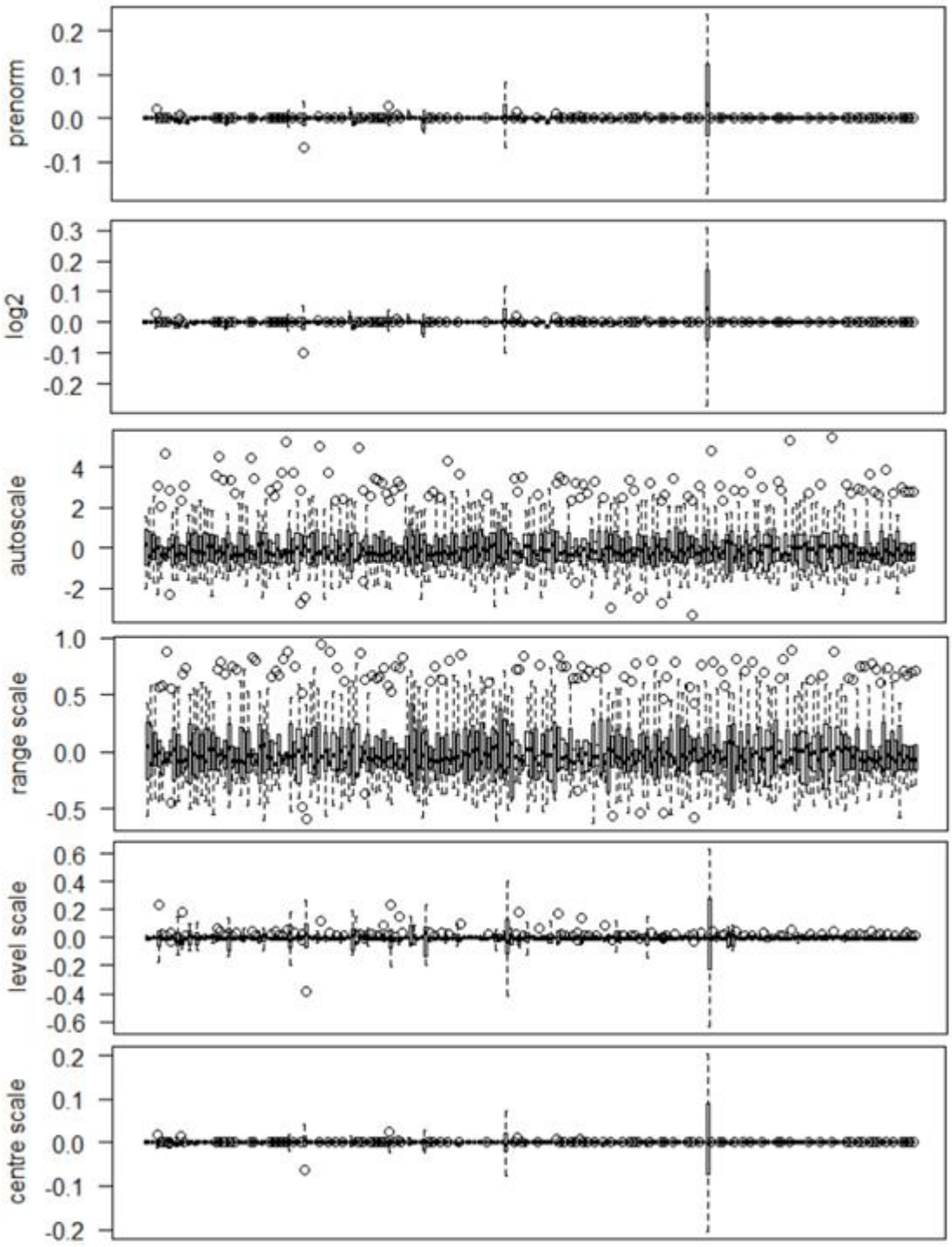

Figure 2.4 A comparison of pre-normalised data, with a range of scaling methods. Data used is the GLDS-138 *Bacillus subtilis* metabolomics dataset.

Scaling influences heteroscedasticity, and this changes the group distribution as well as metabolic ranking (R. A. van den Berg et al., 2006). Van den Bern et al., (2006) also stresses that while the stability of the rank is determined by the standard deviation, and often

abundant metabolites are highly ranked, this does not mean that the biological answer is the most relevant. Since my interest is based on changes in proportion, the scaled data provided from the autoscaling and range scaling where the metabolite abundances are of similar ranges have been levelled out is of my interest. Since range scaling and autoscaling increased the error range when low metabolic abundances are scaled, I have selected autoscaling to minimise the error.

2.2.2 PCA/IPCA

Typical multivariate statistical analyses in metabolomics studies include Principal Component Analysis (PCA) and/or Independent Component Analysis (ICA) (Pan et al., 2007; Scholz et al., 2004). Rather than simply choosing PCA or another method of analysis as a solution for all biological datasets, it's better to understand their assumptions. Both PCA and ICA are unsupervised learning methods, where the underlying structure is estimated without the treatment labels; the labels are added after the structure has been made (Yao et al., 2012).

PCA assumes that a dataset follows a normal (Gaussian) distribution (Jolliffe & Cadima, 2016). ICA targets the non-Gaussian components of the distribution (Hyvärinen & Oja, 2000; Yao et al., 2012). While ICA has been seen as a replacement to PCA, it has its own limitations. It is difficult to tell how many components are required, and the averaging of multiple runs is required for consistency (Engreitz et al., 2010; Yao et al., 2012). Independent Principal Component Analysis (IPCA) is where ICA is used to denoise PCA (Yao et al., 2012). Kurtosis values, first proposed for ICA, will be used in IPCA for selecting the number of components to use (Hyvärinen & Oja, 2000; Yao et al., 2012). It is not uncommon to see super-Gaussian distributions in metabolomics (Liu et al., 2016; X. Li et al., 2012; Scholz et al., 2004). IPCA offers a better visualisation of the super-Gaussian

distributions than ICA and with a reduced number of components than PCA (Yao et al., 2012).

Regardless of whether we use PCA or IPCA, one should note the true use of these analyses. PCA is used to find differences between groups, so we should be careful not to use PCA for biomarker discovery (Hendriks et al., 2011).

2.2.2.1 Kurtosis

In Figure 2.5, we can see a distribution of the principal component (PC) loadings for the *B. subtilis* metabolomics dataset. Kurtosis values for this dataset: 1st IPC **-0.31**, and 2nd IPC **-0.68** (graph not included). Kurtosis is a measure of Gaussianity, a highly positive value indicates hyper-Gaussian, whereas highly negative values indicate sub-Gaussian (Hyvärinen & Oja, E, 2000). Although we can see that the 1st IPC is slightly negative, it is close to zero, and visibly a normal distribution. From this, it is appropriate to use PCA, IPCA is not required.

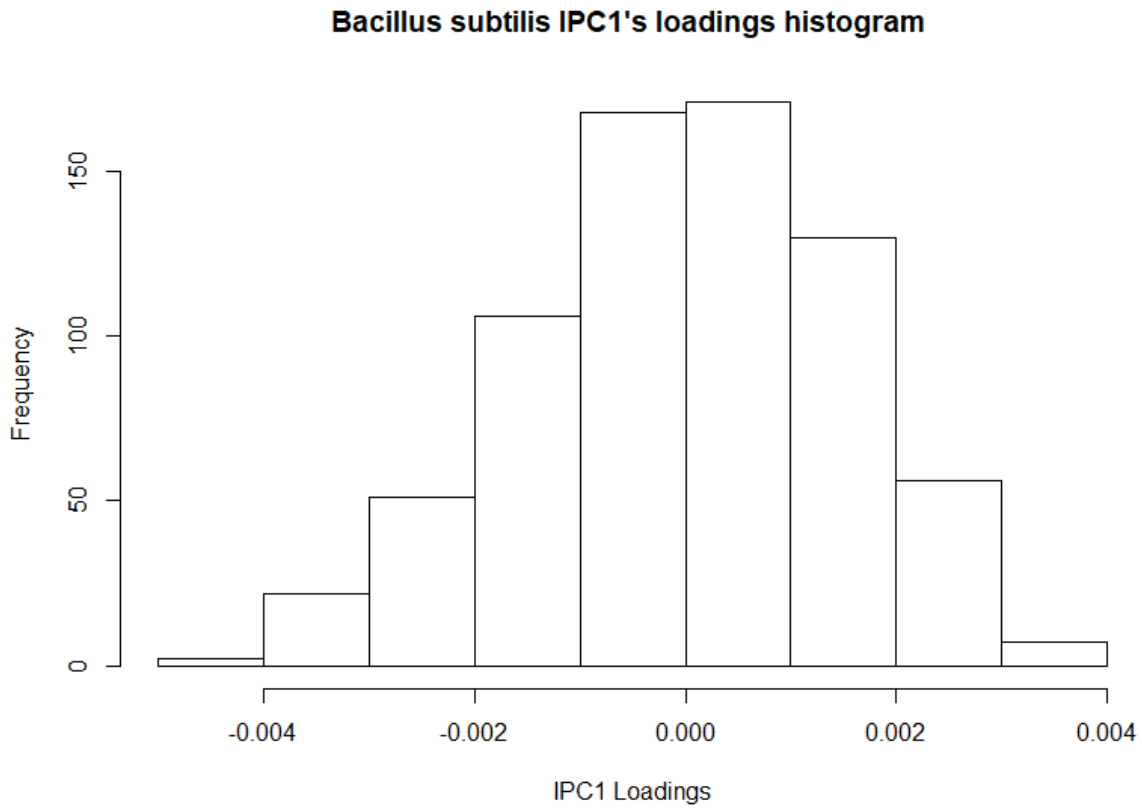

Figure 2.5 A histogram visualisation for *Bacillus subtilis*'s IPC1's loading distribution. Data used is the GLDS-138 *B. subtilis* metabolomics dataset. IPC stands for Independent Principal Component.

The Kurtosis value for *S. aureus* pellet metabolic expressions, we see a value of 88.24, and 8.40, for the 1st (Figure 2.6) and 2nd principal component (Figure 2.7) respectively. The highly positive value also tells us to quantify how hyper-Gaussian the histograms are. A lot of the values are situated quite close.

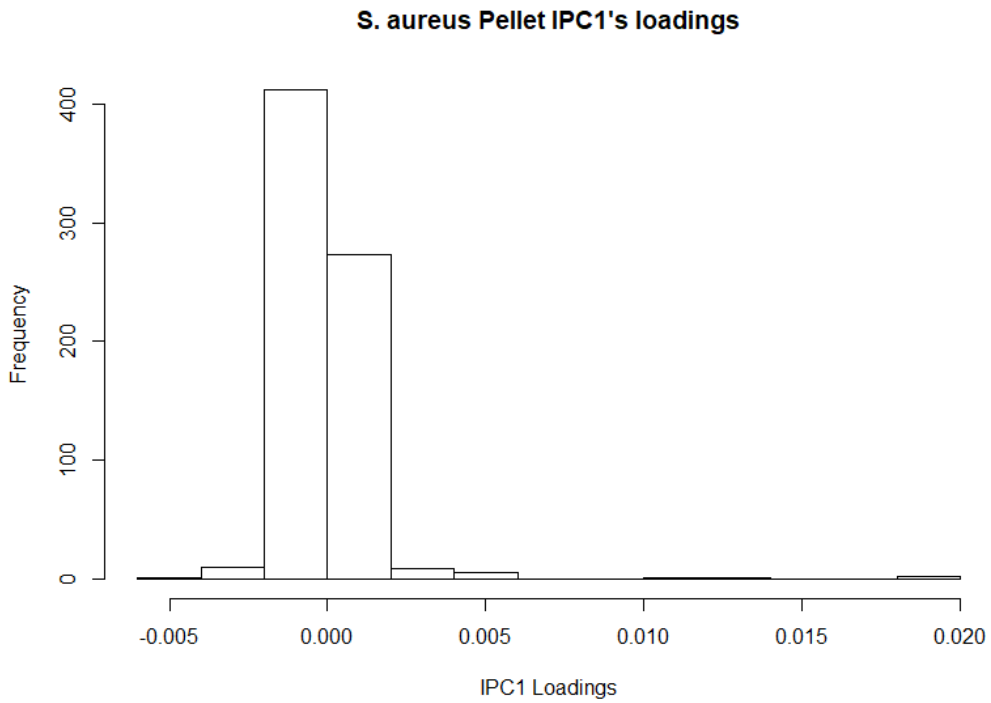

Figure 2.6 A histogram visualisation for *Staphylococcus aureus*'s IPC1's loading distribution. Pellet is the term for bacteria, as metabolomics can involve metabolites from the bacteria or the extracellular media metabolomics. Data used is the GLDS-145 *S. aureus* metabolomics dataset.

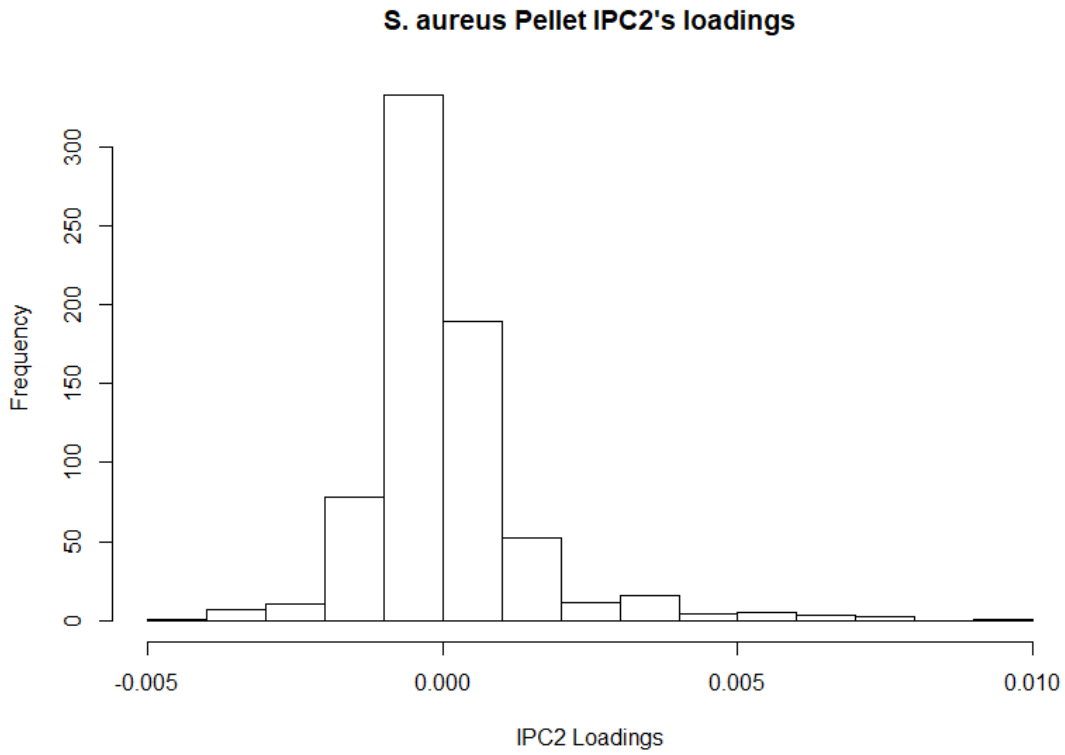

Figure 2.7 A histogram visualisation for *Staphylococcus aureus*'s IPC2's loading distribution. Data used is the GLDS-145 *S. aureus* metabolomics dataset.

2.2.2.2 Groupings

GLDS-138 and GLDS145 are datasets with many different variables. To simplify, we start with a few variables and increase complexity. In Figures 2.8 and 2.9, both have substantial overlaps. The closed curve is the 95% confidence interval for each treatment. This tells us that *B. subtilis* is more or less the same in spaceflight and on ground. In Figure 2.10, we bring in and recalculate the PCA for intracellular and extracellular metabolites together. While the intracellular and extracellular metabolites are in general different, some overlaps occur indicating that the content of intracellular and extracellular metabolites are similar. Extracellular media are the metabolic content of the media, it can express a lack of change, metabolic release or absorption from the bacteria. Figure 2.10 shows more clearly than Figure 2.9 that the spaceflight extracellular metabolites have greater level of variation than the ground control.

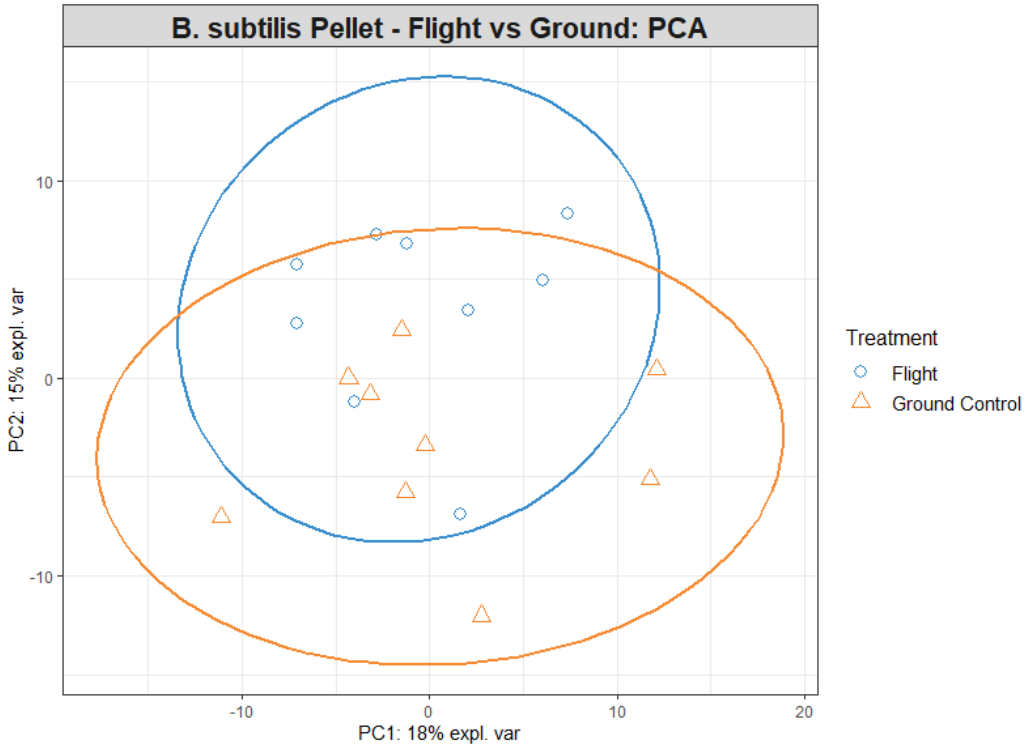

Figure 2.8 A PCA comparison of *B. subtilis* intracellular metabolites with the spaceflight and ground control treatment. There are 9 biological replicates for each group.

