STUDYING THE INTERACTIONS BETWEEN MONOCYTES AND STAPHYLOCOCCUS AUREUS IN A MODELLED MICROGRAVITY ENVIRONMENT

Macauley Green B.Sc. M.Res.

DOCTORAL THESIS

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Abstract:

Since the onset of space exploration, challenges to human health have been reported. Astronauts have suffered from various illnesses and ailments and prokaryotes have been found to thrive aboard spacecraft. Due to proposed missions to Mars and other long-term space habitation, exposure to the space environment is increasing. This leads to new biological, engineering and pharmaceutical challenges. By investigating changes to monocytes (first responders of the immune system) and *Staphylococcus aureus* (one of the most commonly found prokaryotes aboard spacecraft) in a proxy microgravity culture, the reasons for the decreased immune response and increased infection rate during spaceflight can begin to be understood. The aims in this thesis were to investigate the pro-inflammatory response of monocytes in microgravity to bacterial stimulation and understand how this affects their ability to deal with bacterial infection, investigate how changes to *S. aureus* virulence and colonisation phenotype regulation and expression are changed in microgravity culture, and how these microgravity changes interplay with each other in an *in vitro* infection model. It was first shown that microgravity culture leads to a reduction in cytokine production upon bacterial stimulation from both Gram-positive and Gram-negative sources and that an altered response, shown by upregulation of NF-κB translocation, occurs with regards to the transcription factors that regulate this from the Gram-negative stimulation. Inhibition of cytokine secretion was ruled out for the decreased cytokine production observed. Further studies showed that there was increased internalisation of *S. aureus* into monocytes in co-culture. Building on these findings, changes to *S. aureus* in microgravity were investigated and it was shown they have an increased colonisation factor phenotype expression and reduced virulence factor production due to reduction and delay of induction of the *agr* (accessory gene regulator) operon that regulates this. *agr* is part of the quorum sensing system in *S. aureus* and it was subsequently shown that the inducing molecule for this system, autoinducing peptide (AIP), had reduced production in microgravity culture. It was further shown however that interactions between this molecule and its' receptor,

AgrC, are unaffected in microgravity culture. Finally, the impact of microgravity induced changes on these two cell types were investigated with respect to interactions between each other in a microgravity co-culture, forming an *in vitro* infection model. From this the increased internalization of *S. aureus* was explored and visualised with fluorescent microscopy. From these results it was shown that both microgravity induced changes to monocytes and *S. aureus* are needed to cause the increased internalisation of *S. aureus* in monocytes that is previously reported. This was further confirmed with an *S. aureus agr* mutant strain. The novel findings of this work add to pre-existing knowledge of bacterial infection during spaceflight and highlights mechanisms such as *agr*-quorum sensing that have not been investigated previously.

1. Introduction

The last century has seen the frontier of space become accessible by human beings. From Yuri Gagarin's initial flight into space, through NASA's (National Aeronautics and Space Administration) moon landing in 1969, to ventures in space tourism and planned manned missions to Mars from 2030, space has become an environment that is habitable for periods of time by humans. As missions to space become longer and further away in distance, the time spent in the space environment increases, and with it the associated health risks [1] [2].

A main stressor of the space environment is the absence of gravitational force in the frame of reference of the object. On the Earth's surface, gravity applies a force to objects causing an acceleration of 9.8 ms^{-2}, which is known as '1 *g*'. As objects move further from the Earth's surface, this force is reduced. For reference, when going from sea level to orbit, a distance of 400 km, the acceleration due to gravity is reduced to 8.7 ms^{-2}. When in orbit, the object is in free fall and constantly accelerating towards the Earth's centre due to this gravitational force. The forward motion of the object causes its path to be curved, and if sufficient, the object orbits the Earth. When in orbit, therefore, an object's frame of reference is accelerating at the same rate as the force of gravity would produce. The object experiences no net gravitational force. Microgravity is where the acceleration imparted on (parts of) the object due to gravity is slightly different than the acceleration of the frame of reference. During spaceflight, this additional gravitational acceleration can be from the spacecraft, the Moon, the Sun, and other celestial bodies. Astronauts experience slightly different gravitational acceleration forces across their body.

From the very onset of life on Earth, gravity has been the one constant factor. This is one of the only factors that has influenced evolution of every single organisms to have ever existed on Earth, along with other factors, such as life being carbon based and needing water as a solvent. Therefore, when the

gravitational forces are reduced or removed, unknown adaptations and responses may occur to organisms and biological processes.

Bacteria can rapidly adapt to changes in their environment due to their fast replication time, and therefore have the potential to rapidly adapt to microgravity. This could cause significant problems to human health during spaceflight as eukaryotic cells tend to be slower in adapting to differing environments, and the changes imparted by microgravity to biological processes may impact their function. Microgravity-induced immunological changes could impact the ability of astronauts to deal with pathogens in this environment.

The aims of this thesis are to investigate microgravity-induced changes on a commonly found prokaryote both on Earth and in space vessels; namely *Staphylococcus aureus*. It will also be investigated how these changes affect one of the cells of the immune system, namely monocytes and how the microgravity environment impacts their ability to fight bacterial infection. Monocytes were chosen due to their vital role in the immune response and activation of the pro-inflammatory response. Finally, investigations into if these microgravity-induced changes in *S. aureus* and monocytes affect interactions between the two cell types, and if *S. aureus* infection in the presence of monocytes is altered in the microgravity environment.

The following introduction to this thesis is split into two parts. The first part will explain the stressors experienced in spaceflight and overall physiological and psychological changes to human health. This section will then focus on spaceflight-induced changes to prokaryotes and overall changes to the human immune system. Introduction sections in the relevant results chapters will focus more specifically on microgravity induced changes to *S. aureus* and monocytes.

The second part of this introduction will introduce the bioreactor used in this thesis that allows for ground-based investigations into a modelled microgravity environment. The theory behind this bioreactor will be explained

and its use justified. It will then be compared with other ground-based microgravity analogues, and each methodology will be discussed with regards to its uses and limitations.

1.1 The Space Exposome

The space exposome is the collective term for the stressors spaceflight and the space environment imparts onto biological systems [3]. These stressors include microgravity, cosmic/ionizing radiation, possibly elevated CO_2 levels inside spacecraft on all biological organisms and disruption of circadian rhythm, and other mental health stressors on humans specifically. These factors individually or in combination can have significant impact on human health and pose significant problems for long term habitation/travel in the space environment.

1.1.1 Physiological Changes

The stressors of spaceflight can impart physiological changes onto astronauts. The major physiological changes include bone and muscle mass loss, cardiovascular deconditioning, neuro-ocular syndrome, and space adaptation syndrome (leading to space sickness) [4].

Bone and muscle degradation/loss pose significant health risks for astronauts during long term space habitation/exploration. The major effects of muscle loss amongst astronauts are post-flight weakness and lower back pain both during and after spaceflight [5]. NASA reports that astronauts experience up to 20% muscle mass reduction on spaceflights lasting 5-11 days. It is not just the reduced mechanical load of reduced gravity that causes the muscle atrophy and bone loss [6]. Indirect muscle atrophy is also induced by altered levels of circulating growth hormone, glucocorticoids, and anabolic steroids [7]. By applying mechanical loading, bone loss can be recovered but muscle loss cannot [6]. Bone loss is driven by the lack of mechanical loading mentioned through reduced loading stimuli which leads to a loss of

equilibrium in which bone resorption dominates the bone formation process [8]. This bone degradation has an approximate rate of ten times that seen in osteoporosis patients on Earth [8]. This can also cause indirect consequences such as increased risk of renal stones due to elevated calcium.

Microgravity exposure induces haemodynamic changes, such as blood volume shift and altered cardiac function, which can be classified as cardiovascular deconditioning [9]. Spaceflight has been shown to cause a blood shift of approximately two litres from lower body segments to upper body segments resulting in an increased cardiac output approximately 20% without increasing the heartrate [10]. However, blood volume overall is decreased in space [11]. This decrease in volume along with a decrease in arterial pressure leads to a decreased heart rate in microgravity [12]. There are also different timings of these effects. Significant shifts in the absorption, distribution, metabolism, and excretion occur due to the initial fluid shift in the upper body after 1-2 days of spaceflight and then the rebound decrease in blood volume to counter this [13].

One potential countermeasure to microgravity induced cardiovascular deconditioning is exposure to artificial gravity [14]. This negates the blood volume shift and returns the body to a more normal response to that seen on Earth.

Spaceflight-associated neuro-ocular syndrome (SANS), also known as space blindness, does not have a definitive pathogenesis and is theorised to potentially be a combination of inflammation and fluid displacement in the optic nerve sheath and both inflammation and fluid displacement in the cerebrospinal fluid [15]. A valid clinical case definition of SANS is yet to be defined and accepted [16].

Space adaptation syndrome is experienced by 60-80% of subjects within the first 2-3 days of microgravity exposure and a similar portion of people experience this upon return to Earth [17]. There are two hypotheses for space motion sickness, which are the fluid shift hypothesis and sensory conflict

hypothesis [17]. The sensory conflict hypothesis is that there is discontinuity between visual, proprioceptive, and somatosensory input, or semi-circular canal and otolith input [18]. This hypothesis can also be referred to as the 'neural mismatch' hypothesis and may involve alterations to the limbic system which is hypothesised to be the neural mismatch centre of the brain [18]. The fluid shift hypothesis is that the cranial shift of fluids experienced in microgravity induces the sickness [17].

1.1.2 Psychological Changes

The focus of space related health research is studying physiological effects of long-term spaceflight. However, the research area of psychological effects of spaceflight is becoming another prominent area of research. Psychological well-being should not be an overlooked factor with regards to human health during long-term space missions.

Circadian rhythm disruption is one major factor that can affect astronauts physiologically and psychologically. Circadian rhythm can be described as an internal, 24-hour clock with which the brain regulates alertness and tiredness by responding to changes in light [19]. Circadian rhythm can be disrupted by altered exposure to light different to the natural light cycles experienced in a 24-hour period on Earth. Spacecraft are sealed vessels and the inside of the spacecraft are not usually exposed to natural light from the Sun, apart from windows in viewing modules on space vessels such as the International Space Station (ISS). These viewing modules are usually Earth facing, however. Therefore, lighting inside space vessels are from artificial sources and long exposure to artificial lighting has been known to disrupt circadian rhythm [20]. On board the ISS, the lights were changed to LEDs of three different colours to help mitigate this but they are never turned off. For planned missions to Mars, habitation on the surface may also cause changes to circadian rhythm as well. A Martian day, which can be called a 'sol', is approximately 40 minutes longer than a day on Earth. This slight lengthening of a day leading to a chronic shift, where midnight and midday on the 24-hour

clock have reversed after two weeks, could be enough to disrupt the circadian rhythm of any astronauts on Mars.

From a psychological perspective, circadian rhythm disruption has been strongly linked with mental health issues and mood regulation [20]. The disruption has also been shown to affect the processing speed of disrupted subjects and impacted their working memory [21]. Sleep deprivation also causes reduced blood-flow to the brain [22] which compounded with microgravity induced fluid-shift, can lead to cognitive impairment.

Physiologically, the circadian rhythm regulates the production of melatonin and glucocorticoids [20]. Melatonin is said to have its own rhythm and effects on the body with cells exposed to melatonin responding in a 'night-mode' and cells not exposed to melatonin responding in a 'day-mode' [20]. Alterations to melatonin secretion could pose issues for astronaut health. One emerging health benefit is in its role in potentially preventing bone loss during spaceflight [23].

Research is ongoing into negating the disruption into circadian rhythm. This area of research is not only focused on spaceflight but other situations where the only light exposure is artificial, such as habitation on submarines or remote polar outposts. As far back as the early 90's, melatonin was being tested as a potential counter-measure to jet-lag; another circadian rhythm disruption induced phenomenon [24]. Space analogue missions have been undertaken to test potential circadian disruption countermeasures such as dynamic lighting schedules to replicate the diurnal cycle experienced naturally with promising results [25].

Circadian rhythm disruption is not the only psychological disruption incurred from spaceflight. Cognitive dysfunction, emotional instability, visual phenomena and morphological changes to the brain have all been reported [26].

Emotional instability can be induced from the extreme environments experienced during long-term spaceflight. The importance of potential

emotional research and emotional training has been highlighted for planned missions to Mars [27]. Emotional and mental health issues, such as depression, may have played a role in the abortion of the Soyuz T14-Salyut 7 mission and highlights potential problems for future missions [26]. Negative emotional impacts may derive from the disrupted circadian rhythm, lack of privacy, excessive noise from the spacecraft/machinery, and homesickness [26, 28]

1.1.3 The Immune System

The immune system is composed of two different major systems: the innate immune system, and the adaptive immune system. The innate response is commonly referred to as the non-specific response and usually occurs immediately or within a few hours after the appearance of an instigating antigen [29]. The innate immune system consists of physical barriers, such as the skin and mucus, and cells: monocyte, macrophage, neutrophil, natural killer, mast, basophil, and dendritic [30]. The adaptive immune system is commonly referred to as the acquired immune system and occurs at a later timepoint than the innate immune system. The adaptive immune system consists of lymphocytes, known as T-cells and B-cells, which specifically target the pathogen and provide future protection against that pathogen [31].

1.1.4 Generic Immune Responses to Microgravity

Major changes in immune system function occur during spaceflight [32]. During flight, levels of circulating monocyte, T cell, B cell, and neutrophil levels are all increased, whereas that of natural killer cells is decreased [33]. Distribution of peripheral leukocytes is altered with specific subpopulations showing diminished function [3]. Latent viruses, such as herpes, reactivate [34, 35] and indicate compromised adaptive immune function [3]. Epstein-Barr virus, cytomegalovirus, and VZV (human neurotrophic alpha herpes virus) have also been reported to be reactivated during spaceflight, and correlated with stress due to increase in cortisol [33]. Hypoplasia of the spleen can also occur with an increase in peripheral blood neutrophils [36, 37]. Natural killer

cells exhibit lower cell cytotoxicity, and there is also a delayed response to hypersensitivity skin tests [36]. One reason for the inhibition of natural killer cell toxicity is reduced production of granzyme B and perforin with effects being reported up to 60 days after spaceflight [38]. B cell activation in microgravity is still largely unknown, but short term flights have shown no significant changes [36] with long-term spaceflight showing maintained B cell homeostasis [39]. This is an interesting finding as the short-term flight study suggests a Th2 (T helper 2) shift occurs in microgravity, which may affect immunoglobulin production. However, this is only a hypothesis and only postulated due to a decrease in Th1 (T helper 1) cytokines being present [36]. Figure 1.1.4.1 shows the normal differentiation pathways for naïve T cells.

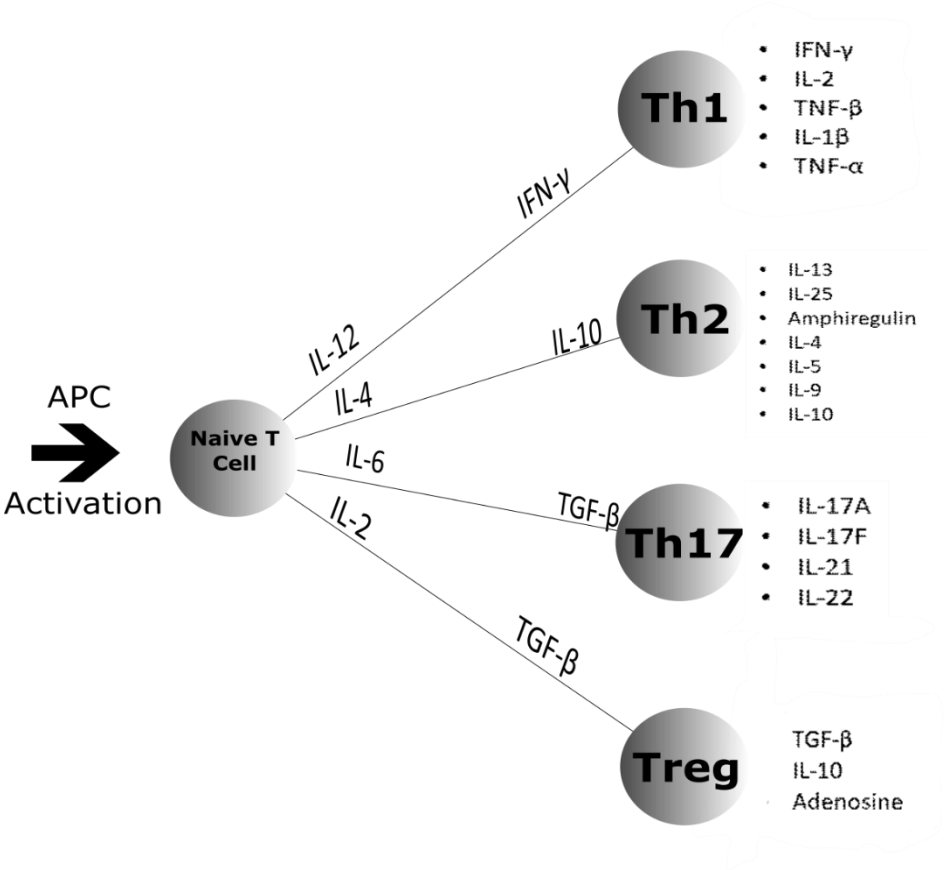


Figure 1.1.4.1: Naïve T cell differentiation after Antigen Presenting Cell (APC) activation. The naïve T cell differentiates into four different classes of T cell: Th1, Th2, Th17 and the Treg. APC activation causes the release of the cytokines shown along the lines which signal for which class the naïve T cell will differentiate into to. Once differentiated, the T cells secrete the cytokines shown by the bullet points to the right [40-42].

Studies of long-term missions on B cell activation and immunoglobulin production is thus far inconclusive [43-46].

Due to the nature of the low-shear environment during spaceflight, motility of immune cells is greatly reduced [36]. Combined with reduced monocyte motility and cytoskeletal modifications this may lead to the reduced interactions between monocytes and lymphocytes, which has been shown to be essential for costimulatory signalling [36]. Early proteomic changes in mammalian cells have been found in proteins that regulate cell motility and cytoskeletal organisation [47]. These early proteomic activations suggest the

gravitational environment activates cell motility and that these cellular events may be a eukaryotic gravity sensing mechanism [47].

1.1.5 Immune Cell Differentiation

Differentiation inhibition has been reported by a plethora of immunological studies in a low-shear environment created by both microgravity and ground-based analogues [48-54].

Various reasons for differentiation inhibition have been proposed. One school of thought is that non-differentiated monocytes are suspension cells that become adherent upon differentiation. Future investigations into whether the low-shear environment prevents adherence and therefore the differentiation of the cells could warrant interesting results.

More recent studies have delved into altered pathways due to low-shear forces to shed light on the differentiation inhibition problem [52]. RAS/ERK/NF-κB pathway was shown to be a low shear-regulated pathway, which showed exogenous ERK and NF-κB activators were able to counteract the effects of microgravity on macrophage differentiation in both microgravity and ground-based analogues [52]. This study also verified *via* qPCR and western blot that the p53 pathway was also affected by the low-shear environment. This concurs with older studies that also concluded altered genetic pathways cause immune cell differentiation inhibition [55, 56]. Furthermore, cell cycle 'arrest and progression' proteins have been shown to be altered. P21 increases 4.1-fold in 20 seconds of spaceflight microgravity culture in primary cells and 2.9 times in Jurkat T cells, compared to ground controls. These results suggest that cell cycle progression is gravity dependent in T cells, and can halt the progression of differentiation[57]. These results were confirmed by other studies [58].

Differentiation into effector T-cells is also driven *via* dendritic cells through the production of IL-2. The alterations in IL-2 production that mimic T cell exhaustion also provides an explanation for T cell resistance to differentiation into effector T cells [59].

The surrounding microenvironment provided by the connective tissues can also have immune-regulatory effects. Mesenchymal stem cells (MSCs) are stromal cells that can differentiate into connective tissues, and are integral to some specific immune responses. They produce cytokines and molecules such as but not limited to: PGE2, nitric oxide, FasL, PD-L1/2, IDO, and IL-6 [60]. Culture in a low-shear environment maintains the undifferentiated state of MSCs [54] as mechanical loading is an important determining factor for osteogenic differentiation [61]. This may potentially be due to the down-regulation of the master osteogenic transcription factor Runx2 and main osteogenic differentiation markers ALPL and OMD in long term microgravity analogue culture [49]. Low-shear culture also effects myogenic differentiation [61]. During spaceflight, 1599 genes have been shown to have an altered expression with important changes being reduced expression of cell-cycle genes, which leads to cell proliferation inhibition [62].

1.1.6 Prokaryote Responses to Microgravity

Prokaryotes have evolved and adapted to survive in a plethora of different environmental conditions [63]. Due to advances in technology, prokaryotes have been exposed to a new novel environment, namely microgravity. This new environment has the potential to generate a plethora of research avenues. This area of research could be beneficial for combatting infection during long term manned space missions and may provide novel insights into prokaryote adaptability and evolution. The following sections review research into the prokaryotic response to the low-shear environment created by microgravity and ground-based analogues.

1.1.7 Prokaryote Viability and Diversity

The human body itself contains a substantial number of bacteria from the bacteria covering the skin to the microflora of the gut [64, 65]. NASA have set acceptability limits for bacteria numbers in the air, on surfaces, and in water for all space bound equipment and vessels.

Table 1.1.7.1: Bacterial acceptability limits outlined by NASA [66].

Time Taken	Air	Surface	Water
Preflight	300 CFU m^{-3}	500 CFU $100cm^{-2}$	50 CFU mL
Inflight	1000 CFU m^{-3}	10000 CFU $100cm^{-2}$	50 CFU mL

As shown in Table 1.1.7.1, it is expected that bacteria during spaceflight will survive and proliferate in microgravity/spaceflight conditions. This is shown by the higher bacterial acceptability limits for air and surfaces inflight compared to pre-flight.

Vessels from different locations unsurprisingly show differences in bacterial population. The Russian Mir space station reported the most dominant genera of airborne and surface bacteria to be *Staphylococcus* with *Sphingomonas* and *Methylobacterium* to be the most dominant genera in the potable water [67]. When water was collected from the ISS between 2009-2012, it was found the most common organisms were *Burkholderia multivorans* and *Ralstonia picketti*, with air and surface dominance of *Bacillus*, *Micrococcus* and *Staphylococcus* species [66]. Overall, the most common phylum was Actinobacteria [68].

1.1.8 Overview of Prokaryote Studies

The following studies have been undertaken to investigate the effects of a low-shear environment on bacteria with a few studies also investigating archaea. The first studies found common responses to spaceflight bacteria with emphasis on the phenotypic responses including but not limited to: changes in growth rate; resistance to external stresses; varying effects on

bacterial conjugation [69]. Table 1.1.8.1 and 1.1.8.2 are a summary of the major studies investigating individual species and strains of bacteria and their critical findings.

Table 1.1.8.1: Summary of bacterial response in microgravity analogue studies.

Name	Type of Microgravity	Studies	Major Findings
Mycobacterium marinum	Rotating Cell Culture System	[70]	562 genes altered transcription level after short growth, 328 after long growth. Down regulation of Metabolism. Increases sensitivity to hydrogen peroxide.
Ralstonia pickettii	Spaceflight samples in Rotating Cell Culture System	[68]	Increased growth rate
Escherichia coli	Rotating Cell Culture System	[71-75]	Shorter replication time, increased survivability in J774 macrophages, increased resistance to osmotic stress, heat and acid. Increase in biofilm thickness and biomass. Deregulation of Hfq
Salmonella enterica serovar typhimurium	Rotating Cell Culture System	[76]	Shorter replication time, increased survivability in J774 macrophages, increased resistance to osmotic stress, heat and acid.
Streptococcus mutans	Rotating Cell Culture System	[77-79]	153 genes upregulated two-fold or more, 94 genes downregulated two-fold or more 100-day evolution experiments
Lactobacillus acidophilus	Rotating Cell Culture System	[80]	Shortened lag phase, increased growth rate, increased antibiotic resistance, increased

			acid and bile resistance.
Lactobacillus reuteri	Rotating Cell Culture System [81]		Reuterin production and stress gene response changes
Klebsiella pneumoniae	Rotating Cell Culture System	[82, 83]	Enhanced biofilm formation, thicker biofilms, increased cellulose production.
			Chromosomal structure changes
Vibrio fischeri	Rotating Cell Culture System	[84, 85]	Hfq mutant studies.
			Altered lipopolysaccharide and vesicle production
Staphylococcus aureus	Rotating Cell Culture System	[87]	Antibiotic resistance increases. Cell wall changes.
		[88]	
	Random Positioning Machine [86]		

Table 1.1.8.2: Summary of bacterial response in spaceflight studies.

Name	**Type of Microgravity**	**Studies**	**Major Findings**
Ralstonia pickettii	Spaceflight samples in Rotating Cell Culture System	[68]	Increased growth rate
Salmonella enterica serovar typhimurium	Spaceflight	[89]	Intestinal infection models
Bacillus subtilis	Spaceflight	[90, 91]	55 genes upregulated (biofilm formation associated genes), 36 genes downregulated (anaerobic respiration associated genes).
			No changes to final cell concentration
Pseudomonas aeruginosa	Spaceflight	[92-94]	Different biofilm architecture to that formed under Earth gravity.
Staphylococcus aureus	Spaceflight	[88]	Cellular envelope changes.

1.1.9 Prokaryote Transcriptomic Changes

To summarise the transcriptomic results from the studies shown in Table 1.1.8.1 and 1.1.8.2, common dysregulated genes have been identified and their expression postulated to be altered because of microgravity.

The global post-transcriptional regulator Hfq is one of these genes that has been identified to show altered levels of expression across multiple pathogenic species of bacteria in both microgravity and microgravity analogues [84, 89, 94]. This gene is found in approximately half of all known bacterial genomes and plays an important role in bacterial stress responses [84]. Hfq is an RNA-binding chaperone protein whose activity regulates bacterial protein expression via small (typically 50 to 500 nucleotides in length) bacterial RNAs (sRNAs), which regulate many bacterial processes [95, 96]. They act via antisense mechanisms on multiple target mRNAs and exert global effects on factors such as virulence, stress response and adaptive metabolic changes [97].

The ferric uptake regulator (Fur) and its homologues; the zinc uptake regulator (Zur), the manganese uptake regulator (Mur) and peroxide stress defence control (PerR) [98] are required in some microgravity analogue stress responses in e.g. *Escherichia coli* [99]. Fur is a transcription factor that represses siderophore synthesis in pathogens by utilising Fe^{2+} as a corepressor [98]. Fur also controls the expression of enzymes that protect against reactive oxygen species damage, which is one defence mechanism of the host immune system in infection [76]. Due to these two processed, Fur is involved in iron homeostasis and reactive oxygen species defence [76].

Many genes that respond to a low-shear environment are found in clusters or operons [76] and upstream of many of these operons is a Fur binding site. Therefore, it can be theorised that the expression and/or activity of Fur may be related to microgravity induced changes due to the reoccurrence of this binding site being found near the operons of ‘gravity-sensitive’ operons.

Regulation of the low-shear response via a Fur binding site has been shown with a *Salmonella fur* mutant which alludes to Fur transmitting the microgravity analogue signal [76]. For the acid resistance response to microgravity analogue operon, *fur* is found upstream of this. When exposed to a low-shear environment, the *Salmonella* strain used in the study shows increased acid resistance whereas the *fur* mutant strain shows no increase in acid resistance [76]. This strengthens the hypothesis of the Fur protein regulating a microgravity stress response operon, however more studies are needed to confirm if the observation of Fur being found upstream of operons that alter in induction and/or expression in microgravity environments is coincidental or has some functional correlation.

General stress responses In *E.coli* and many other bacteria are regulated by the sigma subunit of RNA polymerase known as RpoS [100] [101]. Interestingly, this is not the case for microgravity analogue response via a rotating cell culture system in *Salmonella enterica serovar Typhimurium*, which adapts in an RpoS-independent manner to environmental stresses [71].

1.1.10 Antibiotic Resistance

A major finding of note for bacteria grown under microgravity is the increase and difference in biofilm formation, architecture, and the development of antibiotic tolerance.

Antibiotic resistance poses a severe risk to health both in spaceflight and once the astronauts return to Earth. Upon return to Earth the antibiotic resistant strain may spread through the population. Furthermore, microgravity is just one factor during spaceflight that has been shown to increase antibiotic resistance [102]. The bacterial adaptive response, which is the exposure to a sub-lethal stressor which induces resistance to a lethal level of the same or different stressor [103], can also be triggered by ionising [104] and non-ionising radiation [105] found as part of the cosmic radiation [102]. The radiation may cause changes to antibiotic efflux pumps and sensitivity to

chemicals [104]. Antibiotic resistance profiles (see later) and biofilm formation are not generic responses to extreme environments. A comparative study of *Staphylococcus* and *Enterococcus* isolates from the ISS and the Antarctic Research Station Concordia were compared, and the ISS isolates were found to be more resistant to antibiotics tested for [106]. This could indicate non-space extreme environment studies may not be good substitutes and not have comparable data to the study of the extreme environment of space. This could also mean microgravity and/or other space stressors may trigger the expression of different genes in the response to extreme environments. More comparisons are needed.

Long term microgravity analogue studies have been performed to simulate long term manned missions to try and predict antibiotic resistances that could potentially evolve. One such study used the rotating cell culture system (RCCS) for 1000 generations of *E.coli* over which it became tolerant to cefuroxime, chloramphenicol, cefalotin, cefuroxime axetil, tetracycline, and cefoxitin [107]. Interestingly, after 110 further generations in Earth gravity conditions, chloramphenicol and cefalotin resistance was retained. This could, however, be due to an accumulation of mutations.

During spaceflight, and especially on a long-distance manned mission to Mars, there will be a finite amount and diversity of medications. Especially with the longer manned missions, there is no feasible way to restock the vessels or send new medications/antibiotics. Therefore, if a multidrug resistant strain develops and becomes resistant to the antibiotics onboard the vessel then all the passengers' lives are at risk as the infection may not treatable.

Additionally, this can also pose a threat to health on Earth. In microgravity, the bacteria may develop antimicrobial resistances that are different to what develops in Earth gravity. This could enable widespread infection and disease on return to Earth if the pathogenic bacteria have infected one of the passengers and this has gone unnoticed. Amongst Gram-positive bacteria, the most resisted antibiotic was sulfamethoxazole, and amongst Gram-negative bacteria the most resisted antibiotic was gentamicin [108].

Virulence is defined as the ability of the bacteria to cause disease and can also be referred to as pathogenic potential [109]. Increases in virulence have been reported in both analogue and spaceflight microgravity [110-112]. With regards to spaceflight, this is especially of concern due to the constant close contact with other astronauts in the relatively small space vessel. Increased virulence combined with antibiotic resistance poses a massive health risk and will greatly increase the dangers of both acute and chronic infections.

1.1.11 Archaeal Response

Archaea are distinct from bacteria and are prevalent in extreme environments and are also a natural component of the microbiota of humans [113]. However, no known pathogenic archaea exist [113].

Table 1.1.11.1: Response of Archaea to a low-shear environment.

Name	Type of Microgravity	Studies	Major Findings
Haloferax mediterranei	Rotary Cell Culture System	[114]	Increased resistance to bacitracin, rifampicin and erythromycin
Halococcus dombrowkskii	Rotary Cell Culture System	[114]	Reduced cell aggregation
Haloarcula argentinesis RR10	Rotary Cell Culture System	[115]	Increased production of ribosomal proteins, became multi-drug resistant, evidence of antibiotic efflux pump

Haloarchaea are the most studied area species that live/survive in aqueous environments. Some haloarchaea show increase in antibiotic resistance which may be a problem as archaea and bacteria can undergo horizontal gene transfer, especially from archaea to bacteria [116, 117]. Horizontal gene transfer is the acquisition of new genetic material from another organism, this is a major driver of bacterial pathogen evolution and antibiotic resistance [118].

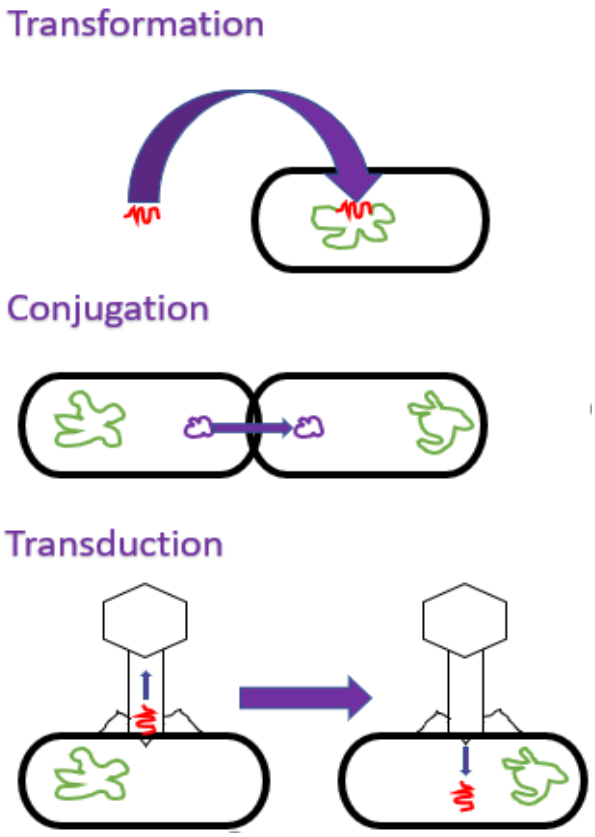


Figure 1.1.11.1: The three mechanisms of horizontal gene transfer. Transformation is where foreign genetic material is acquired from the environment. Conjugation is the direct cell to cell contact of two prokaryotes in which genetic material is transferred through a pilus. Transduction is the transport of genetic material from one prokaryote to another via a virus. These viruses are called bacteriophages.

To conclude the first part of the introduction, it has been discussed how microgravity and the space exposome negatively affects human physiology and impacts astronauts mentally. It has then been discussed how major components of the immune system are impacted by exposure to microgravity. Furthermore, cells of the immune system have been discussed with regards to the impact caused by microgravity exposure as well. Finally, it was discussed how prokaryotes adapt to microgravity and the potential hazards to human health they could cause.

What is apparent from the studies discussed is that not all the studies were performed in spaceflight. Due to the financial resources needed for spaceflight along with experiment justification and feasibility issues that arise with spaceflight, the majority of biological microgravity research is performed on Earth using microgravity analogues. The second part of this introduction reviews these microgravity analogues and explains the culture method chosen for this thesis.

1.2 The Rotating Cell Culture System

The rotating cell culture system (RCCS) was a bioreactor employed in this project to provide an analogous culture environment to that of the one created by microgravity. Every experiment in this project used the RCCS as its form of '0 *g*' culture. As this bioreactor was integral to the project, the following sections explain the theory behind the RCCS, the other ways to perform microgravity culture on the ground, and how this method of simulating microgravity conditions compares with true spaceflight.

1.2.1 RCCS Function

The rotating cell culture system is a bioreactor created and sold by Synthecon (Texas, USA). It composes of a base with 1-4 motorized attachment points for vessels, and a power and control unit to vary the rotation speed of the attachment points.

The microgravity environment of spaceflight creates a low-shear environment. Through the use of fluid shear forces, a low-shear environment can be created on Earth. This low-shear environment created through low fluid shear forces is analogous to the low-shear environment created by the near zero net gravitational force on the frame of reference of the object in orbit or during spaceflight. This allows for ground-based research into the effects of a low-shear environment, and by proxy, microgravity.

To do this, a vessel on the RCCS must be filled with liquid with no headspace. This liquid has an acceleration which changes due to gravity. This is dependent on rotational speed of the vessel and how far away from the axis it is. Cells inside the liquid do small orbits of which the radius is inversely proportional to the speed of rotation. This spinning of the liquid imparts fluid shear forces onto the objects inside the vessel. Slower spinning results in the cells falling through the moving media and faster spinning results in centrifugal force pushing the cells to the outside edges of the vessel. When at a suitable rotational speed, the cells are constantly falling through the circulating liquid. This speed is dependent on the size of the cells. This form of microgravity analogue is called low-shear modelled microgravity or LSMMG. The important difference to note is that during spaceflight both the cells and fluid are falling together and do not move relatively with each other whereas in the RCCS, it is just the cells that are falling inside the circulating liquid.

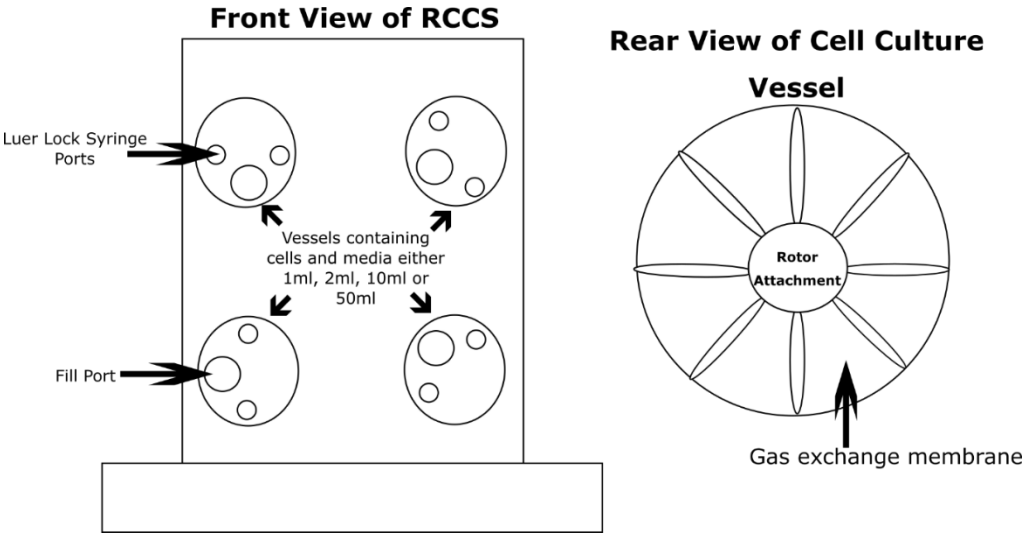


Figure 1.2.1.1: The 2D Clinostat known as the Rotating Cell Culture System (RCCS). The RCCS is capable of rotating up to four individual cell culture vessels. These cell culture vessels carry media containing cells in 1 mL, 2 mL 10 mL or 50 mL formats. The cell culture vessels come in two varieties, disposable and autoclavable. The vessels compose of two Luer Lock syringe ports for small additions or extractions to the cell media and a larger fill port for ease of filling and emptying. The rear of the vessels composes of a gas exchange membrane to allow the diffusion of gases. The vessels rotate clockwise at independent or synchronous speeds [119]. The schematic shows the parts of the RCCS and names the individual components. The photo shows the RCCS set-up used for the studies in this thesis with one RCCS being in a horizontal 'control' orientation and the other RCCS in the '0 *g*' analogue orientation. Attached are 2 mL autoclavable HARV vessels.

1.2.2 RCCS Limitations

The RCCS, like all other microgravity analogues, has its limitations. The main limitation of the RCCS is that the vessels must have zero headspace. For zero headspace, there can be no room for air and each vessel must be 100% full. This poses a problem, especially with microbiology research, with sampling the contents of the vessels over time. Whatever volume of sample is taken out of the vessels must be replaced. When taking a sample, a certain number of cells will be extracted along with the spent media (the 'spentness' of the media depending on the timepoint), and waste products produced by the cells. When media is added back to maintain the zero headspace, it is impossible to replace the identical extracted sample. Any re-added cells may not be in the same growth phase as the ones extracted, so the cell population is changing, and the concentration of nutrients and waste products in the media also varying. The main workaround for this problem is to reduce the sample taken to the minimum amount possible whilst maximising the vessel size used and to reduce the number of samples taken to the minimum amount that will still provide statistically relevant results. A secondary issue with sampling in the RCCS is continuous and discontinuous sampling. There are minimal reported studies and guidelines into this which could be a cause for concern. Discontinuous sampling is where the operator will pause the rotation of the vessels to extract a sample. For these few seconds the samples will not be inside a low-shear environment and depending on the sensitivity of the cells it could result in changes to the response being monitored. Continuous sampling is possible with experience.

Another major issue with the RCCS is bubble formation. Bubbles in the media will result in disturbances in the micro-orbit of the cells inside the vessels resulting in shear forces being applied to the cells and negation of the analogous environment. Media may be degassed in advance, but as this reduces the amount of dissolved gasses in the media it may lead to altered phenological responses in biological experiments. These bubbles can form overtime so long experiments, such as overnight, become particularly

susceptible to this if continuous observation of the experiment is not possible. Bubble formation can be reduced by performing the experiments within a humid environment.

A low-shear environment is not the only factor microgravity culture creates. Due to the lack of gravity in spaceflight, microgravity liquid cultures do not experience convection. Convection is the movement and mixing of liquids due to their differing densities caused either by their properties or temperature changes, and gravity causing the higher density liquids to sink below the lower density liquids. This is one important way in which nutrients and solutes move about and are distributed during cell culture. As the RCCS is in a gravity environment when used on Earth, the liquids inside are subject to convection. Furthermore, the rotation of the vessels themselves move the liquid cultures and induce further movement of the culture medium and its components that would not be seen in true microgravity culture.

A final limitation of the RCCS is culturing on mixed populations. Different sized cells require different rotational speeds for low-shear culture. However, this range is not strictly specific and a generic rotation rate of 25 rpm keeps both eukaryotes and prokaryotes in low-shear culture. The issue arises when culturing suspension and adherent cells. To culture adherent cells in the RCCS the introduction of attachment material (such as a polymer bead) to the vessel is required. As these will have a sedimentary velocity higher than the cells attaching to them, they require a faster rotational rate to keep in suspension. The problem with this is that whilst the attached cells will be in low-shear culture in the accelerated rotation, the increased rotation rate will apply increased shear forces through centrifugal and centripetal forces to any cells still in suspension, weakening the accuracy of the analogue culture on these cells. This is not a problem, however, if cells are pre-attached to the polymer beads or just the attached cells are the targets of interest. This is only an issue when studying a mixed population of suspension and adherent cells which, for instance, can make studying an infection model in an RCCS a difficult task.

1.2.3 Microgravity Analogues

Although the RCCS is the most commonly used microgravity analogue, other microgravity analogues are also widely accepted and produce published results. Table 1.2.3.1 provides a brief summary of the main ways the effects of microgravity is studied on the Earth. These microgravity analogues will then be discussed in detail in the following sections.

Table 1.2.3.1: Microgravity analogues employed in ground-based studies.

Microgravity Analogue	In vivo/ In vitro	Analogous Aspect	Limitations
RCCS	In vitro	Low-shear	Convection present
Diamagnetic Levitation	Both	Low-shear	Increased convection
Parabolic Flight	Both	Low-shear	Varying acceleration from 0 *g* to 1.8 *g*
Random Positioning Machine	In vitro	Low-shear	Convection present
Drop Tower	Both	Low-shear	Short time frame
Dry Immersion	In vivo	Mechanical unloading	Limited use, no low-shear
Wet Immersion	In vivo	Mechanical unloading	Short time frame, limited use, no low-shear
Supine bed rest	In vivo	Mechanical unloading	Limited use, no low-shear
Head down tilt bedrest	In vivo	Mechanical unloading, fluid shift	Limited use, no low-shear
Hind limb suspension	In vivo	Mechanical unloading, fluid shift	Limited use, no low-shear, not suitable for human studies

1.2.4 *In Vitro* Microgravity Analogues

Random positioning machines (RPMs) reposition samples upon two axes (x and y) so that shear forces are created away from the centre of the device where the samples are present. Over long periods of time, the net influence of gravity is said to be zeroed. This occurs when the RPM is used as a clinostat. For use on cells, the RPM works similar to an RCCS but spins on two axes, causing 'tumbling' of the cells, which are three dimensional orbits. The benefit of the RPM is that due to its set up you can insert different samples as opposed to just cells in media. For example, some RPMs can hold multi-well plates or bioreactors so they are subjected to the low-shear environment generated.

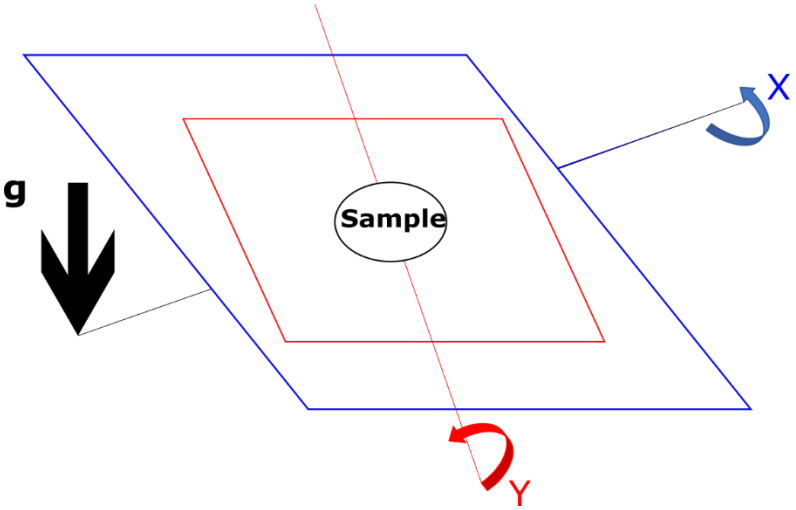


Figure 1.2.4.1: A schematic of how the Random Positioning Machine (RPM) simulates microgravity. This schematic is based upon the illustration from Wuest et al, 2017 [120]. The sample in the centre of the device is constantly repositioned both in the direction of the x axis and the y axis, giving an overall net gravity effect of zero [119].

Parabolic flights are one of the commercially accessible options for microgravity research and are also accessible to research institutes through campaigns such as 'Fly Your Thesis' run by the European Space Association (ESA). Parabolic flights involve an aeroplane that climbs to a desired height

and then flies in parabolic arcs causing the gravity experienced to cycle between 1 *g* to 1.8 *g* to 0 *g* to 1.8 *g* and finally back to 1 *g* with 30 seconds of 0 *g* being experienced between each cycle. Depending on flight plan, the experiment is subjected to multiple parabolas and periods of 0 *g*, each lasting around 30 seconds. There are two major issues with his method: To begin with, the 0 *g* experienced is not constant as it is interposed with 1.8 *g* 'hyper' gravity periods. Therefore, any changes observed over the course of the flight are gravi-sensitive changes and not necessarily microgravity changes. The other issue is experiment duration. The maximum experiment duration time is determined by the maximum flight time of the aircraft, which can make it difficult to justify for biologicy-related experiments. Therefore, this method is applied for more short-term investigations and a typical total parabolic flight is 30 to 60 minutes.

A very short form of 0 *g* analogous culture is the use of drop towers. These involve dropping samples from a height inside a capsule within an evacuated column (tower), and more recently catapulting them to the top of the tower to drop back down for extended time of microgravity culture, resulting in the contents of the capsule being in an inertial frame of reference, or perceiving to be weightless due to the acceleration of the capsule being the same as the acceleration of gravity for the period. The main issue with this method is the duration of a drop within is in the range of seconds. The 146 m Bremen drop tower, for example, provides 4.74 seconds of freefall when the sample is dropped, and 9.3 seconds when operated in the catapult mode. The small period of freefall makes the use of drop towers virtually redundant for biological studies.

