IMPACT OF THE EXPOSURE TO SIMULATED MICROGRAVITY CONDITIONS ON THE PROBIOTIC *LACTOBACILLUS REUTERI* DSM 17938

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LIST OF ABBREVIATIONS

µg; microgravity

RWV; Rotating Wall Vessel

RPM; Random Positioning Machine

LSMMG; Low Shear Modeled Microgravity

EPS; Extracellular Polymeric Substances

HSP; Heat Shock Proteins

GI; gastrointestinal

LAB; Lactic Acid Bacteria

GSS; Simulated Gastric Solution

ISS; Simulated Intestinal Solution

RT-qPCR; Reverse Transcriptase-quantitative PCR

COG; Clusters of Orthologous Groups

KEGG; Kyoto Encyclopedia of Genes and Genomes

CHAPTER 1

Brief thesis presentation

1.1 Short overview

This PhD thesis is a part of MELiSSA (Micro Ecological Life Support System Alternative) space research program aiming to develop an artificial ecosystem for regenerative life support systems for long-term space missions. The goal of MELiSSA project is the understanding, modelling and controlling of five main sub-processes called compartments based on an "aquatic" ecosystem, to obtain the closure of the bio-regenerative life support system.

Designing an efficient life support system is needed to maintain the minimum life requirements for humans in space. For this reason, the health status of astronauts has been extensively investigated. In particular, many researches were focused on the study of astronauts' immune system changes induced by simulated or real spaceflight conditions. On the contrary, very few investigations can be found in the literature that address the issue of the intestinal microbiota and its significant role in the astronaut's health. Moreover, little attention has been given to the study of spaceflight effects on probiotic bacteria, that may be administered to space crew for the purpose to help and improve both innate and adaptive immune responses and in the same time prevent or reduce these effects. The principal environmental stressors of spaceflight have harsh changes in microbial response to the space environment is the microgravity (Senatore *et al*., 2018). This condition could increase the pathogenicity characteristics of some microorganisms and in the presence of more resistant bacteria, the natural terrestrial diversity of the gastrointestinal microflora could be reduced (Saei and Barzegari, 2012). The increased pathogenicity characteristics of some microorganisms, the spacecraft environment with high bacterial contamination due to reduced ventilation and a little varied diet could cause a reduction of the number and/or diversity of beneficial bacteria and, consequently, a decline in immune responses, representing a risk to the health of astronauts, already compromised by psychological stress.

This PhD thesis deals with this context treating some aspects of astronauts' safety in the space with the aim to cultivate a probiotic bacterium in one of bioreactor of MELiSSA loop to use as a direct source of supply for the astronauts' diet.

1.2 Aim and thesis outline

We assessed the role of a probiotic microorganism in a simulated microgravity environment and evaluate whether this spaceflight condition affect the metabolism of this bacterium and modify its probiotic features.

We focused the study on the probiotic strain *Lactobacillus reuteri* DSM17938, that is a principle component of some commercial products, and evaluated its transcriptome and proteome under simulated microgravity conditions to improve prediction of enzymatic pathways present in a well-defined environment. The investigations in the microgravity could highlight the mechanisms of regulation and adaptation of this probiotic bacterium, which will be helpful in uncovering the interactions between this bacterium and a confined environment, including human gut.

CHAPTER 2

General introduction

2.1 Effects of spaceflight conditions

Over the past 50 years, microorganisms were used in over 100 spaceflight experiments (Thirsk et al., 2009). The aim of these experiments was to have a better knowledge about the impact of the space environment on the microbial ecology, genotypic and phenotypic characteristics and interaction with the host. An extensive list of changes, in a wide variety of microbial cell characteristics, has been observed during spaceflight compared to ground controls. Many microbial parameters, like growth, morphology, metabolism and genetic transfer have shown strong changes during numerous in-flight studies. It should be highlighted that all the experiments should be distinguished in two main groups, those in which microorganisms are cultured during spaceflight and those in which microorganisms are subjected to spaceflight conditions but in resting state.

Overall, these studies showed that spaceflight causes increased final cell population coupled to a shortened lag phase, alteration in the secondary metabolism and increased conjugation efficiency. Moreover, during spaceflight experiments, a decreased antibiotic effectiveness and an increased contamination level compared to similar confined environments on Earth were assessed (Lapchine *et al*., 1985; Tixador *et al*., 1994). Therefore, the closed environment of spacecraft could increase, especially during long missions, the risk of infectious diseases (Ilyin *et al*., 2005). In fact, most of the studies have been focused precisely on some opportunistic microorganisms with the aim to overcome and prevent healthcare problems. Even though these experiments evaluate the effect on microorganisms of general spaceflight conditions (e.g. altered gravity, radiations, pressure and temperature), all the authors agree that they are mainly imputable to microgravity, because most of the experiments are carried out in the confined spacecraft environment in which mainly the gravity force is significantly altered (Nickerson *et al*., 2004; Li *et al*., 2015). These studies are focused to evaluate effects of spaceflight on growth kinetic, antimicrobial and biofilm activity and general stress response, of bacteria and yeast cultures.

2.1.1 Effect on growth kinetic

Pioneering investigations on board of various space missions showed that bacteria and fungi remained viable, physiologically active and capable of reproducing. These studies showed basically that microorganisms grew more densely, with a growth curve characterised by a decreased lag-phase and a prolonged exponential phase. Authors used in these experiments microorganisms that

highlight faecal environmental contamination and that are recognised as usually involved in infectious disease on Earth, like *Escherichia coli*, *Salmonella* Typhimurium and *Bacillus subtilis* (Klaus *et al*., 1997; Volkmann *et al*., 1988; Klaus *et al*., 1994; Kacena *et al*., 1997; Kacena *et al*., a1999; Kacena *et al*., b1999; Bouloc *et al*., 1991; Thévenet *et al*., 1996; Mennigmann *et al*., 1986; Mattoni *et al*., 1968). A representative investigation is that by Kacena *et al.* (a1999), who found that cultures of *Escherichia coli* and *Bacillus subtilis*, grown in liquid medium at different temperatures during a Space Shuttle mission, showed a significant more performing growth ability compared to ground controls, reaching a cell density of about 300-400% higher. Leys *et al.* (2004) reviewed the literature on the spaceflight effects on bacterial physiology and we refer to its reading for a comprehensive view of the field at that time.

More recently, experiments carried out in space, confirmed that microorganisms grow better in space then on Earth. For example, *Escherichia coli* cultured on board the International Space Station (ISS) showed a 13-fold increase in the final cell count compared to the ground control (Zea *et al*., 2017).

Interestingly, Kim *et al.* (2013) tested different growth conditions (i.e. phopsphate and oxygen concentration) of the strain *Pseudomonas aeruginosa* PA14 during the Space Shuttle Atlantis spaceflight. They found an increased final cell density compared to normal gravity controls only when low concentrations of phosphate in the media were combined with decreased oxygen availability. In all other cases no difference was registered. Therefore, authors hypothesized that gap in the final cell density between spaceflight and normal gravity could be due to an interaction between microgravity condition and the availability of substrates essential for growth.

Contrasting finding was reported only in one experiment, recently performed on board of ISS, which showed that the strain *Staphylococcus epidermidis* ATCC12228 grew at a lower final cell density compared to ground control (Fajardo-Cavazos *et al*., 2017).

Other experiments were carried out by a Chinese research group (Li *et al*., 2015; Wang *et al*., 2014; Guo *et al*., 2015) who compared the growth of spaceflight mutant strains of *Serratia marcescens*, *Klebsiella pmeumoniae* and *Escherichia coli* with that of parental strains. However, these experiments were conducted on ground after the growth of parental strains on board of ShenZhou VIII spacecraft. They did not find differences in the growth performance. Surprisingly, they did not report data on final cell concentration of cultures grown during spaceflight, lacking the chance to compare the growth performance in the space to that on Earth.

Experiments were also performed by using mould cultures like *Ulocladium chartarum*, which was cultured during ISS flight for 14 days and then colonies compared to ground control. Authors found

that spaceflight conditions reduced the grow rate of aerial mycelium but stimulated the growth of submerged mycelium and new microcolonies (Gomoiu *et al*., 2013).

2.1.2 Effect on physiological characteristics, mutation frequency and gene transfer

Numerous studies were performed to assess physiological modifications of potential pathogens during space missions to preserve the astronauts' health. During the French-Soviet space mission on July 1982, the Cytos 2 experiment was performed, in which the minimum inhibitory concentration (MICs) of different antibiotics was assessed in *Staphylococcus aureus* and *Escherichia coli* (Tixador et al., 1985). Results indicated an enhanced resistance of both microorganisms to all antibiotics tested. Similar analyses were performed in subsequent flight experiments to confirm this effect, and they have repeatedly shown increased antibiotic resistance in these organisms (Lapchine *et al*., 1986). Moreover, a more recent study (Fajardo-Cavazos *et al*., 2016) showed an increased frequency of mutations for *rpoB* gene involved in rifampicin resistance.

Saccharomyces cerevisiae has been also extensively studied during space programs. Some experiments demonstrated that this yeast responded to microgravity through both metabolic and phenotypic changes. One of these modifications regards the bud scar pattern that showed a switch toward more random budding in a spaceflight experiment (Van Mulders *et al*., 2011).

There is a great interest to study the effect of microgravity on fungi, not only because some of these species are expected to be possible contaminants of the spacecraft, giving rise the possibility to infect all crew members, but especially because they produce a variety of secondary metabolites (SMs) with many potentially useful biological activities. SMs, e.g. polyketides, non-ribosomal peptides and terpenes could contribute to the survival of the producing organism in a defined ecological niche (Boruta *et al*., 2017).

Bacteria or fungi with biodegradative and biocorrosive properties may jeopardize the integrity of the spatial hardware and may have a negative effect on the crew health by causing infections. In fact, these microorganisms, through their life activity, are able to cause biointerference in hardware functioning, degrade various structural materials including synthetic polymers, and produce or provoke corrosion of metals (Novikova *et al*., 2004).

Observations of spacecraft contamination include the discovery of biofilms in the water system and other surfaces (Novikova *et al*., 2004; Ott *et al*., 2004; Schultz *et al*., 1989; Son *et al*., 2005). Some studies are focused on this microbial property that could be involved in increased pathogenesis and affected by space conditions. Wilson *et al*. (2007) reported that *Salmonella* Typhimurium cultured in space exhibited clear differences in cell aggregation and clumping, which they saw was associated with an extracellular matrix accumulation consistent with biofilm formation.

On the other hand, Kim *et al.* (2013) did not observe cell clustering on *Pseudomonas aeruginosa* cultured in space, which they suggested was likely due to differences in the extracellular polymeric substances of the strain they used. In fact, unlike *Pseudomonas aeruginosa* PAO1 cultured under simulated microgravity, *Pseudomonas aeruginosa* PA14 cultured during spaceflight did not form cellular aggregates in planktonic cultures. This observation is likely due to differences in the extracellular polymeric substances (EPS) that are responsible for cellular aggregation (Kim *et al*., 2013). Furthermore, different results in simulated microgravity and spaceflight condition may be due to the fact that the spaceflight environment has far lower shear than the low shear modelled microgravity (LSMMG) encountered in simulated microgravity (Crabbé *et al*., 2011).

In another experiment of Kim *et al.* (2013), *Pseudomonas aeruginosa* was cultured during two Space Shuttle missions (STS-132 and STS-135) and the biofilms formed during spaceflight were characterized. The biofilms exhibited an atypical structure composed by columns overlaid with a canopy that has never been observed before on Earth.

Enhanced cell aggregation was registered also in *Candida albicans* during short-term spaceflight (Crabbé *et al*., 2013).

Also the virulence of some bacteria were altered under spaceflight condition. In fact, experiments with mice infected with *Salmonella* Typhimurium grown in-flight displayed an increased percent mortality with a shorter time to death and showed a decreased LD50 (Wilson *et al*., 2007). Conflicting findings showed that some pathogenic microorganisms resulted less virulent than ground controls when grown under microgravity conditions. They were tested against both larval and adult stage of nematode *Caenorhabditis elegans* both under spaceflight condition on the ISS and LSMMG condition, demonstrating a reduced virulence of pathogens (Hammond *et al*., 2013). Even though under LSMMG the decrease in the virulence of pathogens was less pronounced, the overall effect of actual and simulated microgravity was comparable.

There is general agreement that microgravity represents the major influence on bacterial growth kinetics and bacterial cell behaviour during short orbital flights, although radiation may increase microbial mutation rates during flight. Accordingly, after 40 days aboard Mir, mutation rate for a cloned bacterial gene was 2-3 times higher than the ground control (Fukuda et al., 2000). Moreover, the confined environment may create a particular niche for bacteria with increased infectious potential that could be linked to the transfer of plasmids. Bacteria from the *Bacillus* genus present in confined places, such the ISS, showed the capability to perform horizontal gene transfer by acquiring new DNA and sharing genetic material (Timmery *et al*., 2011). The effect of spaceflight on genetic transfer has been investigated numerous times and has indicated increased conjugal transfer rates in *Escherichia coli* (Cifferri *et al*., 1986) and changes in phage induction in

Escherichia coli and *Salmonella* Typhimurium (Alpatov *et al*., 1968; Mattoni *et al*., 1968). Gene transfer via plasmid-mediated conjugation was also assayed in spaceflight within the experiment MOBILISATSIA. Aboard the ISS during the Soyuz Mission 8S two triparental conjugation experiments were performed for studying the impact of spaceflight conditions on plasmid-mediated conjugation: the first experiment was performed with three different strains of *Bacillus thuringiensis* and a second experiment with two strains of *Escherichia coli*, and *Cupriavidus metallidurans* as the final recipient. The plasmid exchange occurs more efficiently between the Gram-positive strains than in the ground control experiment. On the contrary, no significant differences could be observed between spaceflight and ground control for the Gram-negative bacteria (De Boever *et al*., 2007).

Not only potential pathogenic bacteria, but also other model microorganisms were chosen to investigate the spaceflight effects on survival rates, cell structure modifications, and genomic damages in ISS within the frame of the Italian ENEIDE mission, from Feb to Oct 2005 (Canganella and Bianconi, 2007). These microorganisms belong to three taxonomic Domains and to different ecosystems (food, soil, human, intestinal tract, plants,deep-sea) in order to investigate the response to spaceflight conditions, even of microorganisms potentially useful for the astronaut's health in space (i.e. *Lactobacillus acidophilus*, *Streptococcus thermophilus*, and *Enterococcus faecium*) and of microorganisms typically representative of aquatic and soil ecosystems or naturally resistant to adverse conditions (i.e. *Pseudomonas fluorescens, Rhizobium tropici, Bacillus subtilis)* of which it would be interesting to study genes of resistance to space conditions. They observed that a prolonged exposure of microorganisms aboard the ISS resulted in cell mortality and cell morphology alterations according to the specific species investigated, in some cases even to the specific strain.

2.1.3 Effect on gene expression

Some studies have been based on omic approach (i.e. proteomic and expressomic studies) to reveal the effect of space conditions at transcriptional and transductional level. A representative study describing the whole-genome transcriptional and proteomic analysis of *S.* Typhiurium growing in liquid media in spaceflight, revealed that 167 transcripts and 73 proteins altered their expression level compared to ground cultures. Interestingly, of the 69 and 98 up- and downregulated genes, many were found to be part of the *hfq* regulon (Wilson *et al*., 2007). Following spaceflight, a *Pseudomonas aeruginosa* gene expression study revealed that *hfq* was downregulated. Hfq protein,

encoded by the *hfq* gene, is a small RNA binding protein that globally regulates gene expression. In general, transcripts under positive control by Hfq were found to be downregulated while transcripts under negative control by Hfq were upregulated (Crabbé *et al*., 2011). Furthermore, it appeared that Hfq could potentially be a broad global regulator of spaceflight-responsive genes, at least in related Gram-negative bacteria. In another study two spaceflight mutants of *Escherichia coli* isolated after 17 days' growth on spaceflight were characterized by transcriptional and proteomic analysis (Li *et al.,* 2015). Strains diverged from the control one in the expression of many genes and proteins. Genes involved in lipid metabolism, chemotaxis and cell motility were differentially expressed. They also noticed significant changes in the expression of transporter genes as previously found by other authors (Vukanti *et al*., 2008; Arunasri *et al*., 2013). The proteomic analysis identified different regulation level in seven proteins involved mainly in lipid and phospholipid biosynthesis. After the same flight, two mutants from a starting strain of *Serratia marcescens* were selected and analysed for transcriptome and proteome (Wang *et al*., 2014). Authors found changes in metabolic profiles and in the expression level of metabolic genes, comparing the two spaceflight strains among them, and them to the ground control strain. Specifically, changes found at proteomic level were associated to some metabolic functions, like glycolysis/gluconeogenesis, pyruvate metabolism, arginine and proline metabolism and degradation of valine, leucine and isoleucine.

Another microorganism, *Rhodospirillum rubrum* S1H, was sent twice to the ISS within the MESSAGE 2 (Microbiological Experiment on Space Station About Gene Expression) and the BASE-A (Bacterial Acclimation and Adaptation to the Space Environment Conditions-A) experiments. This microorganism was analysed post-flight using a newly developed *Rhodospirillum rubrum* whole genome oligonucleotide microarray and high throughput gel-free proteomics with Isotope-Coded Protein Label technology. The data from transcriptomic and proteomic analysis were compared with that from modeled microgravity experiment and simulation of ISS-ionizing radiation (SimRAD) experiment. The experiment in BASE-A gave the highest number of significant differentially expressed genes with 169 up- and 41 downregulated genes and of the 65 significant differentially expressed genes, 11 were confirmed at the proteomic level. Genes that were upregulated coded for functions involved in transport, cell envelope biosynthesis or maintenance, redox balance, transcriptional regulation, amino acid metabolism and stress response. Moreover, the two spaceflight experiments shared only five genes that were commonly differentially expressed within the threshold limits including the genes encoding for the NADH dehydrogenase subunit L, a ribosomal protein and the putative transcriptional regulator (Mastroleo *et al*., 2009). Results of an on satellite experiment showed that space condition (microgravity as interpreted by the authors) is "sensed" by yeast cells as a stress condition and several mitogen-activated protein kinases (MAPK)

signaling pathways are activated, including the cell wall integrity (CWI)/protein kinase C (PKC), the high osmolarity glycerol (HOG) and the target of rapamycin (TOR) pathways. Authors concluded that their results show that microgravity imposes a stress condition that has the characteristics of an osmotic stress. Indeed, cellular energy is directed towards protective measures such as cell wall biosynthesis (CWI pathway activation) and the production of compounds (glycerol, trehalose) that increase the osmotolerance (HOG pathway) (Willaert *et al*., 2013).

2.2 Systems to mimic or reproduce space microgravity

Ground-based facilities have been used in space research for studying the cellular and molecular biology of different microorganisms giving new insights into the behaviour of microbial cells under altered gravity conditions. Some platforms in actual microgravity were used for exposing cells to effects of reduced gravity. These platforms impose different times of microgravity. Out of the orbital platforms like satellites or space stations that mimic microgravity from weeks to some years, ground-based systems are: i) sounding rockets that determine from 3 to 13 min of microgravity; ii) parabolic flight campaigns that permit a microgravity time of about 20-30 s and iii) drop towers with a microgravity time of about 5 s. The parabolic free-fall flights reach reduced *g* levels, in the range of 10^{-2}-10^{-3} *g*, providing the possibility of performing microgravity experiments within 20 to 30 seconds (Herranz *et al*., 2013).

The microgravity environment of spaceflight is simulated with numerous ground-based methods in order to overcome the limitations of biological gravitational research. Numerous ground-based methods have been developed to simulate the condition of weightlessness in laboratories on Earth, starting from the classical clinostat introduced in 1879 by Julius Sachs. The clinostats are represented by two main systems, the rotating-wall vessel (RWV) and the random positioning machine (RPM). Clinostats with one rotation axis are called 1-D (rarely used) or 2-D clinostats, which run perpendicular to the direction of the gravity vector. Those with two axes, also known as 3-D clinostats (most common), are represented by the RPM (Nickerson *et al*., 2004; Leys *et al*., 2004; Herranz *et al*., 2013). The RWV bioreactor is a 2-D clinostat designed at the NASA Johnson Space Centre (Houston, Texas, USA). Unlike the normal gravity orientation in which the axis of rotation is parallel to the gravity vector, in the 2-D clinostat the rotation occurs on an axis perpendicular to the direction of the gravity force vector (Nickerson *et al*., 2004). In this system, cells grow with low-fluid-shear (<1 dyn/cm2) under a controlled environment, where the liquid counteracts the slow sedimentation, creating a constant "free-fall" of the cells through the culture medium. Therefore, a constant state of suspension in which cells living in tranquil surroundings

without turbulent forces was reproduced (Leys *et al*., 2004; Hammond *et al*., 2001). This bioreactor is composed by a vessel, the rotation base unit and the power supply. The culture media is introduced through a port of vessel positioned on the bioreactor perpendicularly to the ground. The bioreactor is then placed in an incubator at a fixed temperature for optimal cell growth. A cell within this environment encounters the sedimentation effect because when the rotation of the full vessel increases its velocity is transferred radially inward until relative fluid motion ceases (Klaus *et al*., 2001; *Nauman et al*., 2007) . As the cells drop to the bottom of the vessel, they are brought back upwards by the solid body rotation of the media and then remain suspended in the fluid in an orbital path (Fig. 2.1).

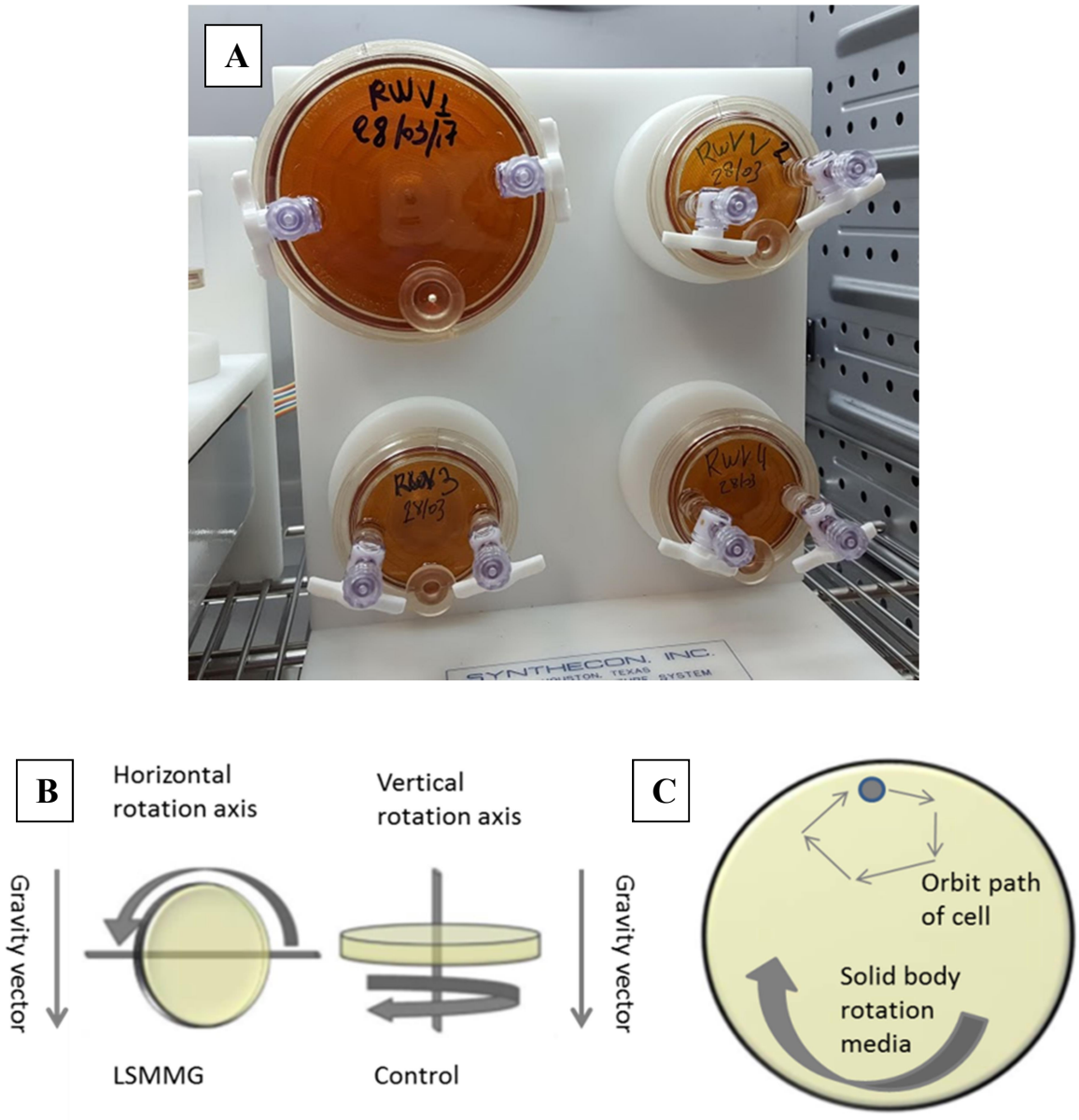


Fig. 2.1: Rotating wall vessel apparatus.

A: The vessel is completely filled with media containing cells (no air bubbles) and rotated at 25 rpm. Aeration is provided via an air-permeable membrane located in the back of the vessel. The apparatus is located in an incubator with fixed temperature.
B: The two orientations of the RWV for experimental set up. In the LSMMG orientation, the axis of rotation of the RWV is perpendicular to the direction of the gravity force vector. In the normal-gravity (control) orientation, the axis of rotation is parallel to the gravity.
C: In the pictured orientation of the vessel (LSMMG condition), this creates a low shear, low turbulence suspension culture environment for cell growth. This is obtained by the solid body rotation of the media in the apparatus in such a way that sedimentation is counterbalance to allow suspension with a minimum of fluid shear forces. Images B and C modified from Yamaguchi *et al.*, *Microbes Environ*. 29 (3), 250-260 (2014). Microbial Monitoring of Crewed Habitats in Space-Current Status and Future Perspectives.

The RWV bioreactor was initially used for eukaryotic cells (Anken, 2013) and then for examining bacteria (Nickerson *et al*., 2000; Crabbé *et al*., 2008; Mastroleo *et al*, 2013), fungi (Johanson *et al*., 2006) and archaea (Dornmayr-Pfaffenhuemer *et al*., 2011) in response to this environment.
The most commonly used microgravity simulator is the RPM apparatus developed by Takayuki Hoson at the Osaka City University. This machine changes the position of an accommodated experiment in three-dimensional space (Hoson *et al*., 1992; van Loon, 2007; Borst *et al*., 2009) with two independently rotating frames (Fig. 2.2) operating with different speeds and directions. This generates positive and negative acceleration forces reproducing microgravity in the order of 10^{-3} *g* (Hoson *et al*., 1992). However, the RPM simulation system has rarely been used for experiments on microorganisms (Mastroleo et al., 2009); instead, many are performed using this device to investigate microgravity-analogue-induced alterations in eukaryotic cells (Meloni *et al*., 2004; Bizzarri *et al*., 2015).

2.3 Effect of simulated microgravity on microbial responses

In an effort to reproduce the low-shear condition that could model aspects of spaceflight (i.e. microgravity), the clinostats RPM and RWV were used for Earth-based studies with the aim to learn how the environment of space could impact both eukaryotic and prokaryotic physiology. There is increasing evidence not only that entire organisms sense and respond physiologically to low-shear, buoyant environments but also that cells respond at the molecular level to this environment. In fact, mechanical shear forces have been demonstrated as having an impact on bacterial physiology and pathogenesis in several studies but in general the effects are comparable to that of actual microgravity (Nickerson *et al*., 2004; Thomas *et al*., 2002).

Fig. 2.2: Random positioning apparatus.
This device has independent motor drives for each frame and these are constantly rotated at 60 rpm. In a fast rotating clinostat running at 60 rpm the residual g is between 10^{-3} and 10^{-2} g.

2.3.1 Effect on growth kinetics

Experiments conducted in microbial liquid cultures under simulated microgravity conditions have shown a shorter lag phase, as well as longer log phase (Brown *et al.*, 2002; Shao *et al*., 2017) and in most studies the bacterial final cell density was greater than those conducted under normal gravity (NG) (Nickerson *et al.*, 2004; Wilson *et al.*, 2002). Moreover, the lack of sedimentation determines a uniform distribution of cells with higher accessibility to nutrients, allowing bacteria to reach higher final density. Furthermore, some results demonstrated that bacterial physiological responses to microgravity condition depend on growth phase but also on growth medium proving that nutrient resources are modulators of response. Some zones of nutrient depletion developed around bacteria stimulate the entrance into stationary phase and led to overexpression of starvation inducible genes associated with nutrient transport system, stress resistance and metabolic enzymes (Vukanti *et al*., 2008).

Like in spaceflight condition, changes in nutrient composition and then changes in pH of media may determine, under LSMMG conditions, different metabolic responses (Kim *et al*., 2014). In fact, the growth curves of three different *Escherichia coli* strains under microgravity and control conditions are very different depending on the growth media, but in every case optical density measurements under LSMMG were significantly higher than those under NG. (Kim *et al*., 2014). Moreover, glycerol is a modulator that in the medium could help to pass multiple stressors due to nutrient limitations in microgravity conditions because it could enter the cell through simple diffusion and get utilized as a carbon source.

Yeast cells grown under LSMMG conditions, contrarily to *in vivo* study (Liu *et al*., 2008), did not differ in growth rate, cell size and shape, or viability from the controls but, likewise to actual microgravity effect, differed in the polarity as exhibited by random budding compared to the usual bipolar pattern of control. It was hypothesized that this disturbance may possibly be the result of the influence of microgravity on the cytoskeleton, influencing the position of the bud scars. In fact, under microgravity conditions there was a significant increase in expression of budding-related genes (Purevdorj-Gage *et al*., 2006)

2.3.2 Effect on physiological characteristics and gene transfer

Microgravity can also affect the production of SMs, as assessed for the first time by Fang *et al.* (1997) in antibiotic producer microorganisms cultivated in a RWV apparatus. They reported that RWV environment decreased in *Streptomyces clavuligerus* the beta-lactam production (Fang *et al*., 1997), in *Streptomyces hygroscopicus* inhibited the rapamycin production (Fang *et al*., a2000) and in *Escherichia coli* prevented micocin B17 production (Fang *et al*., b2000). The reason of the reduced secondary metabolite production could be due to reduced shear stress encountered in the RWV bioreactors (Demain *et al*., 2001).

RWV creates a low level of fluid shear that is physiologically similar to the brush border of the intestine in which some microorganisms are induced to produce attachment-independent biofilms, as the copious amounts of EPS encasing LSMMG-cultured bacterial cells. Bacterial biofilm alterations as a result of the low fluid shear in the LSMMG environment have been demonstrated. This is in contrast with some results in spaceflight condition where *Pseudomonas aeruginosa* seems to produce less cellular aggregates in planktonic culture (Kim *et al*., 2013), whereas *Salmonella* grown during spaceflight (Wilson *et al*., 2007) is able to produce extracellular matrix consistent with a biofilm.