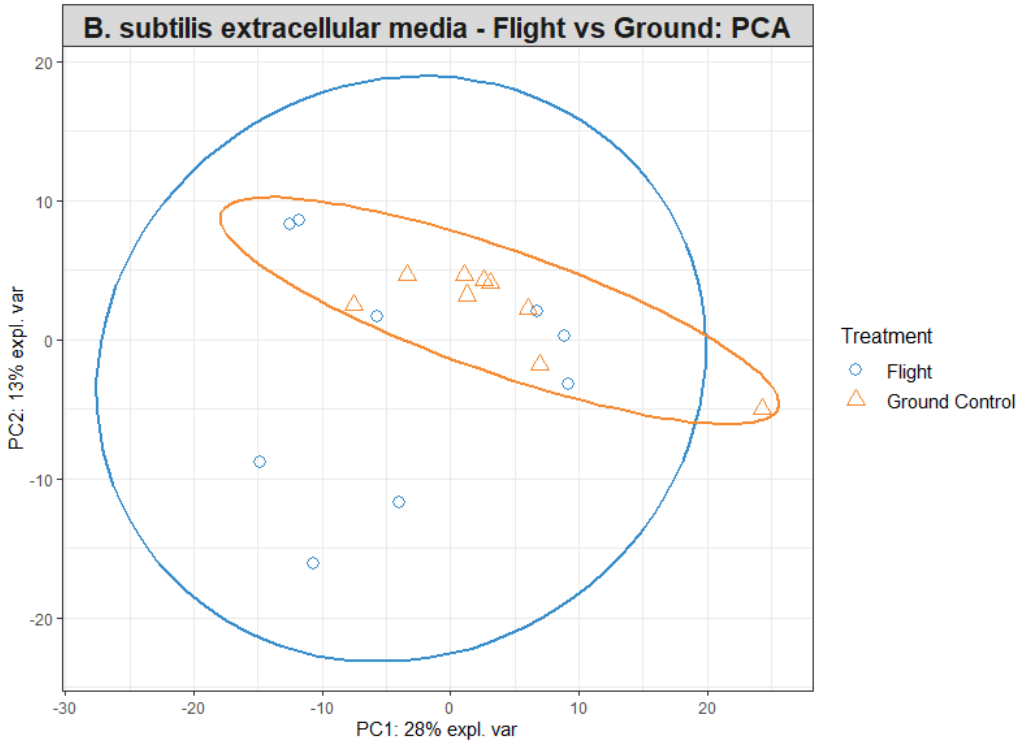

Figure 2.9 A PCA comparison of *B. subtilis* extracellular metabolites with the spaceflight and ground control treatment. There are 9 biological replicates for each group.

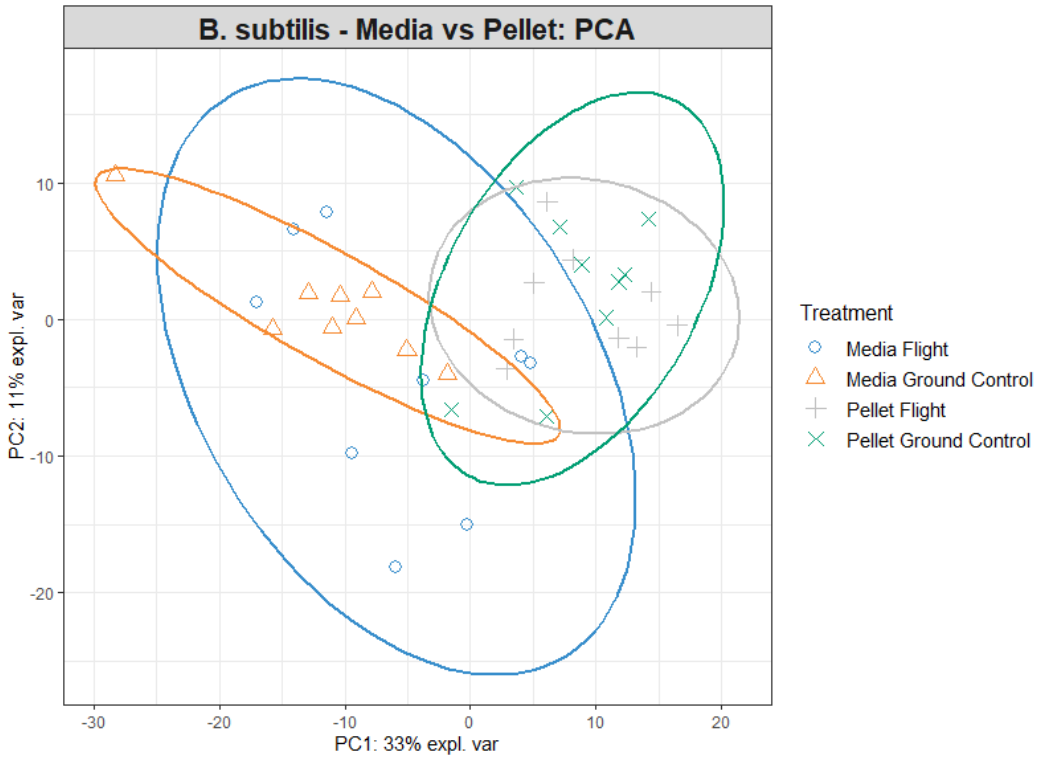

Figure 2.10 A PCA comparison of *B. subtilis* of intracellular and extracellular metabolites with the spaceflight and ground control treatment.There are 9 biological replicates for each group.

The CTRL ASAP and CTRL Frozen results seen in Figure 2.11 were conducted because the freezing protocol for the Bacterial Culture in Biological Research in Canisters (BRIC) hardware showed a slow freezing profile due to thermal inertia (J.M. Galazka, personal communication, May 23, 2020). This was done to compare the effects of fast-freeze and slow-freeze protocols on metabolites. The changes caused to metabolites in the slow-freeze protocol have less variation than fast-freeze protocol. The Positive CTRL Frozen and Ground Control seen in the left side of Figure 2.11 are the same treatment. However, they are separated from one another; this is likely due to batch effects of sample processing on a different date, which was confirmed by J.M. Galazka (personal communication, May 23, 2020). Batch effects will be explored in Chapter 3.

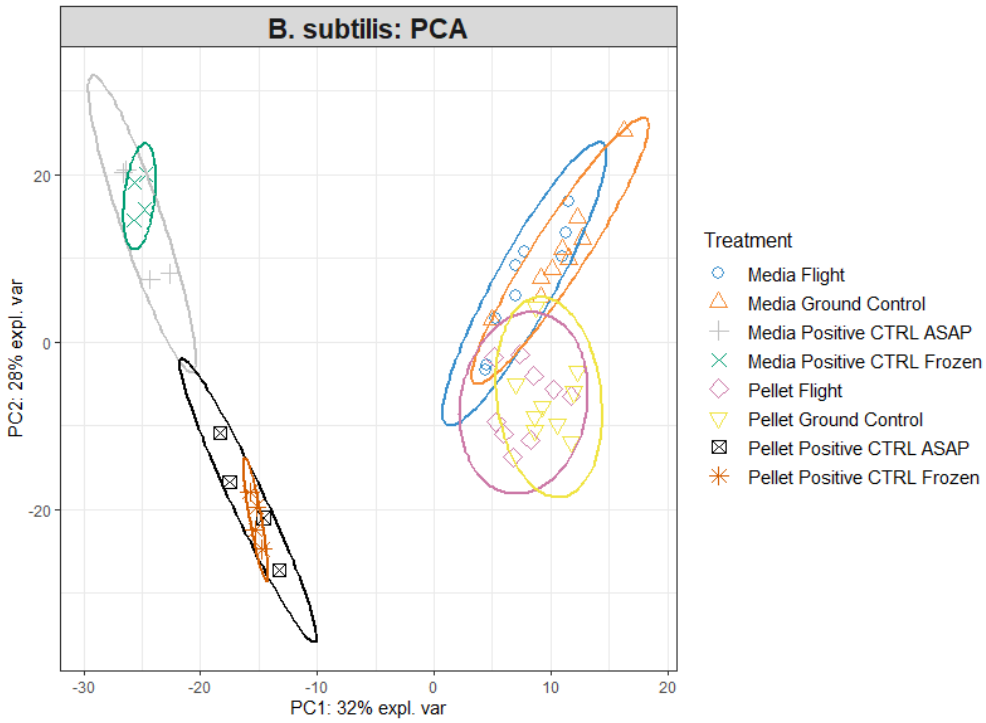

Figure 2.11 A PCA comparison of *B. subtilis* metabolites with the spaceflight, Ground control, and ASAP and Frozen treatment. There are 4 biological replicates in the CTRL treatments. The other treatments have 9 biological replicates.

In Figure 2.12, we can see that the extracellular metabolites, regardless of treatment, are mostly on the negative values of the principal component 1, and the intracellular metabolites

have positive values. The separation between the top, middle, and the bottom clusters are likely due to batch effects from different processing dates.

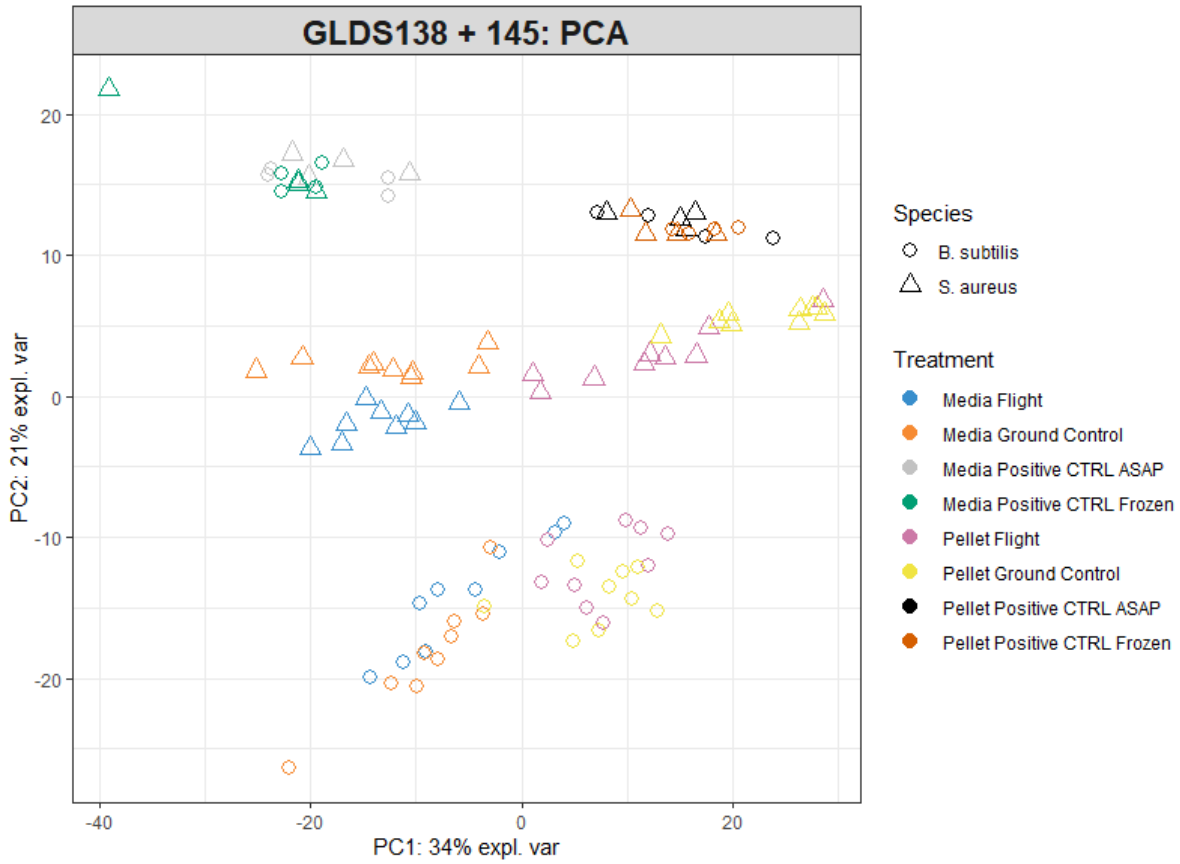

Figure 2.12 A PCA comparison of *B. subtilis* and *S. aureus* metabolites with the spaceflight, ground control, and ASAP and frozen treatment. There are 4 biological replicates in the CTRL treatments. The other treatments have 9 biological replicates.

2.2.3 PLS-DA

Originally, statistical discrimination had not been the intention of the analysis Partial Least Squares (M. Barker & Rayens, 2003). Partial Least Squares-Discriminant Analysis (PLS-DA) is now routinely employed across many different large biological datasets such as transcriptomics and metabolomics (Szymańska et al., 2012; Y. Tan et al., 2004). One problem of PLS-DA is that it aggressively overfits models (Saccenti et al., 2014). This does

mean that it should not be used as a representation of class differences between groups. PLS-DA is an example of a supervised learning algorithm. This is where known responses (or labelled data) with a corresponding set of input data are mapped to see which features of the input data are key descriptors of the labelled data (Yang et al., 2019). This is useful to see which molecules are highly active in response to a treatment. Appropriate use of cross-validation on PLS-DA does allow its use for biomarker discovery (Szymańska et al., 2012). PLS-DA's origin is briefly discussed in section 2.2.5.

In comparison to the PCA in Figure 2.8, PLS-DA (Figure 2.13) aggressively overfits, and it is well documented to do so (Saccenti et al., 2014). PLSDA should not be used for inferring group differences (Westerhuis et al., 2008). Conversely, by noticing that variables or biomolecules exist in the separation of treatments, then those variables will be of interest.

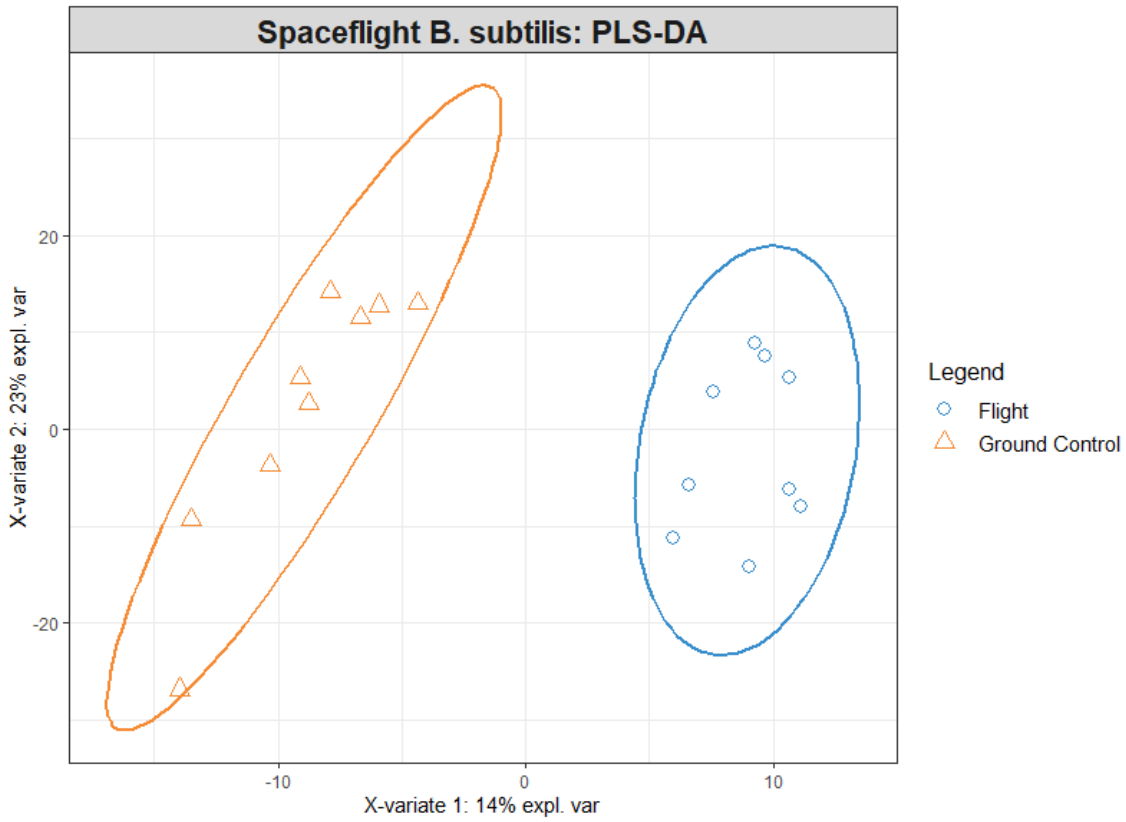

Figure 2.13 A PLS-DA comparison of *B. subtilis* intracellular metabolites with the spaceflight and Ground control treatment.

2.1.3.1 Sparsity and Cross Validation

Due to PLS-DA's aggressive overfitting, attention has been paid towards validating PLS-DA models (Westerhuis et al., 2008). This has resulted in the development of cross-validation strategies (Saccenti et al., 2014). Cross-validation is a method to test the accuracy of a model, and to ensure that it is not noise that defines the model (Piryonesi et al., 2020).

Due to the cost of omics studies, some only have a small number of samples available. A low number of samples means that the separation of data into training, validation, and test sets are often not possible, cross-validation makes better use of having few samples (Westerhuis et al., 2008). The cross-validation (CV) method used is the leave-one-out CV (A. C. Tan et al., 2005). This is where one sample was left out in each iteration as the test sample, and the analysis repeated until each sample has taken the role of the test sample (S. Kim et al., 2016; A. C. Tan et al., 2005). The quality of the models is measured with the balanced error rate (BER), which is the average proportion of wrongly classified samples, and weighting based on the number of samples (Rohart et al., 2017).

The number of repeats in Figure 2.14 was applied so that the classification error rate is a good estimate, whereas the CV-folds are determined in a random manner. From this, we can decide on the number of components to keep in the final model. In the performance plot of Figure 2.14, we observe that all the different methods of calculating error rates overlapped one another, and there is a decrease from 1 to 2 components. The average error rate rises at the 4^{th} component. The different components have overlapping error rates, and the standard deviation of each error rate crosses zero.

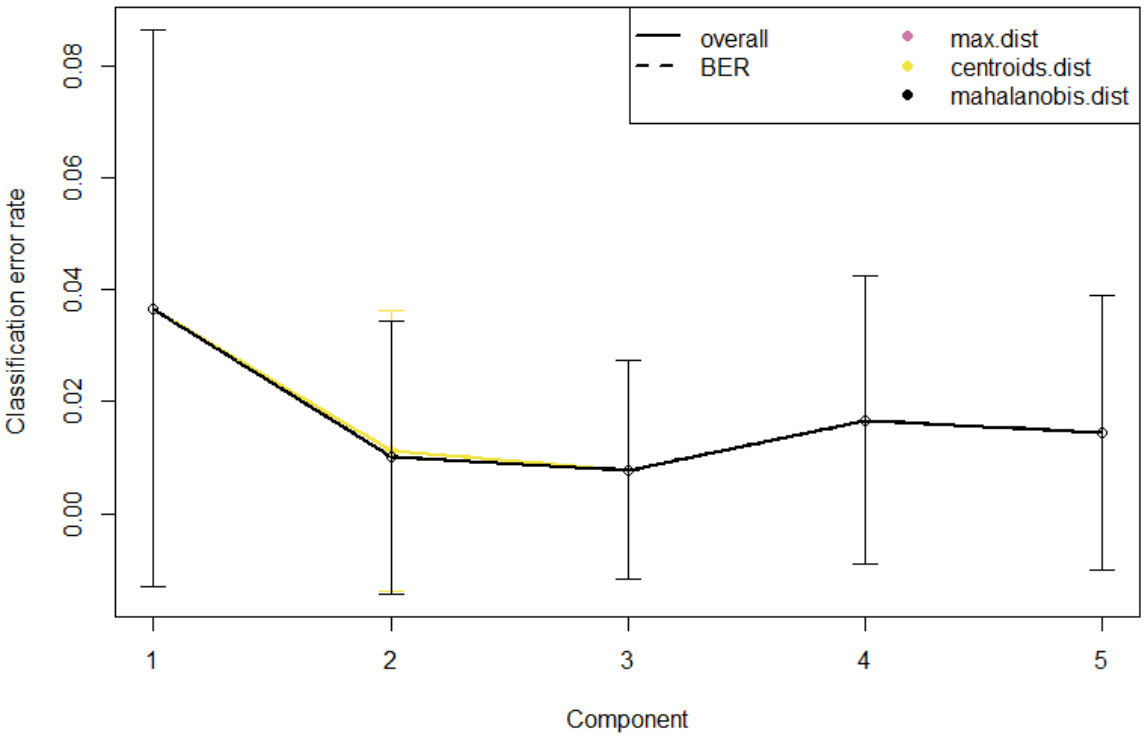

Figure 2.14 A classification performance plot of our PLS-DA model. A 5-fold cross-validation repeated 50 times was applied.