Diamagnetic levitation can be used to levitate small biological samples and even whole small organisms. Diamagnetic levitation replies on the repulsion of diamagnetic substances to a magnetic field. In the case of biological experiments, it is the diamagnetic properties of water that makes this possible. The main issue with diamagnetic levitation is that the magnetic field increases convection due to the non-uniformity of the magnetic field. This

increase in convection, which is not present in true microgravity, has been shown to increase oxygen availability in cultures and affect the outcomes such as bacterial growth [121].

1.2.5 *In Vivo* Microgravity Analogues

So far, all these analogues focus on in vitro style experiments with focus on small cell cultures. Methods have been developed and tested to simulate the weightlessness and/or immobilisation caused by spaceflight microgravity on whole organisms, including humans. There are five commonly used analogues for this which are: dry immersion, wet immersion, supine bed rest, unilateral lower extremity limb suspension, head down tilt bed rest [122].

Dry immersion simulates microgravity induced factors such as lack of support, mechanical and axial unloading and physical inactivity and has been used in studies ranging from a few hours up to 56 days to simulate the effects of short term spaceflight [123]. Dry immersion supports subjects without pressure points to simulate the weightlessness experienced in spaceflight. This is done inside a bathtub where the participant lays on a hydro-isolating elastic fabric that is then submerged into the bathtub. This fabric then folds around the participant, usually up to their neck, enveloping the participant in a zero-buoyancy environment. This is due to the elasticity of the fabric [124].

Wet immersion (also referred to as water immersion), where the subject floats, is similar in effect to dry immersion, although due to how water immersion can induce subacute dermatitis starting from 72 hours, is restricted to short-term studies [122].

Supine bed rest is long term bed rest with the participant facing upwards. Although the participants are subject to the forces of gravity still, the physical inactivity and movement of the participant helps to simulate muscle and bone loss that can be induced in an accelerated rate by the microgravity environment. Head down tilt (HDT) is similar to this, but with the addition that the participants are tilted negatively in the upper body direction at 6

degrees [125]. This tilt induces a fluid-shift towards the head of a degree similar to that seen in spaceflight, and is beneficial for studies into neuro-ocular phenomenon such as visual impairment and intracranial pressure syndrome, increased intracranial pressure and spaceflight associated neuro-ocular syndrome (known commonly as space blindness) [126].

Finally, unilateral lower extremity limb suspension, or hind-limb suspension, is the suspension of the lower limbs of the body to immobilise them and induce an upwards fluid shift as seen in true microgravity. Hind limb suspension is a NASA developed microgravity analogue usually used in rodents by suspending them by the tail to impart mechanical unloading in the hindlimbs and a cephalic fluid shift [127]. Unilateral lower limb suspension is the term when a similar method is employed on humans. This usually involves only the suspension of one leg using either crutches or a singular platform shoe [122]. However, this method only shows the effects of muscle and bone loss and does not induce the fluid shift seen in microgravity and that of HDT.

1.2.6 Comparisons with Spaceflight

The main ongoing debate with all microgravity analogues is how accurately they replicate the true microgravity environment. Ground based analogues have their benefits with their cost-effectiveness, ease of access and reusability whereas launching experiments into space is extremely resource intensive and has many logistical and safety issues, especially with biological research. However, there are few studies comparing microgravity analogues to spaceflight results [128].

Spaceflight missions, however, have become more accessible with the use of CubeSats and facilities onboard the International Space Station (ISS), such as IceCube, and organisations such as ESA run open calls for student-led University teams to send experiments into space. With the emergence of spaceflight companies such as SpaceX and Blue Origin as well, the

commercialization of space missions makes them more accessible to research institutions.

As highlighted with the RCCS and diamagnetic levitation, one factor of true spaceflight that is not replicated in ground-based analogues is the absence of convection. Convection does not occur in spaceflight microgravity as a higher density (or colder) liquid will not sink as there are no gravitational forces. As nearly all microgravity analogues require movement of culture to negate gravitational effects or induce a magnetic field which increases convection, there is culture movement induced that would not be seen in true microgravity. Thus, microgravity analogues are not true representations of microgravity and are analogous to the low-shear environment created by true microgravity but cannot replicate all its effects such as the lack of convection.

1.3 Aims

The aims of this research are to add to knowledge of immune response deficiencies by investigating the mechanisms behind the pro-inflammatory response in monocytes and how microgravity alters it. Another major investigation is based upon one of the most commonly found pathogens, *Staphylococcus aureus* in a proxy microgravity environment. Since microgravity has been reported to influence virulence and as virulence is regulated via *agr*-dependent quorum sensing, investigations were performed to assess the impact of the proxy microgravity environment on *agr* induction, the phenotypes it regulates and what factors may be altering this induction by focusing on interactions between the regulatory molecule, AIP, and its' relevant receptor, AgrC. Finally, it was investigated how these two different cell types interact with each other in an *in vitro* infection model to further understanding of bacterial virulence and subsequent immune response in microgravity.

The results of this thesis will add to pre-existing knowledge of prokaryotic and immune cell changes in microgravity and contribute to the collective effort of

the Astropharmacy/Astromedicine field of keeping astronauts safe on ever increasing duration space missions such as planned manned missions to Mars and even beyond.

Not only will this research provide benefits for space healthcare, by looking at problems from a novel viewpoint, this thesis aims to increase understanding for Earth healthcare as well by further understanding the mechanisms behind pro-inflammatory response inhibition and the colonisation and virulence changes that can occur in *S. aureus*.

1.4 Thesis Overview

The aims set out in this thesis are achieved by first investigating alterations to monocytes. It was first established, using cytokine production results, that the microgravity set-up employed for this investigation is consistent with that published. Once confirmed, it was then investigated how microgravity culture impacts the ability of monocytes to deal with an *S. aureus* infection. Based on these results, pro-inflammatory response alterations were investigated through sequestration inhibition and nuclear factor kappa-light-chain-enhancer of activated B cells (Nf-κB) translocation alterations. Finally, it was investigated if these microgravity induced changes lead to increased bacterial invasion of monocytes due to the pro-inflammatory response alterations.

Monocyte co-culture with bacteria requires a medium that can both facilitate monocyte and bacteria culture. The purpose of Chapter 3 is to address this. This is achieved by investigating multiple cell culture media to justify and rationalise why the co-culture media used in the previous chapter was selected. This chapter focuses on *S. aureus* growth, protein production and activation of the *agr* operon, which is the main focus of the following results chapter. This optimisation chapter also investigated the importance of culture movement and effects of gas exchange on the bacterial cultures to determine a suitable 'normal' gravity control culture for the *S. aureus* investigations by

looking at the same tested factors used in determining the correct culture media.

Chapter 4 focuses on microgravity alterations to *S. aureus* phenotype. This revolves around cell colonisation factor and virulence factor production, of which both factors are regulated by the *agr-dependent* quorum sensing. Alterations to *agr* operon activation with specific focus on AgrC-AIP interactions will then be investigated and conclusions made based upon this.

The purpose of Chapter 5 is to bring together all the previous results chapters and focus on co-cultures between the *S. aureus* and monocytes. Building upon the co-culture results of Chapter 2 and the microgravity induced alterations to bacterial invasiveness of monocytes, it was investigated how the microgravity induced alterations to the *agr* operon activation and quorum sensing may also contribute towards this. This was done through manual counts of internal and external *S. aureus* to the monocytes and confirmed through flow cytometry and confocal microscopy. The impact of *agr* was further investigated through the use of *agr* mutants in co-culture.

The thesis concludes by drawing together and discussing common findings, such as how both monocytes and *S. aureus* have upregulated and down regulated responses in microgravity proxy culture, and how they may synergistically work together. This final chapter also addresses the limitations of the work and suggests future investigations.

The aims set out in this thesis are set out to investigate the following three key hypotheses. The first hypothesis is that microgravity induced changes to cytokine production in monocytes will result in reduced bacterial clearance by said monocytes. The second main hypothesis is that the reported changes in virulence and colonisation factor expression of *Staphylococcus aureus* in microgravity are due to alterations to the *agr* quorum sensing system. The final main hypothesis to be investigated is that microgravity induced changes to monocytes and *S. aureus* will work synergistically together to result in increased invasion of *S. aureus* into the monocytes in a co-culture model.

2. Pro-inflammatory response and ability of monocytes to clear bacterial infection in microgravity

2.1 Introduction

Immune dysfunction has long been reported in astronauts [32, 119]. With longer planned missions such as landing humans on Mars and potential deep space exploration, this observed immune deficiency is a real cause for concern and is thought to at least in part explain why astronauts are more susceptible to opportunistic infections [48]. The ability of immune cells to recognise pathogens is vital in triggering an appropriate immune response to prevent infection [129]. Endotoxins such as lipopolysaccharide (LPS) are expressed on the surface of Gram-negative bacteria [130] such as *Escherichia coli* (*E. coli*) and are the major component responsible for innate immune cell activation [130]. These endotoxins are recognised by toll-like receptors (TLRs) on the surface of innate immune cells such as monocytes and macrophages [131]. Once recognised, these TLRs activate the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) signalling pathway [132] which causes NF-κB to translocate from the cytoplasm of the cell into the nucleus [133]. After translocating, NF-κB binds to promoter regions of the DNA and activates various responses such as cell survival and the pro-inflammatory cytokine production [134].

Given its important role in regulating cell responses to different stimuli, there is substantial interest in better understating the role of NF-κB in regulating cell responses in microgravity [135]. Figure 2.1.1 shows how NF-κB translocation occurs and activates the response genes. However, results have been inconsistent across different cell types [135]. Microgravity analogue cultured RAW264.7 cells showed increased p65 protein levels, one of the two subunits that forms a heterodimer which binds to DNA and is responsible for

the strong transcription activating potential of NF-κB [136], and DNA binding activity [137] along with rat cardiac cells which also showed increased p65 DNA binding activity [138]. Interestingly, human Jurkat T cells cultured in a microgravity analogue environment showed decreased translocation of the p65 protein [139], and downregulated NF-κB gene pathway expression has been reported in space-flown and simulated microgravity cultured macrophages [140]. The p65 subunit is one of five NF-κB signalling subunits and forms a heterodimer with the p50 subunit in the canonical NF-κb signalling pathway[141]. Canonical NF-κb signalling (Fig. 2.1.1) is the most widely studied activation pathway in microgravity research as this is the pathway activated by external stimuli, such as bacterial surface proteins and endotoxins (LPS) or cytokines (TNFα and IL-1), binding to their relevant receptors[142]. This regulates many pro-inflammatory gene expressions and is well known as a mediator of the overall pro-inflammatory response[143].

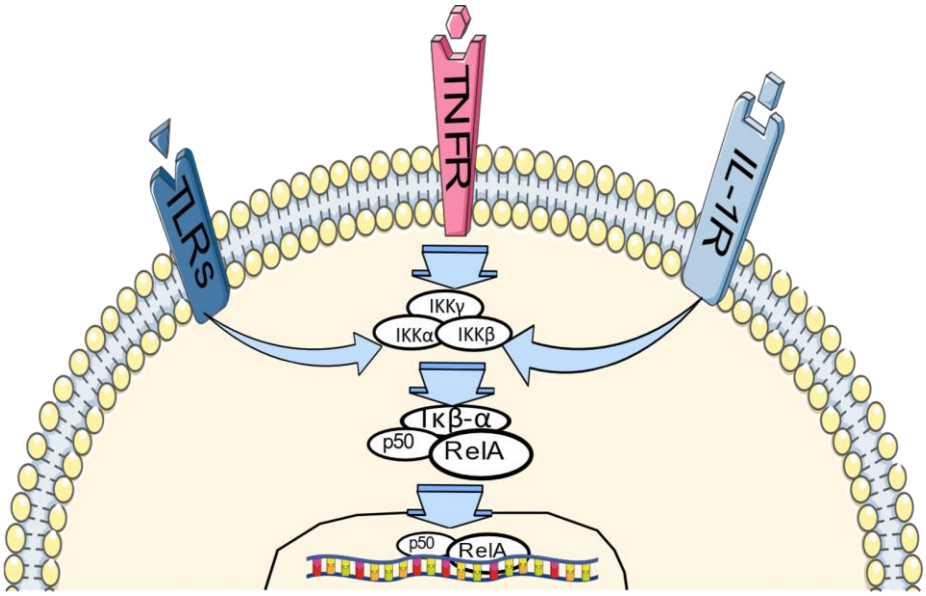


Figure 2.1.1: Activation of NF-κB translocation and subsequent gene transcription from external stimuli. Stimuli external to the cell are recognized by membrane bound cell surface receptors. This recognition then triggers a phosphorylation cascade and translocation of the p50 subunit of the NF-κB complex to travel from the cytoplasm of the cell into the nucleus and bind to the transcription site on the nuclear DNA. This causes transcription of the response genes such as those in the pro-inflammatory response. This is referred to as the canonical activation pathway.

Cytokine production in spaceflight has also been reported to be altered. TNFα expression in mouse bone marrow derived macrophages has shown to be suppressed in rotating cell culture system (RCCS) culture when stimulated with bacterial LPS [144]. This corroborates with short term spaceflight results of whole blood stimulated with LPS in which monocytes showed decreased expression of TNFα along with IL-6 and IL-10 [145]. Additionally, analysis of various spaceflight samples across multiple investigations and missions shows dysregulation of immune signalling[146-150]. However, like NF-κB studies, published results contradict each other. Other studies have shown that TNFα secretion varies based upon the stimulant used and can be increased in simulated microgravity and varies between donors [151].

The aim of this study is to investigate how LSMMG culture affects the ability of human monocytes to respond to bacterial infection using LPS and *Staphylococcus aureus* as surrogates for infection with Gram negative and Gram positive bacteria.

2.2 Methods

Cell Culture:

Peripheral blood mononuclear cells (PBMCs) were isolated from the buffy coat (a separated fraction of the donor blood after centrifugation that contains the white blood cells and platelets) of donated human blood from healthy donors. The buffy coats were obtained from healthy donors (National Blood Service, UK) after obtaining informed written consent and following ethics committee approval (Research Ethics Committee, Faculty of Medicine and Health Sciences, University of Nottingham). Monocytes were isolated to >90% purity using Miltenyi Biotec positive monocytes isolation kit as previously described [152, 153]. Monocytes were cultured in RPMI-1640 supplemented with 10% FBS, 1% pen/strep and 1% L-glutamine at 37^{o} C in 5% CO_2 at a cell density of $1x10^6$ cells per mL. For co-culture studies with bacteria monocytes were cultured in X-Vivo 15 serum free media.

Co-Culture:

Staphylococcus aureus strain USA300 JE2 was used for co-culture manual count studies and cultured in X-Vivo 15 serum free media at 37^{o} C in 5% CO_2. *S. aureus* Xen29 was also cultured in X-Vivo 15 serum free media at 37^{o} C in 5% CO_2. Xen29 was added to monocytes for 4 h at a 20:1 ratio and 200 µL of co-culture was put into a 96 well black chimney plate in a TECAN plate and luminescence readings were taken of each well.

Manual Counts of viable *S. aureus*:

Co-culture was serially diluted by a factor of 10 six times. Ten microlitres of this dilution was added in triplicate to a trypticase soy agar (TSA) plate and incubated at 37^{o}C overnight. The lowest dilution in which individual colonies could be visualised for all three replicates was counted and multiplied by the dilution factor to calculate colony forming units per millilitre (CFU/mL).

For intracellular counts of viable *S. aureus*, Gentamicin was added to co-culture after 4 h at 50 µg/mL for 1 h. Cells were then centrifuged at 13000

RPM and supernatant removed. Cells were then resuspended in 1mL PBS and 1% Triton-X-100 was added for 15 min. This was then centrifuged for 1 min at 13000 RPM and pellet resuspended in 1 mL PBS. Previous manual count method was then followed.

Monocyte Stimulation:

Cells were cultured for 24 h before stimulation. Static cells were cultured in ultra-low attachment plates. Low-shear environment cells were cultured in 2mL vessels on a rotating cell culture system (RCCS) at 10 RPM. After 24 hours, cells were stimulated with lipopolysaccharide from E. coli (O111:B4) at either 10 ng/mL or 1 µg/ml.

Monocyte Staining:

Cells were stained using the NF-κB translocation kit (Luminex Corp.). Cells were permeabilised and NF-κβ stained with Alexafluor 488 and the nucleus with 7-AAD following manufacturer's instructions. Samples were fixed in 4% Paraformaldehyde at 4^{o}C.

For intracellular TNFα flow cytometry, cells were permeabilised and stained with TNFα-PerCP antibody (ThermoFisher). A positive control was created by blocking protein secretion with brefeldin A (BioLegend). Samples were fixed in 4% Paraformaldehyde at 4^{o}C.

Flow cytometry:

Monocytes stained for NF-κB were run on an Imagestream (Luminex corporation) flow cytometer for 1000 events per sample. Cells were gated by forward and side scatter analysis to eliminate non-singular cells. Unstained controls were used for reference to gate for positive staining. This data was then analysed with IDEAS software using the nuclear localisation wizard.

Cytokine quantification:

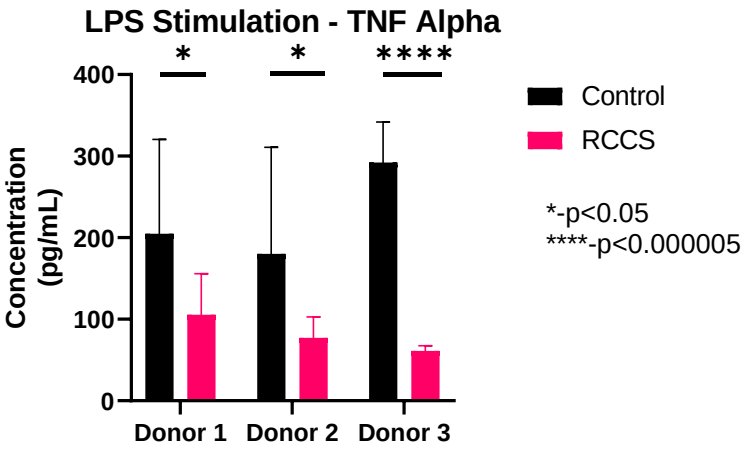
Supernatant from each monocyte stimulation was collected via centrifugation (350 xg) and stored at -20^{o}C. Supernatant was then analysed via ELISA

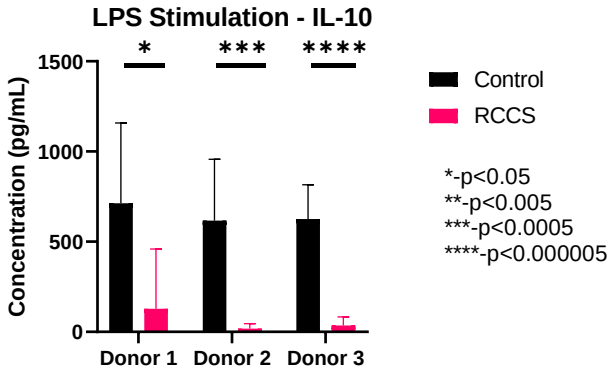
(enzyme-linked immunosorbent assay) on a 384 well plate at 450 nm and 560 nm absorbance on a plate reader.

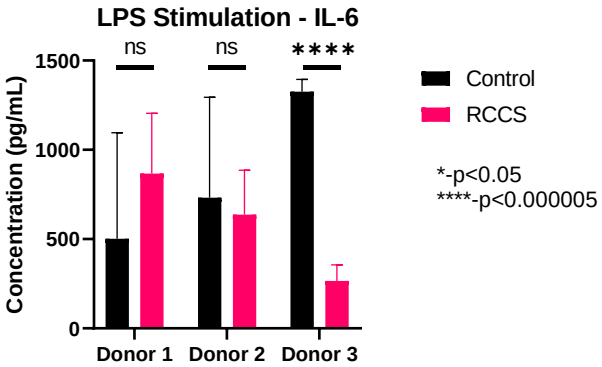
For intra cellular TNFα, samples were run on a FACS Canto II and data analysed using Kaluza software [153, 154].

2.3 Results

First, to confirm the microgravity culture set-up was consistent with results published in the literature [144, 145, 155], known cytokine responses from LPS stimulated monocytes and macrophages were compared with this system to validate the use.







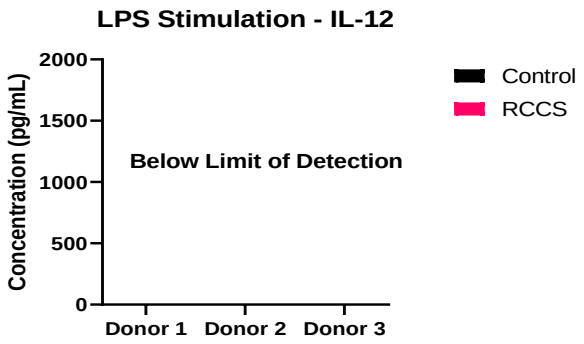
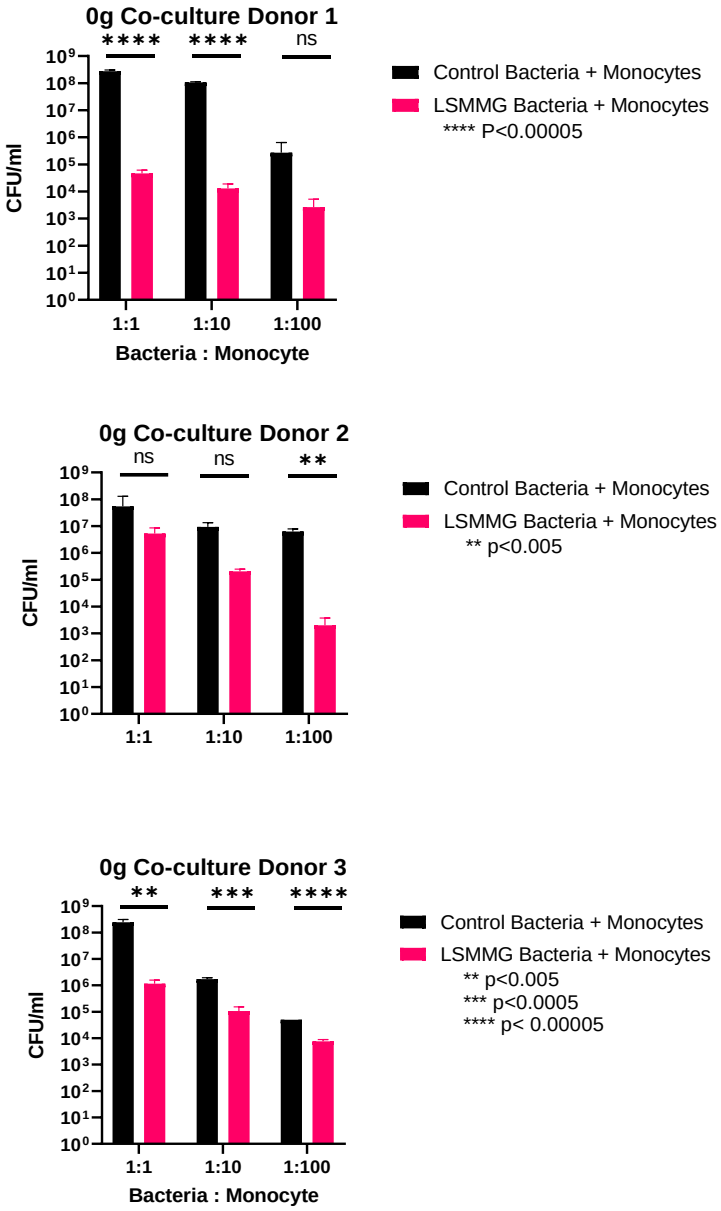


Figure 2.3.1: Cytokine production in LPS (*E.* coli) stimulated monocytes. Monocytes were cultured in an RCCS in either LSMMG orientation (RCCS) or a 'normal' gravity orientation (Control). LPS stimulation was applied for 24 hours. Supernatants were taken and cytokine concentrations quantified via ELISA. t-tests were performed using GraphPad for statistical significance. For each donor, n=3. Error bars represent the standard deviation of the mean.

Figure 2.3.1 shows that for TNFα and IL-10 there is a significant reduction in cytokine production in LSMMG culture compared with control culture. IL-6 also shows this reduction for donor 3 but is inconclusive for the other two donors. IL-12 was below the limit of detection for this assay. Cell viability was confirmed via Trypan Blue analysis to confirm cytokine production changes were not due to cell death being induced (Appendix).

As these results are consistent with what has been reported, it was confirmed that the LSMMG set-up was consistent with that used in the literature. To test the hypothesis that this reduced cytokine production leads to decreased bacterial clearance, USA300 JE2 (CA-MRSA, *Staphylococcus aureus*) was co-cultured with monocytes and the colony forming units were manually counted after 4 h of co-culture. USA300 JE2 is a community acquired methicillin resistant *S. aureus. S. aureus* classifications are later discussed in Chapter 5. This was also confirmed using a luminescent reporter strain Xen29, for all three donors. As Xen29 has a *lux* cassette on a constitutive promoter, light measured from the co-culture could be used to show bacterial cell viability.


0g Co-culture Donor 1
****
****
ns
CFU/ml
1:1
1:10
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Bacteria : Monocyte
Control Bacteria + Monocytes
LSMMG Bacteria + Monocytes
**** P<0.00005
0g Co-culture Donor 2
ns
ns
**
CFU/ml
1:1
1:10
1:100
Control Bacteria + Monocytes
LSMMG Bacteria + Monocytes
** p<0.005
0g Co-culture Donor 3
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CFU/ml
1:1
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1:100
Bacteria : Monocyte
Control Bacteria + Monocytes
LSMMG Bacteria + Monocytes
** p<0.005
*** p<0.0005
**** p< 0.00005

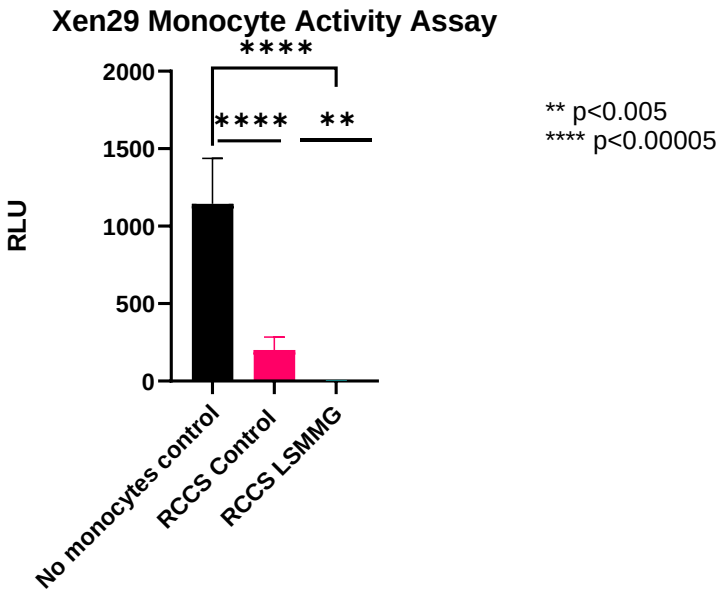


Figure 2.3.2: Co-culture assays investigating ability of monocytes to clear *S. aureus* infection. USA300 was co-cultured at three different multiplicities of infection with monocytes from three different donors for 4 hours. After 4 h, co-cultures were plated for 24 hours and colony forming units manually counted. (n=3). For Xen29 assay, Xen29 was co-cultured with monocytes at a 20:1 ratio for 4 h. After 4 h, light output from Xen29 was quantified using a TECAN plate reader. Independent t-test was performed using GraphPad on manual count samples. ANOVA analysis was performed using GraphPad on Xen29 assay (N=3, n=3). Error bars represent standard deviation of the mean.

Figure 2.3.2 shows that the manual counts for all three donors show a reduction in *S. aureus* counts in LSMMG culture compared with control culture. This finding is confirmed by the Xen29 assay in which control culture has measurable luminescence, and hence live Xen29, compared with LSMMG culture in which no luminescence is emitted.

Intriguingly, the data from Figure 2.3.2 shows that the decreased cytokine production reported in Figure 2.3.1 did not lead to decreased bacterial clearance as an increase in bacterial number was observed as opposed to a decrease. The next step was to confirm decreased pro-inflammatory cytokine

production was occurring in this co-culture due to the differing nature of stimulation *S. aureus* provides in comparison with LPS from *E. coli* to rule out differing cytokine production changes. This is due to LPS stimulating toll-like receptor 4 and *S. aureus* stimulating toll-like receptor 2 and 6. IL-10 (anti-inflammatory) was also included for comparison across the stimuli

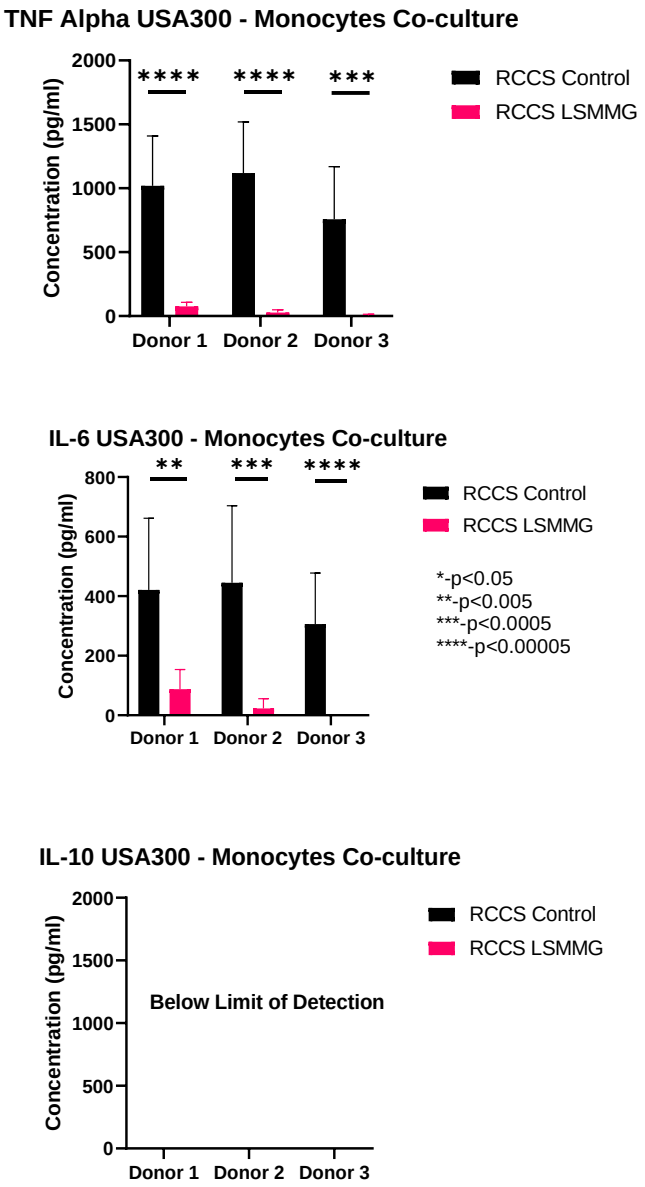


Figure 2.3.3: Cytokine production of monocytes co-cultured with USA300 for 4 hours at an MOI of 20. *S. aureus* (USA300) was co-cultured with monocytes at a 20:1 ratio for 4 h. Supernatants were collected and cytokines were quantified via ELISA analysis. Independent t-tests were performed using GraphPad for statistical significance. For each donor, n=3. Error bars represent standard deviation of the mean.

Figure 2.3.3 shows that in LSMMG co-culture, there is a significant reduction in TNFα and IL-6 production in comparison with control co-culture. For IL-10, both LSMMG and control co-culture was below the limit of detection.

As cytokine production was still reduced in LSMMG culture but bacterial clearance was not, it was confirmed that the reduced cytokine production in LSMMG does not lead to decreased bacterial clearance by monocytes. To better understand the changes to cytokine production, with focus on the main pro-inflammatory cytokine TNFα, it was investigated whether there was an inhibition of secretion of TNFα in the stimulated monocytes. This was to begin to determine if the pro-inflammatory response was being activated in the stimulated monocytes and that the reduced supernatant cytokine was not due to decreased pro-inflammatory activation, but an inhibition of secretion.

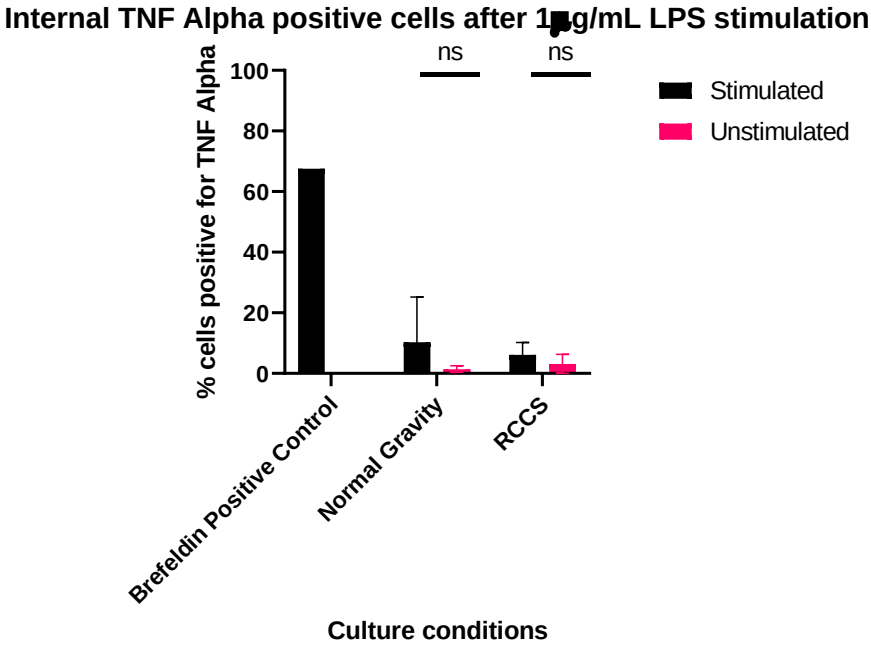
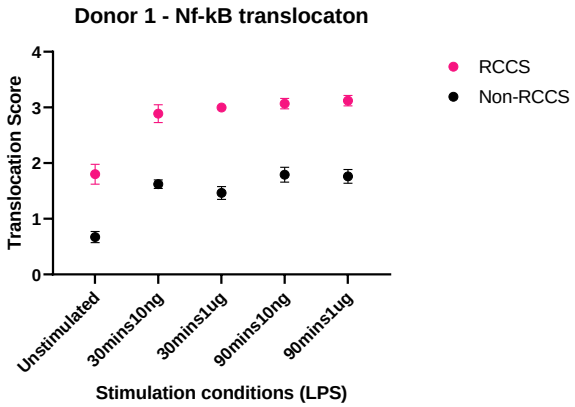


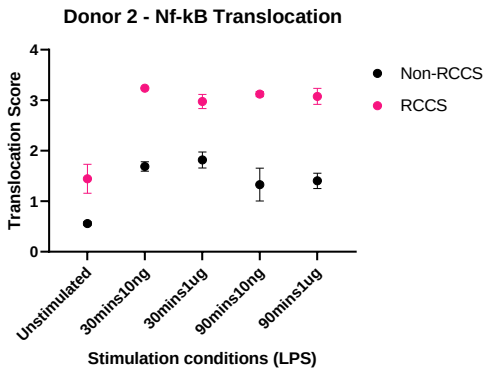
Figure 2.3.4: Primary monocytes stimulated with 1 µg/mL LPS for 90 minutes and stained for intracellular TNFα and analysed via flow cytometry. Brefeldin A was used as a positive control as this prevents secretion of internal proteins. Independent t-test was performed on 'Normal Gravity' and 'RCCS' samples using GraphPad. N=3, n=3. Error bars represent standard deviation of the mean.

Figure 2.3.4 shows that both 'normal' gravity and RCCS culture have significantly fewer positive cells than the brefeldin positive control and

therefore are not inhibiting secretion of cytokines. Both 'normal' gravity and RCCS conditions show an increase between stimulated and unstimulated conditions meaning there is a slight increase in intracellular cytokine. However, there are no significant differences between 'normal' gravity culture and RCCS culture for both stimulation conditions.

As the reason for the decreased cytokine production is not due to inhibition of secretion, the next investigation was to see if there were any alterations to the activation of the pro-inflammatory response. To do this, changes to NF-κB translocation at different timepoints and stimulation conditions were investigated to look for any LSMMG induced changes at both 'high' and 'low' stimulation conditions. Again, cytokine production reduction was confirmed for these conditions.





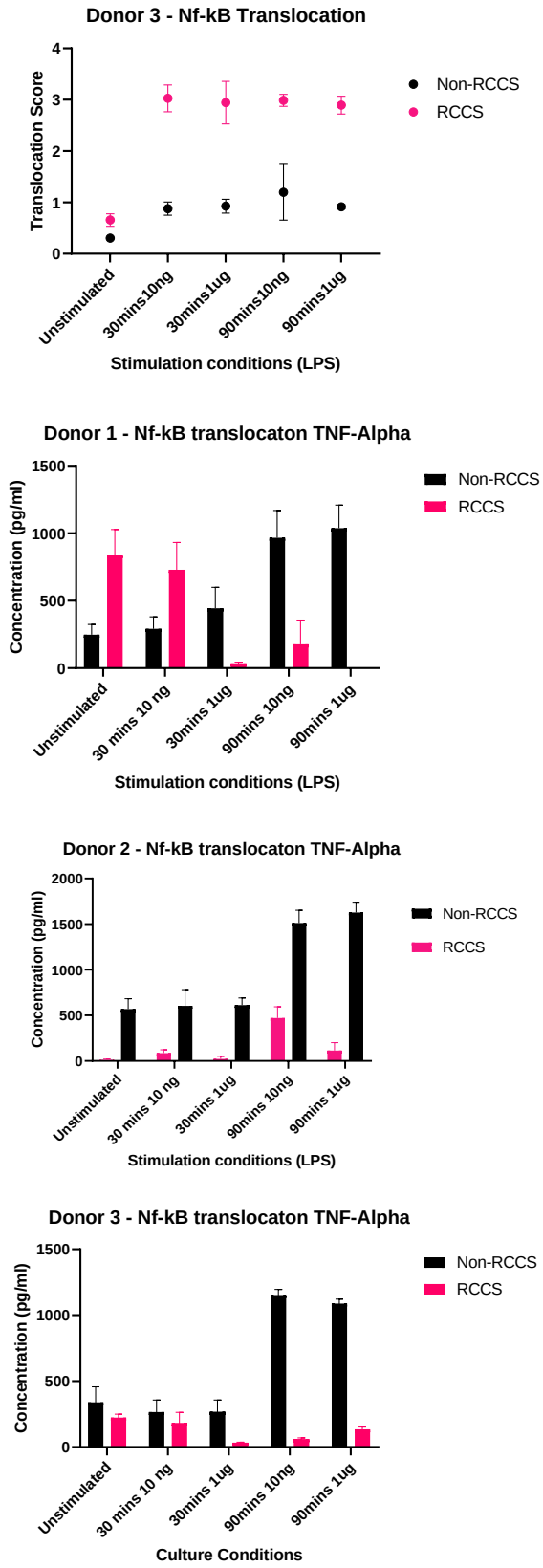


Figure 2.3.5: Nf-κb translocation for LPS stimulated primary monocytes from three unique donors. For each donor n=3. TNFα ELISA for LPS stimulated

primary monocytes from three unique donors. For each donor n=9. Error bars represent +/- standard deviation of the mean.

Figure 2.3.5 shows a clear increase in NF-κB translocation in both unstimulated and stimulated conditions for LSMMG culture in comparison with control culture. It is once again shown that for these conditions, there is an LSMMG induced TNFα production reduction.

It is clear that in LSMMG culture there is an altered pro-inflammatory response. This pro-inflammatory response may lead to changes in bacterial clearance and may not have been discovered by the investigation in Figure 2.3.1. Based upon this, it was hypothesised that there may in fact still be decreased bacterial clearance in LSMMG culture, it is just that the bacteria are invading the monocytes due to the decreased cytokine production. To test this, manual counts from Figure 2.3.2 were repeated but the external bacteria were removed and the monocytes lysed to count the intracellular bacteria.

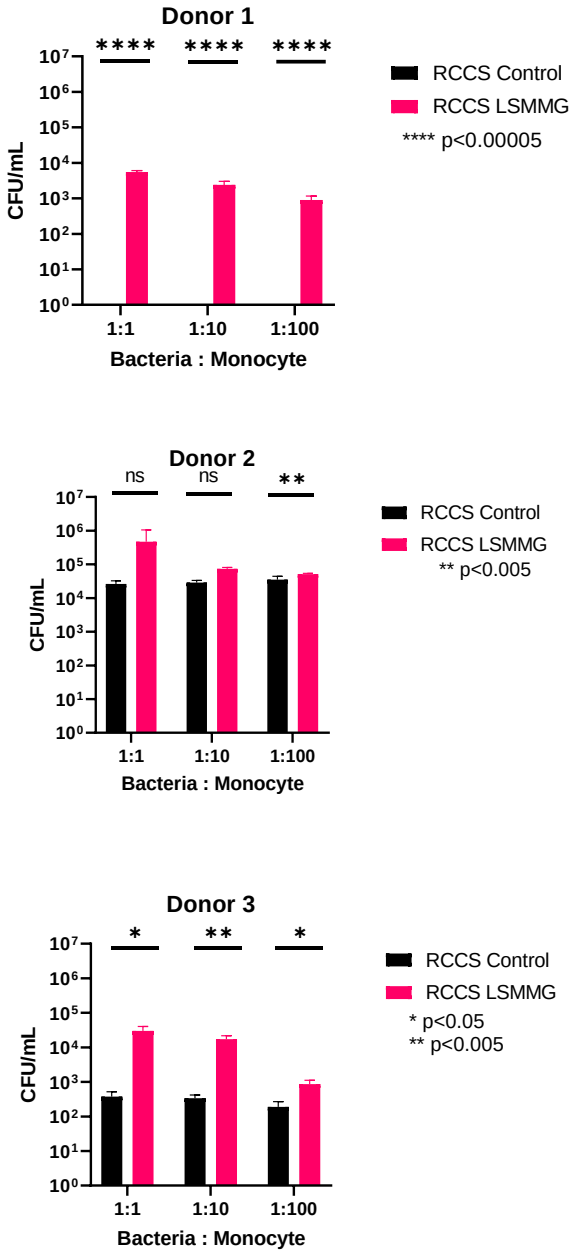


Figure 2.3.6: Manual count of USA300 internalized inside monocytes from 4 hours co-culture in LSMMG conditions. Both monocytes and USA300 were pre-cultured in LSMMG conditions for 24 hours. Afterwards, they were co-cultured at three different multiplicities of infection. Co-cultures were then subjected to gentamicin to kill extracellular *S. aureus* and lysed so only intracellular *S. aureus* remained. These remaining bacteria were plated for 24 h and colony forming units counted. Independent t-test was performed using GraphPad. (n=3). Error bars represent standard deviation of the mean.

Figure 2.3.6 shows that for all three donors for at least one MOI, there is an increase in bacteria in LSMMG culture inside the co-cultured monocytes. This result shows that the decreased 'external' bacteria counts in LSMMG culture was not due to increased clearance (killing/phagocytosis) by monocytes, but was in fact due to increased invasion by the bacteria inside the monocytes causing a decreased number of external bacteria that lead to the initial rejection of the hypothesis that bacterial clearance is reduced in LSMMG culture.

2.4 Discussion

TNFα is the main effector cytokine of the pro-inflammatory response to bacterial factors such as LPS stimulation. As this cytokine has been investigated already in microgravity culture, this cytokine could be used to check the validity of the microgravity set-up [144, 155]. The inhibition of this cytokine production in the RCCS suggests an abnormal pro-inflammatory response to LPS stimulation and that RCCS culture may be causing inhibition of the pro-inflammatory response. The simplest explanation for this phenomenon is that there is decreased cell viability in the RCCS leading to decreased cytokine production. However, it is well reported in literature that cell viability in microgravity culture is unaffected in the timeframes used in this investigation [119, 156, 157] which is in line with data supplied in the Appendix. Preliminary experiments were performed on monocytes cultured for 24 hours in the RCCS and showed that there were no changes in cell viability in comparison with standard culture controls. IL-6 was also included in this initial cytokine investigation as it is another major effector of the pro-inflammatory response. However, as shown, these results were inconclusive.

Cytokines work synergistically with each other and can influence each other's production. IL-12 is known to influence the production of TNFα [158] and therefore LSMMG induced changes to this cytokine could cause changes to TNFα production. This possibility was quickly rejected with IL-12 production levels being negligible. As LPS stimulation activates toll-like receptor 4 (TLR4), it also activates the production of the anti-inflammatory cytokine IL-10. When activated, monocytes can differentiate into either pro-inflammatory or anti-inflammatory subsets [159], therefore IL-10, an anti-inflammatory cytokine, was also measured to see if LSMMG culture induces changes in this and begin to highlight if the cytokine reduction in LSMMG culture is pro-inflammatory specific or spans other responses. IL-10 has been shown to have increased LSMMG production in M1 and M2 macrophages but not in M0 macrophages [155] which when combined with our LSMMG induced reduction in monocyte finding, highlights a potentially significant pattern. Monocytes differentiate

into M0 macrophages and these then differentiate into M1 and M2 macrophages. Following this from an LSMMG cytokine production perspective, this goes from reduction to neutral to increase along the differentiation pathway. Further investigation could be undertaken to see if this pattern holds true and what mechanisms are behind it.

From the observed reduction in cytokine production, it was hypothesised that this reduced cytokine production is representative of a reduced pro-inflammatory response and therefore the monocytes would have reduced ability to clear a bacterial infection. *Staphylococcus aureus* was chosen for the bacterial co-culture due to its prevalence aboard space vessels, highlighting how during space missions it will be a common pathogen that the immune system needs to respond to [119]. USA300 was the specific strain chosen as it is a community acquired 'hyper'-virulent strain of *S. aureus* in which it expresses a large amount of virulence factors leading to easier recognition by the immune system than a more colonising strain of *S. aureus* [160-163]. Based upon the hypothesis, an increased amount of colony forming units from the manual count and an increase in relative light units (RLU) from the Xen29 assay in LSMMG culture compared with control culture was expected.

As this was not the case and there was an actual decrease in both manual counts and RLU in LSMMG culture, the hypothesis was initially rejected as both assays indicate increased bacterial survival and a decreased bacterial clearance by the monocytes. Macrophages, a derivative of monocytes, have been reported to have reduced ROS production which again would be indicative of a reduced pro-inflammatory response and reduced bacterial clearance [164].

As Figure 2.3.1 and Figure 2.3.2 used two different stimulation methods, confirmation of LSMMG induced cytokine reduction for the co-culture with *S. aureus* was needed. LPS from *E. coli*., a Gram-negative bacterium, was used for the initial stimulation and as *S. aureus* is a Gram-positive bacterium, it stimulates the monocytes through different pathways [165, 166] and therefore there may be differing cytokine responses. Once more, TNFα

production was suppressed in LSMMG culture and there was now a clear IL-6 suppression also which was not apparent in the LPS stimulated monocytes. Due to the different stimulation mechanisms, the anti-inflammatory IL-10 was not stimulated with this co-culture and no observations could be made on this. LPS from the Gram-negative *E. coli* stimulates the cells via TLR4 whereas *S. aureus* stimulates the monocytes through TLR2 which recruits TLR1 and TLR6 to recognise a more diverse array of pathogen associated recognition molecules[167]. This confirmed that the initial hypothesis that microgravity induced cytokine reduction would lead to decreased bacterial clearance could be rejected. As stimulation of monocytes with both a Gram-positive and Gram-negative has been performed, a universal response in pro-inflammatory cytokine production inhibition in LSMMG culture to bacterial stimulation irrespective of Gram-type has been potentially highlighted. This would need confirmation with co-culture with *E. coli* and potentially a diverse array of other bacterial species before this claim can be conclusively made. It should be noted however, Gram negative infections show peak cytokine concentrations between 1-5 hours after stimulation whereas Gram positive infections show peak cytokine production approximately 50-75 hours after stimulation when planning future investigations [168].