Wang *et al.* (2016) showed that the ability of biofilm formation is enhanced in *Klebsiella pneumoniae* grown under simulated microgravity in RWV. In a more recent work, heterogeneous morphology in *Klebsiella pneumoniae* continuously cultured in RWV is reported (Wang *et al*., 2017). Indeed, two physiologically distinct subpopulations were isolated, based on biofilm formation, cellulose production, type 3 fimbriae expression and antibiotics resistance.

Self-aggregative biofilm phenotypes of bacterial cultures (*Pseudomonas aeruginosa, Escherichia coli, Staphylococcus aureus* and *Streptococcus mutans*) were also reported after growth in RWV (Castro *et al*., 2011; Crabbé *et al*., 2010; Lynch *et al*., 2006; Tucker *et al*., 2007; Orsini *et al*, 2017). *Staphylococcus aureus* grown in a low fluid shear environment was evaluated for biofilm formation. This microorganism has shown a new attachment-independent biofilm phenotype and a

distinct extracellular polymeric substance, which encased the bacterial cells. These aggregates of cells showed slower growth but were more resistant to antibiotic stress and possessed repressed virulence characteristics compared to NG control (Castro *et al*., 2011). *Pseudomonas aeruginosa* growing in the RWV determined a self-aggregation and increased alginate production conferring a typical phenotype of the cystic fibrosis pathology in the lung (Crabbé *et al*., 2010). Additionally, *Escherichia coli* after 24 h of incubation in LSMMG vessel is also capable of forming a thick biofilm exhibiting a high tolerance to salt, ethanol and two antibiotics in comparison to the normal gravity control (Lynch *et al*., 2006; Tucker *et al*., 2007). Also, *Streptococcus mutans* showed to form more compact biofilm when cultured in RWV compared to NG growth, increasing its cariogenic potential (Orsini *et al*., 2017).

LSMMG could also determine the change of biofilm formation in an eukaryotic cell type, like *Candida albicans*. The growth of the pathogenic strain SC5314 of *Candida albicans* in simulated microgravity resulted in a morphogenic switch that is correlated with enhanced pathogenicity (Altenburg *et al*., 2008). These authors observed an increase in filamentous form of the organism and changes in the expression level of two genes associated to yeast-hyphal transition. This finding was confirmed for the same strain (Searles *et al*., 2011) and more these authors found that the strain enhanced the ability to form biofilm and the resistance to antifungal action of Amphotericin B.

Like in spaceflight condition, also in simulated microgravity *Salmonella* Typhimurium was more virulent in mice, compared to when bacterium grown in normal gravity were used. Remarkably, Chopra *et al.* (1999) reported that three logs less of a *Salmonella* Typhimurium strain grown in microgravity were required to kill 50% of mice, compared to when microorganism were grown under NG.

Moreover, changes in membrane fluidity are observed in some bacterial mechanism of adaptation to a variety of environmental stresses like temperature and acidity. A variation on membrane potential and integrity in *Escherichia coli* and *Staphylococcus aureus* grown in modeled reduced gravity was demonstrated (Vukanti *et al*., 2012). In this study the higher membrane potential under microgravity conditions suggested that the more active bacterial membranes under these conditions may be due to even distribution of cells that proves the higher accessibility of nutrients.

Another study showed a correlation between the increase in membrane fluidity and the heat stress sensibility in different four strains of *Escherichia coli* O157:H7 (Kim *et al*., 2016). They found that exposure to LSMMG conditions reduced the heat resistance and changed membrane fatty acid composition increasing the fluidity. Accordingly, LSMMG determined under expression of eight heat stress-related genes in all the four different strains.

Some ground laboratory investigations showed high probability of transfer of large fragments or even a whole chromosome in spaceflight condition, but the probability is higher between Gram-positive bacterial strains than Gram-negative ones. In the case of Gram-positive bacteria, this ability to exchange plasmids in weightlessness, as occurs under Earth's conditions, should be seen as particularly relevant in the scope of spread of antibiotic resistances and bacterial virulence. In fact, like in spaceflight conditions, plasmid transfer and plasmid-mediated mobilization between *B. thuringiensis* strains were also observed in simulated microgravity experiments (Horneck *et al*., 2010; Beuls *et al*., 2009).

2.3.3 Effect on gene expression

Gene and protein expression profiles were evaluated on *Salmonella* Thyphimurium after growth in liquid medium under simulated microgravity. The expression of 163 genes belonging to different clusters and operons was altered under LSMMG effect and also multiple differences were observed in the total protein and immune-reactive protein profiles (Wilson *et al*., 2002). A more recent work indicated that *Salmonella* Thyphimurium exhibited higher resistance to hydrogen peroxide when cultivated under LSMMG and two catalases enzymes were upregulated demonstrating their involvement in hydrogen peroxide resistance induced by LSMMG (Pacello *et al*., 2012). Furthermore, as in the space environment, also in response to LSMMG there was a down regulation of the conserved RNA-binding protein Hfq. This protein seems to be implicated in modeled microgravity response in both *Pseudomonas aeruginosa* and *Staphylococcus aureus* confirming a Hfq link with fluid shear not only for Gram-negative organism but also for Gram-positive one (Crabbé *et al*., 2008; Castro *et al*., 2011; Crabbé *et al*., 2010).

Another potential pathogenic bacterium studied in simulated microgravity is *Streptococcus mutans,* an oral bacterium responsible of dental caries. An omic approach, by metabolomic and RNA-seq, were used for evaluating the effects of simulated microgravity on gene expression and metabolites production. A total of 247 genes were differentially expressed, 153 were upregulated and 94 were downregulated during simulated microgravity experiment: these included a number of genes involved in carbohydrate metabolism, translation, and stress response (Orsini *et al*., 2017).

In two works (Purevdorj-Gage *et al*., 2006; Sheehan *et al*., 2007) the behaviour of *Saccharomyces cerevisiae* grown in a high-aspect ratio vessel, which mimics the low-shear condition encountered in spaceflight, was examined. This organism differentially expressed over 1000 genes many of which were involved in cell signalling pathway that allow to detect the environment change. Authors found that the expression of genes involved in cell polarization and bud formation was altered.

2.4 Microbial mechanisms of responses to space radiation

Space radiation is composed by *i)* galactic cosmic rays, *ii)* solar particles and *iii)* geomagnetically trapped particles. Group *i)* is composed by high-energy protons (90%), α-particles (9%) and heavy particles (1%); *ii)* is primarily composed by protons and electrons, α-particles and heavy particles; finally, *iii)* is composed by protons and electrons (Ohnishi *et al*., 2004). As well known, ionising radiations of space can transfer energy (linear energy transfer, LET) enough to ionize, or to eject at least one electron from a neutral atom or a molecule. Consequently, they can interact with DNA and cause reparable or irreparable damage, depending on the type and energy of the radiation. Such damages have the potential to modify the genetic code through mutations, alter the way DNA functions, and transfer mutations to the next generation(s) (Horneck *et al*., 2010). Nevertheless, a high dosage of ionizing radiation can force organisms to develop mechanisms that enable them to survive in extreme conditions. Many cells naturally resist the ionizing effects of radiation, particularly in cases of the low exposure levels commonly found in the environment (Matallana-Surget and Wattiez, 2013).

The mechanism of radiation resistance of cells depends on their capacity to repair DNA (Cadet *et al*. 2005). However, others suggested that the mechanism of radiation resistance is due to the ability to neutralize oxygen free radicals (Shrivastav *et al*., 2009). At time, there are some difficulties to mimic and reproduce different radiation components at the same time over a wide energy range because no simulations set-up on the Earth would be able to reproduce all the stress factors related to a spaceflight mission. On the contrary, many experiments were performed in spaceflight by using many radiation detectors and software containers connected to each other, as the BIOSTACK apparatus designed by the European Space Agency (ESA). These instruments were used in a large number of experiments, for example by the Cosmos series satellites and on the MIR space station. The results of these experiments lead to the conclusion that biological effects of ionizing radiation in space are different from the effects on Earth (Horneck *et al*., 2010). In fact, a greater incidence of mutations has been observed (i.e. genetic alterations) on samples in flight, a greater difficulty of cells in reacting to radiation. Ionizing radiation, in fact, can cause DNA/RNA damage such as double/single strand breaks and base modifications. Moreover, UVA and visible light can activate the production of reactive oxygen species (ROS) that interact with DNA, proteins and lipids, or could be involved in a mechanism of synthesis of a specific light-dependent repair enzyme (photolyase), called photo-reactivation. However, the bacterial radioresistance mechanism depends on the level of accumulation of damaged proteins (Daly *et al*., 2007). Radiation−resistant organisms could experience various genomic damage following irradiation like sensitive organisms, but they could survive the formation of hundreds of IR−induced DNA double-strand breaks (DSBs) per

genome. These microorganisms, belonging to thermophilic bacteria, possess various stress adaptation mechanisms which enable them to bypass multiple physical and chemical barriers for survival (Ranawat *et al*., 2017).

A radiation sensitive bacterium is *Shewanella oneidensis* that is killed by low level of ionizing radiation (D_{10} is 0.07 kGy). *Shewanella oneidensis* MR-1 is an environmental gamma proteobacterium strain capable to reduce a variety of compounds, such as toxic metal ions and radionuclides (Middleton *et al*., 2003) but it is very sensitive to all wavelengths of UVR, solar UV, and ionizing radiation (Qiu *et al.*, 2005). This microorganism possesses some important DNA damage repair and damage tolerance systems like the more radiation resistant bacterium *Escherichia coli* and in particular these mechanisms include SOS system, mutagenic and mismatch repair and a DNA photolyase (Qiu *et al*., 2005). Two transcriptomic studies found that after exposure to UVA light treatment *Shewanella oneidensis* MR-1 expressed many genes twice than after exposure to UVC light. *Shewanella oneidensis* experienced a greater oxidative stress during the irradiation treatment that induce a nearly activation of catalase. The genomic response to UVA was greater than UVB and UVC, with about 8% of the genome expressed differentially and most of the significant genes distributed in 16 functional categories (Qiu *et al*., 2006).

On the other hand, the life support organism *Rhodospirillum rubrum*, exposed to a low cumulated dose of ionizing radiation (2 mGy) on board of ISS, activated a significant response at the transcriptomic level. Moreover, the different simulations suggested that when *Rhodospirillum rubrum* was cultivated in minimal medium the radiation had the greater impact on the bacterial response; in contrast, when cultivated in rich medium, the microgravity had more effect on bacterium (Mastroleo *et al*., 2009).

A radiation resistant bacterium is *Acinetobacter radioresistens* 50v1, isolated from the surface of the pre-flight Mars Odyssey orbiter, and studied by microbiological, enzymatic, and proteomic methods. In short, the microorganism showed resistance against hydrogen peroxide and exposure to desiccation, vapour and ultraviolet irradiation. The survival is due to the activation of the enzyme-based degradation of H_2O_2, energy management and modulation of the membrane composition (McCoy *et al*., 2012).

Three bacteria best known for their resistance to the fatal effects of ionizing radiation are *Deinococcus radiodurans*, *Deinococcus gobiensis* and *Deinococcus geothermalis* (Ranawat *et al*., 2017; Daly *et al*., 2009). They are thermophilic bacteria that seem to overcome DNA damage caused by high temperature using the same mechanism of repairing of damage caused by desiccation and radiation (Beblo *et al*., 2009). These radiation resistant thermophiles are able to tolerate both acute and chronic exposure to high levels of ionizing radiation. They are resistant to

desiccation and capable to maintain their homeostasis through DNA repair mechanisms, ROS detoxification system and accumulation of compatible solutes (Middleton *et al*., 2003). *Deinococcus radiodurans* is the most extensively studied radioresistant organism. This microorganism can survive 5,000–15,000 Gy of acute ionizing radiation without loss of viability and it can grow continuously under 60 Gy/hr (Minton, 1996; Mattimore *et al*., 1996). The resistance to radiation and to other DNA-damaging conditions (e.g. UV light and hydrogen peroxide) and desiccation is due to its efficient DNA damage repair (Beblo *et al*., 1996). Enhanced recombinational DNA repair pathways, like mechanisms limiting DNA degradation and restrict the diffusion of DNA fragments, are observed (Cox, 2005).

Bacterial spores are highly resistant to a wide variety of physical stresses such as: wet and dry heat, UV and γ radiation, oxidizing agents, chemicals, and extremes of both vacuum and ultrahigh hydrostatic pressure. Space exposure experiments have demonstrated a strong capacity of survival of bacterial spores in spaceship, mostly when they are protected against solar radiation (Horneck *et al*., 1994). The increased spore γ-radiation resistance is associated to the low level of core water which may reduce the possibility to produce hydroxyl radicals (Nicholson *et al*., 2002). Spores of various *Bacillus* species are generally 10- to 100-fold more resistant to UV than are the corresponding vegetative cells. Spores of *Bacillus* sp. 34hs1, isolated from the spaceship, were the most resistant to the exposure to UV (three times greater) and radiation (10 times greater), when compared with the well-studied spores of *Bacillus subtilis* 168 or its derivatives (Riesenman *et al*., 2000).

Many fungal species also have very high radiation resistance. Like the other eukaryotic organisms, the fungi have two different mechanisms to repair the DNA double-strand breaks. One of these mechanisms, called illegitimate recombination, involves end joining in the absence of DNA sequence homology and sometimes in presence of few basepairs (bp) of homology shared at the ends of the two recombination junctions (Schiestl *et al*., 1993). This second pathway *in Saccharomyces cerevisiae* irradiated with 50 Gy γ-rays could lead to large-scale genomic rearrangements after a single DSB providing some benefits to evolve genetic variants that have growth advantages under genotoxic stress (Chan *et al*., 2007).

A genetic study, focused on *Saccharomyces cerevisiae* exposed to X-rays and γ-rays, revealed some upregulated genes related to cell cycle and DNA processing, cell rescue defence and virulence, protein and cell fate, and metabolism. The downregulated genes belonged to transcription and protein synthesis, cell cycle and DNA processing, control of cellular organization and cell fate (Kimura *et al*., 2006).

Novikova *et al.* (2005) described the morphological and functional changes of *Aspergillus versicolor* and *Penicillium expansum* located on the outside surfaces of the craft for several months in order to survive in the extreme environment. The electron-microscopy investigation revealed many morphological changes, which apparently allowed those fungi to survive.

In recent years, some studies about the effects of microgravity on some biological responses induced by radiation in space experiments have been presented (Moreno-Villanueva *et al.*, 2017). One of these works demonstrated that microgravity environment may inhibit the repair of DNA damage caused by the radiation. In contrast, some space experiments have shown that mutation frequency and DNA repair activity are not impaired by microgravity in *Escherichia coli*, *Bacillus subtilis* (Horneck *et al.*, 1997; Yatagai *et al.*, 2000), and *Saccharomyces cerevisiae* (Pross and Kiefer, 1999). In fact, no significant differences in the kinetics of DNA strand break rejoining were discovered between the *Escherichia coli* microgravity samples and the controls providing evidence that in the microgravity environment cells are able to repair radiation-induced DNA damage almost normally (Horneck *et al.*, 1997).

Finally, space radiations may significantly increase the mutation frequency of certain genes in microorganisms exhorting the study of the space environment effects on microorganisms as an important part of space missions.

2.5 Alterations in astronauts' immune response during space missions

The types and extent of stressors encountered during space missions, are likely to contribute to the variability of immune responses during and after spaceflight. That stress plays an important role in the effects of spaceflight on immunologic parameters is suggested by the frequent findings that stress hormones are upregulated during and after spaceflight (Borchers *et al.*, 2002).

The study of the human reaction to the absence of the ever-present gravitational interaction not only provides information on how gravity has shaped life on Earth, but also provides key insight into potential mechanisms to mitigate the negative effects of microgravity during spaceflight (Foster *et al.*, 2014). Microgravity conditions, in fact, result in bone loss (Lynch *et al.*, 2005; Stein *et al.*, 2013), variations to both the adaptive and innate immune systems (Guéguinou *et al.*, 2009), and an increased potential risk of bacterial and viral infections (Mermel *et al.* 2013).

Both short and extended duration spaceflight missions have been associated with reduced immune responses and altered production of cytokines (Crucian *et al.*, 2014).

Many researchers, in fact, have shown that important immune parameters are reduced during spaceflight through the reduction of the number and proportion of lymphocytes, the depression of dendritic cells function and T-cell activation, and reduction in numbers of monocytes and precursors of macrophages (Gueguinou *et al.* 2009; Saei and Barzegari 2012).

In one study, stresses associated with spaceflight were shown to alter important functions of neutrophils and monocytes (Kaur *et al.*, 2004).

In another study, the astronauts' monocyte functions showed reductions in their ability to overcome *E. coli*, elicit an oxidative burst and degranulation (Kaur *et al.*, 2005). Non-MHC-restricted (CD56) killer cell cytotoxicity tends to decrease after short-term spaceflight (Mehta *et al.*, 2001). Decreased natural killer cell cytotoxicity in cosmonauts after short- and long-term spaceflights have also been described (Konstantinova *et al.*,1993; Lesnyak *et al.*, 1996; Meshkov and Rykova, 1995). Reductions in absolute numbers of lymphocytes, eosinophils and natural killer cells, diminished delayed-type hypersensitivity, (Taylor and Janney; 1992) changes in CD4+:CD8+ ratios and reduced production of IL-2 and IFN-γ have also been reported (Taylor *et al.*, 1997).

A recent study documenting immune changes during six month ISS missions indicated that astronauts experience increases in white blood cells (WBC). The WBC increase was suggested to be an innate immune compensation for decreases in some adaptive immune responses, such as CD4+ T-helper cell subsets and cytotoxic CD8+ T-cell subsets (involved with antigen specific responses) (Crucian *et al.*, 2015). However, another study reported both increased WBCs and CD8+ T-cell subsets in an astronaut with a persistent skin rash throughout a six month mission (Crucian *et al.*, 2016).

The immune system changes of astronauts as well as environmental stress may have been a factor in known incidents of infections illness in crew members. The alteration of immune cells and cytokines could cause cases of gastrointestinal distress (Antonsen *et al.*, 2017), symptoms of anxiety and depression (Slack *et al.*, 2016), skin rashes (Crucian *et al.*, 2014), and viral reactivation (Cohrs *et al.*, 2008; Mehta *et al.*, 2014).

Moreover upper respiratory problems, influenza, rhinitis, pharyngitis or mild dermatologic problems were among the illnesses that astronauts faced during Apollo spaceflights (Taylor *et al.*, 1974). During the Apollo 8 preflight period for instance, all crew members suffered viral gastroenteritis (Taylor *et al.*, 1974). Reactivation of herpes simplex virus 1, varicella zoster virus and Epstein-Bar virus (Stowe *et al.*, 2001; Mehta *et al.*, 2014) was also found in astronauts during

short and long missions. In astronauts of the Mir station, analyses demonstrated a significant number of episodes of microbial infections, including conjunctivitis, acute respiratory events and dental infections (Ball *et al.*, 2001).

Crew in spaceflight have reported increased incidence of skin rashes: in one case a crewmember with documented immune alterations suffered from a skin rash and rhinitis that varied in extremes throughout a six-month ISS mission (Crucian *et al.*, 2014).

All these problems occurred in spaceflight lead to thinking that even if an opportunistic pathogen is dormant or present at trace, undetectable levels in an astronaut before they embark on a mission, reduced immune function may create a favourable environment for their reactivation or growth, becoming a potential health risk. Moreover pathogens can be transmitted between crew members in the confined environment of a spaceship as it was revealed for certain pathogens like *Serratia marcescens* and *Staphylococcus aureus* after spaceflight showing that these bacteria were transferred from astronaut to astronaut (Taylor *et al.*, 1971). This problem has attracted much research in identifying the microbes associated with astronauts and space vehicles with the aim to develop and implement specific measures to monitor, control, and counteract biological contamination in closed-environment systems (Yamaguchi *et al.*, 2014).

2.6 Gut microbiome changes in space and strategy to alleviate its perturbations

Astronauts will face many challenges during long-space missions that test their healthy and balanced microbiome. The human microbiota is an ecological community of all microorganisms residing in and on the human body that includes bacteria, archaea, single-celled eukaryotes and viruses. The synonymous term microbiome describes either the collective genomes of the microorganisms that reside in an environmental niche or the microorganisms themselves (Backhed *et al.*, 2005; Ley *et al.*, 2006; Turnbaugh *et al.*, 2007). Our microbiome helps us break down food, protects us from infection and supports the immunologic, hormonal and metabolic homeostasis of their host. Imbalance in commensal microbiome have been associated with wide range of medical conditions, from irritable bowel syndrome (Saulnier *et al.*, 2011) to diabetes (Giongo *et al.*, 2011), different type of cancer (Schwabe and Jobin, 2013), and even depression (Luna and Foster, 2014). Stressful conditions encountered during the long space missions, including microgravity or cosmic radiations could determine microbial dysbiosis and changes in bacterial physiology (Nickerson *et al.*, 2000; Wilson *et al.*, 2007; Castro *et al.*, 2011). In particular, the microgravity could determine a

reduction in number and diversity of bacterial flora in gastric intestine tract (GI) (Saei and Barzegari, 2012).

Spaceflight and the changes in lifestyle are concurrent with changes in the GI microbiome on both short and long duration missions (Lizko *et al.*, 1979; Nefedov *et al.*, 1971; Taylor *et al.*, 1977).

The compositions of intestinal (Lencner *et al.*, 1984) oral (Decelle *et al.*, 1976; Brown *et al.*, 1976) and nasal (Decelle *et al.*, 1976) flora have been revealed to change even during short spaceflights. Many microbiology researches have been done about the Apollo, Skylab, Space Shuttle, and International Space Station (ISS) programs. Microbiological findings of ground based simulations were supported by results of investigations in real spaceflights. For instance, according to Taylor (a1973, b1974), examination of the Apollo and Skylab crews evidenced loss in anaerobic and gain in aerobic microorganisms of various types. The astronauts were methodically sampled before, during and after missions and it was found that the diversity of GI microbial community went down following the spaceflight. Specimens were obtained for mycological examination from the skin, throat, urine and feces of the six astronauts who conducted the Apollo 14 and Apollo 15 lunar exploration missions both before and after flight (Taylor, b1974). Analysis of pre-flight data demonstrated that the process of severely restricting opportunities for colonization for 3 weeks before flight resulted in a 50% reduction in the number of isolated species (Taylor *et al.*, a1973). Post-flight data indicated that exposure to the spaceflight environment for up to 2 weeks resulted in an even greater reduction with a relative increase in the potential pathogen *C. albicans* (Taylor, c1974). In another study, a reduction in the number of nonpathogenic bacteria and an increase in the number of opportunistic pathogens has been reported in the nasal flora of cosmonauts (Nefedov *et al.*, 1971). It has also been shown in another work a significant reduction in the number of bacterial species of the intestine after 2 weeks of spaceflight (Hales *et al.*, 2002).

In a more recent experiment with medical human subjects during one year spaceflight on Salyut and Mir orbital stations, the intestinal bifidobacteria and lactobacilli were found to hard decrease and then disappear, enzyme and antagonistic activities were reduced and some organisms exhibited increased toxicity (Ilyin et al., 2005). In a similar way, the number of distinct bacterial species within the GI tract of astronauts on board Apollo and Skylab was markedly reduced and richer in Gram-negative aerobic species such as potentially pathogenic *Klebsiella* and *Pseudomonas* emerged (Taylor, c1974). Significant reductions in beneficial intestinal lactobacilli from cosmonauts prior to launch have been recorded (Lencner *et al.*, 1984), an indication that preflight stress may drive changes in the composition of the gut microbiota, a view supported by a study under simulated

Skylab conditions (Holdeman *et al.*, 1976). There is an indirect interplay between the gut microbiota and the immune and endocrine systems in the maintenance of homeostasis (Moloney *et al.,* 2014); stress and other potential disrupters of the microbiome-brain-gut axis will impact on the composition of the microbiome and are likely to clarify for preflight and in-flight changes.

Recently, Turroni *et al.* (2017) published a paper on the variation of the gut microbiota of some people during a long period in a confined environment like during a spaceflight. They evaluated the intestinal microbial communities of the six crewmembers of the MARS500 project over time during the 520 days of ground-based space simulation. They conclude that sharing life in a confined habitat does not alter the flexibility of the individual gut microbial ecosystem, even in the long term, although some microbiota components should be controlled when programming future mission simulations and real spaceflights, to prevent failures in the metabolic and immunological homeostasis of the crewmembers.

The study of the microbial contamination in the MARS500 facility revealed that the most abundant microbial families were represented by typical members of the human microbiota. Opportunistic pathogens, stress-tolerant or potentially mobile element-bearing microorganisms were predicted to be prevalent throughout the confinement, while the overall microbial diversity dropped significantly over time (Schwendner *et al*., 2017).

A NASA's Microbiome experiment on nine astronauts that spent six to twelve months in the ISS demonstrated that the composition of the intestinal microbiome became more similar across astronauts in space, mostly due to a drop in the abundance of a few bacterial taxa, some of which were also correlated with changes in the cytokine profile of crewmembers (Voorhies *et al.*, 2019).

In another important and recent work published in Science (Garrett-Bakelman *et al.,* 2019) the metagenomic sequencing of faecal material allowed the monitoring of changes in the gastrointestinal microbiome of an astronaut in orbit for 340 days. These data were analyzed and compared with those of his homozygous twin left on Earth. Three hundred and seventeen total samples, collected respectively before departure, during the mission and one year after their return, allowed to collect and integrate an important amount of data, from genomics to psychology to microbiome analysis. Many changes were observed in gene regulation, gut microbiome composition, body weight, carotid artery dimensions and serum metabolites. Most of the biological and human health variables remained stable, or returned to baseline, after a 340 days Space mission, these data suggest that human health can be mostly sustained without problems over this duration of spaceflight.

In particular, the gut flora of the twin in space was found to be profoundly different during flight from pre-flight. The microbiome has acquired new species of bacteria but many of these changes seemed harmless and disappeared on Earth. These changes in microbiome composition could be due to the food he consumed while on the space station (mainly freeze-dried or thermo-stabilized prepacked food) although other space-specific environmental factors may have also contributed. When he landed back on Earth, his microbiome returned to pre-flight state.

Findings from this study may provide researchers with a better understanding of how to help improve overall health, such as adjusting astronauts' diets to help beneficial bacteria thrive.

2.7 Probiotics and their recommended use during spaceflight

Methods to modulate microbiota perturbations and associated physiological symptoms could play a role in preventing disease, especially during long mission in which the possibility for external medical intervention decreases. Astronauts, in fact, enter space in top health and probiotics may be a method in preventing or attenuating a decrease in immune-competence. Probiotics are defined as live microorganisms conferring a health benefit on the host when ingested in adequate amounts (WHO/FAO, 2006). Many probiotics are accepted as generally recognised as safe (GRAS) within defined conditions for human ingestion by the Food and Drug Administration, or are regarded as GRAS either due to a long history of safe use or due to expert agreement on strain safety based on scientific evidence (FDA, 2016; Wassenaar and Klein, 2008). Probiotics belong to the genus *Lactobacillus* includes various Gram-positive facultative anaerobic or microaerophilic rod-shaped bacteria. They are a major part of the lactic acid bacteria (LAB) group (including *Lactobacillus, Lactococcus, Enterococcus, Oenococcus, Pediococcus, Streptococcus and Leuconostoc* species) that can convert hexose sugars to lactic acid thus producing an acid environment which inhibits the growth of several species of harmful bacteria (Makarova *et al.*, 2006). In humans, *Lactobacilli* are normally present in the vagina, gastrointestinal tract (Walter *et al.*, 2008) and are together with *Bifidobacterium* one of the first bacteria to colonize the infant gut after delivery (Walker *et al.*, 2013). Studies have shown that certain strains of *Lactobacilli* are effective in preventing antibiotic-associated diarrhoea (Johnston *et al.*, 2011; Hempel *et al.*, 2012). *Lactobacilli* species are commonly selected as probiotics since they express many crucial properties such as: provide several benefits to human health, including competition against pathogens, strengthening of tight junctions of the intestinal epithelial layer, reduction of gastrointestinal distress, production of beneficial metabolites, high tolerance to acid and bile, capability to adhere to intestinal surfaces, withstanding

low pH, interactions with host cells that promote immune and psychological health, and protection from infection (O'Flaherty and Klaenhammer, 2010; Turroni *et al.*, 2014), producing exopolysaccharides and removing cholesterol (Tulumoglu *et al.*, 2013; Lee *et al.*, 2013; Ruiz *et al.*, 2013). Lactobacilli such as: *L. acidophilus*, *L. reuteri*, *L. casei*, *L. paracasei*, *L. rhamnosus*, *L. delbrueckii* subsp. *bulgaricus*, *L. brevis*, *L. johnsonii*, *L. plantarum* and *L. fermentum* are commonly used as probiotic products.

The administration and/or consumption of probiotics is supposed to have immune-enhancing effects, hinder alterations in the human microbiome to a large extent and prevent colonization of potential pathogens. The periodic consumption of probiotics during a space mission, such as *Lactobacillus* and *Bifidobacterium*, could reduce the incidence of negative disorders induced by space conditions.

Despite the positive health benefits attributed to many probiotic strains on Earth, their potential to promote human health and reduce symptoms of illness in spaceflight has not been investigated.

The effectiveness of probiotics for astronauts, in fact, is difficult to measure because the probiotic intervention is short and the studies do not account for confounding factors like subject diet and lifestyle. Documented illnesses and conditions in spaceflight for which probiotics have demonstrated alleviation in human studies include diarrhoea, constipation, skin rashes and infections, respiratory infections, and psychological distress.

Several incidences of diarrhoea, in fact, have been reported in spaceflight and antibiotics were prescribed without probiotics (Antonsen *et al.*, 2017), although increasing evidence supports that a variety of probiotic strains, in combination, or separately will reduce the incidence, duration, and symptoms of antibiotic associated diarrhoea (AAD) in adults receiving antibiotics.

Despite crew quarantine prior to spaceflight, respiratory infections have been documented (Antonsen *et al.*, 2017). Respiratory health is another area with a significant body of evidence supporting a benefit from probiotic consumption (King *et al.*, 2014).

The addition of probiotics should be considered as an indispensable part in the astronaut diet to promote women' health and potentially reduce urinary tract infection, bacterial vaginosis, anxiety, osteoporosis and breast cancer development (Urbaniak and Reid, 2016).

It has been suggested that some species of *Lactobacillus* like *L. acidophilus* CL1285 and *L. casei* LBC8OR in fermented milk reduced incidence and duration of AAD in multiple clinical trials

(Beausoleil *et al.*, 2007; Gao *et al.*, 2010; Sampalis *et al.*, 2010). Moreover, healthy people consuming a tablet containing 5×107 cfu of *Bifidobacterium longum* SP 07/3, and *Bifidobacterium bifidum* MF 20/F (Tribion harmonis™) for up to six months reported reduced durations (by two days), symptoms, and fever duration during common cold episodes compared to a control group (De Vrese *et al.*, a 2005, b 2006). Adult patients with atopic dermatitis who consumed 1×109 cfu/g *L. salivarius* LS01 in maltodextrin twice a day for 16 weeks showed improvement in clinical parameters (Drago *et al.*, 2011). A combination of *L. salivarius* LS01 DSM 2275 and *Bifidobacterium breve* BR03 DSM 16604 delivered at a dose of 1×109 cfu/g twice a day for 12 weeks also improved clinical parameters in adult atopic dermatitis patients (Iemoli *et al.*, 2012).