After estimating the classification performance via the error rate, tuning is performed on 3 components individually to predict treatment across all CV runs. After running CV, the optimal number of components was kept 1. The optimal number of metabolites was kept 5. This can also be observed in Figure 2.15, where the BER gradually lowers to 0 in the 1st component, whereas it starts off at 0 for both 1-to-2 component, and 1-to-3 component. The classification error rate is the lowest with just 1 component. So the final model uses the 1st component with the top 5 metabolites.

Having a model does not automatically mean that it is accurate, so this helps to understand the adjustments needed to improve the accuracy of our model. It can also be said that there are only a few key descriptors defining a trend.

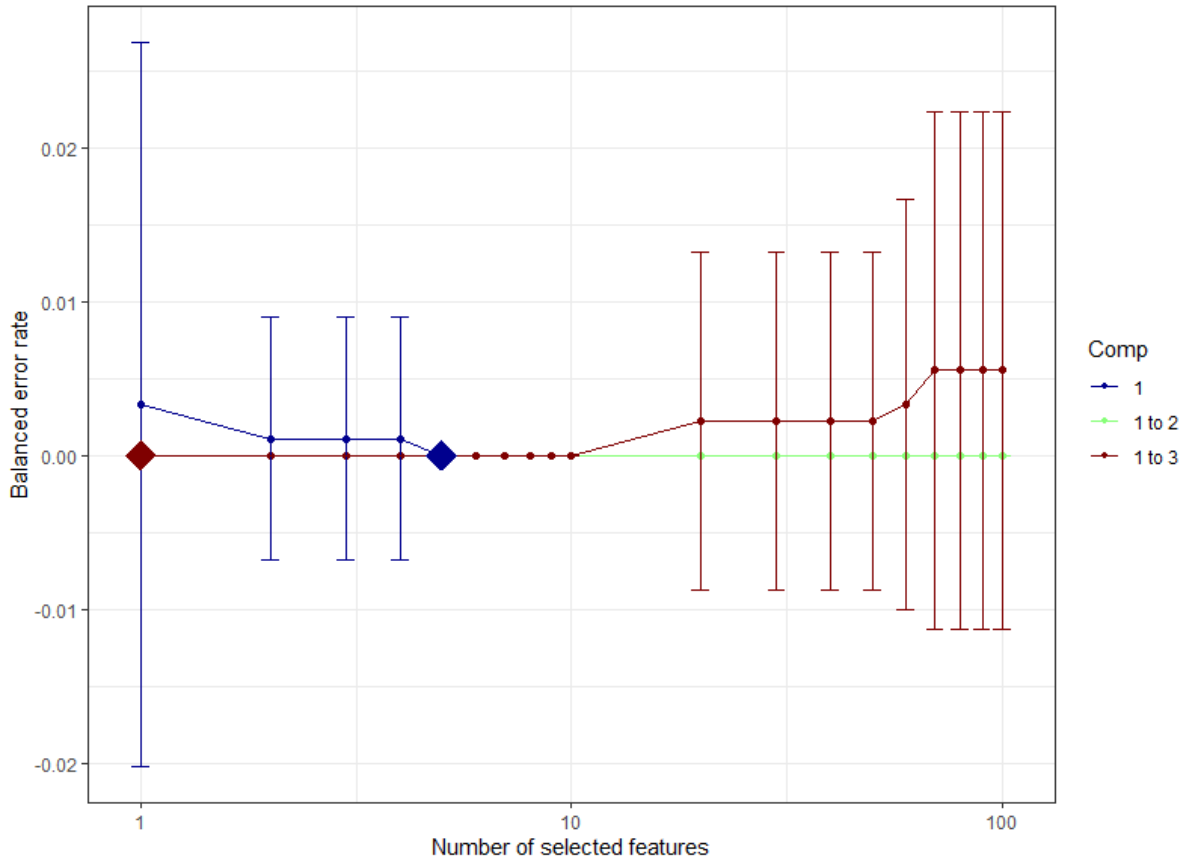

Figure 2.15 A classification error rate plot of our PLS-DA model. Across 3 components, a 5-fold cross-validation repeated 50 times was applied for tuning.

The PLS-DA model from Figure 2.13 was made from all metabolites in the *B. subtilis* flight vs ground control dataset. The sPLS-DA analysis (Figure 2.16) identifies a small subset of metabolites that best discriminate the treatments. We can see with just 5 metabolites, we can observe a similar trend in the 1st component. The top metabolite in the 2nd component has been included, but we can see that it does not define a trend as there is overlap between the flight and ground control data.

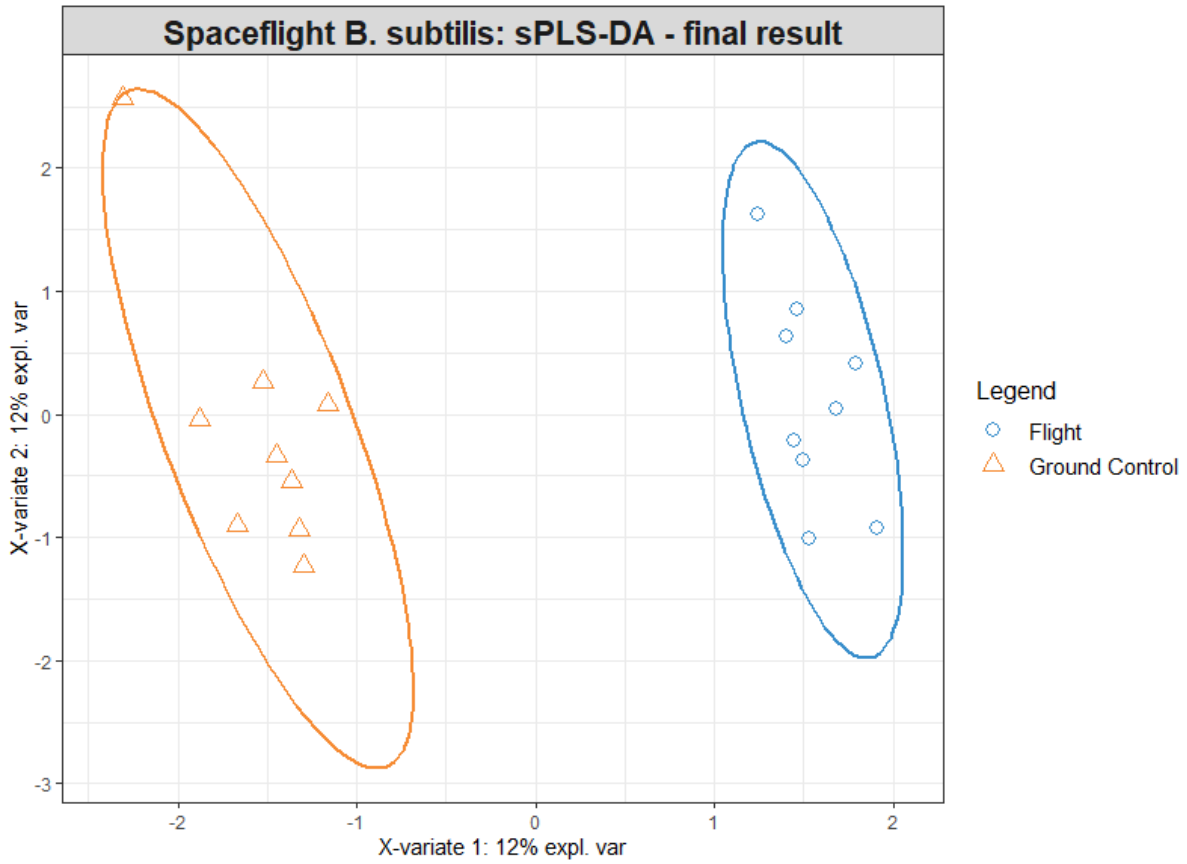

Figure 2.16 A sPLS-DA comparison of *B. subtilis* intracellular metabolites with the spaceflight and ground control treatment.

From Figure 2.17, after CV, the top 2 metabolites from the 1st component are ribonic acid and methionine, the next three are unknown metabolites. Consistent with Figure 2.18, ribonic acid and methionine have a lower and a higher regulation in spaceflight as compared to ground control respectively.

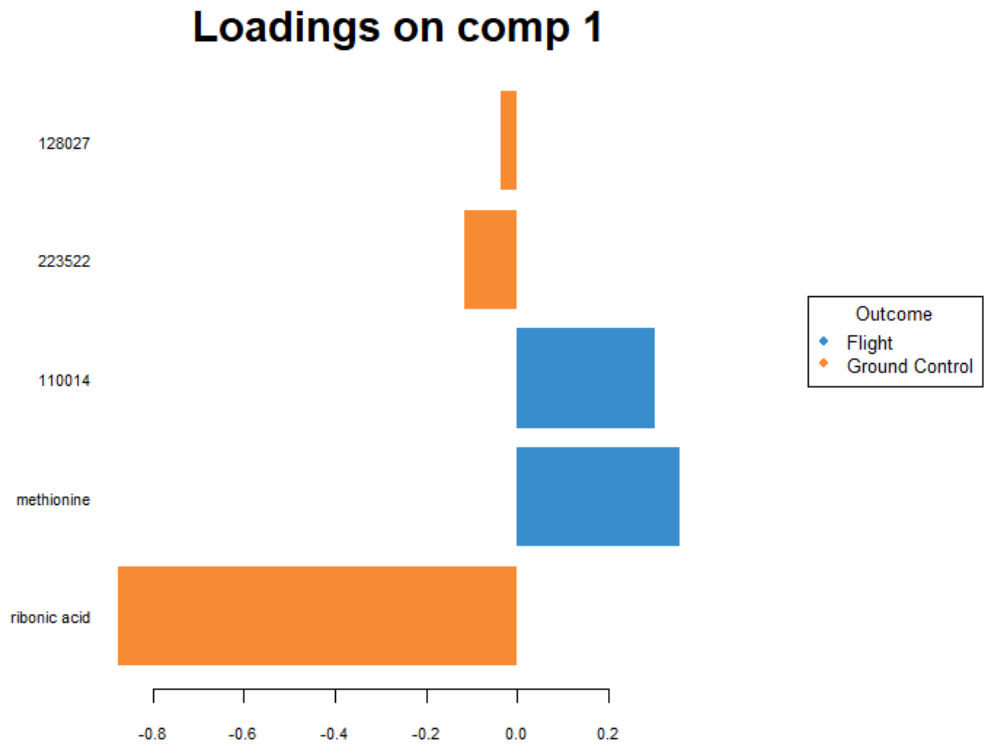

Figure 2.17 A sPLS-DA loadings plot on the top 5 metabolites of the 1st component.

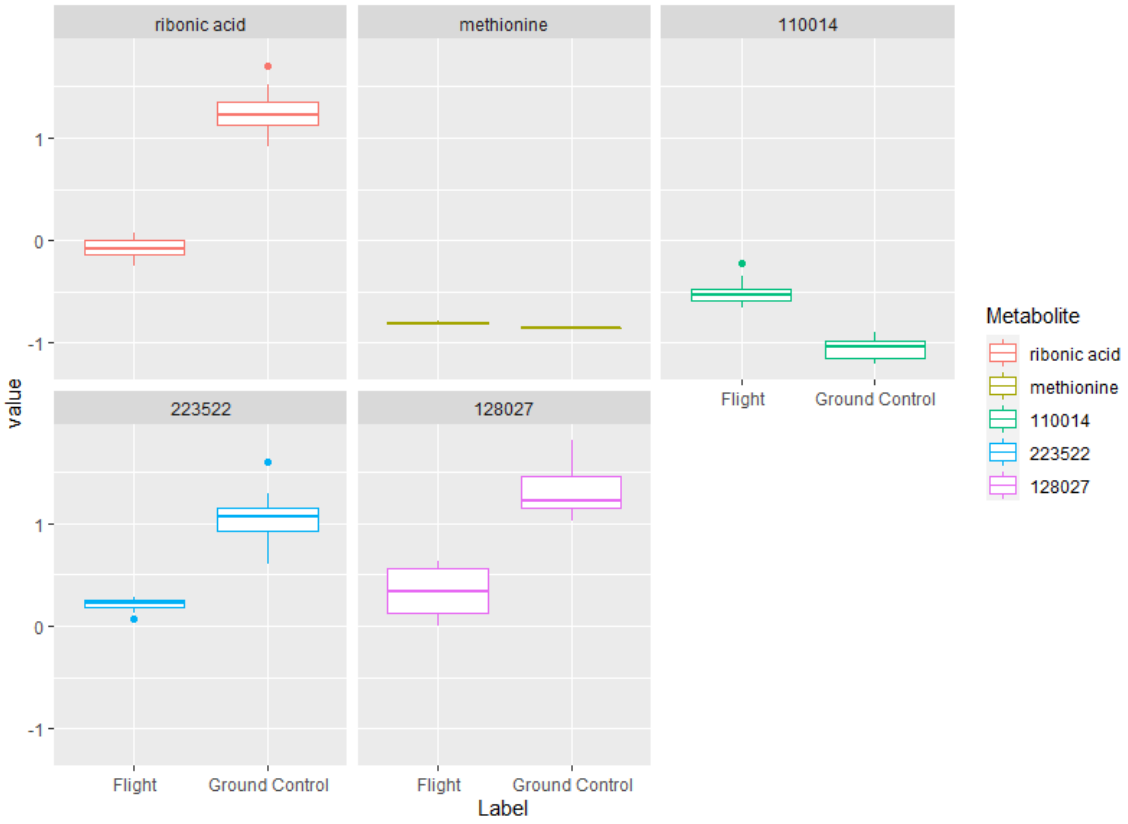

Figure 2.18 Box and whisker plot for the top 5 metabolites from the 1st component, using normalised values.

2.2.4 Convex Biclustering

When sufficient samples and features exist, convex biclustering becomes especially powerful in identifying structurally important features. Biclustering in general has successes in identifying important biological features such as gene biomarkers that separate groups of patients (Sørlie et al., 2001; Sorlie et al., 2003). However, findings are often not reproducible, and this has been speculated to be as a result of ineffective groupings and computational methods for the identification of biclusters (Chi et al., 2017; Sørlie et al., 2001). The clustered dendrogram is the most popular use of biclustering, which utilises hierarchical clustering (Hastie et al., 2009) for both the samples (columns) and features (rows). The problem is the fusing of observations in dendrograms to lower criterions means that the biclustering is only optimal to the nearby criterion, whereas convex clustering obtains the global optima (Chi et al., 2017; Pelckmans et al., n.d.). Instability in the algorithm means that minor perturbations in the data can result in substantial changes in clustering arrangements (Chi et al., 2017). Convex biclustering manages this through Fused Lasso, followed by a simultaneous shrinkage of both the rows and columns (Tibshirani et al., 2005). The end result is a more stable cluster from data perturbations, which also results in a more efficient selection of models (Chi et al., 2017).

On the y-axis in Figure 2.19 identifies the metabolites of *S. aureus* from the GLDS-145 datasets across the different treatments. But our focus will be on how convex biclustering clustered our treatment. Utilising simultaneous clustering of both rows and columns, it was possible to obtain a clustering of treatments that was mostly expected. The exception is in a few of the Pellet Flight (Purple) and Media Control (Green) samples that were clustered together. Visual inspection shows that there are differences between the two. The CTRL ASAP and CTRL Frozen are clustered together, giving greater confidence that the difference in protocol did not have a significant effect on metabolic expression. Convex biclustering

offers an excellent unsupervised learning strategy for accurate clustering (Chi et al., 2017; Pelckmans et al., n.d.).

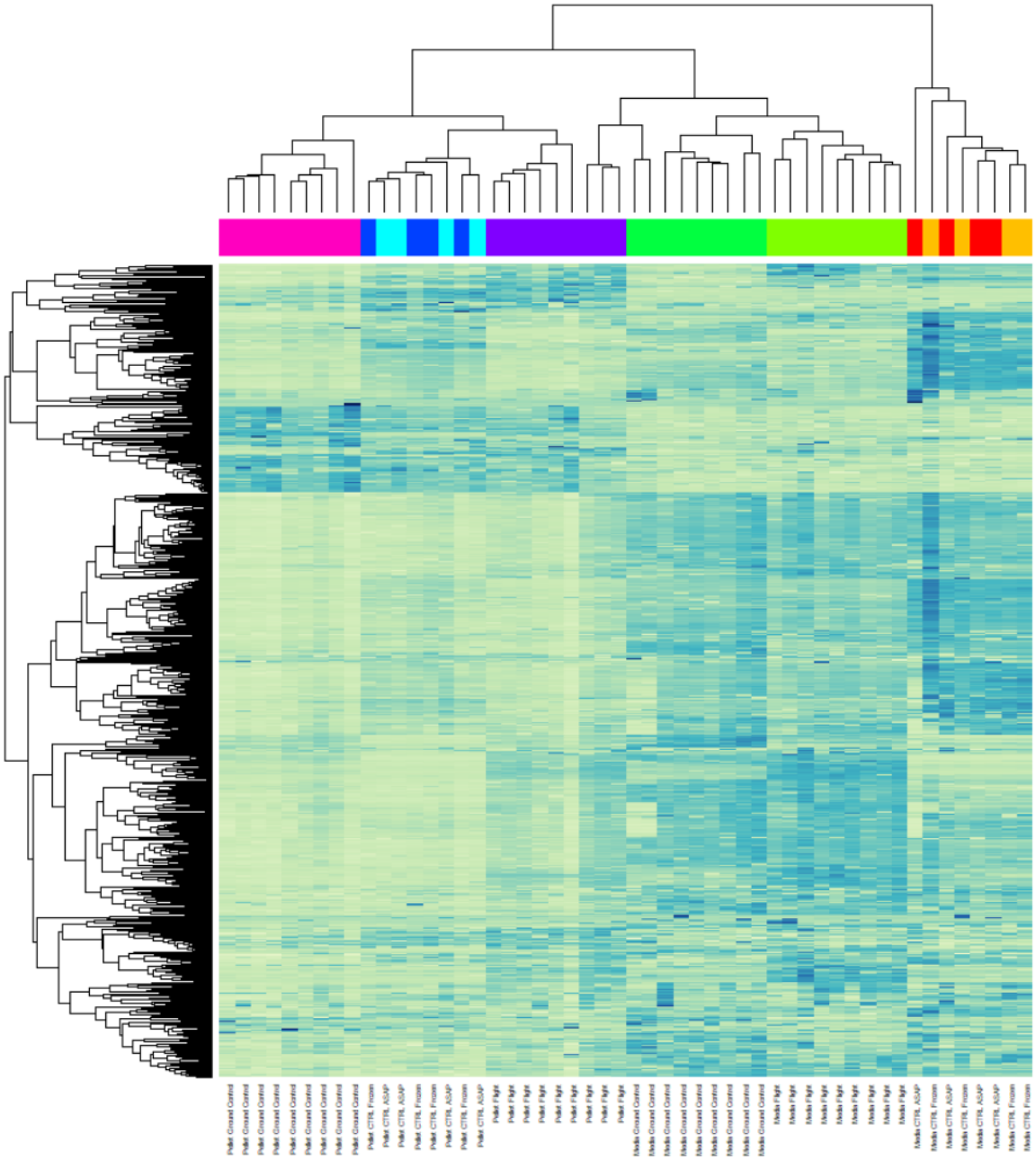

Figure 2.19 A convex bicluster of the GLDS-145 (*S. aureus*) dataset from the cvxbicluster package (Chi et al., 2017). Pink colour corresponds to Pellet Ground Control, blue corresponds to Pellet CTRL Frozen, light blue is Pellet CTRL ASAP, purple to Pellet Flight, green to Media Ground Control, light green to Media Flight, orange to Media CTRL ASAP, light orange to Media CTRL Frozen.