To further investigate the inhibition of cytokine production, it was decided to clarify if the cytokine production reduction is indicative of a reduced pro-inflammatory response. One hypothesis for the reduction in cytokine production is an LSMMG induced inhibition of secretion. This hypothesis is based upon the findings that cells have altered cell membrane permeability in microgravity conditions [169] and therefore biological processes such as protein secretion may be altered. Brefeldin A treated monocytes were used as a positive control for this study and a representation of no cytokine secretion as it prevents transport of proteins from the endoplasmic reticulum to the Golgi apparatus and therefore no protein secretion can occur. As there was no differences in internalised TNFα between LSMMG and control culture conditions, it was determined that secretion was not being inhibited. This is

the first time it has been shown that intracellular TNFα levels in LPS stimulated monocytes do not vary between LSMMG and normal culture conditions.

As secretion has now been shown to not been inhibited, it was decided to look at the activation of the pro-inflammatory response itself. The NF-κB pathway is the main activation pathway for classical activation of the pro-inflammatory response from stimuli binding to cell surface receptors. Based upon this, it was hypothesised that there would be decreased translocation of NF-κB from the cytoplasm to the nucleus, leading to decreased pro-inflammatory response activation, in LSMMG culture compared with control culture when stimulated with LPS. It was also important to investigate if LSMMG culture irrespective of stimulation affected NF-κB translocation to see if the LSMMG environment was triggering any monocyte responses regulated by NF-κB.

A potentially significant finding was made when observing the unstimulated conditions. Without any stimulation, NF-κB has increased translocation in LSMMG conditions. This potentially indicates that LSMMG culture was causing activation of a NF-κB regulated response without any stimuli. The increased translocation may indicate the potential gravi-sensitive nature of NF-κB translocation and activation of the regulated responses and that the lack of force in LSMMG conditions may activate some form of stress response in the monocytes.

Various investigations in LSMMG culture have been done on immune cells (including monocytes)and other cells such as cardiac cells and bone cells on NF-κB translocation with varying results showing increases and decreases compared with a control culture [52, 135, 137-139, 156]. The cause of the differing responses between cell types can only be speculated upon. Across all the investigations, microgravity exposure time varied from 5 minutes to 12 days. NF-κB analysis was also performed by different techniques such as RT-PCR or western blot. Without a ‘gold-standard’ methodology, these differences could be expected and may offer an explanation as to the

diversity of the results. By using the same methodology and quantification as this investigation, future investigations can compare results across cell types to identify any changes to NF-κB translocation in microgravity culture.

The NF-κB translocation data shows that there is an altered LSMMG induced response to this translocation and potentially the responses it regulates. The increased NF-κB translocation may not be indicative of an increased pro-inflammatory response as this complex also regulates other cellular responses. As the RCCS keeps cells in their own suspended micro-orbit, they are unable to attach to a surface and undergo the adhesion interactions (unless adhesion scaffolds/surfaces are added such as polymer beads). NF-κB regulates expression of adhesion molecules such as ICAM-1 (intercellular adhesion molecule 1) and VCAM-1 (vascular cell adhesion molecule 1) [170]. Due to this, it can be theorised that the increased NF-κB translocation could be due to increased expression of adhesion molecules to try and counteract the lack of attachment. This theory would require extensive research and would be its' own standalone investigation. An initial starting point would be to repeat this investigation with the cells either pre-attached to polymer beads or have polymer beads present in the culture to offer an attachment surface. There are positive foundations to build on due to research into rat immune cells finding increased NF-κB activity with increased expression of cell surface adhesion proteins [171-173].

The ImageStream analysis only tracks the p50/p65 heterodimer, which is the main activator of the pro-inflammatory response. The NF-κB complex consists of three other subunits giving a total of five potential subunits to investigate. The ratios in which these subunits translocate to the nucleus of the cell can alter the cellular response they regulate/activate [174]. Therefore, even though the p50/p65 is increased in translocation in LSMMG culture which can lead to an increased pro-inflammatory response, the other three subunits may undergo altered translocation suppressing this activation and/or activating other NF-κB regulated responses. This could be tested by using the ImageStream to also track the translocation of these subunits as well or

directly testing the other responses NF-κB regulates such as cell surface attachment protein expression via PCR or attachment assays.

Finally, it was investigated that the possibility that the decreased cytokine production and altered pro-inflammatory response does in fact lead to decreased ability of monocytes to clear bacterial infection. This would result in that the decreased bacterial viability in LSMMG co-culture was not due to an increased ability of the monocytes to clear bacterial infection, but was in fact that the altered pro-inflammatory response and decreased production of pro-inflammatory cytokines led to increased invasion of the bacteria into the monocytes. This hypothesis was confirmed by the internal counts of bacteria inside the monocytes as there was a greater number in LSMMG culture than control culture. Phagocytosis in monocytes and macrophages has been reported to have decreased function in microgravity conditions with reductions in reactive oxygen species (ROS) production and decreased granulation [175, 176]. Even though this would lead to the monocytes themselves taking in less bacteria, it would lead to a decreased ability to kill the internalised bacteria and therefore increased internal numbers. This increased internal bacteria numbers are what is observed in this study. There also could be increased invasion of the bacteria due to the already discussed LSMMG induced fluidity changes to the monocyte membrane [169]. This increased internal numbers may explain the decreased external numbers as during the initial growth cycle of *S. aureus*, they begin in a colonising, more invasive phenotype and will invade the monocytes more rather than produce virulence factors to 'fight' against the monocytes and the pro-inflammatory response [177-180].

The behaviour of *S. aureus* also raises an interesting avenue of investigation. *S. aureus,* when in low cell-density, expresses colonisation factors and is more likely to invade host cells. It is when it reaches a certain cell density that it switches to a virulence factor producing phenotype and no longer expresses cell colonisation factors and destroys host cells rather than invading them [181-184]. This process is regulated through the *agr* quorum sensing system

[178, 180]. This study only focussed on potential monocyte changes induced by LSMMG culture and does not consider the potential changes to *S. aureus* induced by LSMMG culture. Quorum sensing has been shown in other species of bacteria to be gravi-sensitive [185] and if this is the case with *S. aureus,* then the LSMMG culture may also be causing the bacteria to have a more invasive phenotype than their control culture counterparts. Careful consideration must be taken though when making hypotheses involving quorum sensing as quorum sensing systems are very different from each other. This has already been hinted at with increased cell colonisation factor production reported in LSMMG cultured *S. aureus* [186] and reduced virulence factor production [87, 186] indicating possible LSMMG induced quorum sensing changes. This should be further investigated as the reduced pro-inflammatory response and phagocytic ability of monocytes and macrophages may work synergistically with potential LSMMG induced invasiveness increases in *S. aureus* causing the increased invasion and survivability of the bacteria inside the monocytes indicated by the monocyte intracellular counts. This hypothesis forms the basis of the investigation in Chapter 4 and Chapter 5.

Due to this finding, the initial rejection of the hypothesis that LSMMG culture induces a decreased pro-inflammatory response and therefore decreased bacterial clearance was wrong. The initial finding of decreased extra-cellular *S. aureus* in LSMMG co-culture was not actually indicative of decreased bacterial clearance. Once paired with the internal counts, it was shown that there is a decreased bacterial clearance in LSMMG culture as the bacteria are just invading the monocytes and potentially evading any potential immune response. This may pose substantial problems for human health in spaceflight. If the bacteria are internalised inside the monocytes, they cannot be directly detected by other immune cells through their surface pathogen recognition molecules as they are now inside the monocytes. Furthermore, the monocytes themselves cannot signal to the other immune cells that they are infected due to their suppression of cytokine production in LSMMG

culture. This could lead to long-term persistent infections as the bacteria can thrive and multiply inside the monocytes and evade the host immune response.

Key Findings:

- **Cytokine production reduction in monocytes when stimulated from both Gram-positive and Gram-negative sources in LSMMG culture**
- **Secretion inhibition of cytokines does not occur in LSMMG culture**
- **NF-κB response in stimulated and unstimulated monocytes were altered in LSMMG culture**
- ***S. aureus* were found in increased numbers intracellularly (inside monocytes) in LSMMG culture**

3. Medium and culture condition optimisation for *Staphylococcus aureus* culture and monocyte co-culture

As it has been established how the pro-inflammatory response is impacted by microgravity culture with respect to monocytes, and it was suggested that microgravity culture may be inducing changes in *Staphylococcus aureus,* it was then decided to investigate how *S. aureus* is affected by LSMMG culture and then how these two cell types interact with each other. Both cells require certain growth condition requirements and thus suitable media and growth conditions needed to be found.

3.1 Introduction

Staphylococcus aureus is a Gram-positive bacterium that grows in a coccoid shape and forms 'bunches of grapes'. Like all bacteria, they have optimal growth conditions and require certain nutrient availability for growth. Monocytes also have specific culture conditions, requiring growth factors found in serum and a 5% CO_2 environment, and like *S. aureus,* have different levels of suitability to different culture media.

The purpose of the investigations in this chapter are to find a culture media that is suitable for monocyte culture but can also facilitate *S. aureus* growth to a suitable enough level in which the *agr* operon is activated and an analysable amount of secretome is produced. Future chapters will investigate the quorum sensing regulated outcome, virulence factor production, and production of the quorum sensing molecule, autoinducing peptide (AIP) Therefore, this chapter will focus on these outcomes and how they are affected by differing culture conditions.

S. aureus grows between the temperature range of 15-45^{o}C and grows on all rich media, the most commonly used being brain heart infusion (BHI), tryptic soy agar/broth (TSA/B) and Luria Bertani (LB) agar [187]. *S. aureus* is a

facultative anaerobe, in which it can follow the aerobic or anaerobic respiration pathway depending on conditions, but grows slower anaerobically than aerobically, with anaerobic mid-log phase generation time of 80 minutes, compared to 35 minutes for aerobic conditions [188]. *S. aureus* usually grows 1 μm in diameter in a coccoid shape which clumps with other cocci during growth.

S. aureus causes a plethora of clinical infections and is the leading cause of infective endocarditis and bacteraemia, whilst also causing skin and soft tissue, osteoarticular, pleuropulmonary and device-related infections [189]. Infections are found both in clinical and community settings and can be challenging to treat due to the emergence of strains that are resistant to multiple classes of antibiotics such as MRSA [190]. *S. aureus* produces a plethora of virulence factors such as leukocidins, proteases, enterotoxins, exfoliative toxins, haemolysins and immune-modulatory factors [191].

In *S. aureus*, production of the majority of virulence factors is regulated and controlled by the quorum sensing accessory gene regulator (*agr*) system [182]. The operon has two transcription units in RNAII and RNAIII which are transcribed respectively from P2 and P3, two divergent promoters [192]. Like most Gram-positive bacteria, the activation of this operon is regulated by short peptide chains known as auto-inducing peptides (AIP) [193]. Quorum sensing, in a basic sense, is a process in which cells communicate surrounding cell density to each other causing the adjustment of gene expression [194]. Quorum sensing is investigated in depth in Chapter 4 and fully elaborated on there.

In staphylococci, and hence *S. aureus,* when high cell density is detected from increased AIP concentration, *agr* expression is altered to decrease cell colonisation factor expression and increase expression of degradative exoenzymes and toxin production [178]. Because of the role of AIP concentration in *agr* regulation, it is vital that any culture media is able to facilitate the detectable production of AIP so the effects of RCCS culture on quorum sensing can be studied in Chapter 4.

However, it should be noted that *agr* is not the only regulator of virulence and host-pathogen interactions and *S. aureus* also contains regulatory systems such as SaeRS, SrrAB, ArlRS two component systems, cytoplasmic SarA-family regulators(SarA, Rot and MgrA) and alternative sigma factors (SigB and SigH) [177]. Once more, these are further elaborated upon in Chapter 4.

For these initial studies, the strain chosen was a methicillin resistant *Staphylococcus aureus* (MRSA) strain called USA300 JE2. JE2 is a community associated MRSA (or CA-MRSA) and in the mid 2000's became the predominant cause of staphylococcal disease in the USA [161]. It has been described as 'remarkable' in its' ability to both cause disease in a community and healthcare settings [160]

USA300 is said to be a hyper-virulent strain, in comparison with other MRSA and MSSA strains [163], and isolates of USA300 are usually found to be carrying genes for arginine catabolic mobile element and Panton-Valentine leucocidin but have been rarely found to carry staphylococcal enterotoxin genes [162].

One of the main features of MRSA strains such as USA300 is their enhanced ability of acquiring antibiotic resistance genes which lead to resistances across multiple antibiotic classes [195]. This can lead to an increasing problem worldwide due to the ability of USA300 not being restricted to clinical infections and being able to cause community infections and being found in the environment [196].

Monocytes are a human immune cell usually sourced for culture from a buffy coat from donated human blood and are cultured at 37^{o}C at 5% CO_2 with the standard used media being RPMI 1640 with 10% foetal bovine serum (FBS) and 1% l-glutamine [197].

FBS is a vital component to the culture of monocytes as it is described as a natural cocktail of nutrients and growth factors containing approximately 1800 proteins and over 4000 metabolites, but has yet to be fully characterised

due to variation in batches due to geographical and seasonal sourcing [198]. L-glutamine is used as an additional energy, carbon and nitrogen source [199].The 5% carbon dioxide is not a metabolic requirement of eukaryotic cells. Carbon dioxide is used to maintain a stable pH for the cell culture media.

3.2 Methods

Bacterial Strains:

For this study, *S. aureus* strains USA300 JE2 [200, 201] and ROJ143 [202] were used.

Media used:

For the standard bacterial growth media, tryptic soy broth (TSB) was used. The standard monocyte culture media consisted of RPMI 1640 (without phenol red) (ThermoFisher) with 10% foetal bovine serum (FBS) and 1% l-glutamine. For conditions with no FBS present, RPMI 1640 (without phenol red) and 1% l-glutamine was used. For the two media containing casamino acids, casamino acids were dissolved in deionised water and added to culture medias to a final concentration of 0.6%. The final medium tested was X-Vivo 15 serum free medium without gentamicin or phenol red (Lonza).

Culture conditions:

Shaker culture conditions were performed in a shaker incubator at 200 RPM at 37^{o}C in 25 mL sealed bijou tubes containing 5 mL of culture media.

Static culture was performed in a 37^{o}C incubator in 25 mL sealed bijou tubes containing 5ml of culture media.

Static (gas exchange) culture was performed in a 37^{o}C incubator with 5% CO_2 in 25 mL semi-open bijou tubes containing 5 mL of culture media.

RCCS culture was undertaken in a 37^{o}C incubator with 5% CO_2 in 2 mL autoclavable HARV vessels rotating at 25 rpm.

USA300 growth:

Cultures of USA300 JE2 were initiated in a 1:1000 dilution from a previous overnight culture in the tested media in shaker culture conditions. After 24 hours in the tested conditions, samples were measured in a 1:10

dilution in the tested media in a quartz cuvette on a spectrophotometer at 600 nm absorbance.

Protein analysis:

Cultures from USA300 JE2 growth assays were centrifuged at 13000 RPM for 1 minute to pellet cells. Supernatant was then collected and stored at -20^{o}C.

Sample loading buffer stock was made by mixing together; 2.5 mL 1M Tris-HCl (pH 6.8), 0.5 mL deionised water, 1g SDS, 0.8 mL of 0.1% bromophenol blue, 4 mL 100% glycerol, 2 mL β-mercaptoethanol to make a 2x concentration. 5 µL of sample was added to 5 µL of loading buffer for each sample well.

For protein standard ladder, see Appendix.

SDS-PAGE gels used were Mini-PROTEAN TGX precast gels, 15 wells, 4-20% (Bio-Rad) and stored at 4^{o}C.

Electrophoresis of gels was performed in the Mini-PROTEAN Tetra-cell. Samples were loaded at 10 µL and the apparatus was filled with SDS-running buffer, which consisted of a 1:10 dilution of the 10x stock.

The x10 stock of SDS-running buffer is made of 30.3 g tris base, 144.4 g glycine and 10 g SDS. Deionised water is added until the solution makes up 1 L.

SDS-PAGE was performed at 150 V for 45 min or until the loading dye reached the bottom of the gel.

Gels were then stained overnight in colloidal Coomassie blue (0.5 g Coomassie brilliant blue in 250 mL of ethanol) on a shaker platform. Gels were then de-stained in de-staining solution until banding was visible. De-stain solution was removed and re-added 3-5 times throughout this process.

De-staining solution was made of methanol, deionised water, and acetic acid in a 5:3:1 ratio.

Gels were then imaged on the gel documentation system (Gel Doc) (Bio-Rad).

AIP production:

The same supernatants collected from the USA300 growth assay were used for this.

Overnight shaker culture of ROJ143 was performed in brain-heart infusion medium (BHI) with 10 µg/mL chloramphenicol. Overnight culture was then diluted 1:100 and 195 µL of this dilution was added to each well of a 96 well black microplate (Greiner). 5 µL of sample was then added to each well.

The plate was then put into a TECAN plate reader. The TECAN was set to 37^{o}C and to run for 16 h. Every 15 min each well had its' luminescence and absorbance at 600 nm recorded. Luminescence divided by OD_{600} was plotted on GraphPad (Prism) over time and the area under the graph calculated.

3.3 Results

To begin with, the growth of USA300 after 24 hours was recorded by using a spectrophotometer to measure the OD_{600}. Various media and culture methods were tested to begin the optimisation process for *S. aureus* monoculture and eventual co-culture with monocytes. For the culture conditions, all experiments were performed at 37°C. Static gas exchange was performed in a 5% CO_2 incubator as was the RCCS condition which was also rotating at 25 rpm in the LSMMG orientation.

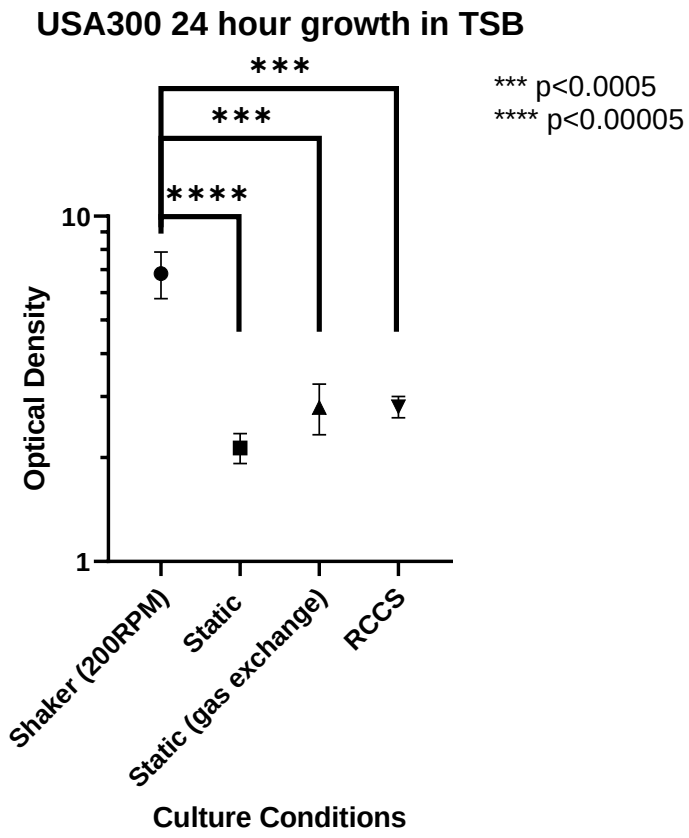
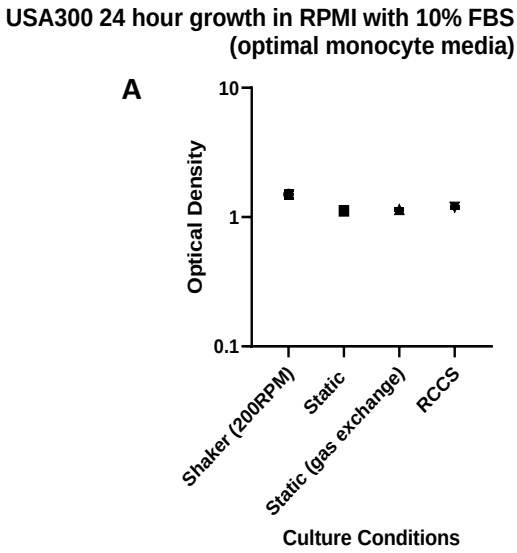


Figure 3.3.1: Final OD_{600} optical density after 24-h growth of USA300 in TSB in four different culture conditions. Optical density was calculated by using the OD_{600} of a diluted sample multiplied by the dilution factor. Error bars show standard deviation. ANOVA analysis was performed using GraphPad. For relationships showing a $p>0.05$, lines were not drawn. N=3, n=3. Error bars represent +/- standard deviation of the mean.

For TSB medium, shaker incubation showed a significantly increased growth rate in comparison with the other three culture conditions which showed no significant difference between each other.

The next two media conditions tested contained foetal bovine serum (FBS), a vital component in eukaryotic cell culture. The first tested media is the optimal culture medium for monocytes and the second test contained the addition of casamino acids, a supplement that promotes bacterial growth.



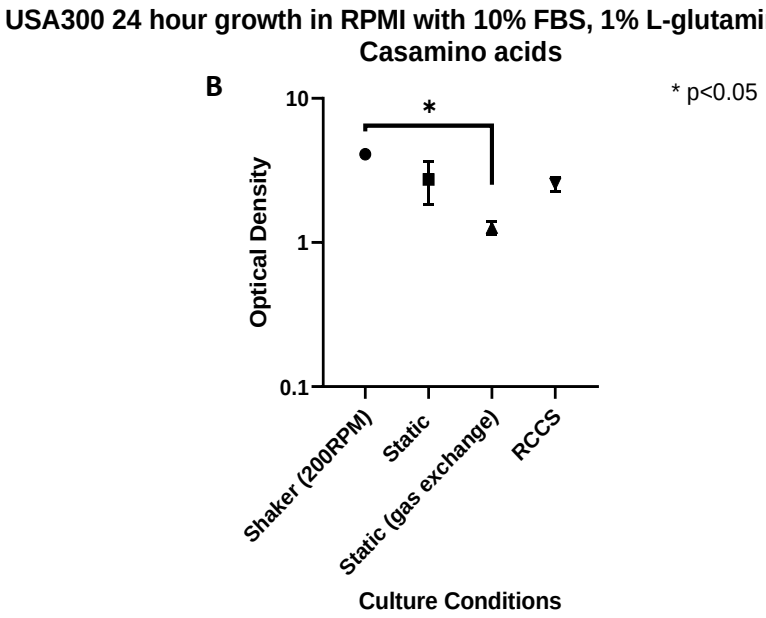


Figure 3.3.2: Optical density for 24-hour growth of USA300 in RPMI with 10% FBS, 1% L-glutamine and with (**B**) or without (**A**) 0.6% casamino acids in four different culture conditions. Optical density was calculated by using the OD_{600} of a diluted sample multiplied by the dilution factor. ANOVA analysis was performed using GraphPad. For $p>0.05$, lines were not drawn. N=3, n=3. Error bars represent +/- standard deviation of the mean.

Figure 3.3.2A shows minimal differences between all four culture conditions with all OD_{600} being approximately 1. Figure 3.3.2B shows there is a significant

increase in growth in comparison to A with shaker, static and RCCS all increasing to above 4. It is also shown that shaker has the highest growth with static and RCCS being indistinguishable from each other but not significantly lower than shaker culture. The OD_{600} of static (gas exchange) samples remained at 1.

To determine the effect of FBS, this experiment was repeated on the two media without the presence of FBS.

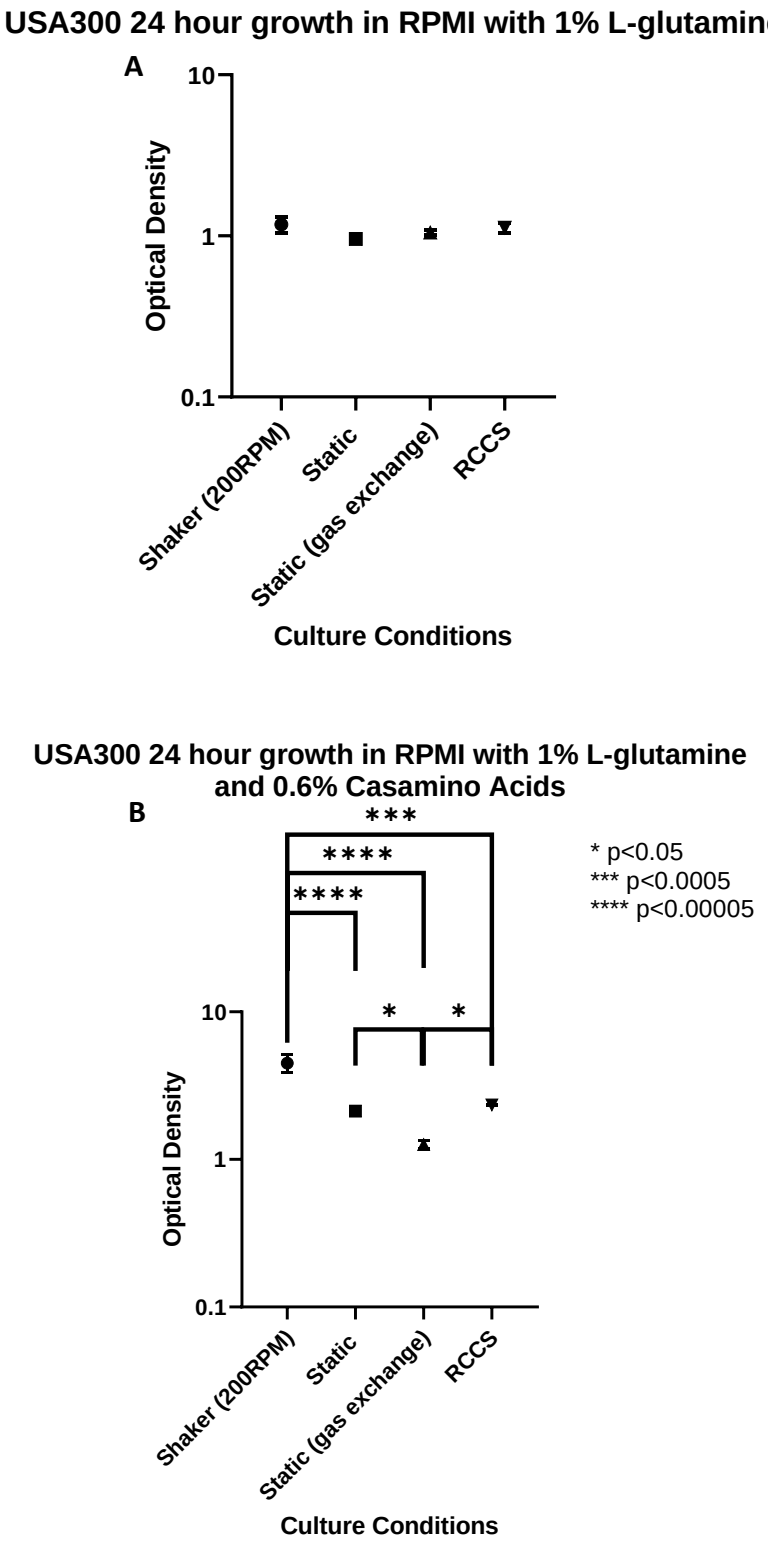


Figure 3.3.3: Optical density for 24-hour growth of USA300 in RPMI with 1% L-glutamine and with (**B**) or without (**A**) 0.6% casamino acids in four different culture conditions. Optical density was calculated by using the OD_{600} of a diluted sample multiplied by the dilution factor. Error bars show standard

deviation. ANOVA analysis was performed using GraphPad. For $p>0.05$, lines were not drawn. N=3, n=3.

Figure 3.3.3A shows little difference between all four culture conditions with each result having an approximate optical density of 1 at 600nm. Figure 3.3.3B shows that with the addition of casamino acids, shaker culture shows the highest growth whereas static (gas exchange) culture remains at 1 optical density at 600nm. Once more, both RCCS and static have increased to an amount that was still lower than shaker culture, however this time, RCCS was significantly higher than static culture.

The final medium tested was X-Vivo 15 serum free medium. This medium is suitable for monocyte culture and more closely mimics the *in vivo* ~~s~~ environment due to its' composition.

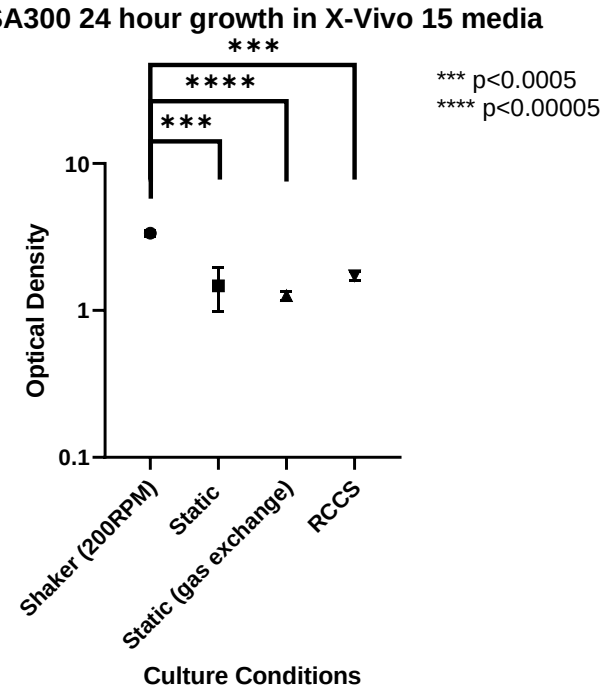


Figure 3.3.4: Optical density for 24-hour growth of USA300 in X-Vivo 15 medium in four different culture conditions. Optical density was calculated by using the OD_{600} of a diluted sample multiplied by the dilution factor. Error bars show standard deviation. ANOVA was performed using GraphPad. For $p>0.05$, lines were not drawn. N=3, n=3.

Figure 3.3.4 shows shaker culture having the highest 24-hour growth of USA300 at OD_{600} 5.0. All three of static, static (gas exchange) and RCCS cultures showed substantially lower growth, falling between 2-3 optical density at 600 nm with no significant difference between them.

S. aureus growth is not the only factor to be investigated in future chapters. Virulence factor production and potentially other secreted components will be investigated in subsequent chapters. Therefore, a medium was needed that facilitated an environment for this production and allows them to be analysable. This section shows the SDS-PAGE results from overnight growth in different media in. For the molecular weight protein ladder used, please see Appendix.

The order of media will follow the previous results, starting with TSB.

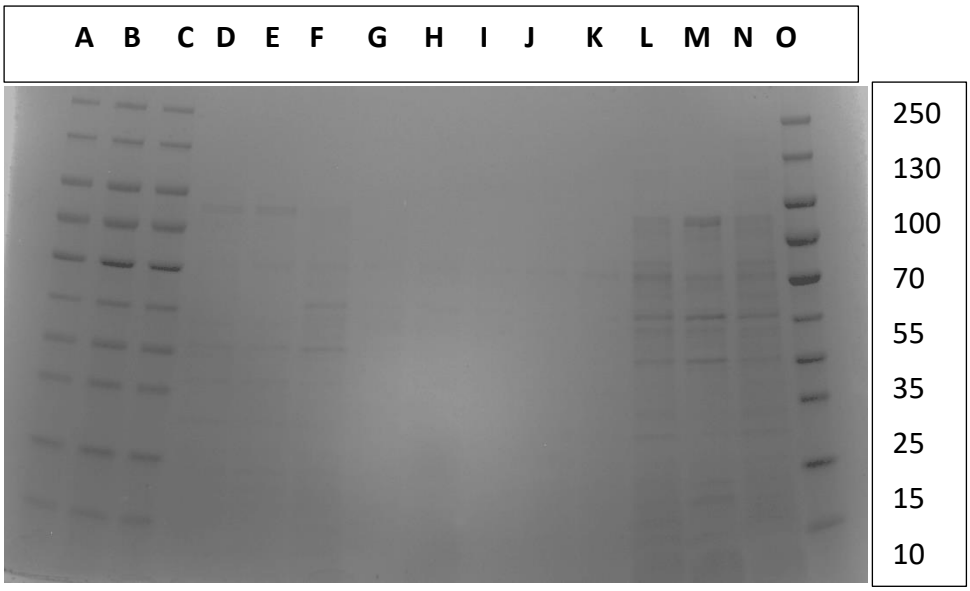


Figure 3.3.5: SDS-PAGE of the supernatant from *S. aureus* overnight growth in TSB. (**A-C, O**) Protein markers, (**D-F**) RCCS, (**G-H**) Static gas exchange, (**I-K**) Static, (**L-N**) Shaker. Numbers at the side of the image represent order and weight of the molecular markers in kDA.

Figure 3.3.5 shows that for TSB culture, only shaker culture (L-N) and RCCS culture (D-F) provides an environment in which USA300 can produce many extracellular products in levels that can be visualised by SDS-PAGE. The secretome itself between the RCCS and shaker culture is different as shown by the differing pattern of bands.

Next, RPMI with 10% FBS and 1% L-glutamine (optimal monocyte media) was investigated.

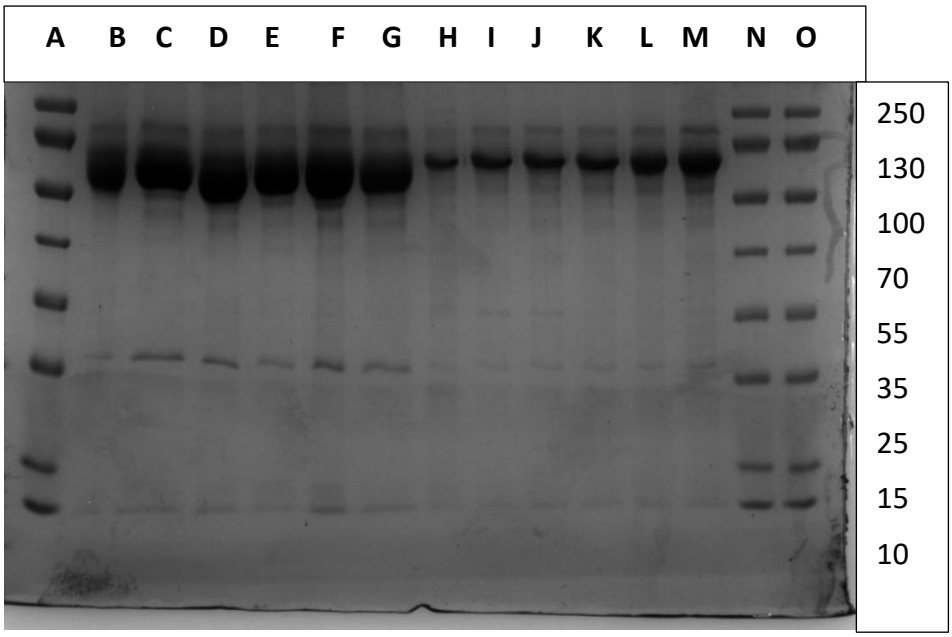


Figure 3.3.6: SDS-PAGE of the supernatant from *S. aureus* overnight growth in RPMI with 10% FBS and 1% L-glutamine. (**A, N-O**) Protein markers, (**B-D**) Shaker, (**E-G**) Static, (**H-J**) Static gas exchange, (**K-M**) RCCS. Numbers at the side of the image represent order and weight of the molecular markers in kDA.

Figure 3.3.6 shows an identical secretome pattern for all four conditions. Both shaker and static, the conditions not cultured in the presence of CO_2, show an increased intensity on the 3 distinct bands (approximately 130 kDa, 35 kDA and 10 kDA) compared with the CO_2 conditions of RCCS and static (gas exchange).

The next investigated medium was the same medium but with 0.6% casamino acids added.

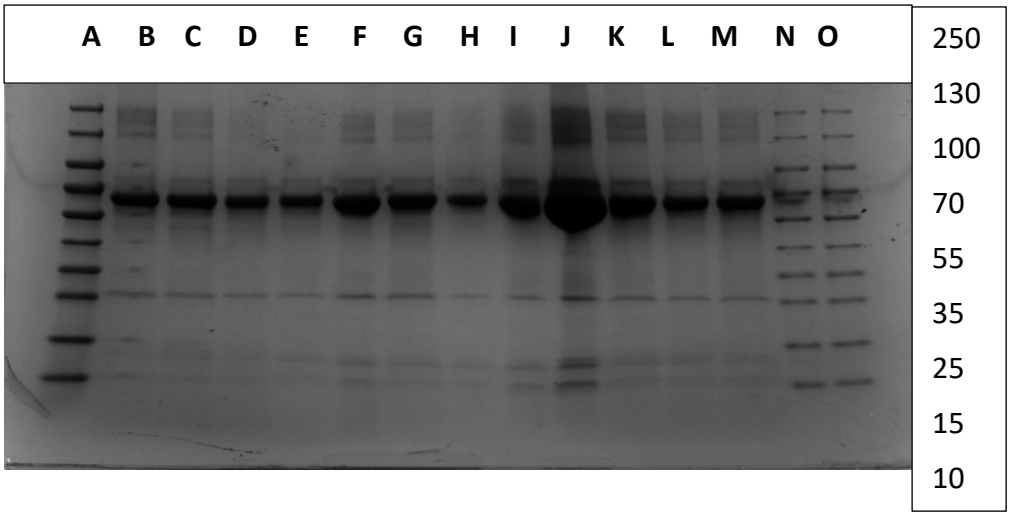


Figure 3.3.7: SDS-PAGE of the supernatant from *S. aureus* overnight growth in RPMI with 10% FBS, 1% L-glutamine and 0.6% Casamino acids. (**A, N-O**) Protein markers, (**B-D**) Shaker, (**E-G**) Static, (**H-J**) Static gas exchange, (**K-M**) RCCS.

For this medium, every culture condition has the same banding pattern and intensities of each band showing no difference between all conditions.

The next two gels are repeats of RPMI and RPMI with casamino acids but both without the addition of FBS.

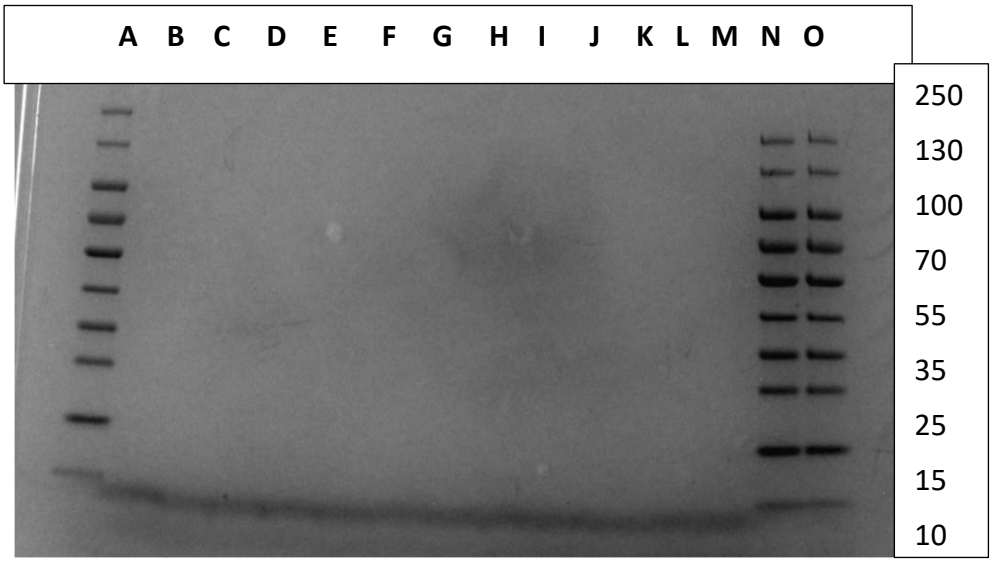


Figure 3.3.8: SDS-PAGE of the supernatant from *S. aureus* overnight growth in RPMI + 1% L-glutamine. (**A, N-O**) Protein markers, (**B-D**) Shaker, (**E-G**) Static, (**H-J**) Static gas exchange, (**K-M**) RCCS.

With the medium being RPMI and 1% L-glutamine alone, there was no analysable secretome for any of the conditions.

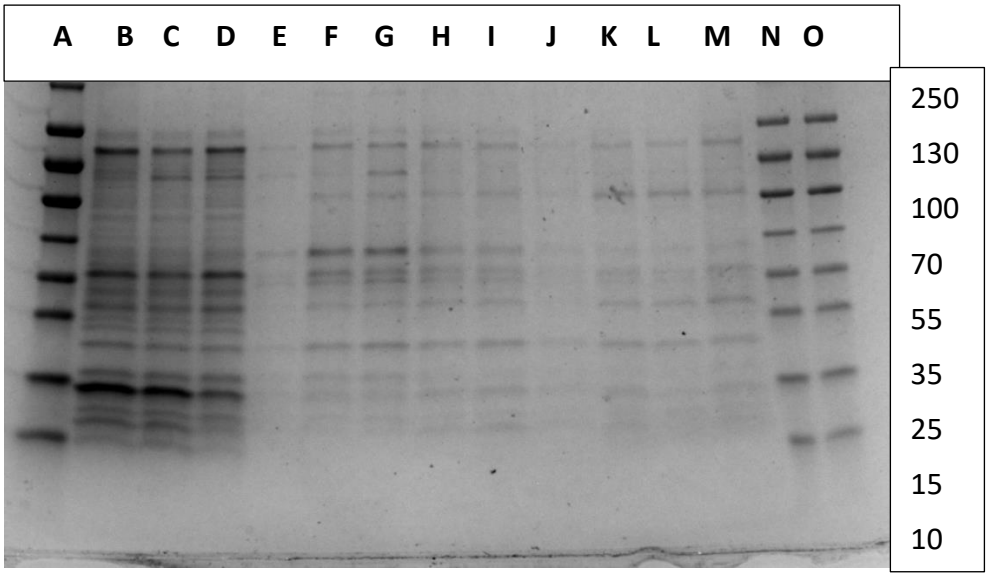


Figure 3.3.9: SDS-PAGE of the supernatant from *S. aureus* overnight growth in RPMI with 1% L-glutamine and 0.6% Casamino acids. (**A, N-O**) Protein markers, (**B-D**) Shaker, (**E-G**) Static, (**H-J**) Static gas exchange, (**K-M**) RCCS.

Figure 3.3.9 shows that for shaker culture, this media has the most intense banding and therefore produces the most analysable proteins. Shaker culture also has a different banding pattern intensity compared with the other three conditions. The other three conditions have very similar band intensities and patterns so it can be said there is no discernible difference.

The final gel is from the culture in X-Vivo 15 media.

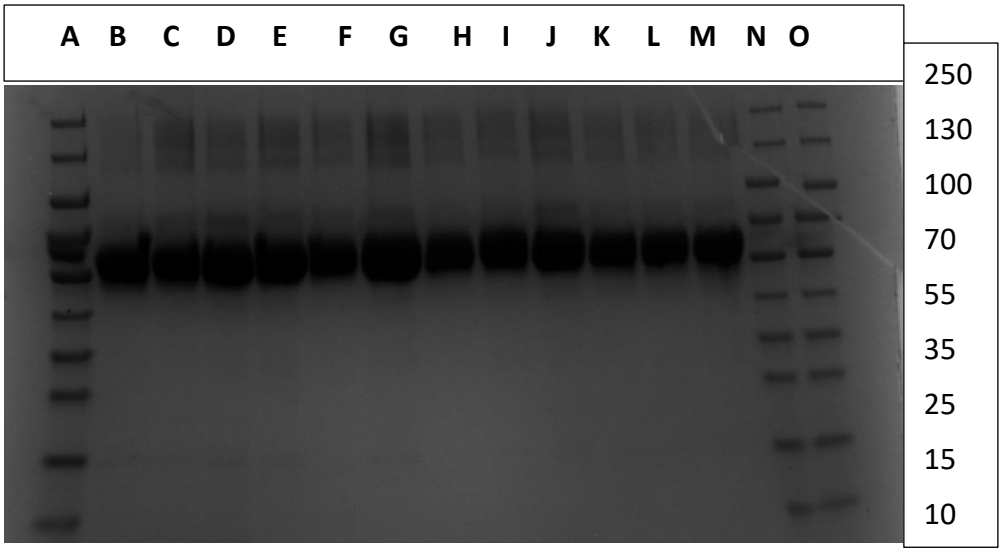


Figure 3.3.10: SDS-PAGE of the supernatant from *S .aureus* overnight growth in X-Vivo 15 media. (**A, N-O**) Protein markers, (**B-D**) Shaker, (**E-G**) Static, (**H-J**) Static gas exchange, (**K-M**) RCCS.

Figure 3.3.10 shows that all culture conditions show the exact same banding pattern with one very large high-weight band dwarfing everything else in the gel.

Now that the overall secretome in the varying medias and culture methods, the production of auto inducing peptide, the regulatory molecule for quorum sensing, was investigated. This was due to the importance of being able to analyse AIP production as part of the investigations undertaken in Chapter 4.

The ROJ143 reporter strain was employed to investigate the amount of AIP produced in the culture media from the growth studies in this chapter. ROJ143 is a strain of *S. aureus* that has a *lux* cassette on the P3 promoter of the *agr* operon but cannot produce its' own AIP [202]. When AIP is added, this activates the *agr* operon and subsequently the *lux* cassette causing the strain to emit light. The next figure is the maximum, saturated response for this assay achieved by adding synthetic AIP-1.

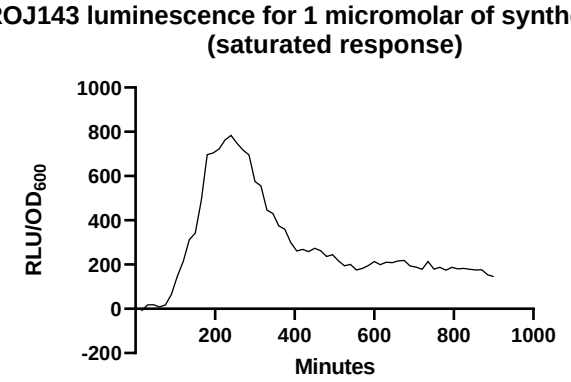


Figure 3.3.11: AIP reporter assay for a saturated maximal response using 1 micromolar of synthetic AIP-1. Relative light units, a measure of luminescence, is divided by OD_{600} to show *lux* expression of cells independent of cell density.

Figure 3.3.11 shows the maximum output of this reporter assay over time. As such, this will become the 100% saturated response when saturated response is being referred to in the subsequent figures in this section.

The same pattern was followed as previous investigations with regards to media investigated. The focus of this sub-set of investigations was on AIP production.