One clinical study on atopic dermatitis provided a perspective that may support easier implementation of the delivery of probiotics on long duration missions. The study reported improved clinical parameters after delivering heat killed *L. acidophilus* L-92 in tablet form to adult patients with atopic dermatitis for eight weeks (Inoue *et al.*, 2014).

Other species of *Lactobacillus* could be helpful for counteracting some psychological distress status affecting astronauts on during long-duration space missions because these bacteria are able to produce some neuroactive compounds including serotonin and small chain fatty acids that have the ability to reduce symptoms of stress, depression, and anxiety in human studies (Rao *et al.*, 2009; Akkasheh *et al.*, 2016; Messaoudi *et al.*, 2011). In one of these works patients with major depressive order consumed a capsule containing *L. acidophilus*, *L. casei*, and *B. bifidum* each at a dose of 2×109 cfu/g daily for eight weeks and experienced a reduction in depression scores, decreased serum insulin levels, and decreased concentrations of the inflammatory marker, C-reactive protein (Akkasheh *et al.*, 2016). The probiotic consumption in space is unfortunately only limited for study purposes in spite of there are many studies providing evidence that consuming certain live microbes can convey positive health benefits. One of these studies was conducted by Human Space Safety and Mission Assurance Office of the Japan Aerospace Exploration Agency (JAXA) and regarded a space experiment involving crew members of ISS regularly consuming probiotics. They consumed capsules containing freeze-dried live probiotic bacterium *L. casei* strain Shirota, present in Yakult drink. This is part of a study on the effect of probiotics on the immune system and on the intestinal microbiota of astronauts. They evaluated that the viability and basic probiotic properties of *L. casei* Shirota stored as a Probiotics Package on the ISS were maintained. Moreover they reported that in outer space there is a higher presence of harmful bacteria in intestine and that immune system functions are less efficient (Sakai *et al.*, 2018).

Another possibility to provide probiotic bacteria to space crew is to use them in soy-based products. Fermentation of soy in astronauts' nutrition, in fact, could add the following health benefits: enhanced bone metabolism, improved nutritional value, stimulation of the immune system, diversification of soy foods with respect to flavor and texture, reduced problems associated with flatulence and better preservation of foods (Buckley *et al.*, 2011).

As the duration of spaceflight increases, the astronaut diet becomes more and more important (Pingannaud, 1982) and can even influence astronaut morale. Fermentation of soy provides the opportunity to diversify the sensory attributes of soy-based foods because lactic and probiotic cultures produce various aromas, which significantly contribute to the flavor of products and because fermentation changes the flavor attributes of the original soy matrix itself. Thus, fermentation by some lactobacilli can significantly reduce levels of volatile components naturally present in the soybean, which are associated with its 'beany' flavor (Blagden *et al.,* 2005). In summary, astronauts can use the different fermentation strategies to diversify the flavors and textures of soy-based foods, reducing monotony, providing food choices to the astronaut with the net result that the psychological welfare of astronauts can be enhanced.

All these studies provide evidence for selecting probiotic strains to support specific attributes of crew health in spaceflight. Upon observation of possible benefits, probiotics can be incorporated into astronauts' food or supplied periodically as a probiotic kit. Design of a personal probiotic kit is recommended to improve the health status of astronauts.

2.8 *Lactobacillus reuteri* DSM 17938: principal genes and proteins involved in functional probiotic features and in general stress conditions

Lactobacillus reuteri (*L. reuteri*) is considered one of the true autochthonous species of the human gastrointestinal (GI) tract (Hou *et al.*, 2015).

Lactobacillus reuteri DSM 17938, in particular, is a generally recognized as safe (GRAS) probiotic strain deposited by BioGaia in the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ) and referenced in the scientific literature also as SD 2112, ING 1 and MM53. *L. reuteri* DSM 17938 derives by the depletion of two plasmids encoding for antibiotic (lincomycin and tetracycline) resistance (Roos and Rosander, 2005; Rosander *et al.*, 2008) from the strain ATCC 55730, isolated in Perù from human breast milk (Johnson *et al.*, 2006).

There are evidence favouring beneficial effects attributed to *L. reuteri* DSM 17938, including improvement of intestinal health, enhancement of the immune response and possible role in the reduction of serum cholesterol, and cancer prevention. While some of the health benefits are well-documented others require additional studies in order to be established. In fact, there is substantial evidence to support *L. reuteri* DSM 17938 used in the treatment of infantile colic, infantile regurgitations, functional abdominal pain and functional constipation and emerging evidence in the improvement of lactose metabolism, *H. pylori* infection and NEC.

Furthermore, its selective ability in binding intestinal cells of IBD affected patients, more than that affected from diverticulitis or rectal carcinoma (Ouwehand *et al.*, 2003) encouraged the selective use of probiotic strains in the treatment of specific syndromes. The probiotic effect of *Lactobacillus reuteri* DSM 17938 has been widely investigated both in *vitro* and in *vivo* on animals and humans (adults and childrens). Besides the efficacy on adults in the suppression of *H. pylori*, reduction of gingival inflammation and plaques and IBS symptoms (Niv *et al.*, 2005; Krasse *et al.*, 2005; Caglar *et al.*, 2006), *L. reuteri* DSM 17938 is highly recommended for paediatric use beyond its proved efficacy in limiting the relapse to eczema in infants related to cow's milk consumption, reducing acute diarrhoea and diarrhoea from rotavirus in children and in other conditions (Szajewska *et al.*, 2014; Urbanska and Szajewska, 2014). Since also its safety and tolerability has been widely confirmed in *vivo* (Wolf *et al.*, 1995, 1998; Mangalat *et al.*, 2012), this probiotic strain is included in many pharmaceutical formulations (BioGaia ProTectics drops, BioGaia ProTectics chewing gum, BioGaia ProTectics straws) while its spreading in foods is still only confined to research level.

The genome of *Lactobacillus reuteri* DSM 17938 isolated from the gastrointestinal tract has been recently sequenced as part of the Human Microbiome Project. This strain contained candidate genes involved in the survival and persistence in the gut such as mucus-binding proteins and enzymes scavenging reactive oxygen species. The possible mechanism involved in adhesion of *L. reuteri* DSM 17938 on intestinal epithelial cells has been linked to surface protein (Wang *et al.*, 2008), mucus-binding protein (Mackenzie *et al.*, 2010), exopolysaccharide (Wang *et al.*, 2010), inulosucrase (Walter *et al.*, 2008), D-alanyl-LTA (Walter *et al.*, 2007), and glucosyltransferase A. Mechanism of action of *L. reuteri* DSM 17938 has been studied extensively as shown in Figure 2.3 (da Srinivasan *et al.*, 2018).

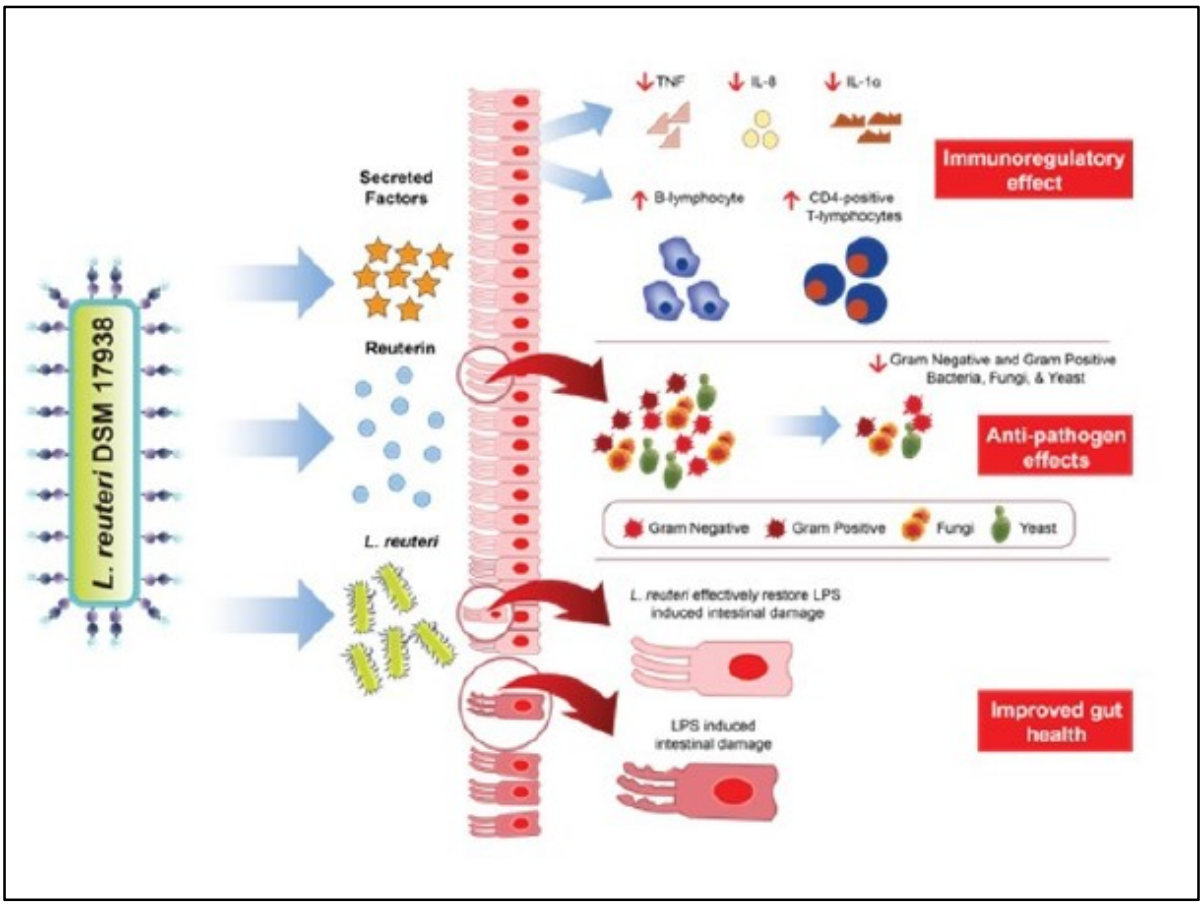


Fig. 2.3: Mechanism of action of *L. reuteri* DSM 17938.

(*L. reuteri* - *Lactobacillus reuteri* ; TNF- Tumor necrosis factor; IL- Interleukin; LPS- Lipopolysaccharide). Image by Srinivasan *et al*., 2018.

This strain was predicted to produce health-promoting factors, including antimicrobial agents and vitamins (folate, vitamin B_{12}). Candidate genes responsible for immunomodulatory properties of this strain were identified by transcriptomic comparisons (Saulnier *et al.*, 2011). Genome analysis revealed that the strain ATCC 55730 may be especially able to synthesize vitamins essential to humans. *In silico* genome analysis revealed that this strain has complete pathways for folate and vitamin B_{12} biosynthesis. This bacterium is predicted to be able to synthesize 11 amino acids (arginine, alanine, asparagine, aspartate, cysteine, glutamine, glycine, proline, serine, lysine and threonine), including three amino acids considered to be required by young children (Saulnier *et al.*, 2011). Moreover this strain of *L. reuteri* can produce the short chain fatty acid (SCFA), acetate, a substrate that is absorbed in the GI tract and enters the circulation to be metabolized by peripheral tissues (Scheppach, 1994).

This bacterium, like other strains of the same species, produce a variety of antimicrobial substances such as hydrogen peroxide (Martinez *et al.*, 2009), lactic acid, reuterin (Bian, 2008; Morita *et al.*, 2008), and reutericyclin (Gänzle *et al.*, 2000). These substances display inhibitory activity against both gram-positive and negative bacteria, yeast, fungi, as well as parasites (Chung *et al.*, 1989).

Similarly to other strains of this species, *L. reuteri* DSM 17938 is a producer of antimicrobial substances (reuterin) and its consumption has been correlated to a stimulation of immune system ad exemplum through i) increasing of anti-inflammatory interleukin 10 in pregnant women (Jacobson *et al.*, 2005); increasing of B and T-lymphocytes and decreasing of gastric mucosal histiocytes (Valeur *et al.*, 2004); iii) reducing fecal calprotectin (Mangalat *et al.*, 2012).

Reuterin, a derivative of glycerol, is produced under anaerobic conditions in the presence of the latter and has broad-spectrum antimicrobial effects against Gram-positive and Gram-negative bacteria including *Escherichia*, *Salmonella*, *Shigella*, *Pseudomonas*, *Clostridium* and *Staphylococcus* (Axelsson *et al.*, 1989), is also able to inhibit the growth of fungi, yeasts and protozoa (Chung *et al.*, 1989). *L. reuteri* DSM 17938 in presence of 250 mm glycerol was successfully used to reduce by 6 logs UFC/g *Escherichia coli* O157: H7 present in minced meat in 20 days to 4° C (Muthukumarasamy *et al.*, 2003). Reuterin has been used to inhibit *Listeria monocytogenes* and *E. coli* O157: H7 in vacuum ham, ricotta and milk (El-Ziney & Debevere *et al.*, 1998; El-Ziney *et al.*, 1999).

Previous literature studies show that reuterin is produced by a two-stage fermentation process: the first phase involves the growth in anaerobic condition with glucose to obtain a maximum concentration of biomass, in the second phase the stationary phase cell pellet is resuspended in a solution containing glycerol for conversion to 3-HPA (Talarico *et al.*, 1988). In other studies it has also been shown that *L. reuteri* cells have the ability to produce reuterin in the growth phase during glucose/glycerol cofermentation under anaerobic conditions (El-Ziney *et al.*, 1998). The fermentation of glycerol by *L. reuteri* occurs in two phases: the first phase is catalyzed by a coenzyme B12-dependent glycerol dehydratase converting glycerol to reuterin and the second phase involves an aldehyde dismutation of reuterin due to a glycerol hydroxide reductase forming 1,3 propanediol (1,3 PPD) (Schutz and Radler *et al.*, 1984; Talarico *et al.*, 1989).

The genes responsible for reuterin production such as the glycerol dehydratase (*gdh*) are part of the propanediol utilization (*pdu*) operon. The gene (1,3 *pdo*) encoding the enzyme responsible for the conversion of reuterin into 1,3 propanediol is 1,3 propanediol dehydratase.

Because of its stable association with vertebrate hosts, *L. reuteri* has been used as a model organism for the study of host-microbe interaction and host-specific adaptation (Walter *et al.*, 2011). Biofilm formation by probiotic bacteria, such as *Lactobacillus* spp., is considered a beneficial property because it could promote colonization and longer permanence in the mucosa of the host, avoiding colonization by pathogenic (Terraf *et al.*, 2012). The capacity of *Lactobacillus* to form biofilms on abiotic surfaces (glass or polystyrene) has been studied during the last years, and the results indicate that only some strains have this property (Kobuta *et al.*, 2008; Kobuta *et al.*, 2009; Jones *et al.*, 2009; Ambalam *et al.*, 2012; Terraf *et al.*, 2012; Fernández *et al.*, 2015). It has also been demonstrated that the EPS produced by some biofilm forming strains is able to inhibit the formation of biofilms by certain pathogenic bacteria (Ramos *et al.*, 2012). The genome of the immune stimulatory strain ATTC 55730 encodes the synthesis of EPS that can be pro-inflammatory (Saulnier *et al.*, 2011). Besides this EPS, a gene encoding a cell surface protein containing five repetitive Rib motifs was identified in strain 55730. This gene has similarity with other genes previously known to play a role in suppression of immune responses. In fact, EPS from *L. reuteri* DSM 17938 was able to influence on adherence of enterotoxigenic *Escherichia coli* (ETEC) to IPEC-1 cells and pro-inflammatory gene expression (Ksonzekova *et al.*, 2016). This probiotic strain is characterized as gastro-intestinal resistant (Wall *et al.*, 2007) and several studies have demonstrated *L. reuteri* capacity to colonize, and their ability to adhere to mucin and intestinal epithelial cells (Miyoshi *et al.*, 2006; Yu *et al.*, 2007; Li *et al.*, 2008; Wang *et al.*, 2008). It was found to encode for components involved in adherence and biofilm formation on epithelia such as: Collagen-binding protein (*CnBP*/Map) (Miyoshi *et al.*, 2006), Mucus-binding protein (*Mub*) (Roos and Jonsson, 2002), Mannose-specific adhesin (*Msa*) (Juge, 2012), Large Surface protein *(Lsp*) and Glycosyl-transferases (*Gtf*) (Walter *et al.*, 2011). *MapA*, a mucus adhesion promoting protein, is able to bind mucus and intestinal epithelial cells Caco-2 having affinity to molecule in the host GI tract (Miyoshi *et al.*, 2008). *Msa* is a gene encoding a mannose-specific adhesin protein that is also involved in the interaction of bacteria with its host in the intestinal tract (Pretzer *et al.*, 2005).

Proteins such as *Mub* and *Lsp* contain LPXTG cell wall binding motifs, are extremely large, contain multiple repeated motifs, and resemble adhesins of pathogenic microbes (Roos and Jonsson, 2002; Walter, 2005).

Two key enzymes involved in EPS synthesis in *L. reuteri* are Ftf and GtfO: *ftf* encodes a fuctansucrose in *L. reuteri* 100-23 (Sims *et al.*, 2011) and allows this strain to adapt to different environments; *gtfo* is a glucosyl-transferase that contributes to cell aggregation, *in vitro* biofilm

formation and colonization of the mouse gastrointestinal tract (Arskold *et al.*, 2006; Walter *et al.*, 2008).

This bacterium, like other LAB bacteria, encounters various environmental conditions upon ingestion by the host and during transit in the gastro-intestinal tract. In fact, these bacteria are continuously exposed to various environmental stresses such as extremes in temperature, pH, osmotic pressure, oxygen, high pressure and starvation which may affect the physiological status and properties of the cells. In *L. reuteri* DSM 17938 were identified and characterized many proteins involved in different stress conditions, like heat stress or ethanol and acid stresses. Firstly, it needs to survive the harsh conditions of the stomach. Humans secrete approximately 2.5 liters of gastric juice each day, generating a fasting pH of 1.5, increasing to pH 3 to 5 during food intake (Franks, 1985). The principal genes in *L. reuteri* involved in the stress response are mostly in common with numerous Gram-positive species. Some of these belong to a group of evolutionary conserved heat shock proteins (HSPs) with chaperonine function which promote the correct folding of nascent polypeptides (Gross *et al.*, 1996; Hecker *et al.*, 1996), assembly of protein complexes, degradation and translocation of proteins (De Angelis *et al.*, 2014). The two major groups of HSPs consist of 70 (*DnaK*) and 60 (*GroEL*) kDa families which function, with accessory proteins, as chaperone machines. The components of the *DnaK* chaperone typically consist of *DnaK*, *DnaJ* and *GrpE*, while that of *GroEL* is composed of *GroEL* and *GroES* (Bukau and Horwich, 1998; Hendrick *et al.*, 1993). Another chaperone, belonging to major group of HSPs, *Ctsr* (class three stress gene repressor) regulates the expression of gene encoding the *ClpATPase* and Clp-protease that assist to re-folding of damaged proteins after stress (Russo *et al.*, 2012). One of this *ClpATPase* is *clpL*, gene putatively involved in alterations of the cell membrane and the cell wall that encodes a transcriptional regulator (Wall *et al.*, 2007). *Ftsh* codifies another chaperone-protease involved in the quality check of proteins in stress environment (Bove *et al.*, 2012). A gene responsive of acid stress and of bile salt stress is *dps*, a putative esterase involved, like *ClpL*, in oxidative stress and cell wall damage effects of bile (Whitehead *et al.*, 2008).

RpoD (RNA polymerase sigma 70 factor) is a sigma factor that respond to generic stress putting the bacterium in a "protective mode": the expression of this gene in *E. coli* is increased under simulated microgravity stress condition (Vukanti *et al.*, 2008) encouraging the expression study also in *L. reuteri*. A key enzyme involved in oxidative stress is *Rex* (redox sensing transcriptional repressor) that is found over-expressed in *Streptococcus mutans* cultivated under simulated microgravity condition (Orsini *et al.*, 2017) promising a similar result also in *L. reuteri*.

Integrated knowledge of genes and proteins expression of this bacterium under simulated microgravity conditions with the functional biological information of other model strains will improve not only the understanding of the interactions between this bacterium with the host but also with other microbes and with the environment such as the space environment.

2.9 MELiSSA project

The acronym MELiSSA means 'Micro Ecological Life Support System Alternative'. It refers to a space research program aiming to develop an artificial ecosystem for bio-regenerative life support systems for long-term space missions to lunar bases or flights to Mars. The project started 30 years ago and is based on a collaborative development program between 15 partners and a number of supporting sub-contractors.

The principal aim is to produce food, water, and oxygen starting from the organic wastes of the mission (e.g., urine, CO2,). The MELiSSA process is inspired by a terrestrial “aquatic” ecosystem and it is composed by five main sub-processes called compartments colonized, respectively, by thermophilic anoxygenic bacteria, photo-heterotrophic bacteria, nitrifying bacteria, photosynthetic bacteria, higher plants, and the crew (Fig. 2.4 by Lasseur *et al.,* 2010). The system uses the combined activity of different living organisms: microbial cultures in bioreactors, a plant compartment and a human crew. Each recycling step has been inspired by the natural organic waste reconversion cycle taking place in a natural lake ecosystem (Mergeay *et al.*, 1988; Hendrickx *et al.* 2006).

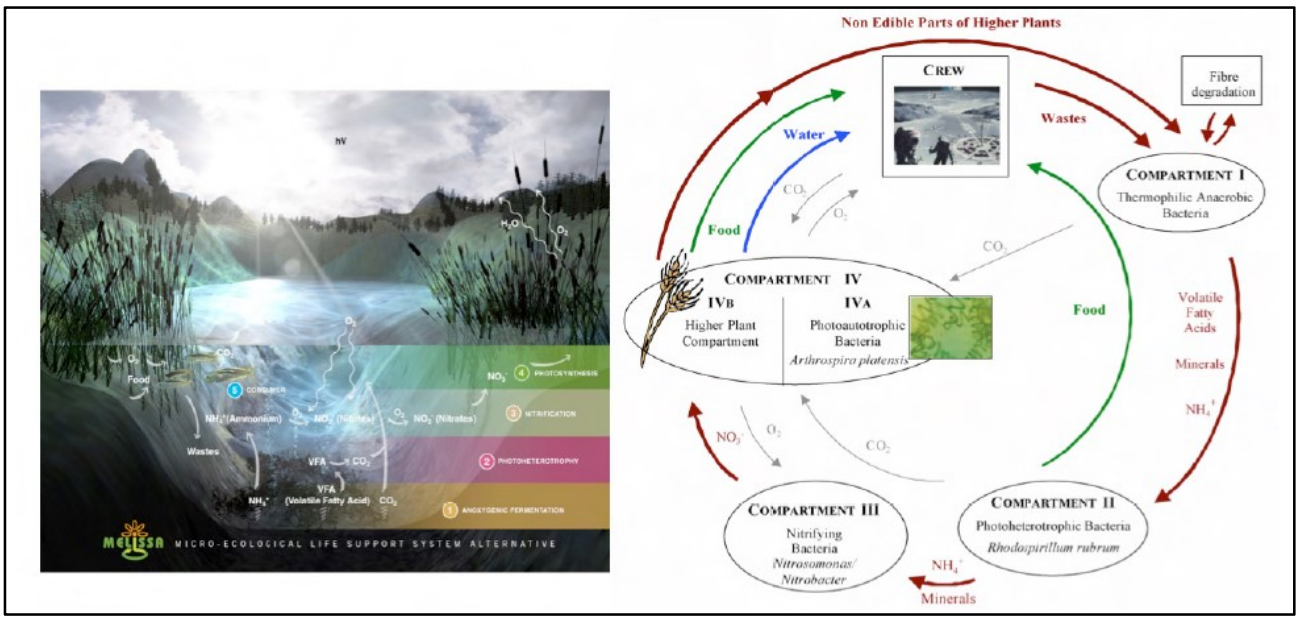


Fig. 2.4: Concept of the MELiSSA loop.

Left-hand side: an aquatic ecosystem. Right- hand side: the compartmentalized structure. Image by Lasseur *et al*., 2010.

The first compartment is the collection point for all mission waste (i.e. urea, kitchen waste) as well as the non-edible parts of the higher plant compartment (i.e. straw and roots). The compartment's aim is to anaerobically transform this waste to ammonium, H_2, CO_2, volatile fatty acids and minerals.

Fermentation, acidogenesis, acetogenesis and methanogenesis of precipitated waste deposited from plants and animals occurs in the anoxic environment. For biosafety reasons and for optimum degradation efficiency, the compartment operates in thermophilic conditions (55°C) anaerobic reactor with inhibited methanogenesis, for the purpose of total carbon conversion to CO_2 and volatile fatty acids. The process of degradation in this compartment is carried out by proteolysis, saccharolysis and cellulolysis.

For the elimination of the terminal products of the liquefying compartment volatile fatty acids can further be converted in the second compartment using purple non-sulphur organisms (in MELiSSA depicted as a photoheterotrophic compartment inhabited by *Rhodospirillum rubrum*). The purple non-sulfur a-proteobacterium, *Rhodospirillum rubrum* S1H is used to convert volatile fatty acids released from the upstream raw waste-digesting reactor to carbon dioxide and biomass, and to complete the mineralization of amino acids into free ammonium that will be forwarded to the nitrifying compartment (Mastroleo *et al.*, 2009).

The nitrifying compartment main function is to cycle NH_4 produced from waste to nitrates, which is the most favourable source of Nitrogen for higher plants. Nitrification, wherein NH_4 is oxidized into NO_3^- through NO_2^-, is performed in MELiSSA with a co-culture of *Nitrosomonas europaea* and *Nitrobacter winogradskyi* (Hendrickx *et al.*, 2006).

Food and oxygen are produced in a photosynthetic compartment consisting of the higher plant compartment and an *Arthrospira* species cultivation unit to produce biomass and O_2 from CO_2, H_2O and minerals likewise to as it would occur on the surface of a lake where sunlight-derived energy is harvested by cyanobacteria, algae and plants. *Arthrospira* species is considered the main oxygen producer and, because of its high levels in proteins, essential fatty acids (linoleic and g-linoleic acid), vitamins (provitamin A, vitamin B_{12}) and minerals (iron), *Arthrospira* is quite suitable as a

food supplement (Garcia-Pichel, 2000). This plant has been commercialized and is sold as 'Spirulina'.

From the starting of the project many studies have been conducted for simulating the spaceflight conditions and for checking the adequacy of bacterial and plant species to use in MELiSSA loop (Crabbe *et al.*, 2008; De Gelder *et al.*, 2009; Beuls *et al.*, 2009; Mastroleo, 2009; Crabbe *et al.*, 2010; Leroy *et al.*, 2010). In parallel, simulations of the space-radiation environment were investigated at SCK-CEN, based on dosimetry experiments performed onboard ISS (Baatout *et al.*, 2007). These studies on Earth converged and allowed the scientific exploitation of spaceflights experiments carried out in 2002 (MESSAGE1), 2003 (MESSAGE 2), 2004 (MOBILISATSIA), 2006 (BASE-A) and 2008 (BASEB& C, BASE-D). These microbial experiments were brought to ISS by Soyuz flights for exposure to the ISS environment for about 10 days before return to Earth (De Boever *et al.*, 2007; Leys *et al.*, 2009; Mastroleo *et al.*, 2009). The bacterium used for MESSAGE and BASE experiments included *R. rubrum* S1H as MELiSSA bacterium (Mastroleo *et al.*, 2009) and *Cupriavidus metallidurans* CH34 (Leys *et al.*, 2009) as an example of a bacterium adapted to a variety of harsh environments, including the clean rooms where satellites are built (Mergeay *et al.*, 2009).

All the strains chosen within the MELiSSA loop have been studied more or less extensively and literature provides us with information on their metabolism, growth requirements, genetics and stress response. The basic knowledge of MELiSSA microorganisms took advantage of the availability of fully sequenced genomes for *Rhodospirillum rubrum* (Reslewic *et al.*, 2005), the nitrifying bacteria of C3: *Nitrosomonas europaea* (Chain *et al.*, 2003) and *Nitrobacter winodgradsky* (Starkenburg *et al.*, 2006), and *Arthrospira* PCC 8005, the edible cyanobacteria of the C4a compartment (Morin *et al.*, 2010). Making use of pangenomic taxonomy signatures for a large set of publicly available cyanobacterial genomes, strain PCC 8005 was allocated as the type strain to a new species named *Arthrospira nitrilium* (Walter *et al*, 2017). Recently, mass produced *Arthrospira* have been placed into a new genus, Limnospira, mainly based on 16S rRNA phylogenetic analysis but also taking into account morphological and ecological data (Nowicka-Krawczyk *et al.*, 2019).

The knowledge of genome of these strains or their closely related relatives offered in this way an abundance of information at the genetic and proteomic level (Hendrickx *et al.*, 2006; Leys *et al.*, 2004). However, their 'space robustness', i.e. stress response in microgravity and resistance to

radiation exposure, has never been tested. In particular, the effects of microgravity, long-term space radiation exposure and changed electromagnetic fields are not fully understood.

In space missions it is expected to test the effects of spaceflight-related stress conditions, thereby providing us with clues to the appropriateness of the MELiSSA organisms.

In this context, *Lactobacillus reuteri* could be cultivated in one of the compartments in loop MELiSSA, like the consumers one, using this bacterium as a direct source for the astronauts. The knowledge of its fully genome is fundamental for the study of its regulation and adaptation to spaceflight condition and for discovering the degree of genetic robustness.

CHAPTER 3

Growth of *Lactobacillus reuteri* DSM17938 under two simulated microgravity systems: changes in reuterin production, GI passage resistance and stress genes expression response

3.1 Introduction

The spacecraft environment could be considered a special ecological niche where some conditions like gravity, radiation and acceleration are very different from that on Earth. The focus of recent researches has been on the effect of space environment on the human, vegetable and microbial life (Löbrich and Jeggo, 2019; Huang *et al.*, 2018; Strauch *et al.*, 2018; Lionheart *et al.*, 2018). The latter is more suitable than human and vegetable because they are easy to manipulate and stable within a wide range of environmental conditions (e.g. temperature, pressure, etc.) and they are able to reproduce in a very short period (Senatore *et al.*, 2018). On the other hand, microorganisms negatively or positively affect human life in space, exactly like on Earth. Therefore, the study of the space environment effects on microorganism's life is crucial to assess the possible unprecedented interactions between human and microorganisms. It is nowadays well known that some alterations under spaceflight conditions could cause phenotypic and genotypic variations in microorganisms, concerning modifications on cell morphology and physiology (Nickerson *et al.*, 2004; Li *et al.*, 2015; Huang *et al.*, 2018). Accordingly, altered level of pathogenicity, viral reactivation, host interaction and production of metabolites under space conditions have been reported (Leys *et al.*, 2004; Horneck *et al.*, 2010; Taylor, 2015). It is generally believed that the main factor that determines changes in microbial response to the space environment is microgravity (Senatore *et al.*, 2018), a term frequently used as a synonym of 'weightlessness' and 'zero-g', which indicates that the g-force is not completely neutralized but almost zero (Klaus *et al.*, 2001; Herranz *et al.*, 2013).