Figure 2.20 and 2.21 are obtained from the ClustRviz package (Weylandt et al., 2020), which is an interactive version of the cvxbiclustr package. Figure 2.20 is a holistic view of the clustering. This allows us to navigate around to look at specific areas of the cluster. Figure 2.22 takes the top 4, and 2 named metabolites from Figure 2.21. The top 4 metabolites are all unknown metabolites, so it's difficult to draw any biological conclusions, but it does indicate that there are metabolites of significant differences when changing from spaceflight and ground control. I've chosen to look at 2-deoxyerythritol, also known as 1,2,4-butanetrio as it has a lighter colour on the scale and thus greater differentiation. The biological significance for *S. aureus* 2-deoxyerythritol is uncertain, but it is useful for the production of polymers (Saha et al., 1997). Figure 2.22 shows that spaceflight does not give *S. aureus* a platform for generating 2-deoxyerythritol.

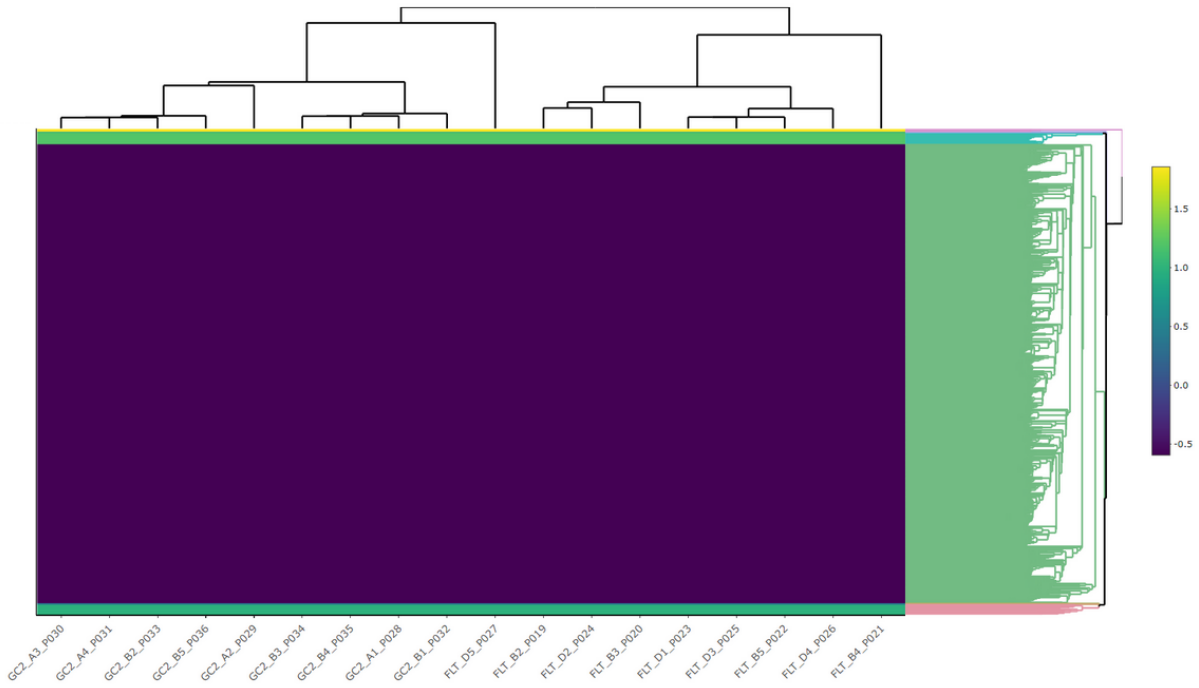

Figure 2.20 A convex bicluster of the GLDS-145 (*S. aureus*) dataset from the ClustRviz package. Observing only the Ground and Flight Pellet dataset.

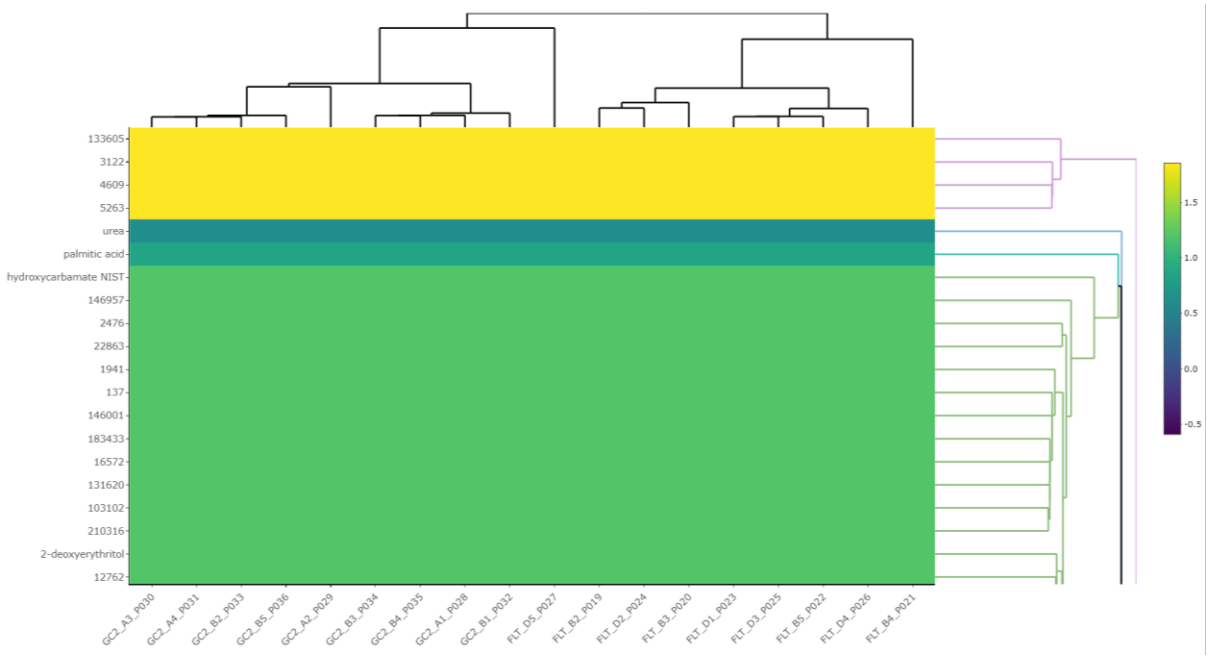

Figure 2.21 A convex bicluster of the GLDS-145 (*S. aureus*) dataset from the ClustRviz package. Observing only the Ground and Flight Pellet dataset. Zoomed in.

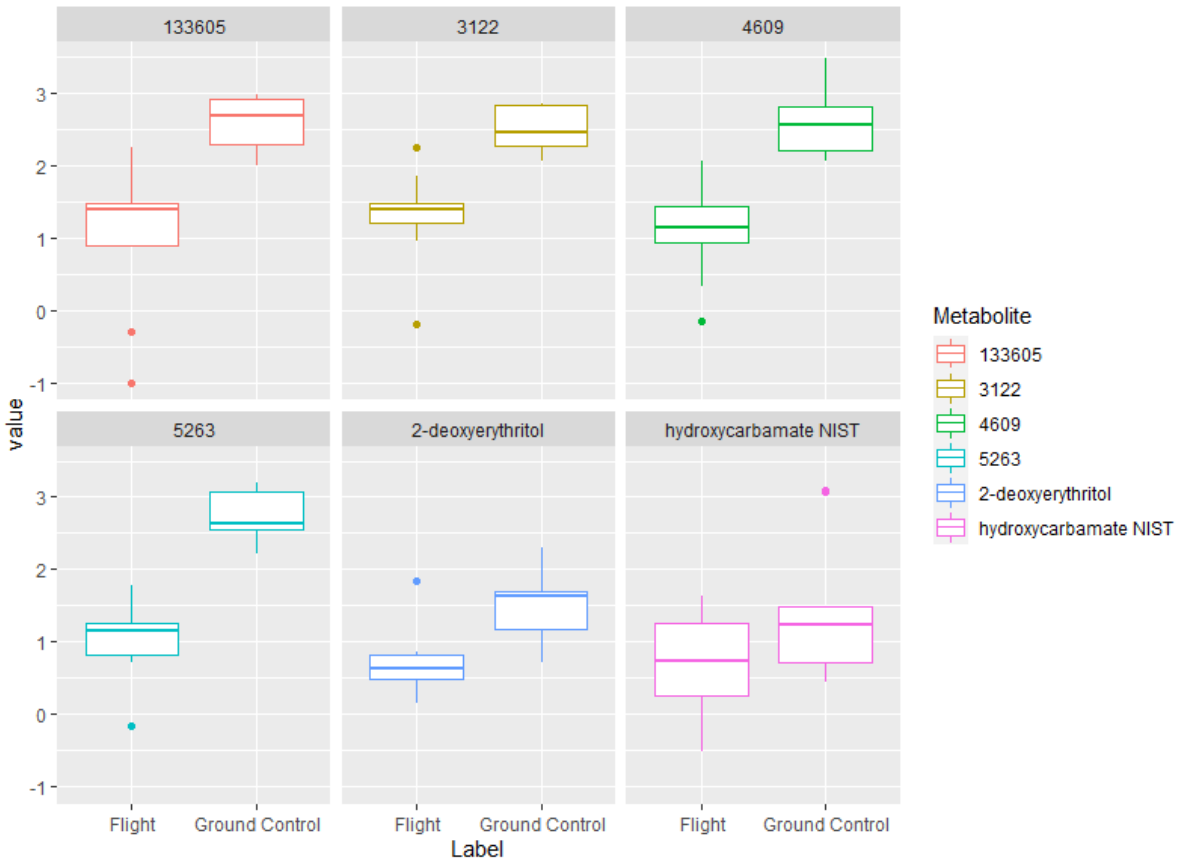

Figure 2.22 Boxplot of top and featured metabolites identified from Figure 2.21.

2.2.5 PLS/DIABLO - Multiomic

With different types of omics data (transcriptomics, proteomics, and metabolomics), one potential analysis of interest is to understand how the different datasets interacted with one another. Having multiple biological compartments has helped to improve biological insights that traditional single omics dataset miss out (D. Kim et al., 2013). Singh et al., (2019) suggests that this may be due to the fact the interactions between the different biological layers are being taken into account. Molecular networks are often not confined to a single omic type. While many approaches exist to handle single omic datasets, current approaches for multi-omics datasets are mostly unsupervised methods, which is good for categorising samples on the basis of phenotype or trait (A. Singh et al., 2019). These include Bayesian Consensus Clustering (Kirk et al., 2012), Similarity Network Fusion (B. Wang et al., 2014) for the identification of novel phenotypic clusters. Joint and Individual Variation Explained (JIVE) (Lock et al., 2013), and generalised canonical correlation analysis (GCCA) (A. Tenenhaus & Tenenhaus, 2011) are useful for extracting variation with common sources (A. Singh et al., 2019). Reconstructing Integrative Molecular Bayesian NETworks recontructs biological networks from experimental data by using curated data (J. Zhu et al., 2012). But for researchers looking for predictors of disease from multi-omics data, supervised learning methods are usually used (A. Singh et al., 2019).

One of such supervised methods is the Partial Least Square regression (PLS) (Ahrens, 1971), or more recently, the authors have preferred to refer to it as Projection to Latent Structure (Abdi, 2010). *D*ata *I*ntegration *A*nalysis for *B*iomarker discovery using *L*atent c*O*mponents (DIABLO) is based on PLS to generate useful visualisations (A. Singh et al., 2019).

Projection to Latent Structure (PLS) regression generalises and combines linear regression and principal component analysis (Abdi, 2010). PLS is useful for analysing two different omics datasets. This is because the relationship between two matrices, X and Y is modelled via a two-block PLS model (Wold et al., 2001). It finds the covariances in the two spaces using a latent variable (LV's) approach where the number of LV's is unknown. Linear combinations of the X and Y variable with the largest covariance are found with PLS (González et al., 2012). When the Y becomes a categorical variable and is used to analyse matrix X, it is referred to as Partial Least Square Discriminant Analysis (PLS-DA) (Wold et al., 2001). PLS-DA is useful to analyse single-omics. Using a set of independent variables, the LV's of PLS are orthogonal factors, which are extracted to obtain a predictor or to analyse a set of dependent variables (Abdi, 2010). The predictive power is strong, and is computationally fast (Wold et al., 2001). This makes PLS useful in finding covariates across different large datasets.

DIABLO uses a consensus class prediction via a weighted majority as different omics datasets may not agree on a predicted class for each sample (A. Singh et al., 2019). The more predictive dataset is up-weighted over omics datasets with weaker correlations between its respective latent components and the outcome of each sample (A. Singh et al., 2019).

Considering the multi-omics problem, we will look at the interactions between polar and non-polar metabolites from the GLDS-145 study. The optimal number of components to keep is indicated once the Q2 value falls below threshold of 0.0975 (Cao et al., 2008; M. Tenenhaus, 1998). However, looking at Figure 2.23, the threshold of 0.0975 is nowhere to be found. This could mean that our model is not a good predictor, or the number of samples might be too small for the CV process. We default to 2 components in this case. After tuning, we are given 1 metabolite for the first component, and 20 metabolites for the second

component. It should be noted that the tuning was restricted to 1 to 20 metabolites, so perhaps there are no good predictors for the 1st component.

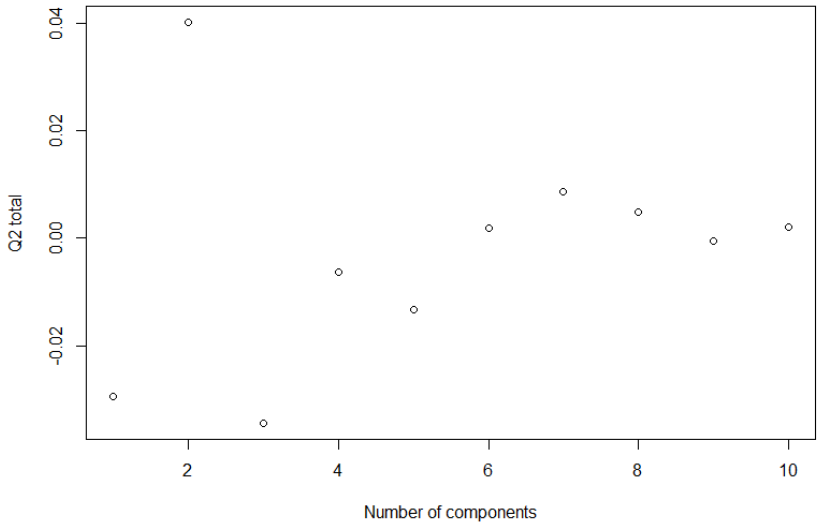

Figure 2.23 Q2 plot on the GLDS-145 (*S. aureus*) dataset. Observing only flight and ground features.

Referring to Figure 2.24, it appears that regardless of whether we are looking at polar (Block: X) or nonpolar metabolites (Block: Y), we have approximately the same capability of determining whether we have a Flight or Ground control. It should be noted that these methods are exploratory, so there is not a good case for significance.

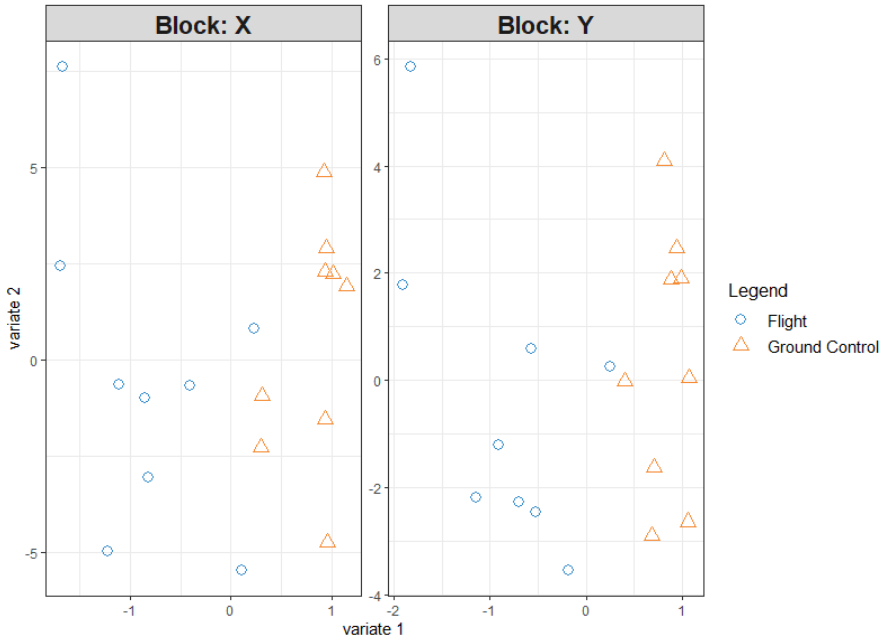

Figure 2.24 sPLS plot of GLDS-145 dataset, focusing on the flight and ground control variable, the X represents polar metabolites, and the Y Block represents non-polar metabolites.

Figure 2.25 is the average of the X and Y variable from Figure 2.24. While there is not much more to gain, it is a capability that could be added if appropriate. It can be used to figure out what variables have the greatest influence in the model. This does not apply as recognised from Figure 2.24, both X and Y have approximately the same scale and similar distribution.