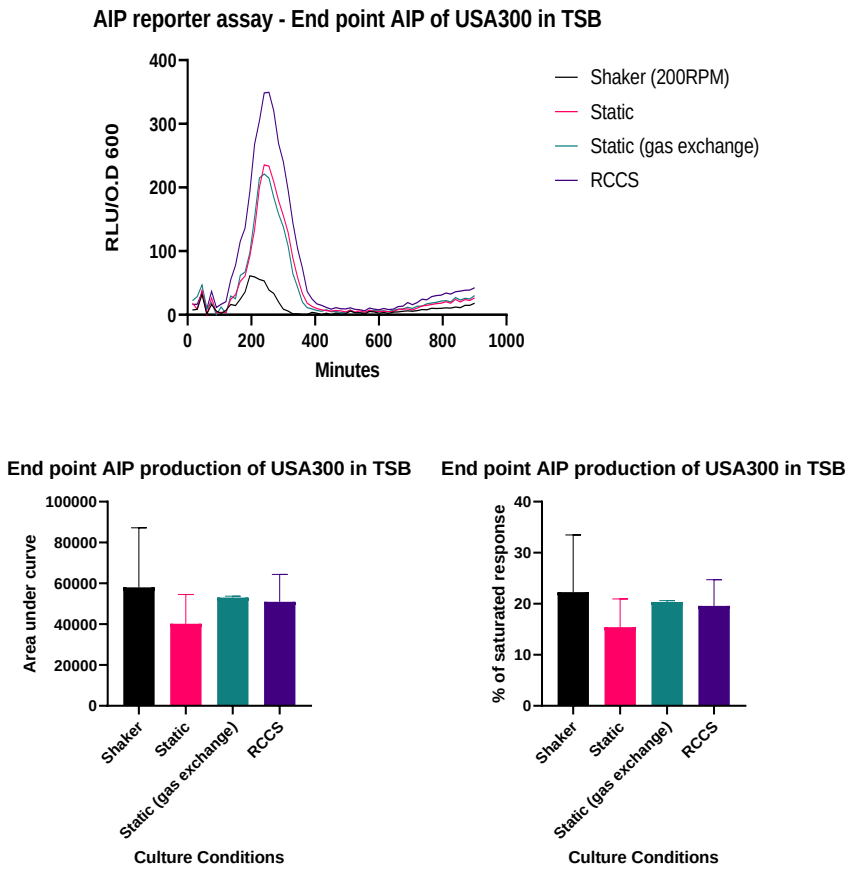


Figure 3.3.12: ROJ143 AIP reporter assay for AIP present in TSB medium after 24 hours growth of USA300. N=3. Light output with respect to cell density was recorded over the culture period. The area under the curve for $RLU/_{OD600}$ was then calculated for total light output over the course of the experiment.

For TSB, shaker culture produced the largest amount of AIP compared to the other three conditions. The other three conditions show a similar amount of AIP. All conditions produce less than half of the saturated response in Fig 3.3.11.

AIP production in the 'optimal' monocyte media of RPMI with 10% FBS and 1% L-glutamine was next investigated.

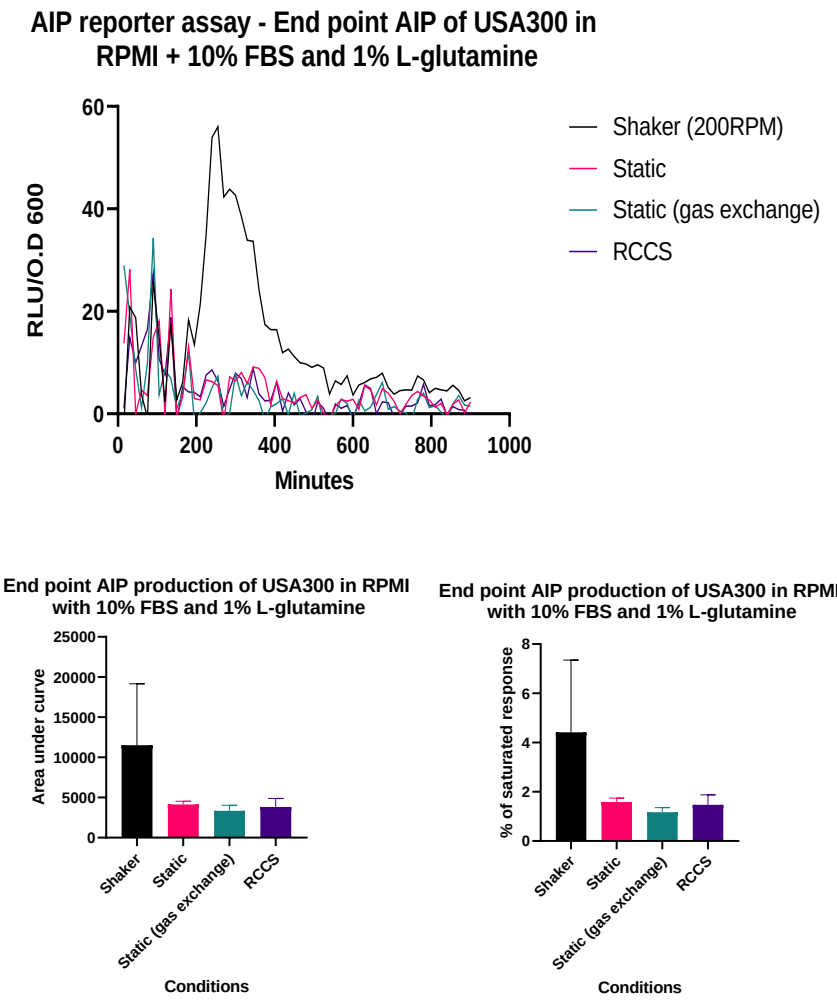


Figure 3.3.13: ROJ143 AIP reporter assay for AIP present in RPMI with 10% FBS and 1% L-glutamine after 24 hours growth of USA300. N=3. Light output with respect to cell density was recorded over the culture period. The area under the curve for $RLU/_{OD600}$ was then calculated for total light output over the course of the experiment.

Figure 3.3.3.13 shows that for the optimal monocyte medium, only shaker culture is eliciting a non-basal response with approximately a response of 5% of the saturated one in figure 3.3.3.11. The other three culture conditions have a response low enough that it is more than likely below the limit of detection for the assay and therefore a null response.

This medium was then further investigated but with casamino acids added to it.

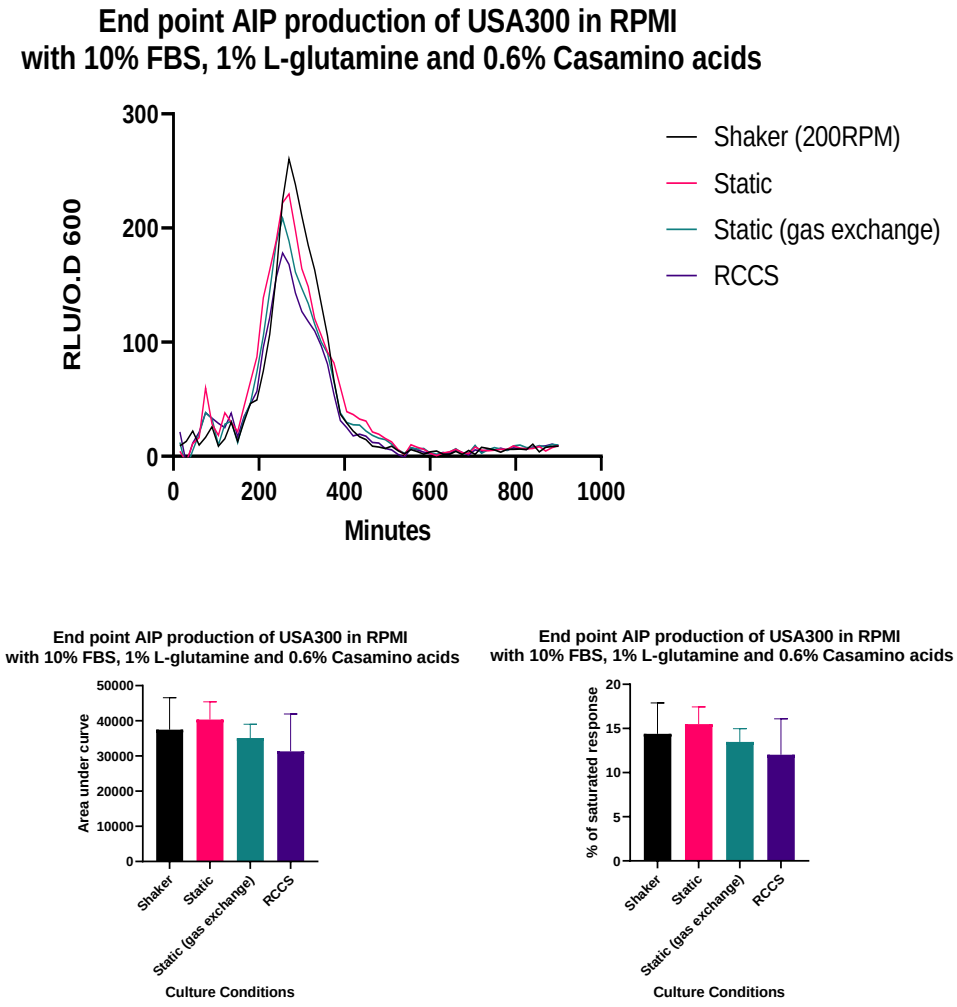


Figure 3.3.14: ROJ143 AIP reporter assay for AIP present in RPMI with 10% FBS, 1% L-glutamine and 0.6% casamino acids after 24 hours growth of USA300. N=3. Light output with respect to cell density was recorded over the culture period. The area under the curve for $RLU/_{OD600}$ was then calculated for total light output over the course of the experiment.

Interestingly, for this medium there were no significant differences between the AIP production of all four culture conditions with all four responses being between 10-15% of the saturated response.

The two previous media but without the presence of FBS was then investigated.

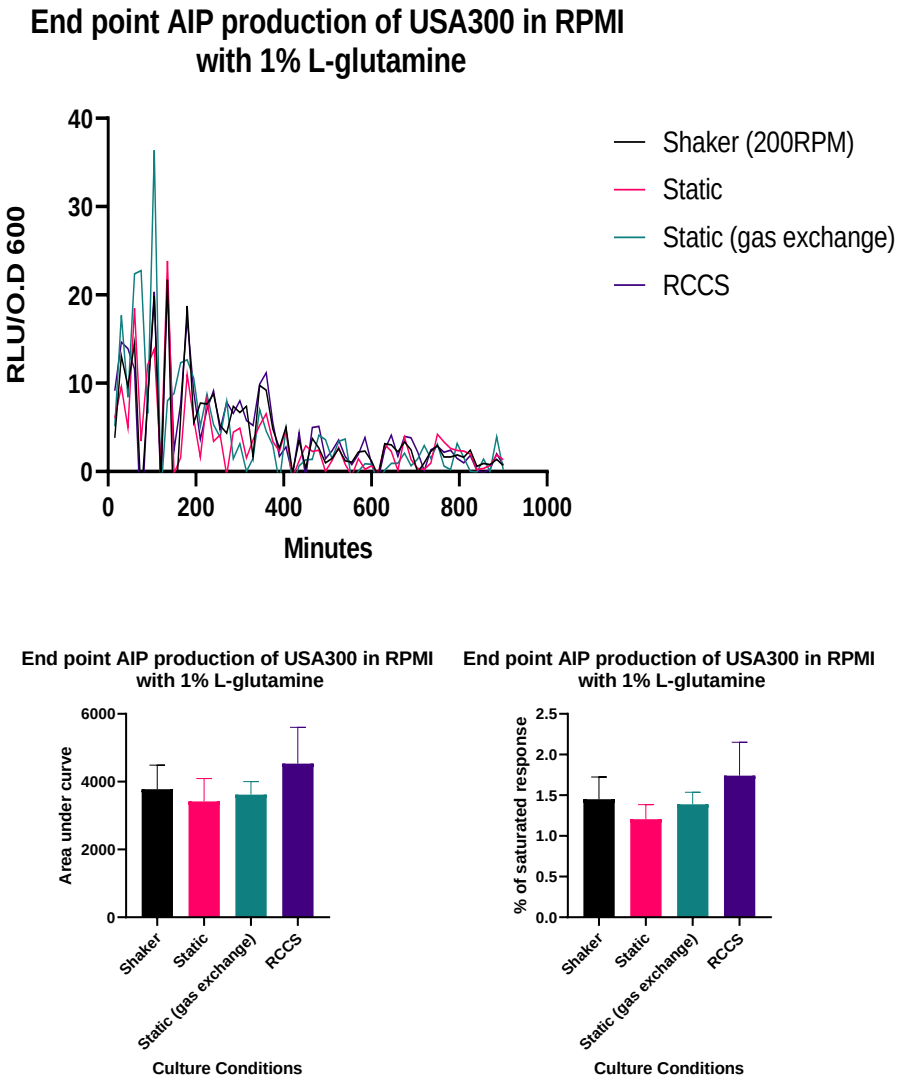


Figure 3.3.15: ROJ143 AIP reporter assay for AIP present in RPMI with 1% L-glutamine after 24 hours growth of USA300. N=3. Light output with respect to cell density was recorded over the culture period. The area under the curve for $RLU/_{OD600}$ was then calculated for total light output over the course of the experiment.

For RPMI and L-glutamine only, all four conditions produce an identical, basal response that is likely to be below the limit of detection of the assay and therefore is a negative result.

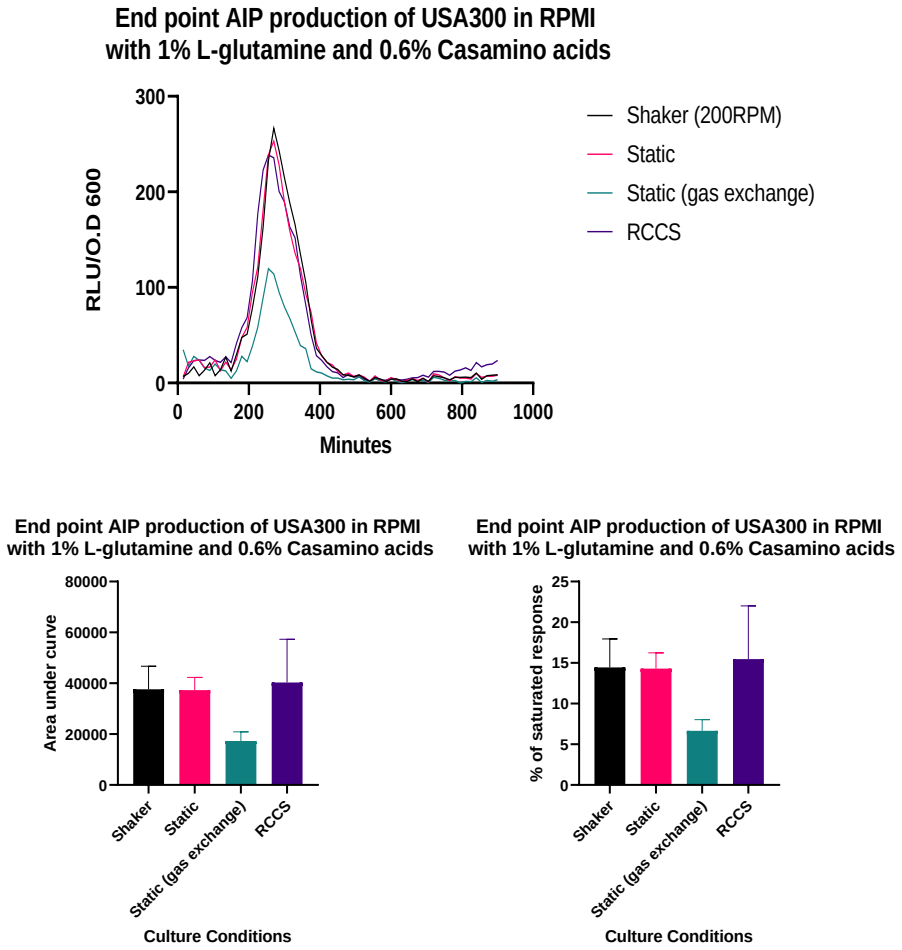


Figure 3.3.16: ROJ143 AIP reporter assay for AIP present in RPMI with 1% L-glutamine and 0.6% casamino acids after 24 hours growth of USA300. N=3. Light output with respect to cell density was recorded over the culture period. The area under the curve for $RLU/_{OD600}$ was then calculated for total light output over the course of the experiment.

As Figure 3.3.16 shows, shaker, static and RCCS produce approximately 15% of the saturated response. Interestingly, static (gas exchange) is the only condition to produce a lower amount of AIP although it is still above the basal amount.

Finally, AIP production in X-Vivo 15 medium was investigated.

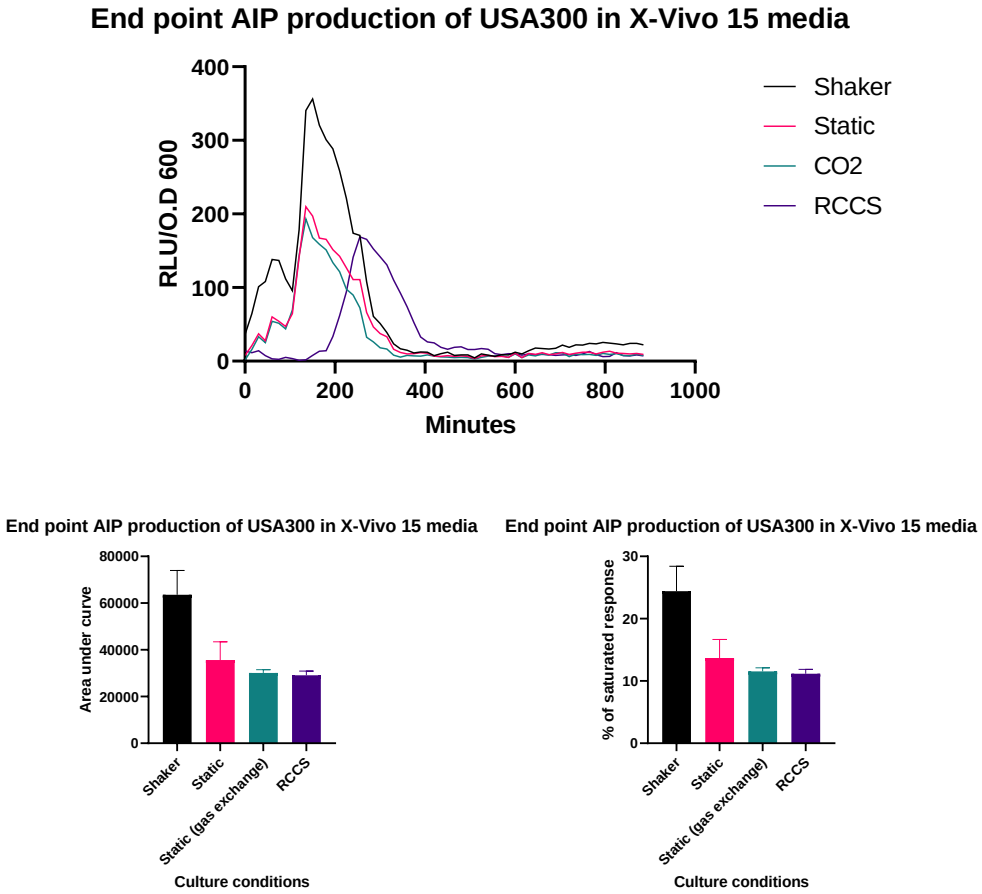


Figure 3.3.17: ROJ143 AIP reporter assay for AIP present in X-Vivo 15 media after 24 hours growth of USA300. N=3. Light output with respect to cell density was recorded over the culture period. The area under the curve for $RLU/_{OD600}$ was then calculated for total light output over the course of the experiment.

Figure 3.3.17 shows that shaker culture produced the most AIP with approximately 25% of the saturated response whilst the other three conditions showed minimal difference between them at 12-13% of the saturated response.

Finally, the results were coalesced to decide on the optimal culture conditions for *S. aureus* monoculture and co-culture with monocytes. Figure 3.3.18 summarises the findings from all the previous results of this chapter. This heatmap uses a subjective ranking system based comparing these findings to each other.

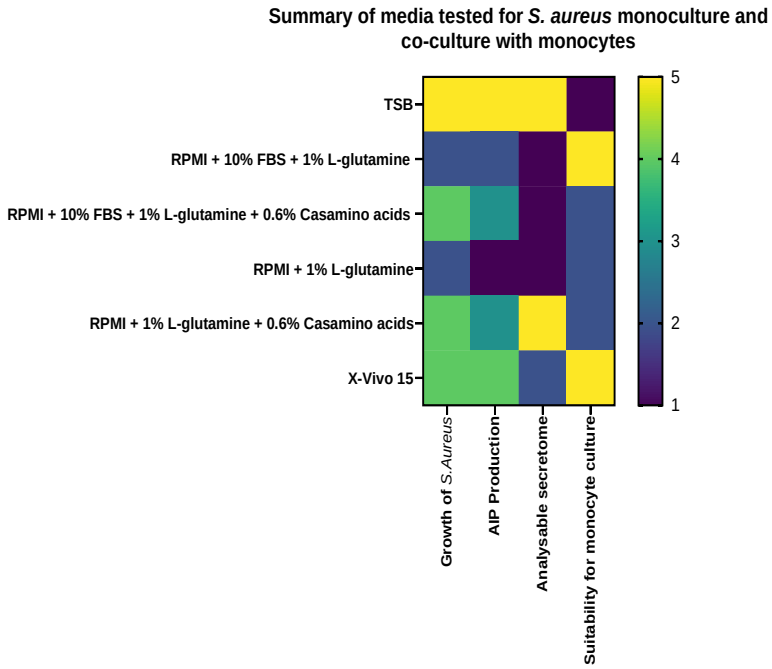


Figure 3.3.18: Summary heatmap for the six media tested in Section 3.3 and that will determine the suitability of the media for *S. aureus* monoculture and co-culture with monocytes. Monocyte culture effectiveness was determined from prior knowledge of monocyte responses and literature. Scale of 1-5 is a subjective scale with 5 being optimal 'for variable investigated and 1 being sub-optimal.

Each variable tested had a desired-outcome that influenced the ratings given. Growth of *S. aureus*'s desired outcome was substantial *S. aureus* OD_{600} at the end of one growth cycle. As TSB is one of two optimal growth media for this outcome, a rating of 5 was given to this medium and ratings for this outcome were compared against this. As TSB is the optimal media, it was given a rating of 5 for the other two *S. aureus* related responses as well (AIP production and secretome analysis). This logic was also applied with regards to the optimal monocyte media with regards to suitability to monocyte culture and was given a rating of 5 with other medias being compared against this.

Figure 3.3.18 does not show a definitive result for an optimal media for both *S. aureus* monoculture and monocyte co-culture. The optimal cell culture medium would facilitate high *S. aureus* growth, with a desirable OD_{600} of

above 5, production of AIP being facilitated, a clear analysable protein production shown by multiple clear bands on the SDS-PAGE, and viable, un-activated culture of monocytes. TSB ranks highest for three categories, but it is completely unsuitable for monocyte culture. The opposite is shown with RPMI with 10% FBS and 1% L-glutamine with this ranking highest for monocyte culture but being unable to facilitate substantial bacterial growth and having almost zero AIP production.

From these findings X-Vivo 15 was chosen. The following section will build upon how this choice came about and its benefits compared with other tested media. It will also be discussed as to which culture conditions were chosen and maintained in all future experiments.

3.4 Discussion

The investigations in this chapter were to determine the optimal culture media for *S. aureus* monoculture and co-culture with monocytes. The other aim was to determine how different culture conditions affect the growth and response of *S. aureus* to determine a suitable '1g' control to compare with RCCS culture.

The initial discussion will focus on the impact of different culture conditions on the response of USA300 and the conclusion it led to.

The first variable to be investigated was the impact of differing culture conditions. As the RCCS LSMMG culture imparts movement onto the culture through 25 rpm rotation and is gas permeable with incubation in a 37^{o}C incubator at 5% CO_2, it was vital to determine how both movement and presence of CO_2 effects *S. aureus* culture to determine a suitable ground '1g' control culture to make valid observations in future chapters. By identifying these effects and finding a suitable '1g' control, any observed changes in future chapters in RCCS LSMMG culture should be due to the LSMMG nature of the culture and not differences in movement or CO_2 presence.

S. aureus are cocci shaped and do not have the ability for movement due to the lack of flagella [203]. Therefore, they rely on the culture vessels to provide the movement for them and the circulation of nutrients and waste products, hence why standard culture is performed at 200 rpm in a shaker incubator.

To see the effects of movement or shaking of culture vessels on *S. aureus* growth and outcomes, shaker culture and static culture can be compared as they were performed in the same incubation conditions, static (gas exchange) can be compared with the RCCS culture as they were performed in the same incubator also. However, caution must be taken with this comparison as differences may not be movement induced changes but could be LSMMG induced changes instead. As the purpose of this initial investigation is to see if any changes are induced though, valid observations can be made and may start to highlight LSMMG changes in *S. aureus.*

When comparing growth across the non-CO_2 conditions, it is shown that for all medias where growth above an approximate OD_{600} of 1 occurs, shaker culture provides significantly higher growth than static culture and has higher growth than the other two remaining conditions as well. This shows why shaker culture is the optimal condition for *S. aureus* growth. Except for TSB, RCCS culture also showed increased growth in comparison to static (gas exchange) when the culture media facilitates growth above 1 O.D. at 600nm.

Both results combined highlight the importance of media movement for increasing culture growth. However, this highlights a potential flaw with the suitability of the RCCS as a true representation of microgravity culture.

The major difference between LSMMG culture in the RCCS and microgravity experienced during spaceflight is that in true microgravity there is no convection. By showing the effects of culture movement on the *S. aureus* outcomes investigated, it has been shown how movement of these solutes influences these outcomes. This shows the impact convection may have on growth, AIP production and secretome expression and how movement increases all of this. Therefore, as true microgravity culture lacks convection, it can be hypothesised that true microgravity culture could have reduced growth, AIP production and secretome production compared with the microgravity analogous culture provided by the LSMMG conditions in the RCCS, which is subject to convection.

Static culture, although subject to gravity and therefore convection, has some similarities to true microgravity culture. Although the culture media is subject to convection and the cells have the force of gravity impacting on them, the cells themselves sediment at the bottom of the culture vessel and do not move. This lack of movement of the cells themselves is like the lack of movement of cells due to lack of convection shown in microgravity.

A perfect true spaceflight microgravity analogue would combine the two spaceflight similarities between static culture and RCCS LSMMG culture. Keeping the cells immobile in a culture vessel with no convection would

mimic the lack of convection experienced in microgravity and isolate this as a variable to test. Theoretically, this could be achieved by making sure the culture liquid is consistent in density throughout the vessel and every part of the culture is identical in temperature. By keeping this stationary it would be analogous of the no convection environment created by microgravity. This has practical issues however requiring perfect uniform heating of culture and a perfect insulator to ensure no heat is lost or it is completely uniform across the culture vessel. This does not address the low-shear environment created by microgravity either as to induce this it would either require movement of the culture vessel (RCCS, random positioning machine, drop towers) or would re-introduce convection (diamagnetic levitation). This highlights why ground-based analogues cannot be truly compared to spaceflight results as there is not a way to truly mimic all the conditions caused by true microgravity in spaceflight.

In standard culture, increased growth rate leads to an increased population of cells and usually increased levels in proteins produced. It is hard to deduce results from the conditions containing FBS (to be later discussed) but when looking at the two medias with the clearest results, (TSB, RPMI with 1% L-glutamine and 0.6% casamino acids) it is once again shown that shaker culture has the strongest intensity of banding and therefore the highest concentration of secretome. It is also shown that for TSB, but not RPMI with casamino acids, that RCCS culture has a higher intensity of banding than static (gas exchange) culture. Once again, these results highlight the importance of culture movement to response of the bacteria.

What is also apparent is that the difference in movement creates different banding patterns. Both shaker compared with static, and static (gas exchange) compared with RCCS have different banding intensity patterns suggesting alterations to their secretome. This could cause differences in assay results when looking for specific products of the bacteria and needs to be noted.

Finally, effects of movement on the production of the quorum sensing molecule AIP can be examined. Once again, in standard conditions, increased

growth leads to an increase in AIP production as more bacteria are present to produce the molecule. However, this is also dependent on other nutrients and environmental conditions.

Interestingly, when enough growth was facilitated for AIP production, only in some of the media was shaker culture producing more AIP than static culture. In the two conditions containing 0.6% casamino acids, shaker and static culture showed similar production of AIP. Further testing is needed for the following hypothesis but the addition of casamino acids may negate the need for movement when it comes to quorum sensing studies. This could be tested by monitoring AIP production over time in static conditions with and without the addition of casamino acids and comparing this to shaker culture in media without the casamino acids to compare the AIP production over time profile and total amounts of AIP produced.

In conclusion, movement of the culture is a factor that affects growth, and production of AIP and proteins, of which all these outcomes will be analysed in the following chapters of this thesis. From this it can be determined that as the RCCS rotates at 25 rpm, any '1g' control will also need to rotate at 25 rpm to keep movement of culture a consistent variable across both culture conditions.

Now the effects of movement on the outcomes to be future analysed have been determined, investigation of the effects of 5% CO_2, which is needed for monocyte culture and hence will be used in the co-culture studies, was undertaken.

As the importance of movement to the observable outcomes has already been determined, it would be an inaccurate comparison to use RCCS culture and shaker culture due to the rotational speed in the RCCS being 8 times lower than that of the rate in the shaker incubator. Therefore, any accurate comparisons can only be made between static and static (gas exchange) culture.

For growth conditions, there was only a noticeable difference in media containing 0.6% casamino acids. When the casamino acids were present, static culture showed increase growth compared with static (gas exchange) culture. This may be due to the function of carbon dioxide in cell culture. Carbon dioxide in cell culture is used to stabilise the pH of cell culture medium. It does this through a reaction with water forming carbonic acid and interacts with a bicarbonate ion. The addition of casamino acids increases the acidity of the culture medium and therefore lowers the pH. This lower pH will be regulated by the carbon dioxide in the gas exchange culture but would not be in the static conditions and it may be that this lower pH is more suitable for the growth of the tested USA300.

When looking at the analysable secretome via SDS-PAGE, there are no clear differences between static and static (gas exchange) for all the media. There could be a case to be made that for RPMI with 1% L-glutamine and 10% FBS that the two non-CO_2 conditions show stronger banding than the CO_2 conditions, but there is minimal banding for each with one being a large high weight band (later discussed) and no differences in the banding patterns for this one. However, to make valid conclusions based on this, determining what proteins the band represented would have to be performed.

When looking at AIP production, there was also major differences between static and static (gas exchange) apart from RPMI with 1% L-glutamine and 0.6% casamino acids which showed lower production in the gas exchange condition. This matches the reduction in growth seen in the 0.6% casamino acids media and leans into the previously highlighted hypothesis.

In conclusion, the presence of 5% CO_2 seems to only matter in the presence of media containing 0.6% casamino acids. As there was no negative effect in X-Vivo 15 and that future investigations will be using a 5% CO_2 incubator for co-culture studies when introducing monocytes, the '1g' control for the RCCS studies should also be in a 5% CO_2 incubator allowing gas exchange like the RCCS vessels to keep results consistent.

To summarise, the importance of movement in the outcomes to be analysed has been shown along with how carbon dioxide presence does not affect the culture. Due to this, the conclusion was made to have the '1g' control for all experiments henceforth, unless stated otherwise, to be an RCCS turned horizontally in the same 5% CO_2 incubator and have the vessels at the same rotational speed of 25RPM. This ensured any growth or secretome changes due to movement speed are negated and the only experimental variable being the orientation of the RCCS which will either create a '1g' culture environment or a LSMMG culture environment.

The basic requirement for any culture medium is that it can facilitate growth. For experiments later in this thesis, a medium in which the bacteria can grow to a suitable population density that allows for production of an analysable quantity of proteins, which is shown by SDS-PAGE band visualisation, and allows for enough growth for activation of the *agr* operon is required.

TSB is one of two standard growth media (the other being brain heart infusion medium, or BHI) and is used as the 'gold-standard' for these comparisons. It was shown that from the end point growth studies that TSB has the highest growth, with the two media containing 0.6% casamino acids coming a close second and X-Vivo 15 following closely behind, with all these media being well above OD 5.0 at 600nm for the optimal shaker culture. Both the RPMI media without 0.6% casamino acids only facilitated growth to an OD_{600} of 1.0 across all four culture conditions and can be considered a basal amount. Therefore, four possible media for *S. aureus* growth were ; TSB, RPMI (with and without) 10% FBS and 1% L-glutamine and 0.6% Casamino acids, and X-Vivo 15.

Bacteria produce various proteins based on the response to their environment and these can be analysed to determine the phenotype of the bacteria and how it could, for instance, affect human health. Being able to analyse the proteins of *S. aureus* in the RCCS compared to a '1g' control may shed light on how microgravity induces changes in the bacteria.

The one thing apparent is that for media containing FBS, and the serum-free X-Vivo 15 media, is that a very large high molecular weight band on the gel that reduces the migration of the smaller molecular weight proteins. This is especially apparent when comparing the RPMI conditions with 0.6% casamino acids as with FBS there was reduced small weight bands but without FBS there are a plethora of smaller weighted bands. This band due to its' molecular weight is more than likely to be serum albumin. Interestingly, X-Vivo 15 is a serum free medium and has a similar high molecular weight band and also appears to have the lower weighted bands blocked from migrating through the gel by a heavier weighted band. This may be due to the alternative growth factors used instead of serum albumin being similar in weight and having this same effect on SDS-PAGE gels. If analysing whole secretome through electrophoresis and western blot for specific factors is to be employed, this left TSB and RPMI with 1% L-glutamine and 0.6% casamino acids as the two suitable medias.

However, secreted factors can be examined other ways. Instead of western blotting for alpha haemolysin, which is a low weight protein that could have been blocked by the FBS in an SDS-PAGE gel, spent media can be applied to THP-1 cells which are sensitive to alpha haemolysin and measure the viability to determine changes in secreted alpha haemolysin instead. Alternatively, albumin could have been removed from the supernatants through the use of organic solvents or spin columns [204]. Although having a whole picture of secretome changes would be ideal for an in-depth comparison in the future experiments comparing RCCS control to RCCS LSMMG cultured *S. aureus*, it is not a vital deciding factor for the medias.

Although analysing whole secretome is not a deciding factor for media selection, the ability to produce the quorum sensing molecule AIP is due to the nature of the studies in subsequent chapters.

As previously explained, AIP production and growth are usually directly related and so the media that facilitate suitable growth should also facilitate suitable AIP production. Due to the RCCS having decreased growth than

shaker culture and being the culture condition being studied, a media was needed that can facilitate AIP production in the RCCS and hence only the media that facilitated AIP production in the RCCS were considered for future *S. aureus* monoculture and co-culture with monocytes for the purposes of this thesis.

When analysing the results, only two media did not produce measurable amounts of AIP. These conditions were RPMI (with and without) 10% FBS and 1% L-glutamine. The addition of 0.6% casamino acids to these conditions does allow the production of AIP however and makes them suitable with regards to this outcome. TSB and X-Vivo 15 also produce a substantial amount of AIP and make them suitable for quorum sensing studies.

As only X-Vivo 15 and RPMI with 0.6% casamino acids qualify for all the criteria needed. It was decided to use X-Vivo 15 medium due to its' published use in literature [205-207] and its suitability to be used to create a more in vivo style environment.

Key Findings:

- **Standard bacterial culture media are not suitable for monocytes**
- **Standard monocyte media are not suitable to facilitate bacterial growth**
- **Culture movement and CO_2 presence effect bacterial growth and responses**
- **X-Vivo 15 Is the most suitable tested medium for co-culture studies**
- **Any '1g' control culture needs to be rotating at the same speed as the RCCS (25 rpm) and in the same culture conditions to be comparable when studying bacterial response**

4. Quorum sensing and induction of *agr* in *Staphylococcus aureus* in microgravity

4.1 Introduction

Since early space exploration, it has been reported that many prokaryotes adapt and thrive in space vessels [119]. The space exposome subjects prokaryotes to conditions unusual to regular Earth conditions, such as increased radiation from cosmic rays, elevated carbon dioxide levels from human habitation of small enclosed vessels and microgravity. As gravity has been the one constant factor throughout the evolution of every single organism on Earth, the near-absence of gravity is a completely novel environment for any organism that is exposed to it.

It is not feasible to completely sterilise an entire vessel for spaceflight and combined with the natural flora of the human microbiome, a sterile environment is impossible. Hence, NASA have set microbial acceptability limits for space missions [119]. As prokaryotes can have short growth cycles and quick replication times, they adapt to changes in environment relatively fast. As space is a novel environment for prokaryotes, it is hard to predict how they will adapt and if they will pose a threat to human health. This need to understand adaptation becomes especially important as mission duration extends, such as future manned missions to Mars or longer term habitation of the International Space Station (ISS) and its potential future iterations.

In over 60 years of space exploration there have been many missions in which the behaviour of prokaryotes has been observed and researched [119]. By combining ground based analogue data and spaceflight data we can observe how different species of prokaryotes adapt to microgravity and other factors, and begin to investigate the mechanisms behind these adaptations. However, one major factor in researching prokaryote adaptations to microgravity is that there is no universal response of bacteria to microgravity [208]. Surprisingly, the majority of microorganisms thrive in microgravity and spaceflight [209].

Bacterial contamination is reduced for space missions with countermeasures such as pre-flight quarantine of astronauts and food being freeze dried [210]. Because of this, organisations such as NASA set bacterial acceptability limits for pre-flight bacterial counts in the air, water, and on surfaces and a maximum density of bacterial contamination is allowed during the mission at any time [119]. This is tested by random swabs and/or sampling of the three different categories and counting how many colony forming units grown on an agar plate from this swab/sample. Data from over 100 missions have been recorded to enable analysis of the diversity of microbial contaminants found upon/in spacecraft[211]. The clean rooms of the European Space Agency (ESA) member states in which space vessels are assembled were found to contain 298 bacterial strains, and present a diverse array of potential contaminants for missions [212].

Staphylococcus aureus is a Gram positive bacteria that is commonly found in the environment and can cause serious infections [190]. *S. aureus* is also found in normal human flora, mainly residing on skin and in mucous membranes, such as the nasal passage [190]. It has been estimated that around 30% of the human population is colonised with *S. aureus* [213]. *S. aureus* strains can be distinguished by various molecular typing methods such as spa typing - single locus sequencing typing [214], multilocus sequencing typing [215] and other methods using PCR amplification of the coagulase gene and restriction fragment length polymorphism of the product [216]. Other typing methods are around phenotypical changes such as antibiotic resistance. A common typing revolves around its methicillin resistance, and can be classified as methicillin resistant (MRSA) or methicillin susceptible (MSSA). The strain is said to be methicillin resistant if it has a minimum inhibitory concentration of oxacillin equal or greater than 4 μg/mL [217]. MRSA can be further sub-divided into two other classifications of hospital acquired (HA-MRSA) and community acquired (CA-MRSA) [217]. CA-MRSA is when a member of the public, who is not undergoing hospital or outpatient care and is usually healthy, acquires an MRSA infection [218]. HA-MRSA is

when a patient who is in hospital or receiving outpatient care acquires a *S. aureus* infection [218].

S. aureus is the leading cause of bacteraemia (viable bacteria in the blood [219]) and causes other infections, such as pneumonia, bone and joint infections, and is also the leading cause of skin and soft tissue infections [189]. *S. aureus* usually begins by entry into an open wound or colonising a mucous membrane such as the nasal passage [220]. Colonisation does not necessarily lead to infection, but does predispose the host to infection by the bacteria [221]. Established colonisation of *S. aureus* leads to presence of bacteria in close proximity to ears, mouth, throat and sinuses of the host organism [221]. Infection is said to occur when the bacteria are exposed to host tissues other than the mucosal membranes, which causes down-regulation of colonisation factor production and upregulation of virulence factor production [179].

S. aureus produces a plethora of virulence factors such as protein A, haemolysins, leukotoxin, enterotoxin, and toxic shock syndrome toxin-1 [191, 222]. These contribute to infection in different ways. Staphylococcal cell wall Protein A captures IgG Fc domains and blocks phagocytosis by innate immune cells [223]. This allows for persistence of the *S. aureus* and infection to establish. Haemolysins are responsible for lysing host cell membranes. This damage to host cell membranes can impede the host immune response by allowing evasion of the host immune cells due to recognition cells being destroyed or destroying the responder cells, impacting on the ability of the immune response to clear the infection. This lysing of host cells also creates vital nutrients for survival of the *S. aureus.* The best characterised virulence factor of *S. aureus* is α-haemolysin [224]. α-haemolysin binds and oligomerises into a heptameric structure on the host cell membrane [225, 226]. The result of this molecular transformation is the formation of a membrane perforating β-hairpin lined amphipathic pore through the lipid bilayer allowing low molecular weight molecules (under 4kDA) through the pore such as ATP and calcium ions [226, 227]. This results in the lysing of the

cell. Leukotoxins also work by lysing cells. Leukotoxins lyse cells through the formation of β-barrel pores [228]. The four main leukotoxins associated with human infection are Panton-Valentine Leukocidin (PVL), gamma-haemolysin, Leukotoxin ED and Leukotoxin AB/GH [228]. PVL is reported to be the most researched leukotoxin due to its' prevalence amongst community acquired MRSA strains [229]. PVL itself causes a range of pathologies known as PVL-SA disease [230]. Staphylococcal enterotoxins are responsible food poisoning and toxic shock syndrome [231]. Enterotoxins can be referred to as 'superantigens' due to their ability to bind to MHC Class II molecules allowing for their function as T cell mitogens [232]. This mitogenic function causes the stimulation of non-specific T-cell proliferation [231]. Staphylococcal enterotoxin B binds to the T-cell receptor and costimulatory receptor CD28 on T-cells and MHC class II and B7 molecules on antigen presenting cells to activate the proliferation and inflammatory response [233]. However, Toll-like receptor 2 ligands on the staphylococcal cell wall can induce IL-10 production and apoptosis of antigen presenting cells preventing the development of toxic shock syndrome, a disease that arises from toxic shock syndrome toxin-1 activating non-specific T-cell proliferation [234, 235]. These ligands include lipoteichoic acid and peptidoglycan [235].

The immune-modulatory molecules lipoteichoic acid and peptidoglycan are not the only cell wall associated molecules that contribute to *S. aureus* virulence. *S. aureus* expresses cell colonisation factors that allow for the bacteria to invade and/or colonise a host organism. Cell wall anchored proteins (CWA) have distinct domains and bind different ligands. In *S. aureus*, CWAs support survival and proliferation in the commensal state and their ability to bind to desquamated epithelial cells is vital during colonisation [236]. They also play a key role in invasive infections due to their ability to bind fibrinogen, which has significance during pathogenesis [236]. There are two fibronectin binding adhesins which are Fn-binding proteins A and B (FnBPA and FnBPB) [237]. These adhesins can bind ligands such as fibronectin, elastin and plasminogen, as well as histones which neutralises their

antimicrobial activity [237]. Fibronectin forming a bridge between α5β1 on the host side and FnBPs on the bacterial side is a prerequisite for any invasion/internalisation into non-phagocytic host cells [238]. FnBPs are not the only CWAs that are involved in *S. aureus* colonisation and virulence. Clumping factor A (ClfA) is another CWA that acts as a virulence factor due to the promotion of adhesion to fibrinogen, a blood plasma protein, causing clumping of *S. aureus* in the blood. [239]. All clinical strains of *S. aureus* carry the ClfA gene[240]. SdrC is another staphylococcal cell wall anchored protein that is involved in virulence and colonisation. SdrC forms low affinity homophilic bonds that promote intracellular adhesion and therefore biofilm formation, and it also forms strong hydrophobic bonds with surfaces, emphasising its role as a multifunctional adhesin [241].

Staphyloxanthin and other carotenoid pigments also have the potential to be virulence factors. Bacterial carotenoids have been shown to protect the bacteria from immune cells such as macrophages [242, 243]. The antioxidative activity of staphyloxanthin specifically also makes it a potential virulence factor as it promotes neutrophil resistance and development of subcutaneous abscesses in mouse infection models [244, 245].

With regards to antibiotic resistance, exposure to cosmic radiation causes increased mutation rates, which can lead to changes in antibiotic resistance [102]. Studies have also shown that simulated microgravity culture alone causes antibiotic tolerance (a temporary resilience to antibiotic treatment that inhibits the effect of usually bactericidal doses of antibiotic) to occur in *E.coli*, with some tolerances persisting once returned to 'normal' gravity culture [107]. These antibiotic resistance and tolerance changes have been thoroughly investigated [102, 107, 246-249].

It has been shown that both simulated microgravity grown [186] and spaceflight flown [249] *S. aureus* develop antibiotic resistance. One mechanism for this increased antibiotic resistance is the promotion of horizontal gene transfer in microgravity [246]. The question remains is that if growth and antibiotic resistance is increasing, how are these prokaryotes

affecting their surrounding environment through cell or surface colonisation and subsequent biofilm formation or virulence factor deployment, which can all have negative effects on human health and cause damage to air and water filters aboard spacecraft.

Bacterial virulence has also been investigated to ascertain how this is altered in microgravity and spaceflight to understand better the illness-causing potential of the causal microorganisms. Virulence can be defined as the ability of bacteria to invade and persist inside a host-organism, and to subvert the host defence mechanisms [250]. Interestingly, different bacteria have differing microgravity induced responses to each other. *Salmonella typhimurium* has shown increased virulence in microgravity culture through increased survival in an infection model by modulating its metabolism for enhanced survival [111, 251], increased virulence and recovered numbers from a murine spleen and liver following oral infection in modelled microgravity [252]. *S. aureus* has been reported to have reduced virulence in microgravity culture through reductions in alpha haemolysin production [87] and in a Caenorhabditis elegans killing model [253].

Staphylococcus was one of the species most commonly found in the air and upon the surface of manned space vessels [66]. In *S. aureus,* both virulence and cell colonisation are co-ordinately controlled by the *agr* (accessory gene regulator) quorum sensing system. Quorum sensing has been investigated in other prokaryotes, such as *Stenotrophomonas maltophilia* [185], and other genetic regulation mechanisms, such as RpoS in *S. enterica* Serovar Typhimirium [254], with regards to the space context. Research into these genetic regulation mechanisms is based on the finding that microgravity culture effects stress-response and virulence factor production [252].

Quorum sensing is a cell to cell communication mechanism in which cells can communicate information regarding local cell density and adjust their gene expressions accordingly [194]. This cell to cell communication works through the production of diffusible chemical signal molecules sometimes called autoinducers [255]. When a minimal threshold stimulatory concentration of

these signal molecules is achieved in the local environment and detected by the bacteria, gene expression is altered [255]. Table 4.1.1 below outlines some examples of the quorum sensing systems in different bacteria.

Table 4.1.1: Summary of different quorum sensing mechanisms in prokaryotes.