In the last few years there has been a growing interest in the effect of microgravity on pathogenic bacteria, more than on other microorganisms. In fact, many researches documented that this environmental stressor could alter the virulence of some potential pathogens like *Salmonella* Typhimurium, *Escherichia coli* and *Staphylococcus aureus* (Nickerson *et al.*, 2000; Wilson et al., 2002; Vukanti *et al.*, 2008; Rosenzweig *et al.*, 2009; Castro *et al.*, 2011; Vukanti *et al.*, 2012). Few and dated investigations have been dedicated on how much spaceflight can affect human associated microorganisms, as reviewed by Saei and Barzegari (2012). Recently, Turroni *et al.* (2017) published a paper on the abundance and diversity of the gastro-intestinal (GI) microbiota of

volunteers during a long period in a confined environment like during a spaceflight. Furthermore Voorhies and Lorenzi (2016) reported changes in the composition and diversity of gut microbiota of astronauts during long period space missions. Moreover, several studies have shown that important immune parameters are reduced during spaceflight through the reduction of the number and proportion of lymphocytes, the depression of dendritic cells function and T-cell activation, and reduction in numbers of monocytes and precursors of macrophages (Saei and Barzegari, 2012). It has been suggested that continuous immune alterations in otherwise healthy subjects may have unknown health consequences on long duration missions (Guéguinou *et al.*, 2009; Crucian *et al.*, 2015). Changes in the GI microbiota and dysregulation of immune cells and cytokines regard cases of gastrointestinal distress (Antonsen *et al.*, 2017), symptoms of anxiety and depression (Slack et al., 2016), skin rashes (Crucian *et al.*, 2014), and viral reactivation (Cohrs *et al.*, 2008). In addition, antibiotic resistance may be increased in spaceflight making treatment of infections difficult (Taylor and Sommer, 2005).

In this scenario, a regular ingestion of probiotics by space mission crew members should be considered to reduce the risk associated to the almost inevitable presence of potential microbial pathogens in the spaceflight environment and reduce the antibiotic associated diarrhoea and in the meantime increase crew resistance to stress and microgravity-induced physiological changes on long duration space missions (Ventura *et al.*, 2012). Indeed, antibiotics are currently prescribed without probiotics to US crews in spaceflight, although increasing evidence supports that a variety of probiotic strains, in combination, or separately will reduce the incidence, duration, and symptoms of antibiotic associated diarrhoea (AAD) in adults receiving antibiotics (Hungin *et al*., 2013).

Interestingly, the physical forces produced in spaceflight environment are similar to that encountered by the microorganisms in gastrointestinal, urogenital and respiratory tracts of the host. In particular, some low-fluid-shear areas of the body, like between the brush border microvilli of epithelial cells, seem to reproduce the condition of spaceflight (Nickerson *et al.*, 2004). Accordingly, to investigate the effects of microgravity environment on microorganisms some methods using different technologies like clinostats have been used (Crabbé *et al.*, 2008; Herranz *et al.*, 2013; Rosenzweig *et al.*, 2014). These ground-based methods have been developed to simulate the condition of weightlessness in laboratories (Briegleb 1992).

One of these bioreactors is the Rotating Wall Vessel (RWV), a clinostat with two rotating axes used instead of one designed at the NASA Johnson Space Centre (Houston, Texas, USA), in which the rotation occurs on an axis perpendicular to the direction of the gravity force vector (Nickerson et al.,

2004). The other commonly used simulator is the Random Positioning Machine (RPM), apparatus developed by Hoson at the Osaka City University, which changes the position of an accommodated experiment (Hoson *et al.*, 1992; Hoson *et al.*, 1997) with two independently rotating frames operating with different speeds and directions and reproducing microgravity in the order of 10-3 g. These two simulated microgravity systems have in common that the sample is constantly rotated perpendicularly to the gravitational field in order to hinder a biological system in perceiving the gravitational acceleration vector (Anken, 2013). However, the mixing level differs with respect to the number of rotation axes, the speed and the direction of rotation (Briegleb *et al.*, 1992; Klaus *et al.*, 2001). During random movement of the RPM, in fact, continuous acceleration changes underwent by the vessel and consequently by the medium and cells that determine an immediate mixing level (Crabbé *et al.*, 2010). The RWV device is able to reproduce a constant state of suspension in which cells living in tranquil surroundings are indirectly impacted by changes in the fluid boundary layer surrounding them (Klaus, 1994; Hammond and Hammond, 2001; Yamaguchi *et al.* 2014). In this system, the gravity vector is randomized and cells grow with low-fluid-shear (<1 dyn/cm2) under a controlled environment, where the liquid counteracts the slow sedimentation, creating a constant "free-fall" of the cells through the culture medium. The term Low-Shear Modeled Microgravity (LSMMG) has been used to describe the environment produced by the RWV bioreactor in which a dramatic reduction in fluid shear is obtained creating a low-shear stress environment for cell culture, as hypothesized to be a limitation of extracellular mass transport due to the lack of gravity-driven convective flows (Zea *et al.*, 2017). The RPM system was less used than RWV for the study of indirect bacterial responses to microgravity but it is especially suited for studies on plants or mammalian cells in suspension, because this system may provide an environment to successfully expand stem cell populations in vitro. In fact, it has been reported that this system may provide an environment to expand human stem cells populations in vitro (Anken, 2013). In this context, these two bioreactors are ideal not only for studying the behaviour of cells under simulated microgravity but also for evaluating the interactions between the probiotic bacteria and the microvilli of epithelial cells in the intestine and other tissue (Guo *et al.*, 2000; Cervantes and Hong, 2016).

Lactobacillus reuteri DSM17938 has been described to possess several properties believed to be important for its capacity to colonize and adhere to epithelial cells and produces from glycerol metabolism an antimicrobial compound known as reuterin, 3-hydroxypropionaldehyde. A lot of studies have demonstrated the probiotic ability of this microorganism (Lebeer *et al.*, 2008) that was recently reviewed by Srinivasan *et al.* (2018). Moreover, some papers describe the food

probiotication by using this microorganism (Malmo *et al.*, 2013; De Prisco *et al.*, 2015; De Prisco *et al.,* 2016) that is usually ingested by commercial supplements. It is expected that all probiotic properties could be obtained also in spaceflight environment, and potentially offer a precaution to gut microbiota imbalance and to some aspects of immune dysregulation.

The aim of this work was to investigate the stress response of *Lactobacillus reuteri* DSM17938 at metabolic and transcriptomic level when cultured under simulated microgravity conditions using the RWV and RPM technologies in order to evaluate if the microgravity condition may influence the capability of this probiotic bacterium to exert its positive effect on a number of human gut disorders. We investigated growth performance, cell morphology, reuterin production and resistance to simulated gastrointestinal passage. Moreover, the expression of generic stress genes (*groEL, hsp20, dnaK, clpL, ftsH, ctsR and rpoD*), specific stress (i.e. acidic, salt and bile stress) genes (*dps* and *rex*), genes involved in the intestinal epithelial cell adhesion (map and msa) and genes involved in the synthesis of Exopolysaccharides (EPS) (*ftf* and *gtfO*) was studied. List and function of selected genes are reported in Table 3.1. We have focused on these genes because they are expressed by *L. reuteri* during gastrointestinal transit and are involved in its probiotic capabilities.

Table 3.1: Selected genes, functional category and coding protein functions.

Gene	Functional category	Coding protein function	References
groEL	Generic stress	Heat shock protein (HSP 60 chaperonine) Gastrointestinal homeostasis	Bergonzelli *et al*., 2006
hsp20		Heat shock protein (chaperonine) Correct folding of damaged proteins	Capozzi *et al*., 2011
dnaK		Heat shock protein (HSP 70 chaperonine) Adhesion to the intestinal mucus	Izquierdo *et al*., 2009; Bove *et al*., 2013
clpL		ATPase with chaperone activity Cell membrane and cell wall alteration	Wall *et al*., 2007
ftsH		A chaperone-protease Quality check of proteins in stress environments	Bove *et al*., 2012

ctsR		Regulator of generic stress (control of *Clp* gene expression)	Fiocco *et al*., 2010; Russo *et al*., 2012
rpoD		RNA polymerase, sigma 70 (sigma D) Response to generic stress	Vukanti *et al*., 2008
dps	Specific stress	Putative esterase Oxidative stress	Whitehead *et al*., 2008
rex		Redox sensing transcriptional repressor Oxidative stress and microgravity stress condition (*Streptococcus mutans*)	Orsini *et al*., 2017
map	Intestinal epithelial cell adhesion	Mucus adhesion promoting protein Adhesion factor	Miyoshi *et al*., 2006
msa		A mannose-specific adhesion protein Interaction with intestinal tract	Pretzer *et al*., 2005
ftf	EPS synthesis	A fructosyltransferase Production of fructose polymers (fructans) from sucrose and of a high molecular weight inulin	van Hijum *et al*., 2002; Sims *et al*., 2011
gtfO		A glucosyltransferase Cell aggregation, *in vitro* biofilm formation and colonization of the mouse gastrointestinal tract	Arskold *et al*., 2006; Walter *et al*., 2008

3.2. Materials and methods

3.2.1 Bacterial strain and growth conditions

The strain *Lactobacillus reuteri* DSM17938 was kindly provided by BioGaia (Noos S.r.l.; BioGaia AB, Stockholm, Sweden) and was previously characterized in our laboratory for probiotic properties (De Prisco *et al.*, 2015). It was routinely aerobically cultured in MRS Broth (Oxoid Ltd., Basingstoke, Hampshire, England) at 37°C.

3.2.2 Simulated microgravity conditions

An overnight culture of *L. reuteri* DSM17938 was diluted in fresh MRS to rich a cell concentration of approximately 10^6 CFU/mL. Eleven 50 mL rotatory cell culture bioreactors (Synthecon, Houston, TX) were filled with the cell suspension taking care that they were completely filled and no air bubbles were present to compromise the low-shear condition. Rotating bioreactors were attached to their base units with the membrane positioned up and rotated in horizontal axis at 25

rpm at 37°C, here designated as rotating wall vessel (RWV) for microgravity treatment (Senatore *et al.*, 2018). Three other vessels were mounted at the centre of bench-top of a microgravity simulator through random positioning (RPM 2.0, Dutch Space, Leiden, The Netherlands), composed of two separated motors that rotate in different directions reaching an angular rotation velocity of 60 deg s-1, here designated as random positioning machine microgravity treatment (Senatore *et al.*, 2018). Finally, the control cultures (1xg) of RPM and RWV experiments were represented by four rotatory bioreactors installed on the base unit of the RWV in vertical axis.

3.2.3 Growth kinetic

The growth kinetic of bacterium was monitored measuring absorbance at 600 nm over 72 h.

During the cultivation, 0.1 mL samples were collected from the sampling port and the same volume was replaced with sterile broth to avoid air bubbles formation. Optical density was recorded at 1 or 2 h intervals up to 30 h and then further two samples were collected at 48 and 72 h. Growth curves were fitted by using DMFit based on the model of Baranyi and Roberts (1994) to calculate maximum growth rate, lag phase duration, and maximum population at stationary phase.

All results are reported as averages ± standard deviation of four replicates treated with RWV, three replicates treated to RPM device and four replicates for control treatment. Experiments described below on cell morphology, reuterin production and gastro-intestinal passage were performed on cultures after 18 h of simulated microgravity exposure.

3.2.4 Cell morphology by Scanning Electron Microscopy (SEM)

Samples were analysed in duplicate through scanning electron microscopy instrument (SEM-Evo 40, Carl Zeiss, Oberkochen, Germany) to examine the morphology of cells after both simulated microgravity treatments. Sample preparation was carried out according to Shao *et al.* (2017) with little modifications. Two mL from the 1xg, RPM and RWV cultures were fixed for 2 h at room temperature by adding 1 mL of 2.5% glutaraldehyde. After centrifugation at 6500 g for 5 min cell pellet was washed in 1 ml of 0.1 M phosphate buffer (PBS) and cell pellet dehydrated in an ethanol gradient (30, 50, 70 80, 90 and 100% v/v) for 20 min each. Samples were then processed along 3 cycles of 30 min contact with 1 mL tert-butyl alcohol (TBA) and centrifugation (6500 g for 5 min). Cell pellet was then resuspended in 100 µl of TBA and incubated overnight at 4°C. After centrifugation the cell pellet was resuspended in 200 µl of PBS, for a better dissolution of the pellet, and finally 200 µl of TBA were added. A portion of 20 µL was dropped on a mica plate, air dried at room temperature, coated in gold and finally observed by SEM.

3.2.5 Reuterin production assay

L. reuteri was tested for its ability to produce the antimicrobial compound reuterin after RPM and RWV treatments compared to controls. Briefly, pellets from 6 mL of cultures were suspended in 250 mmol/L glycerol and incubated at 37°C for 3 hours. After that, samples were centrifuged at 6,500 g for cell separation and 20 µl of supernatant were spotted on TSA plates previously inoculated with Staphylococcus aureus DSM20231. Antimicrobial activity was observed as a growth inhibition zone of the indicator strain, measured by using a calliper and expressed as average of millimetres of the three replicates ± standard deviation.

3.2.6 Measurement of survival to gastro-intestinal passage

The ability of the cultures to survive to simulated gastro-intestinal transit was tested according to Vizoso *et al.* (2006) adapted by De Prisco *et al.* (2015). Briefly, 2 mL of cultures were centrifuged (6,500 g for 10 min), washed twice with 0.1 M of PBS and suspended in 8 ml of gastric simulated solution (GSS: 5 g/l NaCl, 2.2 g/l KCl, 0.22 g/l CaCl2 and 1.2 g/l NaHCO3, 3g/l pepsin, pH 2.5) and incubated at 37°C for 120 min under gentle agitation (200 rpm) to simulate peristalsis. After centrifugation, the pellet was suspended in 10 ml of intestinal simulated solution (ISS: 6.4 g/l NaHCO3, 0.239 g/l KCl, 1.28g/l NaCl, 0.5% bile salts and 0.1% pancreatin, pH 7.0) and incubated at the same condition of GSS. Cells were enumerated before the start of simulated digestion (T0) and after GSS and ISS steps, by pour plating on MRS Agar. Results are expressed in percentage of cell survival.

3.2.7 RNA extraction and Reverse Transcriptase-quantitative PCR (RT-qPCR)

One mL of *L. reuteri* cultures at 7 h (initial exponential growth phase), 15 h (end of exponential growth phase) and 24 h (stationary phase) from the RPM and RWV treatments, were collected and immediately incubated with a 1:10 ratio (w/v) of RNAlater solution (Ambion, Austin, TX) and stored at -70°C until the RNA isolation.

Three biological replicates of both simulation systems and three control replicates were prepared for the RNA extraction following the Ribo Pure Bacteria kit protocol (Ambion). Cell pellets were washed two times with 1 mL of ice-cold PBS and centrifuged and the pellets were solubilized in 1 mL of RNA WIZ and transferred in screw-cap tubes containing approximately 250 µl of ice-cold zirconia beads (Ambion) and homogenized at maximum speed for 10 min using the Mini-Beadbeater 8 (Biospec Products, Bartlesville, OK).

Each RNA sample was then subjected to a DNase treatment using the TURBO DNA-free™ Kit (Ambion) repeating the treatment with 2U of DNase I two times for 30 minutes. The absence of contaminating genomic DNA in each sample was then determined using PCR and *L. reuteri* 16S rRNA-specific primers (Table 2). RNA quality and concentration were determined using a Qubit® 2.0 Fluorometer (Life Technologies, Grand Island, NY) and Qubit RNA HS assay kit (Life Technologies) along with NanoDrop 1000 (Thermo Fisher Scientific, Waltham, MA). Synthesis of the cDNA were carried out starting from 1 μg of total RNA and using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystem, Foster City, CA) according the manufacturer's instructions.

Reverse Transcriptase-quantitative PCR (RT-qPCR) was performed in 96-well plates on a RT-qPCR Chromo4™ System (Biorad, Hercules, CA) using SSoAdvanced Universal SYBR Green (Biorad) as fluorophore. The expression of a total of 13 genes was evaluated.

Amplifications were performed under the following conditions: 95°C for 3 min, 40 cycles of 95°C for 15 s, 58°C for 30 s, and a final step at 72°C for 60 s. At the end of the amplification cycle, a melting analysis was conducted to verify the specificity of the reaction. Differences in gene expression were calculated using the △△CT method after normalizing based on 16S rRNA expression. Data were obtained from three reactions using RNA from the three independent replicates from each treatment. Data are plotted as average and standard deviation. Genes were considered differentially expressed in comparison to the control whose expression value is considered equal to 1 when showed a fold change below 0.5 or above 2.0 and the P-value was lower than 0.05.

The primers for RT-qPCR were constructed with the primer express 3.0 software package (Applied Biosystems, Foster). All primers were verified and aligned with the genome of *L. reuteri* DSM17938 using Tool BLAST (URL: http://img.jgi.doe.gov/cgi-bin/pub/main.cgi). Genes, sequence of primers, melting temperatures and amplicons' size are listed in Table 3.2.

Table 3.2: Primers used for Reverse Transcriptase-quantitative PCR (RT-qPCR) analysis.

Genes	Primers	T°m	Fragment length (bp)
Housekeeping gene			
16S rRNA	f: GGGTTAAGTCCCGCAACGAG r: CCTCCTCCGGTTTGTCACC	63	81
Target genes			
groEL	f: GCTGCAGATGGCAAGTTTGAAG r: GAAGCAGCGTTTTGAAGAGC	63	89

hsp20	f: GTCATCGGGATACCTTTGATG r: GGTAAACGATATTGCCGACTCA	62	98
dnaK	f: CCAACCAGCTGTTGACATTCA r: GATTTGAGGAACACCACGAG	62	121
clpL	f: GGTGCTACTGCTGGTTATGT r: AGAGGGTGATAACTTGTGGGTTAG	62	130
ftsH	f: ACCGGGTAATTGCTGGACCA r: TCGTGTGACCTGCTTCGTGG	62	90
ctsR	f: CGTGGAGGCGGCGGATATAT r: TCACGAACCGAGTCGCCAAT	62	102
rpoD	f: ACACGGACCCTTGAAGAAGT r: AGAACGTGATGGGTGACGT	60	105
dps	f: GACTGGCCAGGAAACTTTGA r: GTCACTTCAATTCCATGCTGATAAA	64	104
rex	f: CCCATCCGAGGATATGGTAAAG r: CCTTCACACCAGCTTCAACTA	62	119
map	f: TTGCTGGGACAGGAACTAATAAT r: CCGACCTTGCTTGATCATATCT	62	107
msa	f: AGATGGCAGCACTAAAGGTAAT r: GTGGGAGTGACCAGCAATATAA	62	100
ftf	f: TTGGCGTACCGCTACTTATTC r: CCAGCAACTTCGCCTCTATT	62	102
gtfO	f: CGTCCGGTGTTCCATGTATT r: CCTCATACGCAATCCTCCTAAC	62	110

3.2.8 Statistical analysis

The statistical differences were evaluated with t-test and with a factorial analysis of variance (ANOVA) and means were compared using the Tukey HSD test by using SPSS software (Ver.17.0, SPSS Inc., Chicago, IL, USA).

3.3 Results

3.3.1 Growth profile and growth rate

Results of monitoring and modelling of bacterial growth (Fig. 3.1) showed that all cultures started the exponential growth phase after about 4 h (lag phase duration). Moreover, all cultures showed a maximum growth rate ranging between 0.472 (±0.016) h^{-1} in RPM and 0.556 (±0.023) h^{-1} in RWV.

Moreover, all samples showed no significant difference of maximum population level.

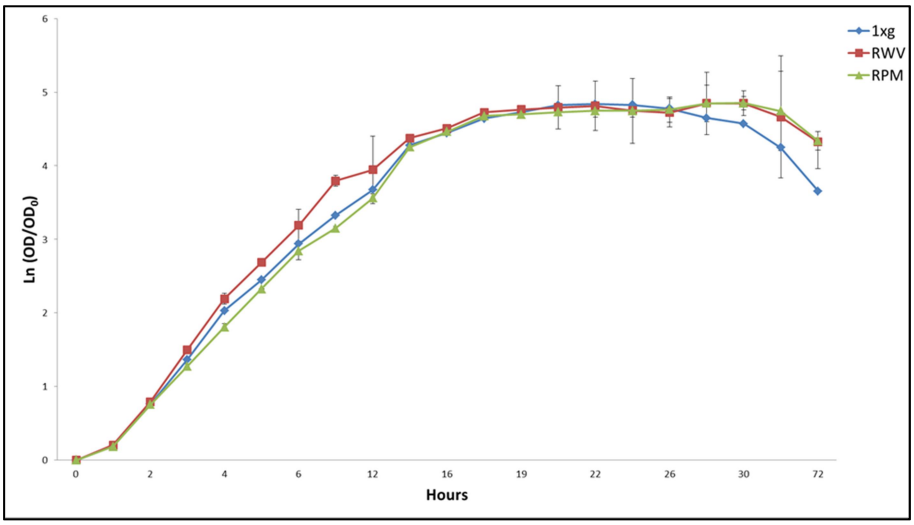


Fig. 3.1: Growth kinetics over 72 hours of cultures under control, RWV and RPM conditions.

3.3.2 Electron microscopy (SEM) analysis

SEM analysis of cultures subjected to RWV and RPM treatments revealed no differences in shape and size of individual cells (Fig. 3.2). However, SEM images showed that cells grown under microgravity (Fig. 3.2 B and 3.2 C) appeared more aggregated compared to control sample (Fig. 3.2 A), in which cells seems well separated. A much more homogeneous distribution of cells within the specimen of control sample was further demonstrated by Atomic Force Microscopy (data not shown) in which no preparation of sample is requested.

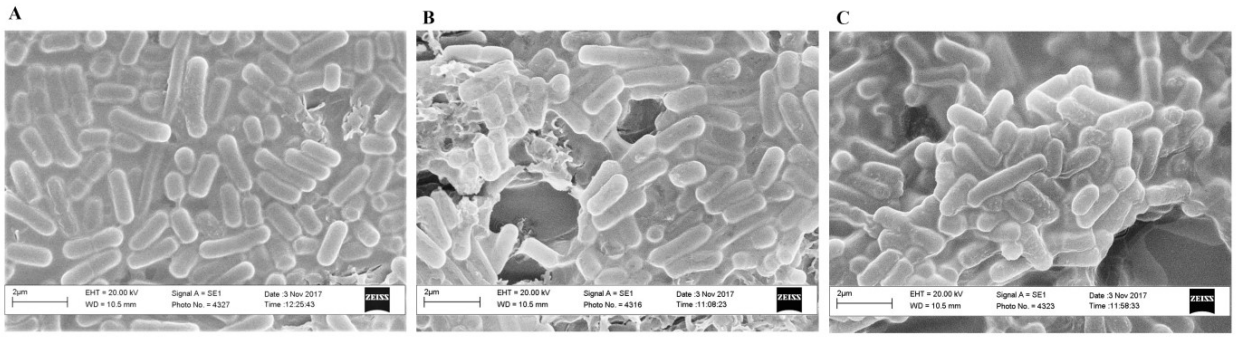


Fig. 3.2: Cell morphology images by Scanning Electron Microscopy (SEM) of *L. reuteri* DSM 17938.

(A) control sample (1xg) , (B) treated sample to RWV, (C) treated sample to RPM.

3.3.3 Reuterin production

Results of antimicrobial test showed that cells grown under both simulated microgravity conditions determined an overproduction of reuterin compared to control culture. Indeed, the indicator strain showed a growth inhibition halo of 32±0.81 and 31±0.28 mm in RWV and RPM, respectively, against a 22±0.95 mm halo of the control culture (Fig. 3.3).

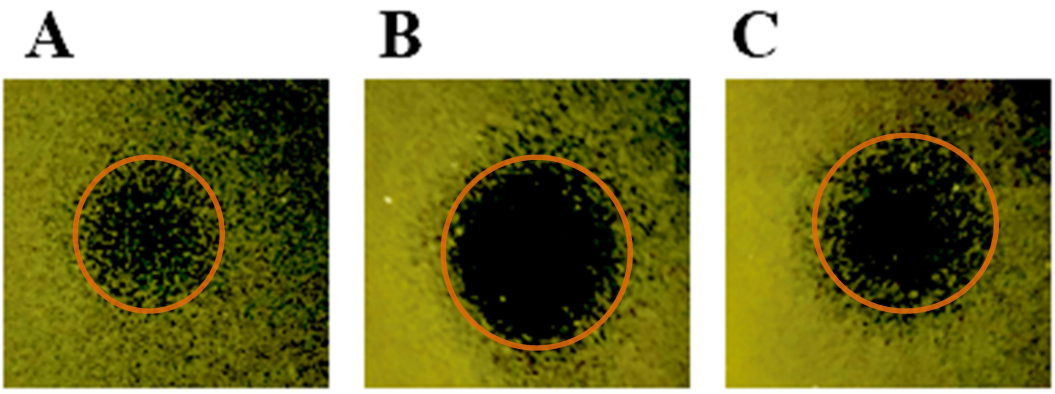


Fig. 3.3: Variation of inhibition halo of *S. aureus* DSM20231 growth on agar plates as effect of simulated microgravity on the reuterin production by the supernatant of *L. reuteri* cultures subjected for 18 hours to control condition (A), to RWV (B) and RPM (C) conditions.

Orange circles limit the areas of the inhibition halos.

3.3.4 Gastro-intestinal stress resistance

The survival rate after 18 h of growing under simulated microgravity and terrestrial gravity conditions was evaluated through the viable counts and the survival percentage was reduced from 71% after gastric simulating step (GSS) to 31% after the intestinal simulating step (ISS) in the 1xg samples and from 100 to 62% in RPM samples. The RWV samples seem to tolerate better the intestine environment because there is less reduction in the survival rate, from 92% to 76% (Fig. 3.4). As a matter of fact, GSS step determines higher survival of cells grown under RPM; whereas ISS exposure determines higher survival of cells grown under RWV. ANOVA test was performed comparing both the three treatments (1xg, RPM and RWV) within the single step of GI passage (time zero, GSS and ISS) and the three steps of GI passage within the single treatment. Results showed no significant difference between the three GI passage steps within RPM samples; on the other hand, a significant difference ($P<0.05$) within RWV samples (Table 3.3) was observed.

Results with t-test showed significant difference in cell survival between control and treated samples after GSS exposure, as well as between RPM and RWV samples ($P<0.05$) (Fig. 3.5). Instead, after ISS exposure, significant difference was shown only between 1xg and RWV cultures ($P<0.05$).

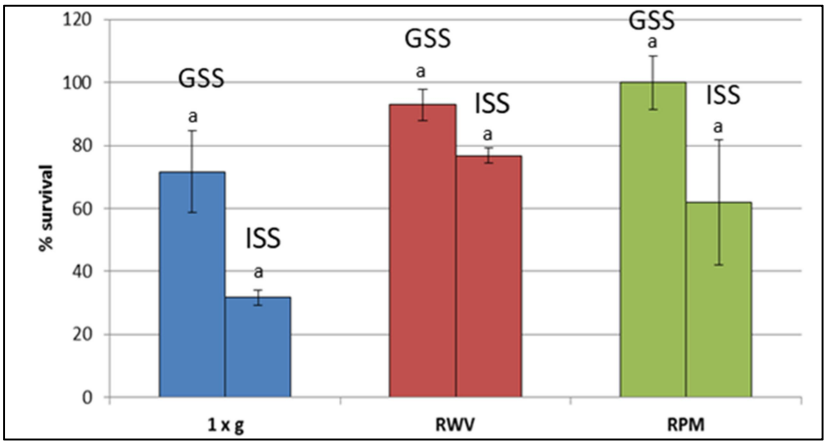


Fig. 3.4: Survival rates of *L. reuteri* exposed to gastric simulated solution (GSS) and intestinal simulated solution (ISS) after 18 hours of growth under simulated microgravity conditions and control condition.

Different letters indicate significant difference ($P<0.05$) between mean values of GSS and ISS as determined by t-test.

Table 3.3: Test ANOVA between the treatments (1xg, RWV and RPM) for every step (time 0, GSS and ISS) and between the three steps within single treatment.

One or two asterisks indicates significant differences between the three treatments or within the single treatment at $P<0.05$ or $P<0.01$, respectively.

Treatments/Steps	T_0	GSS	ISS
Between treatments	9.8522E-11**	0.000556**	0.010676*
within 1xg		0.0613519	
within RWV		0.0123332*	
within RPM		0.0599454	

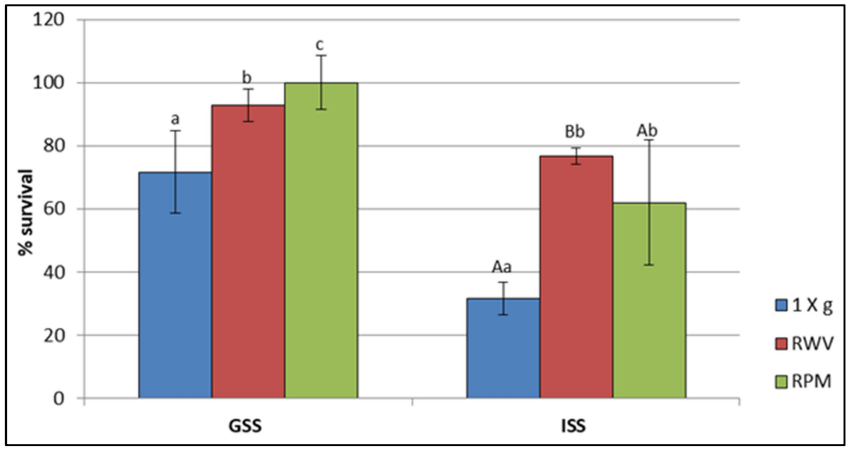


Fig. 3.5: Survival rates of cultures exposed for 18 hours to simulated microgravity (RWV and RPM) in comparison to control culture (1xg) after exposure to gastric simulated solution (GSS) and to intestinal simulated solution (ISS).

Different letters indicate that mean values of GSS and ISS exposure between treatments are significantly different ($P<0.05$), as determined by Tukey HSD test.

3.3.5 Gene expression

Results of gene expression level experiments are depicted in Figure 3.6. RT-qPCR results at 7 h (Fig. 3.6 A) show that most of investigated genes are not differentially expressed because the fold change remains within the limit of 0.5 and 2, even though all of them showed a significant difference compared to control. However, under RPM microgravity, down-regulation of *clpL*, *ctsR* and *rpoD* genes and over-expression of *rex* and *gtfO* genes were shown.

Our results show that picture totally changes after 15 and 24 h of growth under simulated microgravity (Fig. 3.6 B and 3.6 C). In fact, 6 and 7 genes were over-expressed under RWV after 15 and 24 h, respectively. No gene was under-expressed during the growth in the same condition. Instead, 3 genes were over-expressed and 4 under-expressed after 15 h while 1 gene was over-expressed and 6 under-expressed after 24 h, under RPM. After 15 h of growth under microgravity a significant fold change was registered for most of the genes involved in the generic and specific stress response (*hsp20, dnaK, clpL, ctsR , rpoD* and *rex*) (Fig. 3.6 B), while a significant and out of fold change range of 0.5-2 was shown for most of other genes, like some of them involved in the probiosis (i.e. *map* and *msa*) of the tested microorganism. Moreover, the high overexpression of *gtfO* gene - involved in EPS biosynthesis - under both simulated microgravity systems should be underlined.