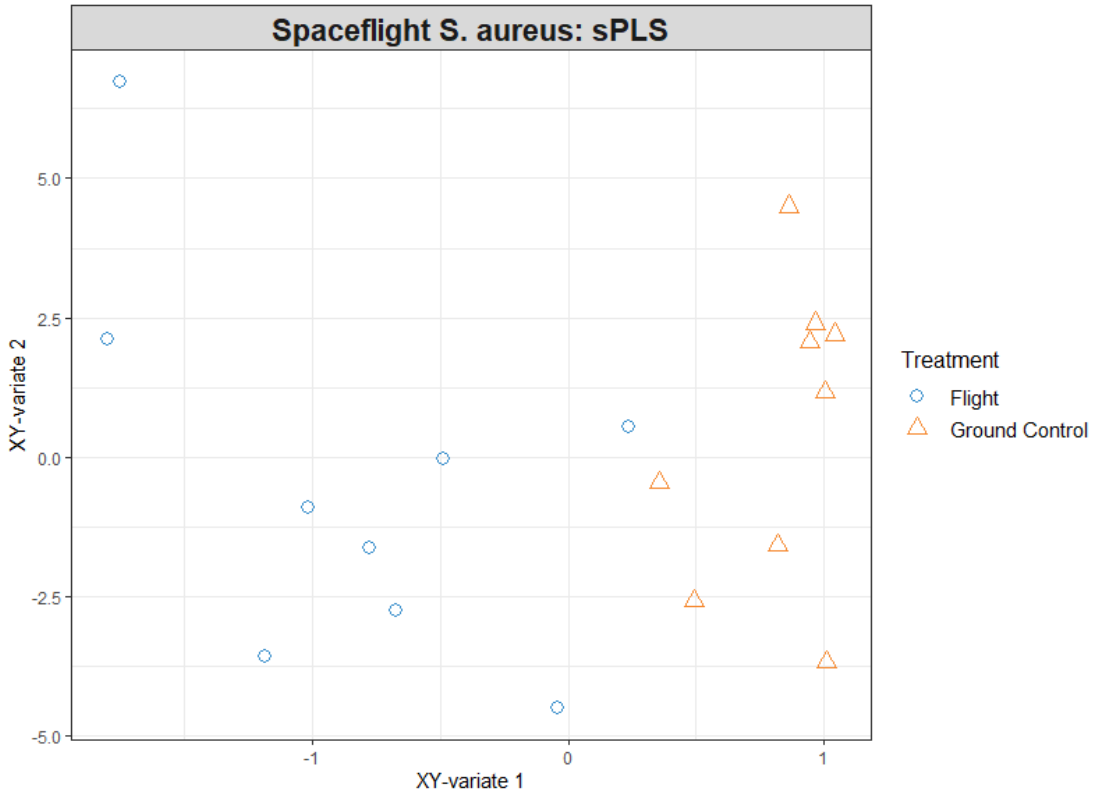

Figure 2.25 sPLS plot of GLDS-145 dataset, focusing on the flight and ground control variables.

In Figure 2.26, the label "metabolite" represents polar metabolites, and "lipid" is used for metabolites towards the nonpolarity of the spectrum. "Metabolite" and "Lipid" represent two different datasets given in GLDS-145 (GLDS-145, 2017). The two datasets represent a difference in protocols. While there are differences in distribution, the differences between spaceflight and ground control are all defined in the first component in DIABLO. The diagnostic plot in Figure 2.27 illustrates that there is a broad agreement between the different omics when looking at only the flight and ground variable. This shows us that spaceflight affects transcriptomics, metabolomic, and lipidomic datasets.

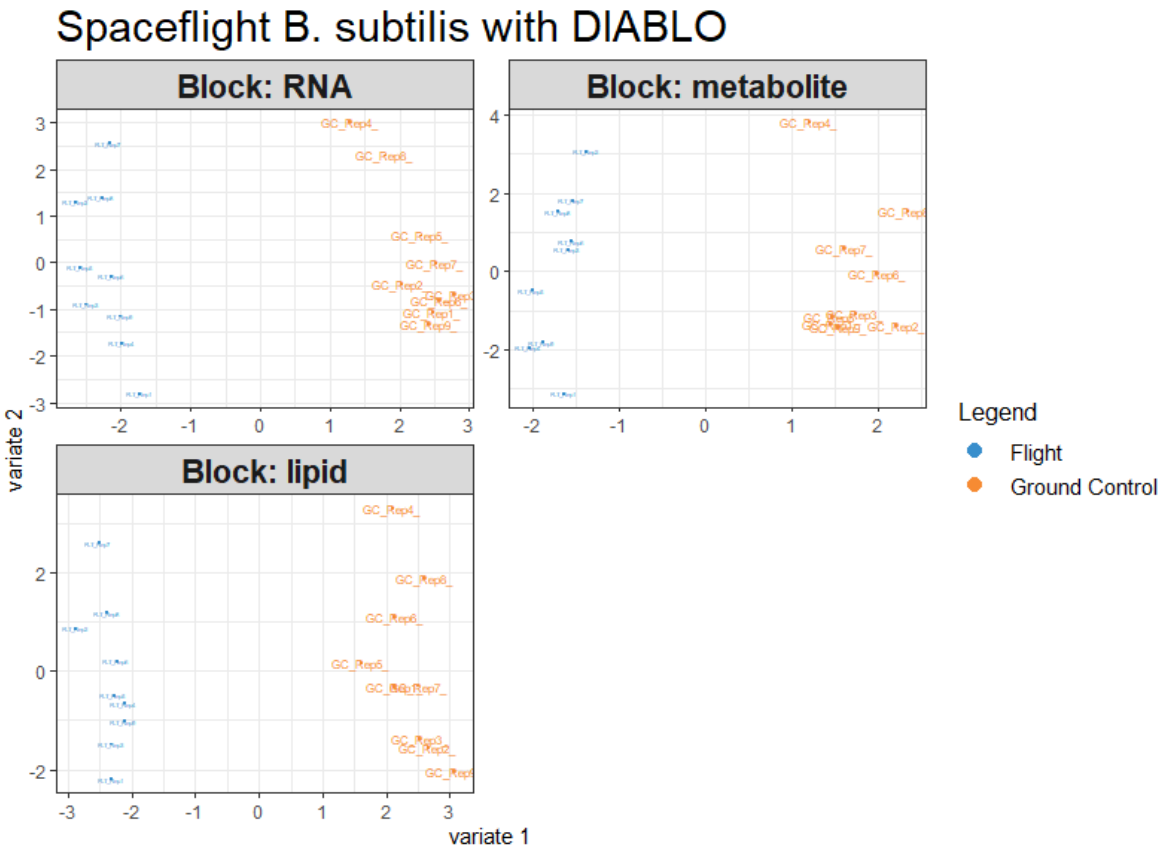

Figure 2.26 DIABLO plot of GLDS-138 dataset, focusing on the flight and ground control variables. Featuring transcriptomics, metabolomic, and lipidomic datasets. Utilising the top 10 and 6 RNAs, top 6 and 5 polar metabolites, top 10 and 6 lipids from the 1st and 2nd component respectively.

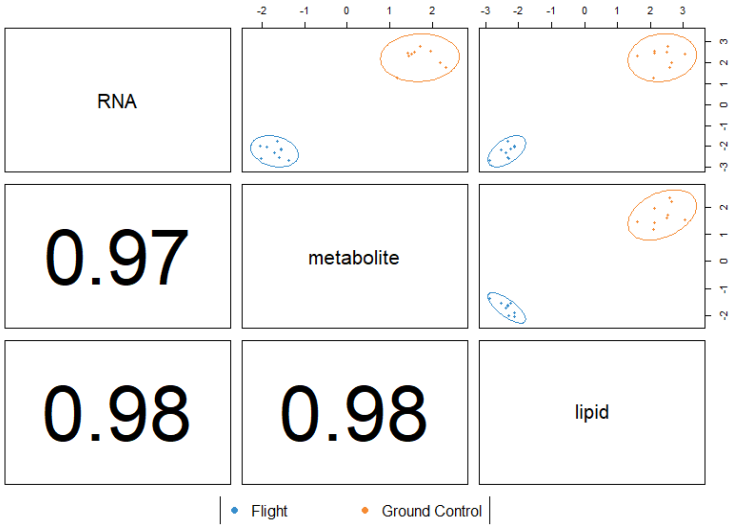

Figure 2.27 Diagnostic plot of GLDS-138 dataset, focusing on the flight and ground control variables.

Figure 2.28 and 2.29 are similar plots but visualised in a different manner. Both show an interaction between the different omics. Figure 2.29 shows that when the correlation cutoff is set above 0.9, there are separate systems affected by spaceflight.

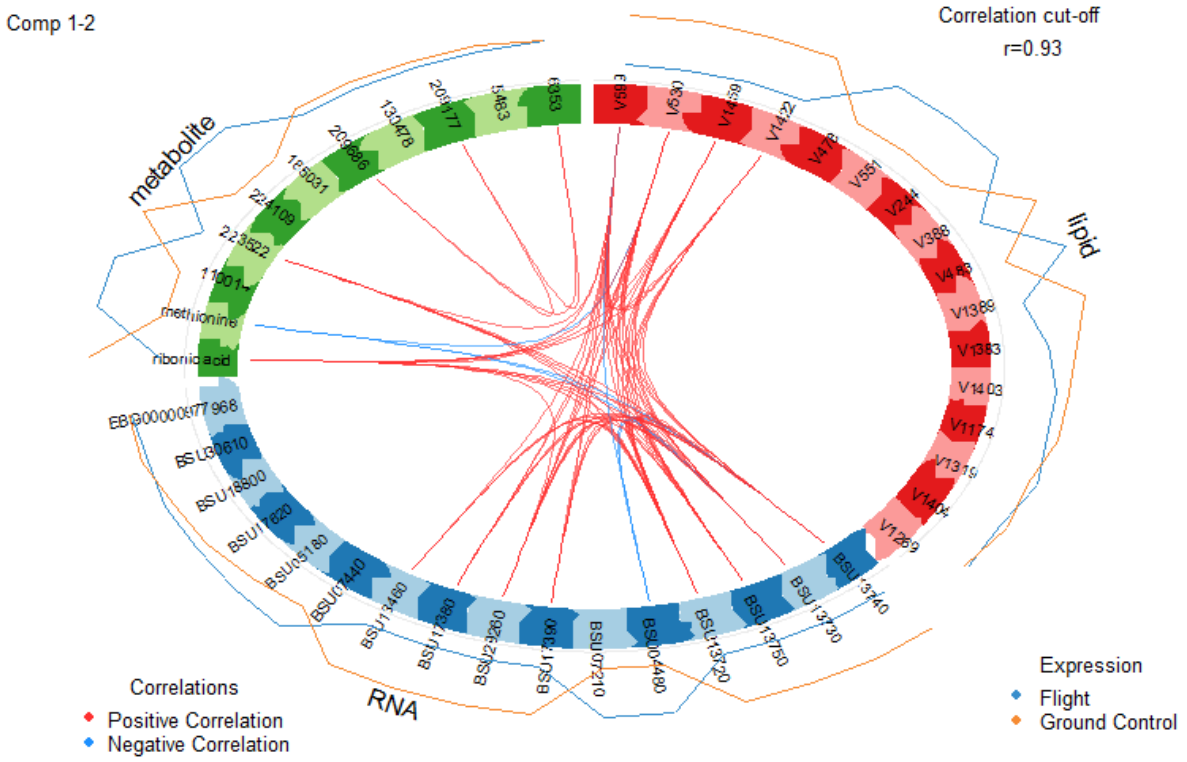

Figure 2.28 Circus plot for the top 2 components. Red lines indicate positive correlation, and blue lines negative correlation. GLDS-138 dataset, focusing on the flight and ground control variables. The correlation cut-off is 0.93.

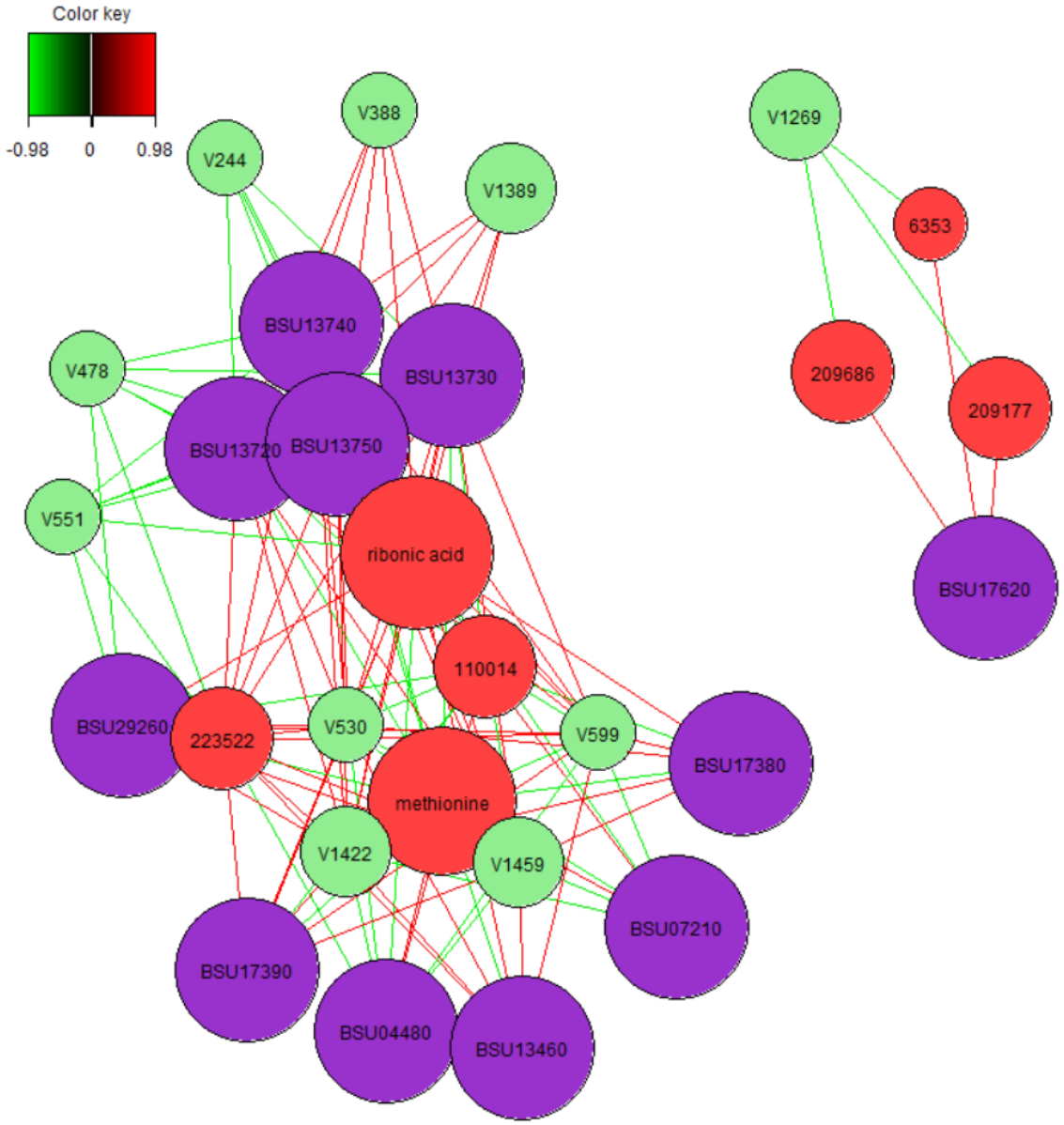

Figure 2.29 Relevance network for the first two components. Green lines for negative correlation, red lines for positive correlation. Red circles are polar metabolites. Green circles for non-polar metabolites. Purple circles for RNAs. Correlation Cutoff at 0.88. GLDS-138 dataset, focusing on the flight and ground control variables.

2.3 Differing PCA Conclusions for Different Omics

Figure 2.8 shows that there is no significant difference between the *B. subtilis* metabolic expression for Flight and Ground Control. However, Figure 2.30 tells us that at the transcriptomic level, there is a discernible difference between Flight and Ground Control. It is possible to imply that a disruption at the transcriptomic level does not equate to a disruption at the metabolomic level. While the genes are expressed quite differently, this does not mean that the phenotypic level, which metabolites are closest to, are different. The alternative explanation is that this is due to the limitation of our ability to detect sufficient numbers of all possible metabolites.

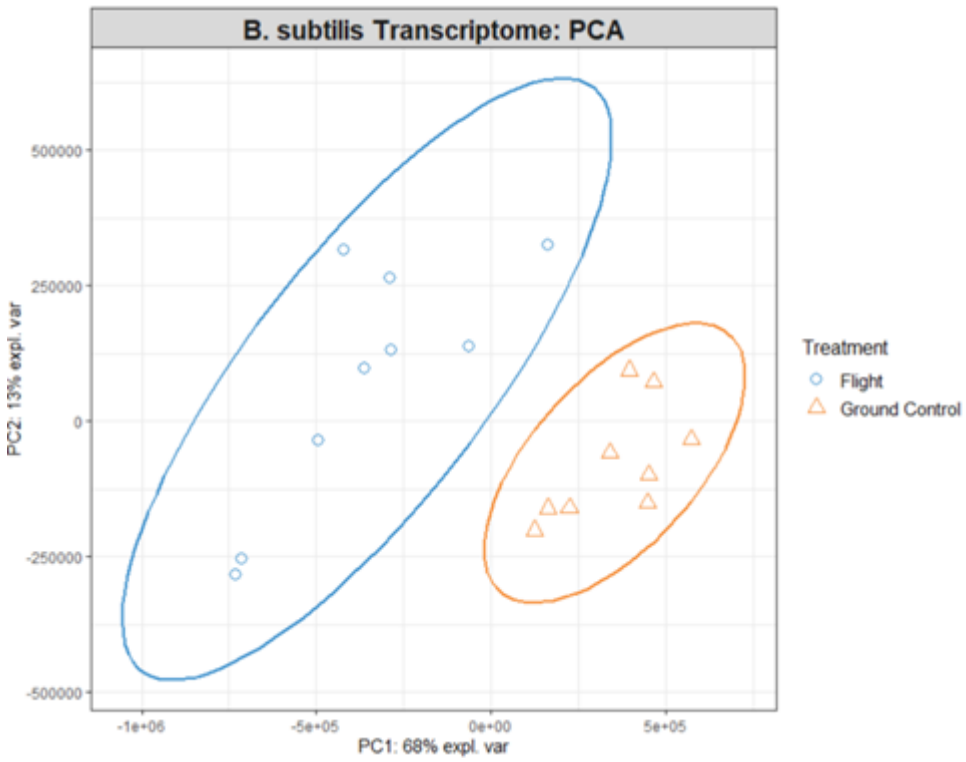

Figure 2.30 A PCA comparison of *B. subtilis* intracellular RNAs with the spaceflight and ground control treatment.

2.4 Capture of fractional gravity component for *Arabidopsis thaliana*

The original study of how *A. thaliana* was affected by changes in gravity and blue light stimulation was carried out with univariate analysis (Herranz et al., 2019). This gives an opportunity to see what else can be gained with multivariate analyses. Figure 2.30 shows that gravity has been captured by the 2nd component, with 1G with positive values, and uG with negative values. The lower gravity (uG, 0.09G, and 0.18G) is captured with the 3rd component, so this could imply a more substantial difference of expression between lower gravity and higher gravity.