Organism	**Gram positive/ Negative**	**Signal Molecule(s)**	**Brief Description**	
Staphylococcus aureus	Positive	Autoinducing peptides 1-4	Regulation of colonisation/virulence through *agr*	
Stenotrophomonas maltophilia	Negative	Diffusible signal factor	*Rfp* cluster for synthesis and perception	[256]
Pseudomonas aeruginosa	Negative	Acyl homoserine lactones, alkyl quinolones	3 systems – PasRI, RhlRI and PqsR/PqsABCDE/PqsH	[257, 258]
Bacillus cereus	Positive	PapR peptide	Activation of virulence regulator PlcR	[259]
Vibrio cholerae	Negative	CAI and DPO autoinducer	Activation represses virulence	[260, 261]

The *agr* quorum sensing in *S. aureus* regulates the expression of virulence and colonisation factors [262], as shown in Figure 4.1.1. It is controlled through cell density dependent mechanisms which is achieved through the production and sensing of an autoinducing peptide (AIP) [263]. The structures of AIPs produced by *S. aureus* are shown in Figure 4.1.2.

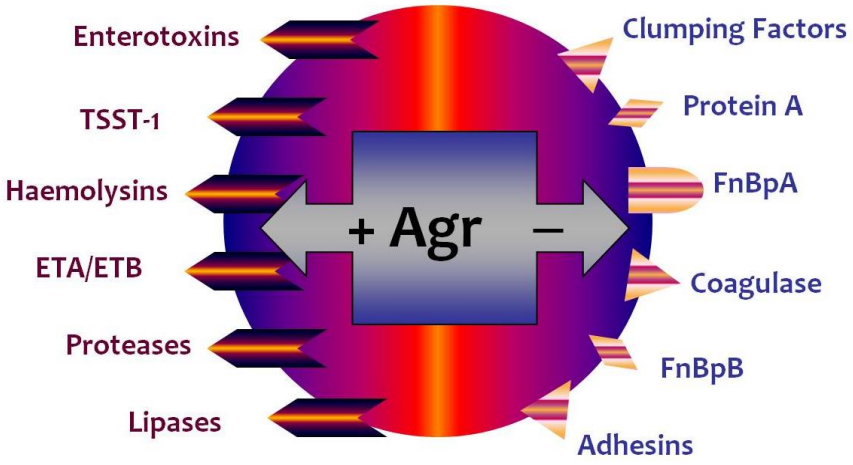


Figure 4.1.1: Regulation of the factors produced by the induction of the *agr* operon. During low cell density, *agr* is not induced and production of colonization factors such as adhesins and Protein A occurs. Once a certain cell density is reached and detected through sensing of the quorum sensing molecule AIP, *agr* is induced and colonization factor production is stopped in favor of virulence factor production of factors such as enterotoxins and proteases.

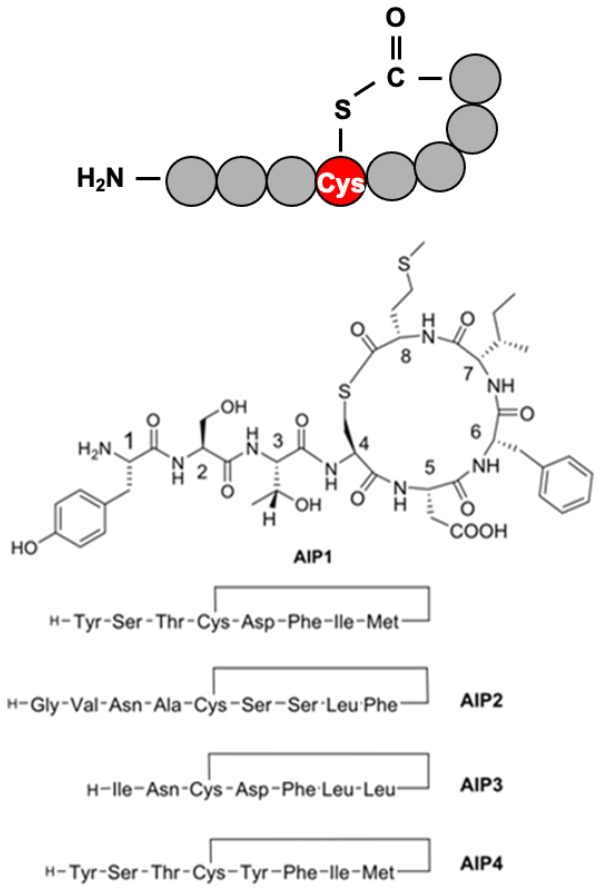


Figure 4.1.2: Structure of the 4 AIPs produced by *S. aureus*. AIP is an autoinducing molecule that is synthesized by *S. aureus* and induces the *agr* operon when a certain local concentration threshold is met and sensed by agrC. Each different AIP has different induction concentrations and can work synergistically or antagonistically to each other.

The *agr* locus consists of four genes, *agrB, agrC, agrD*, and *agrA* [264] in a locus that contains two divergent transcriptional units (*agrBDCA)* and RNAIII controlled by the P2 and P3 promoters respectively. Figure 4.1.3 from Sloan et al illustrates the *agr* gene locus and the components of the *agr*-QS system.

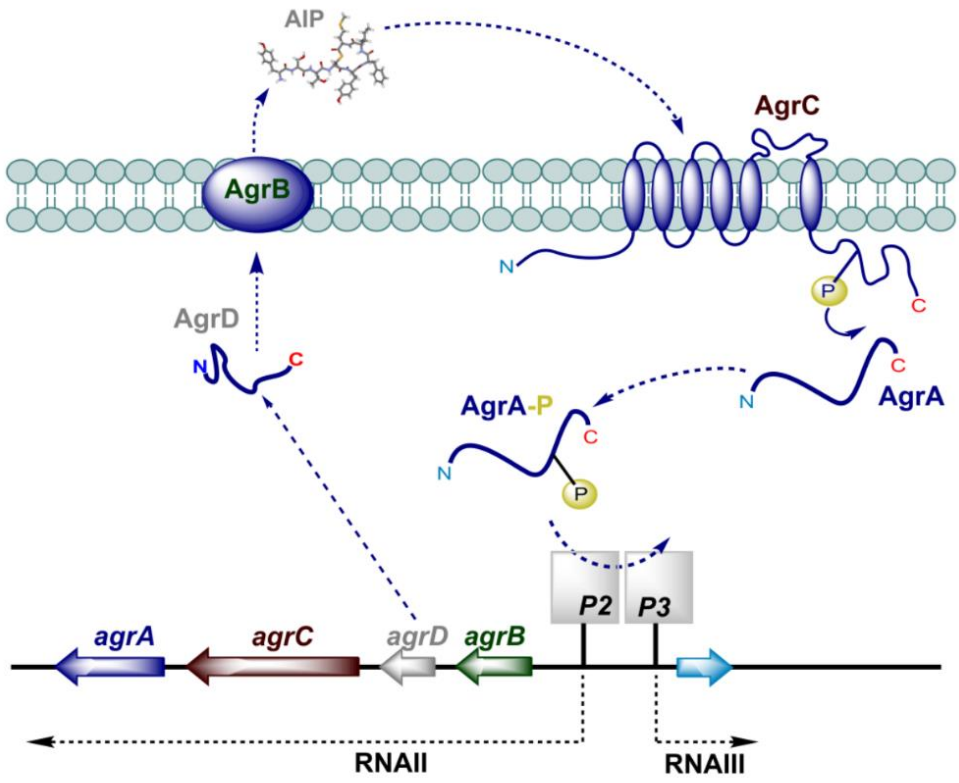


Figure 4.1.3: The *agr* gene locus and components of the *agr*-QS system [181]. The *agr*-QS system is an autoinducing circuit. Transcription of the RNAII genes produces the AgrD precursor of AIP. This is then cleaved and transported across the membrane by AgrB as AIP. AgrC senses AIP and causes phosphorylation of AgrA which in turn binds to the P2 and P3 promoter of the *agr* operon inducing the regulated responses.

The quorum sensing system mechanisms of sensing and production of AIP is a positive feedback loop and is auto-inducing [180]. First of all, AIP is made by AgrB and then the AgrD peptide precursor undergoes MroQ processing [265]. AIP interacts with AgrC, activating the phospho-relay cascade that results in the phosphorylation of AgrA [184]. AgrA then binds to the P2 promoter and upregulates the expression of all four *agr* genes inducing positive feedback leading to the production of more AIP [184].

Starting with AgrB, this is a 22 kDa peptidase, 6 transmembrane domain protein located in the cytoplasmic membrane, and consists of [182, 266].

AgrB is involved in the post-translational modification of AgrD [267]. AgrB proteolytically processes AgrD, removing the C -terminus and generating a thiolactone intermediate [184]. A second cleavage event via MroQ removes the N-terminal peptide and releases the AIP extracellularly [184].

AgrC is the histidine protein kinase that binds AIP and activates the response regulator AgrA [181]. A single point mutation in AgrC, such as tyrosine to cysteine at position 223 of AgrC, can vastly alter the phenotype of *S. aureus* by changing the expression of virulence and colonisation factors [268] and causing differences in the timing of *agr* activation [181].

Finally, AgrA is the response regulator that binds to the P2 and P3 promoters within the RNAIII-*agr* intergenic region [269]. AgrA is activated to bind its target promoters through phosphorylation by AgrC [270].The *agr* operon is not the only regulator of virulence factor production. The SaeRS two component system was discovered in 1994 and controls production of 20+ virulence factors (including leukocidins, haemolysins, superantigens, proteases and surface proteins) [271]. Specifically, SaeRS up -regulates *hla, hlb, hlgABC, lukED and coa* [272]. This regulator is also involved in adaptation, biofilm formation and cell survival [273].

The SrrAB system detects metabolic changes due to infection in the infected host through redox sensitive cysteines and enables further survival of the bacteria at sights of infection by increasing resistance to nitrosative, hypoxic and oxidative stress [274].

Another two-component system is ArlRS and has a 70% identical RNA sequence to that of another regulator, MgrA [275]. Mutants with deletions or insertions in ArlRS and/or MgrA have been shown to be less virulent due to the de-repression of Ebh, a large surface protein [276]. Additionally , these mutants are unable to kill neutrophils and survive direct neutrophil attacks highlighting the importance of these cascades in evasion of the immune response [276].

The SarA family of regulators (which include the previously discussed MgrA) are usually expressed during the exponential phase in which adhesins such as fibronectin are being produced [277]. Interestingly, the transition in virulence from colonisation factor production to virulence factor production, with the activation of *agr,* corresponds to the maximal expression of SarA family of regulators. [277].

SigB is sigma factor that inhibits *agr* activity [177]. SigB silences *agr* allowing for bacterial cells to persist intracellularly in the host cells switching the cells from a virulence factor producing, *agr* active phenotype to an *agr* silent, non-cytotoxic phenotype [278] This factor is crucial for the transcription of heat, oxidative and antibiotic stress resistance [278].

It has been shown that in microgravity *S. aureus* has reduced virulence factor production and an increased host cell colonisation phenotype [253], which is attributed to a downregulation of the RNA chaperone, Hfq [186]. This alteration of Hfq expression has been reported across multiple Gram negative and Gram-positive bacterial species [84, 94, 279].

Both cell colonisation and virulence factor production are regulated in *S. aureus* through the *agr* quorum sensing system [178, 184, 280]. The reported increased cell colonisation and reduced virulence is indicative of either reduced activation of the *agr* operon, causing less switching of the phenotype, or delayed activation of the *agr* operon, in which the colonising phenotype persists longer and the bacterium misses the window of opportunity to express virulence factors by activating the operon too late into the growth phase [181].

Because of the increased cell colonisation phenotype, [186], and reduced production of virulence factors such as alpha toxin [87], reported for *S. aureus* grown in microgravity culture, the function of the *agr* operon was investigated. It can be hypothesised that microgravity analogue culture in an RCCS either reduces or delays *agr* activation as in 'normal' culture conditions,

delayed or reduced *agr* activation would maintain a cell colonisation phenotype while repressing virulence factor production.

It is shown that LSMMG culture does not impact on the growth or morphology of an MRSA and MSSA strain. These strains are shown to have decreased supernatant cytotoxicity and prolonged colonisation factor expression. With bioreporters and analysis of AIP, it is shown in LSMMG culture AIP production is reduced and *agr* induction is delayed and reduced. AgrC-AIP interactions are shown to not be affected by LSMMG culture and it is hypothesised that the most likely reason for the LSMMG induced changes to *agr* induction are due to changes in AIP generation. Finally, supernatant cytotoxicity is shown to be a recoverable function in LSMMG culture with exogenous AIP addition and confirms that AgrC-AIP interactions are not impacted by LSMMG culture.

4.2 Methods

Bacterial strains and growth media

The *S. aureus* strains used in this study are listed in Table 4.2.1. Bacteria were routinely grown in tryptic soy broth (TSB) at 37^{o}C for 16 h with shaking at 200 rpm or on tryptic soy agar (TSA) at 37^{o}C for 24 h. *S. aureus* was also cultured in X-Vivo 15 serum free media without gentamicin or phenol red (Lonza). For RCCS experiments, *S. aureus* was cultured in 2 mL autoclavable vessels (Synthecon) and incubated at 37^{o}C in a 5% CO2 atmosphere. Samples were rotated at 25 rpm in a vertical orientation for LSMMG cultures and horizontal orientation for control cultures. Bacterial growth was recorded by taking a 100 µL sample every hour for the first 8 h and at 24 h. This was added to 900 µL X-Vivo15 media in a quartz cuvette and an optical density at 600 nm ($OD_{600)}$ reading taken in triplicate and recorded.

Table 4.2.1: *S. aureus* strains used in this study.

Strain	Description	Reference
USA300 JE2	Plasmid-cured derivative of the CA-MRSA strain USA300 LAC; *agr* group I	[200, 201]
SH1000	MSSA strain; Functional *rsbU* derivative of 8325-4, *rsbU*$^+$; *agr* group I	[281]
ROJ 143	RN4220 *agr::tetM+P3:luxCDABE pSKermP2:agrC1 agrA*	[202]
ROJ 154	RN4220 *agr::tetM+P3:luxCDABE pSKermP2:agrC3 agrA*	This laboratory; Rasmus Jensen, University of Nottingham, Ph.D thesis 2009
USA300 *gfp*	*attB2: xylA-gfp*	This laboratory
SH1000 *gfp*	*attB2: xylA-gfp*	This laboratory
USA300 *agrP3*::*lux*	Luminescent strain of USA300with *attB2: agrP3::luxCDABE*	This laboratory
USA300 Δagr agrP3::lux	*agr*deletion mutant with *attB2: agrP3::luxCDABE*	This laboratory
TS13	Clinical isolate; ST22 MSSA spa t005 PVL+, leg abscess	[181, 282]
TS14	Clinical isolate; ST22 MSSA spa t005 PVL$^+$, leg ulcer	[181, 282]

Cell attachment:

Bacterial cell attachment assays were performed by adding 1 x 10^6 cells from either 4 h or 24 h growth of *gfp* expressing strains (USA300 and SH1000) to a well in a fibronectin coated 24 well plate. This was incubated at 37^{o}C for 30 min and washed three times with phosphate buffered saline (PBS) OD_{600} and fluorescence at 488nm were recorded in a microplate reader (TECAN).

THP-1 cytotoxicity

Cytotoxicity was measured by culturing THP-1 cells (human leukaemia monocytic cell line) in RPMI-1640 media overnight and adding 1 x 10^6cells per well to a 24 well plate. Supernatants from overnight USA300 or SH1000 cultures (or for AIP addition study, USA300 + AIP overnight culture) were added to the THP-1 cells and cell viability was measured by adding trypan blue in a 1:1 ratio, adding 10 µL of this solution onto a haemocytometer slide and recording percentage viability on an automated cell counter (Countess, ThermoFisher).

Activation of agr:

The activation of *agr* over time was quantified by sampling 100 µL of USA300 *agrP3::lux* culture every h between 2-9 h of growth. 100 µL of fresh medium was added to culture vessels for every sample taken. USA300 *agrP3::lux* was cultured in a 2 mL HARV vessel on an RCCS in either a horizontal or vertical orientation. The medium used for this was X-Vivo 15 serum free medium (Lonza). These samples were pipetted into 96 well plate in a microplate reader (TECAN) where luminescence and OD_{600} were recorded.

AIP detection and quantification:

Cultures containing AIPs were centrifuged after 24 h and the cell-free supernatant collected. For the *agr* bioreporter assay, 5 µL of supernatant was added to a 1:250 dilution of overnight ROJ143 culture in a 96 well plate in triplicate. This was placed in a microplate reader (TECAN) for 16 h at 37^{o}C and luminescence and OD_{600} recorded every 15 min. When required for quantification, the area under the curve for each timepoint was calculated and plotted using GraphPad. Liquid chromatography mass (LC MS/MS) spectrometry was also performed on the supernatant for AIP1 alongside synthetic standards for quantification.

Mass spectrometry:

Chromatography was achieved using a Shimadzu series 10AD VP LC system. The column oven was maintained at 40°C. The HPLC Column used was a Kinetex core-shell XB-C18 (2.6 µm; 50 x 3.0 mm) with an appropriate guard column. Mobile phase A was 0.1% (v/v) formic acid in water, and mobile phase B 0.1% (v/v) formic acid in methanol. The flow rate throughout the chromatographic separation was 450 µL/min. The binary LC gradient initially began at 10% B for 1.0 min, increased linearly to 99% B over 5.0 min. The composition remained at 99% B for 1.0 min, decreased to 10% B over 0.5 min, and stayed at this composition for 3.5 min. Total run time per sample was 10 min.

The MS system used was an Applied Biosystems Qtrap 4000 hybrid triple-quadrupole linear ion trap mass spectrometer equipped with an electrospray ionisation (ESI) interface. Instrument control, data collection and analysis were conducted using Analyst software (v1.6.3). Source parameters were set as: curtain gas: 25.0, ion source potential: 5000 V, temperature: 450 °C, nebulizer gas: 20.0, auxiliary gas: 20.0, and entrance potential: 10 V. The MS detection was conducted with the MS operating in MRM (multiple reaction monitoring) mode. Positive electrospray conditions (+ES) conditions were used based upon the parameters published by Junio et al [283], using a $[M+H]^+$ precursor ion of *m/z*=961 with a product ion of *m/z*=711 following collision induced dissociation (CID). 10 µL of the sterile filtered supernatant samples, without any further sample preparation, were analysed using the developed LC-MS/MS methodology. Biological samples were run in a single batch of samples alongside calibration samples of a synthetic standard of AIP-1 prepared in MeOH, ranging from 0 nM to 1000 nM.

Activation of agr via exogenous AIPs:

For exogenous AIP studies, AIP-1 or AIP-3 was added at a range of concentrations (0.5 nM to 250 nM for AIP-1 and 10 nM – 500 nM for AIP-3) to

ROJ143 and ROJ154, respectively, immediately prior to inoculation in X-Vivo 15 medium (Lonza). Bacteria were cultured in an RCCS in either a horizontal or vertical orientation. ROJ143 is an engineered *S. aureus* strain that emits light when the *agr* operon is activated by AIP-1, the strain cannot produce its own AIP [202]. ROJ154 is identical apart from it is activated by AIP-3. This strain was also engineered by Rasmus Jensen at University of Nottingham. 100 µL samples were taken every h between 2-8 h and pipetted into a 96 well plate in a TECAN plate reader where luminescence and OD_{600} was recorded. For every sample taken, 100 µL of fresh medium containing the same concentration of AIP was added.

4.3 Results

Initially, it was investigated whether LSMMG culture resulted in changes to the phenotypes of USA300 and SH1000 by analysing their growth, cytotoxicity for THP-1 cells, and attachment to a fibronectin coated plate. Growth (Figure 4.3.1A and 4.3.1B) and virulence factor production via aTHP-1 cytotoxicity assay (Figure 4.3.1C) for exotoxins and cell colonisation ability as reflected by the fibronectin binding (Figure 4.3.1D). The monocytic cell line THP-1 is sensitive to *S. aureus* toxins and makes an excellent recipient of the potentially produced toxin for cytotoxicity assays. By applying 10 µL of the overnight medium from the cultures used to produce Figure 1A and 1B to a known amount of THP-1 cells for a small time period, viability of the THP-1 cells was measured to investigate differences in exotoxins between control and LSMMG cultured *S. aureus.*

Fibronectin binding was chosen as the proxy for the colonisation phenotype analysis as *S. aureus* produces two cell wall anchored fibronectin binding proteins, FnbA and FnbB [237, 238].

Additionally, morphological changes to both USA300 and SH1000 was investigated and found no differences by transmission electron microscopy. (Appendix).

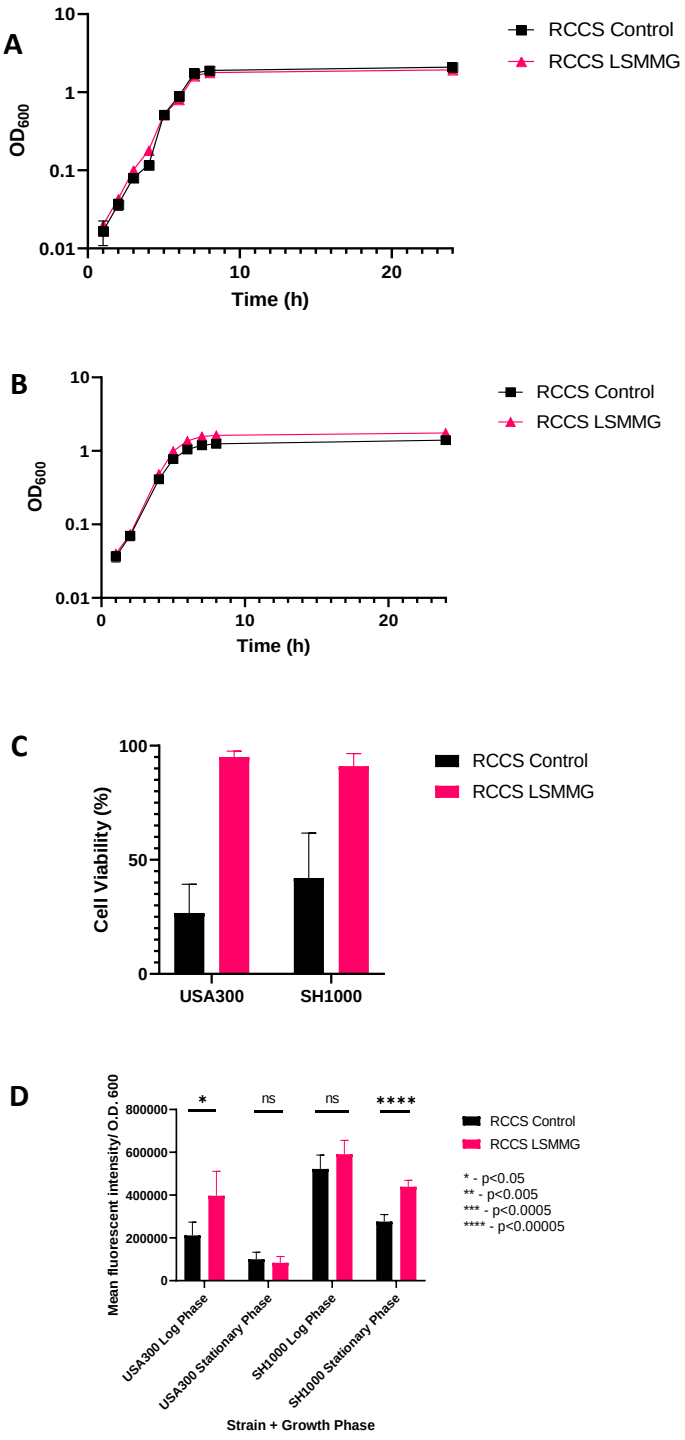


Figure 4.3.1: Growth, cytotoxicity and fibronectin-binding by *S. aureus* USA300 and SH1000 strains grown in RCCS vessels under normal and micro-gravity conditions . (**A)** and (**B**) 24 h growth curves for USA300 and SH1000 respectively. (**C**) cytotoxicity of cell-free culture supernatants, bars show THP-1 cell viability after incubation with bacterial supernatant. (**D**) fibronectin binding. Log phase samples were taken after 4 h of growth and stationary

phase samples were taken after 24 h of growth. Independent t-tests were performed on this data using GraphPad. For all experiments, N=3 and n=3. Error bars show standard deviation of the mean.

Figures 4.3.1A and 4.3.1B show that low-shear culture does not affect the growth of USA300 and SH1000.

Figure 4.3.1C shows that in control conditions, both USA300 and SH1000 secreted exotoxin virulence factors that were released into the supernatant which were cytotoxic for THP-1 cells with USA300 producing a more cytotoxic supernatant than SH1000. When supernatant was supplied from the LSMMG cultures however, both supernatants exerted negligible cytotoxicity for the THP-1 cells and no differences between USA300 and SH1000 were observed.

The production of cell wall colonisation factors was investigated via the proxy of binding to fibronectin. This was measured using a fibronectin coated plate binding assay.

Fig. 4.3.1D shows that for both log phase and stationary phase conditions, SH1000 exhibited increased fibronectin binding compared to USA300. Also, across both strains, there was a decrease in fibronectin binding in the stationary phase compared with the log phase.

Fig. 4.3.1D shows that for USA300, there is an increase in fibronectin-binding after LSMMG culture compared to control culture for the log phase samples. However, the stationary phase samples showed no significant differences.

Interestingly, the reverse occurred for SH1000. The log phase samples showed no significant differences in fibronectin binding between LSMMG and control culture, but for the stationary phase samples there was a significant increase in fibronectin binding for the LSMMG samples compared to the control ones even though the reduction of fibronectin binding from log phase to stationary phase follows the same expected pattern as USA300. This is due to the activation of *agr* switching the phenotype from a colonising factor producing phenotype to a virulence factor producing one.

As cytotoxicity was reduced and fibronectin binding protein expression prolonged in microgravity, it was investigated whether growth under LSMMG conditions impact on *S. aureus agr*-dependent quorum sensing as it regulates the production of virulence factors. Examined first was *agr* expression during growth and quantified AIP production. To begin with, a strain of USA300 that has a *luxCDABE* cassette fused to the *agr*P3 promoter was used. Due to this alteration, this strain emits light as the *agr* system is activated and can be recorded at different time points to investigate timing of *agr* activation and levels of *agr* activation.

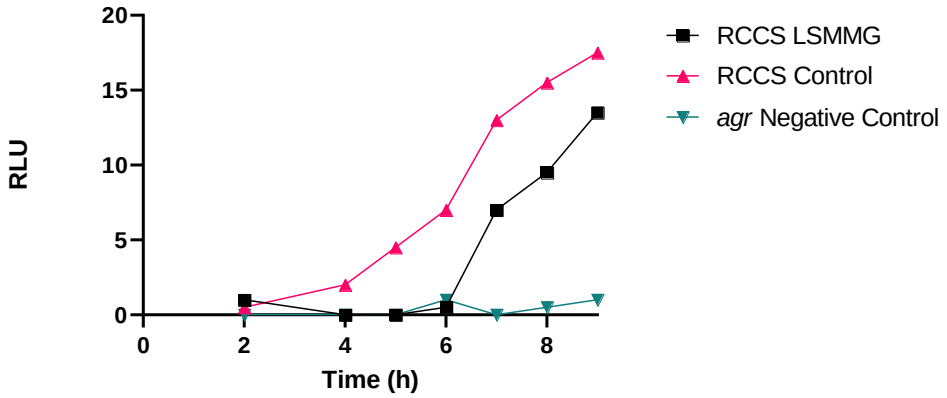


Figure 4.3.2: Growth of USA300 *agr*P3::*lux* under LSMMG conditions influences the timing of *agr* activation. The bioluminescence of USA300 agrP3::Lux over time in X-Vivo 15 media. A USA300 *agr* deletion mutant that retained the *agr*P3:lux cassette was used as a control for background bioluminescence N=3.

Figure 4.3.2 shows that in LSMMG culture in X-Vivo, *agr* activated between 6 and 7 h which is 3-4 h later than the control culture which activated around the 3- and 4-h mark. As a difference in the timing of *agr*P3 activation was observed, this could contribute to the phenotypic differences observed.

To determine whether the differential expression of *agr* in normal vs microgravity is a consequence of delayed AIP accumulation, the *agr* bioreporter assay, ROJ143 and mass spectrometry were both used to quantify production of AIP-1.

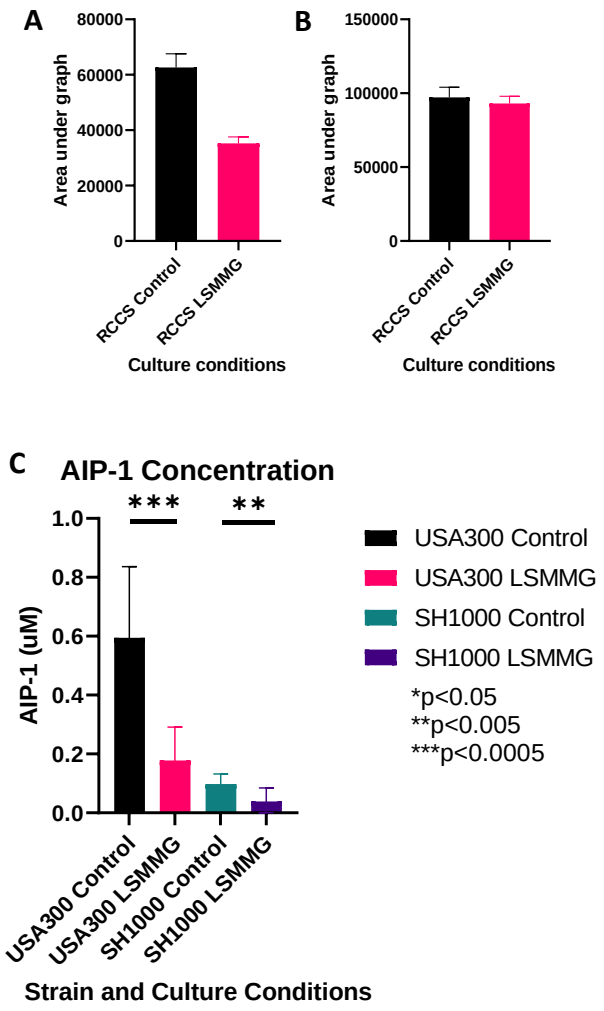


Figure 4.3.3: AIP production by *S. aureus* USA300 and SH1000 grown in X-Vivo 15 medium in RCCS in control and LSMMG orientations to stationary phase (after 16 h growth). (**A**) and (**B**) show AIP concentrations as determined using the ROJ143 reporter assay for USA300 and SH1000 respectively. (**C**) shows LC-MS/MS quantification of AIP produced. Independent t-test was performed using GraphPad. For all experiments N=3 and n=3. Error bars show stanard deviation of the mean.

AIP production in both USA300 and SH1000 was analysed from the cell-free supernatant prepared from overnight growth in X-Vivo 15 medium. Figures 4.3.3A and 3B show the ROJ143 bioreporter assay results for AIP-1 produced. Figure 4.3.3A shows culture in LSMMG reduced the AIP-1 produced by ~45% compared with control culture for USA3000. Figure 4.3.3B shows that for

SH1000 AIP-1 levels reached the same concentration in stationary phase in both culture conditions. LC MS/MS was performed to confirm the results and found a reduction of approximately 30% AIP in LSMMG culture (0.18 µM) compared with the control culture (0.6 µM) in USA300. A smaller reduction to 40% was observed between LSMMG culture (0.04 µM) and control culture (0.1 µM) in SH1000 for AIP production. For an example chromatogram of how this was measured, see Appendix. USA300 produced more AIP-1 than SH1000 in both culture conditions.

As it has now established that the timing of *agr* expression and AIP production differed between normal and microgravity culture, it was next investigated whether the delayed induction of *agr* in LSMMG culture was due to reduced AIP production using reporter strains. These reporter strains cannot synthesise their own AIP but still respond to exogenous AIP allowing for activation of the *lux* cassette on the *agr*P3 promoter. For reference, AIP-1 has a reported EC_{50} of 3.21 nM whereas AIP-3 has a reported EC_{50} of 406 nM [284] for their respective reporter strains (ROJ143 and ROJ154 respectively).

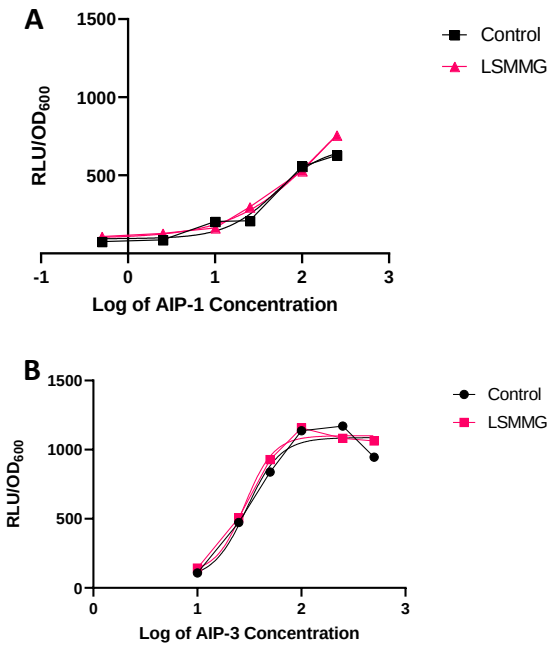


Figure 4.3.4: Addition of exogenous AIP to reporter strain to investigate maximum *agr* activation in control and LSMMG RCCS orientation. (**A**) shows AIP-1 added to ROJ143. (**B**) shows AIP-3 added to ROJ154. Exogenous synthetic AIP was added to the reporter strain at a range of concentrations and subsequent light emission was recorded and peak emission values plotted. N=3, n=3.

Figure 4.3.4 shows that apart from the highest concentration in Figure 4.3.4A, there was no difference between *agr* activation for both AIP-1 and AIP-3 bioreporters in LSMMG culture compared to control conditions.

ROJ143 and ROJ154 are identical except for having either *agrC1* or *agrC3* genes and are activated by either AIP-1 or AIP-3 but cannot synthesize AIPs. To further investigate the role of LSMMG induced quorum sensing changes, two closely related clinical MSSA isolates that differ by a single nucleotide polymorphism in *agrC* that results in differential *agr* activation were cultured. By quantifying AIP production overtime, it can be observed if these SNP-dependent changes in *agr* activation persist in LSMMG culture. These two strains were selected as in 'normal' culture conditions, TS13 cannot properly

autoinduce due to the SNP whereas the SNP in TS14 leaves the quorum sensing mechanism fully functional [181].

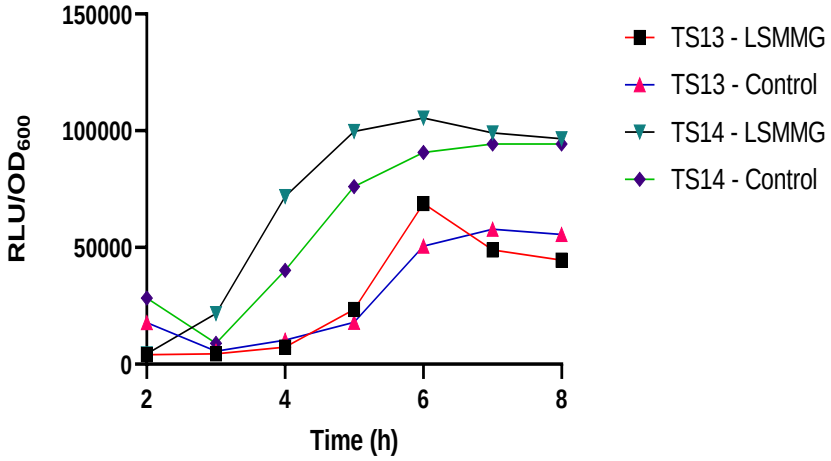


Figure 4.3.5: Differential AIP production as a function of growth in *S. aureus* clinical isolates TS13 and TS14, in control and LSMMG orientation RCCS cultures. AIPs in cell free supernatants from each time point sampled were assayed with ROJ143 and quantified by determining the area under the curve. (N=3, n=3).

As shown in Figure 4.3.5, TS14 begins AIP production at 3 h in control conditions and TS13 begins AIP production between 4 to 5 hours in control culture showing a significant activation delay. For LSMMG culture, these production times remained the same, however, between 4 to 7 hours, LSMMG cultured TS14 produced more AIP than the control culture but by the end of the culture this difference was negligible. The AIP production of TS13 in LSMMG culture remained unaffected.

As Figure 4.3.4 showed that LSMMG culture does not appear to impact on the interaction between AIP and AgrC, which is further supported by Figure 4.3.5, it was hypothesised that adding AIP into LSMMG culture would also recover the cytotoxic control culture phenotype of the strains. To do this 100 µM of AIP-1 was added at inoculation of a USA300 culture and after 16 h the supernatant was harvested and applied this to THP-1 cells as in Figure 4.3.1C.

Fig. 4.3.6 shows that provision of exogenous AIP-1 at the point of inoculation after growth in LSMMG restored the THP-1 cytotoxicity of USA 300 culture supernatants by ~80% to a similar level to that observed for *S. aureus* grown under control conditions in the RCCS.

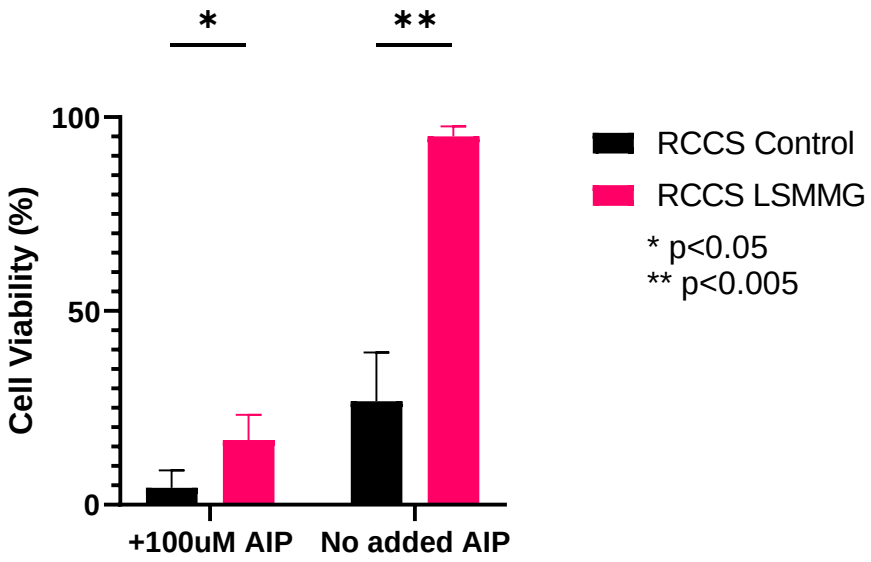


Figure 4.3.6: Viability of THP-1 cells exposed to 16 h cell-free supernatant from *S. aureus* USA300 grown with or without (control) AIP-1 (100 µM). Independent t-tests were performed using GraphPad. (N=3, n=3). Error bars show standard deviation of the mean.

As shown in Figure 4.3.6, there is a substantial increase in cytotoxicity for both control and LSMMG cultured USA300 at the end of one culture cycle. However, when both control and LSMMG culture have the same amount of exogenous synthetic AIP-1 added, there is still a significant increase in cytotoxicity for control USA300 compared with LSMMG USA300. The LSMMG + AIP cytotoxicity is now similar to the control culture without exogenous AIP.

4.4 Discussion

agr is a quorum sensing system conserved in many different Gram-positive bacteria. RCCS culture keeps the bacteria in their own micro-orbit, keeping the cells in suspension in a state of constant free-fall, and inside a closed system not allowing for changes in nutrient access or waste removal which may impact the growth of the bacteria. Growth changes in a microgravity analogue culture for *S. aureus* have already been reported to be minimal [86], when using a random positioning machine, or not significant at all [87], when using an RCCS. However, it has also been shown for a different *S. aureus* strain that growth in an RCCS is reduced compared to control conditions [186]. These growth differences between the studies are possibly due to each study using a different culture medium with tryptic soy broth [186], Müller-Hinton broth [87] and lysogeny broth [86]. Different growth media can result in different growth profiles and other bacterial responses and without standardisation of the growth conditions used it is ill advised to compare the results between these studies. As the LSMMG environment is low-shear, the lack of external forces on the cell may not be able to counter-balance the internal turgor pressure forces of the bacterial cells causing morphological changes. Differences between USA300 and SH1000 were expected to be observed due to USA300 being a CA-MRSA which usually have altered cell walls due to methicillin resistance induced changes compared to MSSAs [285]. These cell wall changes can lead to reduced virulence through interference with the *agr*-QS system but the reduction of penicillin-binding protein 2A expression seen in CA-MRSAs in comparison with HA-MRSAs allow them to maintain a full virulence arsenal [285]. However, this was shown not to be the case for the MSSA and MRSA strains investigated (Appendix). As LSMMG culture did not affect growth or morphology of both USA300 and SH1000, any changes to quorum sensing mechanisms or phenotypes regulated by the *agr*-QS system are therefore not caused by LSMMG induced changes to cell population density. However, consideration must be taken with regards to

this as the size of the quorum is not fixed and can be affected by other environmental factors. For example, AIP-1, one of the QS signal molecules, is rapidly inactivated by oxidation and therefore affects when the cells reach a quorum [286]. Therefore, it is important to investigate if LSMMG culture alters activation of *agr* and/or AIP production.

In *S. aureus,* cell colonisation and virulence factor production are regulated by a sophisticated regulatory network involving multiple regulatory systems including *agr*-dependent quorum sensing.

Both cell colonisation and virulence factor production responses are multi-factorial and can be investigated in a plethora of ways. Virulence factors can be investigated through western-blots, ELISA (enzyme-linked immunoassay), flow cytometry, lysis/killing assays and PCR (polymerase chain reaction) amongst other accepted techniques. Cell colonisation factors can be investigated through specific binding assays, co-culture with epithelial cells, flow cytometry, PCR and various microscopies.

Fibronectin binding proteins FnbA and FnbB play important roles in cell attachment and subsequent colonisation and invasion of human endothelial cells[237]. Therefore, cell colonisation can be investigated through the proxy of fibronectin binding. By showing an increase in binding to a fibronectin coated plate in LSMMG cultured *S. aureus*, it was shown that there may be reduced induction of *agr* which leads to lower repression of fibronectin binding protein expression in LSMMG culture.

Bacterial cell to bacterial cell adhesion in LSMMG culture has also been shown to be increased with a study in which the *S. aureus* MRSA strain N315 was found to have more extra-cellular polymeric substance, polymers involved in cell cohesion and adhesion, present around the bacterial cells in LSMMG culture [186]. This increase in a cell colonisation factor may be one of the reasons that *S. aureus* forms more resilient biofilms aboard vessels such as the International Space Station in a faster fashion than that on Earth [186, 287]. Biofilms (*P. aeruginosa)* aboard space vessels form a column and

canopy structure not seen on Earth and have increased biofilm mass [93]. This novel biofilm structure is dependent on flagella driven motility [93]. Attachment is the first step in biofilm formation, although attachment and colonisation does not always lead to biofilm formation, and fibronectin binding protein expression is one of the main ways *Staphylococci* adhere to biotic surfaces [238]. More work should be undertaken specially tailored to investigating biofilm formation in LSMMG culture and study the expression of all the colonisation factors and other factors required for surface attachment leasing to biofilm formation and processes involved, along with the time to form and architecture of the biofilm itself.

Colonisation factors are primarily expressed when *agr* is not expressed and the cells switch to producing virulence factors when *agr* is expressed. Therefore, the length of time that *agr* is not expressed for directly effects how long the bacteria express cell colonisation factors such as fibronectin binding proteins. As shown, LSMMG culture increased fibronectin binding in the stationary phase in LSMMG culture in comparison with control culture conditions. From this, it can be hypothesised that LSMMG culture may be responsible for the delay in *agr* activation resulting in the retention of a colonization phenotype and a delay in *agr*-dependent virulence factor expression. Additionally, a substantial delay to *agr* activation would lead to the cells missing their 'window of opportunity', a period of bacterial growth in which *agr* is responsive to AIP reaching the threshold concentration, to produce virulence factors [181].

Virulence factor production in *S. aureus* through production of staphyloxanthin and haemolytic toxins has been investigated in LSMMG culture and shows that LSMMG culture causes decreased staphyloxanthin production and decreased erythrocyte haemolysis, indicating decreased exotoxin production [87]. This chapter confirms the findings in the study by Rosado et al by showing a decrease in supernatant cytotoxicity towards human THP-1 cells, a monocyte line which is sensitive to all toxins produced by *S. aureus*. Rosado et al also reported reductions in *hla* expression, an *agr*-

regulated gene that codes for alpha haemolysin production, and a two-fold reduction of the Sae/SaeS regulatory system expression, a two-component system responsible for regulation of over 20 virulence factors including alpha haemolysin [87, 271]. The virulence factors investigated in these studies are also regulated by *agr* expression and support our hypothesis of an *agr* activation delay causing reduced virulence factor production as a sufficiently long activation delay would lead to reduced production of the toxins investigated. Looking at the results of this investigation and Rosado et al highlight how more than one regulatory system may be being affected by LSMMG culture leading to the less virulent phenotype. It is interesting to note that between these two studies, five different strains of *S. aureus* were tested and included both MSSAs and MRSAs and all five strains followed the same response of decreased LSMMG virulence factor production. It is known that MRSAs, especially USA300, produces a greater amount of virulence factors than MSSAs [288], due to increased expression of *agr* and *saeRS* [289], so it is interesting to see in LSMMG culture that both USA300 and SH1000 show almost no supernatant cytotoxicity towards THP-1 cells.

Castro et al reported increased *S. aureus* colonisation and decreased virulence and show the role in which *hfq* downregulation, already reported in Gram-negative organisms [279], contributes to this in the LSMMG response [186]. These Hfq findings are of an interesting nature as it has been shown that Hfq does not play a crucial role in the stress response of *S. aureus* or a crucial role in RNAIII and exoprotein expression [290].

Like *S. aureus,* other species of bacteria also show enhanced colonisation factor production and enhanced biofilm formation (which was not directly tested here) [111, 185]. This could prove significant in highlighting a common prokaryotic response to microgravity. However, some species of bacteria were shown to have increased virulence as opposed to the reduced *S. aureus* virulence [111, 112, 185].

It was further investigated that the possibility of *agr* activation alterations were occurring in LSMMG culture by quantifying *agr* expression over time as

luminescence using an *S. aureus* strain that had a *luxCDABE* cassette fused to the *agr*P3 promoter and by determining the concentration of AIP produced over 9 h growth to stationary phase. This experiment showed delayed and reduced production of AIP in LSMMG culture which is insufficient to activate the downstream target genes. Few investigations of quorum sensing in LSMMG have been carried out [185, 291, 292] with no investigations to date directly investigating quorum sensing of *S. aureus* in LSMMG culture.

Induction of *agr* requires activation of AgrC by AIP. To see if this is altered in LSMMG culture and causing the reduced and delayed *agr* activation, ROJ143 and ROJ154 reporter strains were employed and AIP added to them exogenously. These cannot produce their own AIP and therefore cannot autoinduce the *agr* system but due to them containing a *luxCDABE* cassette fused to the *agrP3* promoter, they produce light when activated by AIP provided exogenously. ROJ143 is activated by AIP-1 and ROJ154 is activated by AIP-3 and these were added respectively. By adding exogenous AIP, it can be investigated whether the AIP-AgrC interactions are being altered by LSMMG culture. As there was no difference in the activation response for both ROJ143 and ROJ154 in LSMMG culture compared to control culture when exogenous AIP was added, it can be determined that the AgrC-AIP interactions are not affected by LSMMG culture. This was further investigated by the addition of AIP-1 to USA300 culture to look at its effects on virulence factor production via cytotoxicity to THP-1 cells. The addition of AIP increased the cytotoxicity of the LSMMG culture supernatant to similar levels of the control culture with no AIP added. This also shows that AgrC-AIP interactions are not affected by LSMMG culture and that LSMMG induced changes to *S. aureus* that are regulated by *agr*-QS may be able to be reduced/recovered by the addition of exogenous AIP.