Results registered after 24 h (Fig. 3.6 C) showed a significant fold change in the expression level of most of tested genes. In particular, the stress genes showed a significant increased and decreased expression level under RWV and RPM conditions, respectively.

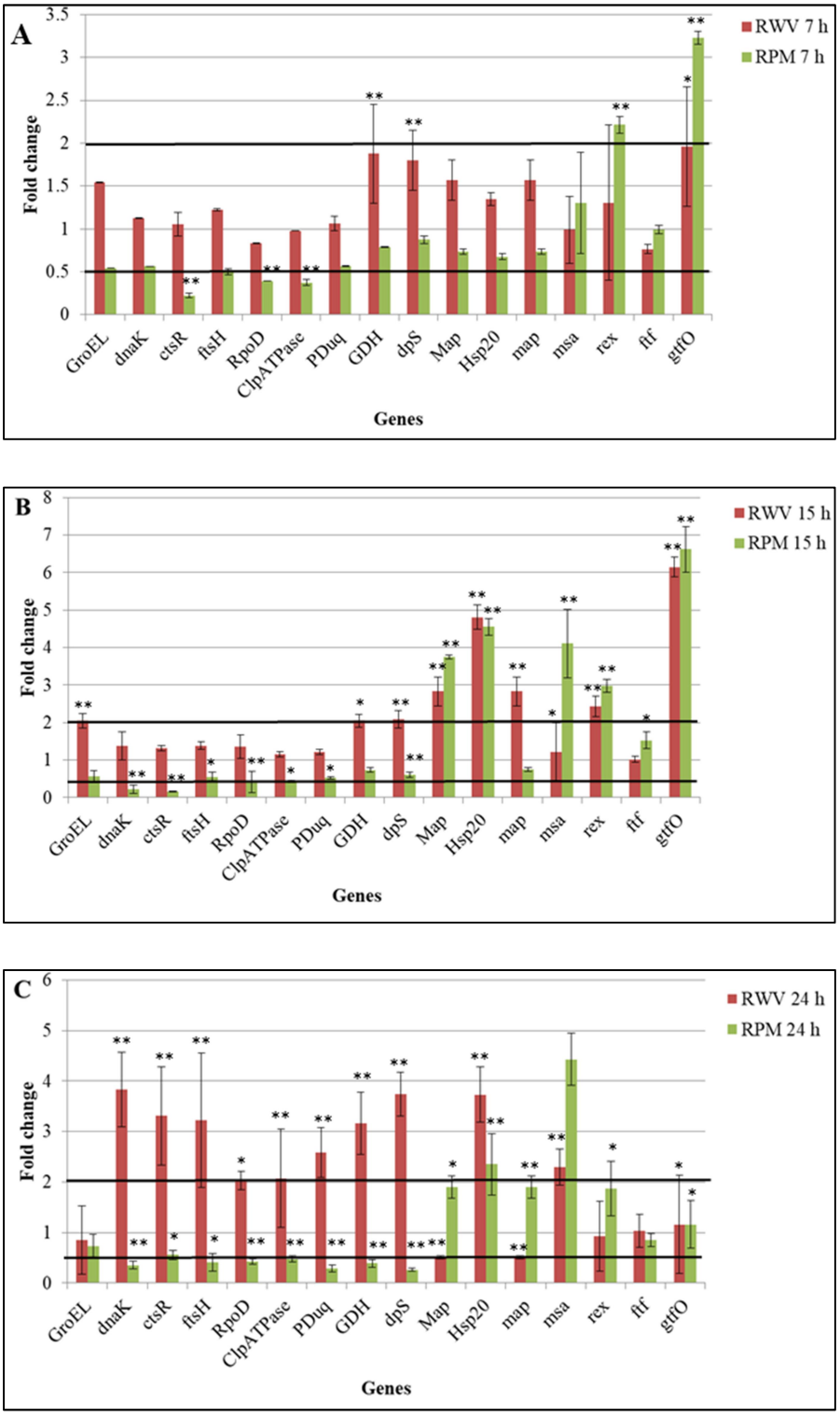


Fig. 3.6: Level of gene expression after 7 (A), 15 (B) and 24 hours (C) under RWV and RPM.

The bold lines show the threshold of upper-regulation (2 folds) and down-regulation (0.5 folds). One or two asterisks indicates significant differences between control and treated samples for $P<0.05$ and for $P<0.01$, respectively.

3.4 Discussion

3.4.1 Growth profile and growth rate

Results of growth experiments are in agreement with a recent study (Castro-Wallace *et al.*, 2017), which demonstrated that another typical probiotic strain for humans, *L. acidophilus* ATCC 4356, exhibited a growth trend, under RWV simulated microgravity, similar to control culture. On the contrary, altered growth profile of the same *L. acidophilus* strain was reported by Shao *et al.* (2017), using a new 3-D RWV system that rotates at 30 rpm. Also, other publications on opportunistic and obligate pathogens showed alterations in growth profiles when cultivated in LSMMG (Leys *et al.*, 2004; Klaus *et al.*, 1997; Kacena *et al.*, 1997; Kacena *et al.*, 1999a; Kacena *et al.*, 1999b). Our conflicting results could demonstrate that every microorganism responds differently to this particular condition.

3.4.2 Cell morphology and surface characterization

SEM observations did not show any changes in cell morphology, both in terms of shape and size of the cultures treated with RWV and RPM compared to 1xg cultures. These results are in agreement with a previous work on *L. acidophilus* in which samples subjected to RWV treatment showed similar morphological and structural features compared to control, indicating that one day LSMMG treatment had no significant effect on the morphology and structure of this probiotic bacterium (Shao *et al.*, 2017). The only difference that could be noticed in both treatments was an alteration of the level of cell aggregation, confirming what reported in the literature for the pathogen *Salmonella* Typhimurium exposed to real microgravity during the space mission STS-115 (Wilson *et al.*, 2007). This bacterium showed the formation of an extracellular matrix and associated cellular aggregation of spaceflight cells that is similar to morphology observed for *L. reuteri* cultures under RPM condition (Fig 1c). Changes in bacterial biofilm characteristics under LSMMG environment have been demonstrated for other microorganisms, for which the low-fluid-shear environment stimulates increased EPS production. Culture of *P. aeruginosa* growing in RWV showed a self-aggregation, an increased alginate production and a phenotype related to cystic fibrosis pathology in the lung (Crabbè *et al.*, 2008). Moreover *E. coli* cultivated under LSMMG environment produced thicker and more resistant biofilms (Lynch *et al.*, 2006).

3.4.3 Reuterin production

The reuterin production is increased under simulated microgravity stress demonstrating that the results are in accordance with that about another probiotic bacterium like *L. acidophilus* treated with a new 3-D RWV system (Shao *et al.*, 2017). LSMMG could also induce increased virulence in pathogenic *Salmonella* Typhimurium grown aboard Space Shuttle mission STS-115 that was able to display an improved percentage mortality in a murine infection model with a shorter time to death and a decreased LD50 (Wilson *et al.*, 2007). In contrast, most studies on bacterial virulence characteristics under low-shear conditions displayed no significant differences in virulence, including decreased secondary metabolites production (Taylor *et al.*, 2015). Indeed, the results obtained in this work are in contrast with previous results on other microorganisms cultured in a RWV apparatus, as assessed for the first time by Fang *et al.* (1997). They reported that RWV environment decreased the beta-lactam and the rapamycin production in *Streptomyces hygroscopicus* and prevented micocin B17 production in *Escherichia coli*. The reason of the reduced secondary metabolites production could be due to reduced shear stress encountered in the RWV bioreactors (Fang *et al.* 2000a; Fang *et al.*, 2000b; Demain *et al.*, 2001). In our case, the higher production of an antimicrobial substance confirms previous results for another lactic acid bacterium (Shao *et al.*, 2017), indicating that the low-fluid-shear in which the bacterium growth is perceived as a stimulus in the production of antimicrobial compound probably because reproduce the same condition of the intestinal tract (Nickerson *et al.*, 2004; Arqués *et al.*, 2015).

3.4.4 Gastrointestinal stress resistance

L. reuteri is a beneficial probiotic organism that inhabits the GI tract. Therefore, it is important to investigate the response of this microorganism to GI passage after the exposure to microgravity condition. The increased resistance of *L. acidophilus* to acid and bile salts stress (Shao *et al.*, 2017) is similar to the response of *L. reuteri* in this work that seems to tolerate the stressful conditions of the stomach and upper intestine. *L. acidophilus* showed greater resistance to low concentrations of bile (<0.05%) rather than high concentrations (0.1%-0.3%). In our case, *L. reuteri* cultures treated for 18 h to RPM and RWV systems were able to resist to high bile salts concentration (0.5%) because, after the intestinal simulating test, a lower reduction of survival was observed for RPM but more for RWV cultures in comparison with control samples. In fact, in comparison to 1xg samples, the treated samples to simulated microgravity showed a greater performance in survival immediately after the simulation step of the gastric environment. Moreover, the RWV samples seemed to tolerate the intestinal treatment (ISS) better than control and RPM samples. These results

are in contrast with what was reported in another work on *L. acidophilus* ATCC 4356 (Castro-Wallace *et al.*, 2017), that highlighted no significant differences in survival between RWV and 1xg cultures treated to simulated gastric or small intestinal juice stress.

3.4.5 Gene expression

Our findings show that the expression level of most tested genes is affected by simulated microgravity. However, as expected, the response is different according to the time of exposure. Moreover, by comparing the RPM and RWV system, we found substantial differences in the expression level of many selected genes.

The unaltered expression of most genes after 7 h of growth is reasonably due to the short period of cultivation, during which the modified environment has not altered the mRNA synthesis respect to control culture yet. This result is supported by the growth curve, which showed that at 7 h all cultures are still at the beginning of exponential phase. Since the results of gene expression under RWV show a minor difference with respect to the RPM condition, we hypothesized that probably RWV is perceived at the beginning of growth as a normal condition of cellular suspension rather than the RPM condition. Results of gene expression after 7 h of treatment to both simulated microgravity conditions are similar to that about other two *Lactobacillus* strains that showed an unaltered or light alteration in the expression level of some stress genes and some key genes of cholesterol metabolism, also after 24 h of growth under simulated zero gravity condition (Shao *et al.*, 2017; Castro-Wallace *et al.*, 2017).

The results at 15 h are analogous to that of two potential pathogens like *E. coli* and *Salmonella* Typhimurium growing under simulated microgravity showed differential level of expression for genes usually known to be involved in thermal, osmotic and acid stress, starting as early as the mid-late log phase (16 h) of growing (Wilson *et al.*, 2002; Wilson *et al.*, 2007; Vukanti *et al.*, 2008).

In general, our results highlight differences between the two simulated microgravity systems: as in RWV condition there was an up-regulation of most of the investigated stress genes, they showed a general down-regulation over time in RPM.

It was noted by microbiologists (Benoit and Klaus, 2007) that the two simulations cannot fully reproduce the effects of actual spaceflight. The gravity vector, in fact, is randomized, but fluid convection and shear stress are not completely avoided. Hence, results obtained from cells incubated in real microgravity are frequently similar, but not completely identical (Hemmersbach *et al.*, 2006). Nevertheless, between two systems, the RWV device is believed to be more appropriate

to study the LSMMG-induced response of cells in a suspension environment, rather than the RPM. In fact, similarities between spaceflight and LSMMG responses were observed and can presumably be ascribed to the analogous low-fluid-shear conditions in-flight and in the RWV. Fluid mixing in both systems is believed to be a pivotal factor contributing to transcriptional differences between *L. reuteri* cultures grown in RWV and RPM. These simulation systems could determine different effects due to the different mixing level. Despite final objectives of these two devices are the same, they may be reached in different ways. Probably, the cells perceive the growth environment differently by showing gene variations based on cultivation conditions. Interestingly, fold change of most stress genes after 15 h and 24 h emphasized differences comparing results of RWV and RPM. In particular, cells grown under RWV microgravity exhibited a tendency to increase the expression level of these genes over time, whereas under RPM microgravity there was an opposite trend.

The low-fluid-shear condition reproduced by RWV may be perceived by the cell as an alteration in oxidative state that involved two specific gene like *rex* and *dps*. The change of oxidative conditions, in fact, is more evident under RWV treatment rather than under RPM treatment determining an increase with time of *dps* expression under RWV environment. This gene is normally involved in different type of stress response in *Escherichia coli* like oxidative stress, irradiation, metal toxicity, heat stress, and pH stress (Martinez and Kolter, 1997; Nair and Finkel, 2004).

Our results about *dps* gene expression not reflect those obtained in another whole transcriptional analysis of a spaceflight culture of *Salmonella* Typhimurium that revealed a down expression of *dps* gene (Wilson *et al.*, 2007). The expression level of *rex* gene underwent an increase from 7 h to 15 h of both growth conditions, in agreement with results of RNA-seq analysis about the bacterium *Streptococcus mutans* subjected for 8 h to simulated microgravity condition (Orsini *et al.*, 2017). In particular, this bacterium showed an overexpression of genes involved in transport of carbohydrates, in biofilm formation and oxidative stress resistance in response to cellular redox, whose expression is regulated by the transcriptional regulator *Rex*. This gene is moreover implicated in the transcriptional response of *Staphylococcus aureus* to LSMMG by the altered expression of a number of *Rex*-regulated genes under this growth condition (Castro *et al.*, 2011).

Moreover, only one gene involved in EPS synthesis i.e. *gtfO*, showed a surprising increase in the expression level already at 7 h after the two treatments. This gene encoding a glucosyltransferase, a sucrose-type enzyme involved in EPS synthesis, is probably more directly responsible for the production of biofilm observed through SEM analysis in both cultures subjected to 18 h treatments.

The *rpoD* gene is up-regulated only after 24 h of RWV condition: this gene codifies a sigma factor that is activated with rpoS upon exposure to oxidative stress signal putting the cell in a "protective state". In *E. coli* AMS6 the stress response to RWV treatment depends on *rpoS* expression that is higher only in stationary phase of growth (Lynch *et al.*, 2004), corroborating our results.

Genes coding for more regulative and/or catalytic functions, i.e. *ctsR* and *ftsH*, exhibited major values of variation in fold change under RWV condition after 24 h of treatment. Recently, *ftsH* was identified as a chaperone-protease belonging to a novel member of the class three stress gene Repressor (*ctsR*) regulon in *L. plantarum* (Fiocco *et al.*, 2009, 2010; Bove *et al.*, 2012; Russo *et al.*, 2012). As *ctsR*, this enzyme is involved in cell protection against environmental stress condition like that encountered during the human oro-gastric-intestinal transit (Bove *et al.*, 2013).

A modification of the expression level of these two genes only under RWV treatment suggests that this system is more similar to that encountered in GI environment.

Interestingly, the high induction of the *dnaK* chaperone gene and the heat shock gene *hsp20* might account for their putative additional roles in mechanism of response to microgravity condition. These two genes are sensitive to the condition of low-fluid-shear reproduced by the RWV system and the strong expression increase is evident at 24 h of treatment. Notably, *hsp20* gene exhibited a marked increase of fold change over time under RWV treatment and a decrease under RPM. This gene, like the other *hsp* genes, responds to different stress conditions like heat, cold, acid, osmotic, oxygen, high pressure and starvation stresses in many *Lactobacillus* strains (De Angelis *et al.*, 2004).

Our results about *dnaK* gene expression are in agreement with a microarray study on *Salmonella* Typhimurium grown under simulated microgravity condition (Chopra *et al.*, 2006) but are in contrast with another transcriptional study on *E. coli* K12 that showed a down-regulation of this gene under modelled reduced gravity (Vukanti *et al.*, 2008).

Considering genes of probiosis, *map* and *msa,* only the latter showed an increase of expression over time under the two different treatments. This increment could be indicative of the major ability of this probiotic microorganism to colonize the intestinal surface and to have a competitive exclusion of pathogens even if this capability is regulated by many other genes.

It is interesting to highlight the differences over time at transcriptomic level of cultures treated to RPM and RWV, which reflect the different way of these devices to reproduce microgravity environment. Differences between RPM and RWV systems were already shown by other authors

that considered the molecular effect induced by both devices (Mastroleo *et al.*, 2013). They evaluated the whole transcriptome of the MELiSSA bacterium *Rhodospirillum rubrum* under both conditions and showed a significant response to cultivation in simulated microgravity at the transcriptomic, proteomic and metabolic level. All 13 genes upregulated under RWV condition were also included in the more pronounced response to RPM cultivation but with higher fold induction in the latter, which was not the case when comparing the response to both devices at the proteomic level. This is in contrast with our RPM results that did not alter the molecular response of *L. reuteri*, demonstrating that the responses to simulated spaceflight conditions are species-specific and depend on the growth conditions. Another work, showing conflicting results of the two microgravity simulation methods, reported the microarray analysis of *Pseudomonas aeruginosa* PAO1 grown in the two different spaceflight-analogue culture systems. Analysis of differential gene expression revealed 134 and 9 genes induced under RWV and RPM, respectively, compared with the normal gravity control indicating that RPM and the normal gravity were sensed in a similar manner by *P. aeruginosa* PAO1 while LSMMG condition reproduced by RWV led to a more divergent transcriptomic profile (Crabbé *et al.*, 2010).

On the contrary, our divergent results regarding the fold change of many selected genes after 24 h of cultivation under two different microgravity environments showed that these conditions and the 1xg controls were recognized in time as different growth conditions by *L. reuteri*.

3.5 Conclusion

In conclusion, this study revealed that simulated microgravity conditions could significantly impact the biological characteristics of the tested *L. reuteri*, in various aspects, including bile tolerance, antimicrobial property and gene expression. Overall, the study offers important information for better understanding the influence of simulated microgravity on probiotic bacteria, and thus may contribute to protect the health status of astronauts in long-term space missions. Moreover, our results confirm that different simulated microgravity systems could have dissimilar effect on bacterial cells depending on their capability to mimic real microgravity condition. These experiments should be considered preliminary to further experiments in spaceflight environment to develop a comprehensive understanding of the impact of the real microgravity condition on the probiotic bacterium with the final aim to provide these microorganisms to the crew members during long term missions.

Notes

This chapter reports the content of original paper entitled “Growth of *Lactobacillus reuteri* DSM17938 under two simulated microgravity systems: changes in reuterin production, GI passage resistance and stress genes expression response” by Giuliana Senatore, Felice Mastroleo, Natalie Leys, Gianluigi Mauriello on ASTROBIOLOGY 2020 20(1):1-14. doi: 10.1089/ast.2019.2082.

CHAPTER 4

Changes in Proteomic expression of *Lactobacillus reuteri* DSM17938 under simulated microgravity conditions

4.1 Introduction

Space is a hostile environment where high vacuum, temperature extremes, microgravity (μg), ultraviolet and ionizing radiation represent risks for astronaut's health.

More than the other factors, microgravity influences the structure, function, evolution and behaviour of all living organisms (Bizzarri *et al*., 2015). Prolonged weightlessness encountered in space can impact the astronaut's health, including bone loss (Ulbrich *et al.*, 2014), cardiovascular variations (Hatton *et al.*, 2002), immune system dysregulation (Crucian *et al.*, 2009), and alterations in sleep and circadian rhythms (Gundel *et al.*, 1999). Microgravity usually refers to a condition of 10^{-6} to 10^{-3} g encountering at the altitude of 300–400 km (LEO) (Herranz *et al.*, 2013). In μg an object is subjected to a free fall status and floats apparently without weight. To study the effects of real μg, different space research platforms are used such as parabolic airplane flights, sounding rockets and orbital platforms such as satellites or space stations (Clement *et al.*, 2006). Due to the high costs of a permanence in real microgravity, some on-ground systems mimicking microgravity can be additionally used (Klaus *et al.*, 2001). Numerous ground-based methods have been developed to simulate the condition of weightlessness in laboratories on Earth.

Numerous studies with simulated microgravity systems have showed that space conditions increase virulence and reduce susceptibility of pathogenic microorganisms against antimicrobial compounds (Senatore *et al.*, 2018). This is probably one of consequences to an impaired function of the immune system of infected humans (Saei and Barzegari, 2012).

In recent years, simulated microgravity experiments have shown many changes of normal biological functions in microorganisms through the alteration in the expression of various genes and the consequent modifications in the expression of proteins (Babbick *et al.*,2007; Grimm *et al.*, 2011; Pietsch *et al.*, 2011). Some alterations of cell wall components, extracellular and intracellular proteins and of some components of signaling pathways were observed (Hughes-Fulford *et al.*, 1996; Schatten *et al.*, 2001; Uva *et al.*, 2002). For this reason, many studies of comparison of proteins, which are present either in cells living under gravity or in cells exposed to microgravity were performed.

In principle, a proteomic response, resulting from the lack of the gravity vector, could determine a physiological impact on microorganisms. A change of the protein production capability of microorganisms must also be considered as a cause of the enhanced infection rate, because incubation of *Escherichia coli* under simulated microgravity showed that the gravity force variation elicits this bacterium to secrete many heat-labile enterotoxins (Chopra *et al.*, 2006). Furthermore, *Pseudomonas aeruginosa*, an opportunistic pathogen that is present in the space habitat, regulated 28 different proteins in response to spaceflight conditions (Crabbé *et al.*, 2011).

Global microarray and proteomic analysis on bacterial pathogen *Salmonella* Typhimurium, growing on Space Shuttle mission STS-115 and compared with identical ground control cultures, demonstrated that 73 proteins changed expression, with the conserved RNA-binding protein Hfq being identified as a likely global regulator involved in the response to this particular environment (Wilson *et al.*, 2007). RWV experiments on the ground, reproducing the condition of low gravity with reduced shearing forces, indicated that under this condition the RNA-binding protein Hfq was downregulated and consequently controls the expression of Hfq-dependent proteins. Moreover, an omic approach used to study the response to simulated microgravity of *Streptococcus mutans*, an oral bacterium responsible of dental caries, showed a total of 153 overexpressed genes principally involved in carbohydrate metabolism, translation, and stress response (Orsini *et al.*, 2017). The research on proteomic response of microorganisms subjected to zero gravity would not only provide new information on pathogenicity mechanisms but would also provide important insight into application-oriented aspects, such as the use of microorganisms as life regenerative support systems to produce biomass and recycle waste in space (Mastroleo *et al.*, 2013).

One of these bacterium, used as a good test microorganism in MELiSSA project for its versatility, *Cupravidius metallidurans* CH34, has been cultivated on two ISS missions (MESSAGE 1 and 2), in parallel with similar control procedures on Earth (Leys *et al.*, 2009). Proteomic analysis revealed the overexpression of different proteins in space conditions, in particular of acetone carboxylase, an enzyme that has never been proved on Earth to be present in the proteome of *C. metallidurans* (Leys *et al.*, 2009). From proteomic analysis on CH34 cells returning from spaceflight 16 proteins were identified which were present in higher concentration in cells developed in spaceflight conditions. These proteins were involved in a specific response of CH34 to carbon limitation and oxidative stress, and included apart from acetone carboxylase subunit, fructose biphosphate aldolase, a DNA protection during starvation protein, chaperone protein, universal stress protein, and alkyl hydroperoxide reductase. The reproducible observation of the over-expression of these same proteins in multiple flight experiments, indicated that the CH34 cells could experience a

substrate limitation and oxidative stress in spaceflight where cells and substrates are exposed to lower levels of gravity and higher doses of ionizing radiation. Another work on the same strain cultivated under RPM and RWV conditions demonstrated that microgravity seems not to be involved in dramatic changes in the bacterial proteome. Significant modifications in the abundance of some proteins were observed in a sample submitted to RPM treatment. Proteins, especially membrane proteins implicated in various cellular functions and stress proteins were significantly more abundant after RPM cultivation (Leroy *et al.*, 2010).

Another microorganism used in the second compartment of MELiSSA loop for converting volatile fatty acids released from the upstream raw waste-digesting reactor to carbon dioxide and biomass is *R. rubrum*. This bacterium was analyzed after two different ISS flights using a newly developed *R. rubrum* whole genome oligonucleotide microarray and high throughput gel-free proteomics with Isotope-Coded Protein Label technology. In contemporary, a simulation experiment of microgravity with RPM system and space-ionizing radiation were performed on Earth under identical culture set-up and growth conditions. Proteomic data in real spaceflight experiments revealed a low overlap with that found in ground simulation experiments. In fact, few significantly over and under-expressed proteins were obtained in the MESSAGE 2 experiment and in BASE-A spaceflight experiment in comparison with the results of simulated microgravity experiment. Therefore, only under RPM treatment, some highly over-expressed unknown-function proteins were observed (Mastroleo *et al.*, 2009).

More than microgravity, cosmic radiation could affect the microbial proteomic response in space. This effect was mainly revealed in the BASE-A spaceflight experiment where various over-expressed genes of *R. rubrum* matched those found during ground tests of ISS radiation. Thus, for the first time studies showed a low dose of ionizing radiation (2 mGy) can induce a significant response at the transcriptomic level, although no change in cell viability was observed (Mastroleo *et al.*, 2009).

In a recent paper (Mastroleo *et al.*, 2013), *R. rubrum* S1H was cultured in a Rotating Wall Vessel (RWV), simulating partial μg conditions on Earth in order to perform proteomic, transcriptomic and metabolic analysis. Regarding the proteomic analysis of the experiment performed with *R. rubrum* S1H in simulated microgravity, 422 proteins were identified: interestingly, among the ten overexpressed proteins, seven were membrane proteins. Furthermore, the translations of 41 hypothetical proteins of *R. rubrum* were identified for the first time. In particular, Rru_A1096 (putative periplasmic protein), Rru_A1353 (putative outer-membrane protein), Rru_A3373

(Conserved protein of unknown function) and Rru_A3662 (Conserved protein of unknown function) have been shown to be significantly downregulated in simulated microgravity.

Here, we studied *Lactobacillus reuteri* DSM17938 at proteomic level using the RWV and RPM systems in order to assess whether some metabolic pathways involved in combating human intestinal disorders can be altered in conditions of microgravity stress. This bacterium, in fact, has been described to possess several properties believed to be important for its capacity to colonize and adhere to epithelial cells (Wadstrom *et al.*, 1987) and produce the antimicrobial substance reuterin (Axelsson *et al.*, 1989). It is expected that these benefits could be obtained also in spaceflight environment, and potentially offer a precaution to gut microbiota imbalance and to some aspects of immune dysregulation.

In this context we evaluated whether the simulated microgravity could affect the proteome of *L. reuteri* and could modify the expression of some proteins involved in probiotic mechanisms.

4.2. Materials and Methods

4.2.1 Bacterial strain and growth conditions

Experiments were performed using *L. reuteri* DSM 17938 kindly provided by BioGaia (Noos S.r.l.; BioGaia AB, Stockholm, Sweden). All cultures were obtained at 37°C in MRS broth and diluted with fresh medium to rich a cell concentration of approximately 10^6 CFU/mL. The bioreactors used in RWV and RPM experiments were filled completely with ca. 55 mL of culture medium taking care to carefully remove all air bubbles through the sampling ports to permit a low-shear condition. Four RWV vessels (Cellon, Luxembourg) attached to their base units and rotated in horizontal axis were used for RWV-simulated microgravity and other four RWV rotatory bioreactors were used for 1xg control installed on the base unit of the RWV in vertical axis. Bacterial growth in RWV conditions was allowed at a rotational speed of 25 rpm at 37°C. Other three vessels were installed in the centre of bench-top of a microgravity simulator through random positioning machine (RPM 2.0, Dutch Space, Leiden, The Netherlands), which works as a random walk 3-D-clinostat (basic mode) with an angular rotation velocity of 60°/s.

4.2.2 Protein extraction and labelling

L. reuteri cultures at 7 h (initial exponential growth phase) and 15 h (end of exponential growth phase) from the RPM and RWV treatments, were collected and immediately incubated with a 1:10

ratio (w/v) of RNAlater solution (Ambion, Austin, TX) and stored at -70°C until the protein isolation. Before protein extraction, about 100 mg of bacterial pellet from treated and untreated culture to microgravity was washed 2 times with 50 mM phosphate buffer saline, pH 7. Cell pellet from all samples were suspended in 0.5% (v/w) of lysis buffer made by 7 M urea (Sigma), 2 M thiourea (Invitrogen) and 2% CHAPS (Sigma) and 0.1 mm Zirconium Silica beads (Biospec products Inc, USA) added in ratio 1:1 (w/v) to the cell pellets. Thereafter, samples were processed along 6 alternated cycles of 1 min in bead beater (Bertin Technologies), 5 min in ice incubation and centrifugation (14000 rpm) for 5 min at 2°C. After all cycles were completed, samples were finally centrifuged (14000 rpm) for 30 min at 2°C. Supernatants were collected and protein concentration was determined by optical density measured at 595 nm using a spectrophotometer (Gene Quant 100, GE Healthcare) and protein concentration was determined against Bovine Serum Albumin (BSA, Thermo Scientific) used as standard.

Relative quantification of proteins was obtained by a tandem mass tagging (TMT)-labelling experiment using a TMT10plex Isobaric Label Reagent Kit (Thermo-Fisher Scientific, USA); protein samples were prepared according to manufacturer's instructions. Thus, an aliquot of each protein sample (100 µg) was adjusted to a 100 µl final volume with 100 mM TEAB, and then reduced with 5 µl of 200 mM tris (2-carboxyethylphosphine), for 60 min, at 55 °C. Protein samples were then alkylated by adding 5 µl of 375 mM iodoacetamide in the dark, for 30 min, at 25 °C. Alkylated proteins were then precipitated by addition of 6 vol of cold acetone to remove chemicals, overnight at -20°C. After precipitation, proteins were pelleted by centrifugation at 8,000 g, for 10 min, at 4 °C, and air-dried. Each sample was digested with freshly prepared trypsin (enzyme to protein ratio 1:50 w/w) in 100 mM TEAB, at 37 °C, overnight. Resulting peptides from each protein sample were labelled with the TMT10plex Label Reagent Set (Thermo-Fisher Scientific, USA), at 25 °C, following manufacturer's instructions, (according to the labelling scheme: Ctrl-TMT10-126, sampleA-TMT10-127, sampleB-TMT10-128 and sampleC-TMT10-129). After 1 h of reaction, 8 µl of 5% w/v hydroxylamine was added in each tube and mixed for 15 min, in order to quench the derivatization reaction. For a set of comparative experiments, tagged peptide mixtures were mixed in equal-molar ratios (1:1:1:1) and vacuum-dried under rotation. Then, pooled TMT-labelled peptide mixtures were suspended in 0.1% trifluoroacetic acid, and fractionated by using the Pierce™ High pH Reversed-Phase Peptide fractionation kit (Thermo-Fisher Scientific) to remove unbound TMT reagents and to reduce sample complexity, according to manufacturer's instructions. After fractionation, eight fractions of TMT-labelled peptides were collected, vacuum-dried and finally reconstituted in 0.1% formic acid for subsequent mass spectrometric analysis.