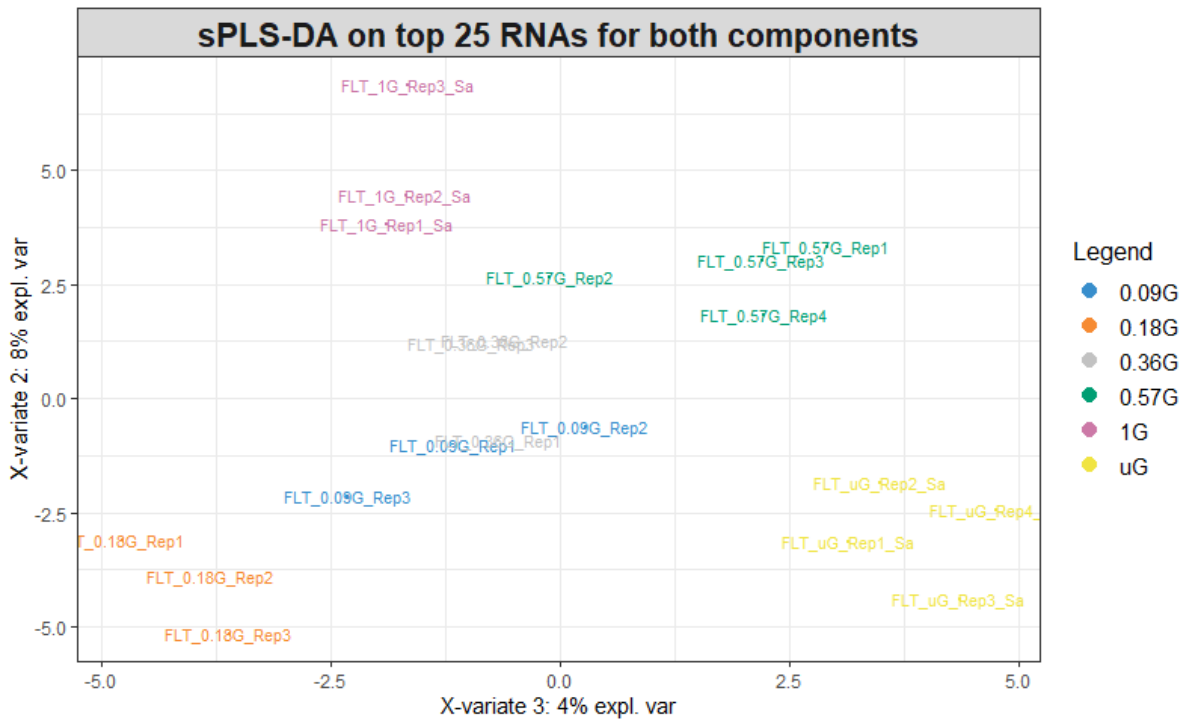

Figure 2.31 A sPLS-DA plot of *A. thaliana* top gene expression under a range of gravity conditions and blue light stimulation treatment.

2.5 Discussion

This chapter has described several different multivariate methods. It is easy to use univariate analyses for simplicity. Omics are large datasets, and this presents opportunities for new methods of analysis. A range of multivariate methods have been used previously, but choosing a method because others use it is not justified, and can lead to misinterpretations. The goal of this chapter was to understand the circumstances in which a particular multivariate method is useful.

PCA works by creating modes that describe variance (Jolliffe & Cadima, 2016). It is possible to see which metabolites or molecules drive variation, but variation does not mean that the molecule is useful in the system. Variance is also useful in assessing whether there is a difference between different groups. PLS-DA, as a supervised learning algorithm, maximises differences from labelled data, and will almost always find a difference (hence overfitting) (Saccenti et al., 2014). Overfitting makes PLS-DA unfit for determining whether there are differences between groups, but its ability to find molecules that drive differences between labelled groups makes it attractive in finding predictive or biologically significant molecules.

The *B. subtilis* PCA in Figure 2.8 tells us there is not a difference between the two groups when judging at the metabolic level. Figure 2.18 demonstrates that no differences between groups does not necessarily mean that there are no differences in metabolic differences. It does tell us that *B. subtilis* as a whole is not significantly affected by spaceflight, but there are a few metabolites affected as identified by the sPLS-DA in Figure 2.17. The few metabolites identified in Figure 2.17 can be seen as connected in Figure. 2.29. Since many of these are unknown metabolites, one can speculate that they have little effect on the physiology of *B. subtilis* overall. The transcriptome in Figure 2.30 tells a different story where there is an obvious difference between spaceflight and ground control groups. A study of *A. thaliana* suggests that some genes are non-adaptive and have little to no consequences to

the organism (Paul et al., 2017). This does not imply that phenotypic changes are more defined at the downstream metabolic level rather than the upstream transcriptomic level. It can mean that greater regulation occurs to fine tune metabolic activity. This implies that care is needed when drawing conclusions from the transcriptome, particularly when certain gene expressions could be misinterpreted.

An issue arising from many studies is however the use of non-conservative cut-off procedures. The paper Paul et al., (2017) presents differentially expressed genes in the 3-digit zone; this can be reduced to two-digit and single-digit when permutations are applied (Roy et al., 2016). It may not be surprising that there are non-adaptive genes in the model. Permutation is often the golden standard applied for univariate analyses. For multivariate analyses, cross-validation is the method required.

When looking for prediction or metabolites of significant differences, PLS-DA in conjunction with cross-validation helps to prevent the requirement of having to look at many different metabolites/proteins/RNAs, and allows a focus on fewer biological objects and assists in the decision on what kind of biological significance a change in environment or treatment causes our organism.

Although not used in this chapter, convex clustering likely would be better utilised in constructing relationships or closeness between different biological organisms. It demonstrates considerable reliability in constructing an accurate cluster as shown in Figure 2.19. It is also capable of finding the top performing metabolites for that particular cluster, as shown in Figure 2.21. The problem is that all the metabolites are unnamed with unknown functions, so it is difficult to tell what the main function of these metabolites are and what kind of metabolites gets sifted through by convex clustering. In Figure 2.3, It has been speculated to be structurally important variates.

DIABLO, as shown in Figure 2.29 allows us to construct multiomic pathways. It can be improved by having easier to understand visualisations. It currently seems to only show interactions between different omics, so it is difficult to discern whether a pathway goes through a series of single omics before traversing different omics, this would likely be the most important development in biological pathways.

In Figure 2.3, a proposed pipeline was made, but ultimately it depends on the ultimate question. If we're looking for differences between groups, then it makes sense to use IPCA/PCA, if the question is about biomarker predictions or finding metabolites of importances, then PLS-DA is the best choice. Convex clustering should be employed when the goal is looking at relationships between biological groups. DIABLO can be used to find closely correlated variates across different omics, a critical improvement is in its visualisation and differentiation between inter- and intra-omic pathways. While univariate methods are easier to apply, interpret, and communicate, omics is a discipline that no longer uses single variables and markers (Saccenti et al., 2014). This chapter has demonstrated that extra information can be obtained from multivariate analyses. Saccenti et al., (2014) points out one should use both univariate and multivariate analyses, but each should be applied appropriately and interpreted correctly.

Chapter 3 - Metabolomics by Gas Chromatography Mass Spectrometry of *Gardnerella vaginalis* cultures in simulated microgravity

3.1 Metabolomics

Since the Viking lander, molecular compounds have been used to link biological activity to the presence of extraterrestrial life (Klein et al., 1976). But this chapter is not about finding extraterrestrial life, it is to assess the metabolic changes to *Gardnerella vaginalis* when subjected to simulated microgravity. Both environmental and biological factors influence the resulting metabolome (Schrimpe-Rutledge et al., 2016). This gives an opportunity to better understand the separation between genotype and phenotype.

Metabolites are separated into primary, and secondary metabolites. Primary metabolites are directly required for an organism's growth and development (Fernie & Pichersky, 2015). Examples being amino acids, carbohydrates, and lipids (Grotewold, 2005). Secondary metabolites are non-essential, but critical for survival, and are often related to signalling and defence mechanisms (Grotewold, 2005; Wolfender et al., 2015).

Metabolomic experiments are often targeted, semi-targeted or untargeted. Targeted metabolomic studies a range of predetermined metabolites selected for analysis (Seyler et al., 2020). Untargeted studies, also known as metabolite profiling, measures as many metabolites as possible without bias (Patti et al., 2012). Untargeted metabolomics are often hypothesis-generating, aiming to be comprehensive, when correlated to databases/libraries, and have relative quantification of metabolite across samples (Schrimpe-Rutledge et al., 2016). Targeted metabolomics are hypothesis driven, analysing fewer metabolites, with a straight

correlation to reference standards of known identity, and are quantified to the absolute values (Schrimpe-Rutledge et al., 2016).

The platforms available for metabolites include gas chromatography (GC), liquid chromatography (LC), nuclear magnetic resonance (NMR), and capillary electrophoresis mass spectrometry (CE-MS). Each of these techniques have their own advantages and disadvantages. The advantages of NMR is that it is easy to identify metabolites with, highly reproducible, and non-destructive (Zeki et al., 2020). However, the number of metabolites that the NMR can detect ranges from 20 to 50 (Wishart, 2008; Zeki et al., 2020). The number of metabolites identified by GC-MS is around 100, while LC-MS can reach close to 500 metabolites (Zeki et al., 2020). CE-MS can exceed LC-MS's separation efficiency, it is not used because of its low reproducibility (Williams et al., 2013). The different methods will have some overlaps in the metabolites they detect, and can complement one another. There is no methodology or technique capable of extracting and/or detecting the entire metabolome of a sample (Wishart, 2011, 2016). This is due to the chemical diversity and variation in the nature of metabolites in any given sample (Beale et al., 2016), as well as the limitation of each metabolomics technique.

GC-MS is widely available, provides high resolution and sensitivity, and is reliable with a relatively low cost (Lei et al., 2011). It is highly reproducible, with available libraries elsewhere (Williams et al., 2013). In this thesis, we use in-house libraries and the NIST reference library. GC requires the analytes to be thermally stable and volatile and so the non-volatile compounds are derivatized before analysis, which can be quite limiting as derivatisation can be a laborious process (Williams et al., 2013). The downsides of limiting to thermally stable and volatile compounds, is that it excludes larger polar compounds (Williams et al., 2013).

This chapter aims to profile bacterial extracellular metabolites. While the decision to utilise extracellular metabolites over intracellular metabolites was due to technical constraints in

culturing the required bacterial load for intracellular metabolomics, it does not mean that there are no advantages to applying extracellular metabolomics.

Extracellular metabolomics is the study of metabolites released by cells into the surrounding environment, and from microbial cultures, this is commonly the culture media (Pinu & Villas-Boas, 2017). This includes all the metabolites of the microbial culture media, and the metabolites released by the microbe into the media itself (Mashego et al., 2007). Separating extracellular metabolites from microbial cells and intracellular metabolites can be achieved with well-established techniques such as centrifugation (Tredwell et al., 2011). While it's difficult to quantify and reproduce the metabolites of the *in vivo* environment, the culture media does give a standard for measurement.

Extracellular metabolomics is also referred to as the metabolic footprint as it is looking at the changes in the extracellular medium due to cellular secretion of metabolites and uptake of nutrients during growth (Villas-Boas et al., 2007). Extracellular metabolites are a mix of consumed metabolites and by-products of microorganisms when grown in a specific environment. Therefore, it is important to control environmental factors as much as possible within a sample in order to reduce 'batch' effects. Environmental factors include pH, temperature, the concentration of nutrients, and among others (Pinu & Villas-Boas, 2017). When comparing samples under different but controlled environments, extracellular metabolomics helps understand the changes on microbial metabolism in response to environmental changes. Thus, microbial metabolomics have direct applications in many different areas, including bioremediation, bioprocess monitoring, functional genomics and biomarker discovery (Allen et al., 2003; Kang et al., 2011; Wu et al., 2010; Zhuang et al., 2010).

The analysis of metabolic activity as it can also be used to elucidate the metabolic cooperation between two microorganisms (Raes & Bork, 2008). An example of this is when

Methanosarcina barkeri and *Acetobacterium woodii* cooperatively degrade glucose with acetate and produce methane at the end (L. Keller & Surette, 2006). Another example utilises proteomics and metabolomics to study ecological succession of biofilms in an acid mine drainage site (Wilmes et al., 2010)

The secretion of metabolites by microorganisms into the surrounding environment also allows the detection of molecules for quorum sensing (QS) molecules, which are the compounds used for communication between cells in microbial communities (Atkinson & Williams, 2009). The cell to cell signalling can include bioluminescence trigger, virulence expression, biofilm formations, coordination of DNA exchange and enzyme expression (Bassler & Losick, 2006; L. Keller & Surette, 2006; Raes & Bork, 2008; Wingreen & Levin, 2006). Metabolomics can also help us understand host-microbe interactions and how that may provoke human disorders (Han et al., 2010). Metabolism has been used to reconstruct feedback loops and metabolic connections in cellular processing (Hollywood et al., 2006). Having a range of metabolites affected means that it is possible to elucidate metabolic pathways. The downstream nature of metabolomics over transcriptomics and proteomics means that it does a better job representing microbial phenotypes (Tang, 2011). Han et al., (2010) mentions that sometimes metabolic pathways, particularly those that involve secondary metabolite biosynthesis, are not constrained to a single organism.

In the previous chapter, I made use of several multivariate methods on open access spaceflight biological data to understand the circumstances they should be applied. This chapter allowed me to better understand the process taken when conducting processing metabolites, and apply multivariate methods onto my own experiments.

Gardnerella vaginalis is found in both healthy women, and women with BV, but it is found in far larger numbers in women experiencing BV (Fredricks et al., 2007a; Shipitsyna et al., 2013). Around 10% of the medical issues occurring during spaceflight are genitourinary (Jones et al.,

2019). Despite having an order of magnitude fewer females than males in spaceflight, the female to male odds ratio for urinary retention is 11:14 (Law et al., 2016). It is possible this is due to sanitary reasons. Urine is collected on the International Space Station to be processed to become drinking water (Jones et al., 2019). Condom-like appliances that interface with astronaut's genitalia are used for the urine collection to prevent spillage and leakage (Jones et al., 2019). For female crewmembers, these include a condom catheter with a metal ring that's pressed in contact with the perineum, or a silicone periurethral device that's inserted into the vaginal introitus (Jones et al., 2019). It is unknown how much reuse is done for females. But the BioDerm wafer that's attached to the male penis is designed to be worn for up to 3 days (Jones et al., 2019). Seeing that urinary tract infection is seen in a higher proportion of females, it is still useful to investigate whether *G. vaginalis* plays a role in these urinary tract infections.

Across the hundreds of datasets in NASA Genelab, there are only two microorganisms with a metabolomics dataset (Fajardo-Cavazos & Nicholson, 2016), both of which were analysed in Chapter 2 (*B. subtilis* and *S. aureus*). Investigations of microorganisms in space with metabolomics are low in numbers. Microorganisms have been documented to have heightened pathogenicity and virulence when subjected to space conditions (Bijlani et al., 2021). Secondary metabolites produced by microorganisms can be used as medicine (Lam et al., 2002), or be toxic for humans (Rosado et al., 2010). It is not believed that there is a common bacterial response towards "spaceflight" (Morrison & Nicholson, 2018).

The goal of this chapter is to conduct a metabolomics experiment using untargeted metabolomics to profile extracellular metabolites from cultures of the human vaginal bacterium *G. vaginalis* under simulated gravity vs simulated microgravity. This study might reveal how microbial metabolism changes in microgravity and how these changes may potentially affect human health. In chapter 1, we established that microgravity has the potential to cause changes to microbial physiology. By subjecting *G. vaginalis* to microgravity, we can figure out

what kind of threat, or lack of a threat it poses in microgravity to human health. See which metabolites are affected, which metabolic networks are affected, are there metabolites effects that are not part of existing networks?

3.2 Methodology

3.2.1 Microbial culture and sample preparation

For our growth media for *G. vaginalis*, it is adapted from ATCC□'s Medium 1685: NYC III medium. The NYC III medium was prepared with 4 grams of HEPES, 15 grams of Proteose Peptone No.3 (BD 211693), 5 grams of NaCl, 10 grams of Yeast extract, 850 mL of distilled water. It was adjusted to a pH of 7.3 with HCl or NaOH. It was then autoclaved at 121°C for a minimum of 15 minutes. After it cooled down to room temperature, 50mL of sterile 10% glucose was added, and 100mL of inactivated horse serum were added to complete the NYC III media.

To avoid growth differences between different samples of *G. vaginalis*, a single 100 mL culture of *G. vaginalis* was grown from frozen in NYC III media overnight in microaerophilic conditions (i.e. Media filled to the top) at 37°C without agitation and 100 x 1mL aliquots were frozen at -80 °C after the addition of 15% glycerol.

From each of my experiments, a vial of *G. vaginalis* was removed from -80 °C, defrosted, and centrifuged to remove glycerol and associated media. After the removal of the supernatant, the formed pellet was broken, new NYC III Media was added into the 15 mL tube with the pellet and was incubated in microaerophilic condition overnight at 37 °C.

After overnight growth, the optical density of the cultured media was measured at 550 nm with NYC III Media as the blank. After measuring the optical density (OD), the cultured media was diluted to 30mL at 0.05 OD with NYC III Media. The diluted culture (10mL) was inserted into the High Aspect Ratio Vessel (HARV). Bubbles were removed from the HARV to minimise turbulence. An additional 10 mL of diluted culture was used for a stationary control in a 15 mL falcon tube. The HARV was attached to the Rotary Cell Culture System (RCCS) to rotate at 28 rpm. The RCCS was placed in a position allowing HARV rotation on the horizontal axis when looking to simulate microgravity, and rotation on the vertical axis when simulating gravity. The diluted cultures were grown for 24 hours.

After the growth period, with a 0.2-µm syringe filter, 10 mL of microbial culture are filtered to remove microbial cells. Three 1-mL aliquots are obtained for three technical replicates. Internal standard D_4-alanine (20µL at 10mM) was added to each sample. The samples were snap-frozen with dry ice or liquid nitrogen and stored at -80 °C until use.

3.2.2 Chemical derivatisation of metabolites

The method of extraction will determine the type of compounds present in the extract (E.g. the use of non-polar solvents can result in a higher abundance of non-polar compounds in the extract) (Atanasov et al., 2021). Many extraction methods involving the use of acidic solutions (hydrochloric acid, perchloric acid) and low temperature (4 °C) in freeze-thaw cycling, which is effective for analysing acid-stable and polar compounds (Faijes et al., 2007; C. Park et al., 2012). This causes the hydrolysis of polymers and proteins, which gives an inaccurate metabolic profile (Beale et al., 2018). The use of alkalis in metabolite extraction requires neutralisation and salt removal steps (Villas-Bas et al., 2007). Cold solvents such as glycerol and cold methanol solutions are also used as metabolite degradation is lower at subzero temperatures, which has shown good reproducibility of organic acids, amino acids, amines

and fatty acids (Beale et al., 2018; Granucci et al., 2015; Jäpelt et al., 2015). This issue with using glycerol is that it can be difficult to remove glycerol and it can pose problems for derivatisation when silylation is considered, which is why Methyl chloroformate (MCF) has been adopted (Jäpelt et al., 2015). Freeze thaw cycles with cold methanol solution (−20 °C) is effective as it uses only one organic solvent, and solvents are passively removed from the sample by evaporation (Hajjaj et al., 1998; Jonge et al., 2012). This method is useful for extracting polar and mid-polar metabolites, but is weak in recovering non-polar metabolites (Villas-Bôas et al., 2005). Later, the chloroform-methanol-water extraction method was adopted as it allowed the extraction of both polar and non-polar metabolites (Koning et al., 1992).

This chapter utilises methyl chloroformate derivatisation, which is good for looking at central carbon metabolism metabolites (fatty acid intermediates, phosphorylated organic acids, amino and non-amino organic acids) (Smart et al., 2010).

3.2.2.1 Methyl chloroformate (MCF) Derivatisation

The frozen samples were put into a pressure of 0.10 pa and freeze-dried at -80 °C overnight. The samples are resuspended in NaOH (400 µL at 1 M), which are then added into a mixture of pyridine (68 µL at 79.1 g / mol) and methanol (334 µL at 32.04 g / mol). MCF (40 µL at 94.5 g / mol) was added to the sample mixtures twice, vortexing for 30 seconds each time. Chloroform (40 µL at 119.38 g / mol) was also added into the sample mixtures and vortexed for 10 seconds. Sodium bicarbonate (800 µL) was added into the mixture and vortexed for 10 seconds as well. Samples were centrifuged at 2500 rpm for 5 minutes at 6 °C. Using a glass pipette and a water pump, the upper layer of the centrifuged samples are removed. To remove residual water, a pinch of sodium sulphate was added into the

remaining sample mixtures. Avoiding visible solids, the remaining supernantants were collected and transferred into a specified vial for GC (Gas-chromatography).