The TS13 and TS14 strains were employed confirm that AgrC-AIP interactions were unaffected by microgravity and to investigate the hypothesis that the delayed and reduced *agr* activation is due to LSMMG induced changes to AIP generation. As these clinical strains only differ by a SNP but have differing *agr*

induction responses (TS14 has unaltered induction whereas TS13 has reduced *agr* induction), it is known that the differences in *agr* induction and subsequent responses are due to this SNP [181]. The major finding from the LSMMG investigation into these two clinical isolates was that TS14, which has 'normal' *agr* induction, shows an altered AIP production response in LSMMG culture compared with control culture whereas TS13, which has reduced *agr* induction, shows no differences in AIP production over time in LSMMG culture compared with control culture. As TS14 showed an altered LSMMG response and TS13 did not, along with the previous investigations into AgrC-AIP interactions with the ROJ143 and ROJ154 reporter strains and recovery of supernatant cytotoxicity in LSMMG culture when exogenous AIP is added to USA300, it can be determined that AgrC-AIP interactions are not responsible for the *agr* activation reduction and/or delay in LSMMG culture and that the most likely hypothesis is that it is due to changes to AIP generation.

Ruling out agrC-AIP altered interactions in LSMMG culture leaves open a variety of possible hypotheses for the reduced AIP production noted in this study. Each stage of the agr system could be affected by LSMMG culture leading to reduced AIP production. The proteolytic processing of AgrD by AgrB into a thiolactone intermediate could be altered along with the second cleavage of this intermediate as well. Additionally, the export of AIP and cleaved N-terminal AgrD peptide across the membrane [293] could also be affected by LSMMG conditions, as it has been shown in eukaryotic cells that cell membranes have altered fluidity in microgravity culture [169]. Whether or not this translates to bacterial membranes remains to be seen. However, due to the nature of the ROJ bioreporters, it has been shown that there are no alterations to AgrC activating AgrA as there are no LSMMG induced differences to activation when exogenous AIP is added therefore this part of the *agr*-QS system can be ruled out regarding LSMMG changes. One way to investigate these possibilities would be to use the TS13 and TS14 investigation in this study as a framework and identify and use clinical isolates, or engineer lab strains that only differ by a SNP in either AgrA, AgrB or AgrD but have

differing *agr* induction responses. If both isolates/strains then respond in the same way to LSMMG culture then this could provide basis for further investigation into how that factor is responsible for the LSMMG observed reduced AIP production and delayed *agr* activation. Additionally, the use of MroQ mutants could also provide insight onto the LSMMG induced changes to *agr*-QS due to its' role in removing the N terminal amino acids of AgrD [294].

In conclusion, it has been shown that in LSMMG culture, both an MSSA strain and MRSA strain show prolonged expression of cell colonisation factors via the proxy of fibronectin binding and decreased virulence factor production. These phenotypes combined can be explained by reduced and delayed *agr* activation during growth in LSMMG culture. It has been subsequently shown that interactions between AIP (inducing molecule) and AgrC (receptor) are not affected by LSMMG culture through the use of two ROJ bioreporters and provide additional insights in this with two clinical isolates that only differ by a SNP but have different *agr* induction responses. This is further backed up by adding exogenous AIP to USA300 in LSMMG culture, which produces a cytotoxic supernatant compared with the non-cytotoxic supernatant in LSMMG culture with no AIP added.

Quorum sensing in microgravity is a novel field and has only been performed on a few bacterial species [185, 291, 292]. Due to the diversity of quorum sensing mechanisms, there are many avenues of investigation to be explored. For the first time, this investigation explored the possibility of quorum sensing changes in *S. aureus* in LSMMG culture and provides evidence for *agr* activation alterations. The investigation also provided evidence for these *agr* alterations to be the reason for the shown and previously reported LSMMG phenotype of increased cell colonisation and reduced virulence factor production in LSMMG culture. This study finished by beginning to eliminate possible mechanistic changes to the agr quorum sensing system and rules out changes to AgrC-AIP interactions whilst suggesting future avenues of

exploration into the other components of *S. aureus* quorum sensing involved in AIP production via processing of AgrD.

Key Findings:

- **LSMMG culture does not alter growth of USA300 or SH1000**
- **LSMMG culture prolongs colonisation factor expression and reduces virulence factor production**
- **LSMMG culture reduces and delays *agr* activation and reduces production of AIP**
- **Exogenous AIP can restore virulence factor production**
- **AgrC-AIP interactions are not affected by LSMMG culture**

5. Interactions between monocytes and *Staphylococcus aureus* in microgravity

5.1 Introduction

It has been established in Chapter 2 that monocytes exhibit a reduced pro-inflammatory response in LSMMG culture which may impact their ability to respond to infection. It has also been established that *Staphylococcus aureus* has reduced and delayed *agr* activation leading to an increase in cell colonisation phenotype and decreased virulence factor production which may cause them to become less cytotoxic and increase their invasiveness of host cells.

Both responses may work synergistically explaining the increased infection rate reported during spaceflight and the persistent chronic infections once returning to Earth [295]. This chapter contains investigations into how monocytes and *S. aureus* interact with each other in LSMMG culture, builds upon findings from the previous chapters and investigated if the reported findings from these chapters of decreased and delayed *agr* activation in *S. aureus* and reduced pro-inflammatory response in monocytes work synergistically together to result in increased bacterial invasion and persistence inside monocytes and how these two previously found results contribute to this.

Pathogen associated molecular patterns (PAMPS) are factors produced by foreign bodies that the host immune system can recognise and respond to. *S. aureus* expresses PAMPs such as lipoproteins, lipoteichoic acid and peptidoglycans [296]. Secreted factors such as phenol-soluble modulin, Protein A and haemolysin are inflammatory to host organisms along with the cell wall components (peptidoglycans and alanylated lipoteichoic acid) [167]. Virulence factor production (haemolysin and Protein A) discussed in detail in Chapter 3 and Chapter 4.

The staphylococcal cell wall composes of murein (peptidoglycan), teichoic acids and wall associated proteins [297]. These cell wall components can all be recognised by host immune cells to trigger an immune response. Cell wall compositions can vary between staphylococcal strains. For example, MRSA strains of *S. aureus* have thicker cell walls than MSSA strains [298].

Peptidoglycan is a dynamic structure and constantly undergoes peptidoglycan turnover where bacteria degrade between 40-50% of their peptidoglycan layer during bacterial growth and reproduction, releasing peptidoglycan fragments into the surrounding environment [299]. These fragments can be subsequently sensed by receptors such as TLRs and NOD-like receptors (NLR) [299]. Interestingly, peptidoglycan recognition protein, or PGRP, is an ubiquitous protein involved in innate immunity as shown from its' conservation from insects to humans [300].

Lipoteichoic acid (LTA) is found in Gram-positive bacteria and is a surface associated amphiphile that is released usually after bacteriolysis from an immune or antibiotic response [301]. LTA plays an important role in activating the immune response as LTA mutants showed over half loss of immune activation activity and therefore is the primary TLR2 activator in the early immune response to Gram-positive pathogens [302].

For both peptidoglycan and LTA, the signalling pathways they activate and/or regulate have been shown to be suppressed in immune cells [303]in microgravity but focussed study on the changes to these in the bacteria themselves are scarce.

The innate immune system, including monocytes, recognises *S. aureus* through pattern recognition receptors such as toll-like (TLR), (NOD)-like (NLR) and C-type lectin (CLR) which activate intracellular signalling pathways to upregulate the transcription of pro-inflammatory response genes [304]. TLR4 has already been discussed in Chapter 2 with regards to monocyte stimulation with LPS from *E. coli* and co-culture with *S. aureus*.

Nuclear oligomerization domain (NOD) like receptors are intracellular proteins that regulate the host innate immune response by acting as scaffolding proteins for signalling platforms that activate mitogen-activated protein kinase (MAPK) pathways and/or the NF-κB signalling pathway [305]. NOD's are highly conserved cytosolic PRR's and check the internal environment for presence of infection [306]. Upon finding a disturbance, the NOD's oligomerize into macromolecular scaffolds that enable fast deployment of signalling cascades to restore internal homeostasis [306].

It has been shown that when macrophages are co-cultured with *E. coli,* TNFα and IL-6 production is suppressed along with the signalling pathways p38 mitogen-activated protein kinase and the Erk1/2 MAPK pathways [303], which are regulated by NOD's [307, 308]. Microgravity changes to NOD's have been scarcely investigated and could be a potential avenue for future research.

CLR's are more involved with the adaptive immune response in comparison with TLRs and NLRs and can induce adaptive immune response signalling either by themselves, through inducing carbohydrate specific signalling pathways or by crosstalk with other PRRs [309]. They are known for their role in antiviral immunity and viral escape [310]. However, with bacterial infection, they sense glycans which are expressed by bacteria [311]. CLR's have been investigated in microgravity with regards to diminished dendritic cell immunogenicity [312] but any LSMMG induced changes in other immune cells has yet to be sufficiently investigated.

Endocytosis is the collective term for processes in which material is brought inside a cell [313]. The two most commonly known processes are phagocytosis and pinocytosis.

Phagocytosis is the ingestion of micro-organisms, apoptotic bodies or other foreign materials by cells classed as phagosomes [314]. The four main steps of phagocytosis are, recognition of target, activation of internal mechanisms via signalling, phagosome formation and finally phagolysosome maturation [315].

There have been investigations into microgravity induced phagocytosis changes. Through diamagnetic levitation it has been shown that the arp2/3 pathway in macrophages is altered in this microgravity analogous culture and may lead to decreased phagocytic ability [176].

Microgravity induced phagocytosis changes can also be inferred from other studies as well. TNF-α production increases macrophage phagocytosis [316] and it has been shown that TNF-α production is reduced in microgravity [144] which could lead to decreased macrophage phagocytosis. Another microgravity induced change is to that of the cytoskeleton, specifically disorganised tubulin expression and disturbed actin cytoskeleton which may cause dysfunctional macrophage responses and therefore reduced phagocytosis [316]. This change and subsequent reduction in phagocytosis has also been reported in neutrophils [317].

Monocytes have also been shown to have reduced phagocytic ability in spaceflight conditions and had reduced ability to engulf *E. coli*, elicit an oxidative burst and degranulate resulting in a reduced phagocytic index [318].

Pinocytosis is the mechanism of up taking liquids and dissolved solutes into the cell through endocytosis. Pinocytosis has not been directly studied in microgravity conditions and is scarcely mentioned in the literature. One of the only studies into the innate immune response of *Drosophila melanogaster* found a change to the *gartenzwerg* gene and is involved in clathrin-independent pinocytosis and suggests there may be a microgravity induced reduction to pinocytosis [319].

Co-cultures and infection models have been studied in microgravity via a plethora of studies. The immune response of macrophages has been investigated with infection from *E. coli* [303], showing a reduction in pro-inflammatory cytokine production.

Using a *Caenorhabditis elegans* model, bacterial infection was analysed by measuring the amount of debris produced by the dead worms, due to infection, in comparison with unaffected healthy worms to see if the bacteria

had increased infectivity or not [253]. This investigation found that the tested bacterial strains of *Klebsiella pneumoniae*, *Staphylococcus aureus*, *Streptococcus pneumoniae*, *Enterococcus faecalis* and *L. monocytogenes* all exhibited decreased worm death and therefore virulence [253]. As already highlighted in Chapter 1 however, there is no clear trend across species of bacteria as infection of *Drosophila melanogaster* with *Serratia marcescens* shows an increase in virulence in both spaceflight and LSMMG conditions [320].

Macrophages co-cultured with *S. enterica serovar Typhimurium* showed activation of the stress associated mitogen activated protein kinase, kinase 4 [321]. Furthermore, the bacteria themselves had an augmented invasive potential and increased tumour necrosis factor alpha (TNFα) production in infected epithelial cells [321]. The same study also found increased production of *E.coli* heat-labile enterotoxin in co-cultures [321]. Finally it was also shown by this study that murine macrophages infected with enteropathogenic *E. coli* also showed increased production of TNFα [321]. Furthermore, co-cultures have also shown that in microgravity monocytes have reduced ability to engulf *E.coli* [36]. CD32 and CD64 which are involved in phagocytosis have also been shown to be reduced in surface expression [318].

Lipopolysaccharides (LPS) are major membrane surface components that are endotoxins present in most Gram-negative bacteria with a few rare exceptions and are strong simulators of innate immunity [322]. Stimulating immune cells grown in microgravity with LPS therefore provides insight into how immunological crosstalk occurs during spaceflight and the response of the immune system to endotoxins. This has already been previously investigated in this thesis with regards to NF-κB translocation in monocytes. A major biochemical response after challenge with LPS is p38 MAP kinase activation via phosphorylation [323]. P38 mitogen activated protein (MAP) kinase are one of four main sub-groups of MAPs that mediate cellular behaviours in response to external stimuli [324], which potentially includes

microgravity. It was hypothesised that p38 MAP kinase would be sensitive to microgravity as there are various genes such as PRKCA that are regulated by p38 MAP kinase and are sensitive to microgravity [325]. However, monocytes exposed to spaceflight did not show impairment of p38 MAP kinase and actually showed a slight increase in activation [326]. C. elegans in analogue culture increased transcriptional expression of three genes that encode the core p38 MAPK pathway and expression of phosphorylated PMK-1/p38 MAPK [327]. These genes were pmk-1, nsy-1 and sek-1 [327].

LPS stimulation also elicits ROS (reactive oxygen species) production in macrophages. This was investigated in a microgravity analogue via Syk phosphorylation [164]. Syk phosphorylation was significantly reduced in microgravity when macrophages were stimulated by LPS, zymosan or curdlan [164], revealing that ROS production in macrophages is sensitive to gravitational forces. Other studies confirm this by showing ROS production in various cell types is increased in a microgravity analogue [328]. The study also found that NF-κB signalling was unaffected by microgravity which is a later step in the signalling cascade than Syk phosphorylation, and inconsistent with the studies previously discussed. This work resulted in the proposal of a hypothesis that during long spaceflights the immune system may be able to adapt to microgravity effects [164].

The issue with space medicine/biology field is the lack of consistency across investigations. Different investigations use different techniques, strains and infection host organisms not allowing for concrete conclusions to be drawn.

The investigations in this chapter centred around alterations to the interactions between monocytes and *S. aureus* in LSMMG culture. Based upon the findings of Chapter 2 and Chapter 4 it was hypothesised that LSMMG co-culture of *S. aureus* with monocytes should lead to an increase of viable internal *S. aureus* inside the monocytes in comparison with control culture. A secondary hypothesis was tested when the first hypothesis was found to be true; that the reason for this increased invasiveness is influenced by the LSMMG changes to *S. aureus*. To do this, *S. aureus* viability was

investigated when co-cultured with monocytes. It was then investigated how LSMMG culture affects phagocytosis and/or invasiveness of the monocytes by counting the number of internal bacteria. This was confirmed with imaging microscopy and further visualised with confocal microscopy. Additionally, as it has been shown the *agr* operon has delayed and reduced activation in LSMMG culture and that this could lead to a more colonising phenotype in *S. aureus,* a final investigation was undertaken to see if LSMMG culture induces differing responses in an *agr* mutant strain of *S. aureus* when co-cultured with monocytes with both external viability and internal counts being reported.

5.2 Methods

Bacterial strains used:

Bacterial strains used are listed in Table 4.2.1.

Monocyte culture:

Monocytes were harvested from donated blood as outlined in Chapter 4. They were cultured at a density of $1x10^6$ mL^{-1} in X-Vivo 15 serum free media (Lonza) at 37°C in a 5% CO_2 incubator.

Pre-culture:

For RCCS LSMMG pre-culture, cells were cultured in a 2 mL autoclavable HARV vessel (Synthecon) at 25 RPM.

Control bacteria (USA300 JE2) were cultured in a shaker incubator at 200 RPM at 37°C in 5 mL of X-Vivo 15 in a sealed 25 mL bijou tube. Control monocytes were cultured in a T25 cell culture flask at 37°C in a 5% CO_2 incubator.

Co-culture:

Control co-cultures were performed in an RCCS in a horizontal orientation in 2 mL HARV's at 25 rpm for 4-hours. Monocyte to bacteria ratios varied between experiments and are declared for each one.

LSMMG co-cultures were performed in an RCCS in a vertical orientation in 2 mL HARV's at 25 rpm for 4-hours. Monocyte to bacteria ratios varied between experiments and are declared for each one.

Manual counts:

Manual counts of external bacteria (USA300 JE2) were performed first by centrifuging the co-culture at 13000 rpm and resuspending the pellet in 1 mL PBS. This was then serially diluted by a factor of 10, six times and 10 µL of each dilution was added three times to an agar plate in separate rows for each dilution. This plate was incubated for 24 hours at 37°C. The first row with three countable repeats was counted and CFU/mL was calculated using the dilution this was from.

For internal counts, samples were centrifuged at 350 xG and supernatant collected. Gentamicin was added to this supernatant at 50 µg/mL for 1 hour. Cells were then centrifuged at 13000 RPM and supernatant removed. Cells were then resuspended in 1mL PBS and 1% Triton-X-100 was added for 15 minutes. This was then centrifuged for 1 minute at 13000 RPM and pellet resuspended in 1 mL PBS. External bacteria manual count method was then followed.

ImageStream analysis:

Co-cultures were performed for the conditions stated in the results using USA300-gfp. Co-cultures were centrifuged at 350 xG for 5 minutes and resuspended in 50 µL of 4% paraformaldehyde. Samples were then run on the ImageStream flow cytometer through a 488 nm laser and brightfield imaging. Images were processed on IDEAS software.

Confocal microscopy:

20 µL of USA300-gfp monocyte co-culture was put onto an APS coated microscopy slide. Slides were imaged on a Leica confocal microscope with a x40 oil immersion lens. Brightfield and 488 nm laser images were recorded, and z-stacks were taken through the visible monocytes. Images were processed on Zen (blue) 3.6. software.

5.3 Results

Both Figure 2.3.2 and Figure 2.3.6 overlap with this chapter and are the initial basis of what this results chapter is based upon. These results showed a reduction in external USA300 counts when cultured in LSMMG conditions compared with control culture but showed an increase in intracellular USA300 in LSMMG conditions compared with control culture. These figures can be found in Chapter 2 Section 3.

These previous results investigated the impacts of LSMMG culture on both monocytes and *S. aureus* at the same time. From both Chapter 2 and Chapter 4, it has been shown that LSMMG culture may impact both of these cell types with regards to interactions in co-culture. To see the impact of LSMMG culture with regards to co-culture interactions on the individual cell types, co-cultures were performed with one cell type pre-cultured in LSMMG and the other cell type pre-cultured in control conditions.

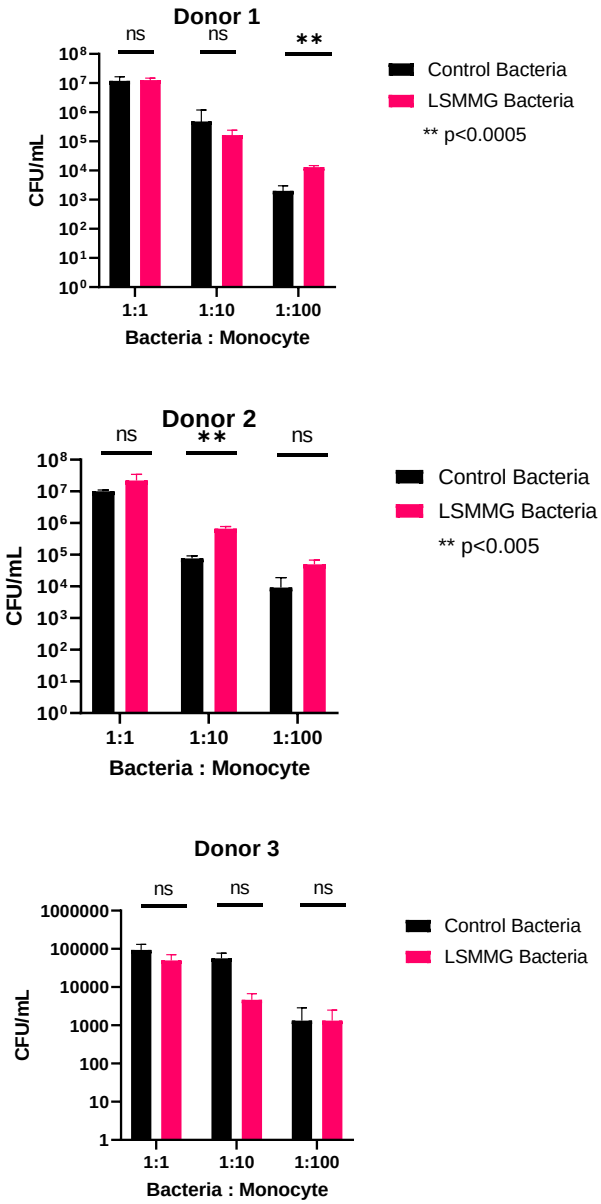


Figure 5.3.1: Manual counts of LSMMG cultured USA300 after 4 hours of LSMMG co-culture with standard cultured monocytes. USA300 was either cultured in an RCCS horizontal (LSMMG) or vertical (control) orientation. These were then added to monocytes grown in '1g' standard conditions in ratios shown on X axis. This co-culture was performed in an RCCS in the LSMMG orientation for 4 h. After 4 h, manual counts of USA300 were performed. Independent t-tests were performed on each donor and MOI using GraphPad. n=3. Error bars show standard deviation of the mean.

Figure 5.3.1 shows no discernible trend between the three donors as to whether LSMMG culture of USA300 affects the interactions with ‘1g’ cultured monocytes.

The same experiment was repeated but with USA300 in standard culture and the monocytes in 24 hours of 0g culture before being added to the 1g co-culture.

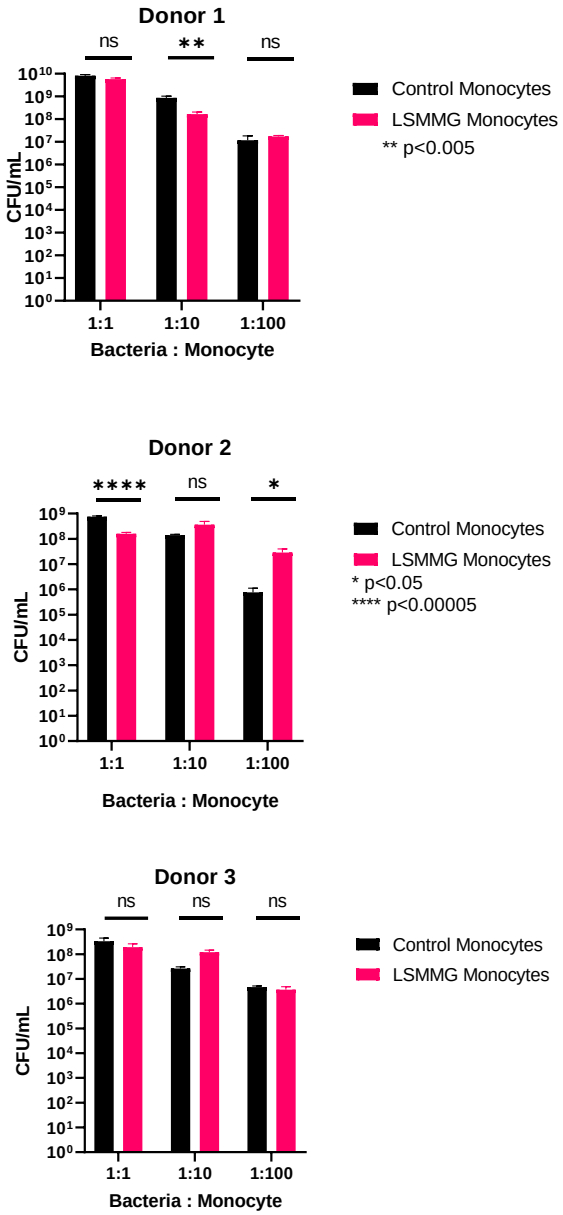


Figure 5.3.2: Manual counts of USA300 cultured for 4 hours with LSMMG cultured monocytes. Monocytes were either cultured in an RCCS horizontal (LSMMG) or vertical (control) orientation. These were then added to USA300 JE2 grown in '1g' standard conditions in ratios shown on X axis. This co-culture was performed in an RCCS in the LSMMG orientation for 4 h. After 4 h, manual counts of USA300 were performed. Independent t-tests were performed on each donor and MOI using GraphPad. n=3. Error bars show standard deviation of the mean.

Figure 5.3.2 shows no discernible trends for differences in counted USA300 across the donors.

Based upon the results of the Figures 5.3.1 and 5.3.2 and the co-culture results from the monocyte chapter (Figure 2.3.2 and Figure 2.3.6), the focus of this investigation was shifted to the LSMMG induced changes to *S. aureus* invasiveness in monocytes. ImageStream flow cytometry was employed to quantify bacterial invasion of monocytes in control culture (1g pre-culture + 1g co-culture), 0g pre-culture alone and 0g pre-culture with 0g co-culture. This was done using a USA300 strain that expresses gfp (green fluorescent protein) to allow it to be visualised. This also potential misinterpretation from clustered *S. aureus* cells that may have been similar in size to that of the monocytes.

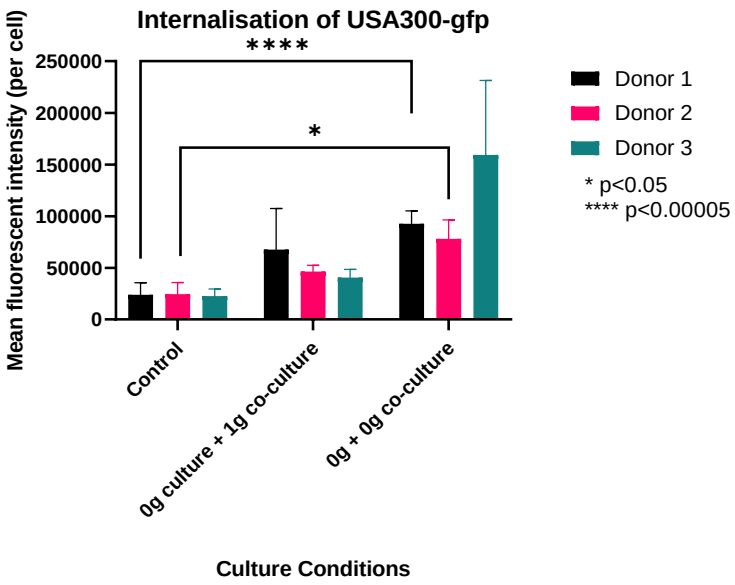
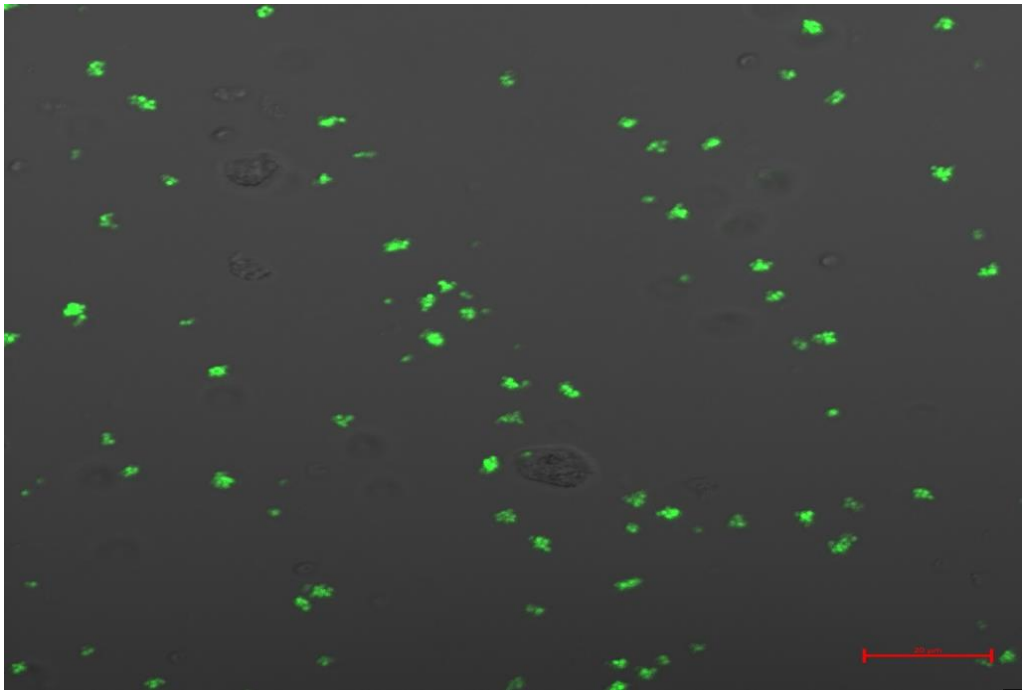
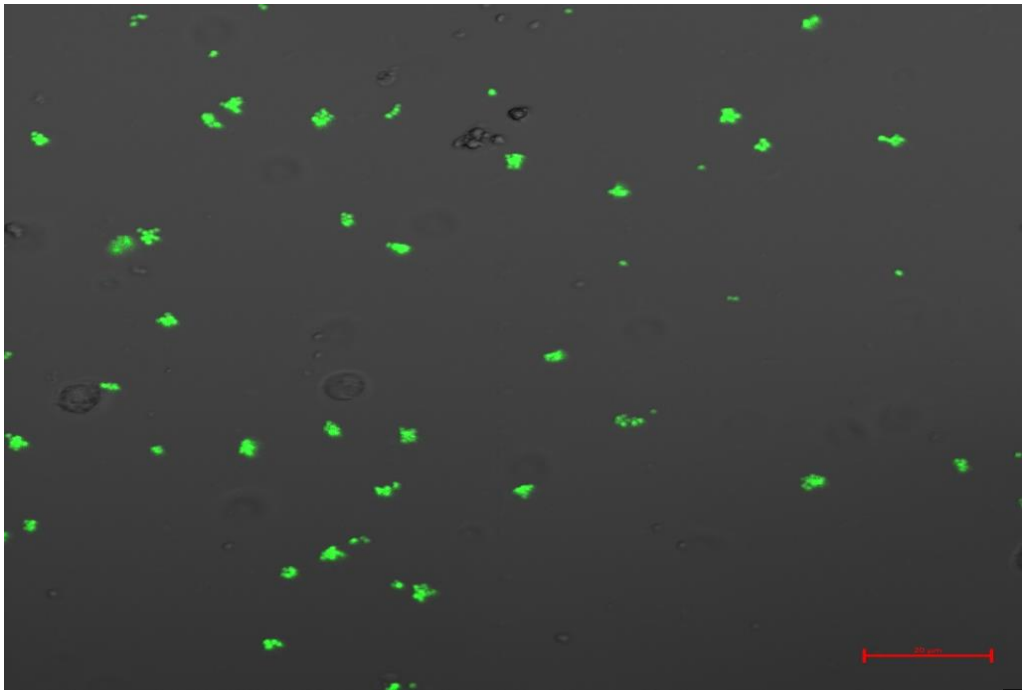


Figure 5.3.3: ImageStream flow cytometry of monocytes co-cultured for 4 h with a gfp expressing USA300 strain. USA300 strain expressing gfp was cultured at a 20:1 ration with primary monocytes for 4 h. After 4 h, co-culture was pelleted via centrifugation and resuspended in 4% paraformaldehyde. Samples were run on an ImageStream flow cytometer and collected cells were gated so that at least 100 monocytes were recorded per sample. Control culture was 1g pre-culture and 1g co-culture. Data was processed with IDEAS software. ANOVA analysis was performed using GraphPad. For $p>0.05$, lines were not drawn. For each donor, n=3. Error bars show standard deviation of the mean.

Figure 5.3.3 shows a significant increase in internalised USA300 for two of three donors when USA300 and monocytes are both pre-cultured and co-cultured in 0g conditions.

To help with visualisation of these results. Confocal microscopy was used for control and 0g pre-culture and co-culture conditions as these had the greatest change in internalised USA300.





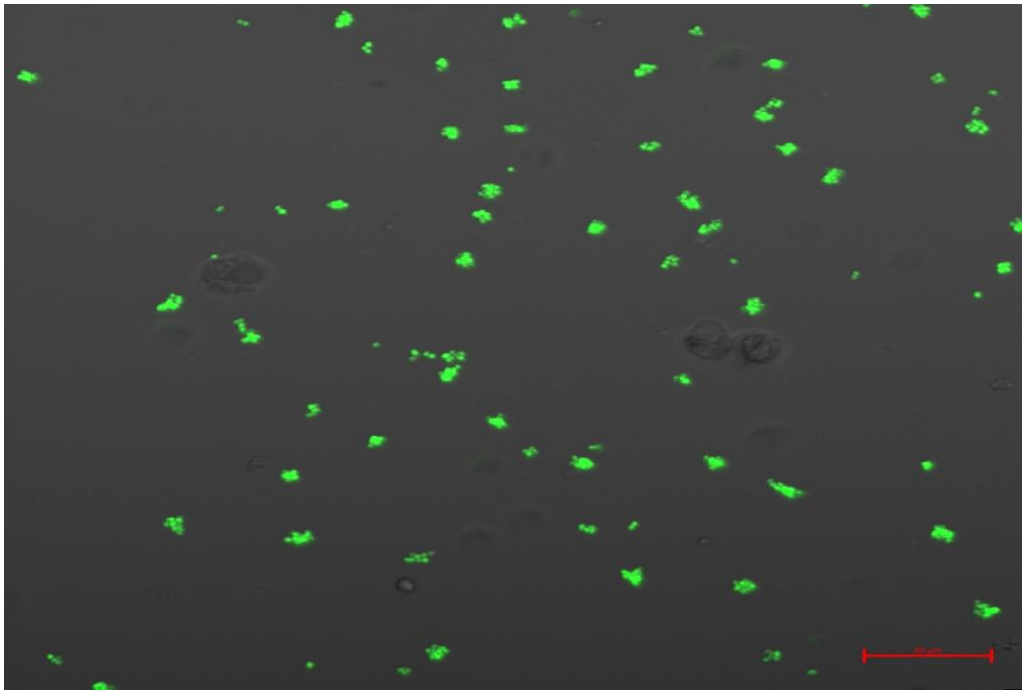


Figure 5.3.4: Confocal microscopy of 1g pre-cultured and co-cultured monocytes (unstained) and gfp expressing USA300 (green) at x40 objective lens. Three images were taken at different locations on the slide Scale bar is 20 µm.

As Figure 5.3.4 shows, monocytes can be clearly seen with little to no USA300 being internalised or associated with them.

For comparison, Figure 5.3.5 shows images from the LSMMG pre-culture and co-culture.

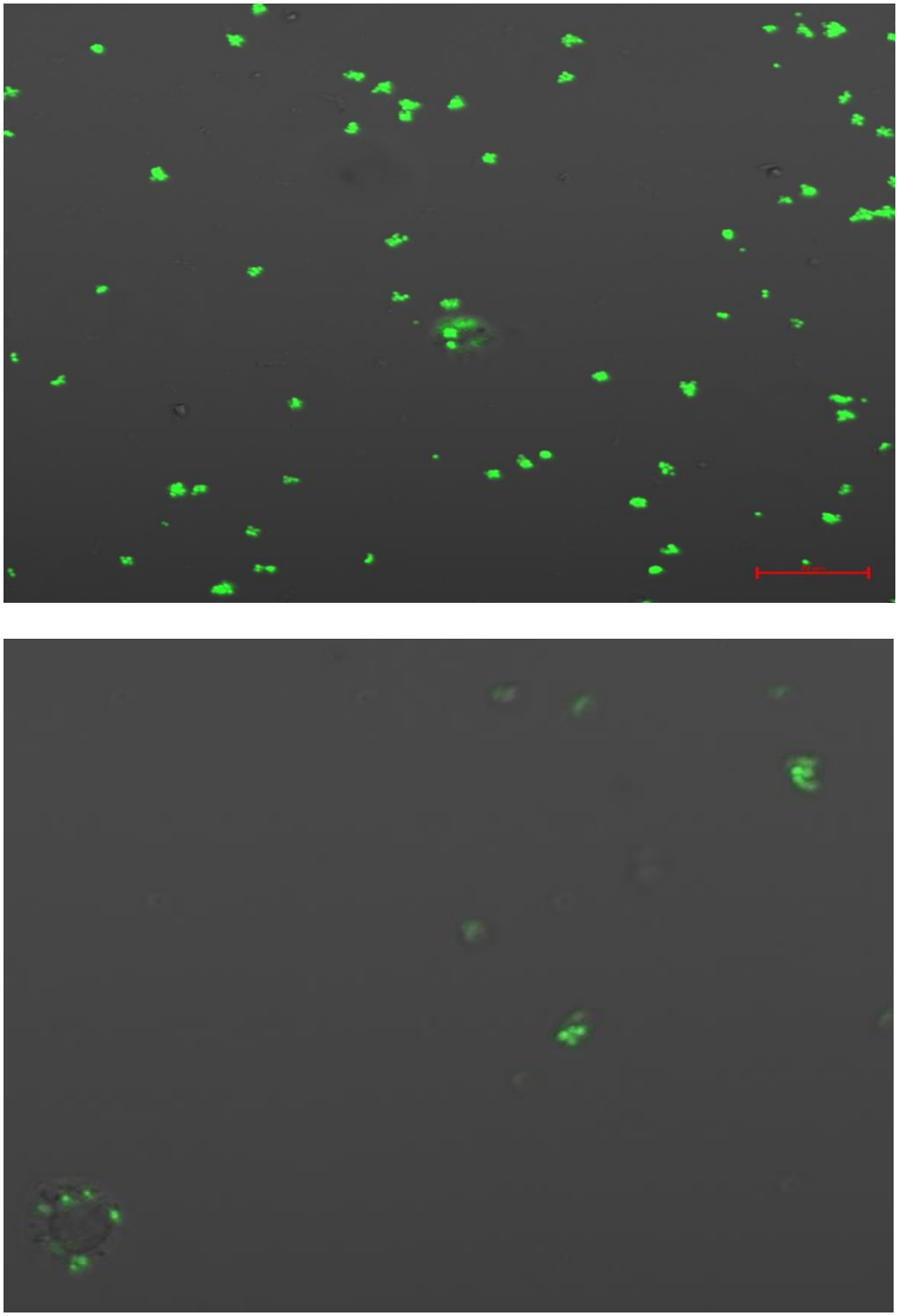


Figure 5.3.5: Confocal microscopy of 0g pre-cultured and co-cultured monocytes (unstained) and gfp expressing USA300 (green) at x40 objective lens. Two images were taken at different locations on the slide. Scale bar is 20 µm.

As shown in Figure 5.3.5, 0g pre-culture and co-cultured USA300 shows internalisation and association with monocytes in the culture.

To show the *S. aureus* was internalised and not just associated with the outside of the monocytes, z stacks were taken at regular intervals through the monocyte to investigate this.

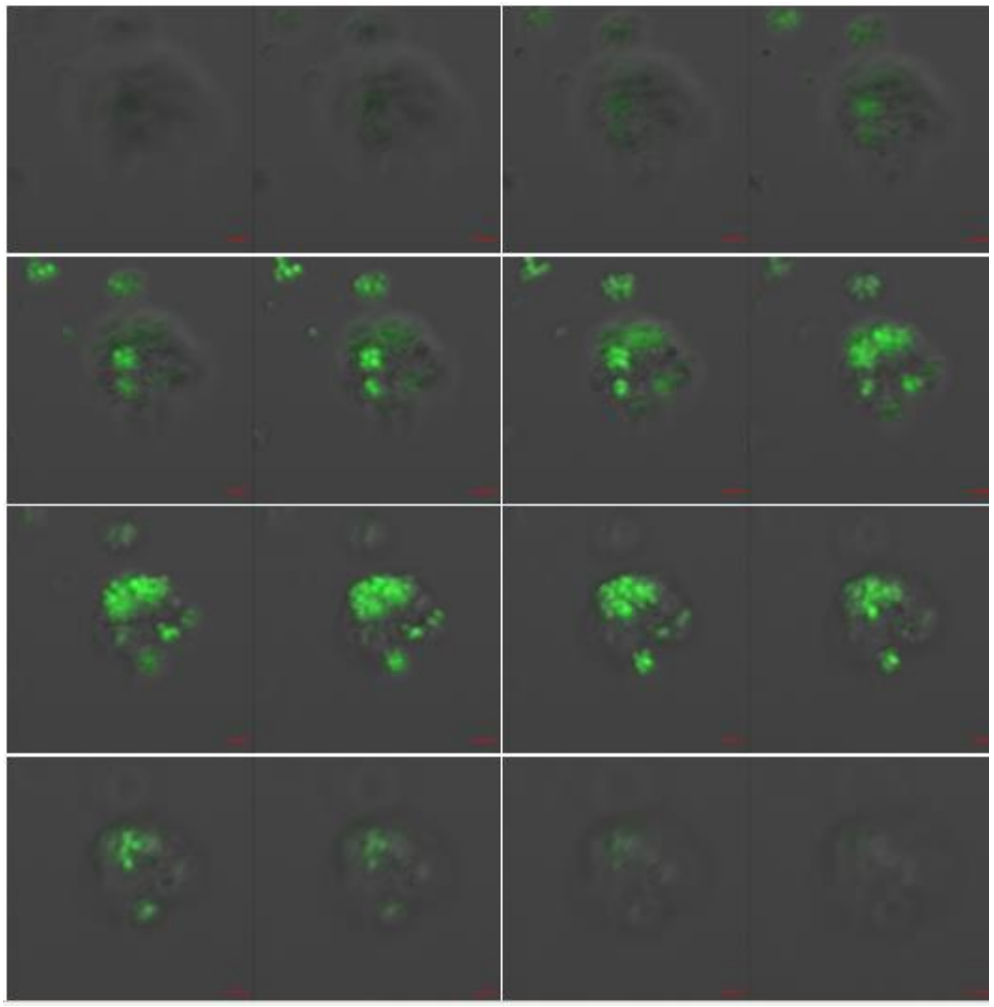


Figure 5.3.6: Confocal microscopy of 0g pre-cultured and co-cultured monocytes (unstained) and gfp expressing USA300 (green) at x40 objective lens. 16 z-stacks were taken through the pictured monocyte at regular intervals. Scale bar is 2 µm.

Figures 5.3.1 to 5.3.6 confirm the hypothesis from the Chapter 2 that LSMMG pre-culture and co-culture cause increased internalisation of USA300. From Chapter 4 it is known that LSMMG culture causes alterations to *agr* activation

and that this contributes to increased colonisation phenotype of USA300. To investigate if these now known *agr* changes are responsible for the increased USA300 internalisation, a USA300 *agr* mutant was used and manual counts were performed for the external and internal bacteria.

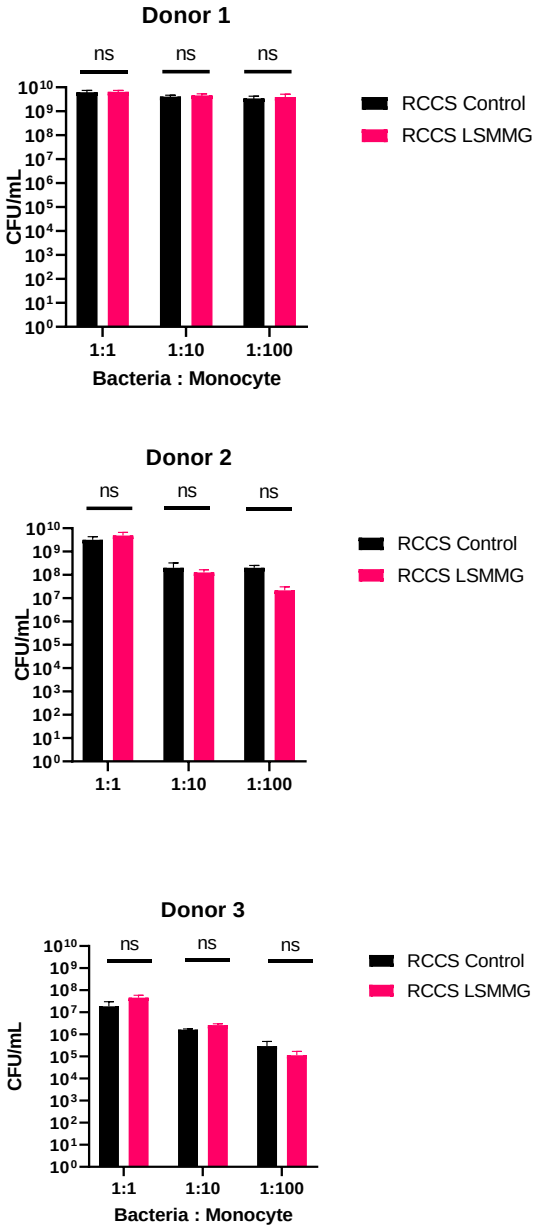


Figure 5.3.7: External manual count of 0g pre-cultured USA300Δagr with 0g pre-cultured monocytes in 4 hours of 0g co-culture. An *agr* negative strain of USA300 was co-cultured for 4 h at the MOIs on the x-axis with 0g pre-cultured monocytes. RCCS control was cultured in the horizontal orientation with LSMMG being in the vertical orientation. After 4 h, co-culture was cultured on a serial dilution plate overnight and colony forming units manually counted. Independent t-test was performed on data using GraphPad. n=3. Error bars show standard deviation of the mean.

Figure 5.3.7 shows no discernible trend between control and LSMMG culture across all three donors. Following this, co-cultures were tested for the internal counts.