4.2.3 NanoLC-ESI-Q-Orbitrap MS/MS analysis of protein digests

TMT-labelled peptide fractions were analyzed with a nanoLC-ESI-Q-Orbitrap-MS/MS platform consisting of an UltiMate 3000 HPLC RSLC nano system (Dionex, USA) coupled to a Q-ExactivePlus mass spectrometer through a Nanoflex ion source (Thermo-Fisher Scientific). Peptides were loaded on an Acclaim PepMapTM RSLC C18 column (150 mm × 75 μm ID, 2 μm particles, 100 Å pore size) (Thermo-Fisher Scientific), and eluted with a gradient of solvent B (19.92/80/0.08 v/v/v water/acetonitrile/formic acid) in solvent A (99.9/0.1 v/v water/formic acid), at a flow rate of 300 nl/min. The gradient of solvent B started at 5%, increased to 60% over 125 min, raised to 95% over 1 min, remained at 95% for 8 min, and finally returned to 5% in 1 min, with a column equilibrating step of 20 min before the subsequent chromatographic run. The mass spectrometer operated in data-dependent mode, using a full scan (m/z range 375-1500, nominal resolution of 70,000), followed by MS/MS scans of the 10 most abundant ions. MS/MS spectra were acquired in a scan m/z range 110-2000, using a normalized collision energy of 32%, an automatic gain control target of 100,000, a maximum ion target of 120 ms, and a resolution of 17,500. A dynamic exclusion value of 30 s was also used.

4.2.4 Bioinformatics for protein identification and quantitation

Raw data files were analyzed for protein identification and relative protein quantification with Proteome Discoverer vs 2.1 software (Thermo Scientific, USA), enabling the database search by Mascot algorithm v. 2.6 (Matrix Science, UK) using the following criteria: NCBI protein database (*Lactobacillus reuteri* (strain ATCC 55730/SD2112)) including the most common protein contaminants; carbamidomethylation of Cys and TMT10plex modification of lysine and peptide N-terminal as fixed modifications; oxidation of Met, deamidation of Asn and Gln, pyroglutamate formation of Gln as variable modifications. Peptide mass tolerance was set to ± 10 ppm and fragment mass tolerance to ± 0.02 Da. Proteolytic enzyme and maximum number of missed cleavages were set to trypsin and 2, respectively. Protein candidates assigned on the basis of at least 2 sequenced peptides and an individual Mascot Score ≥ 30 were considered confidently identified. For quantification, ratios of TMT reporter ion intensities in the MS/MS spectra from raw datasets were used to calculate fold changes between samples. Definitive peptide assignment was always associated with manual spectra visualization and verification. Results were filtered to 1% false discovery rate (FDR).

Three technical replicates were performed for each sample and the differentially expression proteins (DEPs) were selected if they appeared at least twice and the fold change was larger than 1.5 and lower than 0.7 with a P-value less than 0.05.

All identified proteins were distributed over clusters of orthologous groups (COG; http://www.ncbi.nlm.nih.gov/COG) and were subjected to the Kyoto Encyclopedia of Genes and Genomes (KEGG; http://www.genome.jp/kegg) database (Tatusov *et al.*, 1997). Cluster of Orthologous Groups of proteins (COG) is the database for gene/protein orthologous classification (http://www.ncbi.nlm.nih.gov/COG/). Every gene/protein in a COG is supposed to be derived from a single gene/protein ancestor. Orthologs are gene/proteins derived from different species of one vertical family and have the same functions as the ancestor. Paralogs are proteins derived from gene expression and may have new, related functions. The identified proteins were compared with the COG database to predict their hypothetical function.

4.3 Results

4.3.1 Proteomic Analysis/Comparison

In total, 1083 proteins were identified, which cover 49.9% of *L. reuteri* DSM17938 genome. Afterwards, 226 differentially expressed proteins (DEPs) were selected and only 167 had already been noted. These proteins were subsequently categorized based on GO and KEGG classification.

To identify differentially expressed proteins (DEPs) between the treated sample to RWV and RPM technologies and the control sample (1xg), we first screened the 1083 proteins for candidates that showed a fold change in the expression level greater than 1.5 or lower than 0.7 between the treated cultures and the control one for at least two replicates. We then calculated the P-value for each candidate protein and filtered out ones that had a P-value greater than 0.05. A few different proteins were identified as differentially expressed between either of the treated samples and the control samples at 7 h after both treatments. In total, 18 DEPs were discovered between cultures treated to RWV system and the control cultures and 17 DEPs between RPM samples and 1xg samples (Table 4.1). Most of the differences were observed after 15 h of treatment at both ground-based microgravity simulators. Indeed, 126 and 200 DEPs were differentially expressed under RWV and RPM conditions, respectively. About twice of the differentially expressed proteins was represented by the under-expressed ones (Table 4.1).

Table 4.1: Total number of over-expressed and under-expressed proteins under RPM and RWV conditions and for the two collection times.

Samples	Time of exposure	Number of over-expressed proteins	Number of under-expressed proteins
RPM	7 h	3	14
RWV		9	9
RPM	15 h	71	129
RWV		42	84

4.3.2 COG and KEGG analysis of DEPs

DEPs were classified according to COG function category. The enrichment and cluster of DEPs were performed according to KEGG Pathways functional analysis.

In most of cases, the DEPs at 7 h of both treatments were categorized according to the following functions: Purine and Pyrimidine metabolism, Vitamin B_{12} metabolism, Carbon utilization, DNA replication and in Alkaline shock stress. The analysis of 15 h treatment under both systems highlighted the same pathways involved at 7 h and, in addition, another important over-expressed functional category regarded the general stress proteins. Interestingly, under RPM condition many under-expressed proteins affected the tRNA processing, Vitamin B_6 biosynthesis and Mechanosensitive channel and, like the RWV condition, regarded Carbohydrate metabolic process. COG function analysis of differentially expressed proteins for both collection times (7 h and 15 h) and for both growth conditions (RWV and RPM) were showed in figure 4.1 (A-D). KEGG pathways analysis of proteins with different expression (P-value <0.05) were represented in figure 4.2 (A-D).

Venn diagrams showed the relation between differentially expressed proteins at 7 h and 15 h under RPM and RWV treatment (Figure 4.3 A-B). All proteins under-expressed and over-expressed at 7 h in both conditions are also found at 15 h.

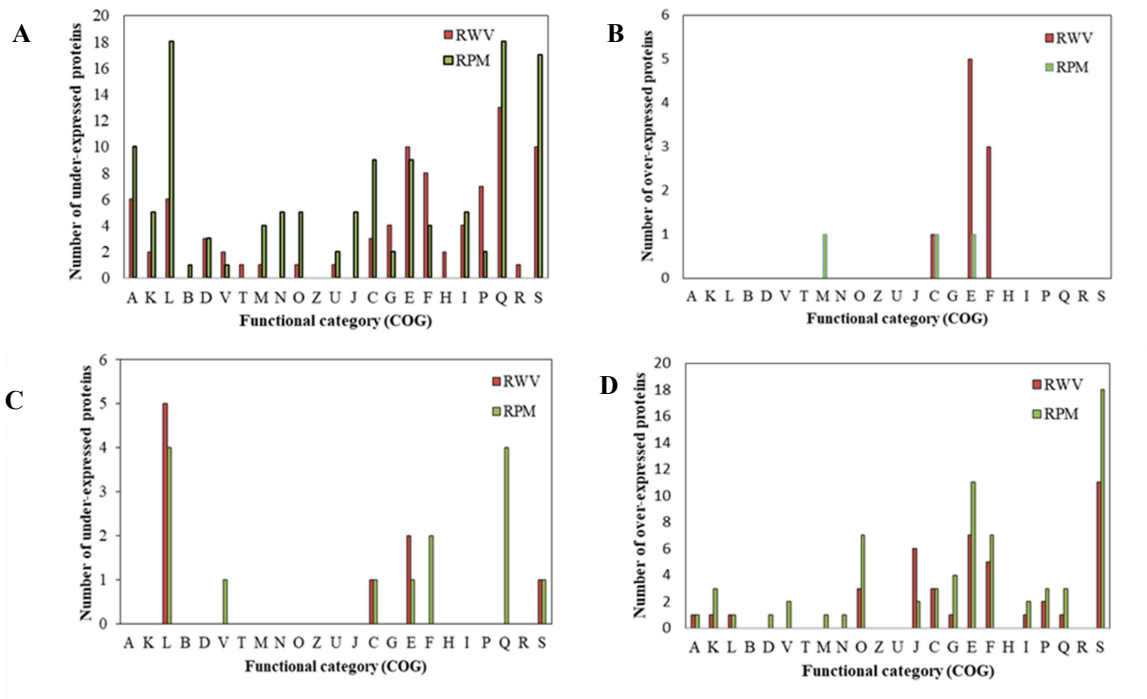
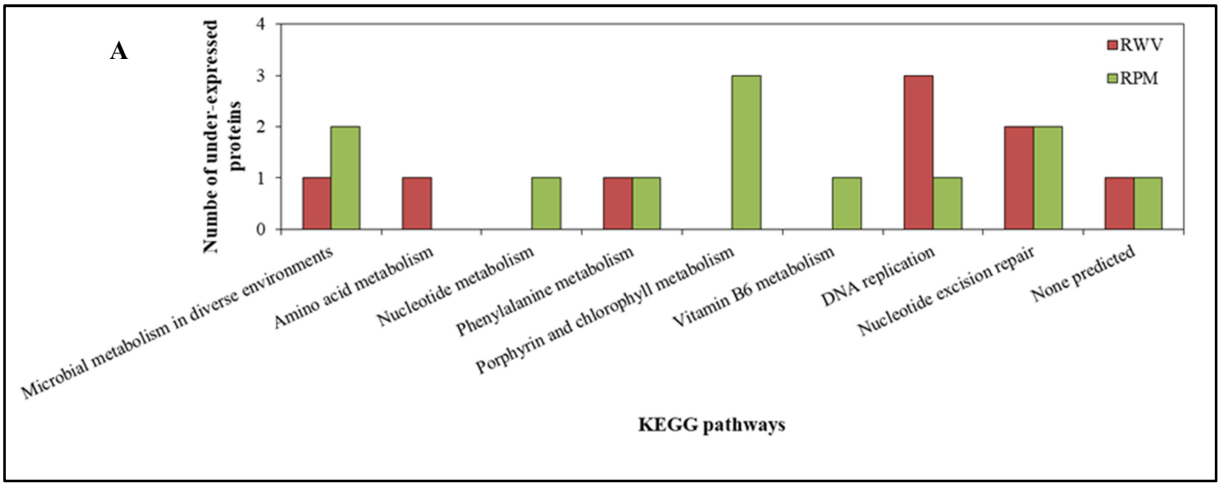
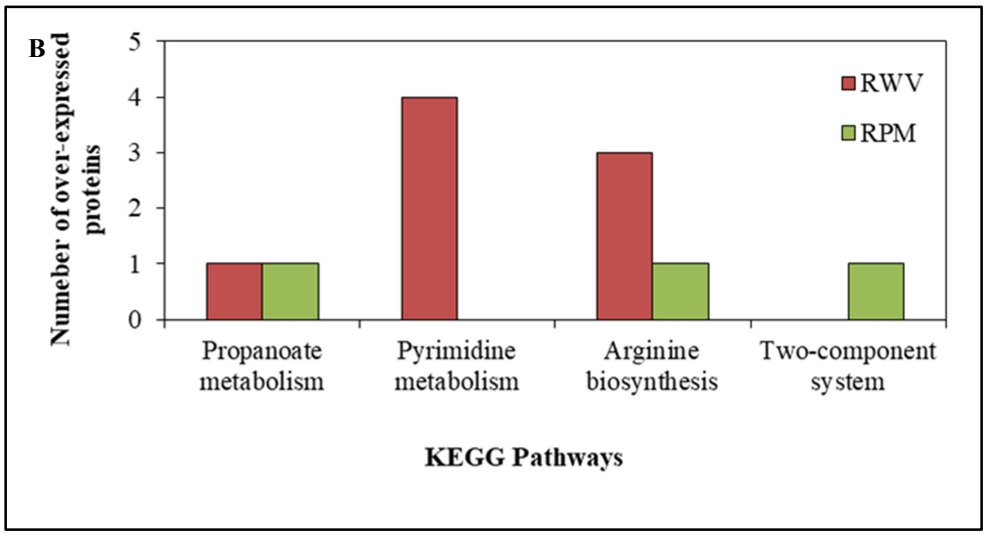


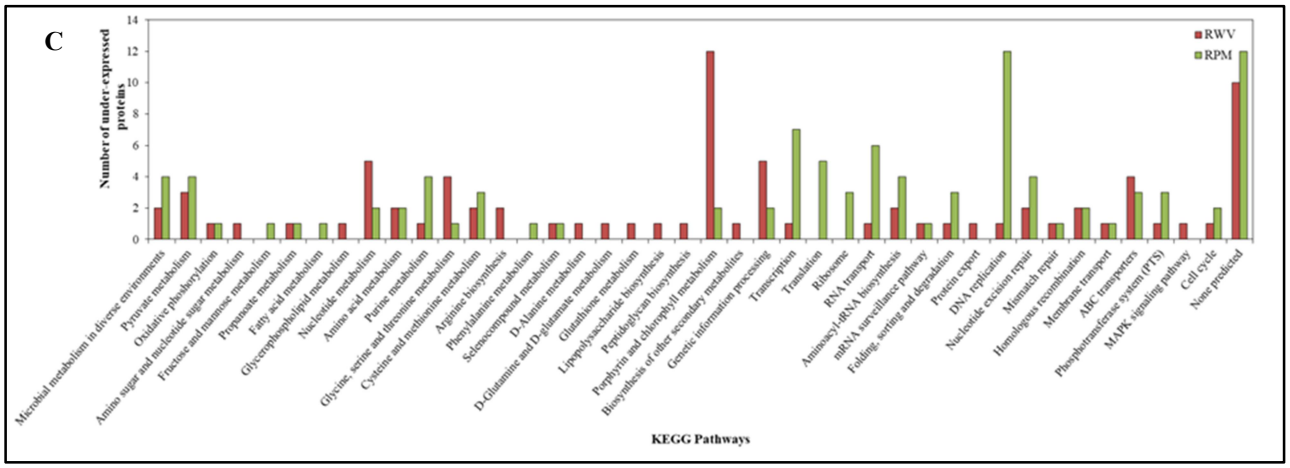
Fig. 4.1: Functional classification of the significant proteins under-expressed and over-expressed at 7 hours (A-B) and at 15 hours (C-D) (0.7 > fold change >1. 5 and P-value <0.05).

Table 4.2: COG functional categories and their nomenclature.

COG functional categories	
Information storage and processing	
A	RNA processing and modification
K	Transcription
L	DNA replication, recombination and repair
B	Chromatin structure and dynamics
Cellular processes	
D	Cell division and chromosome partitioning
V	Defence mechanisms
T	Signal transduction mechanisms
M	Cell envelope biogenesis, outer membrane
N	Cell motility and secretion
O	Posttranslational modification, protein turnover, chaperones
Z	Cytoskeleton
U	Intracellular trafficking and secretion
J	Translation, ribosomal structure and biogenesis
Metabolism	
C	Energy production and conversion
G	Carbohydrate transport and metabolism
E	Amino acid transport and metabolism
F	Nucleotide transport and metabolism
H	Coenzyme metabolism
I	Lipid metabolism
P	Inorganic ion transport and metabolism
Q	Secondary metabolites biosynthesis, transport and catabolism
Poorly characterized	
R	General function prediction only
S	Function unknown


A
Numbe of under-expressed proteins
RWV
RPM
Microbial metabolism in diverse environments
Amino acid metabolism
Nucleotide metabolism
Phenylalanine metabolism
Porphyrin and chlorophyll metabolism
Vitamin B6 metabolism
DNA replication
Nucleotide excision repair
None predicted
KEGG pathways


B
Numeber of over-expressed proteins
RWV
RPM
Propanoate metabolism
Pyrimidine metabolism
Arginine biosynthesis
Two-component system
KEGG Pathways


C
Number of under-expressed proteins
RWV
RPM
KEGG Pathways

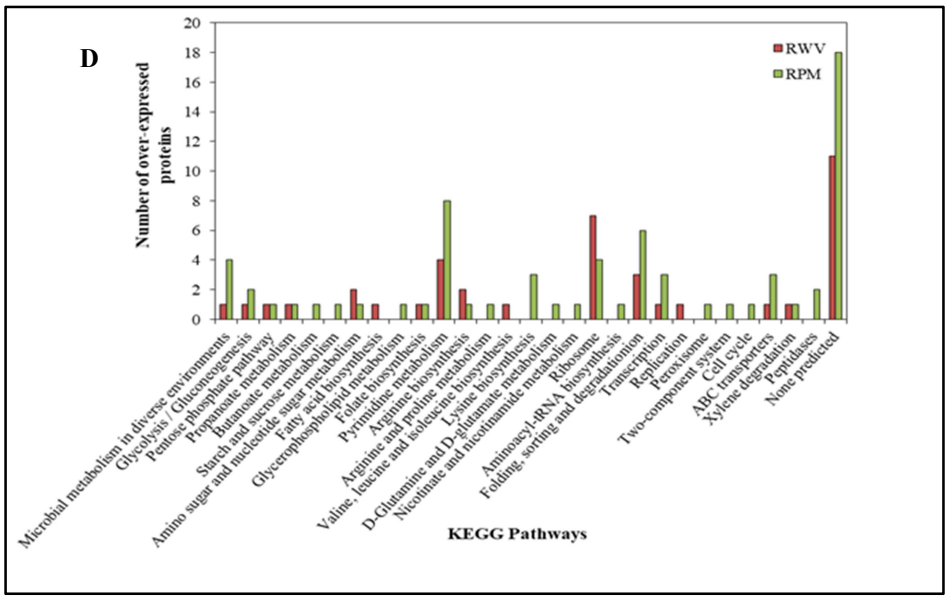


Figure 4.2: KEGG pathways enrichment analysis of significantly under-expressed and over-expressed proteins after 7 hours (A-B) and 15 hours (C-D) of treatments under RWV and RPM (0.7 > fold change >1. 5 and P-value <0.05).

The annotation axis is classified by functional categories. The columns represent the number of all annotated proteins in *L. reuteri* subjected to simulated microgravity systems.

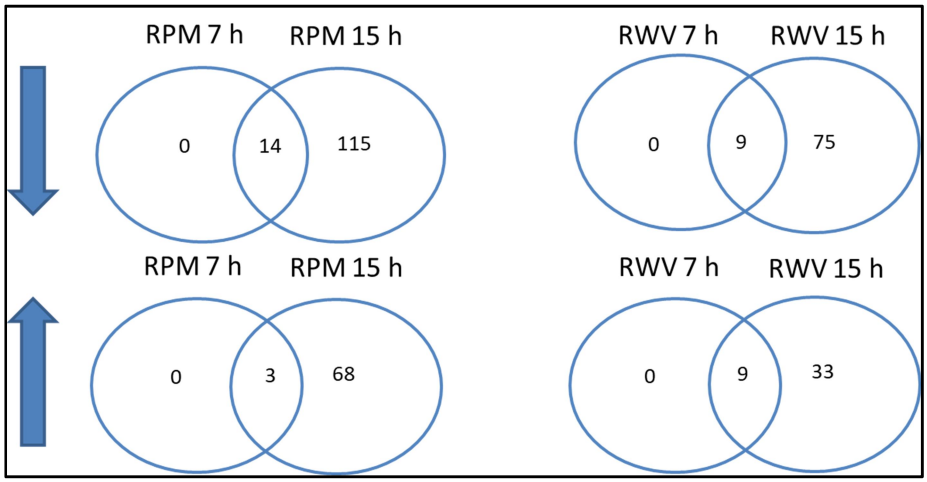


Figure 4.3: Venn diagrams showing the relation between the significant differentially expressed proteins (0.7 > fold change >1. 5 and P-value <0.05) categorized from two simulated microgravity systems.
Arrows indicates under-expression (↓) and over-expression (↑).

4.4 Discussion

Microorganisms are ubiquitous and can be found in almost every habitat and ecological niche on Earth. Microbial survival requires the capability to sense and respond to environmental stress

(Nickerson *et al.*, 2004). Some of these environmental changes are represented by physical or chemical stresses. Only those microbes that quickly reprogrammed themselves will survive in their local environments. It is of great interest to investigate how microbes adapt to different extreme environments, for example, microgravity. In particular, modern proteomics has been successfully applied to microbiological research in order to identify variations that occur during spaceflight that would represent stressors such as physical stress, high G-forces at launch and landing, and microgravity (Grimm *et al.*, 2011). Many proteomic approaches were used for evaluating the microbe response to real and simulated microgravity conditions.

The proteomic analyses of *L. reuteri* DSM 17938 treated for 7 h and 15 h to two different microgravity devices demonstrated that there were significantly more downregulated proteins than upregulated proteins for both times and under both conditions. This results are similar to another study conducted on a "flight strain" of *Klebsiella pneumoniae* demonstrating that there were significantly more downregulated genes and proteins than upregulated genes and proteins (Guo *et al.*, 2015).

The differentially expressed proteins showed, similarly to *K. pneumoniae* protein profile, several functions that included principally nucleotide metabolism, carbohydrate transport and metabolism, protein turnover and translation, ribosomal structure and biogenesis.

Moreover, there is a complete overlap of identified proteins in both times and the samples subjected for 15 h to both simulated microgravity systems showed a greater number of differentially proteins than 7 h probably because the cells are in the phase of maximum division and there is greater transcription of DNA and greater protein synthesis. The similar results obtained under the two different devices demonstrated that the two systems are able to reproduce in similar way the constant state of suspension emulating the real spaceflight condition.

Moreover, our results support the previously proposed hypothesis that under conditions of modeled reduced gravity, zones of nutrient depletion develop around bacteria eliciting responses similar to entrance into stationary phase which is generally characterized by expression of multiple stress responses (i.e. alkaline shock protein, envelope stress response protein, chaperonine) (Vukanti *et al.*, 2008).

A similar stress response is observed in another microorganism *Cupriavidus metallidurans* CH34 after cultivation under RWV and RPM conditions (Leroy *et al.*, 2010). In this study a recent proteomic approach like isotope-coded protein labelling (ICPL) was used. Three different Universal

stress proteins were up regulated and an increased quantity of a nitrite reductase in RPM and RWV conditions was observed. The high concentration of this enzyme could result from anoxic or micro-oxic conditions during growth in simulated microgravity due to decreased homogenization of the medium. Similarly, our results showed a light down-regulation of some enzymes involved in aerobic metabolism (i.e. lactate dehydrogenase), demonstrating that the growth under simulated microgravity conditions occurs in the presence of low oxygen concentration and, thus, under low mixing of medium. Another work on the same microorganism *C. metallidurans* CH34 retrieved from space in the MESSAGE-1, MESSAGE-2 and BASE flight experiments showed the upregulation of a thioredoxin reductase in response to oxidative stress condition. In *L. reuteri* cultures subjected to both simulated microgravity conditions, the expression of the same enzyme was reduced, but other proteins involved in oxidative stress condition were over-expressed (i.e. dps, pdhD).

The analysis of this work also revealed patterns of increased arginine metabolism and increased pyrimidine biosynthesis already after 7 hours of treatment in both simulated microgravity conditions. Similarly another study on two flight strains of *Bacillus cereus* sent on ShenZhou VIII for 16 days showed changes in genes/proteins relevant to metabolism (Su *et al.*, 2014). Likewise to *L. reuteri*, one of these strains of *B. cereus* exhibited an up-regulation of some enzymes involved in the amino acid metabolism and in nucleotide metabolism, displaying in particular an over-expression of pyrimidine-nucleoside phosphorylase. Moreover, the other strain sent in spaceflight over-expressed an enzyme involved in the glucose metabolism, like the samples treated for 7 h and 15 h to RWV device. This result probably suggests that the condition of simulated microgravity by RWV is closer to the real one.

In contrast, other proteomic results obtained for *S. mutans* after cultivation under RWV condition showed a down-regulation of the same metabolic pathways confirming that each strain can behave and feel differently the microgravity stress. In two recent transcriptomic works on a same strain of *E. coli* growing under RWV condition, the results were completely overturned compared to our results (Vukanti *et al.*, 2008, Arunasri *et al.*, 2013). In fact, the *pyr* and *arg* genes were under-expressed from two to three times compared to control sample, while the same gene products in *L. reuteri* were overexpressed from two to three times in comparison with control samples already at 7 h and for both treatments.

Other transcriptomic analysis conducted to date on many potential pathogenic bacteria like *K. pneumoniae*, *E. coli* and *P. aeruginosa* that experienced the spaceflight are in contrast with our

proteomic data because they showed the down-regulation of some key enzymes involved in the synthesis of L-arginine (i.e. *argG*) or in the biosynthesis of pyrimidine (i.e. *pyrB, pyrD, pyrE, pyrF*) (Crabbé *et al.* 2011; Guo *et al.*, 2015; Li *et al.*, 2015). Moreover, these studies underlined the under-expression of the carbamoyl phosphate synthase large subunit (*carB*) which in *L. reuteri* cultures, growing under RWV and RPM treatments, is over-expressed together with the small subunit (*carA*).

Probably the up-regulation of the arginine succinate synthase and arginine succinate lyase demonstrated the necessary to synthesize arginine and fumarate in response to acid stress, as has been amply proved in the past for another strain of *L. reuteri* subjected to a transient acid shock at pH 3.0 for 1.5 h (Lee and Pi, 2010; Teixera *et al.*, 2014). The arginine pathway is most likely the cause of the protective effect of arginine against acid stress, working by releasing ammonia, an alkali-like molecule that helps cells to maintain the internal pH (Teixeira *et al.*, 2014).

Interestingly, a common intermediate of pyrimidine and arginine biosynthesis is carbamoyl phosphate that is sensible to CO_2 concentration: its synthesis in *Lactobacillus plantarum* is inversely dependent from CO_2 concentration (Nicoloff *et al.*, 2004; Bringel *et al.*, a-b 2008).

The up-regulation of these two pathways is probably a consequence of the low concentration of CO_2 which in turn depends on the reduced mixing of gases in the clinostats. It is, in fact, already known that the fluid motion of liquids is reduced, until cancelled, over time in the chambers on a RPM and RWV systems (Klaus, 2001; Wuest *et al.,* 2017). So, also gases dissolved in the liquid are not very accessible to cells.

The down-regulation of Vitamin B_{12} and an up-regulation of pyrimidine and arginine biosynthesis and an increased synthesis of oxidative stress-related proteins are indicative of the fact that the cells would need to cope with the stress condition induced by the two treatments, and need to synthesize new components for the biosynthesis of new nucleic acids and new amino acids, all this at the expense of secondary metabolism, such as that of Vitamin B_{12}.

Finally, the most important proteins involved in protein repair mechanism like GroEL, Dnak and Hsp_{20} are overexpressed after 15 h, as we had already demonstrated at transcriptomic level through RT-qPCR. However, there are still many unknown functions related to microgravity stress response to evaluate. The further deciphering of such unknown functions may shed light on some aspects of *L. reuteri* and in general of bacterial physiology in spaceflight conditions.

4.5 Conclusions

The topic of the present work is the exploitation of the physiology of *L. reuteri* cultures growing for 7 h and 15 h under RWV and RPM conditions by proteomics approach. The results showed different levels of expression of some proteins indicating differential stress response in bacteria subjected to simulated microgravity conditions with respect to their control counterparts. Future findings could open a new area of research elucidating the influence of the stress conditions during a real spaceflight, including not only microgravity but also cosmic radiations, on probiotic cell behaviour. Therefore, additional space experiments and ground simulations (for example, space microgravity and ionizing radiation) in continuous bioreactors are crucial to draw final conclusions concerning the spaceflight impact for MELiSSA project. In this context, *L. reuteri* DSM 17938 plays an important role in its possible cultivation in one of MELiSSA bioreactor with the final aim to take advantage of its natural degrading abilities and simultaneously to use this probiotic bacterium as a direct source of supplying during long-term space missions. New space experiments need to be done in conditions mimicking the future MELiSSA loop conditions (with light and in anaerobic conditions), and should include a detailed metabolic analysis in addition to the proteomic and transcriptomic profiling.

Notes

Experiments described in this Chapter are currently ongoing in collaboration with Dr. Andrea Scaloni and his team of the Institute for the Animal Production System in the Mediterranean Environment (CNR-ISPAAM) of Naples.

CHAPTER 5

Investigation of simulated microgravity effect on the transcriptome of *L. reuteri* DSM 17938

5.1 Introduction

The spacecraft environment consists of microgravity, radiation and magnetic fields. Astronauts living in this environment cannot avoid the presence of bacteria including bacteria carried by astronauts and/or the flight body compartment itself (Horneck *et al.*, 2010).

Recent studies have demonstrated that the virulence and the resistance of some bacteria, that were originally harmless to humans in the space environment, may be increased and may be a serious problem for the humans in long-term space mission (Voorhies *et al.*, 2019).

The features of some bacteria were different after exposure to short- and long-term spaceflight (Juergensmeyer *et al.*, 1999; Taylor and Sommer 2005; Su *et al.*, 2014;Wang *et al.*, 2014). Not only the exposure to real microgravity but also the treatment to ground-based microgravity analogues have been demonstrated to affect bacteria physiological features including morphology, virulence, growth, antibiotics resistance, biofilm formation, the substrate utilization efficiency and gene expressions (Horneck *et al.* 1984; Wilson *et al.*, 2002; Lynch *et al.*, 2006; Crabbe *et al.*, 2010; Rosenzweig *et al.*, 2010; Vukanti *et al.*, 2012; Arunasri *et al.*, 2013). Simulated weightlessness, in fact, altered the gene transcription of *Pseudomonas aeruginosa*, *Salmonella* and *E. coli* (Nickerson *et al.*, 2000; Lynch *et al.*, 2006; Crabbé *et al.*, 2010). Spaceflight and the changes in lifestyle are concurrent with changes in the GI microbiome on both short and long duration missions (Lizko *et al*., 1979; Nefedov *et al*., 1971; Taylor *et al*., 1977).

During space activities, the common intestinal floras are unavoidably taken into space with astronauts, which may increase the risk of infections. Other studies demonstrated that the immune system of astronauts was mis-regulated and the immune ability was greatly impaired during spaceflight (Knight *et al.* 1970; Chang *et al.* 2012).

As consequence of reduced immunity or lack of stimulation in intestinal tract for a long time, some potential pathogenic bacteria may escape the intestinal tract to other organs and may cause serious infections (Croxen *et al.* 2013).

Therefore, to protect the health status of astronauts during spaceflight missions it is necessary to provide probiotics that could alleviate the symptoms of crew-relevant illness.

For this reason, it is critical to understand the effects of spaceflight environment and in particular of microgravity environment, that represent the primary cause of most changes in spaceflight, on both host microbiome and beneficial probiotic bacteria. Probiotics can provide several benefits to human health, including competition against pathogens, strengthening of tight junctions of the intestinal epithelial layer, reduction of gastrointestinal distress, production of beneficial metabolites, interactions with host cells that promote immune and psychological health, and protection from infection (O'Flaherty and Klaenhammer, 2010; Turroni *et al.*, 2014).

Given the limited capacity for crew supplies, probiotics must be carefully selected and evaluated for spaceflight based on their strain-specific benefits and relevance to likely crew conditions.