The GC vials with the sample extracts were loaded into the Agilent 7890B chromatography instrument for metabolite detection. The instrument parameters were based on Smart et al (2010). The Agilent 7890B gas chromatography was coupled with a 5977A inert mass spectrometer and a split/splitless inlet. With an Agilent autosampler, 1 µL of sample was injected into a glass split/splitless 4 mm ID ID straight inlet liner packed with deactivated glass wool (Supelco). The temperature of the inlet was set to 290 °C. Column flow set to 1.0 mL/min, with an initial linear velocity calculated to be 35 cm/s. The purge flow 1 minutes after injection was set to 25 mL / min.

The column consisted of a fused silica ZB-1701 0.25 mm, 30 m long, 0.15 µm stationary phase (14% cyanopropylphenyl, 86% dimethylpolysiloxane, Phenomenex). Instrument grade helium (99.99%, BOC) was used as the carrier gas. The starting temperature for the GC oven was programmed to start isothermally at 45 °C for 2 minutes. This was increased to 180 °C at a rate of 9 °C / min, which was held for 5 minutes; increased to 220 °C at a rate of 40 °C / min, also held for 5 minutes; increased to 240 °C at a rate of 40 °C / min, this was held for 11.5 minutes; increased to 280 °C at a rate of 40 °C / min, and held for 10 minutes. The mass spectrometric detector has a transfer line which was maintained at 250 °C, the source was maintained at 230 °C while the quadrupole was held at 150 °C. Five and a half minutes into the run, the detector was turned on, and it was run in positive-ion, electron-impact ionisation mode, at 70 eV electron energy; the electron multiplier was set with no extra voltage relative to the autotune value. Every 10-12 samples, chloroform blanks were runned to monitor for the instrument carryover. The mass spectra acquired in scan mode between 38 to 550 atomic mass units were used to identify the compounds; it has a detection threshold of 100 ion counts. The AMDIS software was used with an in-house MCF library at the University of Auckland when classifying metabolites.

3.3 Results

3.3.1 Corrections and normalisations

3.3.1.1 Batch Corrections with SERRF

Metabolomics studies can often involve hundreds of samples (Bijlsma et al., 2006; Dunn et al., 2012; Martin et al., 2015). Running these experiments were often done in batches. The data obtained from these batches can differ from days to months. Conducting experiments at different timings have an effect on the instrument sensitivity and differences in tuning, causing errors such as temporal drift, batch effects (Fan et al., 2019). These systematic errors have unwanted variance which drastically affects one's ability to find metabolites associated with the phenotype of interest (Sampson et al., 2013).

In the pursuit of reducing technical errors, many different strategies have been applied onto samples (B. Li et al., 2017; Zacharias et al., 2018). These strategies can be divided into three categories. These normalisations are: data-driven, internal standards-based (IS), and quality control samples (QC)-based (De Livera et al., 2015; Fan et al., 2019). Data-driven normalisations assume that metabolites have properties that are self-averaging, meaning that an increase in concentration in the specific sets of metabolites would result in the decrease of another (Redestig et al., 2009; Sysi-Aho et al., 2007). This assumption may not be valid as the systematic error affects different metabolites can differ (Sysi-Aho et al., 2007). IS-based normalisations make use of either or both internal and external standard compounds to normalise the intensity metabolites in the samples (Fan et al., 2019). Issues arising with IS-based normalisation strategies is that the peak height of IS may not address

all matrix effects, the IS themselves have their own variations, and that changes can occur when mixing with the IS (Boysen et al., 2018; De Livera et al., 2015; Fan et al., 2019). QC aims to have a matrix composition highly similar to the biological samples of interest, which was done by pooling aliquots of the study samples (Fan et al., 2019). WIthin the batches, the QC samples are injected routinely to assess changes to instrument tuning, and the unwanted variations are mitigated via QC-based normalisation methods.

One of QC-based normalisations' primary advantages was that it accommodates both the unwanted technical variation and retains the biological variation of interest (De Livera et al., 2015). The ability to accurately fit the intensity drifts caused by the instruments over time, respond to the outliers within the QC samples, and offsets against overfitting from the QCs are crucial in a reliable QC-based normalisation (Fan et al., 2019). In this chapter, the QC-based normalisation method applied is the systematic error removal using random forest (SERRF) (Fan et al., 2019). Random forest (RF) was applied when the number of variables are higher than samples, and when nonlinear trends are observed, which is often seen in metabolomics datasets (Cocchi, 2019; Fan et al., 2019). RF does not overfit from increasing the number of trees, it can withstand missing values, and is not highly affected by multicollinearity (Fawagreh et al., 2014; Rodriguez-Galiano et al., 2012; Shah et al., 2014).

In figure 3.1, SERRF was applied onto our experimental dataset of *G. vaginalis*'s extracellular metabolites in the different conditions. The QCs in the SERRF dataset were shown to be far closer when compared to the raw dataset. At about 10 units in the SERRF whereas the raw dataset was about 30 units in the 1st principal component respectively.

Figure 3.1 PCA graph of raw and SERRF dataset consisting of the different batches (orange = 1, green = 2, blue = 3) and QCs (purple).

3.3.1.2 Differences from open access datasets

In chapter 2, the open access datasets from NASA Genelab were utilised. This has provided a different source for corrections. In this chapter, we applied a row-wise correction using the internal standards d_4-alanine, this was done by subtracting all rows with d4-alanine values. For the Baseline correction, we had used blanks instead of the media itself.

Three technical replicates were acquired for every sample, and five biological replicates were obtained for each treatment. The scaling applied was autoscaling.

3.3.2 Metabolomic analysis

3.3.2.1 Grouping with PCA

As mentioned in chapter 2, PCA is used to look at differences between different treatments. In a comparison between simulated microgravity (s-µg - displayed as ug) and simulated

gravity (sg) as shown in figure 3.2, there are differences as well as some levels of overlap. Most of the explained differences were in the 1st principal component (PC1).

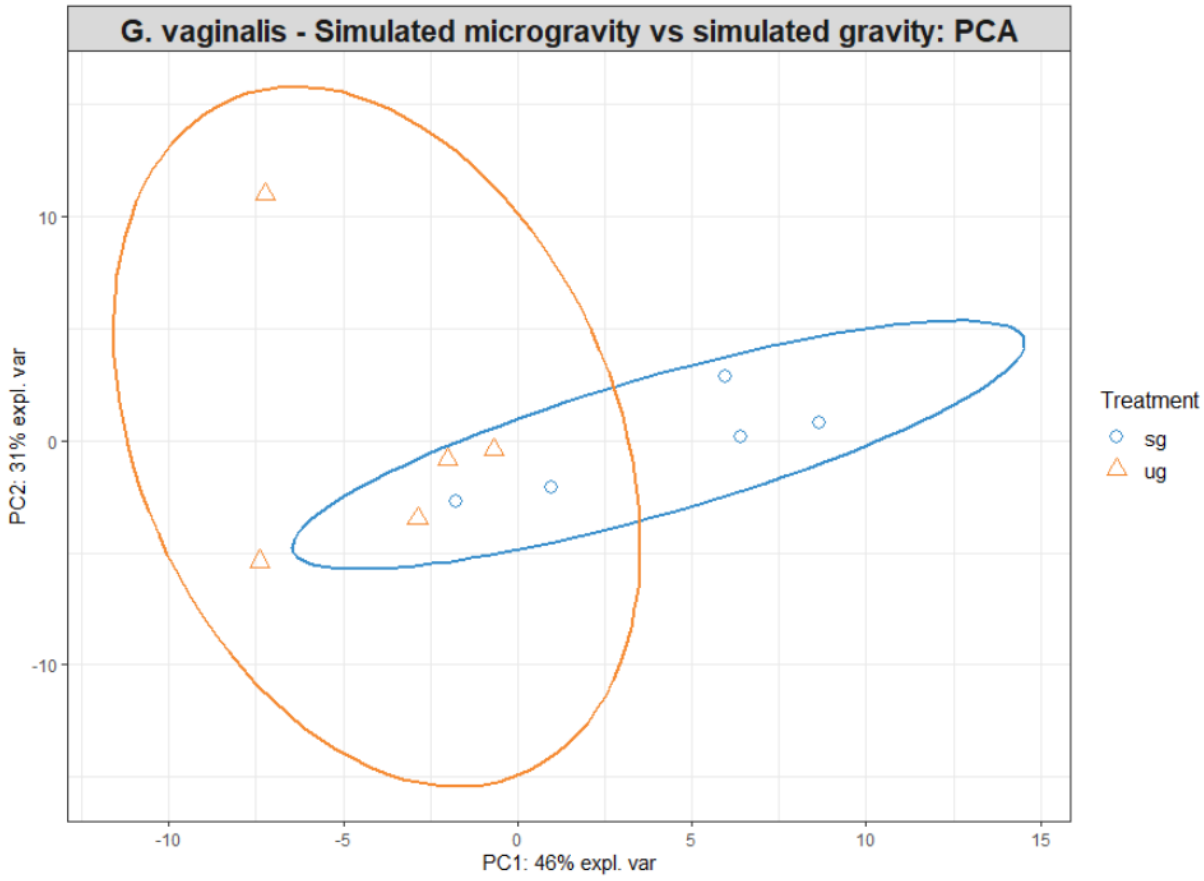

Figure 3.2 A PCA comparison of *G. vaginalis* extracellular metabolites with the simulated microgravity (µg) and simulated gravity (sg) treatment.

3.3.2.2 Top metabolites with PLS-DA

To find the metabolites that are affected the most, we will use PLS-DA, the first "s" in sPLS-DA stands for sparsity, which simply means the use of select elements from PLS-DA, as opposed to the entire datasets. Running cross-validation at 3-folds and 50 repeats resulted in the optimal number of variables to be 8 metabolites from the 1st component. The top 8 metabolites from the 1st component, and the top 100 metabolites that formed the sPLS-DA graph as shown in figure 3.3 were extracted. The top 100 metabolites are determined if two components were kept, but we can see that the 2nd component is undescriptive. Figure 3.3

shows that the top 8 metabolites of the 1st PLS-DA component alone, can be seen to demonstrate a clear separation between the sµg and the sg treatment.

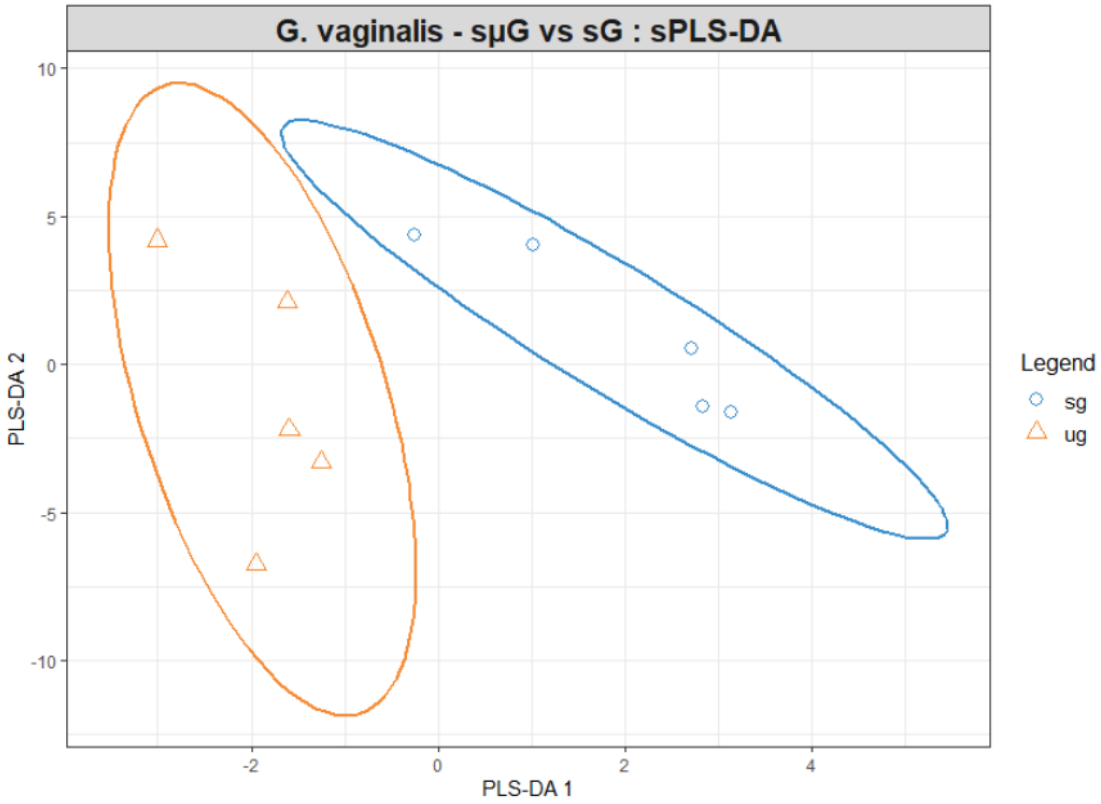

Figure 3.3 A sPLS-DA comparison of *G. vaginalis* intracellular metabolites with the simulated microgravity (µg) and simulated gravity (sg) treatment.

In figure 3.4, the top 8 metabolites are put into a boxplot where all extracellular metabolites are shown to have depressed expression in s-µg. These top 8 metabolites are identified through the cross-validation process. The values are based on normalised values. As multivariate methods from PLS-DA and cross-validation are experimental, we do not describe significance. Hydroxybenzoic acid is identified to show the most difference, with 5-Hydroxy-L-lysine showing the least differences in the top 8 metabolites.

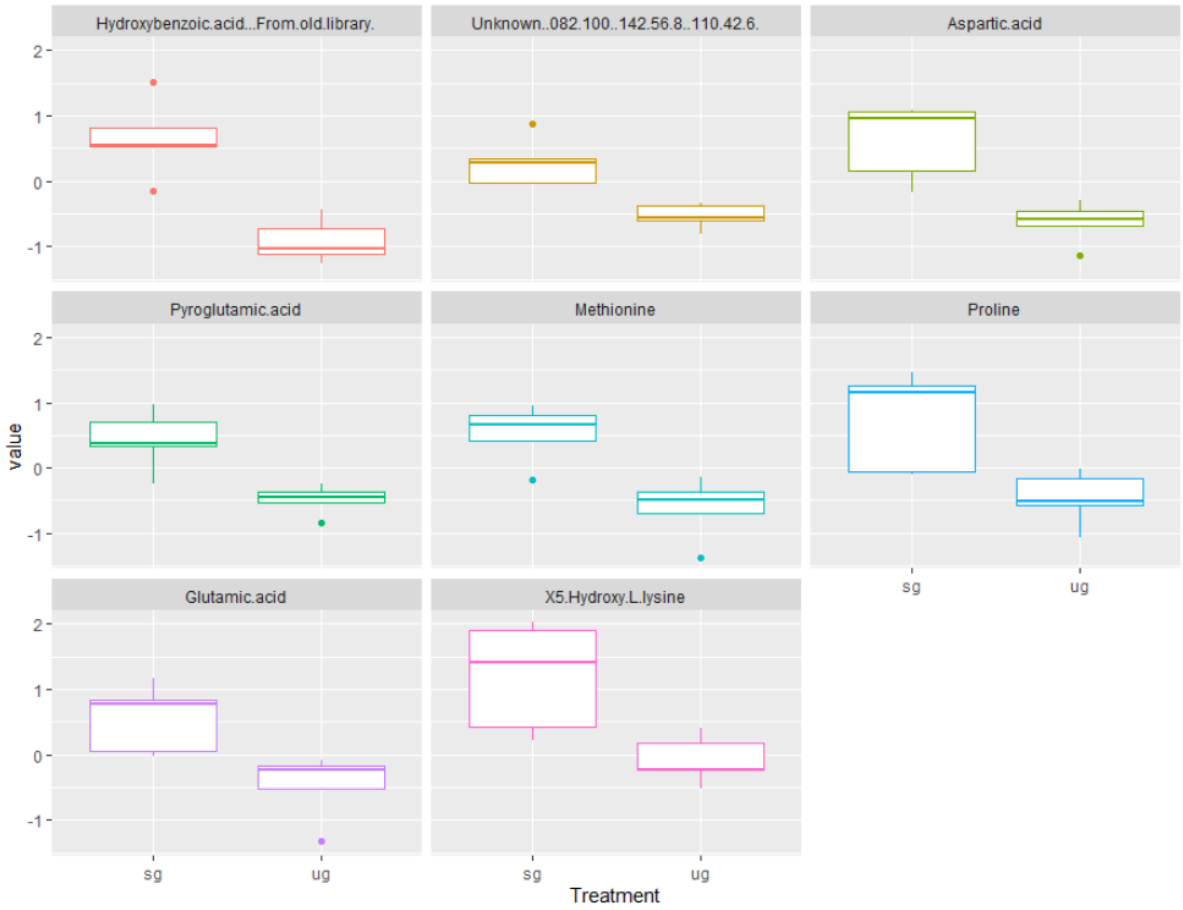

Figure 3.4 Box and whisker plot on the top 8 metabolites from the 1st PLS-DA component, after using normalised values.

3.4 Discussion

To understand how our organism is affected by culturing in suspension, a comparison of metabolic differences between simulated microgravity and simulated gravity was conducted. Extracellular metabolomics is the secretion of metabolites by the microorganisms into the culture media. We have to be careful interpreting changes to extracellular metabolites as a response to environmental factors. When there is a decrease in sets of extracellular metabolites, it is not possible to differentiate if these metabolites are being consumed more or produced in less amounts. Additionally, in a biological system, metabolites are connected to each other via different metabolic pathways and the metabolic flux will depend on the

integration of these various pathways and enzymes. Although important hypotheses may be built from comparing microbial metabolic profiling between different environmental conditions, these must be validated with further experiments. Again, we must be careful and open with our interpretations. With this note of caution, I will discuss the potential implications of our findings with attention to some specific metabolites found in lower concentrations in simulated microgravity relative to simulated gravity. From these alterations, we could envisage some hypotheses to follow in future.

We observed a significant decrease of hydroxybenzoic acid (HA) as a result of microgravity. We envisage a few potential indirect effects. HA is a xenoestrogen, an estrogen mimicking compound whose structural similarity makes it bind to estrogen receptor sites leading to significant effects on the body (Lemini et al., 2003; Paterni et al., 2017; Pugazhendhi et al., 2005). As a synthetic xenoestrogen, *G. vaginalis* was not known to produce HA until we revealed this metabolite in our analysis. Although exhibiting low estrogenic activity, HA has been found to cause positive effectives on gonadal development but also cancer progression (Paterni et al., 2017). HA is uterotrophic and induces vaginal cornification, a terminal cell differentiation program of the human vaginal epithelium that leads to flattened cells with weak intercellular junctions. Imbalance of this process might disrupt vaginal health and immunity (Anderson et al., 2014).