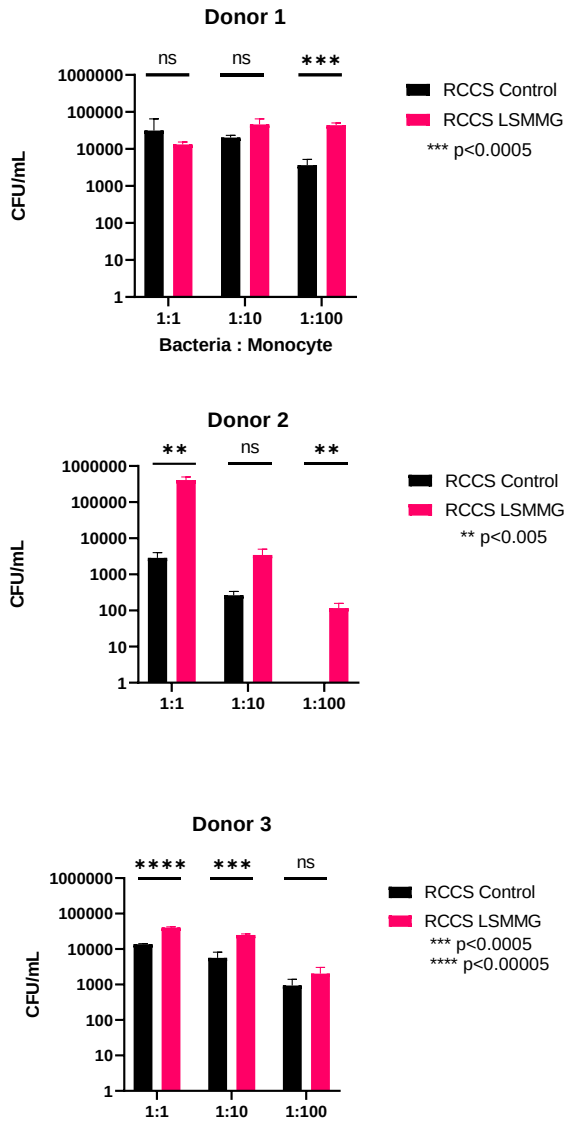


Figure 5.3.8: Manual count of internalized 0g pre-cultured USA300Δ*agr* with 0g pre-cultured monocytes in 4 hours of 0g co-culture. An *agr* negative strain of USA300 was co-cultured for 4 h at the MOIs on the x-axis with 0g pre-cultured monocytes. RCCS control was cultured in the horizontal orientation with LSMMG being in the vertical orientation. After 4 h, co-culture was subjected to Gentamicin and monocytes lysed. Recovered internalised bacterial cells were cultured on a serial dilution plate overnight and colony forming units manually counted. Independent t-test was performed on data using GraphPad. n=3. Error bars show standard deviation of the mean.

Figure 5.3.8 shows that for LSMMG culture, internalised USA300Δagr counts are increased compared with control culture.

To complete the findings from this chapter, the upcoming two figures show changes in cluster formation in control co-culture and LSMMG co-culture respectively from the USA300-gfp and monocyte co-cultures.

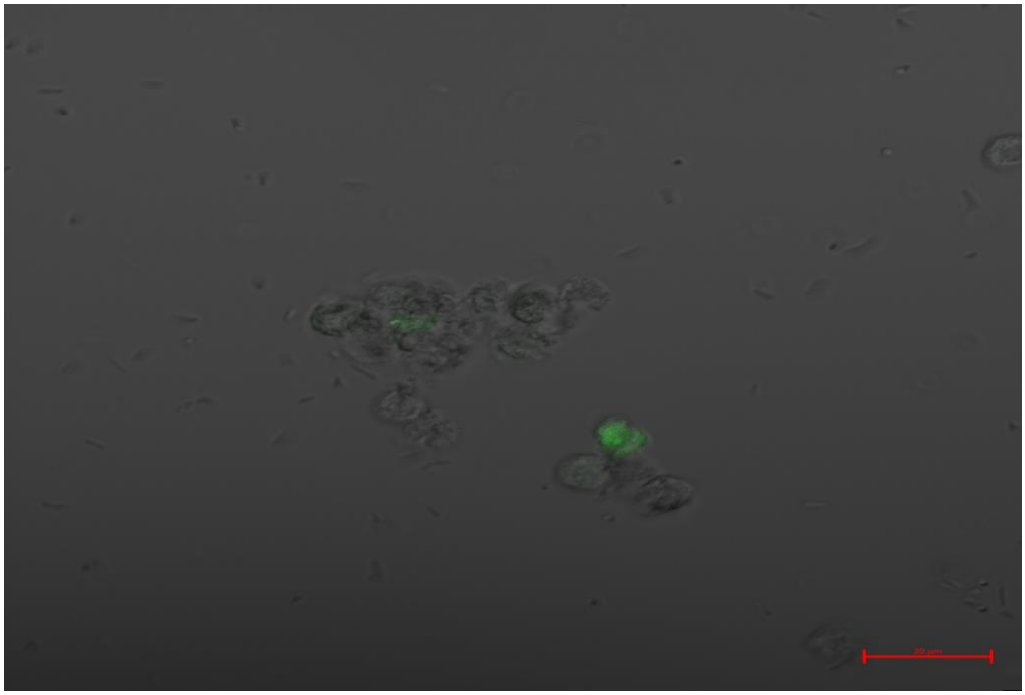


Figure 5.3.9: Confocal microscopy image of a monocyte cluster from 1g pre-cultured monocytes (unstained) and USA300-gfp (green) after 4 hours of co-culture. A x40 objective lens was used. Scale bar is 20 µm.

Figure 5.3.9 shows a small cluster of monocytes from the co-culture with slight association of USA300 shown by the small sections of green. This becomes an interesting phenomenon when compared with the clusters found in 0g co-culture due to clear observable differences.

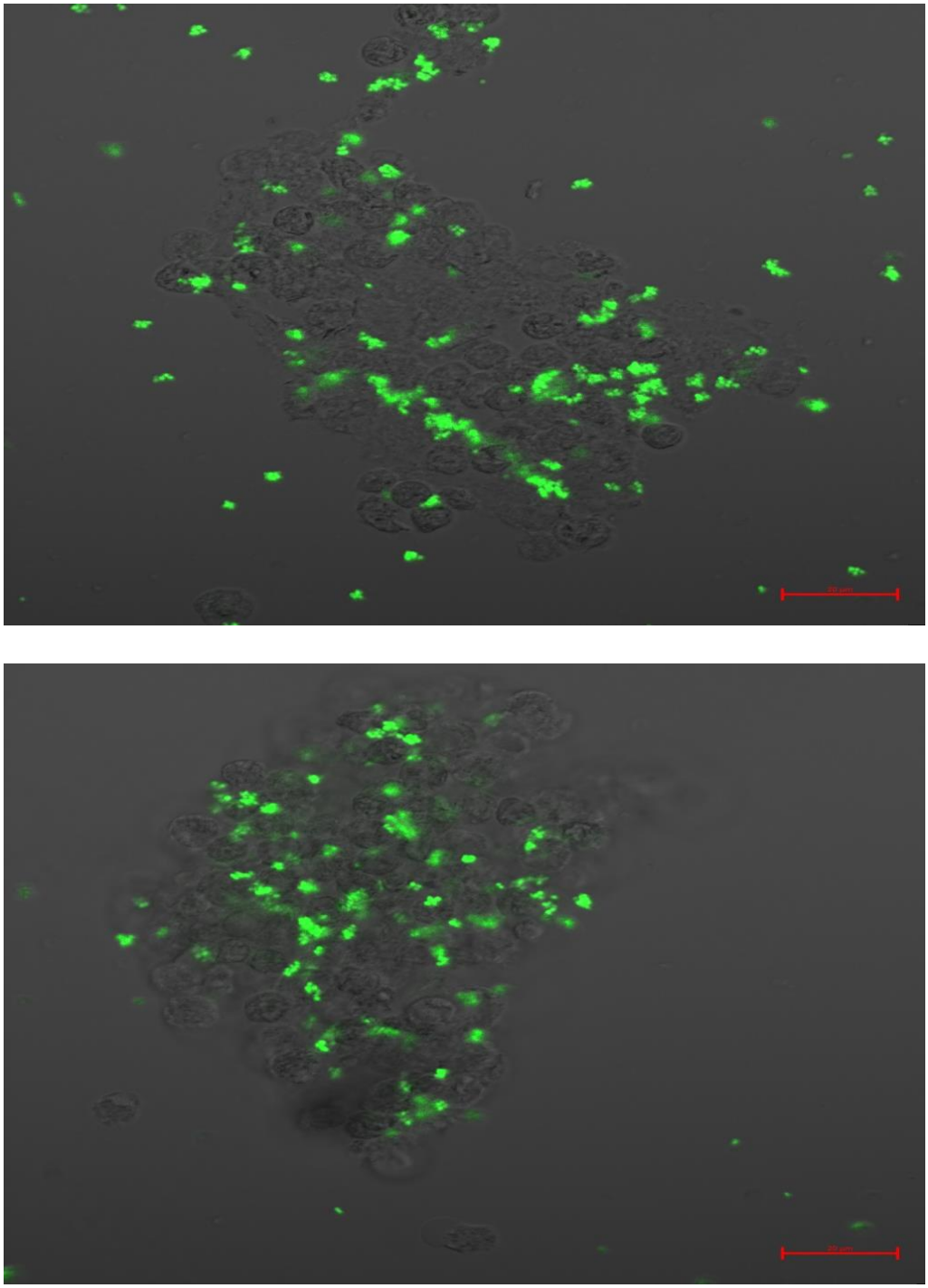


Figure 5.3.10: Confocal microscopy image of two different monocyte clusters from 0g pre-cultured monocytes (unstained) and USA300-gfp (green) after 4 hours of co-culture. A x40 objective lens was used. Both images come from independent co-cultures in the same conditions. Scale bar is 20 µm.

Figure 5.3.10 shows that in the 0g co-cultures, very large clusters of monocytes were found with internalised and associated USA300 as shown by the large green sections running throughout the cluster.

5.4 Discussion

The first stage of the work detailed in this chapter was to investigate the effects of LSMMG culture on the interactions between monocytes and USA300 with a focus on how USA300 survives in the presence of monocytes. It is shown that pre-culturing in LSMMG of either USA300 or the monocytes and adding them to co-culture does not impact on the number of counted external USA300.

However, when incorporating the results of Chapter 2, a clear decrease in the number of external USA300 cells is shown. This suggests that for an observable change in external USA300 counts, both USA300 and monocytes must be pre-cultured in LSMMG conditions to potentially change to a phenotype that causes this decrease.

It is interesting to note that individual LSMMG culture does not cause observable changes. Chapter 2 and Chapter 4 have both reported that LSMMG culture of these cells individually induce changes that should lead to decreased pro-inflammatory response of monocytes and increased invasion by *S. aureus.* Therefore, it would be assumed LSMMG cultured monocytes would have reduced bacterial clearance ability and LSMMG cultured *S. aureus* should have increased invasion due to prolonged expression of colonisation factors that may drive invasion of the monocytes. However, it is not made clear by this investigation if the reported increased invasion in co-culture is monocyte or bacteria driven. Due to this, a hypothesis that the LSMMG induced changes reported in previous chapters may work synergistically to cause this reduction in external USA300 was formed. Chapter 2 shows that monocytes have a reduced pro-inflammatory response in LSMMG culture and therefore it seems illogical that the monocytes have increased ability to clear *S. aureus.* As pro-inflammatory activation usually follows phagocytosis [329], a decreased pro-inflammatory response should be indicative of reduced phagocytosis ability.

It has been reported that monocytes have membranes of increased fluidity in microgravity culture [169] and that *S. aureus* has increased colonisation factor production in Chapter 4 and in the literature [186]. Perhaps these two factors work synergistically to allow for increased invasion of USA300 into the monocytes as opposed to increased cytotoxicity of the monocytes themselves. Therefore, it is logical to then investigate this possibility. It has already been shown how altering the membrane fluidity of host cells changes the adhesion ability of bacteria to these host cells [330].

The next stage of the investigation was to see if the reported changes in LSMMG pre-culture and co-culture cause an increase in monocyte cytotoxicity or *S. aureus* invasiveness. When assessing the results from Chapter 2 and Chapter 4, the more likely hypothesis is that the changes to *agr* activation in USA300 are causing increased invasion by the bacteria into the monocytes through changes to the *agr* regulated phenotype which is also being facilitated by a reduced pro-inflammatory response by the monocytes. However, this decrease in pro-inflammatory response should result in decreased phagocytosis [316] resulting in the cells intaking less bacteria via their own mechanisms. It has also been shown with *E. coli* that monocytes have decreased phagocytic ability in spaceflight culture [318] which means an increase in internal bacterial counts is more likely due to increased invasiveness of the bacteria overcoming the decreased phagocytosis of the monocytes. Interestingly, the decreased oxidative response and degranulation reported in monocytes by Kaur et al would suggest the bacteria can survive better once inside the monocytes.

Chapter 2 already shows that in LSMMG co-culture, there is an increase in intracellular *S. aureus* cells. This led to the hypothesis that the reduced pro-inflammatory response in monocytes in LSMMG culture led to increased invasion of the monocytes. Subsequent investigations into LSMMG induced changes to *S. aureus* in Chapter 4 showed decreased and delayed activation of *agr* in LSMMG culture. This altered *agr* activation may lead to a more invasive and immune response evading phenotype leading to increased

internalisation inside monocytes as shown in Chapter 2. To investigate this hypothesis, a *gfp* expressing USA300 was used in co-culture with the monocytes and ImageStream flow cytometry was used to quantify differences in invasiveness. As the images produced from ImageStream are of low resolution and low quality (Appendix), confocal microscopy was used to visualise the reported differences between LSMMG and control cultures to produce higher resolution and quality images.

Manual counts support the hypothesis that the decreased external USA300 is due to an increase in internal USA300. This is supported by the decreased *agr* activation findings of Chapter 4. However, based upon this, there should have been a decrease in external USA300 for the co-culture of control monocytes with LSMMG USA300. There are possible explanations for this.

The first has already been highlighted in this chapter and that the increased invasive potential of LSMMG cultured USA300 may work synergistically with the LSMMG induced changes in monocytes such as increased cell membrane fluidity and reduced pro-inflammatory response. The other only focusses on the pro-inflammatory response side. Potentially, the control cultured monocytes having a full strength pro-inflammatory response may be enough to overcome the increased invasive potential of the USA300 and protect the monocytes from invasion keeping the external USA300 counts relatively the same. Additionally, these monocytes with an increased pro-inflammatory response (compared with LSMMG monocytes) would have increased phagocytosis [316, 318]. Even though this would initially mean an increase in internalised *S. aureus*, these control cultured monocytes would not have the reported reduction in oxidative stress and degranulation [318] and therefore the bacteria should have reduced survivability once inside the monocytes.

LSMMG pre-culture of both USA300 and monocytes greatly increases the number of internalised USA300. This suggests that the 4 hours of LSMMG co-culture is enough time to induce changes in the interactions between monocytes and USA300 to cause increased internalisation. This is backed up by evidence in both Chapter 2 and Chapter 4. It is shown in Chapter 2 that a

90-minute stimulation with LPS is enough time to observe a marked change in pro-inflammatory response. It is also shown in Chapter 4 that LSMMG culture delays *agr* activation in USA300 from 2-3 hours to 4-5 hours, making *agr* activation during this period of co-culture for control cultures but after this period of co-culture for LSMMG culture. This would ensure the colonising/invasive phenotype is being expressed for the whole of the co-culture period leading to increased invasiveness. With regards to published literature, it has already been reported that *agr* activation delays cause alterations to colonisation and virulence factor expression [181]. Therefore, the delay in *agr* activation in USA300 in LSMMG culture maybe another contributing factor to the observed increased invasiveness.

It is also logical that the LSMMG pre-culture causes a greater increase in monocyte invasion by USA300. This is probably due to the USA300 beginning the co-culture with greater expression of colonisation factors due to the pre-culture and the monocytes starting with a reduced pro-inflammatory response potential and therefore reduced phagocytic ability [316, 318] and increased membrane fluidity [169].

Confocal microscopy helped to clearly visualise the LSMMG induced changes and with the x40 objective lens, it is clearly shown that little to no internalisation occurs and there is little association of USA300 with monocytes in the control culture. Internalisation and association of USA300 in the LSMMG culture is more prevalent than the control culture. This corresponds with the results previously in this chapter shown via the ImageStream and manual count data and provides further novel data and insights into interactions between monocytes and *S. aureus* with regards to invasiveness in co-culture.

Further work could be undertaken into this investigation. Using dual staining methods, it would be interesting and useful to see where in the monocytes the bacteria are residing. By using an endosomal stain, changes in endocytosis

could be tracked to see if this is contributing to the increased invasiveness. An even more powerful investigation would be to live image this but due to the current set-up of the RCCS this would be impractical. The only logistically viable way to do this would be to live image the co-culture on a confocal microscope (or equivalent) during spaceflight so that the microscope itself is in a 0g environment. Live tracking this invasion may shed insights into why the increased invasiveness is occurring. There is potential for setting up a live imaging system perpendicular to the RCCS to live image as it rotates around the axes but as highlighted this may provide substantial logistical problems.

Additionally, if there was a way to live image, employing a USA300 strain with a dual gfp and lux reporter on the *agr* operon could provide vital insights. It is known that in the micro-environment of cells AIP concentration rapidly increases due to the small space and *S. aureus* 'bursts' out of the monocytes due to the fast switching on of the *agr* operon. Similar applications of a dual reporter have been performed in 'standard' culture experiments [331, 332].

Another potential avenue of experimentation could be to see how *agr* activation delays affect invasiveness to see if this is a contributing factor as previously discussed. To do this, both TS13 and TS14 [181] could be co-cultured with monocytes to see if the strain with the later *agr* activation has increased invasiveness to monocytes and behaves the same way as the *agr* delayed LSMMG cultured USA300.

For a more monocyte specific investigation, work could be undertaken to modify the fluidity of the monocyte membranes through the use of temperature or dietary lipids such as cholesterol and see how this impacts the culture. By adding control pre-cultured monocytes with modified membranes to the co-culture, we can see if this is the LSMMG induced change to the monocytes that is causing increased internalisation of the USA300. If it is, we should see an increase in internalised USA300 compared with non-modified monocytes in the same trend as LSMMG vs control pre-cultured monocytes.

It has already been well established throughout this chapter and Chapter 4 that the *agr* operon regulates the expression of colonisation and virulence factors and influences the invasive potential of *S. aureus.* Without *agr* activation, *S. aureus* should remain in a colonising phenotype and not produce virulence factors [333] and can occur naturally during infection due to mutations forming an *agr*$^-$ strain [183]. Therefore, it is crucial to understand how these mutants may behave during an infection in microgravity also. Based upon the known function of the operon, it would be logical to hypothesise that an *agr* mutant would exhibit increased monocyte invasiveness.

When comparing USA300 to the Δagr strain, it is shown that the reduction in external viable cells seen in USA300 LSMMG culture is not present for the Δagr strain. Before conclusions are made based upon this, investigations are needed to take place looking at if the internal counts show any differences.

For both USA300 and the Δ*agr* strain increased internal counts of bacteria in LSMMG conditions compared with control conditions are shown. It is hard to make direct quantitative comparisons due to the nature of monocyte donor to donor variability, but the magnitude of internal cell count increase appears to be similar between the strains.

As internal counts do not differ, but external counts do, it appears that the absence of the *agr* operon has not affected the invasiveness of the *S. aureus* but has given it increased survivability in the presence of monocytes. *S. aureus* secretes Protein A which binds IgG in the wrong orientation and blocks Fc receptor mediated phagocytosis and subsequent innate immune activation [334]. This production of Protein A is suppressed by *agr* activation [335] and hence an *agr* mutant would not have suppressed Protein A production and would therefore have greater evasion of the immune response and may explain the increased external USA300Δagr numbers. This could be confirmed by investigating the differences between two strains where the only difference is one cannot produce Protein A to see if this causes a difference in the external survivability of the bacteria in the presence of monocytes.

Comparing the Δagr results with the USA300 JE2 results from Chapter 2 gives further insight into the role of *agr* and monocyte immune response. As external counts do not change for the *agr* mutant co-culture, this shows that the external count reduction seen with the wild type USA300 in LSMMG co-culture is due to LSMMG induced changes to the *agr* operon.

When analysing co-culture samples via confocal microscopy, a potentially significant observation was found in that LSMMG co-cultures had sporadic large clusters of monocytes with substantial USA300 internalisation whereas the control co-cultures had sporadic smaller clusters of monocytes with little to no internalisation of USA300.

Chapter 2 already discusses how the immune response in monocytes is regulated by cell attachment interactions and how LSMMG culture limits cell attachment leaving cells to only attach to each other unless a scaffold is added. This leads to the hypothesis that the monocytes in LSMMG culture are expressing a greater number of attachment proteins (such as ICAM-1 and VCAM-1) causing these larger macro-structures to form. The literature for cells expressing different levels of attachment proteins in microgravity culture is inconclusive across cell types [171] but increased monocyte attachment has been found in LSMMG cultured rat monocytes [171-173].

In conclusion, the work in this chapter offers additional insights into how the alterations reported in Chapter 2 and Chapter 4 work synergistically. By showing the effects of individual pre-culture on monocytes and *S. aureus,* it was shown how changes to invasion are dependent on LSMMG induced changes to both cell types. The importance of *agr* in *S. aureus* was also investigated through the use of an *agr* mutant and its' influence on bacterial behaviour in co-culture reported on. The work in this chapter also visualised the changes to bacterial invasion in co-culture with monocytes through the use of ImageStream analysis and confocal microscopy.

Key Findings:

- **Both monocytes and *S. aureus* require pre-culture and co-culture in LSMMG to observe noticeable reduction in external bacteria count**
- **In LSMMG culture, *S. aureus* have increased invasiveness of monocytes**
- ***S. aureus* is confirmed to be inside the monocyte and not just adhered to surface**
- **There is a monocyte LSMMG induced change driving the increased uptake of *S. aureus* as well as changes to *agr* induction in *S. aureus***

6. Discussion, Conclusions and Future Work

The work set out in this thesis was for the purpose of achieving the aims set out in Chapter 1. The first aim was to add to the knowledge on already reported immune response deficiencies in microgravity by investigating the mechanisms behind the pro-inflammatory response in monocytes in microgravity culture.

Chapter 2 presented work to achieve these aims through investigations into pro-inflammatory response alterations and the impact of these alterations on ability to clear *S. aureus.* This chapter presented data showing that the already well-known cytokine production reduction in microgravity is apparent for both Gram-positive and Gram-negative stimulations. This reduction in pro-inflammatory cytokines suggested that the monocytes would have reduced ability to clear *S. aureus.* However, this was initially shown not to be the case with a data suggesting an increased ability to clear *S. aureus.* This unexpected result warranted further investigations into how the pro-inflammatory response was being altered in microgravity culture. Based upon the established cytokine production reduction response, novel results were presented showing that this microgravity cytokine production reduction is not due to inhibition of secretion. Activation of the pro-inflammatory response was investigated via NF-κB translocation and differences to this translocation in both stimulated and unstimulated monocytes suggested an altered pro-inflammatory response and alterations of other NF-κB regulated pathways. Alterations to *S. aureus* behaviour were also considered in response to microgravity culture and it was considered that the data that suggested a seemingly initial increased ability of the monocytes to clear *S. aureus* was in fact highlighting a different outcome in which the bacteria were exhibiting increased invasion of the monocytes. This chapter was finalised by confirming this to be the case through increased internal bacterial counts inside the monocytes. Once again, this is a novel finding achieving the aim of adding to

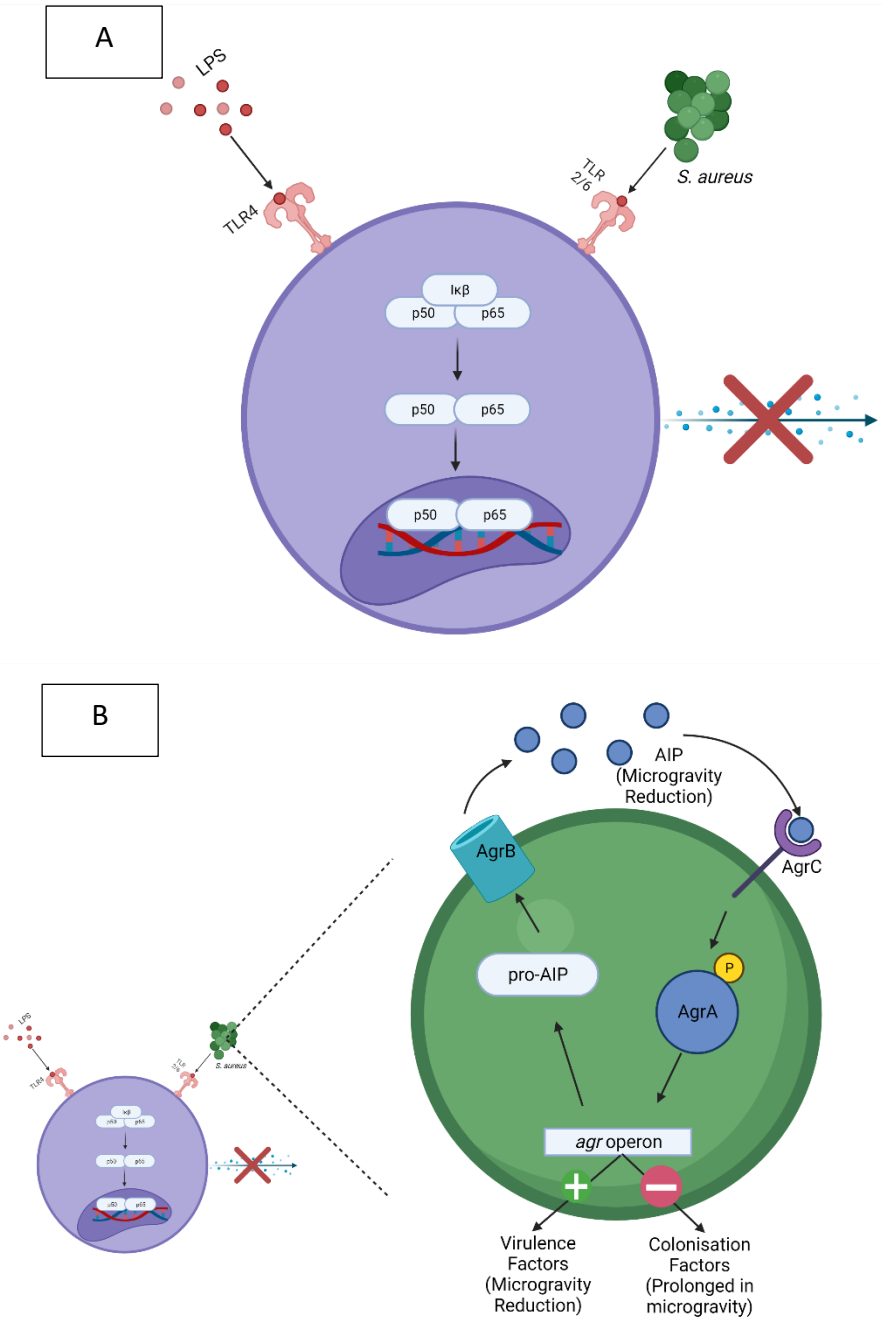
pre-existing knowledge of altered immune responses in microgravity. The work in this chapter also lead to a hypothesis that there was microgravity induced alterations to *S. aureus* that may be making the bacteria more colonising/invasive in microgravity culture. This hypothesis lead to the investigations presented in Chapter 4.

The second aim set out in this body of work was to investigate how microgravity culture affects virulence and colonisation in *S. aureus.* The work presented in Chapter 4 achieved this aim. It was first shown that virulence factor production is reduced, and colonisation factors are expressed for longer in *S. aureus* cultured in the RCCS. Virulence factor production and colonisation factor expression are regulated by the *agr*-QS system and the alterations to the two investigated phenotypes suggested that this system was being altered in microgravity culture. Induction delays and reductions in this system were confirmed and it was also shown that there was a reduction in the production of the regulatory molecule AIP. Further investigations to this system showed that these changes in response were not due to interactions between AIP and AgrC (inducer and receptor respectively). These results combined suggested that it is the generation of AIP that is being altered in microgravity culture and this alteration leads to reduced and delayed *agr* induction. It is then this reduced and delayed *agr* induction that is causing a longer expression of the colonising phenotype leading to a shorter expression of the virulence factor producing phenotype which may cause the bacteria to miss its' window of opportunity to produce virulence factors completely. Virulence and colonisation changes had already been reported in literature. The work in this chapter presented novel findings into alterations into *S. aureus* quorum sensing and for the first time showed how this influences the established phenotypical changes.

It had now been established how monocytes have an altered pro-inflammatory response and how *S. aureus* has a prolonged colonising phenotype in microgravity culture. The work in Chapter 5 was set out to determine which alterations contribute to the increased invasion of

monocytes by *S. aureus* and to further determine the impact of microgravity culture on the interactions between the two cell types. This was achieved through varying 'pre-culture' and 'co-culture' in microgravity and investigating the impact of *agr* in *S. aureus* using an *agr* mutant. The data in this chapter suggested that both alterations to monocytes and *S. aureus* contribute to the differences reported in microgravity culture monocyte invasion and that the greatest invasion differences are seen when both cell types are 'pre-cultured' and 'co-cultured' in microgravity. Once more, the data presented in this chapter are novel and contribute to the existing knowledge of bacterial infections and subsequent immune response in microgravity.

Figure 6.1 is a graphical summary of the findings from Chapter 2, 4 and 5 and shows how these changes may work synergistically to contribute to increased monocyte invasion by *S. aureus* in microgravity.


A
LPS
TLR4
TLR 2/6
S. aureus
Iκβ
p50
p65
p50
p65
p50
p65
B
AIP
(Microgravity
Reduction)
AgrB
AgrC
pro-AIP
P
AgrA
agr operon
Virulence
Factors
(Microgravity
Reduction)
Colonisation
Factors
(Prolonged in
microgravity)

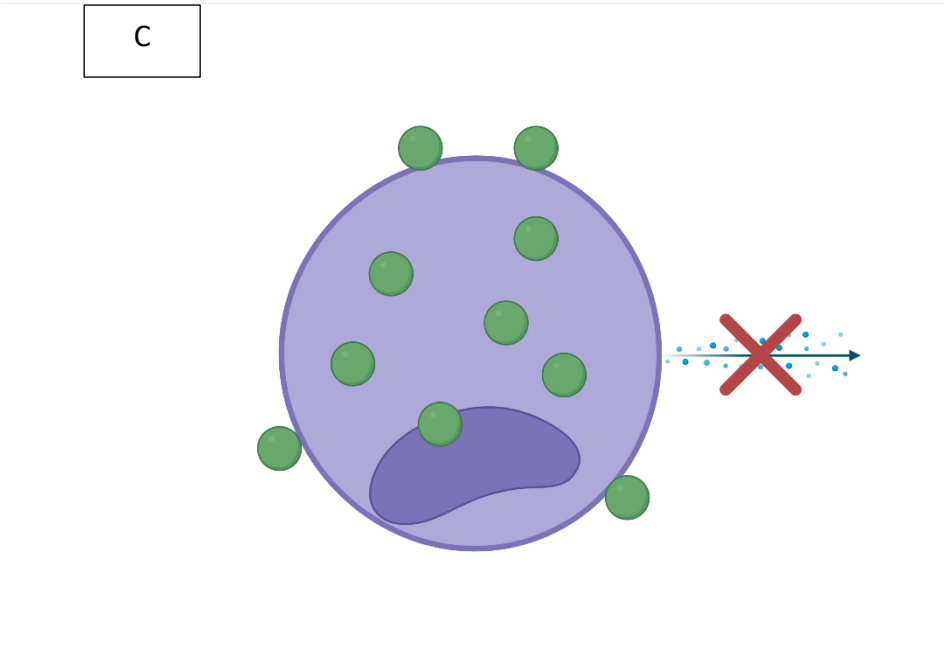


Figure 6.1: A graphical representation of the three major investigations in this thesis. 6.1A is a graphical summary of the pro-inflammatory changes to monocytes in LSMMG culture. The major findings represented are altered NF-κB translocation (LPS stimulation) reported through tracking of the p50/p65 heterodimer and inhibition of cytokine production. 6.1B shows the *agr*-QS system in *S. aureus* and how this is altered in LSMMG culture. The major findings represented are decreased virulence factor production and prolonged colonization factor production due to reduced and delayed *agr* induction and subsequent reductions in AIP production. 6.1C represents the changes from 6.1A and 6.1B combining to result in increased internalization of *S. aureus* inside monocytes in LSMMG culture and a cytokine production inhibition. Created with BioRender.com.

Astronauts on long-term space missions have reported suffering from chronic infections during missions, and months after return to Earth [119]. In one study, 46% of astronauts on board the ISS reported a 'notable event' with regards to illness during their flight [295]. These reported illnesses could be explained by a weakened immune response and/or increased infectivity of pathogens such as *S. aureus*.

Monocytes were found to have a reduced pro-inflammatory response and *S. aureus* has reduced virulence factor production and increased cell colonisation factor production in LSMMG culture. Previous studies have revealed how changes to the *agr* operon in *S. aureus* effects monocyte invasiveness through co-culture studies in Chapter 5 have already been looked at. However, the changes reported in Chapter 2 and 4 could also work synergistically in other ways apart from invasiveness to help explain the reported increases in chronic infection and illness reported during spaceflight.

Innate immune cells such as monocytes can respond to bacterial infection by recognising virulence factors [336]. Therefore, the reported reduction in *S. aureus* virulence factor production via cytotoxicity to THP-1 cells here, combined with those reported in literature through carotenoid and staphyloxanthin production [87, 186] in LSMMG culture may lead to reduced pathogen recognition and therefore a reduced immune response.

The observations of altered colonisation inform our understanding of biofilm formation. Studied elsewhere, biofilms have been shown to form more readily in spaceflight and in LSMMG culture [119, 186]. For the cell colonisation changes in *S. aureus* a fibronectin binding assay was used as a proxy for the colonising ability of the bacteria. This investigation provides insight into changes in biofilm in LSMMG culture as it has been shown in a LAC strain of USA300 that fibronectin binding proteins are required for biofilm formation [337]. Therefore, the prolonged expression of colonisation factors shown here via the proxy of fibronectin binding suggest increased biofilm formation may also occur. Biofilms have immune evasion mechanisms by providing a physical barrier from the immune cells [338] and exporting polymers that constitute the extra-cellular polymeric matrix (EPM) which can shield recognition molecules such as PAMPs and protect the bacteria from processes such as phagocytosis [334]. Therefore, if the LSMMG induced increased fibronectin binding is leading to increased biofilm formation, this will cause increased immune evasion.

If the innate immune cells such as monocyte are able to overcome the reduction in pathogen recognition molecules due to the decreased amount of virulence factors produced by *S. aureus* that is reported here in LSMMG culture, they must still be able to respond to the infection. Cytokine production is vital in the activation of the pro-inflammatory response and it has been shown that a reduction in production of both the pro-inflammatory cytokines TNFα and IL-6 in co-culture with *S. aureus* and a reduction in the anti-inflammatory cytokine IL-10 when LPS stimulated in LSMMG culture occurs. This shows that there is potentially a decreased recognition of pathogens in LSMMG culture shown by the decreased amounts of response cytokine produced. Even at high concentrations of stimuli where cytokine is being produced, this LSMMG induced reduction is still present indicating that there is a mechanistic reason behind a cytokine production reduction as well as the effect of reduced pathogen recognition. Therefore, if the cells can still recognise the pathogens despite the LSMMG induced alterations, their ability to clear the infection is still being reduced. This has already been reported with reduced phagocytosis, with emphasis on reduced oxidative stress and degranulation, in monocytes in LSMMG culture [175, 339].

This potential increased immune evasiveness through decreased recognition in LSMMG cultured *S. aureus* by immune cells and decreased response ability by LSMMG cultured monocytes could help explain why there is an increase in infections and illnesses during spaceflight. These two outcomes should work synergistically together causing increased infection rate, due to decreased recognition, and a longer infection period, due to decreased immune response. This may also be affected by reported microgravity induced changes to the adaptive immune response [32, 340]. This is further supported with the evidence of increased *S. aureus* invasiveness and survivability inside monocytes in LSMMG culture which shows the decreased ability of immune cells to clear infection and the increased potential for chronic infection by *S. aureus*.

This thesis consisted of four main investigations in which three produced novel results with regards to the interactions between monocytes and *S. aureus* in microgravity.. However, each investigation had limitations and with hindsight and/or access to greater resources, could have been performed differently to reduce these issues.

One of the main limitations of the work in Chapter 2 becomes apparent when analysed in conjunction with the other chapters of this thesis. This limitation is that the studies into LPS stimulation in monocytes was performed in the standard monocyte culture media of RPMI 1640 with 10% FBS, 1% L-glutamine and 1% pen/strep. When the monocytes were used for co-culture, they were then cultured in X-Vivo 15 serum free media. Although both media are suitable for monocyte culture it was not investigated as to whether these media induce a difference in response in the monocytes. This study was performed before knowing that this medium would be unsuitable for the planned quorum sensing studies. It was not financially viable to reperform NF-κB translocation studies on the monocytes in X-Vivo 15 media due to the cost of antibodies and cost of running the ImageStream.

NF-κB translocation analysis was not performed on the monocytes co-cultured with *S. aureus*. Without this analysis it cannot be determined if the cytokine suppression seen in both Gram-negative and Gram-positive monocytes is due to the same mechanisms and if the upregulation of NF-κB translocation is playing a role in both cases. As explained, ImageStream analysis is an expensive process and alternative allocation of resources was prioritised.

The control culture for the monocyte cultures in this study when stimulated with LPS was also performed in an ultra-low attachment plate as opposed to the latter which used 'control' orientation of the RCCS. With hindsight, this limitation could have been overcome as at the time of experimentation there was only one RCCS in our possession with three culture vessels and it was not known that there would be access to more culture vessels and another RCCS 12-18 months later. The ultra-low attachment plate provided an environment

where the monocytes could not attach to anything apart from themselves like the LSMMG environment caused by RCCS culture but did not reflect the movement of the culture media in the RCCS nor the cells being kept in their own micro-orbit with little changes in their local environment.

The work in Chapter 3 was based around the optimisation of culture medium and vessels and therefore in itself was about removing limitations from the future studies.

To better establish the effects of movement on the bacterial responses monitored, testing different rotational speeds in the shaker incubator ranging from 0 to the 200 rpm used could have highlighted even more the importance of culture movement to *S. aureus* growth. This was not detrimental to the investigation however as the importance of culture movement was still determined in deciding suitable conditions for a control culture for the RCCS.

With time and resources, additional media could have also been tested. There are other serum free cell culture media that may have facilitated greater *S. aureus* growth allowing for responses closer to that of the optimal medium of TSB. These media are listed in a database [341] and could potentially be screened and tested to find a medium even more suitable than X-Vivo 15.

The major limitation of the work in Chapter 3 was due to difficulties of sampling with the RCCS. As described in Chapter 1, the RCCS must be zero headspace meaning no air bubbles can be in the culture and any samples removed must be added in equal volume. When sampling the bacterial cultures, there is removal of a small portion of the population, spent media and waste products. It is impossible to re-add the sample taken back into the culture because upon removal it has been exposed to gravity. It cannot be recreated the exact stage of growth and phenotype of the cells removed and cannot add back media that has identical nutrient depletion and waste contents. To counteract this, sampling must be undertaken in as minimal intervals as possible, hence why the experiments involving sampling start at 2

hours as to not disturb the culture during the initial phase and occur every hour after that to minimise the above described problems. Ideally, sampling from 1 hour onwards and at every 15 minutes would have been performed, but this was not feasible.

For studies in which AIP was added, it was made sure the medium added back during the sample contained the same concentration of AIP as the culture at the start. This is because AIP is only sensed and not metabolised. Care was taken during sampling to not stop the rotation of the RCCS culture vessels to ensure discontinuous rotation did not occur.

One limitation of the work in this chapter is with the dose-response 'curves' of AIP-1 and AIP-3 addition to ROJ143 and ROJ154 respectively. If more concentrations were used and an accurate maximal response concentration determined, it would have been possible to generate an EC_{50} for both AIP-1 and AIP-3 and look at the differences between these for control and LSMMG culture. However, there was only access to two RCCS's and six culture vessels enabling a maximum of 6 concentrations to be tested from the same starter isolate. Access to more RCCSs and more culture vessels would have allowed for more concentrations to be tested giving a more accurate dose-response curve and the possibility of generating an EC_{50}. The addition of more RCCSs would have also required more incubators.

Co-cultures using an RCCS also posed significant challenges. For internal cell counts, gentamicin was used to kill external USA300. Gentamicin in higher concentrations can permeate a monocyte membrane and therefore could limit the accuracy of this assay. This is why follow up assays of ImageStream analysis and confocal microscopy were employed to analyse the internal USA300 in an unaltered state. There was also no guarantee the gentamicin had a 100% effectiveness on the external USA300 which could have slightly altered the results.

All imaging of co-cultures had to be fixed and could not be live due to the nature of the RCCS. Samples had to be removed and fixed to limit the effects

of exposure to gravity as it was unknown what timeframe it takes for these co-cultures to respond to the introduction of gravity. Therefore, all images are only a snapshot of the responses that were being analysed, and therefore a true representation of the invasion process of *S. aureus* into monocytes was not achieved. The timeframe analysed was only four hours of co-culture and more information could have been gathered with regards to LSMMG induced *S. aureus* invasiveness by looking at different periods of co-culture.

Due to the ability of Protein A to suppress immune function and the already explained reduced immune function from microgravity culture, it would provide vital insight to look at any LSMMG induced changes in Protein A production. Based on results from Chapter 4 it is now known that LSMMG culture delays *agr* activation and therefore this will result in a greater period of growth where Protein A production is not being suppressed. Therefore, based on this, there should be increased production of Protein A in LSMMG culture. By investigating Protein A production in the time period before *agr* activation also, it could be investigated if LSMMG culture effects Protein A production via other mechanisms than *agr* activation delay. Looking at Protein A alterations may shed light on why there are increased chronic infections and suppressed immune response in microgravity.

For monocyte stimulation, the only receptor investigated was TLR, specifically TLR4. As previously explained, TLR's are not the only receptors involved in the signalling cascade for NF-κB as NOD's can also upregulate this process. It was shown that *S. aureus* causes a suppression of TNFα and IL-6 production in monocytes and Chai et al show that *E. coli* causes a suppression in both cytokines as well in macrophages. After this is investigated and confirmed in monocytes, this could lead to some insightful investigations. By investigating changes to NOD's in monocytes in LSMMG culture, this could potentially highlight why cytokine suppression occurs. NODs search the intracellular environment for infection and a dysregulation of these NOD's could also be one of the reasons USA300 was able to invade monocytes in increased numbers in LSMMG culture as if NOD function was impaired, the USA300

would be better able to survive inside the monocytes. This would be because of reduced recognition of the internal infection and a subsequent reduced pro-inflammatory response such as the production of TNFα and IL-6.

Lipoteichoic acid is the main TLR2 ligand in early Gram-positive infection recognition and LTA mutants have been shown to have reduced pro-inflammatory response [302]. Investigating LTA changes in LSMMG culture to *S. aureus* may also provide insight into the suppressed immune response and cytokine release to the bacteria. An increase in LTA could lead to the results observed.

Both NODs and LTA are involved in the regulation of the NF-κB pathway. The experiments detailed in Chapter 2 only looked at changes to this pathway from the Gram-negative stimulation of LPS from *E. coli*. As a similar cytokine reduction is reported from both Gram-negative and Gram-positive stimulations, it would be beneficial to see if the reported increase in NF-κB translocation in Gram-negative stimulated monocytes occurs in the monocytes co-cultured with the Gram-positive *S. aureus*. As mentioned in Chapter 2, it would also be beneficial to see if co-culture with *E. coli* causes the same response in monocytes as LPS stimulation alone does or if this causes a different response.

To conclude, it was shown the impact LSMMG culture has on the pro-inflammatory response in monocytes and subsequent clearance of bacterial infection in an *in vitro* model, how the *agr* quorum sensing and their controlled phenotypes are affected by LSMMG culture, and how these cells interact with each other in LSMMG co-culture.

Discussion revolved around each of these findings in depth and what their potential impact towards human health during spaceflight could be. The limitations of each of these chapters has also been highlighted and suggestions made on how to improve the quality of the research if repeated or followed up.

Suggestions were also made for future work, building upon the findings of this thesis and begin to highlight what mechanisms may be being altered to cause the LSMMG induced phenotype in both monocytes and *S. aureus*.

Overall, there a plethora of immune response related issues when it comes to long-term exposure to microgravity. This body of work provides novel data to achieve the aims of further understanding changes to the pro-inflammatory response of monocytes and subsequent ability to deal with bacterial infection, changes to virulence and colonisation of *S. aureus,* and interactions between these two cell types in microgravity. The reduced immune response and increased pathogen invasiveness may lead to increased long-term infections even in the healthiest of individuals limiting potential for 'space-tourism' to be opened to the general populace. This also causes concern for planned manned missions to Mars and other long-term space habitations where resupply of pharmaceuticals is impossible or limited. Increased chronic infections can spread through the crew causing severe health issues and in the worst cases mission abortion or death of crew members if unable to be treated by the limited medicine supply onboard.

7. References

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8. Appendix

8.1 Green et al – Review Paper

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Review

Immunity in Space: Prokaryote Adaptations and Immune Response in Microgravity

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Abstract: Immune dysfunction has long been reported by medical professionals regarding astronauts suffering from opportunistic infections both during their time in space and a short period afterwards once back on Earth. Various species of prokaryotes onboard these space missions or cultured in a microgravity analogue exhibit increased virulence, enhanced formation of biofilms, and in some cases develop specific resistance for specific antibiotics. This poses a substantial health hazard to the astronauts confined in constant proximity to any present bacterial pathogens on long space missions with a finite number of resources including antibiotics. Furthermore, some bacteria cultured in microgravity develop phenotypes not seen in Earth gravity conditions, providing novel insights into bacterial evolution and avenues for research. Immune dysfunction caused by exposure to microgravity may increase the chance of bacterial infection. Immune cell stimulation, toll-like receptors and pathogen-associated molecular patterns can all be altered in microgravity and affect immunological crosstalk and response. Production of interleukins and other cytokines can also be altered leading to immune dysfunction when responding to bacterial infection. Stem cell differentiation and immune cell activation and proliferation can also be impaired and altered by the microgravity environment once more adding to immune dysfunction in microgravity. This review

1 . Introduction

The immune system is influenced by external stressors and adapts accordingly. The differential immune response under microgravity gives rise to complex immunological issues [1,2] which will be discussed in this review. For instance, 15 of the 29 Apollo mission astronauts incurred viral or bacterial infections during their mission or within a week upon returning to Earth [3]. Many bacteria grown under microgravity experience physiological changes, increased virulence, and differential antibiotic susceptibility amongst other changes [4–6]. Such changes can work synergistically to cause an increased chance of infection with a chance of enhancing the potential for a poor prognosis. However, the microgravity environment may also provide novel insights into different biological phenomena here on Earth such as T cell exhaustion [7] and T cell ageing [8].

The last century has seen the frontier of space become accessible by human beings. From Yuri Gagarin's initial flight into space, through NASA's (National Aeronautics and Space Administration) moon landing in 1969, progressing to ventures in space tourism and planned manned missions to Mars in 2030, space has become an environment that is habitable for periods of time by humans. As missions to space become longer and

further away in distance, the time spent in the space environment increases and with it, the associated health risks [9,10]. One major differential factor from the environment on Earth is the change in gravity to a near-zero state known as microgravity.

Most research on the effects of microgravity on biology either aims to help provide insight on how to keep astronauts healthy on prolonged missions into space or to use microgravity as a research tool to further understand the biology to be beneficial to patients on Earth. The focus of this paper is to summarise the impact of microgravity on the immune response to bacterial infection and the individual changes to bacteria and immune cells.