L. reuteri DSM 17938 is an established probiotic strain that could be provided as food supplement in the diet of astronauts because it has many beneficial effects well documented in a number of clinical trials. Studies have shown that supplementation with *L. reuteri* can alleviate GI tract symptoms and improve colicky symptoms in breastfed infants (Indrio *et al.*, 2014; Savino *et al.*, 2010), reduce infections (Tubelius *et al.*, 2005) and improve feeding tolerance in formula-fed premature neonates (Indrio *et al.*, 2008). *L. reuteri* strains may exert immunoregulatory effects in the human gut through controlling lipopolysaccharide (LPS)-induced TNF-α and intestinal damage (Jones and Versalovic, 2009; Liu *et al.*, 2010). Moreover, this strain has also been reported to prevent oxidative damage caused by free radical (Wang *et al.*, 2008). It is expected that these benefits would translate to the spaceflight environment, and potentially offer a countermeasure to gut microbiome dysregulation and to some aspects of immune dysregulation. However, research concerning how probiotics respond differentially to space environmental conditions is scarce. No specific studies on *L. reuteri* growing under spaceflight conditions were conducted, but some recent studies with another probiotic bacterium, *L. acidophilus* ATCC 4356, were performed (Castro-Wallace *et al.*, 2017, Shao *et al.*, 2017) demonstrating that this bacterium behaved in a similar way in spaceflight.

Many experimental tools and methods have been developed to study microgravity induced physiological changes. Recently, genomic and proteomic approaches have gained a lot of attention.

Understanding gene and/or protein expression could be the key to extricate the mechanisms behind microgravity-induced problems and find effective countermeasures to spaceflight-induced alterations (Nichols *et al.*, 2006).

Due to revolutionary advances in high-throughput DNA and RNA sequencing technologies and computer-based genetic analyses, genome decoding and transcriptome sequencing (RNA-seq) (Wang *et al*., 2009; Lohse *et al*., 2012) analyses are rapid and available at low costs.

In this chapter, *L. reuteri* DSM 17938 was analysed at transcriptomic level by next generation sequencing to identify the changes of this bacterium occurred under two different simulated microgravity conditions.

5.2 Materials and methods

5.2.1 Bacterial strain and growth conditions

The strain *Lactobacillus reuteri* DSM17938 was kindly provided by BioGaia (Noos S.r.l.; BioGaia AB, Stockholm, Sweden) and was previously characterized in our laboratory for probiotic properties (De Prisco *et al.*, 2015). It was routinely aerobically cultured in MRS Broth (Oxoid Ltd., Basingstoke, Hampshire, England) at 37°C.

5.2.2 Simulated microgravity growth conditions

An overnight culture of *L. reuteri* DSM17938 was diluted with fresh MRS to rich a cell concentration of approximately 10^6 CFU/mL. Therefore, twelve 50 mL rotatory cell culture bioreactors (Synthecon, Houston, TX) were filled with the cell suspension taking care that they were completely filled and no air bubbles were present to compromise the low-shear condition. Four rotating bioreactors were attached to their base units and rotated in horizontal axis at 25 rpm at 37°C, here designated as rotating wall vessel (RWV) for microgravity treatment (Senatore *et al.*, 2018). Other four vessels were mounted at the centre of bench-top of a microgravity simulator through random positioning (RPM 2.0, Dutch Space, Leiden, The Netherlands), was composed by two separated motors that rotate in different directions reaching an angular rotation velocity of 60 deg s-1, here designated as random positioning machine microgravity treatment (Senatore *et al.*, 2018). Finally, the control cultures (1xg) of RPM and RWV experiments were represented by four rotatory bioreactors installed on the base unit of the RWV in vertical axis.

5.2.3 RNA extraction

L. reuteri cultures of 15 mL were collected at 7 h (initial exponential growth phase), at 15 h (end of exponential growth phase) and at 24 h (mid-late stationary phase) and immediately incubated with a 1:10 ratio (w/v) of RNAlater™ Solution (Ambion, Austin, TX) for 5 min at room temperature.

Samples were collected by centrifugation at 6500 g for 5 min at 4°C (Eppendorf Centrifuge 5810R). Supernatants were removed and the resulting cell pellets were stored at -70°C until RNA isolation was performed. Two biological replicates of both simulated microgravity devices and control cultures and for all collection times were prepared for sequencing per the following protocol. Cell pellets were resuspended in 8 mL of frozen PBS 0.1 M and 2 mL + 2 mL of cultures were treated separately for the RNA extraction using RNeasy® Mini Kit with some technical precautions (Qiagen, Hilden, Germany). Initially, a volume of 700 µl of RLT buffer was added to every 2 mL of cultures and after the step of the suspension into the 2 mL Safe-Lock tube containing the acid-washed beads and the disruption of cells in the TissueLyser for 5 min at maximum speed, the two separated volumes were collected in a single Eppendorf and treated with an equal volume of ethanol (70%). The remaining protocol was completed per manufacturer's instructions.

The final elution was performed with 40 µl of RNase-free water and treated two times for 30 minutes with 2 U of TURBO DNA-free™ Kit (Ambion).

The absence of contaminating genomic DNA in each sample was then determined using PCR and *L. reuteri* 16S rRNA-specific primers. RNA quality and concentration were determined using a Qubit® 2.0 Fluorometer (Life Technologies, Grand Island, NY), with Qubit RNA HS assay kit (Life Technologies) along with NanoDrop 1000 (Thermo Fisher Scientific, Waltham, MA). The RNA integrity was evaluated by gel electrophoresis (2% of agarose).

5.2.4 Library preparation

Ribosomal RNA was removed from 1 µg of isolated RNA via the Epicentre Ribo-Zero Magnetic Kit (Bacteria) (Illumina, San Diego, CA) per manufacturer's protocol. After the systhesis of single stranded cDNAs, buffer, dNTPs (in which dTTP were replaced by dUTP), DNA polymerase I and RNase H were applied to systhesis the complementary cDNA strands. The double stranded cDNAs were purified using AMPure XP beads (Beckman Coulter, Brea, CA) and tested for the quantity using a Qubit 2.0 fluorometer (Life Technologies), and then diluted to 1 ng/µl before checking insert size on an Agilent 2100 and quantifying to greater accuracy by quantitative PCR (RT-qPCR) (library activity >2 nM).

Purified rRNAdepleted RNA was stored at -70 °C until used for HiSeq library preparation.

5.2.5 Illumina sequencing and Bioinformatic analysis

Libraries are fed into Illumina machines according to activity and expected data volume. HiSeq reads were trimmed with Trimmomatic v0.33 and the filtration consisted of three steps: removing reads with 1 bp of Ns' base numbers, removing reads with 40 bp of low quality (≤Q20) base numbers, and removing adapter contamination.

The reads are then mapped to the *Lactobacillus reuteri* DSM 17938 genome available from NCBI with STAR Aligner. Raw counts were transformed to log Counts Per Million (CPM) for visualisation. To remove differences in library size TMM (Trimmed mean) normalization was performed on the log-CPM transformed gene abundance levels. The HTSFilter package (Rau *et al.*, 2013) was used to remove genes with consistent low expression following normalisation and testing as suggested by the HTSFilter documentation. Normalisation and differential expression testing was conducted using edgeR (Robinson *et al.*, 2010) putting together the various biological replicates and performing the comparison between control samples and treated samples.

Correspondence factor analysis (CFA) and spectral map analysis (SMA) were used to check if the samples could be grouped in different clusters and which genes are responsible for these clusters.

Only genes with a P-value below 0.05 and showing a $\log_2$ of fold change below -1.0 or above 1.0 were considered true differentially expressed genes (DEGs). Differentially expressed genes were identified by analysing the transcriptomic data according to the COG function.

5.3 Results

5.3.1 RNA-Seq alignment and comparative transcriptomic analysis

More than 90% of the reads (paired-end) were mapped on and counted on *Lactobacillus reuteri* DSM 17938 genome available from NCBI (RefSeq: NC_015697.1; 93% similarity with SD 2112).

A number of genes received too low count-values to be considered in down-stream analysis. These genes were removed. As a result, from the 2169 genes we retained 1931 genes.

CFA showed that the largest separation exists between samples measured after 7 h of treatment under both devices and all other samples (squares). The first component explained 74% of the observed variation. Many genes (red circles) were also involved in this separation. The second component explained 8% of the total variation. This component separated samples from the control

group from samples from the RWV and RPM groups. It also suggests that the difference between the RWV and control samples was less that the differences between the RPM and control samples (Figure 5.1).

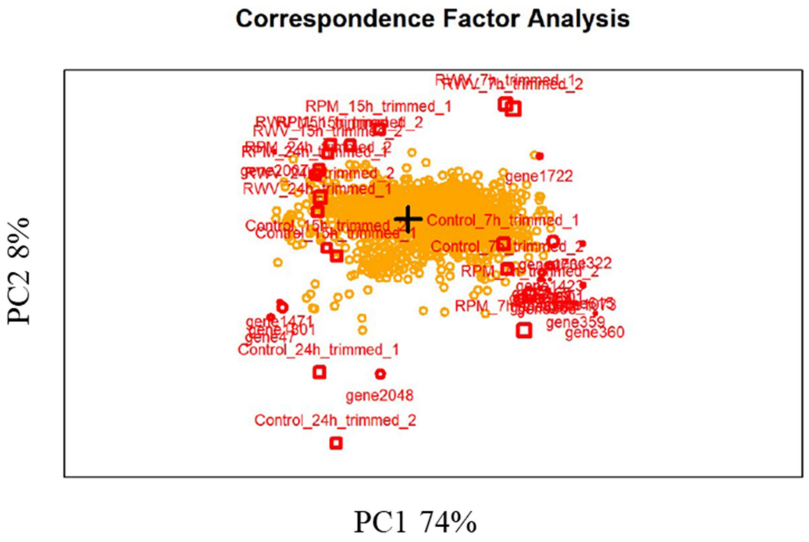


Fig. 5.1: Correspondence factor analysis of all samples (squares) on the basis of the abundance of gene expression (circles). Red circles represent genes involved in the main clustering of sample at 7 hours.

The total number of differentially expressed genes, except for the under-expressed genes in RWV 7 h, gradually increased over time, as it was showed in Figure 5.2. Moreover, more differentially expressed genes after 15 h and 24 h of both treatments was represented by the under-expressed ones.

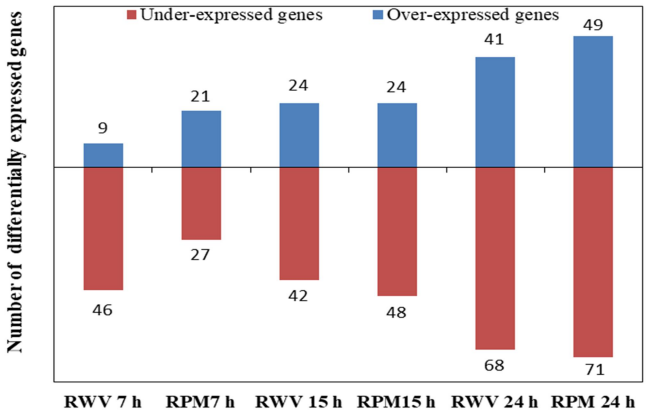
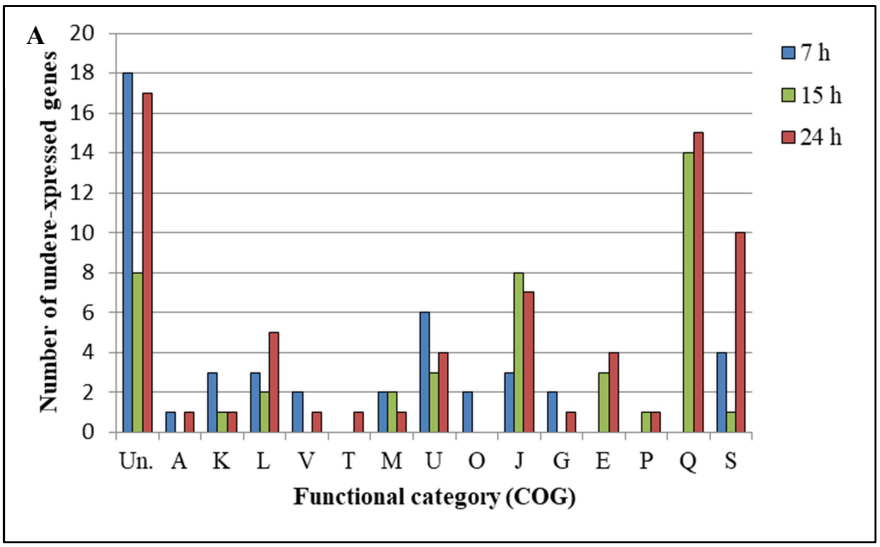
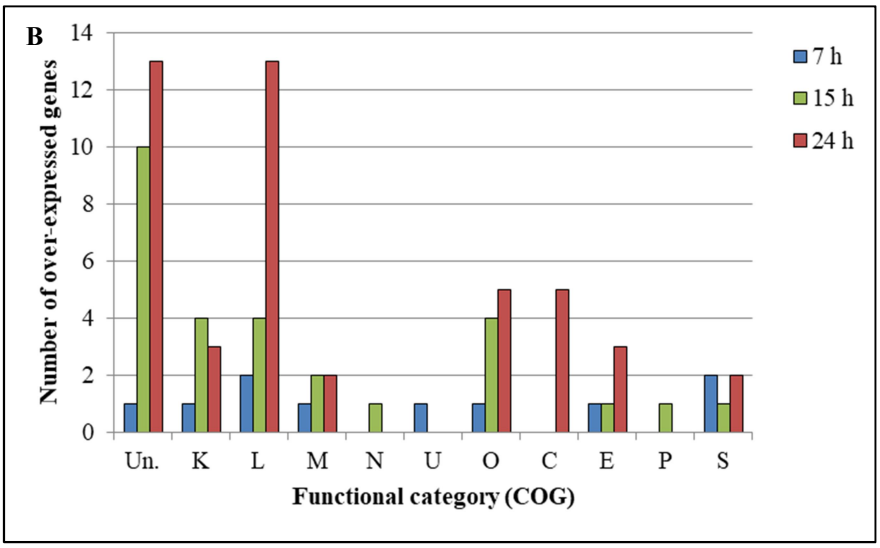


Figure 5.2: Total number of over-expressed and under-expressed genes in RWV and RPM at three collection times (7, 15 and 24 hours).

DEGs were classified according to COG functional category. As it was showed in figure 5.3, the 'unclassified' (Un.) class was the most numerically abundant category in both experiments and for over-expressed and under-expressed genes, the second most under-expressed category was represented in RPM and RWV experiments and for all collection times by the "Secondary metabolites biosynthesis, transport and catabolism" (Q) (Figure 5.3 A-C). Indeed, almost 30% of significant under-expressed genes found at 15 h and 24 h of both treatments encoded for enzymes or proteins involved in Vitamin B_{12} biosynthesis.

Significant over-expressed genes (Figure 5.3 B-D) were principally involved in DNA replication, recombination and repair process. Most of these shared over-expressed genes were characterized by enzymes or proteins responsible for transposon-mediated genetic recombination (i.e. integrase core domain protein, transposase).





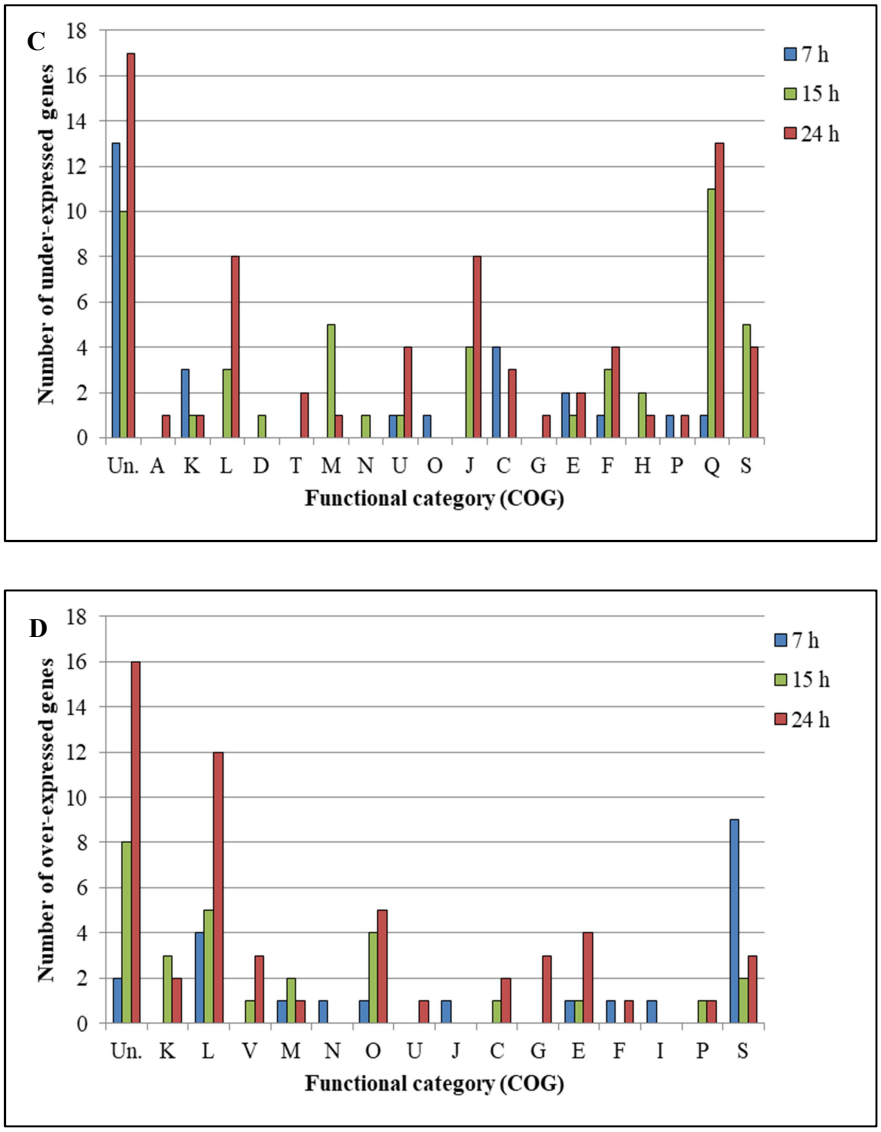


Figure 5.3: Functional classification of the significant genes under-expressed and over-expressed in RWV (A-B) and in RPM (C-D). See Table 4.2 for the COG functional categories.

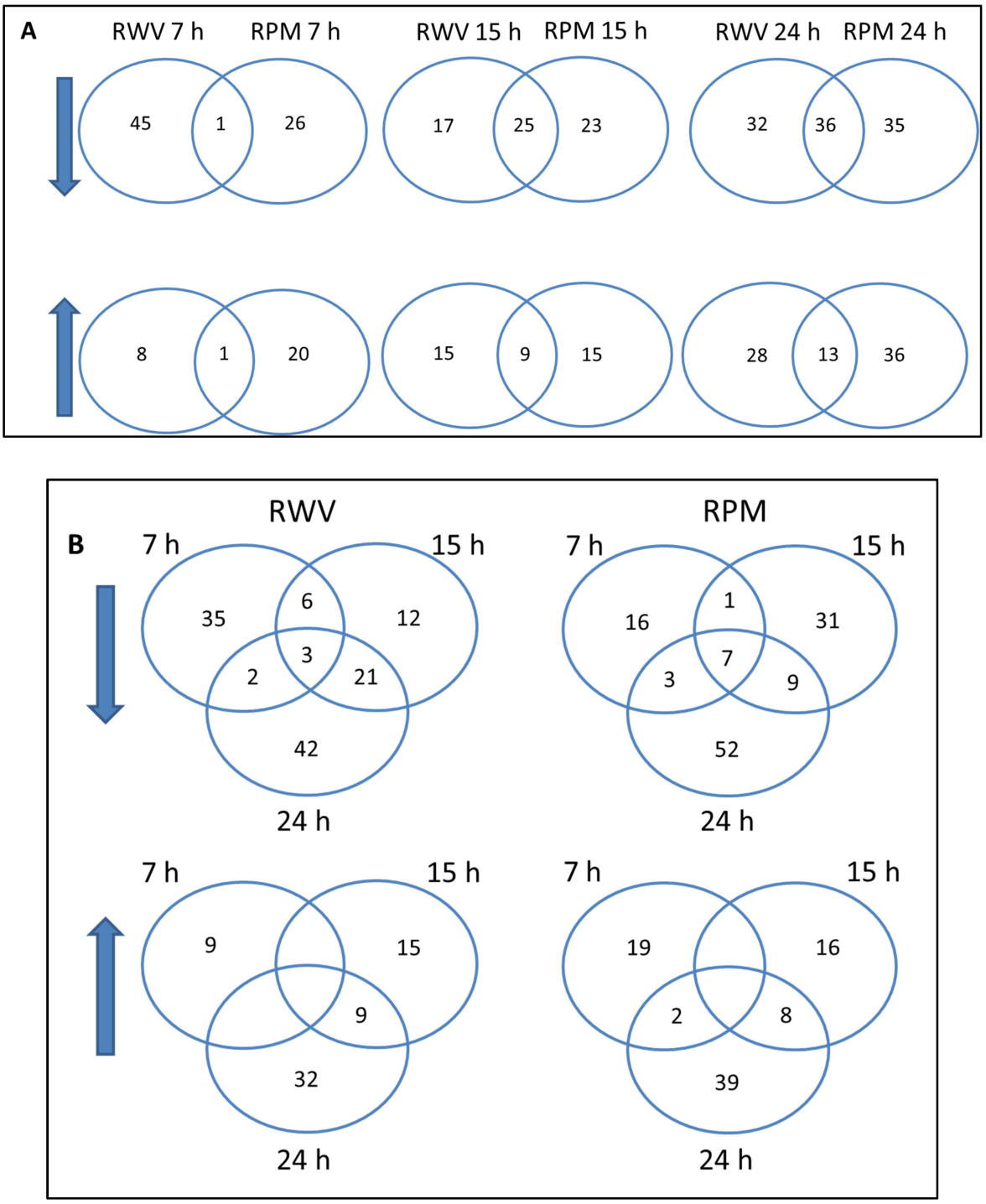


Figure 5.4: Venn diagrams showing the relation between the significant differentially expressed genes (-1 > $\log_2$ fold change > 1 and P-value <0.05) categorized from time of treatment (A) and simulated microgravity systems (B).

Arrows indicates under-expression (↓) and over-expression (↑).

More than half of the total number of under-expressed genes were shared between RWV and RPM samples after 15 h and 24 h and almost the 30% for the over-expressed genes at the same collection

times (Figure 5.4 A). Moreover, under-expressed genes more than over-expressed ones were shared between the three collection times (Figure 5.4 B) and most number of genes were shared between 15 and 24 hours. The most significant under-expressed and over-expressed genes shared between the two different treatments and by the two collection times (15 h and 24 h) are reported in Table 5.1 and 5.2. Most of the under-expressed shared genes were involved in Vitamin B_{12} biosynthesis (i.e. precorrin-3B C(17)-methyltransferase; precorrin-4 C(11)-methyltransferase) and in Amino-sugars metabolism (i.e. glutamine-fructose-6-phosphate transaminase) (Table 5.1). Most of the over-expressed genes shared in at least one collection time between RPM and RWV were involved in membrane transport and in recombination processes (i.e. ABC transporter; integrase core domain protein; resolvase) (Table 5.2). There was a gene in common to all samples coding for a large conductance mechanosensitive channel protein that consists of a membrane protein responsible of response with electrophysiological activities under stretch forces or under osmotic stress condition affecting the cell.

Table 5.1: List of the significant under-expressed genes (P<0.05) shared between the RWV and the RPM experiments and by at least one of the collection times (15 and 24 hours).

Abbreviations: log_2FC, log_2fold change; NS, not significant. See Table 4.2 for the COG nomenclature.

Locus Tag	Product name	COG	Log_2 FC RWV 15 h	Log_2 FC RPM 15 h	Log_2 FC RWV 24 h	Log_2 FC RPM 24 h
HMPREF0538_21631	glutamine-fructose-6-phosphate transaminase	E	-2.152	-1.763	-2.878	-2.569
HMPREF0538_20901	precorrin-3B C(17)-methyltransferase	Q	-1.392	-1.185	-1.177	-1.089
HMPREF0538_20903	precorrin-4 C(11)-methyltransferase	Q	-1.182	-1.172	-1.155	-1.079
HMPREF0538_20904	precorrin-6Y C5,15-methyltransferase (decarboxylating), CbiT subunit	Q	-1.106	1.085	-1.139	-1.094
HMPREF0538_20907	cobalt-precorrin-8X methylmutase	Q	-1.223	-1.284	-1.043	-1.070
HMPREF0538_20905	precorrin-6y C5,15-methyltransferase (decarboxylating), CbiE subunit	Q	-1.106	-1.002	-1.042	-1.070
HMPREF0538_20906	cobalamin biosynthesis protein CbiD	Q	-1.065	-1.026	-1.060	-1.056
HMPREF0538_20829	hypothetical protein (putative protein serine/threonine phosphatase)	Un.	-1.384	-1.476	-1.559	-1.049
HMPREF0538_20243	ribosomal protein L32	J	-1.633	-1.398	-1.393	-1.969
HMPREF0538_20970	hypothetical protein	Un.	-1.429	-1.364	-1.117	-1.026
HMPREF0538_20572	ribosomal protein L30	J	-1.073	NS	-1.404	-1.384
HMPREF0538_22032	hypothetical protein	Un.	-1.432	-1.089	-1.371	-1.072

HMPREF0538_22021	DNA-binding protein HU-beta	L	NS	-1.674	-3.305	-2.693
HMPREF0538_21710	thioredoxin-disulfide reductase	C	NS	NS	-1.084	-1.148
HMPREF0538_21322	hypothetical protein	Un.	-1.568	-1.387	-1.501	-1.257
HMPREF0538_20886	putative adenosylcobinamide kinase/adenosylcobinamide-phosphate guanylyltransferase	Q	-1.082	-1.374	-1.046	-1.234

Table 5.2: List of the significant over-expressed genes (P<0.05) shared between the RWV and the RPM experiments and by at least one of the collection times (15 and 24 hours).

Abbreviations: log_2FC, log_2fold change; NS, not significant. See Table 4.2 for the COG nomenclature.

Locus Tag	Product name	COG	Log_2 FC RWV 15 h	Log_2 FC RPM 15 h	Log_2 FC RWV 24 h	Log_2 FC RPM 24 h
HMPREF0538_21979	arginine ABC transporter, ATP-binding protein ArtM	E	1.747	1.705	3.188	2.516
HMPREF0538_21811	integrase core domain protein	L	1.151	1.272	1.034	1.358
HMPREF0538_21980	ABC transporter, permease protein	M	1.851	1.8092	2.818	2.377
HMPREF0538_21523	large conductance mechanosensitive channel protein	P	2.235	1.348	1.690	1.813
HMPREF0538_20782	hypothetical protein	Un.	1.456	1.276	1.428	1.387
HMPREF0538_21527	aldehyde-alcohol dehydrogenase	C	NC	NC	1.494	1.141
HMPREF0538_20432	resolvase, N-terminal domain protein	L	NC	NC	1.1484	1.798
HMPREF0538_21819	integrase core domain protein	L	NC	NC	3.2845	2.349
WP_003670800.1	partial GroEL	O	1.123	NC	1.349	1.256

5.3.2 Integration of transcriptomic and proteomic analysis

Transcriptomic data were compared with the proteomic results described in the previous chapter.

Of all significant under-expressed genes after 15 h and 24 h in RWV microgravity system, ten were confirmed to be under-expressed protein in at least one of the two collection times for the same treated sample (Table 5.3). Of all significant over-expressed genes in RWV, six were shared with proteomic analysis on the same sample (Table 5.4). For RPM samples, ten significant under-expressed genes had also been found as under-expressed proteins (Table 5.5) and eight significant over-expressed genes had their corresponding protein (Table 5.6).

In Table 5.3 and 5.5 most genes and their corresponding proteins are involved in Vitamin B_{12} biosynthesis and in Amino-sugars metabolism.

Similarly to the expression of the protein coding for D-lactate dehydrogenase, the corresponding gene showed a down-regulation under RWV and RPM treatments.

The results of the comparison between over-expressed genes and proteins confirmed the up-regulation of the large conductance mechanosensitive channel protein (AEI56371, *MscL*).

Moreover, some stress genes that were also found as over-expressed proteins were *groEL* (AAR08285), *hsp20* (WP_072574878*)*, *cpn10* (EDX41887), *asp23/gls24* (AEI58257) and *clpP* (AEI56700). Only *groEL* was in common to RWV and RPM samples.

In RWV samples, there was an up-regulation of the protein carbamoyl-phosphate synthase, (AEI57438, *carB*) that is an important intermediate of pyrimidine and arginine biosynthesis.

Table 5.3: Under-expressed genes and proteins (P-value<0.05) shared by at least one of collection time (15 and 24 hours) in RWV experiment.

Abbreviations: log_2FC, log_2fold change. See Table 4.2 for the COG nomenclature.

Accession Number	Product name	COG	Log_2 FC RWV mRNA	Log_2 FC RWV protein
AEI57840	glutamine-fructose-6-phosphate transaminase	E	-2.878	-1.335
AEI57116	precorrin-6y C5,15-methyltransferase (decarboxylating), CbiE subunit	Q	-1.138	-1.0078
AEI57120	cobyrinic acid a,c-diamide synthase	Q	-1.258	-1.124
AEI57114	precorrin-4 C(11)-methyltransferase	Q	-1.155	-1.5937
AEI57111	precorrin-6A reductase	Q	-1.155	-1.048
AEI56294	D-lactate dehydrogenase	G	-1.261	-1.235
AEI57796	thioredoxin-disulfide reductase	C	-1.084	-1.449
AEI57891	ATP synthase F0 subunit C	U	-1.155	-1.713
AEI57121	aminotransferase, class I/II	E	-1.422	-1.606
AEI57110	uroporphyrinogen-III C-methyltransferase	Q	-1.007	-1.049

Table 5.4: Over-expressed genes and proteins (P-value<0.05) shared by at least one of collection time (15 and 24 hours) in RWV experiment.

Abbreviations: log_2FC, log_2fold change. See Table 4.2 for the COG nomenclature.

Accession Number	Product name	COG	Log_2 FC RWV mRNA	Log_2 FC RWV protein
AEI57736	Aldehyde-alcohol dehydrogenase	C	1.494	1.276
AEI57438	carbamoyl-phosphate synthase, large subunit	E	1.365	1.267
AAR08285	Partial GroEL	O	1.349	1.089
EDX41887	chaperonin Cpn10	O	1.239	1.306
AEI57733	transcriptional regulator, PadR family	K	1.365	1.256
AEI56371	large conductance mechanosensitive channel protein	M	1.690	1.164

Table 5.5: Under-expressed genes and proteins (P-value<0.05) shared by at least one of collection time (15 and 24 hours) in RPM experiment.

Abbreviations: log_2FC, log_2fold change. See Table 4.2 for the COG nomenclature.