Also known as parabens, HA is effective against yeasts and molds at a concentration far lower than weak acids, but they are not as effective against bacteria, particularly Gram-negative bacteria (García-García & Searle, 2016). Thus, HA is used in cosmetics, pharmaceutics and as food preservatives (García-García & Searle, 2016). Possibly, the decrease of this molecule in the extracellular vaginal environment may have implications on the status of the microbiome and in a long-term it could favour the growth of *Candida albicans*, a common fungal pathogen in the vagina. Changes in the concentration of this molecule could have an influence on the development of the polymicrobial biofilm that is

typical of bacterial vaginosis and which *G. vaginalis* is the driver and the main species. Its decrease in concentration may impact on the colonisation of Gram-positive bacteria such as *Atopobium vaginae*, a bacterium also seen in high numbers in women with BV and commonly found in association with *G. vaginalis* (Mendling et al., 2020). Lastly, co-crystalisation studies of metronidazole (MTZ), a drug frequently used to treat vaginal infections by *Trichomonas vaginalis* and *Gardnerella vaginalis*, with hydroxybenzoic acid has been described to improve drug bioavailability, reducing potential side effects on carcinogenesis and genotoxicity in animals and humans (Gentili et al., 2005; J. Li et al., 2021; Y. Lin et al., 2012; Stjern et al., 2017).Therefore, having lower concentrations of this metabolite in microgravity means that treatment with this drug may not be as efficacious and, concerningly, it may enhance its side effects.

We also observed significant decreases of pyroglutamic acid and methionine in microgravity, which we envisaged as an important metabolic connection. Pyroglutamic acid, sometimes known as 5-Oxoproline, is an intermediate substrate in the metabolism of glutathione (Clarkson et al., 2010). Glutathione (GSH) is involved in the defence of cells against toxic xenobiotics, which includes pollutants, drugs, and carcinogens (Pompella et al., 2003). Most importantly, in the biological context of this anaerobic bacterium, GSH is a potent antioxidant and should contribute to maintaining the redox status that favours the dysbiotic vaginal microbiome and BV (Holmes et al., 1985). Disturbed levels of reduced vs oxidised GSH (as well as low levels of ascorbate) had been implicated as a metabolic signature of women with BV (Srinivasan et al., 2015). As seen for pyroglutamic acid, the reduced levels of methionine we found here may also be linked to perturbations on the redox status because the transsulfuration pathway connects methionine and GSH biosynthesis (Sbodio et al., 2019). Part of the transsulfuration pathway, GSH, cysteine, and ascorbic acid are downregulated as well (unpublished data).Together, the reduction of both pyroglutamic acid and methionine reinforces this idea. In addition, S-adenosylmethionine (SAM) is produced from the metabolic cycle that connects these two biosynthetic pathways (Sbodio et al., 2019). SAM

feeds into the synthesis of polyamines (Sauter et al., 2013). Increased polyamines leads to an odorous vaginal discharge that is a signature of symptomatic women with BV and abundant *G. vaginalis* (Srinivasan et al., 2015). These polyamines (such as putrescine, spermine, and spermidine) might help buffer the vaginal pH and counteract with lactobacilli. With the flourishing of *G. vaginalis* in dysbiosis or BV, the vaginal environment is considered anaerobic and reduced. Our study raises the hypothesis that microgravity may affect the redox status of the vaginal environment because of changes in the biosynthesis of GSH. This may be translated to other sites of the body, such as other anaerobic bacteria thriving in the gut microbiome. Although this is still preliminary or speculative, a note of caution is that microgravity in the long-term may significantly affect the composition of microbiomes in our body by altering the redox status and favouring different species of bacteria to thrive.

Although speculative, it is possible to explore the potential of *G. vaginalis* with the ability to regulate glutathione in their human host. By generating GSH itself, *G. vaginalis* can choose the right moment to stop producing it. Fostering a reliance on *G. vaginalis* to produce pyroglutamic acid, can render the host an environment of complacency, and be vulnerable to sudden changes.

Another point about methionine is the point link of this amino acid with elevated immune response in chickens (Mirzaaghatabar et al., 2011) and confirmed to offer a supportive role in immune-compromised or deficient patients (Van Brummelen & du Toit, 2007). If this is a universal response in microbial metabolism under microgravity, reduced levels of methionine may lead to downregulation of immune response.

Amino acids are obviously main constituents of peptides and proteins but some of them have specific roles in human cellular responses and microbial behaviour. Our study found reduced levels for a few amino acids that are implicated with specific cellular responses. For example, glutamate is a very common neurotransmitter (Binder et al., 2008). If reduced

glutamate is an universal response to microgravity, long exposure to a lack of gravity may result in cognitive impairment (Bechtholt-Gompf et al., 2010), making astronauts more vulnerable to making wrong decisions when under pressure .

Aspartate and lysine have been implicated in bacterial quorum-sensing for swarming motility and biofilm formation (Champalal et al., 2018). 5-Hydroxy-L-lysine, or hydroxylysine, is an amino acid specific to collagen and collagen-like proteins (Kraenzlin & Seibel, 2006). Collagen is notoriously important for healthy joints and skin elasticity. Not surprisingly, it is recognised as a marker for bone and skin degradation (Seibel et al., 2006). If reduced hydroxylysine is an universal response to microgravity by microbes of our body and our own cells, this may offer additional explanation to why astronauts when exposed long-term to a lack of gravity experience loss in bone content and skin rashes or hypersensitivities (Braun et al., 2019; Eckart, 2013).

In conclusion, our study revealed some significant changes on metabolites of a human anaerobic bacteria (this is characteristic of vaginal dysbiosis and BV) in response to microgravity. In this study, 8 metabolites, with previously little or no attachment to *G. vaginalis*, have been brought to light. While this study indicates that microgravity does have a significant influence on the metabolism of this bacterium, it will be worth investigating whether these responses are conserved across dysbiotic bacteria of the human body such as in the human gut and skin. It would be interesting to consider host-protective bacteria as well, polymicrobial interactions and even *ex vivo* microbial colonisation systems in future studies. Our preliminary data suggests that some consequential physiological changes may derive from exposure to microgravity, which may serve as hypotheses to be addressed in future investigations. We hope that our study might inform the importance of understanding physiological changes to microgravity. Studies alike will help design strategies towards mitigating negative health impacts on the body and microbiomes, facilitating space exploration for humanity.

Chapter 4 - Discussion

Starting this thesis, I set out to work out how microgravity affects bacterial behaviours. *G. vaginalis*, a critical bacteria involved in bacterial vaginosis, also seen in asymptomatic women (Benito et al., 1986), has yet to be explored in space biological research. Before assessing how *G. vaginalis* was affected by microgravity, I explored the use of different multivariate techniques for analysing biological data. This was done by looking at open access datasets provided by NASA Genelab (genelab.nasa.gov). This helped me identify circumstances when particular multivariate methods should be used or avoided. *G. vaginalis* was investigated for metabolic changes when subjected to simulated microgravity. Some of the top metabolites identified when subjected to simulated microgravity from sPLS-DA had no previous literature relevance to *G. vaginalis*. This study will help us assess the potential health risk of *G. vaginalis* and likely other bacteria pose on astronauts in space, and uncover metabolic pathways that are affected by microgravity. Understanding how bacteria adapt to space conditions help us understand the countermeasures required for development and the unveiling of potential biotechnological properties.

4.1 Biological research in space

After a NASA engineer died from heart failure while waiting for a heart transplant from donors, NASA engineers poured thousands of man-hours to develop a device to assist an irreparably damaged heart (DeBakey, 2008). Figure 1.2 from Chapter 1 demonstrates that spaceflight has profound effects on the human body. Abroad a flight on STS-115 (September 2006), *Salmonella typhimurium* demonstrated first hand, changes in microbial virulence and gene expression (Wilson, Ott, Höner zu Bentrup, et al., 2007). The study by Wilson et al (2007) shows the matrix accumulation like those seen in a biofilm. These unique changes are not just

to show that spaceflight makes bacteria aggressive. Changes in behaviour in conjunction with changes in biological expression (not limited to just genes) means that disease-targets are unveiled. Omics is important because it identifies where those changes occur over a comprehensive set of expressions, and which set of networks are affected. This demonstrated the ability of spaceflight for disease-biomarker discovery, and at the end of the day, we go to space to improve lives on Earth.

The general public will know that astronauts lose bone and experience muscle loss in space. Mechanical loading determines the changes to the bone (Buckey, 2006). Unloading from being in space results in an average bone monthly demineralisation rate of 0.5% and 1.7% on the hip and other bones involved in weight-bearing (Braddock, 2020; Eckart, 2013). Post-menopausal women that experience osteoporosis without treatment demineralised at 1.5% per year (Zea, 2015). This also means that microgravity can serve a novel platform for drug development of osteoporosis (Zea, 2015). AMGN-007, developed by Amgen, was sent on the Space Shuttle Mission STS-108, demonstrating a bone mineral density greater than both untreated mice on flight *and* the ground controls on Earth (Stodieck, 2013; Zea, 2015). Amgen ended up choosing AMG 162 as it had a longer half-life (Lacey et al., 2012). This resulted in two FDA approved drugs, Prolia and Xgeva, marketed to treat postmenopausal osteoporosis and bone metastasis respectively, and are being expanded to look at the treatment on other bone diseases (Zea, 2015). The effective overarching idea is that after one pinpoint how a particular environmental factor affects an organism, a specific application can be envisaged, tested and deployed (such as microgravity for the osteoporosis drug screening process).

Microbes inhabit and adapt to ecological niches of amazing differences and complexity. These niches have a wide range of environmental conditions, such as alteration in pH, oxygen levels, temperature, nutrient availability, and osmotic pressure gradients (Audia et al., 2001; Cavicchioli et al., 2000; Foster & Spector, 1995; Hecker & Völker, 2001; Hengge-Aronis, 2002;

Poolman et al., 2002). Microorganisms have a shorter life cycle and are easier to manage in experimental conditions than higher organisms such as plants and animals. The way microorganisms perceive changes to mechanical forces from a reduction in gravity has the potential to bring novel insights to disease mechanisms, therapeutics, and drug screening processes.

4.2 Concluding remarks and future directions

I will separate the advancing of the field and future directions into three main sections. This will include expanding the knowledge on *G. vaginalis* response to microgravity, other directions in the space sector, and technological advancements.

From the results of chapter 3, it is still preliminary to assume that the top metabolites have therapeutic effects or are targets for drugs. However, hypotheses have arisen on how these metabolites affect may *G. vaginalis* behaviour and how these alterations may impact in dysbiosis and disease. This will help us understand mechanisms and enable targeting and screening of better drugs for the treatment of BV. It would also be useful in conducting a study on intracellular metabolomics to assess how *G. vaginalis* is being affected inside the cell to complement our study in chapter 3 (i.e. extracellular metabolomics). One can also move towards multi-omics applying a multi-omic approach as entailed in chapter 2. This can involve the integration of intracellular metabolomics with transcriptomics, for example. Chapter 2 made use of supervised and unsupervised learning methods, so we can look at incorporating semi-supervised learning methods. Semi-supervised learning methods reduce costs from readily available data (X. Zhu & Goldberg, 2009), and assess whether *G. vaginalis* under the effects of microgravity mirror labelled BV. Our findings may also instigate investigations with other human commensal bacteria. For example, it would be worth

investigating the gut microbiome in which the environment is notoriously reduced and anaerobic.

The main focus of this thesis has been on microgravity, but radiation is also a space stressor (Chancellor et al., 2014). This would allow us to diffuse the differences between how microgravity and radiation affects *G. vaginalis* individually and separately, and then speculate how these space stresses affect other commensal microorganisms.

Space is inherently expensive and can be bureaucratic, so I would like to propose technological advances that would be useful in mitigating these issues.

While this thesis looks at how microgravity affects organism *G. vaginalis* alone. *G. vaginalis in vivo* may not behave the same when interacting with cells. Difficulties arise when attempting to grow human epithelial cells and *G. vaginalis* cells in microgravity. One way to explore this issue is the use of tissue chips.

Tissue chips, sometimes known as “organs-on-chips” or simply microphysiological systems (MPS), are being explored for the purpose of drug-screening (Low & Giulianotti, 2020). Drug development is expensive, has long testing cycles, and attrition rates are extremely high (Low & Giulianotti, 2020). Despite promising results from preclinical studies in animal models and 2D cell culture, toxicity and safety issues lead to intensive attrition to promising compounds (Waring et al., 2015). This attrition has been attributed to speciation differences between animals and humans, and the inability of 2D models to meet *in vivo* cell mechanics (Blumenrath et al., 2020; Low & Giulianotti, 2020). Drug metabolism from animal testing only apply on a case-by-case basis (Kirkland et al., 2007). MPS gives a novel and promising platform to model human diseases and disorders (Low & Giulianotti, 2020).

MPS platforms developed include Lung-on-a-chip, Heart-on-a-chip, Liver-on-a-chip, Vessel-on-a-chip, Tumor-on-a-chip, and others (Alshareef et al., 2013; Huh et al., 2010; Lee et al., 2007; Z. Wang et al., 2016; Werdich et al., 2004; Zheng et al., 2012). MPS has also been used for metabolomics (Kraly et al., 2009; L. Lin & Lin, 2015). Biofilms were recently investigated on “tooth-on-a-chip” (Rodrigues et al., 2021). There is yet development for Vagina-on-a-chip or biofilm-on-a-chip, which is a development required for *G. vaginalis* and BV-related organisms.

The main advantages of tissue chips is its high-controllability, and low-cost, and the mimicking of organs in a functional manner (Bhise et al., 2014; Huh et al., 2011; Moraes et al., 2012). Clinical trials on chips (CToCs) are also being proposed as each chip can be made from an individual, revealing potential toxicity issues for individuals early on, paving a literal path towards personalised models (Blumenrath et al., 2020).

Studies on cosmonauts and astronauts on Space Shuttle missions have largely been limited to easily obtainable samples, such as urine and blood, which prevents the examination of tissue or organs at the molecular level (Yeung et al., 2020).Transcriptomic studies on the effect of microgravity on mice liver and kidneys (T. Hammond et al., 2018), and proteomic studies of a mouse retina after 35-days on the International Space Station (Mao et al., 2018). It is difficult to assess how these findings apply to human health. The closed environment of the International Space Station National Laboratory (ISSNL) means that hazard potential has to be well described, many levels of containment required (sealing and double plastic bags) is required (Yeung et al., 2020). The enclosed nature of tissue chips helps with avoiding contamination. Under these circumstances, it would be useful to utilise tissue chips in space. Urinary tract infections are more common in female astronauts (Jemison & Olabisi, 2021), so there is a case for space in this area. The role of spaceflight for accelerated disease modelling also gives an opportunity to investigate drug testing and disease progression when accounting for both the pathogen and its corresponding host.

Bioprinting offers a solution that mimics human tissues much better than traditional 2D cell cultures (Cubo-Mateo et al., 2020). 3D bioprinting offers a wide range of potential future applications, such as tissue constructs for wound healing and bone defects (Cubo-Mateo et al., 2020). A variety of different methods can be used for bioprinting, which include inkjet, micro-extrusion, and laser-induced forward transfer (LIFT) (Cubo-Mateo et al., 2020; Derakhshanfar et al., 2018).

There is a growing number of researchers investigating bioprinting in space (Cubo-Mateo et al., 2020; Gelinsky, 2020; Sun et al., 2020). Bioprinting in space allows for more fluidity in the bioinks, the construction of complex geometries (such as cavities, voids, tunnels), it also offers label-free, scaffold-free, and nozzle-free magnetic levitational bioassembly (Sun et al., 2020). Space bioprinting is mostly magnetic levitation based, with an example being the Russian company 3D Bioprinting solutions (Sun et al., 2020). A label-free bioprinting means that cellular movements can be tracked without utilising fluorescent probes, which may affect normal cell activity (Fattah et al., 2016). Bioprinting in space would allow the investigation of how *G. vaginalis* interact with human cells at the 3D level.

It can be a bit difficult and a time-consuming process assessing the ISS due to legal and bureaucratic boundaries. Albeit more expensive, having small satellites, commonly known as CubeSats or nanosatellites, launched allows for a shorter wait to launch. Launch schedules, regardless of whether on the ISS or CubeSats, means that payloads require integration up to 1 year or more before the actual launch, which means organisms have to remain viable in stasis during that time resulting in a constraint in pre-launch conditions, rather than by the actual technology (Kanapskyte et al., 2021). Microbes are useful as they require less effort and interaction, and the use of cheaper biochemical assays and instruments (Kanapskyte et al., 2021).

There have been a few CubeSats that have biological payloads as the focus of its mission. *GeneSat-1* successfully observed cell growth of *Escherichia coli* with fluorescence in all nine microfluidic wells, which also demonstrated a system capable of autonomous operations in low Earth orbit (Parra et al., 2008). *PharmaSat* measured the growth of *Saccharomyces cerevisiae* on its fluidic card when subjected to different concentrations of antifungal agents, and measured growth by optical density, and culture viability with a metabolic indicator dye (Diaz-Aguado et al., 2009; Ricco et al., 2011). *PharmaSat* was the first free-flying satellite with an automated pharmaceutical dose-response system (Zea et al., 2021). *SporeSat* utilised a miniature centrifuge originally intended for the *GraviSat* project, where it demonstrated the ability to generate artificial gravity in conjunction with the measurement of calcium signalling in real-time with ion-sensitive electrodes (Fleming et al., 2014; J. Park et al., 2017). *EcAMSat* heavily reused *PharmaSat*'s spare hardware and designs to investigate the effects of microgravity on uropathogenic *E. coli* when subjected to dose-dependent antibiotics (Matin et al., 2017). *O/OREOS* was the only CubeSat mission with an astrobiology focus where it subjected *B. subtilis* spores to microgravity and radiation for 6 months, and measured germination and metabolic activity which were derived from *GeneSat-1* and *PharmaSat* (Nicholson et al., 2011).

In the Artemis era, *BioSentinel* is a secondary payload on the Space Launch System (SLS) Artemis 1 mission where it will be the first deep space biological CubeSat, subjected to radiation beyond low Earth orbit and will look at DNA damage and the survival rate of *S. cerevisiae* over 6 to 12 months (Padgen et al., 2021). The small size and mass of CubeSats makes it quite useful for doing biological research beyond low Earth orbit, and the investigation of radiation in cislunar space (Zea et al., 2021).

The space environment provides a platform for accelerated disease modelling and understanding biological pathways. Understanding cellular responses and the affected pathways to space flight stressors will allow us to assess the potential applications for our life

on Earth and help humanity for safe and long-term space exploration. Microorganisms are very advantageous because of their fast mode of growth, affordability and scalability. However, we envisaged the need to understand these changes in the context of microbiomes and host interactions. Advancing the field should be on a basis of automation, and modern technology. Hopefully, these advancements will facilitate space biology research in the near future.

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Appendix

Email communication with Jonathan Galazka

Galazka, Jonathan M. (ARC-SCR) [redacted]@nasa.gov> Sat, 23 May 2020, 04:27

to me, [redacted]

Hi Yen-Kai,

Regarding batches:

That pattern makes sense, the CTRL and Frozen ASAP were done a separate time. Once we had the Flight and Ground control samples we became aware that the freezing profile was quick slow. The BRIC canisters were simply thrown into the -80 on the ISS and with so much thermal inertia, the samples were effectively slow-frozen. The CTRL and Frozen ASAP samples were designed to determine the effects of this freezing protocol. I can double check is there are any other batches in the metabolomics data.

Jon

Jonathan M. Galazka, PhD
GeneLab Project Scientist
NASA Ames Research Center
Moffett Field, CA

Galazka, Jonathan M. (ARC-SCR) [redacted]@nasa.gov> Sat, 23 May 2020, 04:46

to me, [redacted]

Hi again,

Attached is the freezing profile for the three BRIC canisters (CanA, CanC, CanD) along with three attempts at mimicking this on the ground. A styrofoam box containing heat block placed into the -80 C (Box + Block) is what was used for the CTRL samples.