2. Microgravity Simulation and Applications

Microgravity is the condition when objects appear to be weightless. Studies have been conducted on true microgravity in space since the initial Apollo missions and in microgravity analogues as early as the 1980s. True microgravity has been regularly investigated onboard SpaceLab, the Russian Mir space station, and the International Space Station (ISS). As well as experiencing true microgravity, onboard samples and experiments are subjected to time dislocation, elevated carbon dioxide levels and low dose cosmic radiation [11]. Some of these factors can be synergistic, for instance, radiation induces oxidative stress in the skeletal system and microgravity increases the oxidative stress-induced [12]. However, resources are limited on these stations and sending samples and consumables for experiments to these platforms is expensive and requires substantial time to plan and execute. A solution to this problem is to create an environment analogous to the low-shear environment created by microgravity on Earth. This has the benefit of being more time and cost-effective and the microgravity variable can be separated from other space variables more easily. These microgravity analogues still are subjected to 1 g but create a low-shear environment through various means as discussed in the following sections. In true microgravity, the overall net force of gravity is in the range of 10^{-6} compared to the force of gravity at the Earth's surface.

2.1. Microgravity Analogues—The Common Devices

Microgravity creates a low-shear culture environment since convection currents are absent. By creating a low-shear environment on Earth, low-shear responses of biological samples can be investigated and used to theorise responses in other low-shear environments such as microgravity. One method of creating a low-shear environment is the rotating cell culture system (RCCS) (Figure 1). This bioreactor was designed by NASA and is commonly used across Europe and the USA via production and distribution from Synthecon [13,14]. This bioreactor employs solid body rotation around a horizontal axis to minimise fluid shear forces on the sample whilst keeping it in suspension. This is achieved through rotation at a precise speed where this phenomenon occurs. Shear force is the application of a force perpendicular to a surface. The difference in velocity between the layers in moving liquid result in shear forces being imparted on the liquid and samples contained within. By rotating the liquid containing vessel at a precise speed, shear forces can be minimised, hence 'low-shear'. This needs to be fine-tuned with respect to the weight of the sample to prevent sedimentation and keep the samples in 'freefall' by balancing the net shear force with gravity on the sample. This creates an analogous environment in the low-shear environment created by true microgravity.

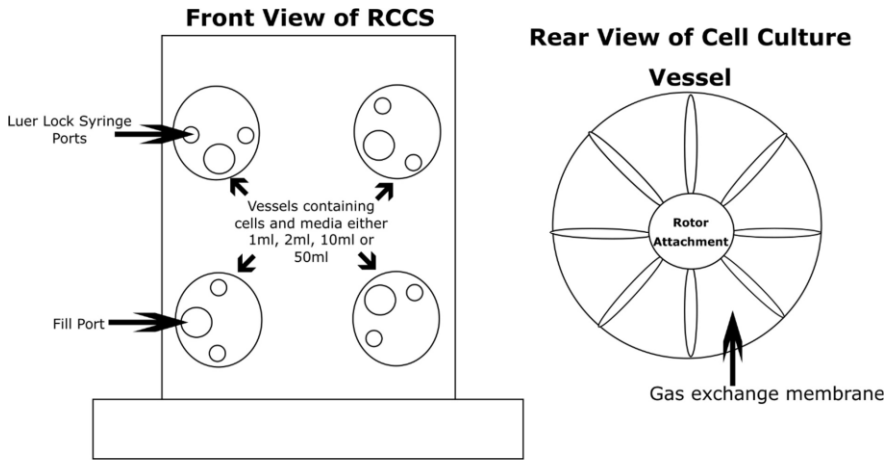

Figure 1. The 2D clinostat; this one is known as the Rotating Cell Culture System (RCCS). The particular version illustrated in this figure is capable of rotating up to four individual cell culture vessels. These cell culture vessels carry media containing cells in 1 mL, 2 mL, 10 mL, or 50 mL formats. The cell culture vessels come in two varieties, disposable and autoclavable. The vessels compose of two Luer Lock syringe ports for small additions or extractions to the growth medium and a larger fill port for ease of filling and emptying. The rear of the vessels composes of a gas exchange membrane to allow the diffusion of gases. The vessels rotate clockwise at independent or synchronous speeds.

The random positioning machine (RPM) (Figure 2) is another common tool for creating a low-shear environment for the biological samples by creating shear forces away from the centre where the sample is. Utilizing constant repositioning upon a dual axis, this instrument causes the overall net influence of gravity to be zeroed at long time scales [15]. Both the RWV and RPM are two examples of a 2D clinostat.

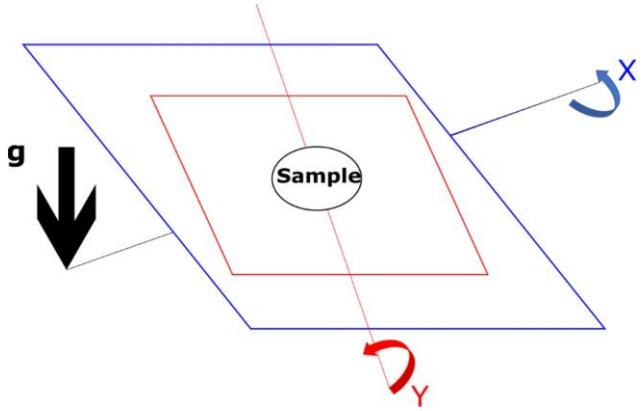

Figure 2. A schematic of how the Random Positioning Machine (RPM) simulates microgravity. This schematic is based upon the illustration from Wuest et al. 2017 [16]. The sample in the centre of the device is constantly repositioned both in the direction of the *x*-axis and the *y*-axis, giving an overall net-zero gravity vector.

2.2. Microgravity Analogues—History of Clinostats and Alternative Methods

Clinostats rotate samples around one or more axes and were developed in the late 1800s when gravity was discovered to be a major factor in plant growth [17]. This goes back to as early as 1806 and the use of a water wheel to generate altered gravity environments [17]. Slow

rotation around an axis (1–2 rpm) was found in the 1980s to induce ultrastructural disturbances not found in the microgravity environment [18]. The first study to use a faster rotation with a clinostat was Briegleb in 1992 [19].

Diamagnetic levitation is another microgravity analogue. This is the use of a high gradient magnetic field that can levitate a biological sample [20]. This method, however, does not negate phenomena not observed in microgravity, such as convective stirring of liquid that increases oxygen availability in the samples, which is a major issue in space as convection is not present [20,21].

Parabolic flight and drop towers are also employed by researchers for short microgravity exposure. During parabolic flight, an aeroplane will fly in parabolic arcs imparting approximately 30 s of free-fall-per-parabola. The nature of the flight path causes the imparted gravitational forces to fluctuate between microgravity during freefall and 1.8 g during the reversal of the flight path at the bottom of the parabolic flight path. These short fluctuating periods of microgravity and hyper-gravity have already been used in immunological research [22,23]. Drop tower samples are dropped from a substantial height in a capsule covered by either an aerodynamic drag shield or in a vacuum to allow free-fall at 9.8 m s^{-2}. This enables the samples to experience near weightlessness as they freefall from a substantial height. NASA, for example, use a 24.1-metre drop tower that enables 2.2 s of microgravity to be experienced.

Microgravity analogues, however, are not an entirely accurate model of orbital microgravity conditions. Gene expression analysis of human renal cortical cells cultured during spaceflight and in an RCCS on Earth show that 700 more genes with a total of 1600 had altered expression levels compared to 900 genes with altered expression in the RCCS [24].

3. Prokaryotic Responses to Microgravity

Prokaryotes have evolved and adapted to survive in a plethora of different environmental conditions [25]. Microgravity is a different environmental condition that, due to advances in technology, is becoming a condition for prokaryotes to adapt to and can be researched and explored. This area of research could be beneficial for combatting infection during long term manned space missions and may provide novel insights into prokaryote adaptability and evolution. The following sections review the research into the prokaryotic response to the low-shear environment created by microgravity and ground-based analogues.

3.1. Cell Viability and Diversity

The human body itself contains a substantial number of bacteria from the bacteria covering the skin to the microflora of the gut [26,27]. NASA has set acceptability limits for bacterial numbers in the air, on surfaces and in water for all space-bound equipment and vessels.

As shown in Table 1, it is expected that bacteria during spaceflight will survive and proliferate in microgravity/spaceflight conditions. This is shown by the higher bacterial acceptability limits for air and surfaces inflight compared to preflight.

Table 1. Bacterial acceptability limits outlined by NASA [28].

Time Taken	Air	Surface	Water
Preflight	300 CFU m^{-3}	500 CFU 100 cm^{-2}	50 CFU mL^{-1}
Inflight	1000 CFU m^{-3}	10,000 CFU 100 cm^{-2}	50 CFU mL^{-1}

Vessels from different locations unsurprisingly show differences in bacterial populations. The Russian Mir space station reported the most dominant genera of airborne and surface bacteria to be *Staphylococcus* with *Sphingomonas* and *Methylobacterium* to be the most dominant genera in the potable water [29]. When water was collected from the ISS between 2009–2012, it was found the most common organisms were *Burkholderia multivorans* and *Ralstonia pickettii* with air and surface dominance of *Bacillus*, *Micrococcus,* and *Staphylococcus* species [28]. Overall, the most common phylum was Actinobacteria [30].

3.2. Overview of Previous Studies

The following studies have been undertaken to investigate the effects of a low-shear environment on bacteria with a few studies also investigating archaea. The first studies found common responses to spaceflight bacteria with emphasis on the phenotypic responses including but not limited to: changes in growth rate, resistance to external stresses and varying effects on bacterial conjugation [14]. Below is a brief summary of the major studies investigating individual species and strains of bacteria and their critical findings.

3.3. Transcriptomic Changes

To summarise the transcriptomic results from the studies in Table 2, common dysregulated genes have been identified and hypothesised as being altered because of microgravity. **Table 2.** Summary of bacterial response in spaceflight and microgravity analogue studies.

Name	Low-Shear Environment	Studies	Major Findings
Mycobacterium marinum	Rotating Cell Culture System	[31]	562 genes altered transcription level after short growth, 328 after long growth periods. Downregulation of Metabolism. Increases sensitivity to hydrogen peroxide.
Ralstonia pickettii	Spaceflight samples in Rotating Cell Culture System	[30]	Increased growth rate
Escherichia coli	Rotating Cell Culture System	[32–35]	Shorter replication time, increased survivability in J774 macrophages, increased resistance to osmotic stress, heat and acid. Increase in biofilm thickness and biomass.
Salmonella enterica serovar typhimurium	Rotating Cell Culture System	[36]	Shorter replication time, increased survivability in J774 macrophages, increased resistance to osmotic stress, heat and acid.
Streptococcus mutans	Rotating Cell Culture System	[37,38]	153 genes upregulated two-fold or more, 94 genes downregulated two-fold or more
Lactobacillus acidophilus	Rotating Cell Culture System	[39]	Shortened lag phase, increased growth rate, increased antibiotic resistance, increased acid and bile resistance.
Bacillus subtilis	Spaceflight	[40]	55 genes upregulated (biofilm formation associated genes), 36 genes downregulated (anaerobic respiration associated genes).
Pseudomonas aeruginosa	Spaceflight	[41–43]	Different biofilm architecture to that formed under Earth gravity.
Klebsiella pneumoniae	Rotating Cell Culture System	[44]	Enhanced biofilm formation, thicker biofilms, increased cellulose production.
Vibrio fischeri	Rotating Cell Culture System	[45]	Hfq mutant studies.
Staphylococcus aureus	Rotating Cell Culture System Spaceflight	[46] [4]	Antibiotic resistance increases. Cell wall changes.

The global post-transcriptional regulator Hfq is one of the genes that has been identified to show altered levels of expression across multiple pathogenic species of bacteria in both microgravity and microgravity analogues [43,45]. This gene is found approximately in half of all known bacterial genomes and plays an important role in bacterial stress responses [45]. Hfq is an RNA-binding chaperone protein whose activity regulates bacterial protein expression via small bacterial RNAs (sRNAs) [47]. The latter regulate many bacterial processes and have a length usually ranging between 50–500 nucleotides [48]. They act via antisense mechanisms on multiple target mRNAs and exert global effects on factors such as virulence, stress responses and adaptive metabolic changes [49].

The ferric uptake regulator (Fur) and its homologues; the zinc uptake regulator (Zur), the manganese uptake regulator (Mur), and peroxide stress defence control regulator (PerR) [50] are required in some microgravity analogue stress responses in e.g., *Escherichia coli* [51]. Fur is a transcription factor which represses siderophore synthesis in pathogens by utilising Fe^{2+} as a

corepressor [50]. Many low-shear environment response genes are found in clusters or operons [36] and upstream of many of these operons is a
Fur binding site. Regulation of the low-shear response via Fur has been shown with a *Salmonella fur* mutant which is consistent with Fur transmitting the microgravity analogue signal [36]. For the acid resistance response to microgravity analogue regulon, *fur* is found upstream. When exposed to a low-shear environment, the *Salmonella* strain used in the study showed increased acid resistance whereas the Fur mutant strain showed no increase in acid resistance [36]. This strengthens the hypothesis of the Fur protein regulating a microgravity stress response regulon; however, more studies are needed.

General stress responses in *E. coli* and many other bacteria are regulated by the sigma subunit of RNA polymerase known as RpoS [52,53].

Interestingly, this is not the case for microgravity analogue response in a rotating cell culture system in *Salmonella enterica serovar Typhimurium* which adapts in an RpoSindependent manner to environmental stresses [32].

3.4. Antibiotic Resistance

A major finding of note for bacteria grown under microgravity is the increase and differences in biofilm formation, architecture, and the development of antibiotic tolerance.

Antibiotic resistance poses a severe health risk both in spaceflight and once the astronauts return to Earth. Upon return to Earth, an antibiotic-resistant strain may spread through the population. Furthermore, microgravity is just one factor during spaceflight that has been shown to increase antibiotic resistance [54]. The bacterial adaptive response, which is the exposure to a sub-lethal stressor which induces resistance to a lethal level of the same or different stressor [55], can also be triggered by ionising [56] and nonionising radiation [57] found as part of the cosmic radiation [54]. Radiation may cause changes to antibiotic efflux pumps and sensitivity to chemicals [56]. Antibiotic resistance profiles (see later) and biofilm formation are not generic responses to extreme environments. A comparative study of *Staphylococcus* and *Enterococcus* isolates from the ISS and the
Antarctic Research Station Concordia were compared and the ISS isolates were found to be more resistant to the antibiotics tested [58]. This could indicate non-space extreme environment studies may not be good substitutes or generate comparable data to the study of the extreme environment of space. However, more comparisons are needed. This could also mean microgravity and/or other space stressors may trigger the expression of different genes in the response to extreme environments.

Long term microgravity analogue studies have been performed to simulate long term manned missions to try and predict antibiotic resistances that could potentially evolve. One such study used the RCCS for 1000 generations of *E. coli* over which it became tolerant to cefuroxime, chloramphenicol, cefalotin, cefuroxime axetil, tetracycline and cefoxitin [59]. Interestingly, after a further 110 generations in Earth gravity conditions, chloramphenicol and cefalotin resistance was retained. This could be due to an accumulation of mutations.

During spaceflight, and especially on a long-distance manned mission to Mars, there will be a finite amount and diversity of medications. Especially with the longer manned missions, there is no feasible way to restock the vessels or send new medications/antibiotics. Therefore, if a multidrug-resistant strain develops and becomes resistant to the antibiotics on board the vessel then all the passengers' lives are at risk as the infection may not be treatable.

Additionally, this can also pose a threat to health on Earth. In microgravity, the bacteria may develop antimicrobial resistances that are different from those that develop in Earth's gravity. This could enable widespread infection and disease on return to earth if the pathogenic bacteria have infected one of the passengers and this has gone unnoticed.

Virulence is defined as the ability of the bacteria to cause disease and can also be referred to as pathogenic potential [60]. Increases in virulence have been reported in both analogue and spaceflight microgravity [6,61,62]. With regards to spaceflight, this is especially of concern due to the constant close contact with other astronauts in the relatively small space vessel. Increased virulence combined with antibiotic resistance poses a massive health risk and will greatly increase the dangers of both acute and chronic infections.

3.5. Archaeal Responses to Microgravity

Archaea are distinct from bacteria and are prevalent in extreme environments and are also a natural component of the microbiota of humans [63]. However, no known pathogenic archaea exist [63]. Table 3 summarises studies of archaea in a low-shear environment.

Table 3. Response of Archaea to a low-shear environment.

Name	Low-Shear Environment	Studies	Major Findings
Haloferax mediterranei	Rotary Cell Culture System	[64]	Increased resistance to bacitracin, rifampicin and erythromycin
Halococcus dombrowkskii	Rotary Cell Culture System	[64]	Reduced cell aggregation
Haloarcula argentinesis RR10	Rotary Cell Culture System	[65]	Increased production of ribosomal proteins, became multi-drug resistant, evidence of antibiotic efflux pump

Haloarchaea are the most studied area species that live/survive in aqueous environments i.e., water. Some haloarchaea show an increase in antibiotic resistance which may be a problem as archaea and bacteria can undergo horizontal gene transfer, especially from archaea to bacteria [66,67]. Horizontal gene transfer is the acquisition of new genetic material from another organism, this is a major driver of bacterial pathogen evolution and antibiotic resistance [68].

The consequences of infection are not solely dependent on the pathogen trying to infect the host. The immune response is vital in clearing infection and conferring future immunity. Microgravity has a severe impact on the immune system both as a whole and on its individual components and will be discussed in the following sections. Additionally, microgravity and other space stressors such as radiation, sleep deprivation, isolation and microbial contamination have been shown to suppress immune function [54]. This review will summarise how the natural defence against pathogens is affected and how the crosstalk between immune cells and bacterial pathogens is also altered by the microgravity stressor.

4. Immune Cell Responses to Microgravity

The immune system is composed of two different major systems, the innate immune system and the adaptive immune system. The innate response is commonly referred to as the non-specific response and usually occurs immediately or within hours after the appearance of an instigating antigen [69]. The innate immune system consists of physical barriers such as the skin and mucus and cells such as monocytes, macrophages, neutrophils, natural killer cells, mast cells, basophils and dendritic cells [70]. The adaptive immune system is commonly referred to as the acquired immune system and occurs at a later time point than the innate immune system. The adaptive immune system consists of lymphocytes known as T-cells and B-cells which specifically target the pathogen and provide future protection against that pathogen [71]. The following sections of this review will outline how both systems are affected by a low-shear environment and how this impacts on their response to bacterial infection.

Major changes in immune system function occur during spaceflight [72]. Circulating monocytes, T-cells, B-cells and neutrophils are all increased with a decrease in natural killer cells [73]. Distribution of peripheral leukocytes is altered with specific subpopulations showing diminished function [1]. Latent viruses such as herpes reactivate [74,75] and indicate compromised adaptive immune function [1]. Epstein-Barr virus, cytomegalovirus and VZV (human neurotrophic alpha herpes virus) have also been reported to be reactivated during spaceflight [73]. Hypoplasia of the spleen can also occur with an increase in peripheral blood neutrophils [76,77]. Natural killer cells exhibit lower cell cytotoxicity and there is also a delayed response to hypersensitivity skin tests [76]. One reason for the inhibition of natural killer cell toxicity is reduced production of granzyme B and perforin with effects being reported up to 60 days after spaceflight [78]. B cell activation in microgravity is still largely unknown but short-term flights have shown no significant changes [76]. This is an interesting finding as the same study suggests a Th2 shift occurs

in microgravity which may affect immunoglobulin production. Figure 3 shows the normal differentiation pathways for naïve T cells. However, this is only a hypothesis and due to a decrease in Th1 cytokines being present [76].

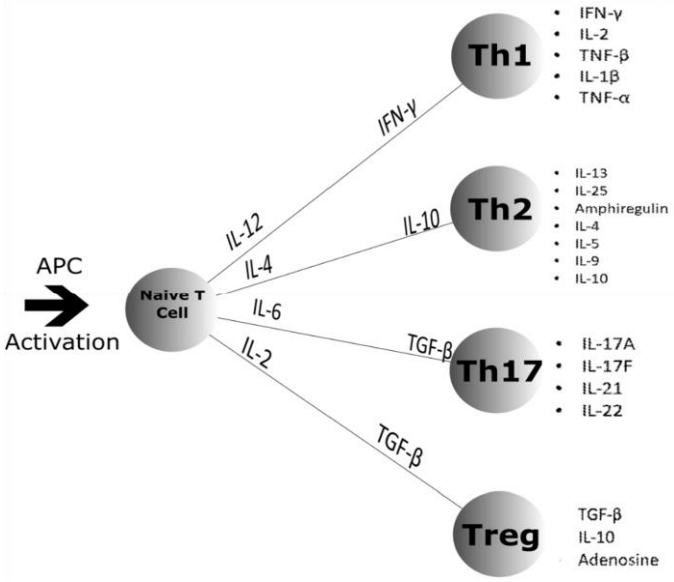

Figure 3. Naïve T cell differentiation after Antigen Presenting Cell (APC) activation. The naïve T cell differentiates into four different classes of T cell: Th1, Th2, Th17 and the Treg. APC activation causes the release of the cytokines shown along the lines that signal for which class the naïve T cell will differentiate into. Once differentiated, the T cells secrete the cytokines shown by the bullet points to the right [79–81].

Studies of long-term space missions on B cell activation and immunoglobulin production is thus far inconclusive [82–85].

Due to the nature of the low-shear environment, motility of immune cells is greatly reduced [76]. This combined with reduced monocyte motility and cytoskeletal modifications may lead to the reduced interactions between monocytes and lymphocytes which has been shown to be essential for costimulatory signalling [76].

4.1. Cell Differentiation

Differentiation inhibition has been reported by a plethora of immunological studies in a low-shear environment created by both microgravity and ground-based analogues [15,86–91].

The reasons for differentiation inhibition have been greatly speculated upon. One school of thought is that non-differentiated monocytes are suspension cells that become adherent upon differentiation. Future investigations into whether the low-shear environment prevents adherence and therefore the differentiation of the cells could warrant interesting results.

More recent studies [89] have delved into altered pathways due to low-shear forces to shed light on the differentiation inhibition problem. RAS/ERK/NF-κB pathway was shown to be a low-shear regulated pathway, where exogenous ERK and NF-κB activators were able to counteract the effects of microgravity on macrophage differentiation in both microgravity and ground-based analogues [89]. This study also verified via qPCR and western blot that the p53 pathway was also affected by the low-shear environment. This concurs with older studies which also conclude that altered genetic pathways cause immune cell differentiation inhibition [92,93]. Furthermore, cell cycle 'arrest and progression' proteins have been shown to be altered. P21 increases 4.1-fold in 20 s of spaceflight microgravity culture in primary cells and 2.9 times in Jurkat T-cells compared to ground controls. These results suggest that cell cycle progression is gravity dependent in T-cells

and can halt the progression of differentiation [94]. Additionally, these results were confirmed by other studies [95].

Differentiation into effector T-cells is also driven via dendritic cells through the production of IL-2. The alterations in IL-2 production that mimic T cell exhaustion also provides an explanation for T cell resistance to differentiation into effector T cells [96].

The surrounding microenvironment provided by the connective tissues can also have immune-regulatory effects. Mesenchymal stem cells (MSCs) are stromal cells that can differentiate into connective tissues and are integral to some specific immune responses. They do this via the production of cytokines and molecules such as but not limited to; PGE2, nitric oxide, FasL, PD-L1/2, IDO and IL-6 [97]. Culture in a low-shear environment maintains the undifferentiated state of MSCs [91] as mechanical loading is an important determining factor for osteogenic differentiation [98]. This may potentially be due to the downregulation of the master osteogenic transcription factor Runx2 and main osteogenic differentiation markers ALPL and OMD in long term microgravity analogue culture [87]. Low-shear culture also affects myogenic differentiation [98]. During spaceflight, 1599 genes have altered expression with important changes being a reduced expression of cell-cycle genes which leads to cell proliferation inhibition [99].

4.2. Pathogen Recognition

A few co-culture studies have been conducted to investigate how immune cells and bacteria respond to each other in a low-shear environment. Macrophages co-cultured with *S. enterica serovar Typhimurium* showed activation of the stress associated mitogenactivated protein kinase, kinase 4 in a ground-based analogue [100]. Furthermore, the bacteria themselves had an augmented invasive potential and increased tumour necrosis factor-alpha (TNFα) production in infected epithelial cells [100]. The same study also found increased production of *E. coli* heat-labile enterotoxin in co-cultures [100]. Finally, it was also shown by this study that murine macrophages infected with enteropathogenic *E. coli* also showed increased production of TNFα [100]. Furthermore, co-cultures have also shown that in a low-shear environment, monocytes have reduced ability to engulf *E. coli* [76]. CD32 and CD64 which are involved in phagocytosis have also been shown to be reduced in surface expression [101].

Lipopolysaccharides (LPS) are major membrane surface components that are endotoxins present in most Gram-negative bacteria with a few rare exceptions and are strong stimulators of innate immunity [102]. Stimulating immune cells grown in a low-shear environment with LPS therefore provides insight into how immunological crosstalk occurs during spaceflight and the response of the immune system to endotoxins. A major biochemical response after the challenge with LPS is p38 MAP kinase activation via phosphorylation [103]. P38 mitogen-activated protein (MAP) kinases are one of four main sub-groups of MAPs that mediate cellular behaviours in response to external stimuli [104], which potentially includes microgravity. It was hypothesised that p38 MAP kinase would be sensitive to microgravity as there are various genes such as PRKCA that are regulated by p38 MAP kinase and are sensitive to a low-shear environment [105]. However, monocytes exposed to spaceflight did not show impairment of p38 MAP kinase and actually showed a slight increase in activation [106]. *Caenorhabditis elegans* in analogue culture increased transcriptional expression of three genes that encode the core p38 MAPK pathway and expression of phosphorylated PMK-1/p38 MAPK [107]. These genes were pmk-1, nsy-1 and sek-1 [107].

Stimulating immune cells with LPS also causes NF-κB (nuclear factor-kappa B) to translocate from the cell cytoplasm to the nucleus. This translocation is altered in many cell types in microgravity and analogues [108]. A study on human Jurkat T cells showed decreased translocation of NF-κB in parabolic flight and ground-based analogues, [109] and two studies on activated human T cells via RT-qPCR and microarray on whole cell lysates [110,111] showed suppressed expression of NF-κB gene targets. An interesting connection can be made between 1g and low-shear environment studies to theorise why the translocation is altered. NF-κB has been shown to be MyD88 dependent [112] and furthermore, potential immune blunting of cells due to the low-shear environment causes suppression of MyD88 [113]. MyD88 encodes for proteins involved in the early uptake of LPS [113]. This may explain why some studies show inhibition of

NF-κB translocation in a low-shear environment from both microgravity and ground-based bioreactors.

LPS stimulation also elicits ROS (reactive oxygen species) production in macrophages. This was investigated in a microgravity analogue via Syk phosphorylation [114]. Syk phosphorylation was significantly reduced in microgravity when macrophages were stimulated by LPS, zymosan or curdlan [114], revealing that ROS production in macrophages is sensitive to gravitational forces. Other studies confirm this by showing ROS production in various cell types increases in a microgravity analogue [115]. The study also found that NF-κB signalling was unaffected by the microgravity analogue which is a later step in the signalling cascade than Syk phosphorylation, and inconsistent with the studies previously discussed. This work resulted in the proposal of a hypothesis that during long spaceflights the immune system may be able to adapt to microgravity effects [114]. Additionally, in macrophages in a microgravity analogue, TNFα but not IL-1β was suppressed following stimulation with LPS [116].

LPS is not the only bacteria-derived stimulus of the immunological response to bacterial infections. This is due to recent findings suggesting that LPS stimulation may not be affected by a low-shear environment [117]. LPS and pokeweed mitogen stimulation both failed to alter levels of TNFα and IL-10 release in whole blood [117]. The overall findings of this study concluded that the IL-2 and interferon-gamma responses to immune cell mitogen and antigen stimulation are inhibited by a microgravity analogue whereas TNFα and IL-10 secretion are greatly influenced by a microgravity analogue [117]. These results also corroborate the spaceflight sample results [2]. Mitogen stimulated immune cells showed reduced production of interferon-gamma, IL-10 and TNFα just like the microgravity analogue results. This study additionally showed reduced production of IL-6 and IL-5 [2]. A major contrast between the findings was that in a microgravity analogue via a random positioning machine, LPS stimulation did not alter levels of IL-10 production compared to ground controls whereas during spaceflight IL-10 production was reduced during LPS stimulation. These variations may be due to differences between microgravity analogues and true microgravity or it could be as a result of the differing conditions of space other than microgravity. IL-8 production was also increased during LPS stimulation in spaceflight which is concurrent with other studies [113]. Transcriptomic analysis of the immune cells during spaceflight shows suppression of MyD88, MD-2, and Lbp which are responsible for encoding [113] proteins that are involved in the early uptake of LPS [113].

The fascinating area of interest arising from comparing these studies is the difference in interleukin expression upon LPS stimulation depending on whether cells were cultured in a microgravity analogue or true microgravity. Transcriptomic analysis of LPS stimulated immune cells grown in a microgravity analogue compared to the spaceflight analysis of Chakraborty et al. 2014 may be able to add clarity to these different results.

4.3. Cell–Cell Interactions

Cell–cell interactions are severely impacted by microgravity and low-shear analogues. Dendritic cells play a vital role in recognising pathogens and activating T-cells. Murine dendritic cells (JAWSII) have recently been cultured in the rotary cell culture system for 2–14 days to determine the impact of microgravitational changes both short term (less than 72 h) and long term (4–14 days) [118]. Short term culture was shown to enhance the T-cell activation of dendritic cells through increased expression of surface proteins that are associated with maturation and interleukin-6 (IL-6) production [118]. Other dendritic cell studies in the rotary cell culture system have shown that T-cell resistance to activation in a microgravity analogue mimics T cell exhaustion found in patients suffering from chronic diseases and/or tumours due to changes in e.g., IL-2 production [96].

Other immune responses are also affected by a low-shear environment; inflammation, specifically adaptation of the vasculature (release of vasoactive factors [119]), is determined by the vessel wall state which composes of endothelial cells and mesenchymal stem cells [120]. Microgravity analogues have been shown to exacerbate the effect of endothelial cell activation by inflammatory mediators [121]. However, endothelial cell adhesive cascade molecule expression is not affected by the low-shear environment [121].

4.4. Cytokines

Cytokines are vital to the immune system and immunological crosstalk. They are small, secreted proteins influencing communication and interaction between cells [122]. The immune response to a pathogen is affected by the low-shear environment which alters the cytokine profile and consequently the function and proportion of leukocytes [73].

IL-6, which is altered in dendritic cells grown under microgravity, plays an important regulatory role in both the innate and adaptive immune system and is produced after stimulation by the majority of nucleated immune cells and plays an important role in the response to bacterial infection [123]. Studies on interleukin production and associated TLRs (toll-like receptors) during spaceflight have given inconclusive results [123]. Studies on samples retrieved after spaceflight has shown that immune cells expressing TLR2 and TLR4 both increase [124] and decrease in expression [125]. IL-6 is one of many cytokines that have been reported to have altered levels during spaceflight, studies have shown that many more cytokines exhibit altered levels depending on host health. For instance, astronauts suffering from latent virus reactivation show elevated levels of IL-1 alpha, IL-4, IL-6, IL-8, IL-10, IL-12p70, IL-13, interferon-gamma, eotaxin, and IP-10 [126]. This illustrates the changes in cell signalling in the microgravity environment and begins to reveal the scope of cytokine changes in this extreme environment [127]. Differences in adaptive reactions (i.e., changes in cytokine production) with various cytokines help to show how different parts of the immune system adapt to spaceflight. IL-4, IL-6, IL-8, and IL-10 adaptive reactions were found six months after spaceflight whereas IL-2, TNF alpha, and interferon-gamma adaptive reactions were found after only 12 days of spaceflight [128].

5. Concluding Remarks

Both bacteria and immune cells can be influenced by growth under a low-shear environment created by microgravity or an analogue. Bacteria exhibit increased proliferation, biofilm formation, and virulence gene expression making them an increased health risk, which when combined with immune dysfunction in microgravity increases the risk of opportunistic infection. The impairment of pathogen recognition and immunological crosstalk impedes and diminishes the immune response from the very early stages of disease progression. Changes in cytokine expression and production in addition to this allow for an increased chance of successful disease progression from initial colonization.

Furthermore, impairment of immune cell function reduces the ability of the immune system to clear an infection, once more promoting chronic disease progression. Prolonged immune repression upon return to Earth gravity conditions is also a significant health concern.

Immune responses in microgravity are an exciting area of research with many unexplored avenues yet to be investigated, especially the effects of long-term spaceflight. It has highlighted many obstacles that will need to be overcome before long manned missions to other celestial bodies and deep space exploration can occur.

The most important and compelling areas of research going forward should be how immune cell differentiation is inhibited. Additionally, the immune response to bacterial stimuli needs to be further elaborated upon to discover as to what extent the recognition of bacteria and subsequent signalling and host response is inhibited.

With respect to bacteria, the development of bacterial antibiotic tolerance and biofilms is a major issue that needs to be addressed for long term space flight to be a safer venture.

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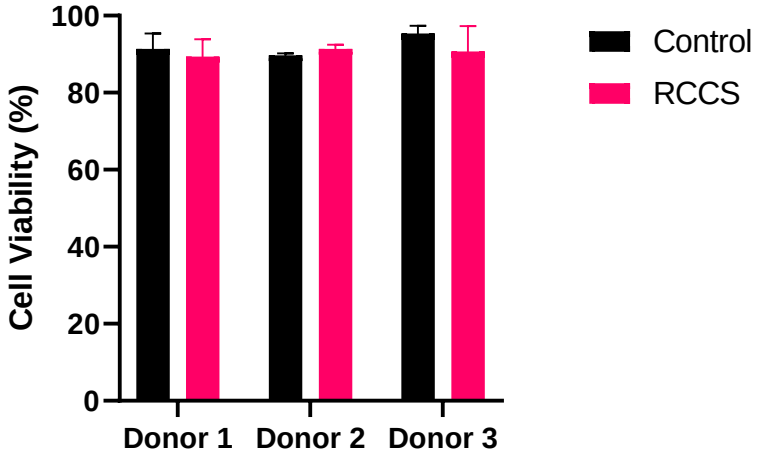
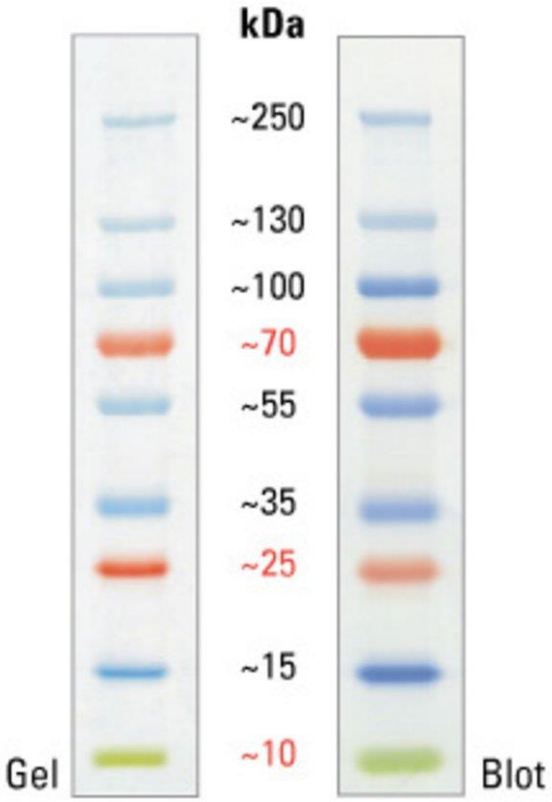
8.2 RCCS Monocyte Cell Viability



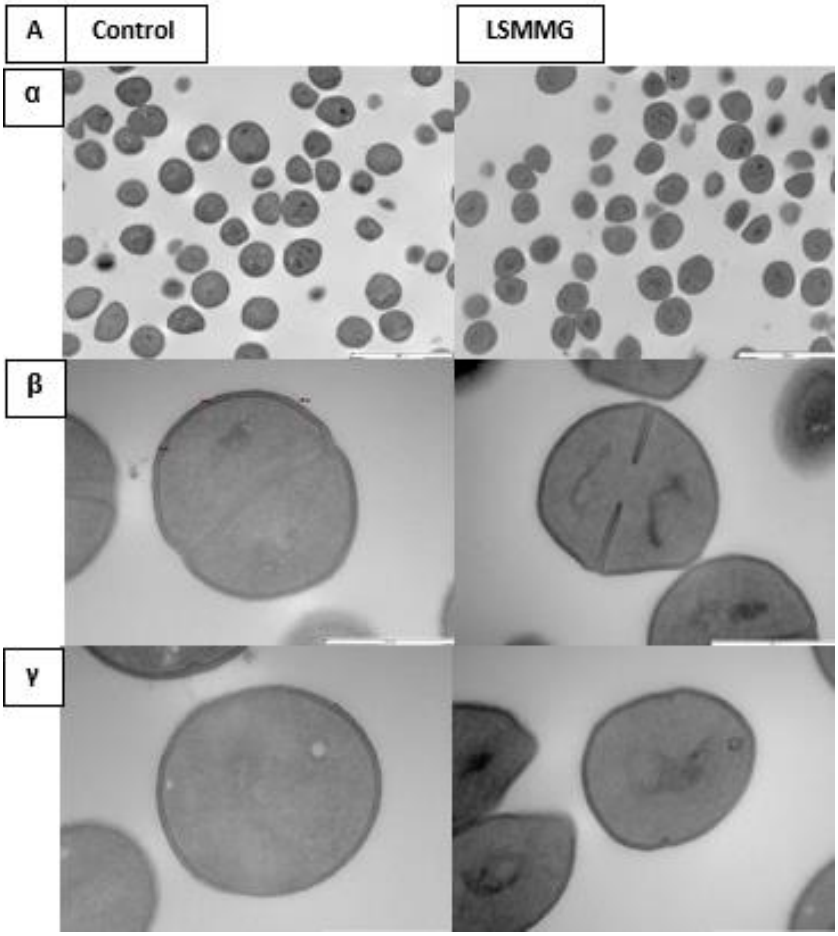
Appendix 8.2: Monocyte Viability from LPS stimulation ELISA assays (Figure 2.3.1). Monocytes were taken after 24 h LPS stimulation and mixed with Trypan Blue in a 1:1 ratio. Cell viability was determined using a Countess (ThermoFisher).

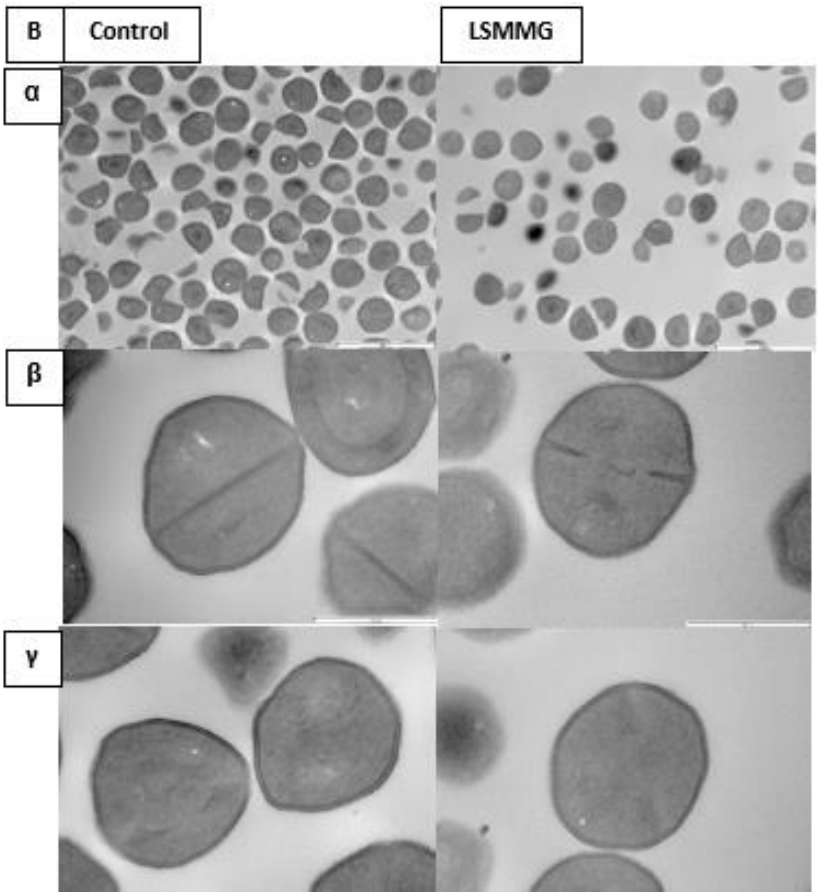
8.3 SDS-PAGE Ladder



Appendix 8.3: Molecular weights of bands in the pre-stained protein marker used in the SDS-PAGE gels. PageRuler pre-stained protein ladder (ThermoFisher).

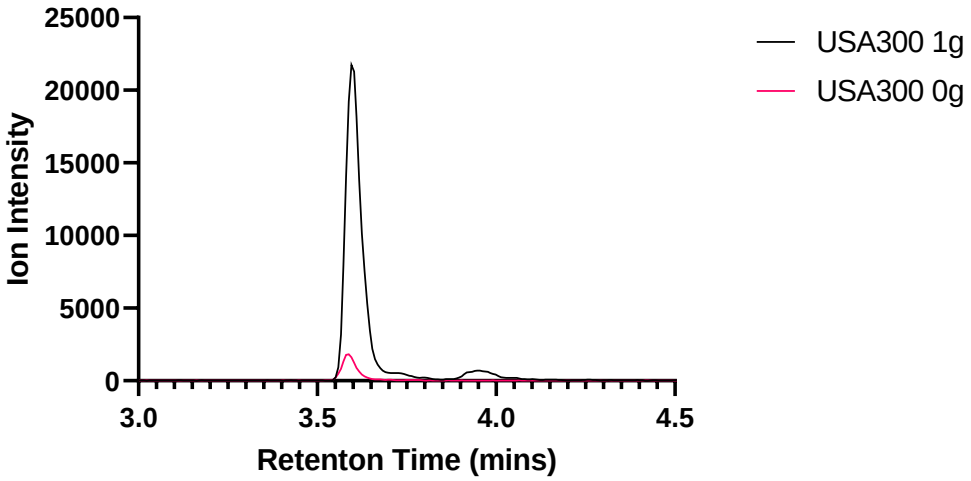
8.4 Transmission Electron Microscopy of USA300 and SH1000





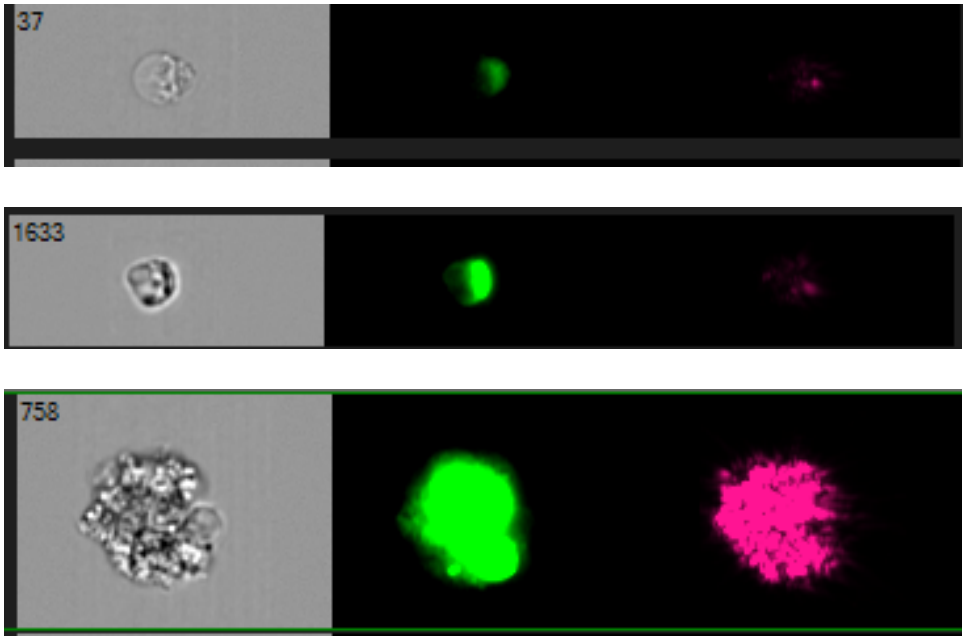
Appendix 8.4: Transmission electron microscopy of USA300 (**A**) and SH1000 (**B**) in the late exponential phase of growth in X-Vivo 15 media. **α** shows 11500 times magnification, **β** shows 60000 times magnification of a dividing cell and **γ** shows 60000 times magnification of a non-dividing cell.

8.5 Chromatogram example from mass spectrometry



Appendix 8.5: Example of chromatogram generated from AIP-1 mass spectrometry. The chromatogram presented is an example of all the chromatograms generated for quantification of AIP-1 from supernatant samples.

8.6 ImageStream Images



Appendix 8.6: Examples of low resolution ImageStream images from co-culture studies. The channels are brightfield, 488 nm (green) and over fluorescence respectively.

8.7 Covid Statement/Impact

This Ph.D. project commenced 1st October 2019 before the onset of the Covid-19 pandemic. The Covid-19 pandemic resulted in a 6 month working from home period during the second half of the 1st year of the project resulting in a loss of laboratory working time.

Upon return to labs, strict Covid regulations resulted in less opportunities for training, limited resources and longer completion times for experiments. In the Biodiscovery Institute 2, where the microbiology work was carried out for this experiment, social distancing protocols resulted in limited space in the lab where a hot-bench policy had to implemented and an enforced one week per month working from home policy was enforced to accommodate all the students. For the year this was implemented, this led to a 25% reduction in work and a reduction in training opportunities due to post-doctoral students and technicians being on different work from home rotation schedules. Additionally, some equipment was in rooms allowing only for a maximum capacity of one due to social distancing guidelines so training on these was impossible for the year and a sample drop off system was implemented. This system was heavily booked. This booking system carried over to other pieces of equipment ands limited their use and accessibility.

In Life Sciences B Floor, where the Immunology work was carried out, social distancing guidelines were also implemented for over a year upon return. This laboratory (B137b) contained four MSC Class II cell culture areas which before Covid could accommodate eight students. After social distancing was implemented, this was reduced to one student per hood and a booking system implemented, once again limiting usage and accessibility to perform cell culture inside these cabinets. This also provided significant impact for performing ELISA assays. The designated ELISA bench in the laboratory was only allowed to be used by one person at a time through the booking system due to social distancing guidelines. ELISA assays can take between 4-8 hours depending on the protocol and before social distancing, three people could use the bench and could be rotated out with other students during incubation

periods. After social distancing was implemented and due to the timescale of ELISA, it was only possible for one student a day to perform ELISA. As ELISA is a staple of immunology research and the research group using this ELISA bench consisting of between 10-20 post-graduate students and staff, availability to perform this vital technique was severely limited.

Finally, the Covid-19 pandemic caused significant supply issues. Commonly used consumables such as foetal bovine serum and goat serum became in short supply and had their prices significantly increased, leading to time and money constraints. Other common consumables such as tissue culture flasks, 24 well plates and syringes became in limited supply. These consumables have high throughput in a biological lab and shortage of these caused significant issues. Additionally, equipment such as the PCR machine was lent to QMC for Covid-19 diagnostics, removing the use of it for our lab.

To conclude, this thesis was successfully able to be completed despite the outlined issues due to the Covid-19 pandemic and social distancing protocols implemented. However, the investigations in this thesis may have been able to be performed in further depth and even an additional investigation may have been able to be performed if the Covid-19 pandemic did not happen.