Accession Number	Product name	COG	Log_2 FC RPM mRNA	Log_2 FC RPM protein
AEI57301	nitroreductase	C	-1.144	-1.666
AEI57840	glutamine-fructose-6-phosphate transaminase	E	-1.763	-1.079
AEI57116	precorrin-6y C5,15-methyltransferase (decarboxylating), CbiE subunit	Q	-1.002	-1.117
AEI57120	cobyrinic acid a,c-diamide synthase	Q	-1.0672	-1.095
AEI57112	precorrin-3B C(17)-methyltransferase	Q	-1.185	-1.007
AEI57097	putative adenosylcobinamide kinase/adenosylcobinamide-phosphate guanylyltransferase	Q	-1.374	-1.228
AEI57107	cobalamin biosynthesis protein CbiM	Q	-1.0626	-1.148
AEI56294	D-lactate dehydrogenase	G	-1.460	-1.046
AEI57796	thioredoxin-disulfide reductase	C	-1.148	-1.033
AEI57806	excinuclease ABC subunit A	L	-1.3380	-1.051

Table 5.6: Over-expressed genes and proteins (P-value<0.05) shared by at least one of collection time (15 and 24 hours) in RPM experiment.

Abbreviations: log2FC, log2fold change. See Table 4.2 for the COG nomenclature.

Accession Number	Product name	COG	Log_2 FC RPM mRNA	Log_2 FC RPM protein
AEI57736	Aldehyde-alcohol dehydrogenase	C	1.141	1.529
AEI58257	Asp23/Gls24 family envelope stress response protein	M	1.064	1.274
WP_072574878	Hsp20/alpha crystallin family protein	O	1.134	1.687
AAR08285	Partial GroEL	O	1.256	1.003
AEI56700	endopeptidase ClpP	O	1.227	1.088
WP_065533227	DNA helicase PcrA	L	1.087	1.376
AEI58186	arginine ABC transporter, ATP-binding protein ArtM	E	2.516	1.247
AEI56371	large conductance mechanosensitive channel protein	M	1.813	1.089

5.4 Discussion

Assessing the probiotics responses to spaceflight and characterizing their altered physiology and gene and protein expression in the microgravity environment are of critical importance when evaluating the risk for infectious disease in spaceship and the possibility to use probiotics for preventing these infections.

Our transcriptomic results on the effects over time of two simulated microgravity conditions on *L. reuteri* DSM 17938 showed that in the initial exponential growth phase there was a low significant differences in gene expression, confirming the proteomic data described in the previous chapter. This results are also agree with the RT-qPCR results and could be due to the short period of cultivation during which the cell has not yet had time to respond to stress conditions through the alteration of the whole transcriptome and proteome.

The analyses after 15 h and 24 h of treatment under both simulated microgravity systems demonstrated that there were significantly more downregulated genes than upregulated genes, as it was previously demonstrated for the proteins. In both simulated microgravity conditions the most numerically abundant functional category was represented by the 'unclassified' (Un.) class. This result suggests that the stress condition induced by simulated microgravity influences gene expression levels through many genes and proteins regulation and modification. On the other hand,

another work evaluating the effects of real and simulated microgravity on the transcriptome of another microorganism has reported the same result of a strong abundance of unclassified proteins underlining the need to continue the studies for identifying the specific role of these hypothetical proteins (Mastroleo *et al.*, 2009). These studies indicate that a substantial part of the genetics of these microorganisms is not well known. The expression of these genes in response to environmental parameters not encountered on the Earth raises the question of how these genes have been kept during evolution.

Most of the differentially under-expressed genes result in metabolic pathway, in particular in cobalamin biosynthesis. Moreover, the enzyme glutamine-fructose-6-phosphate transaminase (*glmS*) was down-regulated under both growth conditions, also confirming the proteomics data. This enzyme is a precursor in the peptidoglycan biosynthesis pathway and its regulation has a direct impact on the overall production of peptidoglycan. In lactobacilli, the down-regulation of *glmS* in response to cytoplasm acidifying agents, such as bile salt in *L. casei*, is observed. Under this kind of stress, there is an increasing demand for ATP from the F0F1 extrusion pumps to maintain the internal pH. Hence, the down-regulation of *glmS* gene decreases the level of glutamine-fructose-6-phosphate aminotransferase activity and reduces the flux of carbon to the peptidoglycan biosynthetic pathway. As a consequence, all fructose-6P is driven towards glycolysis for higher production of ATP (Alcantara *et al.*, 2012). In our case, there was a down-regulation of this gene and of its corresponding protein, indicating that the accumulation of lactic acid produced from the sugar fermentation of this bacterium determined a pH decrease and also consequently an up-regulation of some enzymes involved in arginine and fumarate biosynthesis, as it was previously demonstrated by proteomic results. As the first barrier against the stressing condition, *L. reuteri* cells could respond to medium acidification by modifying the properties of their cell walls, as it was demonstrated for another bacterium like *Lactobacillus vini* (Mendonça et al., 2018). In the context of the Chapter 3 of this thesis, we have studied the expression of some specific genes in EPS synthesis, like *ftf* and *gtfO*, and few differences have been detected, as, moreover, it also emerged from the transcriptomic analysis.

So, more other studies on composition and remodelling of cell wall is needed because it can be considered to be the first barrier of the cells against stressful environmental conditions.

The under-expression of thioredoxin-disulfide reductase gene and its corresponding protein in both growth conditions it could be indicative of the fact that growth is taking place in anaerobic conditions and therefore it is not necessary to use this enzyme, which is in general indispensable for

the aerobic growth in facultative anaerobe lactobacilli (Serata *et al*., 2012). Other works on a microorganism growing under simulated microgravity condition demonstrated that this condition induces mainly genes involved in anaerobic metabolism, which was reinforced by a lower expression in spaceflight samples of a cytochrome with high affinity for oxygen that is typically induced under microaerophilic conditions growth under oxygen-limiting conditions (Crabbé *et al.*, 2011).

Many over-expressed genes in *L. reuteri* cultures growing under both microgravity conditions belonged to mobile genetic elements, like insertion sequences (IS). The mobility of insertion sequences has been linked to various bacterial stress responses (Foster, 2007). Also the low fluid shear environment represents a condition of high stress for the cells that activate some mechanisms of genetic mobility for their survival. In general, mobile genetic elements in bacteria like IS elements, transposons and pro-phages (Frost *et al*. 2005) play a prominent role in genetic mobility within a strain's genome (intracellular mobility) and form one of the driving forces in the evolution of organisms (Liu *et al.* 2009).

The spaceflight condition could determine an increase in the plasmid-mediated conjugation, as it was demonstrated in the MOBILISATSIA experiments, aiming to investigate the impact of spaceflight conditions on different microorganisms sent aboard the International Space Station during the Soyuz Mission 8S. These experiments demonstrated that plasmid exchange between the Gram-positive bacterial strains occurred in the spaceflight experiment more efficient than in the ground control experiment (De Boever *et al.*, 2007).

The over-expression of phage-associated genes may be due to activation of the integrated phage as an escape mechanism to the low pH. In fact, the activation of phage-associated genes were observed in the same strain of *L. reuteri* after a sudden shift in environmental acidity to a pH close to the conditions in the human stomach (Wall *et al.*, 2007).

These results are corroborated by the over-expression of some different stress proteins expressed under both treatments that represent the first response to microgravity stress condition, as it was previously demonstrated for some of the same stress genes through RT-qPCR. Moreover, the cell wall motif LPXTG of binding to epithelial cell is over-expressed in both of treated samples, indicating that the microgravity condition may activate the adhesion promoting proteins, as for some of them it has been demonstrated via RT-qPCR.

The contemporary over-expression of some ABC transporters under both microgravity treatments, principally involved in specific carbohydrates transport, highlights that these transport systems may play more essential role in the adaptability to environmental conditions likely contributes to its competitive ability for limited availability of carbohydrate sources in low fluid shear condition.

Another interesting over-expressed gene in all samples treated for 15 and 24 h to RWV and RPM codifies for a large conductance mechanosensitive channel protein. A similar protein, the mechanosensitive channel MscS, was found in the MESSAGE 2 experiment on the bacterium *Rhodospirillum rubrum* SH1 (Mastroleo *et al.*, 2009). The over-expression of MscS was attributed to an hypo-osmotic shock due to a sudden reduction of the external osmolarity, that may induce the activation of this channel as an emergency safety valve (Booth *et al.*, 2007). In response to the alteration of the cellular homeostasis, other genes involved in carbon utilization and energy production, like the aldehyde-alcohol dehydrogenase, were up-regulated in cultures growing after 24 h of RWV and RPM treatments, for coping to the decrease of the external osmolarity and for restoring the homeostasis.

Considering the substantial transcriptional and translational changes in some metabolism pathways in *L. reuteri* DSM 17938 treated to simulated microgravity conditions, it would be interesting to test in future whether these changes will affect the interaction between this bacterium and the environment. More importantly, it is crucial to understand in future how these gene and protein expression changes may alter the relationship between *L. reuteri* and its human host.

Notes

Experiments described in this Chapter are currently ongoing in collaboration with Dr. Felice Mastroleo and Dr. Natalie Leys of the Interdisciplinary Biosciences (BIO), Institute for Environment, Health and Safety, SCK-CEN, Belgian Nuclear Research Centre, Boeretang (BE).

CHAPTER 6

General discussion

The lifestyle in spaceship and the relative sterility associated to the extreme environmental stressors, such as microgravity and cosmic radiations, could compromise the balance between the human body and its microbiome. An astronaut's body during spaceflight encounters increased risk for microbial infections and conditions because of immune dysregulation and altered microbiome, i.e. dysbiosis. This risk is further affected by increase in virulence of pathogens in microgravity. Health status of astronauts might potentially benefit from maintaining a healthy microbiome by specifically managing their diet on space in addition to probiotic therapies (Cervantes *et al.*, 2016). The microbiome status of astronauts and the human immune system could be altered by the exposure to microgravity (Crucian *et al.*, 2018), as well as to the accelerating forces during launch and landing (Blue *et al.*, 2012).

Immunological and physiological problems of astronauts could compromise the duration of spaceflights. A wide range of diseases that could potentially affect astronauts in extended missions have already been described (Saei *et al.*, 2012). Only recently has attention been paid to the astronauts' intestinal microbiome through the study of composition and diversity of its microbial communities (Saei *et al.*, 2012; Turroni *et al.*, 2017; Voorhies *et al.*, 2019). The first barrier to various diseases in humans is represented by the microbiome. The term "human microbiome" refers to the collective genomes of the complete microbiota present in the human body (Cox *et al.*, 2013; Zhu *et al.*, 2010). The microbiome directly influences biochemical, physiological pathways and assists to shape the gastrointestinal immune system (Hooper *et al.*, 2012).

In fact, the interactions between the gastrointestinal barrier and the microbiome appear to be a complex mechanism that helps in maintaining proper immune function in the gut in which healthy and pathogenic bacteria compete for dominance (Kamada *et al.*, 2013; Kamada *et al.*, 2014). A continuous balance between beneficial and potentially harmful bacteria is considered normal and contributes to a dynamic and healthy human gut (Figure 6.1) (Kamada *et al.*, 2014). There is increasing evidence of the association between the changes in the balance of gut flora and several diseases (Kinross *et al.*, 2011).

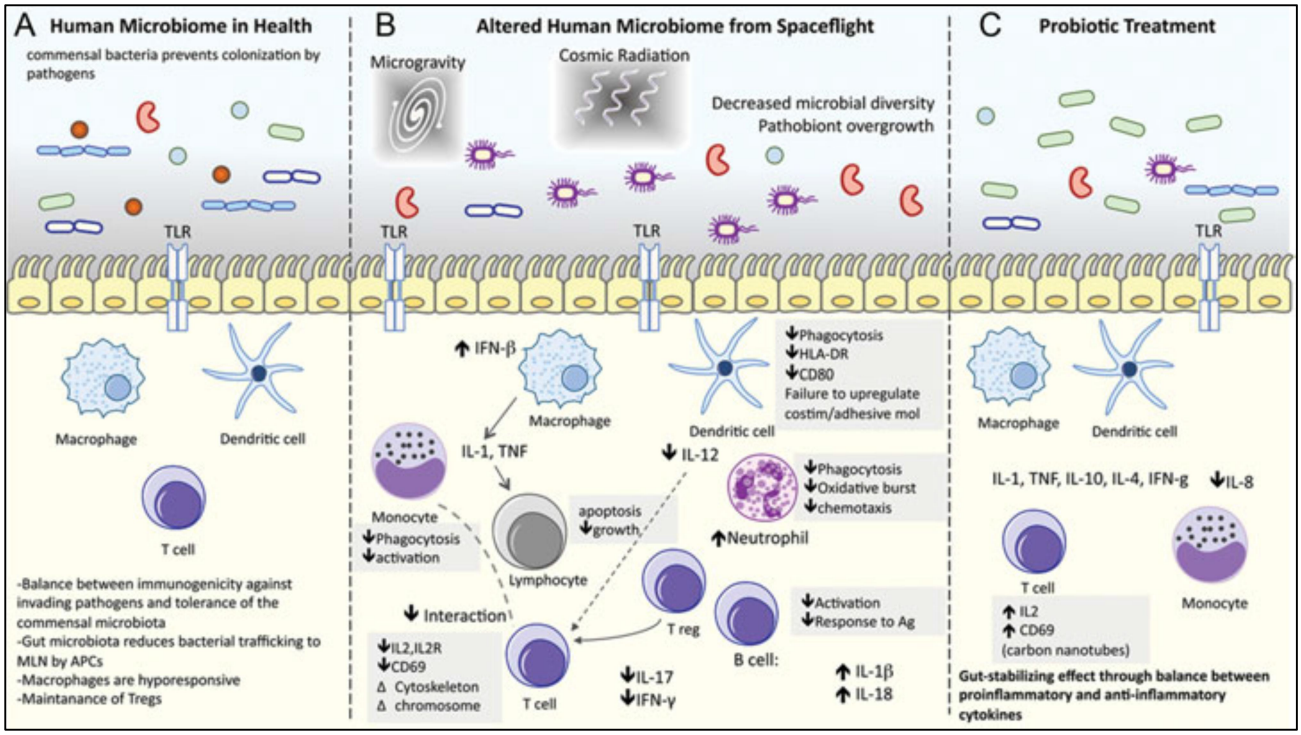


Figure 6.1: Dysbiosis and Immune Dysregulation in Outer Space.

The environmental stress of traveling can cause changes in the microbiome composition or its gene expression (Dumas *et al.*, 2006), which may lead to the transient dominance of pathogenic gut bacteria that can cause disease such as travelers' diarrhea (Gorbach *et al.*, 1975).

Spaceflights, in fact, can induce dysbiosis in the human microflora, which can result in the reduction of the defence group of microorganisms (bifidobacteria and lactobacilli) and appearance of opportunistic pathogens such as *Escherichia coli*, enterobacteria and clostridia (Lizko *et al.*,1984). Subsequently, this procedure can lead to accumulation of potentially pathogenic species and their long-term persistence (Ilyin *et al.*, 2005). Pioneering studies revealed a reduction in the number of non-pathogenic bacteria and an increase in the number of opportunistic pathogens in the nasal flora of cosmonauts (Nefedov *et al.*, 1971). A significant reduction in the number of bacterial species in the intestine after spaceflight and terrestrial analogs has also been reported (Taylor *et al.*, 2005). In addition, a decrease in lactobacilli, with concurrent replacement with pathogens, has been observed utilizing three different models simulating space environment (Ritchie *et al.*, 2014). It was demonstrated that microgravity stress can alter bacterial virulence, and promote antibiotic bacterial resistance. The low-fluid shear dynamics associated with microgravity were proved to play a key role in the regulation of microbial gene expression, physiology and pathogenesis (Mishra *et al.*, 1992; Kacena et al., 1999; Klaus *et al.*, 2001; Nickerson *et al.*, 2004). Other studies have shown that microgravity is associated with changes in virulence factors (Nickerson *et al.*, 2000; Nickerson *et*

al., 2004; Wilson *et al.*, 2002; Wilson *et al.*, 2007; Rosenzweig *et al.*, 2010) and increased biofilm formation (Mauclaire *et al.*, 2010). Certain bacteria acquire increased pathogenic features, such as changes in growth modulation and alterations in response to antibiotics after exposure to simulated microgravity or spaceflight (Wilson *et al.*, 2007; Chopra *et al.*, 2006). Higher minimal inhibitory concentrations to various classes of antimicrobial agents (Tixador *et al.*, 1985; Lapchine *et al.*, 1986) and increased production of quorum-sensing molecules that trigger bacterial communication and physiological changes in some bacterial species have been described (Crabbé *et al.*, 2008). Microgravity may also influence the cell interaction between mutualistic bacteria and their hosts, accelerating bacteria-induced apoptosis in host tissues (Foster *et al.*, 2014; Ilyin *et al.*, 2005). Moreover, microgravity could be associated with impaired human immune response and with reactivation of latent infections , such as herpes virus (Rooney *et al.*, 2019). Immune cells subjected to conditions of altered gravity manifest a number of changes in structure and function (Figure 6.1 b) (Bakos *et al.*, 2001; Cubano *et al.*, 2006). Studies have revealed a decrease in total white blood cell (WBC) count, lymphocytes, monocytes, and eosinophils, and a slight increase in neutrophils in rats flown on a nine-day mission (Allebban *et al.*, 1994). Similar results showing a decrease in total leukocytes, lymphocytes, monocytes and elevated neutrophils were later reported in rats flown on a 14-day mission (Ichiki *et al.*, 1996). Both studies demonstrated a decrease in the absolute number of CD4 and CD8 as well as B-lymphocytes.

The microbiome somehow contributed to stressor-induced immune-enhancement and the intestinal microflora appeared to be necessary for the increase in circulating cytokines (Bailey *et al.*, 2012). In fact, after exposure to microgravity, macrophages express high levels of inflammatory receptors, and produce large quantities of pro-inflammatory cytokines and mediators (Saei and Barzegari, 2012).

The administration or consumption of probiotics is supposed to have immune-enhancing effect, hinder variations in the human microbiome, and prevent colonization of potential pathogens. Lactobacilli stimulate Peyer's patches macrophages and/or DCs to release inflammatory cytokines such as TNF-α, IFN-γ and IL-12, and regulatory cytokines such as IL-4 and IL-10. Lactic acid bacteria can downregulate production of the pro-inflammatory cytokine IL-8 (Buckley *et al.*, 2011), which has been reported to increase during long-term spaceflight (Crucian *et al.*, 2014).

The availability of a variety of probiotic foods already compliant with space food can support the astronauts in unconsciously growing consumption of probiotics during spaceflight with the aim of improving the immune defences already so weakened by the factors listed above.

Elucidation of the mechanisms by which microgravity interacts with probiotics and with their physiological and molecular characteristics may provide clues for developing rational strategies to improve the immune responses of astronauts during long space missions and reduce the incidence of diseases. For this reason, the study of the microgravity effects on metabolism, transcriptome and proteome of *L. reuteri* DSM 17938 may be useful for improving knowledges about its positive effect on a number of human microbiome alterations and on possible gut colonization by potential pathogens. The study was conducted by using the two most commonly used microgravity simulators, RPM and RWV.

Prior to conducting studies reported in this thesis, we did not have any experience with these two simulating systems and with the bacteria cultivation under these two conditions. Different strategies of bacteria cultivation and various experiments were performed using for first the RPM provided by prof. Pippia at the Department of Physiology of the University of Sassari. These experiments were performed growing some colonies of *L. reuteri* in solid media for 3 days and evaluating the reuterin production, the resistance to GI simulated passage and the expression of some stress genes. This first experiment showed different results in comparison to the same experiment conducted growing bacteria in liquid media, emphasizing how different growth methods can influence the bacterial response. We decided to continue with the experiments performing in liquid media that best reproduce a real situation of bacteria cultivation in space. In fact, based on the extensive literature and on our personal experience, the cultivation in liquid media reproduce better the low-fluid shear condition found in space.

Some metabolic and physiological aspects were first investigated in Chapter 3 of this thesis. No alterations in growth profiles under both simulated microgravity conditions were observed, encouraging the possible cultivation of this bacterium in spaceflight. The results of growth kinetic are in good agreement with another study on a probiotic bacterium, but are in contrast with many other studies on potential pathogenic bacteria grown in simulated microgravity.

Moreover, cell size and shape in RWV and RPM treatments revealed no significant differences than control. On the contrary, a significant enhanced production of reuterin and a greater tolerance to GI passage compared to control were observed for both treatments. The capacity to produce an antimicrobial compound is essential to perform the growth inhibiting action of pathogens which may increase their pathogenesis under microgravity conditions, as has been widely demonstrated in the literature.

Another important probiotic characteristic is the GI resistance that not all probiotic strains possess. With the results reported in Chapter 3, we confirmed this evidence for *L. reuteri* and we also demonstrated that both simulated microgravity conditions can confer the GI resistance to our probiotic strain. In our opinion, more attention has to be given to an accurate simulation of gastrointestinal environment in its composition and physiology. In this regard, a better simulation of human digestive tract, including the intestinal microbiota, can be achieved through the use of computer-controlled human GI models (e.g. Simulator of the Human Intestinal Microbial Ecosystem, SHIME), in order to have more information regards the real effect of spaceflight condition on astronaut's microbiota.

Similarly, to the best of our knowledge, a study at molecular level about stress-response genes was conducted in Chapter 3 showing that all investigated genes were upregulated in the RWV experiment, in particular after 15 h and 24 h of treatment, whereas the RPM experiment seems to determine the down expression of most of them. These differences at transcriptomic level between cultures subjected to RPM and RWV treatments reflect the different way of these devices to reproduce microgravity environment, as has been extensively discussed in other works (Crabbé *et al.*, 2010; Mastroleo *et al.*, 2013).

These results, in fact, are in good agreement with other studies that used RWV system for evaluating stress genes expression of other bacteria confirming that the two systems have dissimilar effect on bacterial cells depending on their capability to mimic real microgravity condition.

Experiments described in Chapter 4 and 5 of this thesis produced results which showed different levels of expression of some genes and proteins. The identification of these genes and proteins should be performed in order to fully understand which pathways are potentially influenced by the microgravity environment created by RWV and RPM systems. The two analysis have led to similar results, highlighting a down-regulation of Vitamin B_{12} and an upregulation of pyrimidine and arginine biosynthesis and an increased synthesis of some stress-related proteins suggesting the necessary by the cultures to face the stress condition induced by the two treatments through the biosynthesis of new nucleic acids and new amino-acids against of secondary metabolism like that of vitamin B_{12}. The biosynthesis of arginine and pyrimidine is also probably due to the necessary to respond to acid stress, as it was widely already demonstrated for other lactobacilli (Rollan et al, 2003; Teixeira et al., 2014). These analysis highlighted the over-expression of a mechanosensitive channel protein, of some ABC transporters and some general stress proteins and chaperones to repair and stabilize proteins and DNA, suggesting that *L. reuteri* possess a number of strategies to

adapt to this particular environment. Moreover, the proteomic analysis found out many proteins involved in DNA repair and recombination mechanism(s) utilized by *L. reuteri* under both systems, underlining the importance of gene-acquisition and loss within the *L. reuteri* strain diversification that is required to adapt to a large range of environments.

This study represents an important step in understanding the response of a probiotic bacterium to a simulated microgravity environment with the view of considerate the possible use of this strain in a bioreactor of MELiSSA loop as a direct source of procurement for the astronauts.

Furthermore, it allows assessment of the role that low fluid shear regions found in the human body play in the regulation of probiotic and molecular characteristics of this bacterium.

Future perspectives

The aim of MELiSSA project is the understanding, modelling and controlling of every single compartment based on an "aquatic" ecosystem, to obtain the closure of the regenerative life support system for long-term manned space mission; consequently, many processes have to be coupled together. The key issue is to be able to guarantee food production in a future mission, stable both in quantity and in quality. Regarding food sources, mainly higher plants and probiotics, this level of characterization is far from being complete and from allowing a good understanding and a proper modelling approach.

In the future, new food sources of probiotics will likely play a significant role in the astronauts' diet. However, to fully realize the possible cultivation of probiotics and in particular of *L. reuteri* DSM 17938 in one of bioreactor of MELiSSA loop, a range of multidisciplinary actions should be pursued as part of a cooperative model to address the following points:

- evaluation of possible cultivation of this strain in a thermophilic anaerobic compartment (Compartment I) and study of its capacity to degrade the insoluble fibers and non-edible parts of plant and to produce a volatile acid like acetic acid and CO_2;
- study of the expression of genes and proteins under different bacterial growth conditions (thermophilic cultures versus photoheterotrophic cultures, thermophilic culture in space condition, etc.);

- performing of flight experiment to understand culture behaviour in real reduced gravity with the aim to develop a model that will be based on *L. reuteri* metabolism and its regulation by more space stressors.

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Scientific Curriculum

Current Occupation

PhD candidate January 2016-March 2020

In Food and Agricultural Sciences at University of Naples, Federico II, Dept. of Agricultural Sciences, Portici (Na). Division of Microbiology.

Research topic: Study of the exposure to simulated microgravity conditions on the probiotic *Lactobacillus reuteri* DSM 17938.

Work experience January 2014-January 2016

- **Research fellow**

  Application of stable and transient genetic transformation methods to evaluate the function of genes involved in controlling the shape and size of tomato and pepper fruit.

  Activities within the program PON Research and Competitiveness GenHORT for the enhancement of excellent vegetable production in Campania with advanced genomics tools.

  Institute of Biosciences and BioResources, National Research Council of Italy (CNR-IBBR) Portici, NA, via Università 133, 80055 Italy

- **Research fellow** January 2012- January 2014

  Study of seawater and sediment microbial diversity in tourist port areas of Mediterranean basin, samplings and surveyes in study areas and bioindicator monitoring of pollution state of different habitat.

  Activities within MAPMED research project (Management of port areas in the Mediterranean sea basin).

  University of Florence, Department of Evolutionary Biology "Leo Pardi" – Florence, Via Romana 17-19.

- **Research fellow** December 2010-December 2011

  Study of rizosphere microbial diversity of some indicator plants in areas exposed to genetically modified crops (OGM).

  Activities within for LIFE+ 2008 DEMETRA (DEvelopment of a quick Monitoring index as a tool to assess Environmental impacts of Transgenic crops) research project.

  University of Florence, Department of Evolutionary Biology "Leo Pardi" – Florence, Via Romana 17-19.

Education and training

June 2010

Master degree in Agri-food Biothecnology, with honours

with a thesis on the study of transient and stable transformation of tobacco plant with a viral gene to increase the endogenous defense to biotic stress. Title: "Study of the *TnBVank*1 gene to increase the endogenous defenses of tobacco from biotic stress".

University of Naples, Federico II, Dept. of Agricultural Sciences, Portici (Na). Division of Molecular biology.

December 2007

Bachelor degree in Agri-food Biothecnology, with honours

with a thesis on the expression study of a gene involved in increasing the tobacco defence to biotic stress. Title: "Expression study of a viral gene".

University of Naples, Federico II, Dept. of Agricultural Sciences, Portici (Na). Division of Molecular biology.

List of Publications

- Giuliana Senatore, Felice Mastroleo, Natalie Leys, Gianluigi Mauriello. (2020). Growth of *Lactobacillus reuteri* DSM17938 under two simulated microgravity systems: changes in reuterin production, GI passage resistance and stress genes expression response. *Astrobiology*. 20 (1):1-14. doi: 10.1089/ast.2019.2082.
- Giuliana Senatore, Felice Mastroleo, Natalie Leys, Gianluigi Mauriello. (2018). Effect of microgravity and space radiation on microbes. *Future Microbiology*. 1 (13):831-847. doi: 10.2217/fmb-2017-0251.
- Francesco Vitali, Manolis Mandalakis, Eva Chatzinikolaou, Thanos Dailianis, Giuliana Senatore, Enrico Casalone, *et al*. (2019). Benthic prokaryotic community response to polycyclic aromatic hydrocarbon chronic exposure: importance of emission sources in Mediterranean ports. *Frontiers in Marine Science*. 6, 590.
- FrancescoVitali, Giorgio Mastromei, Giuliana Senatore, Cesarea Caroppo, Enrico Casalone. (2016). Long lasting effects of the conversion from natural forest to poplar plantation on soil microbial communities. *Microbiological Research*. 182: 89-98.

Conference participation and contributions

- Giuliana Senatore, Francesco Vitali, Cesarea Caroppo. "Evaluation of microbial biodiversity in rizospheric soils of wild plants in the risk assessment of unintended negative environmental impacts of transgenic crops", 29th National Meeting of the Italian Society of General Microbiology and Microbial Biotechnology. National Research Council of Italy, Pisa, 22-24 September 2011. Abstract and poster.
- Giuliana Senatore, Francesco Vitali."Characterization of bacterial communities in tourist ports in the Mediterranean Sea Basin", XII Congress of the Italian Federation of Life Sciences (FISV). University of Rome, La Sapienza. 24-27 September 2012. Abstract and poster.

- Giuliana Senatore, Francesco Vitali. “Fingerprinting characterization of prokaryotic communities in sediments from three tourist ports in the Mediterranean area”, Microbial Diversity 2013- Microbial Interactions in Complex Ecosystems” of Italian Society of Agri-Food and Environmental Microbiology (SIMTREA). University of Turin, 23-25 October 2013. Abstract and poster.
- Giuliana Senatore, Maria Cammareri. “Identification and Characterization of Candidate Genes controlling Fruit Shape and Size in Tomato”, 58th Annual Conference of the Italian Society of Agricultural Genetics, Quarté Sayàl, Alghero (SS), 15-18 September 2014. Abstract and poster.
- Giuliana Senatore. “Probiotics beyond the terrestrial limits: transcriptomic and proteomic approach to investigate the effect of simulated microgravity on the probiotic *Lactobacillus reuteri* DSM17938”, MELiSSA Workshop, University of Lausanne, 8-9 June 2016. Abstract and poster.
- Giuliana Senatore. “Probiotics beyond the terrestrial limits: transcriptomic and proteomic approach to investigate the effect of simulated microgravity on the probiotic *Lactobacillus reuteri* DSM17938”, 21th Workshop “Developments in the Italian PhD Research on Food Science, Technology & Biotechnology”, University of Naples, Federico II. Dept. of Agricultural Sciences, Portici (Na), 14-16 September 2016. Abstract and poster.
- Giuliana Senatore, Felice Mastroleo, Natalie Leys, Gianluigi Mauriello. “Phenotypic and Transcriptomic changes of *Lactobacillus reuteri* DSM17938 under simulated microgravity conditions”, 22th Workshop “Developments in the Italian PhD Research on Food Science, Technology & Biotechnology”, University of Bozen, Dept. of Food Technology, 20-22 September 2017. Abstract and poster.
- Giuliana Senatore. “Metabolic, transcriptional and proteomic changes of the probiotic *Lactobacillus reuteri* DSM17938 under simulated microgravity”, 1st Workshop Joint Agrospace

MELiSSA, National Research Council of Italy, Rome, 18 May 2018. Abstract and oral communication.

Research and educational experience

PhD guest at the Department of Physiology, University of Sassari September 2016

Investigation of some metabolic charactersistic of *L. reuteri* DSM 17938 cultivated for 5 days on solid media under RPM.

PhD guest at the Institute of Environment Health and Safety of SCK-CEN, Belgian Nuclear Research Centre, Boeretang (BE). January-April 2017

Investigation of behaviour of *L. reuteri* liquid cultures cultivated for 3 days under RWV and RPM, with special attention to growth kinetics, reuterin production and simulated GI passage.